VIRAL AND CELLULAR DETERMINANTS OF REOVIRUS-INDUCED NF-κB ACTIVATION
AND APOPTOSIS

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology and Immunology

December, 2006

Nashville, Tennessee

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Dedicated to My Parents

Thomas and Shirley Hansberger

ACKNOWLEDGEMENTS

I have had the privilege of meeting so many unique and wonderful people during the course of my life. Each and every one of those people had an influence on the choices I made and the direction my decisions took me. I could not have made it this far without the support of so many people. I would like to especially thank the following for making it possible for me to have the opportunity to train in such a great environment provided by Vanderbilt University:

- My parents for the infinite amount of guidance and support that they have provided me. You mean the world to me and I hope I make you feel as proud as you have made me feel loved.

- My brother for being my best friend and always being there to listen.

- Scott Lyons for his eternal friendship and for teaching me how valuable life is. I hope to be half the person you were.

- Jackie Campbell for being one of my favorite people. Without you, I never would have made it this far.

- The entire Campbell family. You welcomed me into your family with open arms and I'll never forget that.

- Terry Dermody for being a great mentor and friend during my time at Vanderbilt. I will always be grateful to you for the training and counseling you gave me and the fun times we had.

- Past and present members of the Dermody lab. I've enjoyed the friendship and the atmosphere in which you all have contributed. I especially would like to thank Craig

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AC   Ammonium chloride

AO   Acridine Orange

CNS   Central nervous system

CHO   Chinese hamster ovary cells

CAR   Coxsackievirus and adenovirus receptor

DNA   Deoxyribonucleic acid

dsRNA   Double-stranded ribonucleic acid

EMSA   Electrophoretic mobility shift assay

FFU   Fluorescent focus unit

GAPDH   Glyceraldehyde-3-phosphate dehydrogenase

H   Hours

H&E   Hemotoxylin and Eosin

HLL   HIV long-terminal repeat luciferase

HTLV   Human T-cell lymphotropic virus

IFN   Interferon

IκB   Inhibitor of κB

IKK   IκB kinase

IL   Interleukin

IPS   Interferon promoter stimulator

IRF   Interferon regulatory factor

ISG   Interferon stimulated gene

ISVP   Infectious subvirion particles

HIV	Human immunodeficiency virus
JAK	Janus-associated kinase
JAM	Junctional adhesion molecule
L	L929
mAb	Monoclonal antibody
MEF	Murine embryo fibroblasts
Min	Minute
MDCK	Madin-Darby canine kidney
MOI	Multiplicity of infection
mRNA	Message ribonucleic acid
NF-κB	Nuclear factor-κB
NGS	Normal goat serum
NIK	NF-κB inducing kinase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCFC	Primary cardiac fibroblast culture
PCMC	Primary cardiac myocyte culture
PCR	Polymerase chain reaction
PFU	Plaque forming units
PKR	RNA-dependent protein kinase
PSL	Photostimulus luminescence
RNA	Ribonucleic acid
SA	Sialic acid

SDS   Sodium dodecyl sulphate

SEC   Seconds

STAT   Signal transducer and activator of transcription

T1   Type 1

T2   Type 2

T3   Type 3

TNF   Tumor Necrosis Factor

TUNEL   Terminal dUTP nick-end labeling

UV   Ultraviolet

Wt   Wild-type

CHAPTER I

BACKGROUND AND LITERATURE REVIEW

Overview of virus-induced apoptosis

Processes by which viruses injure and kill host cells are essential determinants of

viral pathogenesis. Apoptosis is a genetically programmed form of cell death that plays an

important regulatory role in many biological processes such as organ development, cellular

homeostasis, and immunity. Apoptosis can be triggered by a variety of environmental

stimuli and results in numerous morphological and biochemical changes including cell

shrinkage, vacuolization, membrane blebbing, condensation of nuclear chromatin, and

cleavage of chromosomal DNA into oligonucleosome-length fragments (3, 126, 167, 218).

Membrane-bound fragments of apoptotic cells are efficiently removed by phagocytes without

inducing an inflammatory response.

Many viruses are capable of inducing apoptosis of infected cells (126, 140, 153, 167,

188). In some cases, apoptosis triggered by virus infection may serve as a component of host

defense to limit viral replication or spread (162). This defense mechanism is mediated either

directly by self-destruction of host cells prior to completion of the viral replication cycle or

indirectly through immune recognition of infected cells by cytotoxic T lymphocytes (126,

167). In other instances apoptosis may enhance viral infection by facilitating viral

dissemination or allowing the virus to evade host inflammatory responses (37, 126, 188). For

some viruses, cellular factors operant during apoptosis may function to increase the

production of viral progeny (140, 167). The biochemical pathways activated by most viruses

to induce apoptosis are largely unknown.

This thesis was focused on mechanisms used by mammalian reoviruses to induce apoptosis. Reoviruses are highly tractable models for studies of viral replication and pathogenesis. Apoptosis is the principal means of cell killing employed by some reovirus strains and therefore may influence viral virulence (40, 125, 128, 145, 199). My research was directed toward identifying components of the cellular signaling apparatus required for reovirus-induced apoptosis and determining the effector mechanisms that elicit the apoptotic response during reovirus infection. Identifying and understanding the cellular components of the virus-induced apoptotic signaling machinery will help understand disease mechanisms and identify new targets for therapeutic intervention.

Reovirus structure and replication

Mammalian reoviruses are nonenveloped viruses that contain a genome of 10 discrete segments of double-stranded (ds) RNA (reviewed in Nibert and Schiff (122)). These viruses are the prototype members of the *Orthoreovirus* genus of the *Reoviridae* family. Reoviruses exhibit a broad host range infecting most mammalian species, including humans (reviewed in Tyler (195)). Reoviruses were named _r_espiratory _e_nteric _o_rphan viruses on the basis of their repeated isolation from the respiratory and enteric tracts of children with no obvious clinical symptoms of illness (157). Disease associated with reovirus infection is unusual and restricted to the very young. There are three reovirus serotypes, which can be differentiated on the basis of hemagglutination profiles and neutralization assays (149). A major mechanism of cell killing by reovirus is the genetically programmed cell death pathway that leads to apoptosis.

Reoviruses particles are approximately 85 nm in diameter and formed by eight structural proteins arranged in two concentric protein shells (or capsids) with icosahedral symmetry (42, 52) (Fig. 1). The T=1 inner capsid surrounds the centrally condensed 10
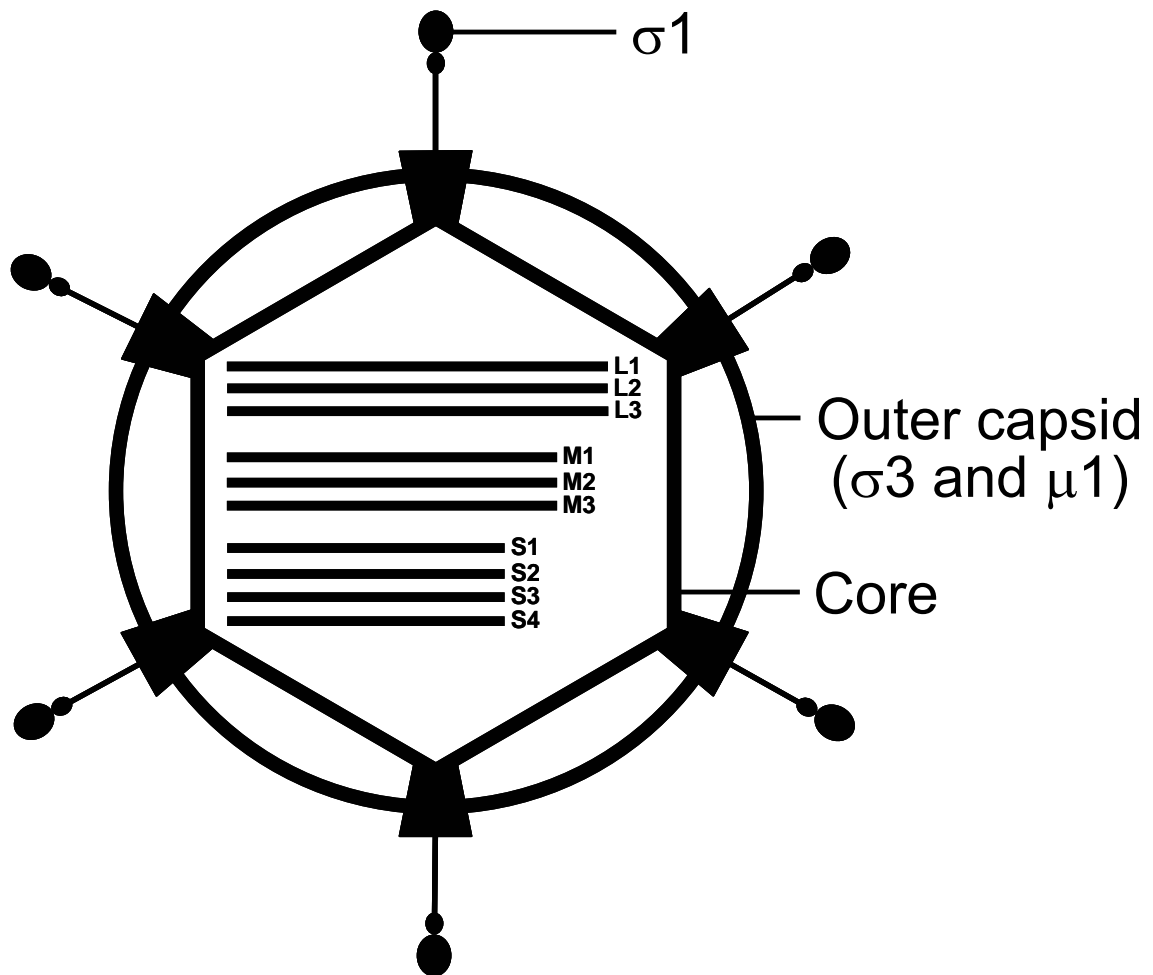
Figure 1. The reovirus virion. The reovirus virion contains two concentric protein shells termed the outer capsid and core. The core contains 10 dsRNA gene segments that are classified by size as large (L), medium (M), or small (S). The viral attachment protein, $\sigma 1$, is depicted as a ball and stick model protruding from the outer capsid. Viral proteins $\sigma 3$ and $\mu 1$ form the outer capsid and serve as substrates for endocytic proteases.

dsRNA gene segments and, together with the genomic RNA, forms the tightly packed viral core. It is assembled from two major proteins, λ1 and σ2 (120 and 150 copies each, respectively) (42, 142), and minor core proteins μ2 (~20 copies) and λ3 (~12 copies) (43). The T=13 (left-handed) outer capsid is constructed from complexes of proteins σ3 and μ1 present in 600 copies each. A core spike, formed by pentamers of the λ2 protein (60 copies), projects from the inner capsid through the outer capsid at the 12 axes of five-fold symmetry (142). Partially inserted within a turret-like opening of the λ2 pentamer is the delicate, fiber-like trimeric attachment protein, σ1 (36 copies) (29, 58).

The reovirus replication cycle is entirely cytoplasmic (reviewed in Nibert and Schiff (122)). Reovirus virions are capable of binding to cell-surface carbohydrate (11), and junctional adhesion molecule-A (JAM-A) (11). Following viral attachment, reovirus virions enter cells by receptor-mediated endocytosis (6, 20, 155, 183), in which β1 integrins mediate internalization (102). Within an endocytic compartment, the viral outer capsid is removed to generate infectious subvirion particles (ISVPs) (Fig. 2). During virion-to-ISVP disassembly, σ3 is degraded and lost from virions, σ1 undergoes a conformational change, and μ1 is cleaved to form particle-associated fragments δ and ϕ. Removal of σ3 exposes hydrophobic domains in μ1 that facilitate interactions of ISVPs with endosomal membranes (26, 27), leading to delivery of core particles into the cytoplasm and concomitant activation of the viral transcriptase (122). Transcription of the viral genome occurs within the core, and nascent mRNAs are released through turret-like openings at the viral vertices. Viral mRNAs are used as template for viral protein synthesis and replication of viral genomic dsRNA (90, 161). Particles containing dsRNA segments are termed replication particles. These particles are transcriptionally active and yield additional viral mRNAs resulting in an amplification of

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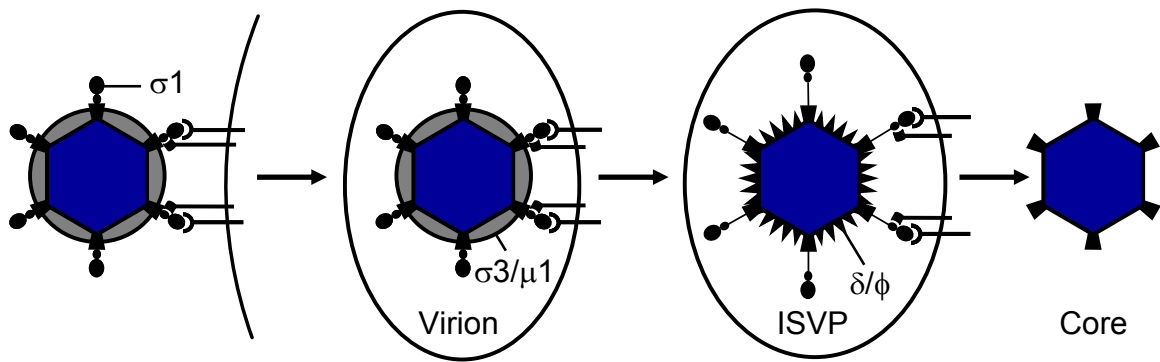
Figure 2. Entry of reovirus into cells. Following attachment to cell-surface receptors, reovirus virions enter cells by receptor-mediated endocytosis. Within an endocytic compartment, the viral outer capsid undergoes acid-dependent proteolysis resulting in generation of ISVPs. Steps in viral entry are completed by interaction of ISVPs with endosomal membranes, leading to delivery of transcriptionally active core particles into the cytoplasm.

viral protein synthesis (81, 117). Following addition of viral outer-capsid proteins to the replication particles, progeny virions are assembled and exit the cell.

Reovirus pathogenesis and diseases

The pathogenesis of reovirus infection has been studied most extensively using newborn mice, in which serotype-specific patterns of disease have been identified (reviewed in Virgin et al. (207)). The best characterized of these models is reovirus pathogenesis in the murine central nervous system (CNS). The segmented nature of the reovirus genome has allowed the determination of the genetic basis for complex viral phenotypes by analysis of reassortant viruses containing mixtures of gene segments derived from parental strains that exhibit a biological polymorphism of interest. Using this strategy, strain-specific differences in the pathogenesis of reovirus CNS infections have been genetically mapped (50, 211). This work has provided important insights into mechanisms of reovirus tropism and virulence.

Following oral inoculation of newborn mice, reovirus is internalized by intestinal M cells (216) and undergoes primary replication in lymphoid tissue of Peyer's patches (83, 118, 154). Virus then invades the CNS, yet strains of different serotypes use different routes of dissemination and manifest distinct pathologic consequences. Serotype 1 (T1) reovirus spreads to the CNS hematogenously and infects ependymal cells (197, 211), resulting in hydrocephalus (210). In contrast, serotype 3 (T3) reovirus spreads to the CNS through nerves and infects neurons (118, 197, 211), causing lethal encephalitis (187, 210). Analysis of reassortant viruses obtained by coinfecting cells with prototype strains T1 Lang (T1L) and T3 Dearing (T3D) demonstrated that the pathways of viral spread in the host (197) and tropism for neural tissues (50, 211) segregate with the viral S1 gene, which encodes the viral

attachment protein, σ1 (89, 209). These studies suggest that σ1 determines the CNS cell types that serve as hosts for reovirus infection.

Reovirus infection causes pathology and physiologic dysfunction in a wide range of organs and tissues, including the hepatobiliary system, the myocardium, lungs, and endocrine tissues (207). Of these, myocarditis (174) has become a particularly well-established experimental model of reovirus-induced disease. Myocarditis caused by reovirus infection is unusual in comparison to other viral etiologies of myocarditis in that the pathogenesis is not immune mediated. Instead, reovirus cytopathicity is a direct cause of myocyte injury, which results from a complex interplay of the interferon (4, 124, 175) and apoptosis pathways (44). Efficiency of viral RNA synthesis is a key factor in determining the extent of myocardial injury. Accordingly, viral gene segments encoding proteins involved in viral transcription and genome replication play important roles in determining strain-specific differences in the capacity of reovirus to induce myocarditis (171, 172, 175).

Genetics of reovirus-induced apoptosis

Reovirus induces the biochemical and morphological characteristics of apoptosis both in cultured cells (40, 145, 199) and in the murine CNS (125, 128) and heart (44, 128). Insights into mechanisms by which reovirus induces apoptosis have emerged from studies using strains T1L and T3D, which differ in the capacity to induce apoptosis in a variety of cell types (Fig. 3). Reovirus strain T3D induces apoptosis much more efficiently than T1L in murine L929 (L) cells (199), Madin-Darby canine kidney (MDCK) cells (145), and HeLa cells (38). These differences have been analyzed genetically using T1L x T3D reassortant viruses and indicate that the S1 gene is the principal determinant of differences in the
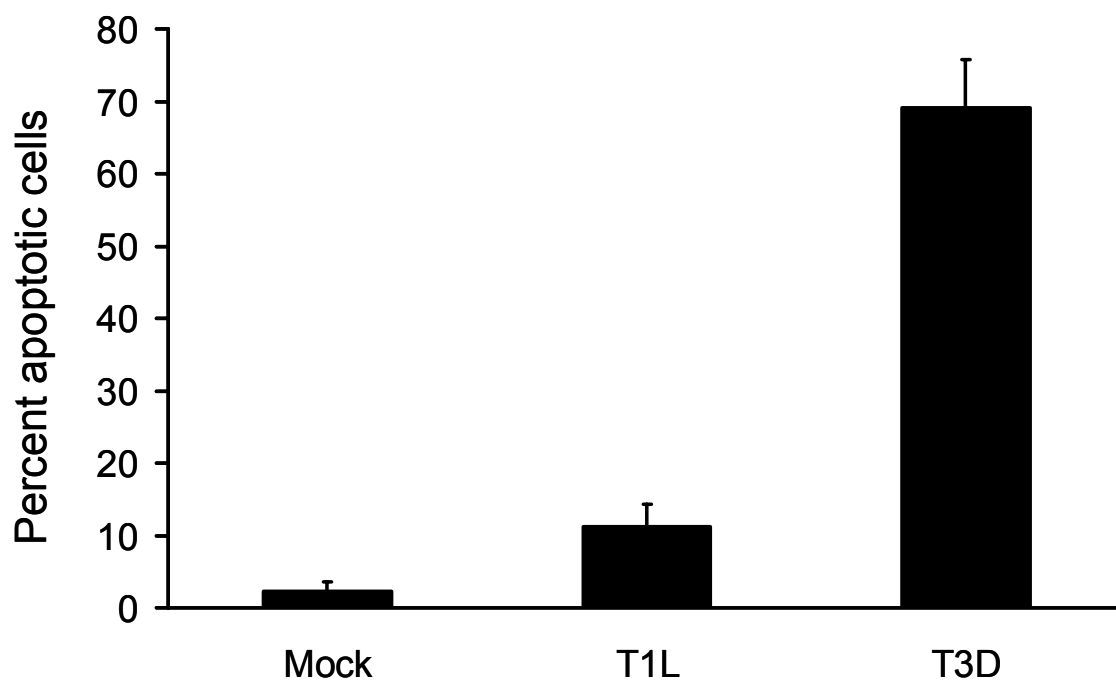
Figure 3. Reovirus-induced apoptosis. HeLa cells were adsorbed with either gel saline (mock), T1L, or T3D at an MOI of 100 PFU/cell and incubated at 37°C for 48 h in medium. Cells were harvested, stained with acridine orange (AO), and scored for apoptosis using morphological criteria. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations.

capacity of T1L and T3D to induce apoptosis (38, 145, 199). However, a smaller, independent contribution is made by the M2 gene segment.

The S1 gene is bicistronic and encodes the nonstructural protein σ1s, along with the attachment protein σ1 (55, 75, 158). The role of σ1s in reovirus-induced apoptosis was tested using strains T3C84 and T3C84-MA, which vary in the expression of σ1s. Results from these experiments showed that expression of σ1s is not required for apoptosis induction (146), indicating that the σ1 protein is the S1 gene product responsible for mediating the strain-specific differences in apoptosis by reovirus.

The M2 gene encodes outer-capsid protein μ1 (112, 120), a 76-kDa amino-terminal myristilated protein (123). The μ1 protein is proteolytically cleaved within the endocytic pathway (5, 20), and these cleavage fragments are proposed to interact with endosomal membranes to deliver viral cores into the cytoplasm (70, 71, 101). The exact role of the μ1 protein in apoptosis is unknown, but it may contribute to apoptosis induction by directly interacting with intracytoplasmic sensors of viral infection resulting in proapoptotic signaling.

In addition to receptor binding steps being an important determinant of apoptosis induction by reovirus, viral replication steps following attachment are also required to induce signals that lead to apoptosis. Inhibitors of viral disassembly abolish the capacity of virions, but not ISVPs, to induce apoptosis (39). However, inhibitors of RNA synthesis, particles devoid of dsRNA, and temperature-sensitive (ts) viral mutants arrested at defined steps in reovirus replication have no effect on apoptosis induction (39). Taken together, these results suggest that reovirus disassembly resulting in ISVP formation, but not viral transcription or subsequent steps in viral replication, is required to induce apoptosis.

Apoptosis induced by reovirus requires activation of NF-κB

A critical component of the intracellular signal transduction apparatus that leads to apoptosis following reovirus infection is nuclear factor-κB (NF-κB) (40). NF-κB is a transcription factor family that plays important roles in cell growth and survival. The prototypical form of NF-κB consists of two subunits, p50 and RelA (RelA) (7). NF-κB is retained in the cytoplasm in a nonactivated state by the IκB family of inhibitor proteins. Following signal-dependent phosphorylation on two amino-terminal serine residues, IκB is ubiquitinated and targeted for degradation via the proteasome (7, 132). NF-κB is released from IκB, which exposes a nuclear localization signal on NF-κB and results in its translocation to the nucleus (17). Agonists that stimulate a transient pattern of NF-κB activity, such as TNF-α, promote the selective degradation of IκBα (16). In contrast, agents that elicit a prolonged NF-κB response, including bacterial lipopolysaccharide and IL-1, are associated with degradation of both IκBα and IκBβ (189). There exist two cytokine-inducible IκB kinases (IKKs), termed IKKα and IKKβ, that phosphorylate IκBα and IκBβ at the appropriate regulatory serines (104, 203). IKKα and IKKβ are components of a large (~ 500-900 kDa), multisubunit complex that contains a regulatory subunit called IKKγ (51, 115, 151, 223, 226). The IKK catalytic subunits are activated following stimulation with several NF-κB-inducing agonists, including TNF-α and IL-1 (51, 115, 226).

Two principal signaling pathways are known to activate NF-κB via the IKK complex. The classical NF-κB pathway is stimulated in response to cytokines, LPS, dsRNA, and most negative-stranded RNA viruses. This pathway is dependent on IKKβ and IKKγ for phosphorylation and subsequent degradation of IκBα and nuclear translocation of the p50 and RelA (RelA) subunits of NF-κB (21, 92, 156, 184, 194, 223). The alternative pathway is

dependent on the activation of NF-κB-inducing kinase (NIK), which in turn leads to

phosphorylation of IKKα (220). Activated IKKα phosphorylates the IκB-like molecule,

p100, which is processed to form the NF-κB subunit, p52 (163). The p52 subunit binds to

RelB, translocates to the nucleus, and activates NF-κB-dependent gene expression (179).

The alternative pathway of IKK activation is thought to operate independently of both IKKβ

and IKKγ (47).

NF-κB activation can either potentiate (1, 62, 82) or inhibit apoptosis (15, 100, 202)

depending on the nature of the NF-κB agonist. Many RNA viruses are capable of activating

NF-κB, including dengue virus (77), influenza virus (131), reovirus (40), respiratory

syncytial virus (108), and Sindbis virus (94). Interestingly, activation of NF-κB is required

for the induction of apoptosis by some viruses, including dengue virus (105), reovirus (40),

and Sindbis virus (94).

Reovirus activates NF-κB in cell culture beginning at 2-4 h post-infection and

reaches maximal levels of activation at 6-8 h post-infection (Fig. 4) (40). Electrophoretic

mobility shift assays using antibodies specific for p50 and RelA identified both of these

subunits in the NF-κB complexes activated during reovirus infection. Cells devoid of either

p50 or RelA do not activate NF-κB following reovirus infection (40), providing further

evidence that these NF-κB subunits are in the complexes activated by reovirus. Three lines

of evidence suggest that NF-κB activation is required by reovirus to induce apoptosis (40).

First, treatment of HeLa cells with a proteasome inhibitor blocks NF-κB activation following

reovirus infection and substantially diminishes reovirus-induced apoptosis. Second, transient

expression of a dominant-negative form of IκB that constitutively represses NF-κB activation

significantly reduces levels of apoptosis induced by reovirus. Third, and most convincingly,
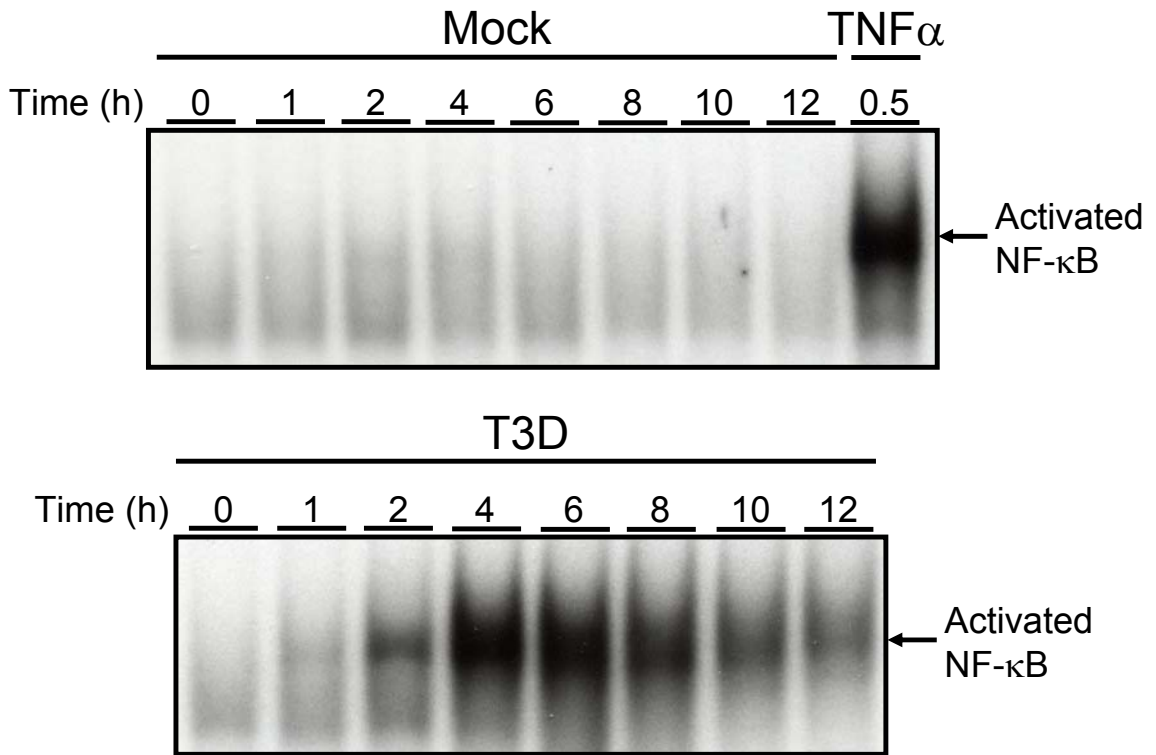
Figure 4. NF-κB activation in response to reovirus. Nuclear extracts were prepared from uninfected HeLa cells (0 h), mock-infected cells (Mock), or cells infected with T3D at an MOI of 100 PFU/cell for the times shown. Cells also were treated with 20 ng/ml of TNFα for 30 min as a positive control. Extracts were incubated with a radiolabeled NF-κB consensus oligonucleotide, and resulting protein-oligonucleotide complexes were resolved by acrylamide gel electrophoresis, dried, and exposed to film. NF-κB-containing complexes are indicated.

apoptosis by reovirus is substantially diminished in mouse embryo fibroblasts lacking either of the NF-κB subunits p50 or RelA. These findings indicate that NF-κB plays an essential role in the mechanism by which reovirus induces apoptosis of host cells.

Significance of the research

Many viruses are capable of inducing apoptotic cell death. However, mechanisms of virus-induced apoptosis are not well understood. The main objective of this thesis research is to understand mechanisms by which reovirus activates NF-κB and induces apoptosis. I describe four main findings that help elucidate these mechanisms. First, reovirus can induce apoptosis in cells lacking JAM-A using a mechanism dependent on the μ1 protein. Second, IKKα and IKKγ are required for reovirus-induced NF-κB activation and apoptosis indicating that reovirus activates a novel NF-κB pathway resulting in cell death. Third, NF-κB functions in a proapoptotic manner in the brain while playing an antiapoptotic function in the heart of mice infected with reovirus. Fourth, IFN-β inhibits reovirus-induced apoptosis by reducing reovirus replication in cardiac myocytes. Understanding mechanisms of NF-κB activation may result in the development of novel pharmacologic methods to combat disease caused by pathological activation of NF-κB. Moreover, data from this research will generate new knowledge about how viruses activate NF-κB and induce apoptosis. Such knowledge may foster development of new antiviral therapeutics that mediate apoptosis blockade.

CHAPTER II

JAM-A-INDEPENDENT, ANTIBODY-MEDIATED UPTAKE OF REOVIRUS INTO
CELLS LEADS TO APOPTOSIS

Introduction

Reovirus infection is initiated by the attachment of virions to cell-surface receptors

via the σ1 protein (89, 209). The σ1 protein engages both JAM-A (11, 24, 57, 137) and cell-

surface carbohydrate (2, 49, 133, 134) to initiate viral entry. Anti-JAM-A mAb J10.4, which

inhibits reovirus binding to JAM-A, also blocks reovirus-induced apoptosis (11), indicating

that JAM-A binding is essential for initiating the apoptotic process. The JAM-A cytoplasmic

tail is approximately 45 amino acids in length, contains 13 potential phosphorylation sites,

and interacts with several PDZ domain-containing proteins, suggesting a role in ligand-

induced cell signaling (14, 54). Inhibition of binding to sialic acid by treatment with

neuraminidase also diminishes the apoptotic response elicited by reovirus (38). Thus,

binding to both JAM-A and sialic acid is required for maximum levels of apoptosis following

reovirus infection.

In addition to receptor binding, post-attachment events are required for the induction

of apoptosis by reovirus (39). Inhibition of viral disassembly using ammonium chloride, a

weak base that increases vacuolar pH (110) or E64, an inhibitor of cysteine proteases

such as those contained in the endocytic compartment (9), abolishes reovirus-induced

apoptosis. On the other hand, interference with steps in viral replication subsequent to

ISVP formation and membrane penetration using ribavirin, an inhibitor of RNA synthesis

(139), does not perturb apoptosis induced by reovirus (39). Thus, in addition to sialic

acid- and JAM-A-mediated attachment of reovirus to cells, replication steps during or

after viral disassembly that occur before the cytoplasmically delivered core becomes transcriptionally active, also contribute to reovirus-induced apoptosis.

In keeping with studies of inhibitors of viral receptor binding, analysis of T1L x T3D reassortant viruses indicates that the σ1-encoding S1 gene is the primary genetic determinant of differences in the capacity of these strains to induce apoptosis in numerous cell types (145, 199, 200). The µ1-encoding M2 gene also has been implicated in the apoptotic response induced by reovirus (199, 200). Since the µ1 protein functions in virus-induced endosomal membrane penetration following disassembly but prior to viral RNA synthesis (93, 121, 127), the deleterious effects of reovirus disassembly inhibitors on apoptosis induction suggest a functional link between the M2 gene segment and differences in the efficiency of apoptosis exhibited by different reovirus strains (145, 199, 200).

In this study, we determined whether reovirus is capable of inducing apoptosis independent of JAM-A and sialic acid binding. We found that antibody-mediated uptake of reovirus into JAM-A-negative, Fc-receptor-expressing cells results in productive infection and leads to apoptosis in a σ1-independent fashion. Moreover, apoptosis induced following this uptake pathway also is dependent on viral disassembly. Analysis of reassortant viruses and an M2 mutant virus demonstrates that the µ1 protein influences the strength of proapoptotic signaling following reovirus infection. These data suggest that signaling induced as a result of σ1 interactions with JAM-A and sialic acid are not necessary for apoptosis induced by reovirus and that the µ1 protein is the viral factor that stimulates the cellular apoptotic machinery.

Results

A JAM-A truncation mutant lacking the cytoplasmic tail is expressed at the cell surface

To determine whether the cytoplasmic tail of JAM-A contributes to reovirus-induced apoptosis by evoking proapoptotic signaling events, chinese hamster ovary (CHO) cells were stably transfected with empty vector or vector encoding full-length JAM-A or a C-terminally truncated form of JAM-A that lacks the cytoplasmic tail (JAM-AΔCT). CHO cells were selected since they are poorly permissive to reovirus infection (57); yields of reovirus following infection of CHO cells are 100- to 1000- fold lower in the absence of ectopic expression of JAM-A {Forrest, 2003 #4656; Campbell, 2005 #5059}. Whole-cell extracts from stably expressing cells were analyzed for expression of JAM-A by immunoblotting (Fig. 5A). While no JAM-A-specific band was detected in the vector-transfected cells, both full-length JAM-A and the faster migrating JAM-AΔCT proteins were expressed to high levels in the cell lines tested. The surface expression of both JAM-A and JAM-AΔCT was assessed by flow cytometry using JAM-A-specific mAb J10.4 (Fig. 5B). Both wild-type and mutant JAM-A proteins displayed approximately equivalent surface expression, suggesting that removal of the C-terminal domain of JAM-A does not prevent transport of JAM-A to the cell surface. These stably transfected cells are therefore suitable for analysis of infection and apoptosis induction by reovirus.

Reovirus induces equivalent levels of apoptosis in CHO cells that express JAM-A and JAM-AΔCT

To determine whether CHO cells stably expressing JAM-AΔCT can support reovirus infection, cells were adsorbed with reovirus strain T3D at an MOI of 10 PFU per cell, and viral infectivity was assessed by using indirect immunofluorescence (Fig. 6A). CHO cells

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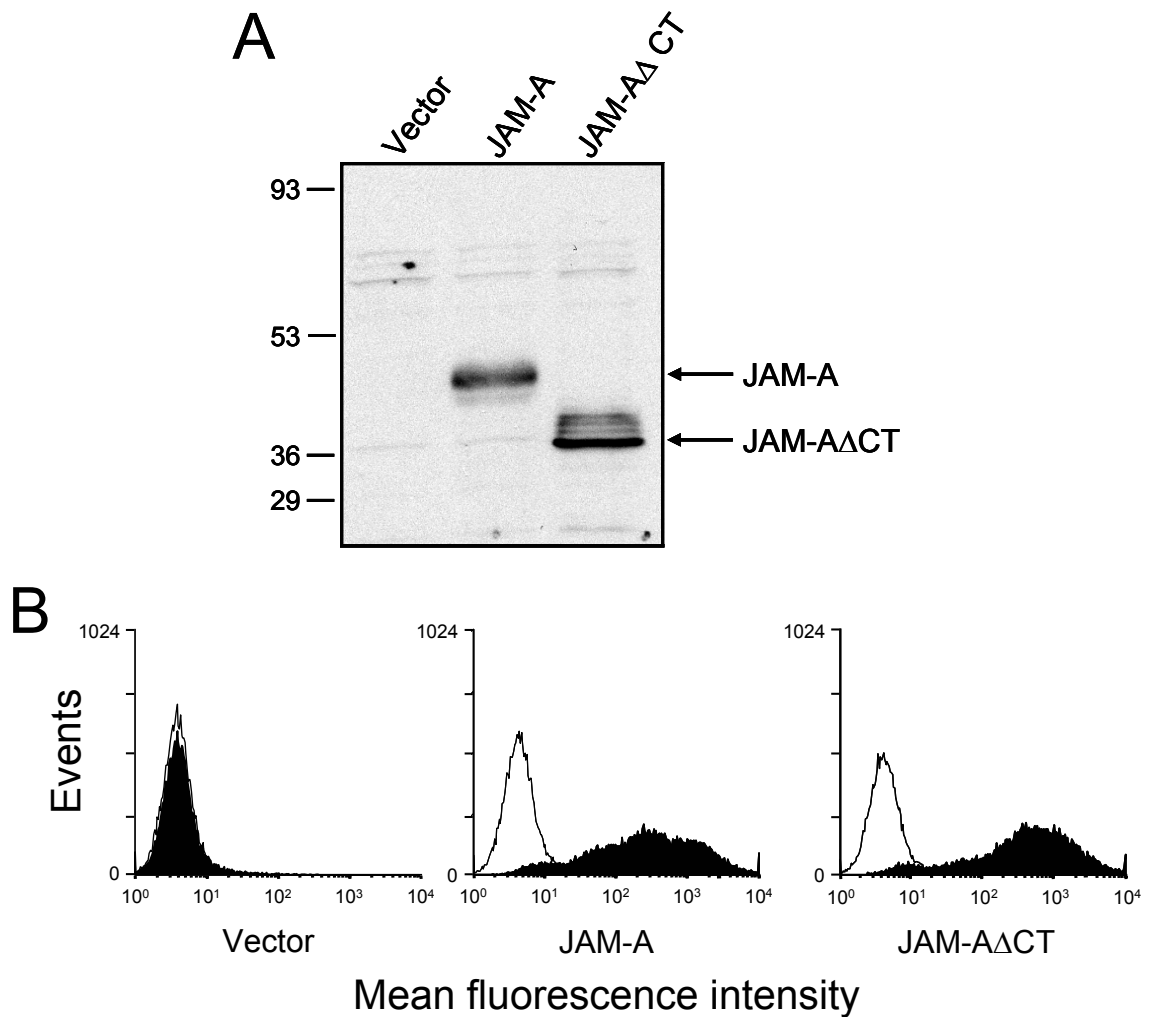
Figure 5. Stable expression of JAM-A and JAM-AΔCT in CHO cells. (A) Whole-cell lysates (1 x 10^5 cell equivalents) were prepared from CHO cells stably transfected with empty vector, JAM-A, or JAM-AΔCT, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anti-JAM-A mAb J10.4. The positions of full-length JAM-A and truncated JAM-AΔCT are shown on the right. The positions of molecular weight standards (in kilodaltons) are shown on the top. (B) Stably transfected CHO cells (1 x 10^6) were incubated with either anti-JAM mAb J10.4 (filled histograms) or an isotype-matched control mAb (open histograms) at 10 μg/ml, followed by incubation with PE-labeled anti-mouse Ig secondary antibody. The results are presented as mean fluorescence intensity.

transfected with either empty vector or those engineered to stably express full-length JAM-A were used as controls. In contrast to vector-transfected cells, cells expressing either JAM-A or JAM-AΔCT were equivalently capable of supporting infection by T3D. These data indicate that although JAM-A expression is required for efficient infection of CHO cells, the JAM-A cytoplasmic tail is dispensable.

To determine whether the JAM-A cytoplasmic tail is required for apoptosis, stably transfected CHO cell lines were adsorbed with T3D at an MOI of 100 PFU per cell, and apoptosis was assessed by using AO staining (Fig. 6B). None of the cell lines tested showed significant apoptosis following mock infection (< 5%). T3D infection of vector-transfected cells induced levels of apoptosis equivalent to those following mock infection of cells. In contrast, T3D infection of either the JAM-A- or JAM-AΔCT-expressing cell lines induced an equivalent percentage of cells to undergo apoptosis (~ 22%). Therefore, analogous to our findings in the infectivity assays, the cytoplasmic tail of JAM-A is not required for reovirus-induced apoptosis.

Antibody-mediated uptake of reovirus into Fc receptor-expressing cells leads to infection and apoptosis

To determine whether the requirement for JAM-A during reovirus infection and apoptosis can be bypassed, we utilized antibody-mediated infection of Fc receptor-expressing CHO (CHO-B1) cells. These cells stably express the B1 isoform of the mouse Fc receptor II (80). Therefore, in the absence of JAM-A, these cells should allow internalization of antibody-reovirus complexes into cells via Fc receptors, resulting in efficient infection of normally non-permissive cells. For these experiments, T3D virions were incubated with increasing concentrations of σ1-specific, neutralizing mAb 9BG5 (23) prior to infection of CHO-B1 cells. HeLa cells were used in parallel to confirm the neutralizing efficacy of mAb

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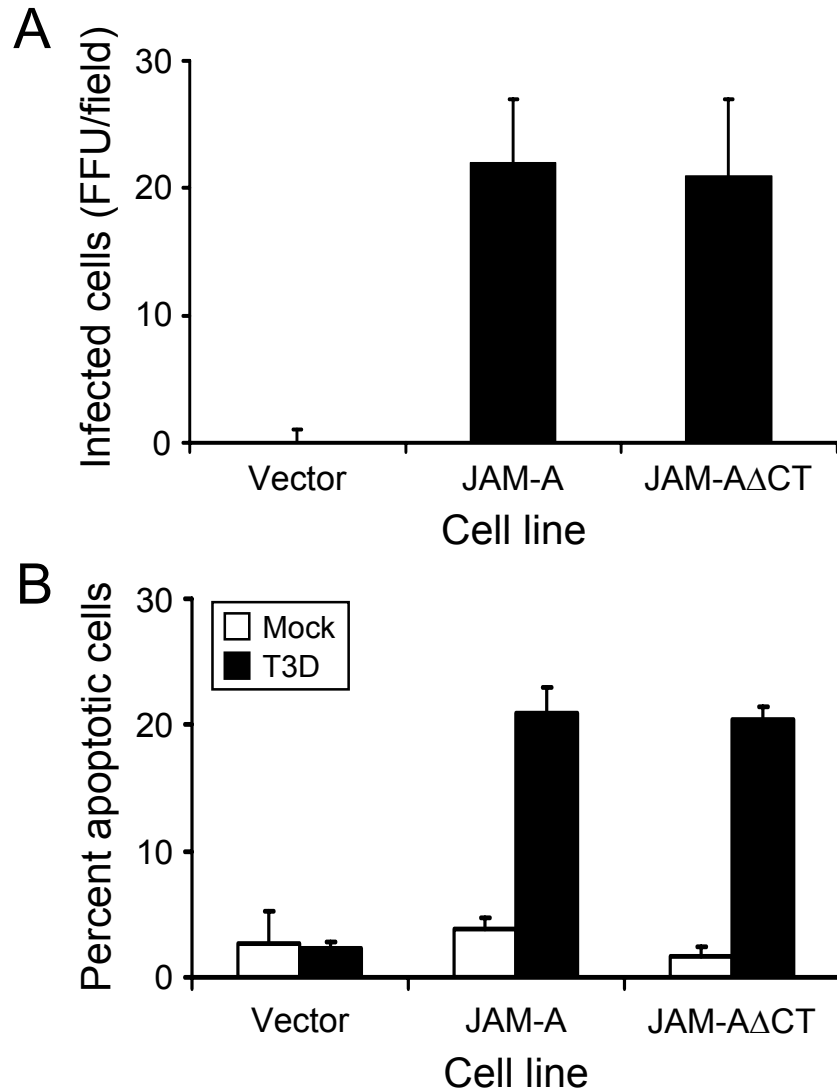
Figure 6. Infection and apoptosis of JAM-A- and JAM-AΔCT-expressing CHO cells. (A) Cells were adsorbed with T3D at an MOI of 10 PFU/cell. After incubation at 37°C for 18 h, cells were fixed using methanol. Infected cells were visualized by immunostaining with polyclonal rabbit anti-reovirus sera, followed by incubation with Alexa546-labeled anti-rabbit IgG. Reovirus-infected cells were quantified by counting fluorescent cells. The results are presented as mean fluorescent focus units (FFU)/field. Error bars indicate standard deviations. (B) Cells were adsorbed with either PBS (mock) or T3D at an MOI of 100 PFU/cell. Cells were harvested at 48 h after infection and stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations.

9BG5 at the concentrations tested. For each cell line, the number of infected cells was assessed 18 h post-infection by indirect immunofluorescence. As anticipated, the efficiency of reovirus infection of HeLa cells decreased in proportion to antibody concentration with little infection detected in the presence of 2.5 μg per ml of 9BG5 (Fig. 7A). We conclude that 9BG5 interferes with σ1-JAM-A interactions, which are critical for reovirus infection of HeLa cells. In contrast, the efficiency of infection of CHO-B1 cells by T3D increased in proportion to 9BG5 concentration, with maximal infection observed in the presence of a completely neutralizing concentration of 9BG5, 2.5 μg per ml (Fig. 7A). These findings demonstrate that reovirus infection can be established in a JAM-A-independent manner if an alternative high-affinity binding moiety is provided. These data corroborate a previous report of antibody-mediated enhancement of reovirus infection of a murine macrophage-like cell line (22).

To determine whether infection initiated in a JAM-A-independent manner also triggers apoptosis, the capacity of reovirus-9BG5 complexes to induce apoptotic cell death of both HeLa cells and CHO-B1 cells was assessed by using AO staining (Fig. 7B). Approximately 35% of HeLa cells showed apoptotic nuclei at 48 h post-infection in the absence of antibody treatment of virions. Consistent with the decrease in the capacity of reovirus to infect HeLa cells in the presence of 9BG5, the percentage of cells undergoing apoptosis also decreased with increasing 9BG5 concentrations. However, in the absence of 9BG5, T3D induced minimal apoptosis in CHO-B1 cells in comparison to mock-infected cells. In concordance with the infectivity data, the percentage of apoptotic CHO-B1 cells increased with increasing concentrations of 9BG5, with maximal apoptosis seen following pretreatment with 2.5 μg per ml mAb (~ 25%). These findings demonstrate that reovirus infection initiated in the absence of JAM-A binding also leads to apoptosis. Therefore σ1-

JAM-A interactions or signaling pathways induced as a consequence of these interactions are dispensable for apoptosis induction by reovirus.

To exclude the possibility that binding of mAb to Fc receptor contributes to apoptosis induction, we tested whether incubation of reovirus with irrelevant anti-Myc mAb 9E10 was capable of inducing apoptosis (Fig. 7C). While incubation of T3D with two different mAbs directed against the reovirus outer-capsid, 7F4 (λ2) and 9BG5 (σ1), induced 21% and 22% apoptosis, respectively following infection of CHO-B1 cells, incubation of T3D with mAb 9E10 failed to induce apoptosis at levels over mock-infected cells. These findings suggest that only antibodies directed against reovirus outer-capsid proteins allow virus attachment to CHO-B1 cells and subsequent induction of apoptosis. To exclude the involvement of signaling induced as a result of Fc receptor crosslinking due to binding of reovirus-antibody complexes, Fc receptors were crosslinked using 2.4G2 (a rat anti-Fc receptor mAb) and an IgM antibody directed against rat IgG. This treatment did not induce apoptosis in CHO-B1 cells in comparison to mock-treated cells. As an additional control for the effect of crosslinking Fc receptors, T1L core particles, which lack outer-capsid proteins σ1, σ3, and μ1, were incubated with mAb 7F4 and added to CHO-B1 cells. Neither untreated cores nor 7F4-core complexes induced apoptosis of these cells. These data suggest that apoptosis does not result from proapoptotic signaling induced as a consequence of Fc-receptor crosslinking but requires binding of reovirus particles containing outer-capsid proteins.

Viral disassembly is required for apoptosis-induced by Fc-mediated uptake of reovirus

To determine whether viral disassembly in cellular endosomes is required for apoptosis following Fc-mediated delivery of reovirus, CHO-B1 cells were treated with AC prior to infection by mAb-treated T3D virions. Treatment of cells with 20 mM AC, a

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Figure 7. Infection and apoptosis of HeLa cells and CHO-B1 cells in the presence of mAb 9BG5. (A) Reovirus particles were incubated overnight with the indicated concentration of mAb 9BG5 and adsorbed to either HeLa cells or CHO-B1 cells at an MOI of 100 PFU/cell. After incubation at 37°C for 18 h, cells were fixed using methanol. Infected cells were visualized by immunostaining with polyclonal rabbit anti-reovirus sera, followed by Alexa546-labeled anti-rabbit IgG. Reovirus-infected cells were quantified by counting fluorescent cells. The results are presented as mean fluorescent focus units (FFU)/field. Error bars indicate standard deviations. (B) HeLa cells or CHO-B1 cells were adsorbed with 100 PFU/cell of either virus or virus-antibody complex and harvested at 48 h after infection and stained with AO. (C) CHO-B1 cells were mock-infected, infected with T3D virions with or without 2.5 μg per ml of Myc-specific mAb 9E10 (antibody control), σ1-specific mAb 9BG5, or λ2-specific mAb 7F4 at an MOI of 100 PFU/cell, or infected with T1L core particles with or without 2.5 μg per ml of mAb 7F4 at an MOI of 10^4 particles/cell. CHO-B1 cells also were incubated with 1 μg/ml anti-Fc receptor rat IgG mAb 2.4G2, followed by incubation with 5 μg/ml of IgM specific for rat IgG (anti-FcR). Cells were harvested at 48 h after infection and stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to T3D incubated with control mAb 9E10.

concentration sufficient to block reovirus disassembly (183, 213), abolished the capacity of

T3D to induce apoptosis (Fig. 8A). Thus, acid-dependent proteolytic disassembly is required

for apoptosis induction by this uptake mechanism. To determine whether the apoptosis-

inhibitory effect of AC is due to blockade of viral RNA synthesis, we tested ribavirin, an

inhibitor of viral RNA synthesis (139), for the capacity to diminish apoptosis. In keeping

with our previously published results (39), apoptosis induced by reovirus infection via Fc-

mediated uptake was unaffected by ribavirin (Fig. 8A). To corroborate these results, we

tested the apoptosis inducing capacity of UV-inactivated reovirus virions, which are

incapable of establishing productive infection (166). We found that UV- inactivated reovirus

is capable of inducing apoptosis following Fc receptor-mediated uptake of a high MOI of

virus (Fig. 8B). These findings are consistent with our previously published observations

(199). Collectively, these results demonstrate that steps in reovirus replication cycle that

occur after attachment but before transcription are required for virus-induced apoptosis,

regardless of the type of receptor used to initiate infection (39). In addition, these results

suggest that death signaling during reovirus infection may occur independently of receptor

engagement.

Fc receptor-dependent infection abolishes σ1-related differences in the apoptosis-inducing capacity of reovirus

Differences in the capacity of some reovirus strains to induce apoptosis are linked to

differences in affinity for sialic acid (38). To determine whether reovirus strains that are

incapable of binding to sialic acid can induce apoptosis when infection is initiated via Fc-

dependent uptake, strains T1L and T3SA-, neither of which is capable of binding to sialic

acid (10, 28, 49), were incubated with σ1-specific antibodies and adsorbed to CHO-B1 cells.

Incubation of T1L virions with type 1 σ1-specific mAb 5C6 (206) and incubation of T3SA-
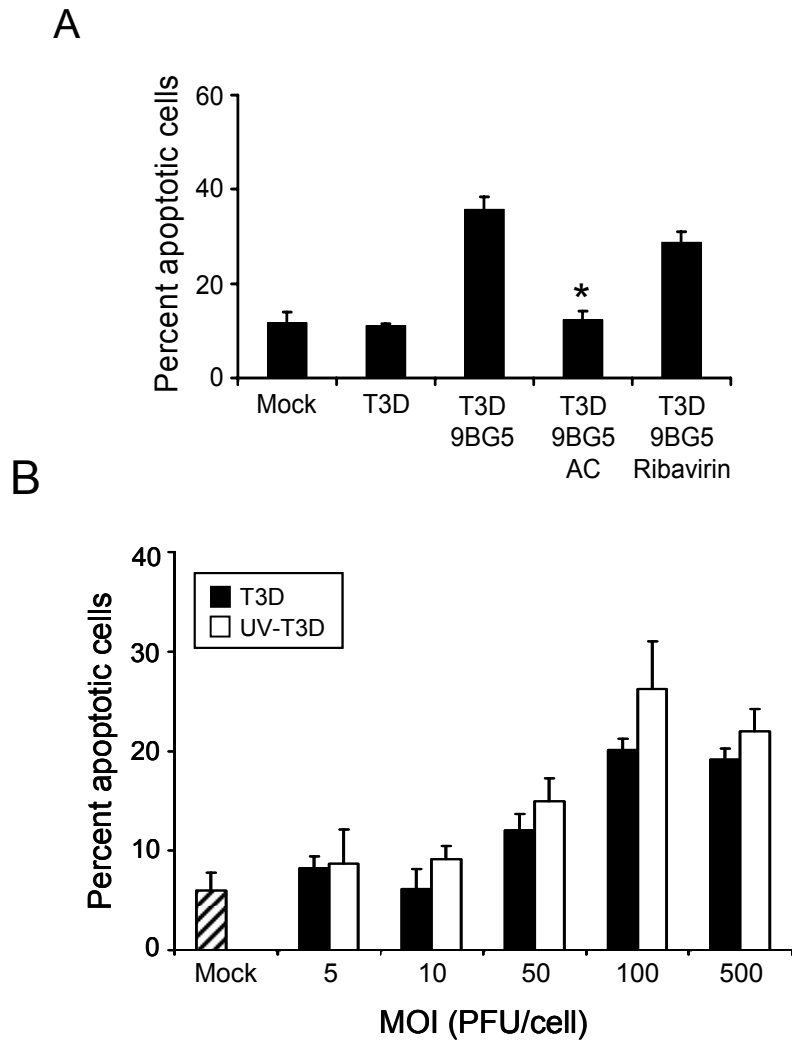
Figure 8. Effect of inhibitors of viral replication on apoptosis induced by reovirus. T3D alone or preincubated with 2.5 μg/ml of mAb 9BG5 was adsorbed to CHO-B1 cells at an MOI of 500 PFU/cell. After incubation at 37°C for 48 h in untreated medium or medium containing either 20 mM AC or 200 μM ribavirin, cells were stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to T3D incubated with mAb 9BG5.

virions with type 3 σ1-specific 9BG5 (23) resulted in significantly higher levels of apoptosis in CHO-B1 cells in comparison to levels observed following infection with T1L and T3SA-virions in the absence of antibody treatment (Fig. 9). The percentage of cells undergoing apoptosis following antibody-mediated infection of non-sialic-acid-binding reovirus strains was at least equal to that observed following infection with T3D. Therefore, σ1-related differences in apoptosis efficiency are overcome when infection is initiated via Fc-mediated uptake. These results make it unlikely that the σ1 protein is the viral effector of apoptosis induction.

Differences in apoptosis induction following Fc receptor-dependent infection of T1L x T3D reassortant viruses are linked to the M2 gene segment

We have demonstrated previously that the viral S1 and M2 gene segments segregate with differences in the apoptosis-inducing capacity of T1L and T3D (145, 199). To ascertain whether any strain-specific differences exist when the effect of the S1 gene segment is circumvented by Fc receptor-dependent uptake, CHO-B1 cells were adsorbed with T1L x T3D reassortant viruses after incubation with σ1-specific antibodies. Type 1 and type 3 strains were incubated with σ1-specific mAbs 5C6 and 9BG5, respectively, prior to infection. Each of the reassortant viruses tested produced an approximately similar number of infected cells as judged by indirect immunofluorescence (data not shown), suggesting equivalent efficiency of antibody-mediated uptake and infection. The percentage of cells undergoing apoptosis as a result of antibody-mediated infection of cells was assessed at 48 h after infection. The reassortant viruses were ranked from highest to lowest by apoptosis-inducing capacity (Table 1). Although the reassortants did not cluster distinctly into two groups with high and low apoptotic potential, six of the seven strains with the highest levels of apoptosis had an M2 gene segment derived from T3D. Conversely, seven of the eight strains with the

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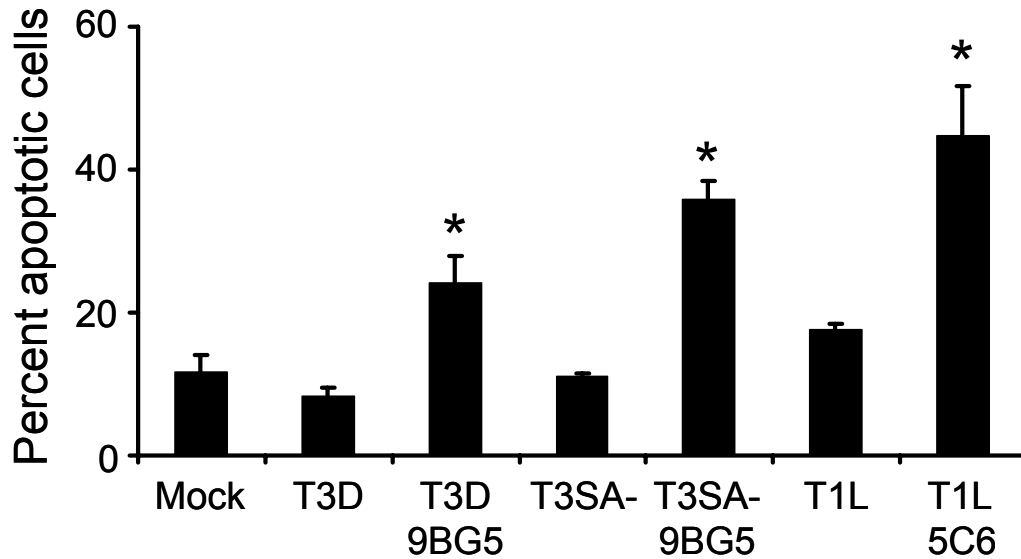
Figure 9. Apoptosis induction in CHO-B1 cells following Fc-mediated infection by T1L and T3SA-. Cells were adsorbed with T3D, T3SA-, or T1L with or without 2.5 μg per ml of mAb 9BG5 (for type 3 strains) or mAb 5C6 (for T1L) at an MOI of 100 PFU/cell. Following infection at 37°C for 48 h, cells were harvested and stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations.

lowest levels of apoptosis had an M2 gene segment derived from T1L. Analysis of the data using the Mann-Whitney test showed that only the M2 gene segregated at a statistically significant level with the capacity of these strains to induce apoptosis ($P = 0.02$). There was not a statistically significant association between apoptosis and the S1 gene ($P = 0.28$), which is the primary apoptosis determinant following infection of JAM-A-expressing cells, when infection was initiated in an Fc-dependent fashion (38, 145, 199, 200). These data suggest that the M2-encoding μ1 protein, which functions in penetration of cell membranes (93, 121, 127), is the primary viral determinant of strain-specific differences in apoptosis induction following infection by Fc-mediated uptake.

Reovirus mutant tsA279 is inefficient in apoptosis induction

To determine whether a mutant virus with a defective μ1 protein is altered in the capacity to trigger apoptosis, we used reovirus strain tsA279.64, which contains a temperature-sensitive mutation that maps to the M2 gene segment (67). This virus was derived from a coinfection of T1L and tsA279, which contains temperature-sensitive mutations in both the M2 and L2 gene segments. The tsA279.64 virus contains the mutant M2 gene segment but not the mutant L2 gene segment, thus facilitating analysis of the contribution of the M2 gene segment to apoptosis induction. When assembled at non-permissive temperature, virions containing the mutant M2 gene segment cannot penetrate membranes due to a misfolded μ1 protein (67). To examine whether μ1-mediated membrane penetration is required for apoptosis induction, HeLa cells were adsorbed with increasing MOIs of tsA279.64 virions assembled under permissive and non-permissive conditions. The percentage of cells with apoptotic nuclei were assessed by using AO staining 48 h after infection at non-permissive temperature (Fig. 10). At all MOIs tested, particles assembled at

Table 1. Apoptosis induction by T1L x T3D reassortant viruses in CHO-B1 cells

Virus strain	Origin of gene segments [a]										% Apoptosis [b]
	L1	L2	L3	M1	**M2**	M3	S1	S2	S3	S4	
EB138	D	L	L	D	**D**	L	D	D	L	L	80.52
KC150	D	L	L	L	**D**	L	D	D	L	D	62.86
EB97	D	D	L	D	**D**	D	D	D	D	L	52.31
EB68	L	D	L	L	**D**	L	L	L	D	D	41.29
1HA.3	L	L	L	L	**L**	L	D	L	L	L	40.63
EB144	L	L	L	L	**D**	D	L	L	D	L	38.27
KC9	D	D	L	D	**D**	D	L	D	D	D	35.06
EB98	L	D	L	L	**L**	L	L	D	L	D	32.62
EB121	D	D	L	D	**L**	D	L	D	D	D	28.94
EB120	D	D	D	L	**L**	D	D	D	L	L	26.69
G16	L	L	L	D	**L**	L	L	D	L	L	24.46
G2	L	D	L	L	**L**	L	D	L	L	L	23.14
EB143	D	L	L	L	**L**	L	D	L	L	L	23.04
EB145	D	D	D	D	**D**	L	L	D	D	D	16.28
EB113	L	L	L	D	**L**	L	L	L	D	L	14.54

[a] Parental origin of each gene segment: L, gene segment derived from T1L; D, gene segment derived from T3D.

[b] CHO-B1 cells (2×10^5) were adsorbed with virus strains at an MOI of 100 PFU per cell. After 1 h, the inoculum was removed, fresh medium was added, and cells were incubated at 37°C for 48 h and stained with AO to assess apoptosis. Shown are the mean percentage of cells undergoing apoptosis for three independent experiments. The M2 gene was the only gene associated with the efficiency of apoptosis as determined by using the non-parametric Mann-Whitney test, without adjusting for multiple comparisons ($P = 0.02$).

Figure 10. Apoptosis induced by μ1 temperature-sensitive mutant tsA279.64. HeLa cells were adsorbed with tsA279.64 grown at permissive or non-permissive temperatures at the MOIs shown. Following infection at 37°C for 48 h, cells were harvested and stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to virions grown at permissive temperature at an equivalent MOI.viations.

non-permissive temperature induced less apoptosis than particles assembled at permissive temperature. These data demonstrate that virions containing a μ1 protein that is inefficient in membrane penetration are less potent inducers of apoptosis, which further highlights a key role for the μ1 protein in apoptosis induction.

Discussion

We have previously shown that binding of reovirus to JAM-A and sialic acid is required for efficient induction of apoptosis (11, 38). In addition, we have reported that viral disassembly in cellular endosomes is also necessary for apoptosis induction by reovirus (39). However, since receptor binding is prerequisite for virus disassembly, these findings do not resolve the question of whether reovirus attachment and disassembly provide two distinct signals, both of which are required for apoptosis induction, or whether the viral disassembly events are sufficient for proapoptotic signaling. To address this question, we uncoupled reovirus attachment to JAM-A and sialic acid from viral disassembly by providing an alternative means of viral entry. We report here that antibody-mediated uptake of reovirus into Fc receptor-expressing CHO cells independent of binding to JAM-A and sialic acid leads to productive infection (22) and apoptosis. Furthermore, we demonstrate that antibody-directed binding of reovirus to Fc receptors expressed on CHO cells is not sufficient for reovirus-induced apoptosis. Analogous to JAM-A- and sialic acid-dependent infection, viral replication steps during or after disassembly in endosomes but prior to RNA synthesis also are required for reovirus-induced apoptosis when infection is initiated by Fc-mediated uptake. Analysis of apoptosis induction by T1L x T3D reassortant viruses following Fc-mediated uptake showed that differences in the efficiency of apoptosis exhibited by type 1 and type 3 strains segregates with the μ1-encoding M2 gene segment. Neither core particles

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that lack the µ1 protein and are therefore incapable of penetrating endosomal membranes, nor

the thermosensitive M2 mutant virus tsA279, which contains a misfolded, penetration-

defective µ1 protein, can efficiently induce apoptosis. These findings suggest that reovirus

membrane-penetration protein µ1 induces proapoptotic signaling events during or after

endosomal membrane penetration.

Our reassortant analysis indicated that only the viral M2 gene segment segregated

statistically significantly with the capacity to induce apoptosis. Although the apoptosis-

inducing capacities of T3D M2 containing reassortants were generally higher than those of

T1L M2 containing viruses, the percentage of cells undergoing apoptosis formed a

continuum rather than two distinct clusters. We think that since apoptosis is induced during

or after membrane penetration mediated by the µ1 protein, steps in the viral replication cycle

preceeding these m1 events, such as viral attachment, internalization and disassembly, also

are likely to affect the magnitude of apoptosis. Thus, viral genes that affect these steps also

might influence the apoptotic response. Minor strain-specific effects of other viral gene

segments may also explain why we observe that Fc-mediated uptake of T1L induces

significantly greater apoptosis that T3D (Fig. 5 and parents in Table 1, Student's t test, p

<0.05). Since we did not find any linkage between the S1 gene and apoptotic potential, we

do not think that the differences in the efficiencies of the two σ1 antibodies utilized for this

analysis contribute to these results. An alternative explanation to account for the unusually

high and low apoptosis inducing capacities of the reassortants may be the presence of

mutations in their M2 gene that alter the capacity of the virus to induce apoptosis. Thus,

although multiple reovirus genes contribute to the capacity of reovirus to induce apoptosis,

based on our statistical analysis of reassortant viruses as well as analysis of an M2 ts mutant,

we conclude that the M2 gene product µ1 is the primary mediator.

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In addition to the findings described in this chapter, three independent lines of evidence support a crucial role for µ1 in reovirus-induced apoptosis. First, differences in apoptosis efficiency displayed by strains T1L and T3D are linked to the µ1-encoding M2 gene (145, 199, 200). Second, studies with pharmacologic inhibitors of reovirus replication place the apoptosis-inducing events subsequent to viral disassembly but prior to RNA synthesis (39), which coincides with µ1-mediated membrane penetration (26, 93, 121, 127). Third, transient transfection of a plasmid encoding T3D µ1 is sufficient to induce apoptosis in CHO cells (36). Interestingly, although plasmid-mediated expression of µ1 neither mimics normal delivery of µ1 via endosomal rupture during viral membrane penetration, nor de novo expression of µ1 in infected cells (since µ1 is always found associated with its protector protein σ3 (168, 169, 190), it leads to the same consequence.

It is not known how the viral disassembly events culminating in µ1-mediated membrane penetration elicit proapoptotic signaling. We envision two possibilities. First, endosomal disruption by µ1 may lead to release of hydrolytic enzymes such as cathepsins, which in turn damage mitochondria and stimulate death signaling (46, 64, 144). Interestingly, mitochondrial injury has been reported as early as 4 h following reovirus adsorption, suggesting the involvement of an early viral replication event (85, 86). It is also possible that release of these enzymes causes apoptosis via their action on death regulators such as Bid (182). Of note, Bid cleavage has been observed during reovirus infection and has been hypothesized to play a role in apoptosis induction (85). Second, fragments of µ1 produced during proteolytic viral disassembly are known to gain access to the cytoplasm (27). These fragments may activate other cellular sensors of viral infection or directly injure mitochondria to induce apoptosis. Concordantly, the µ1 protein localizes to mitochondria during infection or when expressed from plasmids in transfected cells, suggesting a post-

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endosomal site of action. Interestingly, a 30-residue C-terminal fragment of μ1 is sufficient to localize to mitochondria and induce apoptosis in transfected cells (36).

Although our findings point to μ1 as a key viral regulator of proapoptotic signaling, this work does not explain the previously established unequivocal association between the S1-encoded σ1 protein and the efficiency of apoptosis induction by reovirus (145, 199, 200). Strains encoding a σ1 protein capable of binding to JAM-A and sialic acid are the most potent inducers of apoptosis (38). We did not observe efficient infection of CHO-B1 cells in the absence of mAb pretreatment at the MOIs used. Therefore, we think that these cells do not express sufficient quantities of sialic acid or JAM-A on the cell surface to effect productive infection. Thus, infection of these cells appears to be dependent only on the presence of a high-affinity receptor such as the Fc receptor. Since the efficiency of antibody-mediated uptake and delivery of both sialic-acid-binding and non-sialic-acid-binding strains of reovirus via Fc receptors is essentially equivalent in CHO-B1 cells, σ1-related differences are negated. An alternative explanation for our findings is that antibody-mediated attachment of virions to Fc receptors stimulates a signaling cascade in cells that mimics signaling induced as a consequence of σ1 binding to JAM-A and sialic acid and that Fc receptor-mediated signaling acts in concert with the viral disassembly events to elicit apoptosis. However, given the marked differences in functional properties displayed by JAM-A and Fc receptors, this explanation seems less likely. Therefore, our current and previous results suggest that the linkage of σ1 to apoptosis efficiency is not related to σ1-mediated stimulation of the cellular proapoptotic machinery, but rather the capacity of σ1 to efficiently deliver virions into endosomes for disassembly. We hypothesize that as opposed to viral infection, which requires penetration by just one infectious virion, efficient apoptosis induction by reovirus requires endosomal penetration by multiple virions. Since sialic acid

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allows more avid binding of virions to cells (10), we think that this entry route may deliver virions more efficiently into endosomal compartments for uncoating and subsequent membrane penetration, leading to higher levels of apoptosis (38).

This study highlights a new role for the viral membrane-penetration protein μ1 in apoptosis induction. Other viruses such as coronavirus, Sindbis virus, and vaccinia virus also have been reported to require post-attachment cell entry events in endosomes to induce apoptosis (78, 98, 138). However, the viral determinants of apoptosis by these viruses are unknown. Interestingly, entry of Sindbis virus into endosomes induces apoptosis through activation of sphingomyelinases and release of the proapoptotic second messenger, ceramide (76). Although we anticipate that interactions between the enveloped Sindbis virus with endosomes differ from those with μ1, it is possible that ceramide plays a role in reovirus-induced apoptosis. Collectively, these studies point to cellular endosomes as sites from which proapoptotic signaling events are initiated and imply a conserved mechanism by which host cells detect the presence of invading pathogens. Pathogen detection during the entry phase may allow host cells to more efficiently limit the spread of infection by initiating a suicidal response. Alternatively, induction of apoptosis by a virus early in its replication cycle may prevent the development of an inflammatory response, thereby allowing the virus to better evade host defenses.

A fascinating similarity exists between the properties of reovirus protein μ1 and several toxins elaborated by bacteria and viruses. Analogous to the capacity of μ1 to mediate membrane permeabilization (26, 71, 101, 192), α toxin of *Staphylococcus aureus*, lysteriolysin O of *Listeria monocytogenes*, and killer toxins (K1 and K2) of the yeast L-A virus, also can form pores in host cell membranes (107, 191, 212, 215). Interestingly, each of these toxins also has the capacity to induce apoptotic cell death (143, 212). Our ongoing

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studies are focused on understanding the precise mechanism by which µ1 induces apoptosis

during reovirus infection. Through these studies we hope to gain broader insight into events

at the pathogen-host interface that evoke death signaling and cause disease.

CHAPTER III

IκB KINASE SUBUNITS α AND γ ARE REQUIRED FOR ACTIVATION OF NF-κB
AND INDUCTION OF APOPTOSIS BY MAMMALIAN REOVIRUS

Introduction

Transcription factor NF-κB plays an important regulatory role in apoptosis evoked by

reovirus in cultured cells (40) and in vivo (128). Inducible members of the NF-κB family are

sequestered in the cytoplasm by inhibitory IκB proteins, including IκBα, IκBβ, IκBε, and

p100/NF-κB2 (8, 69, 176, 204, 214). In response to a wide variety of NF-κB inducers, IκB

proteins are phosphorylated at specific serine residues, earmarking these molecules for

destruction by the ubiquitin-proteasome pathway (21, 30, 69, 132, 194, 214).

Phosphorylation of IκB proteins is mediated by cytokine-inducible IκB kinases (IKKs) IKKα

and IKKβ (104, 203), which can form higher order complexes containing a regulatory

subunit called IKKγ/Nemo (51, 115, 151, 223, 226). A primary function of IKKβ is to

modulate the inhibitory interaction of IκBα with the prototypical form of NF-κB containing

p50/RelA dimers (48, 51, 115, 141, 177). This regulatory circuit, termed the classical

pathway of NF-κB activation, is strictly dependent on the presence of IKKγ/Nemo (156, 160,

223). In contrast, IKKα functions in an alternative IKKγ-independent pathway of NF-κB

activation that leads to proteolytic processing of p100 and production of a fully functional

p52 Rel subunit (35, 163, 179). Unlike the classical, IKKβ-directed pathway of NF-κB

activation, the alternative pathway involving IKKα is dependent on prior phosphorylation of

this IKK by NF-κB-inducing kinase (NIK) (95, 163, 221).

To better understand the mechanism of NF-κB activation by reovirus, I conducted

experiments to define the NF-κB/Rel, IκB, and IKK proteins that are under reovirus control.

These studies revealed that NF-κB/Rel proteins are mobilized to the nuclear compartment

with biphasic kinetics following reovirus infection. Reovirus-induced activation of NF-

κB/Rel proteins is accompanied by selective degradation of IκBα, suggesting a role for

IKKβ. However, subsequent studies with IKK subunit-deficient cells clearly demonstrate

that IKKα rather than IKKβ plays an essential role in the mechanism by which reovirus

activates NF-κB and downstream apoptotic genes. I also assembled evidence indicating that

the reovirus/IKKα axis is intact in cells lacking NIK, an upstream activator of IKKα, but not

in cells lacking the IKKβ regulatory subunit IKKγ/Nemo. Taken together, these data suggest

that reovirus activates the IKKα pathway of the NF-κB signaling apparatus downstream of

NIK, perhaps via direct interactions with the regulatory subunit IKKγ/Nemo.

Results

Reovirus infection results in the biphasic activation of NF-κB/Rel DNA-binding proteins

In prior studies, we found that reovirus activates the functional expression of

p50/RelA complexes, suggesting the involvement of classical NF-κB signaling (40).

However, it remained unclear whether NF-κB/Rel proteins linked to the alternative pathway

of the NF-κB pathway are activated during reovirus infection. To more completely define

the composition of NF-κB complexes activated by reovirus, we used nuclear extracts from

reovirus-infected HeLa cells and Rel-specific antibodies to monitor the composition of DNA-

binding complexes formed in EMSAs. NF-κB/Rel DNA-binding activity was readily

detected over background levels (mock treatment) within 2 hours after infection with reovirus strain T3D (Fig. 11A), which potently induces apoptosis in cultured cells (40, 145, 199) and the murine CNS (125). Peak levels of NF-κB/Rel DNA-binding activity were observed at 4-8 h post-infection. Supershift analysis of extracts obtained at 4, 6, and 8 h post-infection revealed the presence of DNA/protein complexes containing p50 and RelA, but neither p52 nor RelB (Fig. 11B), suggesting preferential usage of the classical versus alternative NF-κB pathway by reovirus. Complexes containing c-Rel were apparent in supershift assays only after 8 h of infection (Fig. 11B). Thus, reovirus induces a biphasic pattern of NF-κB/Rel activation featuring the initial nuclear translocation of complexes consisting of p50 and RelA, followed by those containing p50, RelA, and c-Rel. Given that the cellular gene encoding c-Rel contains functional NF-κB binding sites (63), this expression pattern may reflect de novo synthesis of c-Rel rather than its mobilization from a latent cytoplasmic pool.

These initial experiments conducted over an 8 h timecourse provided no evidence for the capacity of reovirus to stimulate the nuclear expression of p52, a signature Rel protein involved in the alternative pathway of NF-κB signaling. To further investigate whether reovirus interfaces with the alternative NF-κB pathway, we extended the timecourse of T3D infection to 24 h and monitored extracts for processing of p100 to p52 (163). Levels of p100 were significantly reduced between 16 and 24 h post-infection (Fig. 12). Consistent with a precursor/product relationship, diminution in p100 protein levels were accompanied by a significant increase in the steady-state levels of p52 (Fig. 12, panels A and C). Taken together with the NF-κB/Rel profiling data shown in Fig. 11B, this finding suggests that reovirus engages not only the classical pathway of NF-κB signaling but also the alternative pathway, albeit at much later times of infection.
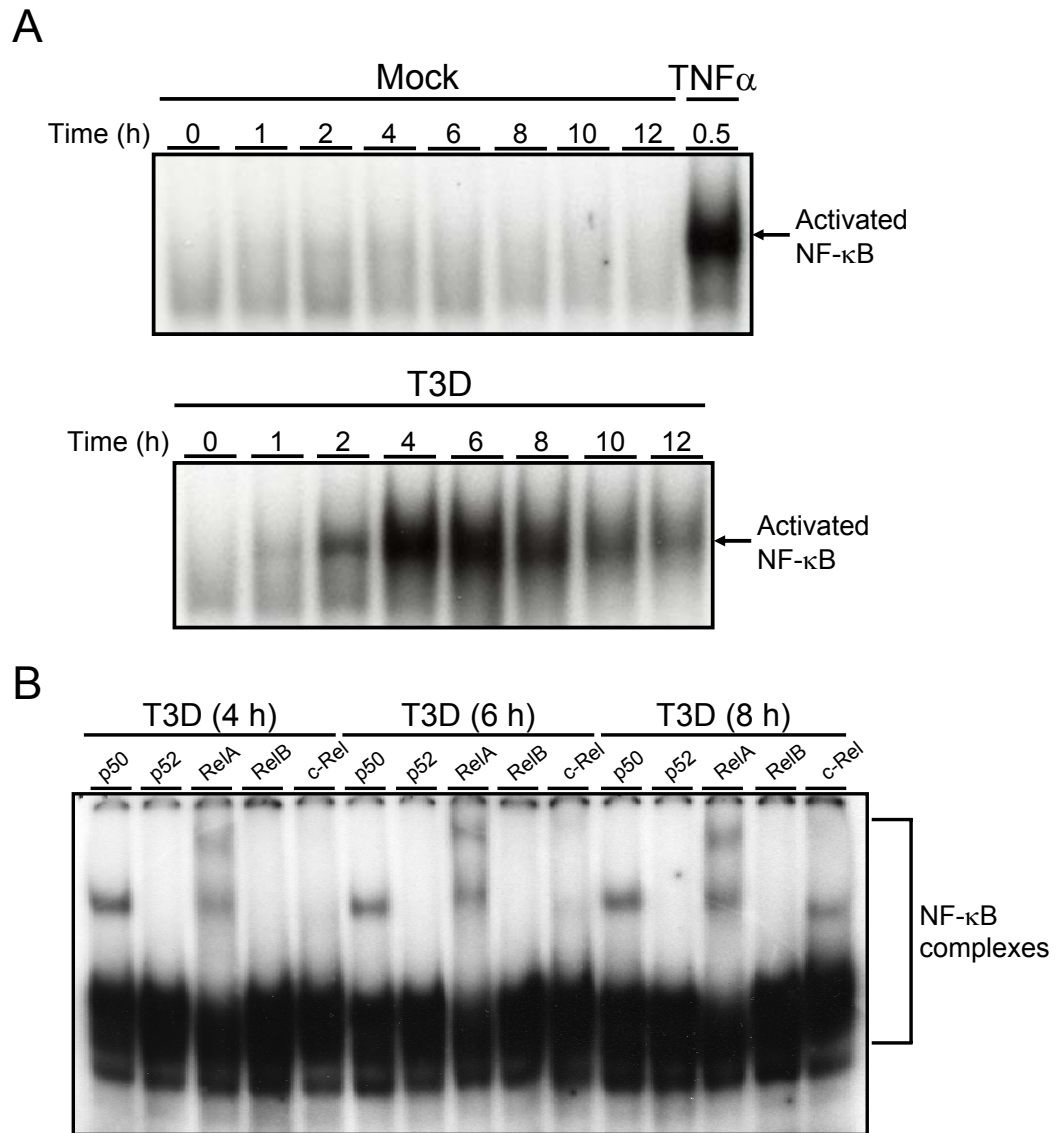
Figure 11. Biphasic activation of NF-κB/Rel proteins in reovirus-infected cells. (A) Nuclear extracts were prepared from uninfected HeLa cells (0 h), mock-infected cells (Mock), or cells infected with T3D at an MOI of 100 PFU/cell for the times shown. Cells also were treated with 20 ng/ml of TNFα for 30 min as a positive control. Extracts were incubated with a radiolabeled NF-κB consensus oligonucleotide, and resulting protein-oligonucleotide complexes were resolved by acrylamide gel electrophoresis, dried, and exposed to film. (B) Nuclear extracts prepared at 4, 6, and 8 h post infection were incubated with antisera specific for p50, p52, RelA, RelB, or c-Rel prior to the addition of a radiolabeled NF-κB consensus oligonucleotide. NF-κB-containing complexes are indicated.

Figure 12. Processing of p100 to p52 during reovirus infection. (A) Whole-cell extracts were prepared from uninfected HeLa cells (0 h), mock-infected cells (Mock), or cells infected with reovirus T3D at an MOI of 100 PFU/cell for the times shown. Cells also were treated with 2 μg/ml of an agonistic lymphotoxin-β receptor antiserum for 8 h as a positive control. Extracts were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted by using an antiserum specific for p100/p52. Band intensity was quantified by using the Image J program. The results are presented as the mean ratio of (B) p100/actin or (C) p52/p100 for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's *t* test in comparison to untreated cells (0 h).

Reovirus infection leads to the selective degradation of IκBα

Activation of the classical NF-κB pathway by physiologic agonists is primarily dependent on degradation of IκBα (reviewed in (19, 59, 66)), an inhibitor that sequesters p50/RelA complexes in the cytoplasmic compartment (8). We have previously shown that degradation-resistant forms of IκBα attenuate reovirus-induced apoptosis, which is critically dependent on NF-κB activation (40). However, mammalian cells express other labile inhibitors that are structurally similar to IκBα, such as IκBβ (96) and IκBε (214). Indeed, prior studies suggest a potential role for signal-dependent degradation of IκBβ (189) and IκBε (214) in the inducible nuclear entry of c-Rel. To determine whether any of these inhibitors is under reovirus control, we monitored their levels in T3D-infected cells in immunoblotting studies using IκB-specific antibodies. The cellular pool of IκBα was significantly reduced within 4 h after infection with T3D (Fig. 13, A and B). In contrast, levels of IκBβ and IκBε were maintained under the same stimulatory conditions over the entire 8 h timecourse (Fig. 13, C-F). We conclude that IκBα is a primary cellular target of reovirus, which is fully consistent with its capacity to stimulate nuclear translocation of NF-κB p50/RelA.

IKKα and IKKγ are required for reovirus-induced NF-κB activation

We next investigated the mechanism by which reovirus destabilizes IκB proteins. Cytokine-induced degradation of NF-κB inhibitors is dependent on their phosphorylation at specific serine residues by IKKs such as IKKα and IKKβ (21, 51, 226). These structurally-related enzymes can interact and form higher-order complexes with other cellular proteins (reviewed in (59, 66)). Integration of the regulatory protein IKKγ/Nemo into such complexes
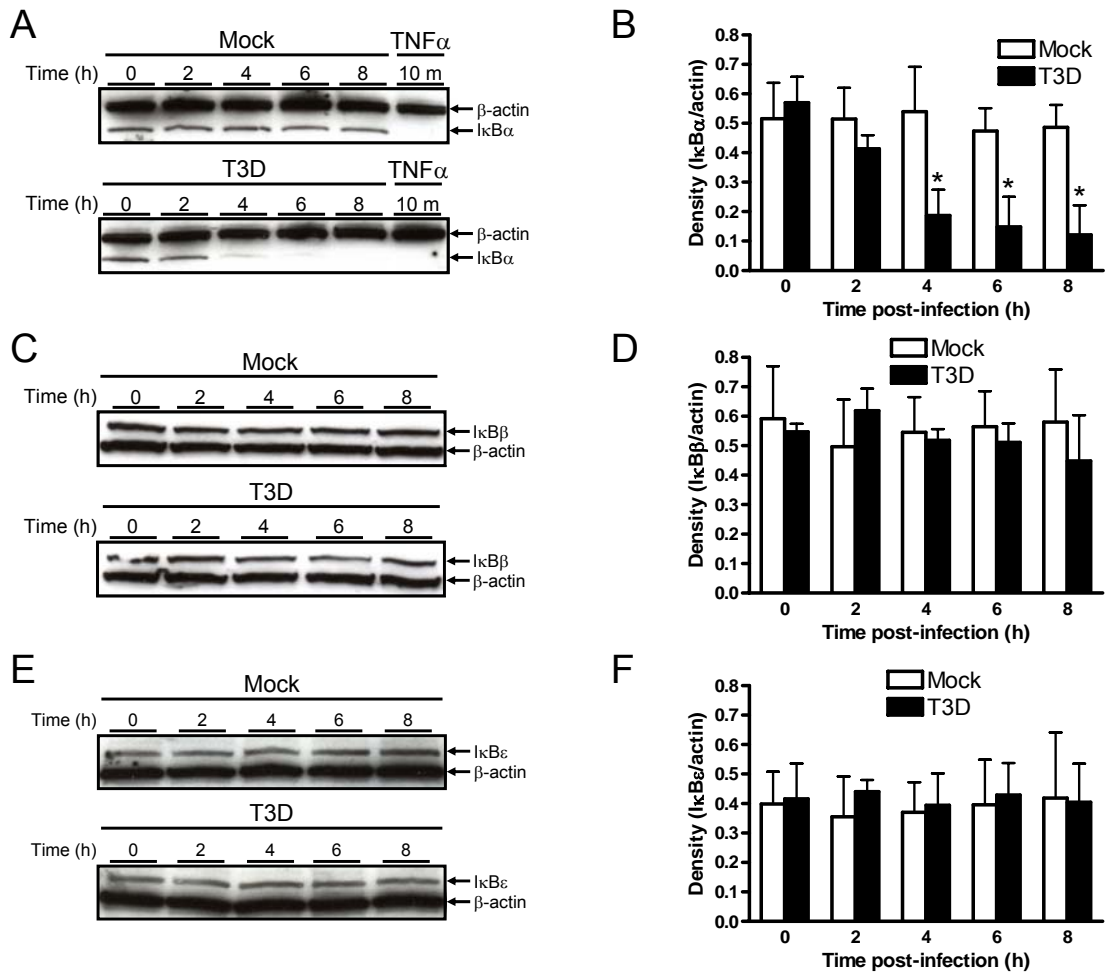
Figure 13. Reovirus infection leads to degradation of IκBα but not IκBβ or IκBε. Cytoplasmic extracts were prepared from uninfected HeLa cells (0 h), mock-infected cells (Mock), or cells infected with reovirus T3D at an MOI of 100 PFU/cell for the times shown. Cells also were treated with 20 ng/ml of TNFα for 10 min as a positive control. Extracts were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted by using antisera specific for (A) IκBα, (C) IκBβ, or (E) IκBε. An actin-specific antiserum was used to detect levels of actin as a loading control. Band intensity corresponding to levels of (B) IκBα, (D) IκBβ, and (F) IκBε was quantified by using the Image J program. The results are presented as the mean ratio of IκB/actin for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to untreated cells (0 h).

is required for the activation of IKKβ (156, 160, 223) but not IKKα (35, 47, 163). The most

well-characterized substrate of IKKβ is IκBα (73, 115), whereas IKKα catalyzes

phosphorylation of p100/NF-κB2 (163, 221).

To determine whether either IKKα or IKKβ is required for reovirus-induced

activation of NF-κB, cellular IKK complexes were immunopurified from HeLa cells either

before or after infection with T3D and monitored for their capacity to phosphorylate IκBα in

vitro. In keeping with the kinetics of IκBα degradation (Fig. 13A) and NF-κB activation

(Fig. 11A), IκB kinase activity exceeding basal levels in uninfected cells was readily detected

within 4 h after exposure to T3D and sustained for at least an additional 4 h (Fig. 14A).

These data suggest that IKKs are critically involved in the mechanism by which reovirus

diminishes the cellular pool of IκBα (Fig. 13A).

To determine whether IKK activation is required for the nuclear translocation of NF-

κB by reovirus, cells were treated with escalating doses of the IKK inhibitor BAY 65-1942

prior to infection with T3D. Importantly, BAY 65-1942 inhibits IKKβ more efficiently than

IKKα (227). As demonstrated in EMSAs, treatment of cells with BAY 65-1942 suppressed

NF-κB signaling induced by reovirus (Fig. 14, B and C), although incompletely, perhaps

reflecting incomplete blockade of IKKα. Immunoblotting studies of nuclear extracts from

the same panel of infected cells indicated that BAY 65-1943 had a profound inhibitory effect

on reovirus-induced nuclear translocation of RelA (Figs. 14, D and E), which is primarily

under the control of IKKβ. These pharmacological data suggest that reovirus activates NF-

κB via a mechanism involving either IKKα, IKKβ, or both of these IKKs.

To identify the IKK subunits responsible for NF-κB activation by reovirus, NF-κB

DNA-binding activity was assessed in murine embryo fibroblasts (MEFs) deficient for
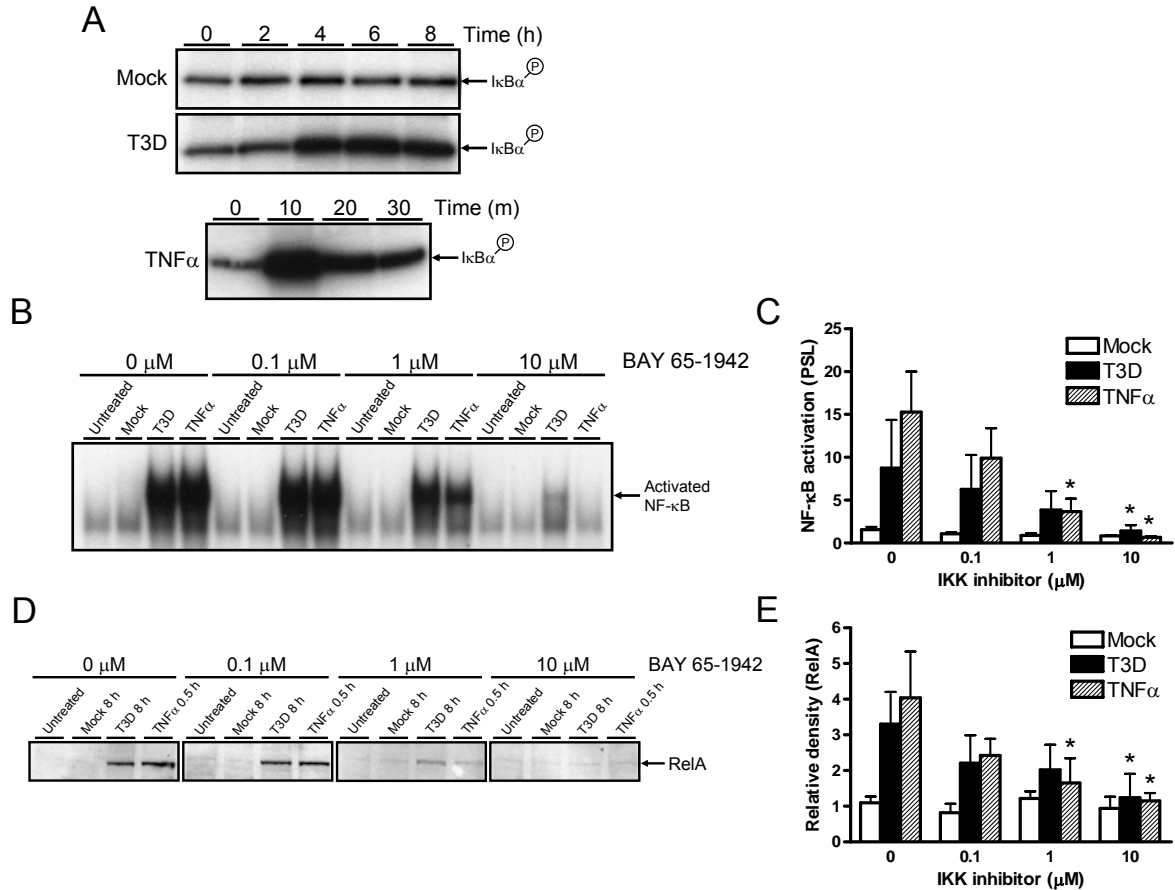
Figure 14. Involvement of IKKs in reovirus-induced NF-κB activation. (A) Whole-cell extracts were prepared from uninfected HeLa cells (0 h), mock-infected cells (Mock), or cells infected with reovirus T3D at an MOI of 100 PFU/cell for the times shown. Cells also were treated with 20 ng/ml of TNFα for the times shown as a positive control. The IKK complex was immunoprecipitated by using an IKKγ-specific antiserum prior to incubation with a GST-IκBα substrate in the presence of $[\gamma\text{-}^{32}P]ATP$. Kinase reactions were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. (B) HeLa cells were pretreated with IKK inhibitor BAY 65-1942 for 1 h at the concentrations shown and uninfected (Untreated), mock-infected (Mock), or infected with reovirus T3D at an MOI of 100 PFU/cell for the times shown. Nuclear extracts were incubated with a radiolabeled NF-κB consensus oligonucleotide, and resulting protein-oligonucleotide complexes were resolved by acrylamide gel electrophoresis, dried, and exposed to film. (C) Band intensity was quantified by determining PSL units relative to uninfected cells for four independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's *t* test in comparison to untreated cells (0 μM). (D) Nuclear extracts from the experiment shown in panel B were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted by using a RelA-specific antiserum. (E) Band intensity was quantified relative to uninfected cells by using the Image J program. The results are presented as the mean RelA band intensity for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's *t* test in comparison to untreated cells (0 μM).

IKKα, IKKβ, or the IKKβ regulatory subunit IKKγ/Nemo. In initial experiments, EMSAs

were conducted with nuclear extracts from wild-type MEFs following infection with T3D for

8 h, which corresponds to peak levels of NF-κB DNA-binding activity (Fig. 11A). Reovirus

induced NF-κB DNA binding activity to levels comparable to or exceeding those observed in

control experiments with wild-type MEFs treated with the cytokine TNFα (Fig. 15A), a

potent agonist of IKK. Similar results were obtained with nuclear extracts from reovirus-

infected MEFs lacking IKKβ (Fig. 15A). However, the capacity of reovirus to activate NF-

κB was completely disrupted in MEFs lacking IKKα (Fig. 15A), indicating preferential

usage of this IKK relative to IKKβ. MEFs lacking the regulatory subunit IKKγ/Nemo (Fig.

15A) also were incapable of reovirus-mediated NF-κB signaling. Immunoblotting studies of

nuclear extracts from the same panel of infected cells confirmed these results (Fig. 15C).

Nuclear translocation of RelA was detected in wild-type MEFs and IKKβ-deficient MEFs,

but not in MEFs lacking IKKα or IKKγ. Differences in reovirus-mediated signal

transduction in IKK-deficient MEFs could not be attributed to differences in viral infection or

growth (Fig. 16). Thus, these findings suggest that IKKα and IKKγ are required for reovirus-

induced NF-κB activation.

NIK is capable of phosphorylating and activating IKKα in response to some agonists

of the alternative NF-κB pathway, such as lymphotoxin β (95, 109). To determine whether

NIK is required for NF-κB activation in response to reovirus, NIK-deficient MEFs were used

to probe for NF-κB induction by EMSA and immunoblotting in response to reovirus T3D

infection (Fig. 17). Reovirus infection resulted in NF-κB activation in both wild-type and

NIK-deficient MEFs, indicating that NIK is dispensable for reovirus-induced NF-κB

activation. Taken together, these data confirm a requirement for endogenous IKK in the

Figure 15. IKKα and IKKγ are required for reovirus-induced activation of NF-κB. (A) Wild-type MEFs or MEFs deficient in IKKα, IKKβ, or IKKγ were uninfected (0 h), mock-infected (Mock), infected with reovirus T3D at an MOI of 100 PFU/cell for 8 h, or treated with 20 ng/ml of TNFα for 1 h. Nuclear extracts were incubated with a radiolabeled NF-κB consensus oligonucleotide. Resulting protein-oligonucleotide complexes were resolved by acrylamide gel electrophoresis, dried, and exposed to film. NF-κB-containing complexes are indicated. (B) Band intensity was quantified by determining PSL units relative to uninfected cells for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's *t* test in comparison to mock-treated cells (0 h). (C) Nuclear extracts from the experiment shown in panel A were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted by using a RelA-specific antiserum.

Figure 16. Cells deficient in IKK subunits are permissive for reovirus infection and growth. (A) Wild-type MEFs or MEFs deficient in IKKα, IKKβ, or IKKγ were infected with T3D at an MOI of 1000 PFU/cell. After 24 h incubation, cells were fixed and incubated with reovirus-specific antiserum. Infected cells were identified by using indirect immunofluorescence. (B) Cells and medium were infected with reovirus T3D at an MOI of 1 PFU/cell and incubated for the times shown. Cells and medium were frozen and thawed twice, and viral titers were determined by plaque assay. The results are expressed as the mean viral titers for three independent experiments. Error bars indicate standard deviations.

A

Wild-type

NIK -/-

	Mock	T3D	TNFα			Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h		0 h	8 h	8 h	0.5 h

← Activated NF-κB

B

Wild-type

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

← RelA

NIK -/-

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

← RelA

Figure 17. Reovirus-induced activation of NF-κB in NIK-deficient cells. (A) Wild-type MEFs or NIK-deficient MEFs were uninfected (0 h), mock-infected (Mock), infected with reovirus T3D at an MOI of 1000 PFU/cell for 8 h, or treated with 20 ng/ml of TNFα for 30 min. Nuclear extracts were incubated with a radiolabeled NF-κB consensus oligonucleotide. Resulting protein-oligonucleotide complexes were resolved by acrylamide gel electrophoresis, dried, and exposed to film. NF-κB-containing complexes are indicated. (B) Nuclear extracts from the experiment shown in panel A were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted by using a RelA-specific antiserum.

mechanism by which reovirus activates NF-κB and strongly suggest that this virus selectively utilizes the IKKα arm of the NF-κB signaling pathway. Surprisingly, IKKγ/Nemo, which is known to regulate IKKβ rather than IKKα, is also required for NF-κB activation by reovirus, but NIK is dispensable.

IKKα and IKKγ are required for reovirus-induced apoptosis

Since IKKα and IKKγ are required for NF-κB activation following reovirus infection (Fig. 15), we examined whether IKK stimulation by reovirus leads to apoptotic cell death. IKK-deficient MEFs were infected with reovirus T3D, and apoptosis was assessed by quantitation of caspase 3/7 activity (Fig. 18A). Levels of activated caspase 3/7 following infection of wild-type and IKKβ-deficient MEFs were substantially greater than those following infection of MEFs deficient in either IKKα or IKKγ. To corroborate these results, we tested wild-type and IKK-null MEFs for viability following infection with T3D (Fig. 18B). In comparison to wild-type and IKKβ-deficient MEFs, a significantly greater percentage of IKKα- and IKKγ-deficient MEFs remained viable during a time course of reovirus infection.

To determine whether NIK is required for apoptosis induction following reovirus infection, NIK-deficient MEFs were infected with reovirus T3D, and apoptosis was assessed by quantification of caspase 3/7 activity (Fig. 18C). Levels of caspase 3/7 activity in MEFs deficient in NIK were equivalent to those in wild-type cells following infection with T3D. In parallel with these results, we observed no significant difference in the viability of wild-type and NIK-deficient MEFs following T3D infection (Fig. 18D). Together, these functional data with IKK- and NIK-deficient MEFs strongly correlate with the capacity of reovirus to modulate IκBα and NF-κB during infection (Figs. 11-16). Our findings suggest that IKKα
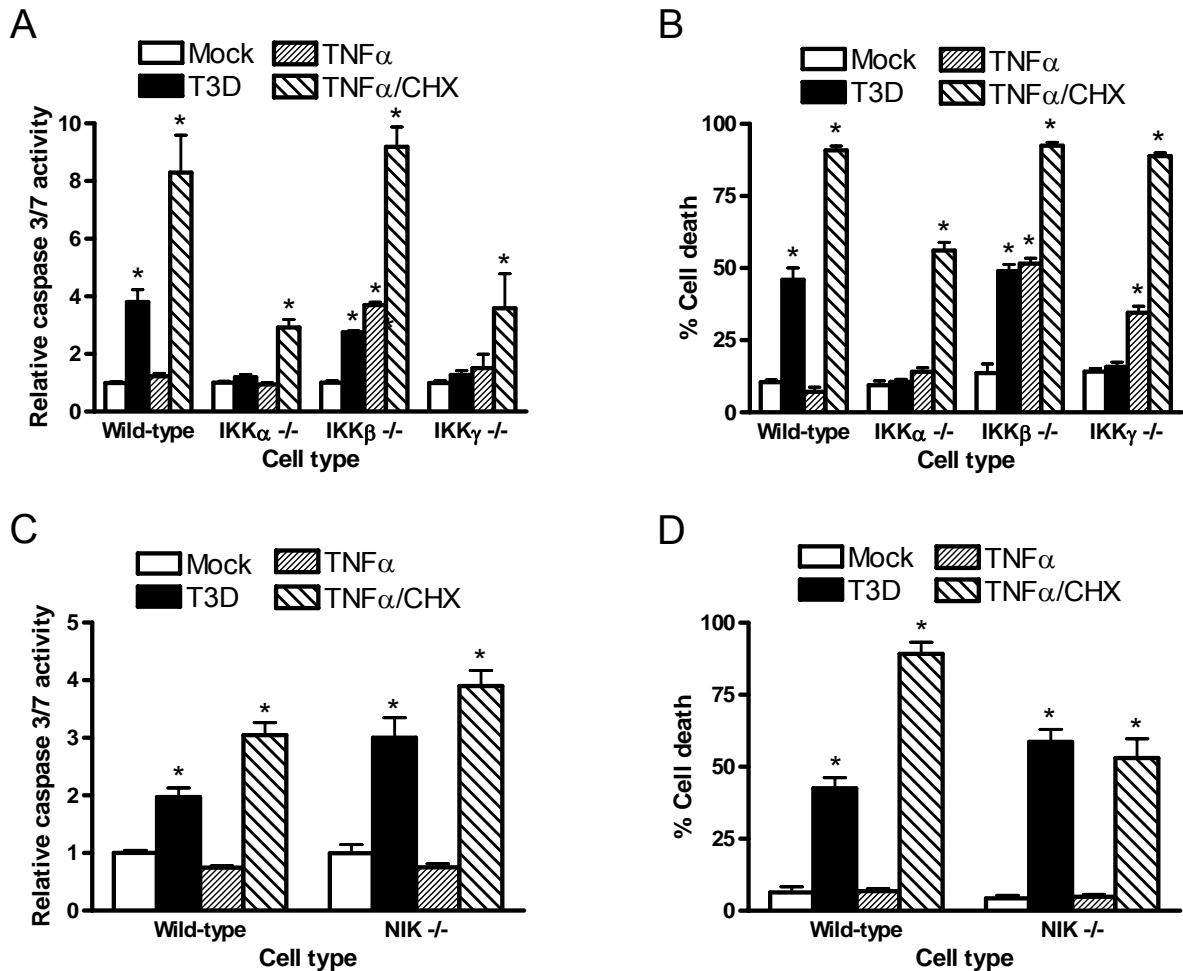
49

Figure 18. IKKα and IKKγ are required for reovirus-induced apoptosis. Wild-type MEFs or (A) MEFs deficient in IKKα, IKKβ, IKKγ, or (C) NIK were mock-infected, infected with reovirus T3D at an MOI of 1000 PFU/cell for 24 h, treated with 10 ng/ml of TNFα for 12 h, or treated with 10 ng/ml of TNFα and 10 μg/ml of cycloheximide for 12 h. Caspase 3/7 activity was quantified by using a luminescent substrate. The results are expressed as the mean caspase activity relative to mock-infected cells for three independent experiments. Wild-type MEFs or (B) IKK-deficient MEFs or (D) NIK-deficient MEFs were mock-infected, infected with reovirus T3D at an MOI of 1000 PFU/cell for 48 h, treated with 10 ng/ml of TNFα for 24 h, or treated with 10 ng/ml of TNFα and 10 μg/ml of cycloheximide for 24 h. Cell viability was quantified by trypan blue exclusion. The results are expressed as the mean percentage of cell death for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to mock-infected cells.

and IKKγ are required for the activation of NF-κB induction of apoptosis in response to reovirus. In keeping with NF-κB activation by reovirus, apoptosis is completely independent of the IKKα inducer NIK, indicating a different mechanism of IKKα activation in response to reovirus.

Discussion

Results reported here identify constituents of the NF-κB signaling apparatus induced by reovirus and provide evidence that NF-κB activation during reovirus infection requires integral components of both the classical and alternative pathways. Using MEFs deficient in the expression of individual IKK subunits, we demonstrate that reovirus-infected cells lacking IKKα are impaired for NF-κB activation (Fig. 15) and apoptotic programming (Fig. 18), whereas both of these processes are operative in cells lacking IKKβ. Despite its preferential usage of IKKα, reovirus retains the capacity to elicit both NF-κB activation and apoptosis in the absence of NIK (Fig. 16 and 19), a known activator of IKKα in cytokine-treated cells (95, 163). Furthermore, targeted disruption of the gene encoding IKKγ/Nemo, which is dispensable for cytokine-induced signaling of IKKα (35, 47), significantly attenuates reovirus-induced NF-κB activation and apoptosis (Fig. 15 and 18). In light of these findings with NIK and IKKγ, the precise mechanism of reovirus action on IKKα remains unclear. The simplest interpretation of these results is that reovirus accesses the cellular NF-κB machinery by directly interfacing with IKKα/IKKγ complexes, with IKKγ serving as an adaptor that docks one or more reovirus gene products. In keeping with this possibility, IKKγ tethers the HTLV1 Tax protein to IKK complexes, resulting in persistent activation of IKKβ and NF-κB (25, 33). In what may be another related finding, IKKγ also is

51

required for Tax-induced activation of IKKα (219). Although data emerging from studies of

IKKβ-deficient mice suggest the presence of functional IKKα/IKKγ complexes (91, 92, 150,

184), direct evidence for the existence of IKKα/IKKγ complexes in wild-type animals has

not been reported. Notwithstanding, our results clearly establish that reovirus activates NF-

κB and downstream proapoptotic genes via a mechanism involving IKKα but not IKKβ.

       The principle in vivo substrate of IKKβ is IκBα (51, 115, 141). This cytoplasmic

inhibitor tightly controls the nuclear translocation of p50/RelA dimers (8), effectors of the

classical NF-κB pathway (reviewed in (19, 59, 66)). The principle in vivo substrate of IKKα

is p100/NF-κB2 (163). An integral inhibitor in the alternative NF-κB pathway, p100

assembles with the transactivator protein RelB (163, 179). Following IKKα-mediated

phosphorylation, p100 is processed to p52 via a proteasome-dependent mechanism,

permitting the nuclear entry of p52/RelB complexes (163, 179). Given these distinct

mechanisms, our findings with reovirus-infected cells suggest an unconventional function for

IKKα in substrate targeting. Specifically, we were unable to detect either p52 or RelB DNA-

binding activity in nuclear extracts from cells following 4 to 8 h of reovirus infection (Fig.

11B). Instead, at these early timepoints the predominant Rel species detected were p50 and

RelA (Fig. 11B), which are primarily under IκBα control (7, 8). Consistent with this Rel

profile, IκBα protein levels were significantly reduced by 4 h post-infection (Fig. 13).

Although p100 processing to p52 was observed at late time points during reovirus infection

(16 h), it seems unlikely that this delayed response contributes to the more rapidly evolving

signals required for apoptosis (39, 40, 145). Accordingly, we propose that IKKα rather than

IKKβ targets IκBα for proteolytic destruction and regulates the nuclear translocation of

p50/RelA complexes in reovirus-infected cells. This working model is fully concordant with

the phenotype of cell lines deficient for either IKKα, p50, or RelA, all of which are impaired

for reovirus-induced NF-κB activation and proapoptotic signaling (Figs. 14, 15, and (40)). In agreement with this model, prior studies with recombinant proteins indicate that IKKα can efficiently phosphorylate NF-κB-bound forms of IκBα in vitro (225).

Both IKKα and IKKβ contain regulatory serine phosphoacceptors in their so-called "T loop" domains (48, 115). Signal-dependent phosphorylation of the T loop serines in IKKα and IKKβ is a prerequisite for their catalytic activation (48). Based on in vitro studies with recombinant proteins, IKKα and IKKβ can autophosphorylate at these T loop serines (48, 160, 186). Physiologic agonists of the alternative NF-κB pathway stimulate T loop phosphorylation and activation of IKKα via the upstream kinase NIK (95, 163, 221). However, NIK is dispensable in the mechanism by which reovirus induces NF-κB activation and apoptosis (Fig. 16 and 18).

What signal transducers couple reovirus to IKK? Experiments using pharmacological inhibitors suggest that NF-κB-dependent apoptotic signaling is triggered by viral replication steps that occur after disassembly but prior to RNA synthesis (39). Strain-specific differences in the capacity of reovirus to induce apoptosis segregate with viral genes encoding the σ1 and μ1 proteins (38, 145, 199), which play important roles in viral attachment (89, 209) and membrane penetration (26, 27, 101), respectively. Importantly, transient expression of μ1 is sufficient to induce apoptosis in cell culture (36), implicating this protein in the reovirus/NF-κB signaling axis. We envision three potential mechanisms by which μ1, or perhaps another viral gene product, initiates NF-κB signal transduction during reovirus infection. First, μ1 may activate viral sensors that mediate the recruitment of the adaptor protein IFN-β promoter stimulator (IPS-1), which leads to the activation of NF-κB in response to viral infections by recruiting TNF-associated factor 6 to the signaling

complex (84, 116, 164, 222). Second, µ1 may activate a novel cellular kinase that phosphorylates the T loop of IKKα. Third, µ1 may interact with IKK complexes directly, leading to conformational changes that stimulate oligomerization and trigger autophosphorylation of the T loop in IKKα (74, 136). Additional studies will be required to precisely define the mechanism by which reovirus hijacks NF-κB to mediate apoptotic cell death.

CHAPTER IV

ORGAN-SPECIFIC ROLES FOR TRANSCRIPTION FACTOR NF-κB IN REOVIRUS-
INDUCED APOPTOSIS AND DISEASE

Introduction

Mechanisms of viral disease involve complex interactions of pathogen virulence

factors and host responses. Perhaps the best-understood basis of organ-specific viral

pathology is the availability of cell-surface molecules required for viral attachment and entry.

Rarely, however, is viral disease ascribable solely to receptor recognition. More commonly,

additional virus-host interactions determine the outcome of infection (198), and these pivotal

steps are of much interest in studies of viral pathogenesis. Factors expected to modulate viral

growth and virulence in an organ-dependent manner include the capacity of virus to

efficiently utilize the host translational apparatus, including strategies to circumvent antiviral

effects of IFN; availability of cellular proteins to facilitate viral replication and gene

expression; and changes in the intracellular signaling dynamic induced by viral infection.

The NF-κB family of transcription factors plays a key role in the regulation of cell

growth, activation, differentiation, and survival. Following exposure of cells to a variety of

stimuli, NF-κB is activated and translocates to the nucleus (17), where it serves as a

transcriptional regulator (111, 204). In systems in which NF-κB is activated during

apoptosis, NF-κB can either prevent (15, 97, 111, 202) or potentiate (1, 53, 82, 94) cell death

signaling. Following reovirus infection of cultured cells, the heterodimeric NF-κB complex

p50/RelA enters the nucleus and activates proapoptotic gene expression (40). When NF-κB

activation is inhibited using proteasome inhibitors or dominant-negative forms of IκBα,

reovirus-induced apoptosis is blocked (40). Moreover, cell lines deficient in either of the p50

or RelA NF-κB subunits do not undergo apoptotic cell death following reovirus infection. These findings indicate that activation of NF-κB in cell culture is required for reovirus-induced apoptosis.

Experiments reported in this chapter demonstrate that the p50 subunit of NF-κB plays an essential role in the development of encephalitis and myocarditis in reovirus-infected mice. Although reovirus infects the intestine and disseminates systemically following peroral inoculation of mice lacking the NF-κB p50 subunit, apoptosis is diminished in the brain yet strikingly enhanced in the heart. These findings suggest a novel role for NF-κB in the pathogenesis of viral infection; it serves a proapoptotic function in the CNS, while mediating a prosurvival function in the myocardium.

Results

Reovirus activates NF-κB in vivo

To determine whether reovirus is capable of NF-κB activation in the intact host, we performed in vivo luciferase assays using transgenic mice engineered to express luciferase under control of an HIV long-terminal repeat promoter that contains NF-κB consensus binding sites (88). These mice were inoculated perorally with either PBS (mock-infected) or 10^4 PFU reovirus strain T3SA+, which was chosen for these studies because of its capacity to activate NF-κB and induce a potent apoptotic response in cultured cells (38). Seven days after inoculation, the mice were imaged for luciferase activity as a marker for NF-κB activation (Fig. 19A and B). Little luciferase activity was detected in the mock-infected mice (Fig. 19A). In contrast, reovirus-infected animals exhibited systemic luciferase activity (Fig. 19B), which indicates that reovirus is capable of NF-κB activation in vivo.
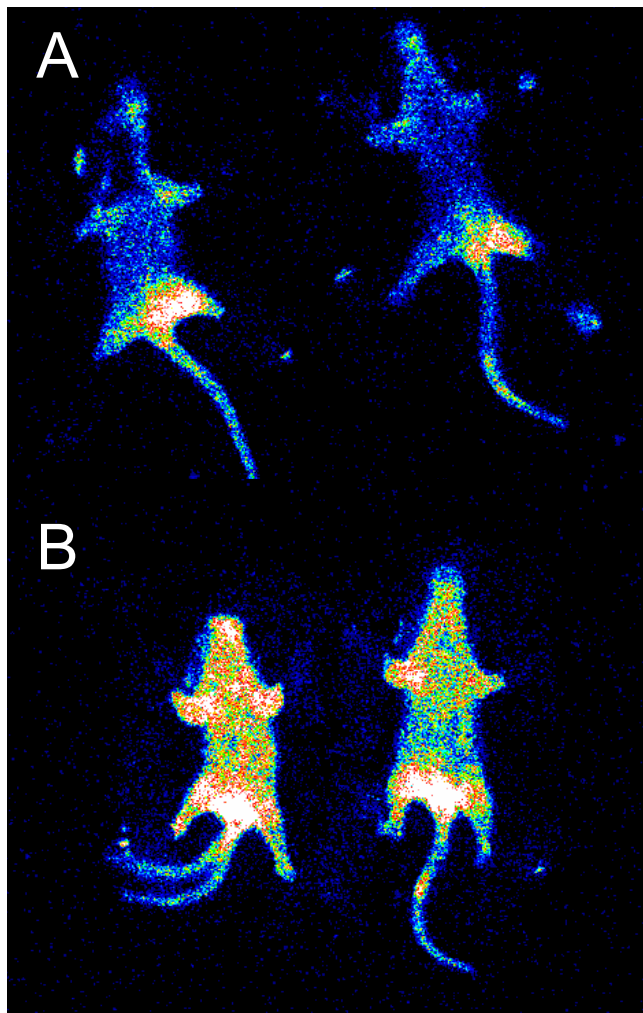
Figure 19. NF-κB activation following reovirus infection of HLL mice. Newborn HLL mice were inoculated perorally with (A) PBS (mock) or (B) 10^4 PFU of reovirus T3SA+. Mice were inoculated intraperitoneally with luciferin 7 days post-infection and imaged for luciferase activity as a marker for NF-κB activation. Bioluminescence indicates areas of NF-κB activation.

Reovirus-induced activation of NF-κB in the murine CNS and heart is dependent on p50

To determine whether reovirus activates NF-κB in the murine CNS and heart, we performed EMSAs using brain and heart extracts prepared from reovirus-infected or mock-infected wild-type and p50-null mice. Newborn p50+/+ and p50-/- mice were inoculated with either PBS or 10^4 PFU reovirus T3SA+. Cell extracts were prepared from brain and heart tissue 12 days after inoculation, incubated with a radiolabeled oligonucleotide consisting of the NF-κB consensus binding sequence, and resolved by PAGE using nondenaturing conditions (Fig. 20A and D). NF-κB DNA-binding activity was detected in extracts from the brain of reovirus-infected p50+/+ but not p50-/- mice (Fig. 20A). Similarly, NF-κB DNA-binding activity was detected in the heart of p50+/+ mice infected with reovirus but not p50-/- animals (Fig. 20D). These findings indicate that reovirus infection in the murine CNS and heart induces nuclear translocation of NF-κB, which is contingent on the expression of the NF-κB p50 subunit.

To confirm the specificity of NF-κB DNA-binding activity in these experiments, we incubated cell extracts from reovirus-infected p50+/+ mouse brain and heart with a ^{32}P-labeled NF-κB consensus oligonucleotide in the presence of excess unlabeled consensus oligonucleotide (Fig. 20B and E). Binding of the radiolabeled probe was competed with that of unlabeled consensus oligonucleotide, which suggests that the gel-shift activity detected following reovirus infection is specific for sequences bound by NF-κB.

In cell culture, reovirus infection results in the nuclear translocation of NF-κB complexes containing subunits p50 and RelA (40). As an additional specificity control in these experiments for the activation of NF-κB, nuclear extracts were prepared from reovirus-infected p50+/+ mouse brain or heart and incubated with an antiserum specific to RelA prior to the addition of the NF-κB-specific oligonucleotide (Fig. 20C and F). Addition of the
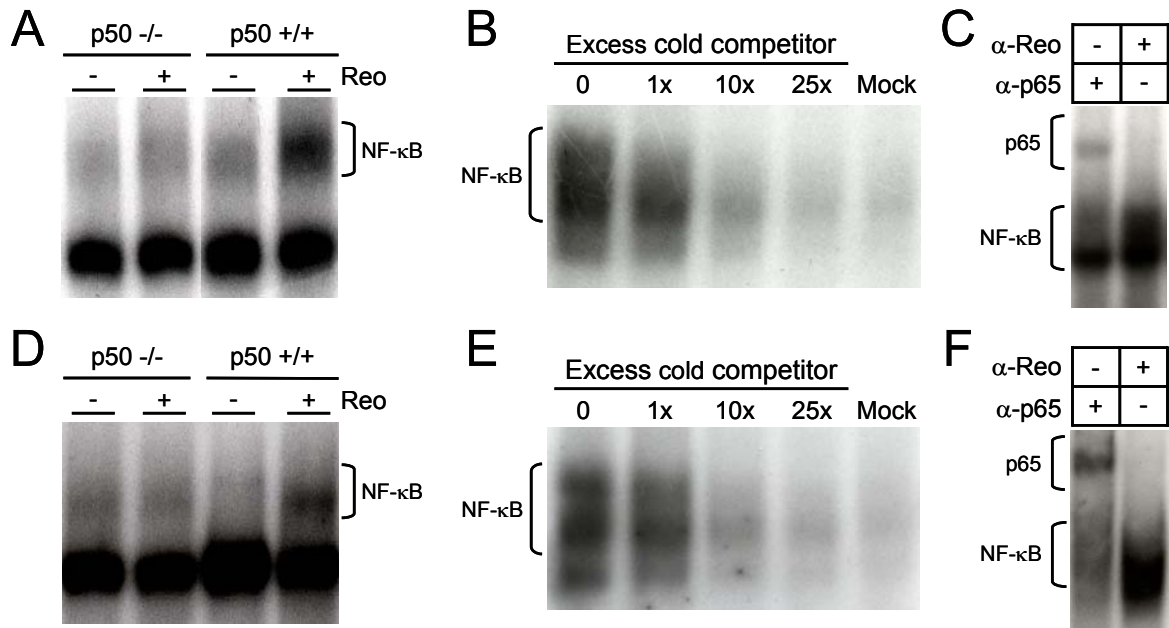
Figure 20. Reovirus-induced NF-κB gel-shift activity following infection of p50 +/+ and p50 -/- mice. (A and C) Newborn p50 +/+ and p50 -/- mice were inoculated perorally with either 10^4 PFU of reovirus T3SA+ (Reo) or PBS. (A) Brains and (C) hearts were resected 12 days post-inoculation, and cell extracts were prepared. Extracts were incubated with a ^{32}P-labeled NF-κB consensus oligonucleotide and resolved by nondenaturing polyacrylamide gel electrophoresis. Activated NF-κB complexes are indicated. Shown is a representative experiment of four performed. (B and D) Extracts were prepared from either (B) brains or (D) hearts of p50 +/+ mice 12 days following peroral inoculation with T3SA+. Prior to the addition of the ^{32}P-labeled oligonucleotide probe, extracts were incubated with either a control antibody specific for reovirus protein σ3 (α-reovirus) or an antibody specific for NF-κB subunit p65 (α-p65). Super-shifted complexes containing p65 are indicated. *, non-specific bands.

RelA-specific antiserum resulted in bands of higher relative molecular mass, which verified

that RelA is present in the NF-κB complexes activated following reovirus infection. These

findings provide strong evidence that reovirus infection of the murine CNS and heart induces

the nuclear translocation of NF-κB and this effect is abolished in mice lacking p50.

NF-κB subunit p50 is not required for efficient reovirus replication or dissemination in the murine host

To determine whether p50 plays a role in reovirus growth in vivo, we inoculated

p50+/+ and p50–/– mice intracranially or perorally with 10^4 PFU reovirus T3SA+. Viral

titers in the brain were determined by plaque assay 2, 4, and 6 days after intracranial

inoculation (Fig. 21A) and in the intestine, liver, brain, and heart 4, 6, 8, 10, and 12 days after

peroral inoculation (Fig. 21B). Following intracranial inoculation, viral titers in p50+/+ and

p50-/- mice were equivalent at all time points tested. After peroral inoculation, virus

replicated efficiently in the intestines of both p50+/+ and p50-/- mice and disseminated to the

liver, brain, and heart. Viral titers in the intestine, liver, and brain did not differ between

p50+/+ and p50-/- mice. In sharp contrast, viral titers in the hearts of p50-/- mice were more

than 1,000-fold higher than those in the hearts of p50+/+ animals. These findings suggest

that p50 is dispensable for reovirus growth in vivo and that the absence of p50 in the heart,

but not in other tissues tested, allows for increased reovirus replication.

NF-κB subunit p50 is required for efficient induction of apoptosis in the CNS following reovirus infection

To assess reovirus-induced pathologic changes in the CNS of p50+/+ and p50-/-

mice, we prepared brain sections from mice euthanized at 12 days following peroral

inoculation with reovirus T3SA+ and examined them after staining with H&E (Fig. 22A and

B). Brain sections from reovirus-infected p50+/+ and p50-/- mice exhibited evidence of
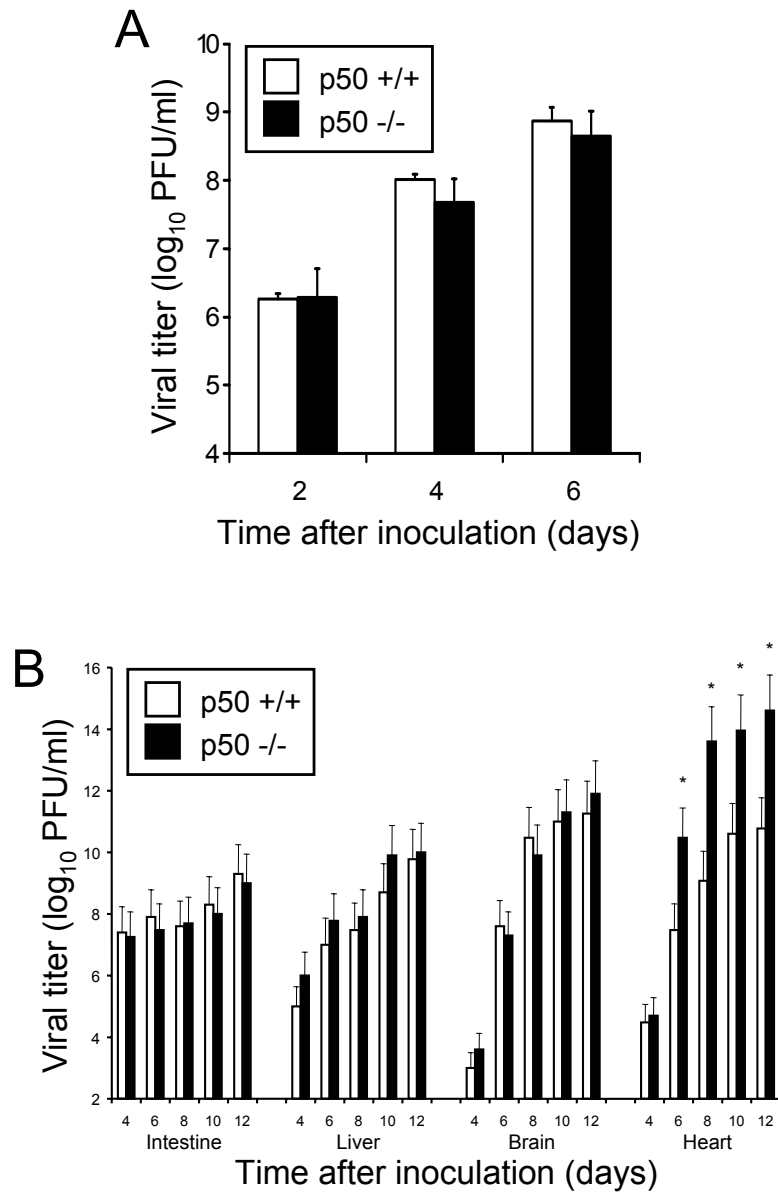
Figure 21. Growth of reovirus in p50 +/+ and p50 -/- mice. (A) Titers of reovirus in brain after intracranial inoculation of p50 +/+ and p50 -/- mice. Newborn mice were inoculated with 10^4 PFU of reovirus T3SA+. At days 2, 4, and 6 post-inoculation, mice were euthanized, brains were harvested, and viral titers were determined by plaque assay. (B) Titers of reovirus in intestine, liver, brain, and heart after peroral inoculation of p50 +/+ and p50 -/- mice. Newborn mice were inoculated with 10^4 PFU of T3SA+. At days 4, 6, 8, 10, and 12 post-inoculation, mice were euthanized, organs were harvested, and viral titers were determined by plaque assay. The results are expressed as the mean viral titers for two to four animals for each timepoint. Error bars indicate standard deviations. *, $P < 0.05$ by T test.
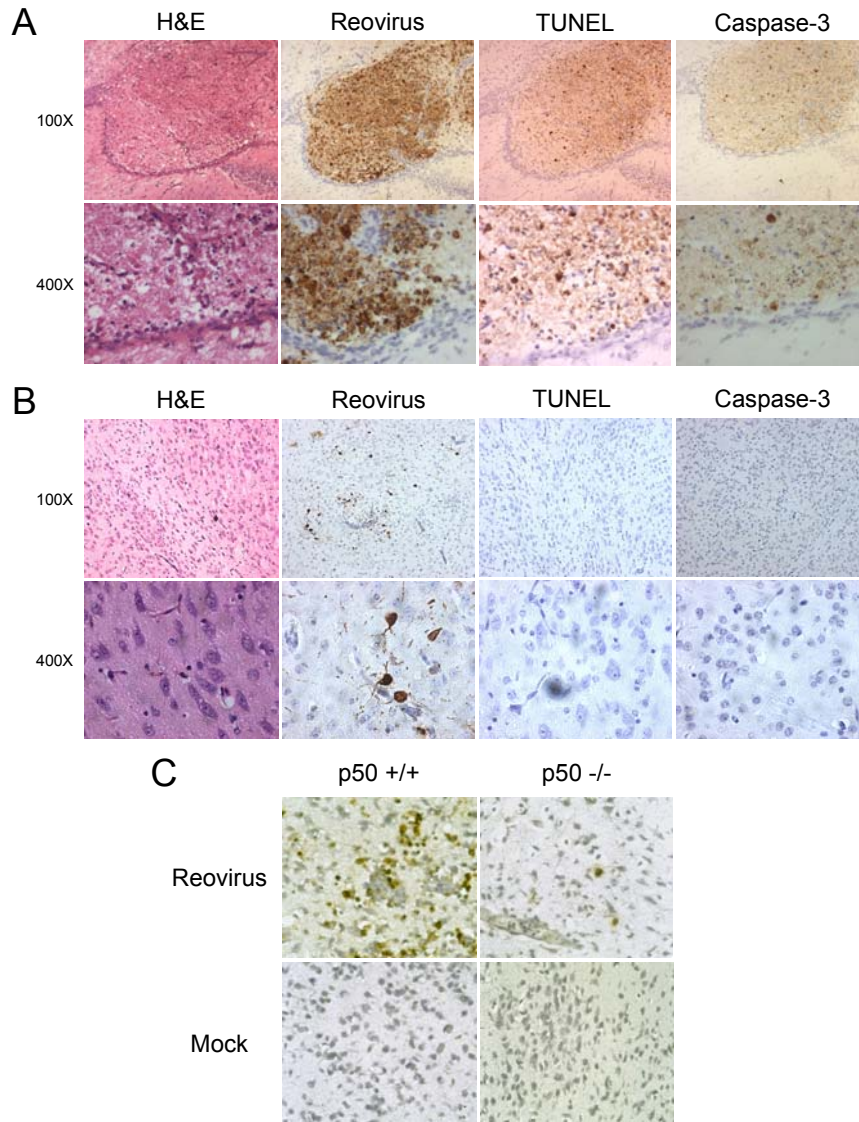
Figure 22. **Inflammation, reovirus protein expression, TUNEL staining, and immunohistochemical detection of activated caspase-3 in the brain of reovirus-infected (A) p50 +/+ and (B) p50 -/- mice.** Newborn mice were inoculated perorally with 10^4 PFU of reovirus T3SA+. At 12 days post inoculation, brains were harvested, paraffin embedded, sectioned, and stained with hematoxylin and eosin, polyclonal reovirus-specific antiserum, TUNEL, or activated caspase 3-specific antiserum as indicated. Shown are consecutive sections of diencephalon. Original magnifications were 100X (top panels) and 400X (bottom panels). (**C**) Newborn mice were inoculated intracranially with 10^4 PFU of T3SA+ or gelatin saline (mock). At 6 days post-inoculation, mice were euthanized, and brain sections were stained using a TUNEL assay. Shown are sections of the upper brain stem. Original magnifications were 200X. Brown staining indicates reovirus protein, fragmented DNA, or activated caspase 3.

meningoencephalitis. Inflammatory infiltrates were detected primarily in the cerebral cortex, hippocampus, diencephalon, and brain stem. Morphologically, inflammatory cells were mostly lymphocytes and macrophages/microglia with some plasma cells and neutrophils. Inflammatory changes were more extensive in p50+/+ mice (Fig. 22A) than in p50-/- mice (Fig. 22B), which suggests that the neurovirulence of reovirus is attenuated in mice lacking an intact NF-κB signaling apparatus.

To assess the distribution of reovirus protein expression in the CNS of p50+/+ and p50-/- mice, we prepared brain sections from mice euthanized 12 days following peroral inoculation and stained them using a reovirus-specific antiserum (Fig. 22A and B). Immunohistochemical staining for reovirus protein demonstrated the presence of immunoreactive neurons in brains of both p50+/+ and p50-/- mice (Fig. 22A and B). Antigen-positive neurons were detected in a pattern recapitulating the inflammatory changes; the cerebral cortex, hippocampus, diencephalon, and brain stem were primarily involved. The number of reovirus-infected cells and their distribution was similar in p50+/+ and p50-/- mice (Fig. 22A and B). These results suggest that the lack of p50 does not alter reovirus tropism for specific neural regions.

To determine whether p50 is required for apoptosis in the murine CNS, we prepared brain sections from reovirus-infected p50+/+ and p50-/- mice 12 days following peroral inoculation (Fig. 22A and B) or 6 days following intracranial inoculation (Fig. 22C) and assayed them for fragmented DNA using the terminal dUTP nick-end labeling (TUNEL) technique. Apoptotic cells were quantified by counting all TUNEL-positive cells in cortex, hippocampus, basal ganglia, diencephalon, and brain stem of each section obtained from mice inoculated intracranially (Fig. 23). Numbers of TUNEL-positive cells in the brains of infected p50+/+ mice were significantly greater than those in the brains of infected p50-/- mice. These findings were the same following both peroral and intracranial inoculation (Fig.

22). Thus, reovirus-induced apoptosis in the murine CNS is dependent on the p50 subunit of NF-κB.

Activation of caspase 3 is a highly specific biomarker of apoptotic cell death (22). To confirm that DNA fragmentation observed in the brains of reovirus-infected p50+/+ mice is due to apoptosis, we stained brain sections with an antiserum specific for the activated form of caspase 3 (Fig. 22A and B). Activated caspase 3 was detected in regions of the brain in which TUNEL-positive staining also was observed. Moreover, cells immunoreactive for caspase 3 were detected at a much higher frequency in the brains of p50+/+ mice. Morphologically, cells immunoreactive for caspase 3 were primarily neurons, and most immunoreactive neurons also exhibited morphologic evidence of apoptosis. These results provide additional evidence that expression of NF-κB subunit p50 is required for efficient induction of apoptosis during reovirus infection in the murine CNS.

Absence of NF-κB subunit p50 leads to enhanced pathology and massive apoptosis in the murine heart following reovirus infection

Since viral titers in the hearts of p50-/- mice were more than 1,000-fold higher than in those of p50+/+ mice (Fig. 21B), we examined heart tissue for evidence of inflammation and tissue injury. Newborn p50+/+ and p50-/- mice were inoculated perorally with either 10^4 PFU reovirus T3SA+ or PBS and weighed daily. Mice were euthanized at various time points after inoculation, and hearts were removed and weighed. There were no significant differences in the heart weights of mock-infected p50+/+ and p50-/- mice (Fig. 24A). Surprisingly, heart weights of reovirus-infected p50-/- mice were significantly greater than those of p50+/+ mice (Fig. 24B). Differences in the percent heart weight (heart weight relative to total body weight) of infected p50+/+ and p50-/- mice became detectable at 8 days after inoculation and continued to increase with time, while there was no significant increase
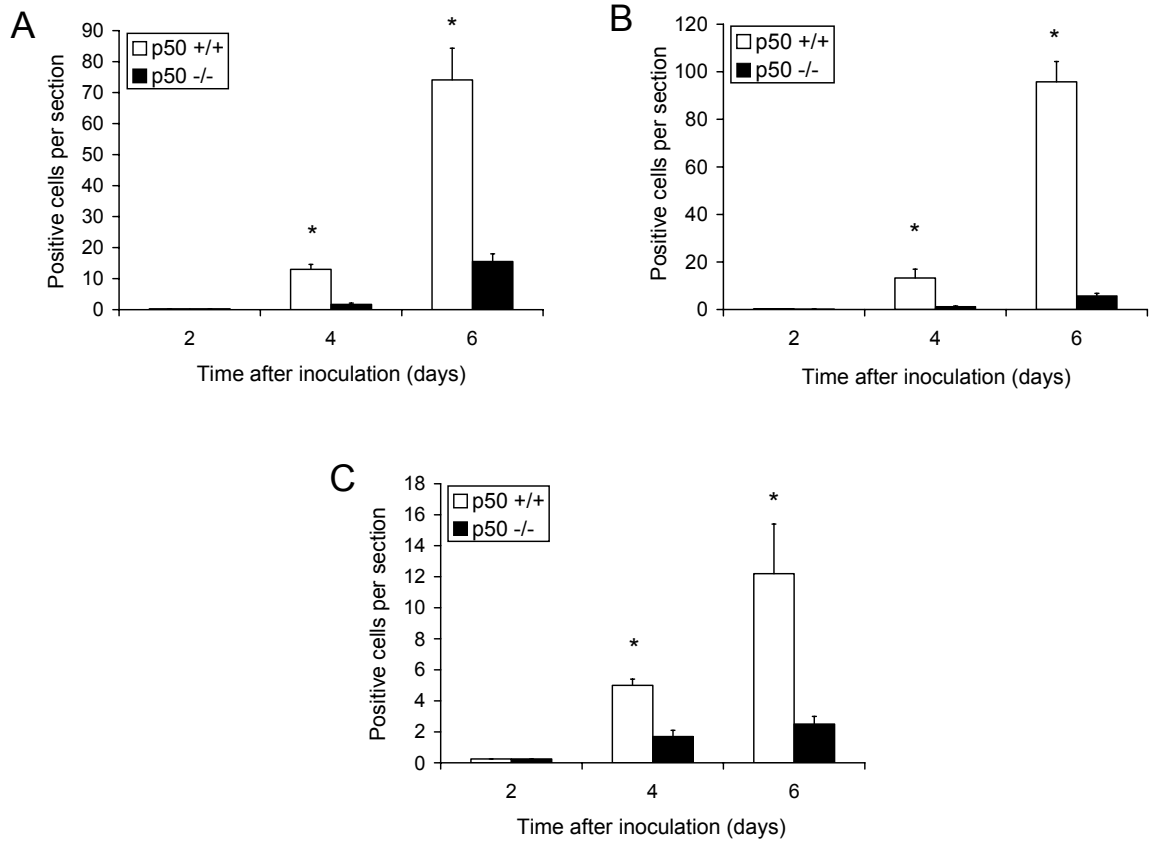
Figure 23. Quantitation of TUNEL staining in (A) cortex and hippocampus, (B) basal ganglia and diencephalon, and (C) brain stem of reovirus-infected p50 +/+ and p50 -/- mice. TUNEL staining was performed using tissue sections prepared 2, 4, and 6 days following intracranial inoculation of p50 +/+ and p50 -/- mice with 10^4 PFU of reovirus T3SA+. For each brain region, all positive cells in a single parasagittal section were counted for four to eight animals. The results are expressed as the mean number of apoptotic cells per region. Error bars indicate standard deviations. *, $P < 0.05$ by T test.

in the percent heart weight of infected p50+/+ mice (Fig. 24B). Dramatic differences were observed in the gross appearance of hearts dissected from p50+/+ and p50-/- animals following infection with reovirus (Fig. 24C). Hearts from reovirus-infected p50-/- mice had a blanched appearance with diffuse surface irregularities corresponding to confluence of purulent lesions, consistent with overt myocarditis. In contrast, hearts from mock-infected p50-/- or p50+/+ mice or reovirus-infected p50+/+ mice displayed no overt abnormalities.

To determine whether reovirus-induced myocardial injury in p50-/- mice is associated with contractile dysfunction, we performed echocardiography on 10-day-old mice after peroral inoculation with either reovirus T3SA+ or PBS. Fractional shortening, assessed by 2-dimensional, directed M-mode measurements, was substantially decreased in reovirus-infected p50-/- mice (<10%; Fig. 24D), while it was preserved in mock-infected p50-/- mice (>40%; Fig. 24E) and reovirus-infected p50+/+ mice (>40%; Fig. 24F). Heart size was also increased in reovirus-infected p50-/- mice compared with mock-infected p50-/- mice and reovirus-infected p50+/+ mice. Intact atrioventricular conduction was observed in all mice, which suggests that the pathologic process was not specifically targeted to the conduction system. These results suggest that the myocardial pathology associated with reovirus infection of p50-/- mice is associated with diminished contractility.

On a microscopic level, hearts of p50-/- animals displayed extensive myocyte destruction with features of apoptotic and necrotic cell death. Affected areas were notable for cell fragments, granular debris, and scattered calcifications. Thorough sectioning of the organ block revealed that pathology was not limited to any particular region of the heart. Hearts from reovirus-infected p50+/+ mice and mock-infected p50-/- and p50+/+ mice demonstrated no significant microscopic pathology. We conclude that reovirus is more pathogenic in the heart in the absence of NF-κB subunit p50.

Figure 24. Heart pathology following reovirus infection of p50 +/+ and p50 -/- mice. (A and B) Newborn p50 +/+ and p50 -/- mice were inoculated perorally with either (A) PBS (mock) or (B) 10^4 PFU of reovirus T3SA+, and heart size was monitored at two-day intervals. Percent heart weight was calculated as the ratio of heart weight to body weight. The results are expressed as the mean heart weights of at least four animals for each timepoint. Error bars indicate standard deviations. *, $P < 0.05$ by T test. (C) Hearts from mice euthanized 12 days following peroral inoculation with reovirus T3SA+ or gelatin saline (mock). (D, E, and F) Electrocardiography and echocardiography of (D) reovirus-infected p50 -/-, (E) mock-infected p50 -/-, and (F) reovirus-infected p50 +/+ mice. Newborn mice were inoculated perorally with 10^4 PFU of T3SA+, and tests were performed 10 days post-inoculation. A P-wave/QRS ECG complex is displayed above the corresponding echocardiographic image. Systolic and diastolic LV cavity dimensions are indicated by bars superimposed on the M-mode images.

To assess the extent and location of reovirus infection in the murine myocardium in the presence and absence of p50, we performed reovirus antigen staining on heart sections from p50+/+ and p50-/- mice euthanized 12 days following peroral inoculation with reovirus T3SA+ (Fig. 25A and B). Immunohistochemical staining for reovirus protein demonstrated immunoreactive myocytes in heart sections prepared from both p50+/+ and p50-/- mice (Fig. 25A and B). However, the number of reovirus-infected cells differed substantially between p50+/+ and p50-/- mice, consistent with the significant difference in viral titer in the hearts of these animals.

To determine whether expression of p50 influences apoptosis in the murine heart, we inoculated p50+/+ and p50-/- mice perorally with reovirus and assessed them for apoptosis using TUNEL staining (Fig. 25A and B). There were rare TUNEL-positive cells in the hearts of p50+/+ mice following reovirus infection (Fig. 25A), whereas numerous foci of apoptosis were present in the hearts of p50-/- mice (Fig. 25B). Interestingly, foci of apoptotic cells in the hearts of p50-/- mice coincided with areas of intense staining for reovirus antigen, which suggests a link between reovirus replication and apoptosis in cardiac myocytes.

To confirm that the absence of p50 leads to enhanced apoptosis in the heart during reovirus infection, we prepared heart sections from p50+/+ and p50-/- mice 12 days following peroral inoculation with reovirus and stained them for activated caspase 3 (Fig. 25A and B). Caspase 3 staining revealed numerous positive myocytes in the same areas of the heart that also were positive for reovirus antigen and TUNEL staining. These results suggest that, in contrast to its effects in the murine CNS, the NF-κB p50 subunit protects against apoptosis induced by reovirus infection in the murine myocardium.
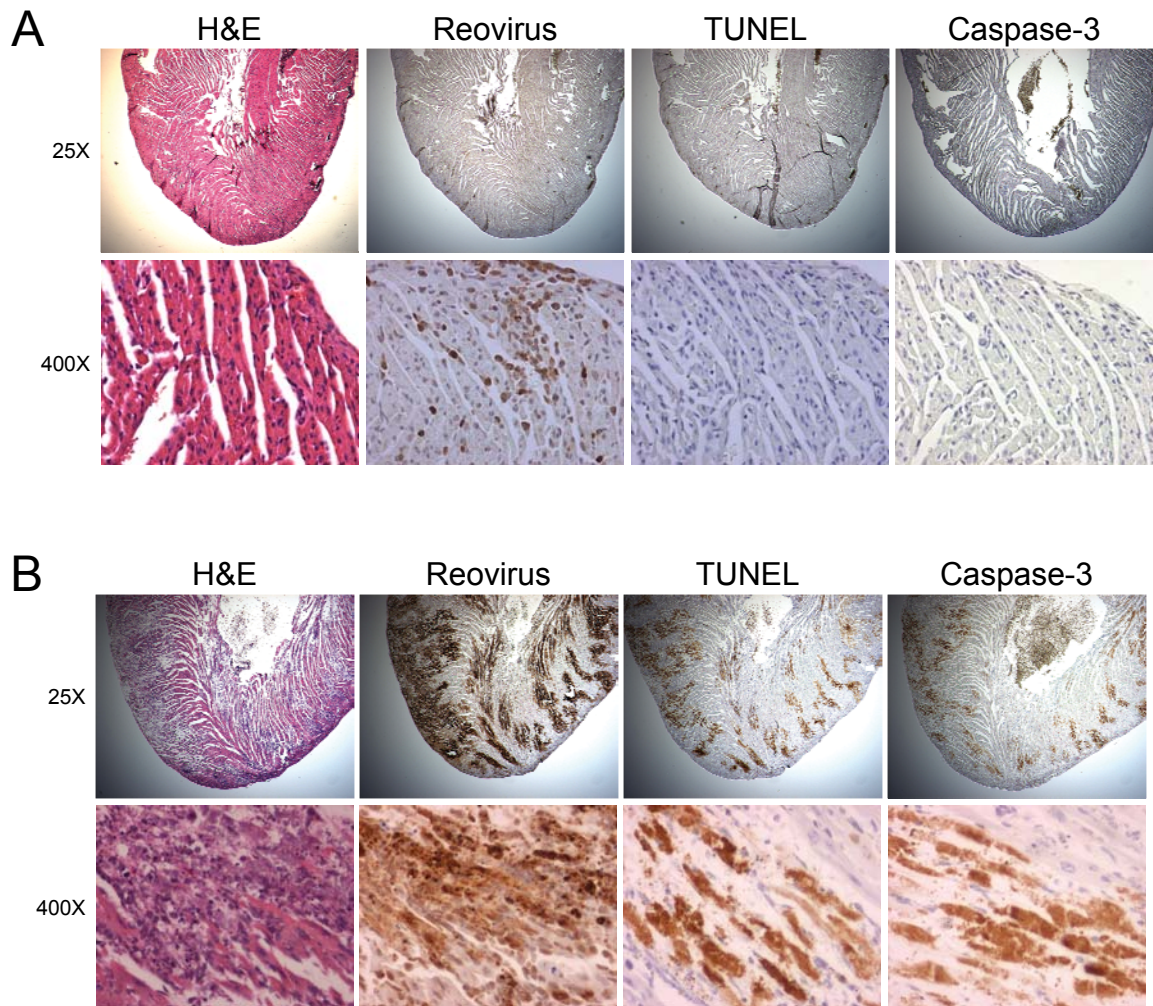
Figure 25. **Inflammation, reovirus protein expression, TUNEL staining, and immunohistochemical detection of activated caspase 3 in the heart of reovirus-infected (A) p50 +/+ and (B) p50 -/- mice.** Newborn mice were inoculated perorally with 10^4 PFU of reovirus T3SA+. At 12 days post inoculation, hearts were harvested, paraffin embedded, sectioned, and stained with hematoxylin and eosin, polyclonal reovirus-specific antiserum, TUNEL, or activated caspase 3-specific antiserum as indicated. Original magnifications were 25X (top panels) and 400X (bottom panels). Brown staining indicates reovirus protein, fragmented DNA, or activated caspase 3.

IFN-β is induced in the heart of wild-type mice following reovirus infection

Results presented thus far demonstrate that enhanced reovirus growth in the heart of p50-/- mice is associated with massive apoptosis. We thought it possible that the absence of NF-κB-mediated activation of innate immune responses might lead to increased viral replication and resultant pathology in the heart. To test this hypothesis, we inoculated p50+/+ and p50-/- mice perorally with reovirus T3SA+ or PBS. Twelve days after inoculation, heart and brain were removed, and levels of IFN-β mRNA were determined using quantitative PCR (Fig. 26). Using GAPDH mRNA as a standardization control, little IFN-β mRNA was induced in the brain of either p50-/- or p50+/+ mice in the presence or absence of reovirus infection (Fig. 26). In contrast, IFN-β mRNA levels were substantially increased in the heart of reovirus-infected wild-type mice compared with p50-/- animals (Fig. 26). These results indicate that IFN-β induction by reovirus in the murine heart is dependent on NF-κB and suggest that IFN-β protects the heart from reovirus-induced apoptosis and disease.

IFN-β treatment of p50-null mice attenuates reovirus-induced myocarditis

To determine whether NF-κB-mediated expression of IFN-β plays a direct role in protection of the heart against apoptosis and disease caused by reovirus, we tested the effect of IFN-β treatment on reovirus infection of p50-/- mice. Newborn p50-/- mice were inoculated intraperitoneally with either IFN-β or PBS 1 day prior to peroral inoculation with reovirus T3SA+ and treated daily for 9 days thereafter. On day 10, the animals were euthanized, and brain and heart were removed for determination of viral titer and histopathology (Fig. 27). IFN-β treatment significantly decreased viral titer in both brain and heart (Fig. 27A). In p50-/- mice treated with IFN-β, viral titers reached only 10^2 PFU in the brain and were less than 10^2 PFU in the heart (Fig. 27A). In parallel with these results,
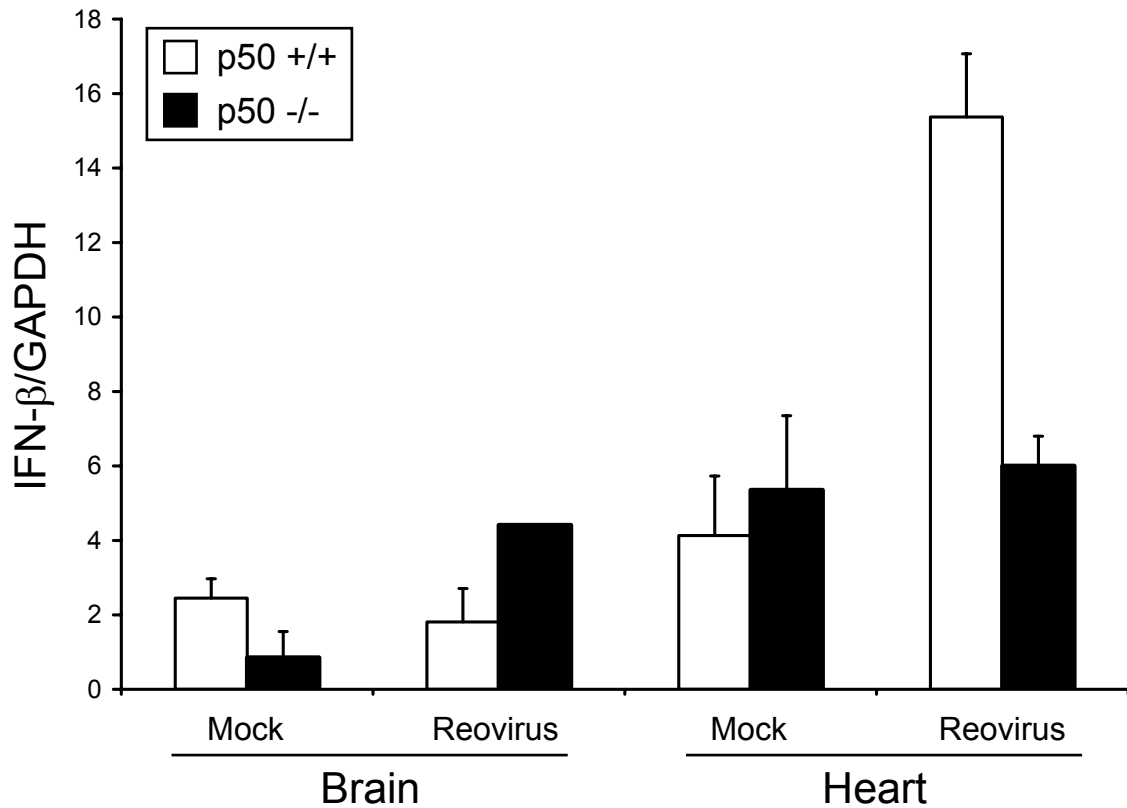
Figure 26. Levels of IFN-β mRNA in brain and heart of p50 +/+ and p50 -/- mice.
Newborn mice were inoculated perorally with either PBS (mock) or 10^4 PFU of reovirus
T3SA+. At 12 days post-inoculation, brains and hearts were resected, and whole-organ
RNA was isolated and used as a template to generate cDNA. Levels of IFN-β and GAPDH
cDNA were assessed by real-time PCR. The results are expressed as the mean ratio of IFN-
β cDNA to that of GAPDH for two animals. Error bars indicate standard deviations.
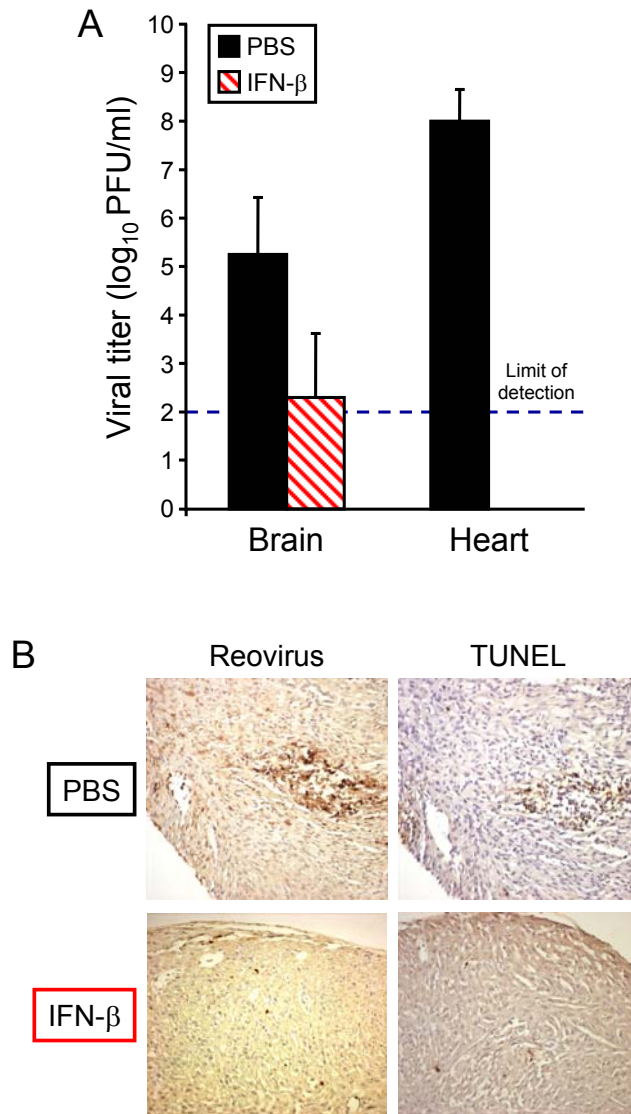
Figure 27. Reovirus replication and apoptosis in infected p50 -/- mice following treatment with IFN-β. Newborn mice were inoculated intraperitoneally with either IFN-β or PBS 1 day prior to peroral inoculation with 10^4 PFU of reovirus T3SA+. Animals were treated with either IFN-β or PBS for an additional 9 days, and brains and hearts were resected. (A) Viral titers in the brain and heart. Organs were homogenized, and viral titers were determined by plaque assay. The results are expressed as the mean viral titers for three animals. Error bars indicate standard deviations. *, $P < 0.05$ by T test. (B) Histopathology of reovirus infection in the heart. Hearts of reovirus-infected p50 -/- animals from (A) were paraffin-embedded, sectioned, and stained with polyclonal reovirus-specific antiserum or processed for TUNEL analysis. Original magnifications were 25X (top panels) and 200X (bottom panels). Brown staining indicates reovirus protein or fragmented DNA.

apoptosis in the heart of IFN-β-treated p50-/- mice was substantially diminished (Fig. 27B).

Thus, a critical component of the underlying mechanism of NF-κB-mediated protection

against reovirus-induced myocardial injury is contingent on IFN-β.

Discussion

Results reported here indicate organ-specific roles for NF-κB in the pathogenesis of

viral disease, which is a heretofore unknown property of this signaling molecule. The key

finding is that marked differences in the pathogenesis of reovirus infection in the CNS and

heart are dependent on the action of NF-κB. Following reovirus infection in the CNS,

p50+/+ mice exhibited significant neuronal apoptosis, while p50-/- mice displayed a minimal

apoptotic response. In sharp contrast, reovirus induced little apoptosis in the heart of p50+/+

mice, whereas extensive apoptosis occurred in the heart of p50-/- mice. These findings

indicate that NF-κB subunit p50 plays two distinctly different roles in reovirus pathogenesis,

serving a proapoptotic function in the brain, while mediating a prosurvival function in the

heart.

Studies using mice with targeted disruptions of specific NF-κB subunits have shown

that NF-κB serves important functions in the development and function of innate and

adaptive immunity (87, 165, 208). Mice lacking p50 have no apparent developmental defects

(165), and immune cells mature normally. However, p50-/- mice display defects in B cell

activation, isotype switching, and antibody production (165). These defects render p50-/-

mice more susceptible to infection by the Gram-positive bacterial pathogen *Streptococcus*

pneumoniae, but they remain capable of efficiently clearing infection by the Gram-negative

pathogens *Escherichia coli* and *Haemophilus influenzae* (165). When p50-/- mice are

infected with encephalomyocarditis virus, they are actually more resistant to infection than

controls. This difference is thought to be due to an increase in apoptosis that leads to a

decrease in viral growth (165). These findings stand in stark contrast to what occurs in the CNS and heart of reovirus-infected mice.

In experiments comparing reovirus infection of p50+/+ and p50-/- mice, we found that the presence or absence of p50 did not alter primary viral replication in intestinal tissue or dissemination of virus to the liver, brain, or heart. Although viral replication in the brain after intracranial inoculation also was independent of p50, replication in the heart was increased in p50-/- mice by approximately 1,000-fold. What might explain the enhancement of reovirus replication in the heart of p50-/- mice? Reovirus strains have been characterized previously as having the capacity to grow in the murine heart and produce cardiac disease (170, 174). In primary cardiac myocytes, nonmyocarditic reovirus strains induce more IFN-β and are more sensitive to the antiviral effects of this cytokine than myocarditic reovirus strains (175). Furthermore, normally nonmyocarditic strains are capable of producing myocarditis in infected IFN-α/β receptor-/- mice (175). Thus, it appears that type I IFNs restrict viral replication in the heart and attenuate cardiac disease.

NF-κB is known to induce the expression of several mediators of innate immune responses including type I IFNs (31, 56, 224). Therefore, absence of p50 may allow reovirus to achieve much higher titers and cause myocarditis. We tested this hypothesis by determining brain and heart levels of IFN-β mRNA in response to reovirus infection of p50+/+ and p50-/- mice (Fig. 26) and by treating reovirus-infected p50-/- mice with IFN-β (Fig. 27). In these experiments, we found a dramatic increase in IFN-β expression in the hearts of wild-type mice but only a minimal IFN-β response in the hearts of p50-null animals. Moreover, reconstitution of p50-/- mice with IFN-β substantially diminished reovirus replication and apoptosis, which resulted in diminished myocardial injury. These results indicate that IFN-β is a necessary component of the NF-κB–mediated protective response against reovirus in the heart. However, it is likely that other components of innate immunity

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are involved in this effect. Preliminary data from our laboratory suggest that in addition to IFN-β, IL-6, macrophage migration inhibitory factor (MIF), and tumor necrosis factor (TNF) are expressed at higher levels in the heart of p50+/+ mice than p50-/- mice (S.M. O'Donnell and T.S. Dermody, unpublished observation). These findings suggest that following reovirus infection of the heart, NF-κB is activated and leads to induction of potent innate immune responses, which in turn attenuate viral replication at that site, resulting in diminished apoptosis and disease.

The enhanced growth of reovirus in the heart of p50-/- mice compared with p50+/+ mice was associated with extensive myocarditis and resultant tissue injury and dysfunction. This result was confirmed by histopathological studies, echocardiography, and physical examination revealing signs of heart failure. The pathology observed in the heart of p50-/- animals was characterized by extensive tissue damage and little inflammatory infiltrate, similar to findings made in previous studies of reovirus myocarditis (174). Therefore, our results suggest that apoptosis is the primary mechanism of cardiac damage in reovirus-induced myocarditis, as reported previously (44). Damage to cardiac myocytes during reovirus infection occurs in the complete absence of adaptive components of host defense (173). It is possible that a similar mechanism occurs in humans, which would explain why some patients with acute myocarditis develop heart failure in the setting of sustained viremia (103).

In contrast to the enhanced growth of reovirus in the heart of p50-/- mice, viral growth in the CNS of p50+/+ and p50-/- mice was equivalent. However, we observed dramatic differences in the number of apoptotic cells in the two mouse strains as indicated by TUNEL and caspase 3 staining. Therefore, the efficiency of viral growth is not strictly correlated with the extent of the apoptotic response. Nonetheless, despite these p50-dependent differences in viral growth, our results suggest that apoptosis is an important

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mechanism of reovirus-induced disease in both the CNS and heart. In the CNS of p50-/-
mice, apoptosis and inflammation following reovirus infection were diminished. However,
in the hearts of these animals, apoptosis and tissue injury were enhanced. This correlation
between apoptosis and pathology lends support to the hypothesis that therapies directed at
blocking programmed cell death might attenuate viral virulence, consistent with results from
previous studies of reovirus-induced myocarditis (44). However, our findings suggest that
pharmacologic inhibition of NF-κB activation may reduce pathologic injury at some sites and
exacerbate disease at others, depending on the nature of the NF-κB agonist.

The precise cell types responsible for the p50-dependent effects on apoptosis in
response to reovirus infection in mice are not apparent from our study. It is possible that
expression of p50 in neurons is required for apoptosis of these cells and expression of p50 in
cardiac myocytes mediates protection of these cells against apoptotic injury. However, it is
also possible that p50-dependent immune responses contribute to the observed differences in
cell fate. For example, NF-κB-mediated release of cytokines such as TNF-α from immune
cells might contribute to the neuronal apoptosis that occurs during reovirus infection of the
CNS, whereas NF-κB-mediated release of type I IFNs from immune cells might mediate a
protective effect in the heart. Since adoptive transfer of immune cells is not technically
feasible in the newborn mice required for studies of reovirus pathogenesis, discrimination
between these possibilities awaits the development of mice with tissue-specific ablation of
NF-κB activity.

The role of NF-κB in response to a variety of cellular stresses has been studied
extensively using cultured cells (60). However, little is known about the contributions of
specific NF-κB subunits in vivo. The extensive array of NF-κB inducers and target genes
(130) suggests that numerous mechanisms exist to direct transcription of appropriate NF-κB-
dependent genes in response to specific stimuli. One such regulatory mechanism is likely to

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be the activation of specific NF-κB complexes (e.g., p50/RelA heterodimers) for each inducing signal. Individual homodimeric and heterodimeric NF-κB complexes exhibit different affinities for target DNA sequences (61), and this provides a potential mechanism by which NF-κB-inducing stimuli regulate transcriptional activity of specific subsets of cellular genes. We showed previously that reovirus requires p50/RelA for efficient apoptosis in cell culture (40). However, we found in the current study that p50 plays organ-specific roles in disease pathogenesis in vivo. These findings emphasize that NF-κB subunits can have different functions following activation with the same stimulus depending on the cellular environment. Continuing studies in this area may reveal new layers of control of NF-κB responses and extend understanding of how viruses cause tissue-specific injury.

CHAPTER V

INTERFERON-β INHIBITS REOVIRUS-INDUCED APOPTOSIS IN CARDIAC
MYOCYTES BY BLOCKING VIRAL REPLICATION

Introduction

Viral myocarditis is often fatal in infants and can progress to chronic myocarditis,

dilated cardiomyopathy, or cardiac failure in adults (217). Many viruses have been shown to

cause myocarditis, but enteroviruses and adenoviruses account for most reported cases (106,

147, 148, 193). Enterovirus-induced myocarditis is immune-mediated (41, 147), while

cardiac damage in response to adenovirus is caused by virus-induced cytopathicity (106).

Mechanisms used by viruses to directly injure the heart are not well understood.

Certain strains of reovirus cause myocarditis in experimentally infected mice (174).

Cardiac damage following reovirus infection is independent of the adaptive immune system

(172, 173), which normally plays a protective role in reovirus-infected animals (173).

Instead, reovirus directly induces apoptosis in the heart resulting in myocarditis (44). An

important determinant of reovirus-induced myocarditis is interferon-β (IFN-β) (128, 175).

Strains of reovirus capable of causing myocarditis (i.e., myocarditic strains) induce less IFN-

β than nonmyocarditic strains and are not as sensitive to its antiviral effects (175).

IFN-β is secreted from infected cells and binds to the IFN α/β receptor resulting in

dimerization and activation of the Janus-associated kinase (JAK) family of kinases. Once

activated, JAKs phosphorylate tyrosine residues on the signal transducer and activator of

transcription (STAT) molecules STAT 1 and STAT2 (68, 135, 159, 180). The STAT

proteins interact with IFN regulatory factor 9 (IRF-9) to form ISGF3, which translocates to

the nucleus and enhances the expression of IFN-stimulated genes (ISGs). Classical ISGs

include double-stranded RNA-dependent protein kinase (PKR) and 2'-5' oligoadenylate synthetase, which both function to inhibit translation in infected cells (180).

Reovirus infection leads to NF-κB activation in numerous cell types (34, 40, 128). However, the outcome of NF-κB activation varies depending on the infected tissue (128). NF-κB activation in the CNS leads to high levels of neuronal apoptosis and resultant encephalitis. In contrast, NF-κB activation in the heart leads to IFN-β production, which limits viral replication and protects against apoptosis. In the absence of NF-κB signaling, reovirus infection induces widespread apoptotic damage to cardiac myocytes, resulting in myocarditis. Cell types involved in these highly divergent responses to reovirus infection in vivo have not been defined. Moreover, pathways that act upstream and downstream of NF-κB in reovirus-infected animals are not known.

In the experiments described in this chapter, I used primary cardiac myocyte cultures (PCMCs) to better understand mechanisms of reovirus-induced myocarditis. First, I tested whether PCMCs are permissive for reovirus infection and growth. Second, I assessed PCMCs for apoptosis in response to reovirus. Third, I determined the effect of IFN-β on reovirus infection, growth, and apoptosis. The results suggest that IFN-β mediates an anti-apoptotic role by diminishing viral infection and growth in cardiac myocytes.

Results

Reovirus infection of primary cardiac myocytes

In a previous study, we found that the p50 subunit of NF-κB is required for induction of IFN-β expression and protection against the apoptotic response elicited by reovirus in the heart (128). To better understand mechanisms of reovirus-induced apoptosis in cardiac

tissue, we generated PCMCs and tested these cultures for the capacity to support reovirus infection. PCMCs were infected with reovirus strain T3SA+, a potent inducer of apoptosis (38), and infectivity was quantified by indirect immunofluorescence (Fig. 28). Approximately 60 cells per visual field were observed to be infected with reovirus 20 h post-adsorption, suggesting that PCMCs are permissive for reovirus infection.

Since IFN-β serves a protective function in the murine heart (128, 173), we next tested the effect of pretreatment with either IFN-β or IFN-β-specific antiserum on reovirus infection in PCMCs (Fig. 28). Treatment with IFN-β significantly reduced reovirus infection in these cultures in comparison to untreated control cells. Similar results were obtained when myocytes were treated with ribavirin, a viral RNA synthesis inhibitor (139), prior to infection. In contrast, treatment with IFN-β-specific antiserum had little effect on reovirus infection in the myocyte cultures, suggesting that cells present in the intact heart other than myocytes are responsible for secreting cardioprotective IFN-β. Together, these data indicate that reovirus is capable of infecting cardiac myocytes and that infection is potently inhibited by the exogenous administration of IFN-β.

Reovirus growth in primary cardiac myocytes

To determine whether PCMCs are capable of supporting the complete reovirus replication cycle, we measured yields of infectious progeny by plaque assay following a time course of reovirus T3SA+ infection (Fig. 29). Reovirus produced high titers in PCMCs at 24 and 48 h post-infection, reaching yields in excess of 100-fold at the later time point. However, reovirus growth in cardiac myocytes was virtually abolished by pretreatment with IFN-β. Similarly, treatment with either ribavirin or E64, a protease inhibitor that blocks reovirus disassembly (5), diminished reovirus growth in PCMCs. In keeping with our
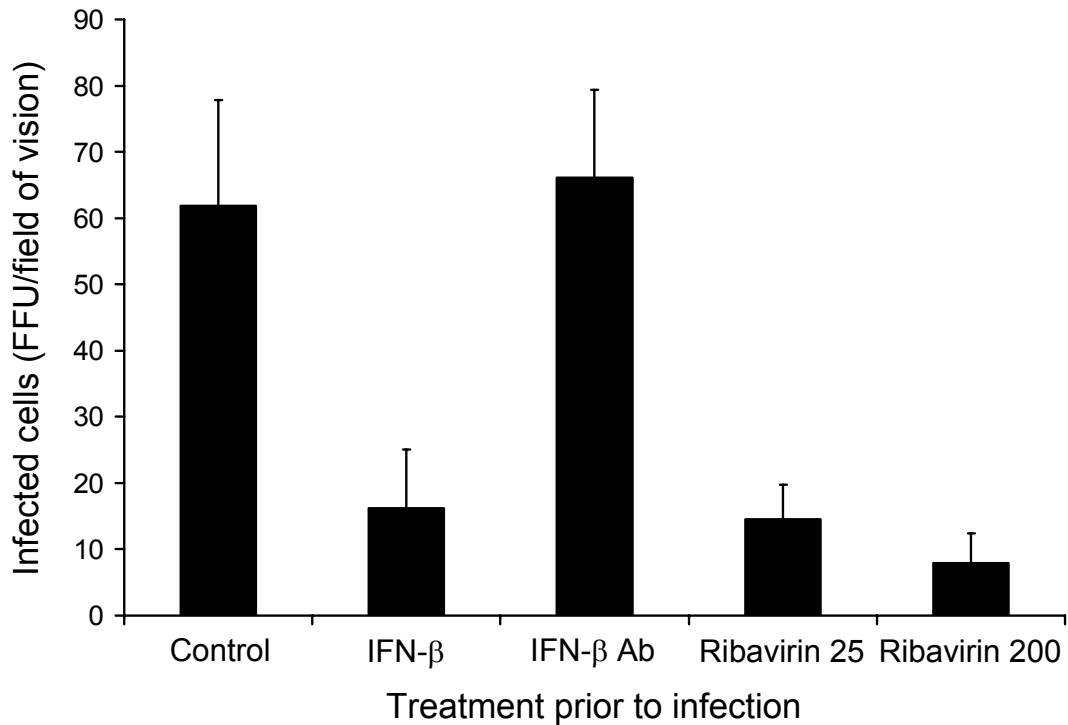
Figure 28. Reovirus infection in PCMCs. PCMCs were pretreated with either fresh medium (control), 1.2×10^4 units of IFN-β, 275 neutralizing units of IFN-β-specific antiserum, or 25 or 200 μM of ribavirin. Cells were then infected with T3SA+ at an MOI of 10 PFU/cell. After 24 h incubation, cells were fixed and incubated with reovirus-specific antiserum. Infected cells were identified by using indirect immunofluorescence. The results are expressed as the mean fluorescent focus units/field of vision for three independent experiments. Error bars indicate standard deviations.
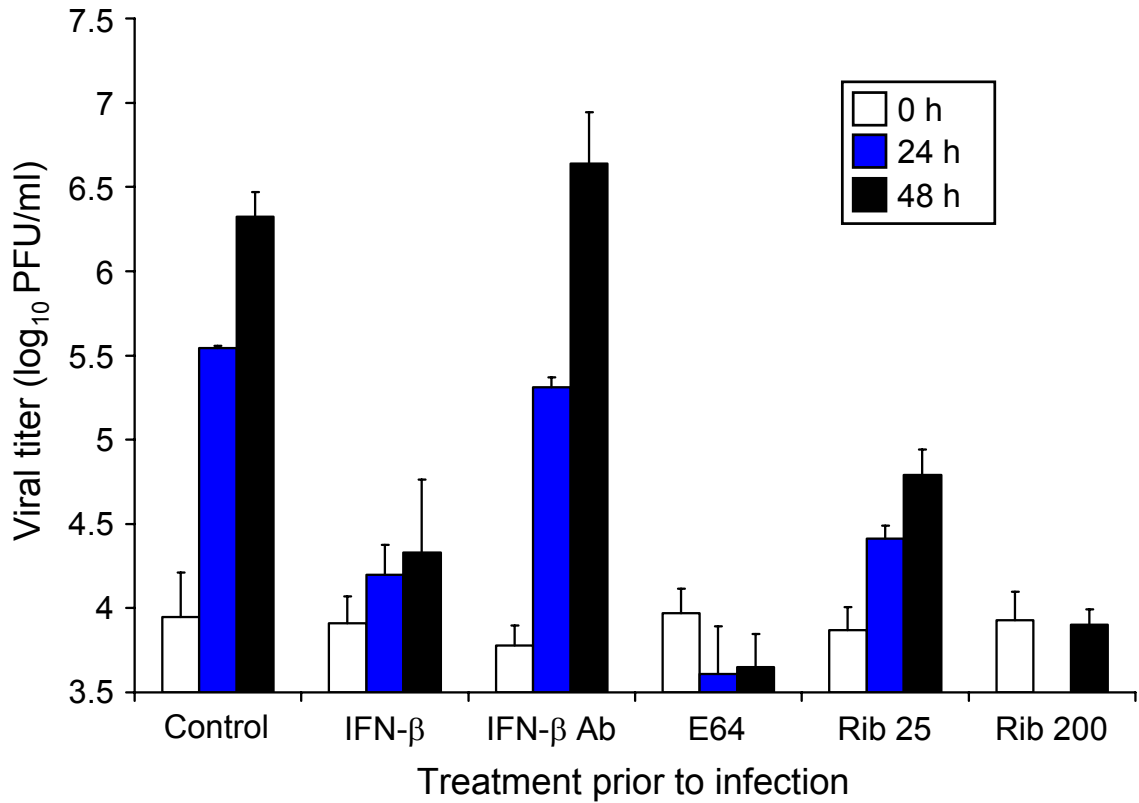
Figure 29. Reovirus growth in PCMCs. PCMCs were pretreated with either fresh medium (control), 1.2×10^4 units of IFN-β, 275 neutralizing units of IFN-β-specific antiserum, 100 μM E64, or 25 or 200 μM of ribavirin. Cells were then infected with T3SA+ at an MOI of 10 PFU/cell and incubated for the times shown. Cells and medium were frozen and thawed twice, and viral titers were determined by plaque assay. The results are expressed as the mean viral titers for three independent experiments. Error bars indicate standard deviations.

findings in the immunofluorescence assays of new viral protein synthesis, IFN-β-specific antiserum treatment had little effect on reovirus growth in PCMCs. From these data, we conclude that PCMCs are capable of supporting reovirus growth and that IFN-β reduces the production of viral progeny in these cultures.

Reovirus-induced caspase 3/7 activation in primary cardiac myocytes

To determine whether reovirus is capable of inducing apoptotis in PCMCs, we infected the cultures with reovirus T3SA+ and assessed apoptosis by quantitation of caspase 3/7 activity (Fig. 30). In comparison to mock-infected cells, levels of caspase 3/7 activity were substantially greater in cells infected with T3SA+. Similar to viral growth assays, caspase 3/7 activity was diminished by pretreatment with IFN-β and completely abolished by either E64 or ribavirin. Caspase 3/7 activity in PCMCs treated with IFN-β-specific antiserum prior to reovirus infection was unaffected in comparison to mock-infected PCMCs. These data indicate that inhibition of caspase 3/7 activity by IFN-β correlates with inhibition of viral growth.

Reovirus-induced caspase 3/7 activation in cardiac myocytes lacking the IFN-α/β receptor

To obtain additional evidence for an antiviral function of IFN-β in reovirus infection of PCMCs, we generated cardiac myocytes from mice deficient in the IFN-α/β receptor (119). These cultures were tested for the capacity to support reovirus growth by plaque assay (Fig. 31). Reovirus produced high titers in IFN-α/β receptor-null PCMCs at 24 and 48 h post-infection, reaching yields in excess of 50-fold at the later time point. Viral growth was
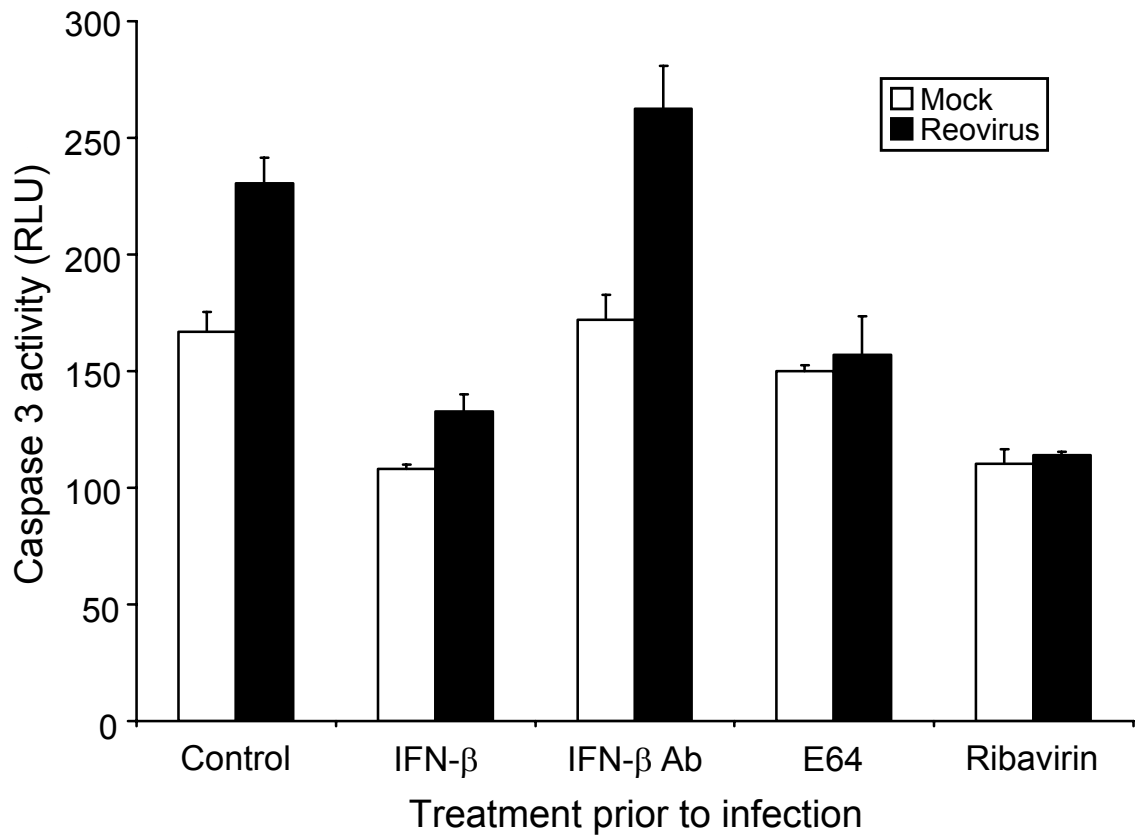
Figure 30. IFN diminishes apoptosis in PCMCs following reovirus infection. PCMCs were pretreated with either fresh medium (control), 1.2 x 10⁴ units of IFN-β, 275 neutralizing units of IFN-β-specific antiserum, 100 μM E64, or 200 μM ribavirin. Cells were then either mock-infected or infected with reovirus T3SA+ at an MOI of 100 PFU/cell for 24 h. Caspase 3/7 activity was quantified by using a luminescent substrate. The results are expressed as the mean caspase activity relative to mock-infected cells for three independent experiments. Error bars indicate standard deviations.
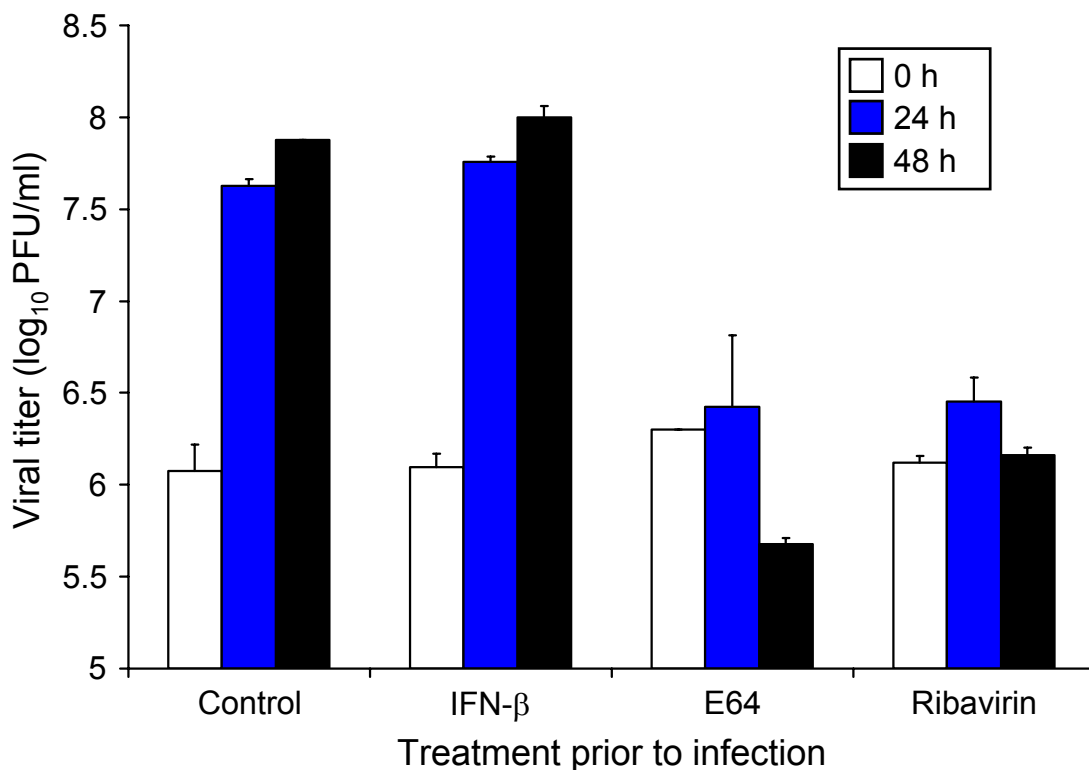
Figure 31. Reovirus growth in PCMCs lacking the IFN-α/β receptor. IFN-α/β receptor-null PCMCs were pretreated with either fresh medium (control), 1.2×10^4 units of IFN-β, 100 μM E64, or 200 μM ribavirin. Cells were then infected with T3SA+ at an MOI of 10 PFU/cell and incubated for the times shown. Cells and medium were frozen and thawed twice, and viral titers were determined by plaque assay. The results are expressed as the mean viral titers for three independent experiments. Error bars indicate standard deviations.
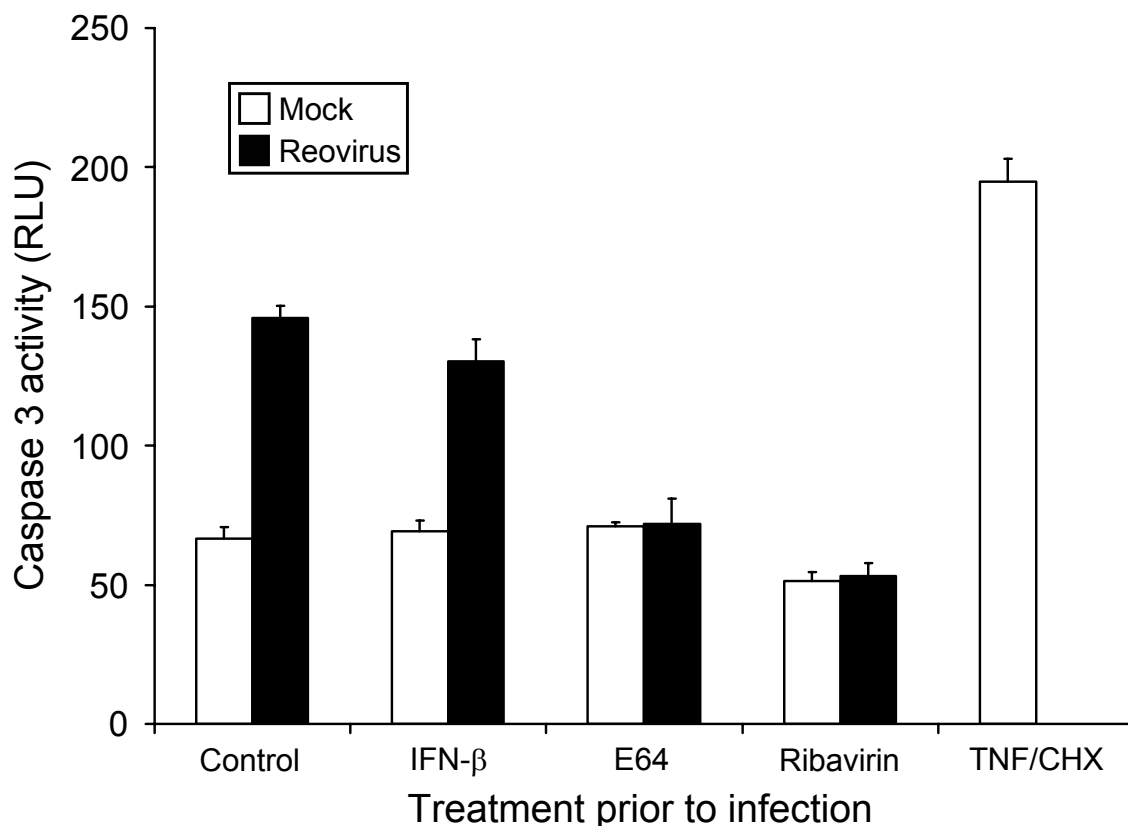
Figure 32. Reovirus induces apoptosis in PCMCs lacking the IFN-α/β receptor. IFN-α/β receptor-null PCMCs were pretreated with either fresh medium (control), 1.2×10^4 units of IFN-β, 100 μM E64, or 200 μM ribavirin. Cells were then either mock-infected, infected with reovirus T3SA+ at an MOI of 100 PFU/cell for 24 h, or treated with 10 ng/ml of TNFα and 10 μg/ml of cycloheximide (CHX) for 12 h. Caspase 3/7 activity was quantified by using a luminescent substrate. The results are expressed as the mean caspase activity relative to mock-infected cells for three independent experiments. Error bars indicate standard deviations.

blocked by treating PCMCs with either E64 or ribavirin prior to infection. As expected, treatment with IFN-β had no effect on PCMCs lacking expression of the relevant receptor.

We next investigated whether reovirus is capable of inducing apoptosis in cardiac myocytes lacking the IFN-α/β receptor. IFN-α/β receptor-null PCMCs were infected with reovirus T3SA+, and apoptosis was assessed by quantitation of caspase 3/7 activity (Fig. 32). Reovirus induced substantial levels of caspase 3/7 activity in the receptor-null PCMCs, and this activity was blocked by treatment of cells with either E64 or ribavirin prior to infection. The increase in caspase 3/7 activity induced by reovirus in IFN-α/β receptor-null PCMCs paralleled that in reovirus-infected wild-type PCMCs (compare Figs. 30 and 31). Therefore, signaling from the IFN-α/β receptor during reovirus infection of these cells does not contribute to apoptosis.

Discussion

This study was designed to determine the relationship of IFN, viral replication, and apoptosis in reovirus-infected cardiac myocytes. Two important conclusions emerge from these experiments. First, reovirus infection of PCMCs leads to increased caspase 3/7 activity, indicative of apoptosis. Second, treatment with IFN-β inhibits reovirus growth and decreases virus-induced apoptosis in PCMCs. Together, these findings suggest that IFN-β-mediated inhibition of reovirus replication in cardiac myocytes diminishes apoptotic cell death.

Myocytes lost or damaged during viral infection are not replenished, which raises the possibility that these cells have a unique response in the presence of virus. Previous reports indicate that basal levels of IFN-β are higher in PCMCs than in primary cardiac fibroblast cultures (PCFCs) (181). Furthermore, PCMCs express more IFN-β than PCFCs when exposed to reovirus (181). This heightened IFN response suggests that these differentiated,

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nondividing, essential cells display an enhanced immune response against pathogens such as reovirus. However, we found that the pretreatment of PCMCs with an antiserum specific for IFN-β had no effect on reovirus infection (Fig. 28), growth (Fig. 29), or apoptosis (Fig. 30). Additionally, PCMCs isolated from IFN-α/β receptor-null mice are fully permissive for reovirus growth (Fig. 31) and undergo apoptosis following reovirus infection (Fig. 32). These findings suggest that IFN-β expressed by PCMCs in response to reovirus is not sufficient to inhibit viral replication or apoptosis. Collectively, these results support the hypothesis that the IFN-β responsible for inducing the antiviral response in PCMCs following reovirus infection is produced by another cell type, perhaps in PCFCs.

NF-κB induces the expression of type I IFNs following stimulation with numerous agonists (31, 56, 224). We and others previously have shown that numerous ISGs are induced in an NF-κB-dependent manner in reovirus-infected cells (45, 129, 178). Since reovirus-induced apoptosis is also NF-κB-dependent, it seemed possible that IFN-β expression as a result of NF-κB activation elicits programmed cell death in infected cells by an autocrine pathway. In support of this possibility, IFN-α/β enhances apoptosis in response to double-stranded RNA and influenza virus (185), suggesting that IFN-α/β release sensitizes cells for apoptosis in response to certain stimuli. This response could aid in viral clearance by earmarking infected cells for elimination by phagocytic cells. However, this mechanism is not involved in the cell death response in PCMCs following reovirus infection. There are three lines of evidence that support this conclusion. First, pretreatment with IFN-β diminishes apoptosis in PCMCs in response to reovirus (Fig. 30). Second, pretreatment with IFN-α/β-specific antiserum had no effect on reovirus-induced apoptosis (Fig. 30). Third, reovirus infection of IFN-α/β receptor-null PCMCs and wild-type PCMCs produced equivalent levels of apoptosis (Fig. 32). It is possible that IFN functions to sensitize some

types of cells to apoptosis following reovirus infection, but this cytokine does not enhance death signaling in cardiac myocytes.

Previous reports indicate that reovirus replication is not required for apoptosis in HeLa cells (39) and L929 cells(199). However, we found that viral replication is required for apoptosis in reovirus-infected PCMCs. Reovirus replication is inhibited in PCMCs following pretreatment with IFN-β, which also diminishes reovirus-induced apoptosis (Figs. 29 and 30). We also observed that pretreatment with RNA synthesis inhibitor ribavirin reduced both viral growth and apoptosis in reovirus-infected PCMCs (Figs. 31 and 32). These data are consistent with data gathered from animals infected with reovirus demonstrating that IFN-β treatment inhibits both viral growth and abolishes apoptosis in the heart (128). Together, these findings suggest that viral replication is required for reovirus-induced apoptosis in cardiac myocytes and that IFN-β functions in blocking apoptosis by reducing reovirus growth.

In this study, we show that reovirus growth in PCMCs is diminished by pretreatment with IFN-β and this blockade of viral replication correlates with inhibition of reovirus-induced apoptosis. We also found that endogenous IFN-β produced by PCMCs is not sufficient to limit reovirus growth, suggesting that PCMCs must be preexposed to IFN-β to inhibit viral infection. Future studies will identify the cell types responsible for producing cardioprotective IFN and elucidate mechanisms of the IFN-mediated antiviral state in cardiac myocytes.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Processes by which viruses injure and kill their host cells are essential determinants of viral pathogenesis. Although general features of virus-induced cell death are understood, little is known about the cellular sensors of viral infection that trigger cell killing. A thorough understanding of mechanisms of virus-induced apoptosis is essential for uncovering a role for this apoptotic process in viral pathogenesis and designing antiviral therapeutics targeting specific signaling pathways elicited by viruses responsible for cell death and disease. The work described in this thesis uses mammalian reovirus, a highly tractable experimental model, to elucidate intracellular signaling pathways activated by viruses to cause cell death and disease.

The data presented in this thesis support the following conclusions: 1) reovirus-induced apoptosis is independent of viral attachment to cell-surface receptors such as JAM-A and sialic acid (Chapter II); 2) reovirus stimulates IKK complexes consisting of the α and γ subunits to phosphorylate IκBα leading to NF-κB activation and apoptosis (Chapter III); 3) NF-κB activation functions in a proapoptotic manner in the brain and an antiapoptotic manner in the heart of reovirus-infected animals (Chapter IV); and 4) IFN-β mediates an antiviral state by inhibiting viral proliferation and apoptosis in cardiac myocytes (Chapter V). This chapter summarizes the data presented in this dissertation and highlights future directions for this research.

The role of µ1 in reovirus-induced apoptosis

Results from Chapter II demonstrate that signaling pathways activated by binding of

σ1 to JAM-A and sialic acid are dispensable for reovirus-mediated apoptosis. Instead, we

found that the µ1 protein plays an essential function in stimulating proapoptotic machinery.

Analysis of T1L x T3D reassortant viruses revealed that the µ1-encoding M2 gene

segment is the only viral determinant of the apoptosis-inducing capacity of reovirus when

infection is initiated via Fc receptors. Additionally, a temperature-sensitive, membrane

penetration-defective M2 mutant, tsA279.64, is an inefficient inducer of apoptosis. The

µ1 protein contains three putative membrane-interaction motifs, including the N-terminal

myristate moiety and two amphipathic α-helices (79, 93, 121, 123), and three known

proteolytic cleavage sites. These include an autocatalytic cleavage site at amino acid 42,

which separates µ1N and µ1C, and a cleavage site at amino acid 580, which releases the δ

and ϕ fragments (27, 121, 127). While cleavage at the autocatalytic site is required for

membrane penetration, the physiologic consequences of the δ-ϕ cleavage are unknown (27,

127). This cleavage occurs concomitantly with the formation of ISVPs and allows release of

δ into the cytoplasm following endosomal membrane penetration (27). A third, less

understood, cleavage leads to release of ~10 C-terminal amino acids (114).

Future studies will identify the mechanism of µ1-induced apoptosis following

reovirus infection. To determine whether µ1 is sufficient for induction of death signaling,

cells will be transfected with plasmids encoding full-length µ1, µ1 cleavage fragments, or µ1

truncation mutants and tested for NF-κB activation and apoptosis induction. This strategy

will allow us to extensively mutagenize regions within µ1 and rapidly screen for domains

that affect its capacity to induce apoptosis.

To define functional domains in µ1 important for proapoptotic signaling, we will use a reverse genetics approach recently established in our laboratory to test viruses containing of specific point mutations in the µ1 protein for the capacity to induce apoptosis. The glycine residue at the myristoylation site will be substituted with alanine, and apolar residues within the amphipathic α-helices will be replaced with polar residues to disrupt helix stability. These mutations will likely alter µ1-membrane interactions. Cleavage sites in µ1 will be altered individually and in combination to assess the role of specific µ1 cleavage events in apoptosis induction. These studies will identify regions in µ1 required for reovirus-induced apoptosis and may allow µ1 cleavage events to be disassociated from µ1-mediated membrane penetration in proapoptotic signaling.

The role of Rel subunits p52 and c-Rel in reovirus-induced apoptosis

We found in chapter III that both p52 (16 h) and c-Rel (8 h) are activated following the initial induction of NF-κB complexes containing p50 and RelA. Processing of p100 results in the formation of p52, but it is unclear how c-Rel becomes activated following reovirus infection. Levels of both IκBβ and IκBε remain unchanged following an 8 h timecourse of reovirus infection, suggesting that c-Rel is not activated via degradation of either of these IκB isoforms. Reovirus-induced c-Rel activation could result from nuclear translocation of NF-κB complexes consisting of p50 and RelA at early times of infection and induction of c-Rel expression leading to its subsequent activation in the form of c-Rel homodimers or RelA/c-Rel heterodimers. Interestingly, previous reports indicate that NF-κB activation is abolished at late times of infection (6-8 h) in both p50-

and RelA-deficient cell lines following reovirus infection (40). These findings suggest that both p50 and RelA are required for c-Rel induction in response to reovirus.

It is possible that initial activation of p50/RelA NF-κB complexes inhibits apoptosis at early times during infection and complexes containing p52 or c-Rel promote apoptosis at later times of infection. To test the role of p52 and c-Rel in reovirus-induced apoptosis, we will use cells deficient in either p52/NF-κB2 or c-Rel to determine whether reovirus is capable of inducing apoptosis in the absence of these signaling molecules. Alternatively, overexpression of the Rel subunits p50 and RelA could be used to assess whether these NF-κB complexes function to inhibit apoptosis when constitutively active during reovirus infection. Results from these experiments will define the role of p52 and c-Rel in reovirus-induced apoptosis.

The mechanism of IKK activation in response to reovirus

Results from chapter III illustrate that reovirus stimulates IKK complexes consisting of the α and γ subunits. Upon activation, IKK phosphorylates IκBα leading to the nuclear translocation of p50/RelA complexes. This novel NF-κB pathway activated by reovirus is independent of NIK, which is responsible for phosphorylating IKKα following the induction of the alternative pathway of NF-κB activation by some agonists(95, 163, 221). The mechanism elicited by reovirus that is responsible for IKK stimulation remains unresolved and is currently being investigated in our laboratory.

Since μ1 plays an important role in reovirus-induced apoptosis, we envision a mechanism in which this viral protein interacts directly with IKK inducing a conformational change in the complex and subsequent autophosphorylation of the catalytic subunits. We have begun to test this hypothesis by transiently expressing μ1 in combination with

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individual IKK subunits and performing immunoprecipitation assays with antiserum specific

for the appropriate IKK molecule followed by immunoblotting using µ1-specific antiserum.

Additional experiments along this line of inquiry will resolve whether µ1 can directly

activate IKK.

An alternative explanation for the mechanism of IKK activation in response to

reovirus is that µ1, or another viral protein, interacts with a kinase or an adaptor protein

upstream of IKK resulting in the phosphorylation of the IKK catalytic subunits. To identify

these possible interactions, yeast-two-hybrid experiments could be utilized to determine

possible µ1-interacting candidates that may potentially function in IKK activation. Mutants

of µ1 will be used to determine the domains that are required for this interaction. These

experiments will elucidate the mechanism of IKK activation in response to reovirus and

further characterize the function of µ1 in reovirus-induced apoptosis.

The role of IKK in reovirus pathogenesis

Findings reported in chapter IV indicate that NF-κB activation in the CNS leads to

high levels of neuronal apoptosis and encephalitis. In contrast, NF-κB activation in the heart

leads to IFN-β production, which limits viral replication and protects against apoptosis. In

the absence of NF-κB signaling, reovirus infection induces widespread apoptotic damage to

cardiac myocytes, resulting in myocarditis. Mechanisms underlying the divergent cellular

fates following NF-κB activation by reovirus in vivo remain unclear. Data from chapter III

suggests that IKKα and IKKγ are required for reovirus-induced NF-κB activation in cell

culture. These findings implicate the alternative pathway of NF-κB signaling in reovirus-

infected cells. However, we also have evidence indicating that p50/RelA heterodimers are

the predominant nuclear species following reovirus infection of cultured cells (Chapter III)

and in vivo (Chapter IV), consistent with the classical mode of NF-κB signaling. In light of these results, we think it possible that the differential effects of NF-κB signaling in reovirus disease could be attributable to tissue-specific activation of different IKK subunits, which in turn influence the composition of nuclear NF-κB complexes. Considering the findings presented in chapters III and IV, these mechanisms may involve tissue-specific activation of different IKK subunits, which in turn may influence the composition of nuclear NF-κB complexes. Experiments using tissue-specific IKKα- or IKKβ-deficient mice should clarify the function of IKK in reovirus-induced disease.

Conclusions

With this research, we set out to establish a better understanding of the apoptotic response induced during reovirus infection that culminates in disease. The studies performed in this thesis carefully dissect mechanisms of reovirus-induced NF-κB activation and apoptosis. The long-term goal of this work is to identify intracellular events elicited by reovirus that activate NF-κB resulting in apoptosis induction and tissue damage in the host. Knowledge gained from this research could lead to the development of therapeutics designed specifically to inhibit viral replication or block signaling pathways required for virus-induced apoptosis. Identification of a novel NF-κB activation pathway elicited by reovirus is particularly intriguing due to the potential impact in the field. These findings set the stage for precise biochemical analysis of the mechanisms employed by reovirus to induce apoptosis and cause disease.

CHAPTER VII

DETAILED METHODS OF PROCEDURE

Cells, viruses, and antibodies

Spinner-adapted murine L929 cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.) supplemented to contain 5% fetal bovine serum (Gibco-BRL, Gaithersburg, Md.), 2 mM L-glutamine, 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 0.25 mg of amphotericin per ml (Gibco-BRL). HeLa cells and MEFs were maintained in monolayer cultures in Dulbecco's minimal essential medium (Gibco-BRL) supplemented to contain 10% fetal bovine serum, L-glutamine, and antibiotics as described for L cells. CHO cells were maintained in Ham's F12 medium supplemented with fetal bovine serum, L-glutamine, and antibiotics as described for HeLa cells.

The prototype reovirus strain T3D are laboratory stocks. Reovirus strain T3SA+ was generated by reassortment of reovirus strains T1L and type 3 clone 44-MA (12). Viral stocks were prepared by plaque purification and passage in L cells (205). Purified virions were prepared by using second- and third-passage L-cell lysate stocks as previously described (58, 155). Viral particles were freon-extracted from infected-cell lysates, layered onto 1.2 to 1.4 g/cm^3 CsCl gradients, and centrifuged at 62,000 x g for 18 h. Bands corresponding to virions (1.36 g/cm^3) were collected and dialyzed in virion storage buffer (150 mM NaCl, 15 mM MgCl$_2$, 10 mM Tris [pH 7.4]). Concentrations of reovirus virions in purified preparations were determined from the equivalence 1 OD$_{260}$ = 2.1 x 10^{12} virions. The particle-to-PFU ratio of stocks used for viral infectivity assays was approximately 10-100 to 1.

Generation and characterization of mAbs 5C6, 9BG5, and 7F4, which are specific for the type 1 σ1, type 3 σ1, and λ2 proteins, respectively, have been previously described (23, 195). The immunoglobulin G (IgG) fractions of polyclonal rabbit antisera raised against T1L and T3D (206) were purified by using protein A-sepharose as previously described (10). A mixture of these sera was capable of recognizing all strains of reovirus used in this study. Protein-A purified, JAM-A-specific mAb J10.4 was obtained from Dr. Charles Parkos (Emory University) (99). Anti-Myc mAb 9E10 and anti-Fc rat mAb were purchased from BD Biosciences (San Jose, CA). Fluorescent dye-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). Antisera specific for IκBα, IκBβ, IκBε, p50, RelA/RelA, RelB, c-Rel, IKKγ, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody antiserum for p100/p52 was purchased from Upstate Biotechnology (Lake Placid, NY). The agonistic lymphotoxin-β receptor antiserum was purchased from BD Biosciences (San Jose, CA). The IKK inhibitor BAY 65-1942 was obtained from Dr. K. Ziegelbauer (Bayer Health Care AG, Leverkusen, Germany). Rabbit polyclonal antiserum specific to the activated form of caspase 3 (anti-caspase 3/Asp 175) was purchased from Cell Signaling Technology (Danvers, MA). IFN-β and IFN-α/β-specific antiserum was purchased from Calbiochem (San Diego, CA).

Generation of CHO cells stably expressing JAM-A or JAM-AΔCT

Human JAM-A was subcloned into expression plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA). Truncation mutant JAM-AΔCT was generated by PCR using full-length JAM-A cDNA as template. Sequences encoding amino acids 1-260 (Δ261-299) were cloned and appended with a stop codon using T7 primer and 5'-TACGGGATCCTCAGGCAAACCAGATGCC-3' as forward and reverse primers,

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respectively. The PCR product was digested with *Bam*HI and introduced into pcDNA3.1

using complementary restriction sites. Fidelity of cloning was confirmed by automated

sequencing. CHO cells stably expressing empty vector alone, JAM-A, and JAM-AΔCT were

generated by transfection of CHO-K1 cells with empty pcDNA3.1 vector, pcDNA3.1

encoding JAM-A, or pcDNA3.1 encoding JAM-AΔCT. Cells were grown to 50%

confluence in 6 cm dishes (Costar, Cambridge, MA) and transfected with 4 μg of each

plasmid by using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's

instructions. Transfected cells were selected by growth in the presence of 1 mg per ml G418.

After the tenth passage in the presence of G418, cells expressing high levels of JAM-A as

assessed by JAM-A-specific mAb J10.4 staining (mean fluorescence intensity > 5000) were

separated using a BD FACsort cell sorter (Becton-Dickinson, Palo Alto, CA).

Immunoblot assays

Following treatment, whole-cell extracts were generated by incubation with lysis

buffer (10 mM HEPES [pH7.4], 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT,

0.1% Igepal, 1 mM $Na_4O_7P_2$, 1 mM NaF, 1 mM $NaVO_3$, and 1 μM microsystin). Extracts

(10-50 μg total protein) were resolved by electrophoresis in 10% polyacrylamide gels and

transferred to nitrocellulose membranes. Membranes were blocked at 4ºC overnight in

blocking buffer (PBS containing 0.1% Tween-20 and 5% BSA). Immunoblots were

performed by incubating the membranes with primary antibodies diluted 1:500 to 1:2000 in

blocking buffer at room temperature for 1 h. Membranes were washed three times for 10 min

each with washing buffer (PBS containing 0.1 Tween-20) and incubated with horseradish

peroxidase-conjugated goat anti-rabbit (Amersham Biosciences, Piscataway, NJ) and bovine

anti-goat antibodies (Santa Cruz Biotechnology) diluted 1:2000 and 1:3000, respectively.

Following three washes, membranes were incubated for 1 min with chemiluminescent

peroxidase substrate (Amersham Biosciences) and exposed to film. Band intensity was

quantified using the Image J program (NIH, Bethesda, MD).

Flow cytometric analysis for JAM-A surface expression

CHO cells stably transfected with empty vector, JAM-A, or JAM-AΔCT were

detached from plates using PBS containing 10 mM EDTA. Cells were washed twice with

chilled PBS prior to incubation with 10 μg per ml of either JAM-A specific mAb J10.4 or an

isotype-matched control mAb at 4°C for 1 h. Cells were stained with a 1:1000 dilution of

PE-labeled anti-mouse IgG at 4°C for 1 h and analyzed using a FACScan flow cytometer

(Becton-Dickinson).

Fluorescent-focus assays of viral infectivity

Monolayers of cells (2 x 10^5) in 24-well plates (Costar) were adsorbed with various

MOIs of reovirus at room temperature for 1 h. Following removal of the inoculum, cells

were washed with PBS and incubated in complete medium at 37°C for 18 h to permit

completion of a single cycle of viral replication. Monolayers were fixed with 1 ml of

methanol at -20°C for a minimum of 30 min, washed twice with PBS, blocked with 2.5% Ig-

free bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS, and incubated at room

temperature for 1 h with polyclonal rabbit anti-reovirus serum at a 1:1000 dilution in PBS-

0.5% Triton X-100. Monolayers were washed twice with PBS-0.5% Triton X-100 and

incubated with a 1:1000 dilution of Alexa546-labeled anti-rabbit IgG. Monolayers were

washed with PBS, and infected cells were visualized by indirect immunofluorescence using

an Axiovert 200 fluorescence microscope (Carl Zeiss, New York, NY). Infected cells were

identified by the presence of intense cytoplasmic fluorescence that was excluded from the nucleus. No background staining of uninfected control monolayers was noted. Reovirus antigen-positive cells were quantified by counting fluorescent cells in at least three random fields of view in duplicate wells at a magnification of 20X.

Antibody-mediated infections

Reovirus virions (1×10^{11}) were incubated at 4°C overnight with various concentrations of mAb in 1 ml PBS. Cells were adsorbed with virus at an MOI of either 100 or 500 PFU per ml at room temperature for 1 h. Following removal of the inoculum, cells were washed with PBS and incubated in complete medium at 37°C for various intervals. For inhibition of viral disassembly and RNA synthesis, cells were maintained in 20 mM AC or 200 µM ribavirin, respectively, throughout the course of infection. For inhibition of disassembly, cells also were pretreated for 1 h with medium containing 20 mM AC.

Quantitation of apoptosis by acridine orange (AO) staining

Cells (2×10^5) grown in 24-well tissue-culture plates were adsorbed with reovirus virions or mAb-virion complexes at various MOIs. Following incubation at 37°C for 48 h, the percentage of apoptotic cells was determined by using AO staining as previously described (199). For each experiment, 200 to 300 cells were counted in three independent wells, and the percentage of cells exhibiting condensed chromatin was determined by epi-illumination fluorescence microscopy using a fluorescein filter set (Carl Zeiss).

Electrophoretic mobility shift assay (EMSA)

Cells (3×10^6) grown in 100 mm tissue-culture dishes (Costar, Cambridge, MA) were treated with 20 ng/ml of TNF-α (Sigma-Aldrich, St. Louis, MO), adsorbed with T3D at a multiplicity of infection (MOI) of 100 plaque forming units (PFU)/cell, or treated with gel saline (mock-infection). After incubation at 37°C for various intervals, nuclear extracts (10 μg total protein) were assayed for NF-κB activation by an EMSA using a ^{32}P-labeled oligonucleotide consisting of the NF-κB consensus-binding sequence (Santa Cruz Biotechnology) as previously described (40). For supershift assays, 2 μg of rabbit polyclonal antiserum specific for p50, p52, RelA, RelB, or c-Rel was added to the binding-reaction mixtures and incubated at 4°C for 30 min prior to the addition of radiolabeled oligonucleotide. Nucleoprotein complexes were subjected to electrophoresis in native 5% polyacrylamide gels at 180 V for 90 min, dried under vacuum, and exposed to Biomax MR film (Kodak, Rochester, NY). Band intensity was quantified by determining photostimulus luminescence (PSL) units using a Fuji2000 phosphor imager and the Multi Gauge software (Fuji Medical Systems, Inc., Stamford, CT).

Kinase assay

Cells (8×10^5) were treated with 20 ng/ml of TNFα, adsorbed with T3D at an MOI of 100 PFU/cell, or mock-infected. Whole-cell extracts were incubated with an IKKγ-specific antiserum in the presence of ELB buffer (50 mM HEPES [pH 7.4], 250 mM NaCl, 5 mM EDTA, 0.1% Igepal). Immunoprecipitates were equilibrated in kinase buffer (10 mM HEPES [pH 7.4], 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 12.5 mM β-glycerophosphate, 50 μM Na_3VO_4, 2 mM NaF) and incubated with 10 μM ATP, 5 μCi of [γ-^{32}P]ATP (Perkin Elmer), and 1 μg of recombinant GST protein fused to amino acids 1-54 of

IκBα (GST-IκBα) at 30°C for 30 minutes (32). Kinase reactions were terminated by heat

denaturation in the presence of 1% SDS. Radiolabeled products were resolved by SDS-

PAGE, transferred to nitrocellulose membranes, and visualized by autoradiography.

Caspase 3 activity assay

Cells (3×10^3) grown in clear-bottom, black-walled, 96-well tissue-culture plates

(Costar) were inoculated with 10 ng/ml of TNFα, a combination of 10 ng/ml of TNFα and 10

μg/ml of cycloheximide (Sigma-Aldrich), T3D at an MOI or 1000 PFU/cell, or mock-

infected. Following incubation at 37°C for 24 and 12 h with respect to T3D- or TNFα-

treatment, caspase 3 activity was quantified by using the Caspase-Glo 3/7 Assay (Promega).

Luminescence was detected by using a Topcount NXT luminometer (Packard Biosciences

Co., Meriden, CT).

Trypan blue exclusion assay

Cells (4×10^4) grown in 6-well tissue-culture plates were inoculated with 10 ng/ml of

TNFα, a combination of 10 ng/ml of TNFα and 10 μg/ml of cycloheximide, T3D at an MOI

of 1000 PFU/cell, or mock-infected. Following incubation at 37°C for 48 and 24 h with

respect to T3D- or TNFα-treatment, cells were collected and washed with PBS. The cell

pellet was resuspended in 50 ml of PBS and stained using 100 ml of a solution containing

0.4% trypan blue (Kodak) in PBS. For each experiment, 200 to 300 cells were counted and

the percentage of cell death was determined by light microscopy (Axiovert 200; Zeiss,

Oberköchen, Germany).

Mice and inoculations

HIV long-terminal repeat luciferase (HLL) mice were generated as described previously (18). Control p50+/+ mice (B6129PF1/J-A^{W-J}/A^W) and p50-/- mice (B6129P-Nfkb1^{tm1Bal}) (165) were obtained from the Jackson Laboratory. Newborn mice weighing 2.0-2.5g (2-4 days old) were inoculated either intracranially or perorally with purified T3SA+ diluted in PBS. Intracranial inoculations were delivered to the right and left hemispheres (5 μl each) using a Hamilton syringe (BD Biosciences) and a 30-gauge needle (196). Peroral inoculations were delivered into the stomach (50 ml) by passage of a polyethylene catheter 0.61 mm in diameter (BD Biosciences) through the esophagus (154). The inoculum contained 0.5% (vol/vol) green food coloring so that accuracy of delivery could be judged. For determination of NF-kB activation, viral titer, and immunohistochemical staining, mice were euthanized at various intervals following inoculation and organs were collected.

Assessment of NF-κB activation by in vivo luciferase activity

Two-day-old HLL mice were inoculated perorally with either 10^4 PFU T3SA+ or PBS. Mice were anesthetized with isoflurane before imaging and immobilized for the duration of the integration time of photon counting (3 minutes). Luciferin (0.75 g/mouse in 0.2 ml isotonic saline) was inoculated intraperitoneally and mice were imaged using an intensified charge-coupled devise camera (C2400-32; Hamamatsu Corp.). For the duration of photon counting, mice were placed inside a light-tight box. Light emission from each mouse was detected as photon counts and a digital false-color photon emission image of the mouse was generated.

In vivo EMSAs

Two-day-old p50+/+ and p50-/- mice were inoculated perorally with either 10^4 PFU T3SA+ or PBS. Mice were euthanized 12 days after inoculation. Brains and hearts were aseptically removed, snap frozen on dry ice, and stored at -70°C. Organs were weighed, placed in a mortar with liquid nitrogen, and ground into a powder. Lysis buffer (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) was added in a ratio of 1 ml per 200 mg of tissue. Samples were frozen and thawed 3 times and centrifuged at 12,000 g for 10 minutes. The supernatant was used as the whole-cell extract.

Whole-cell extracts (10 μg total protein) were assayed for NF-κB activation by EMSA using a ^{32}P-labeled oligonucleotide (1 ng) consisting of the NF-κB consensus binding sequence (Santa Cruz Biotechnology Inc.) as described above and previously (40). For competition experiments, unlabeled consensus oligonucleotide at various concentrations was added to the reaction mixtures along with radiolabeled oligonucleotide. For supershift experiments, 1 μl of a rabbit polyclonal antiserum specific to RelA (250 μg/ml; Santa Cruz Biotechnology Inc.) was added to the binding reaction mixtures and incubated at 4°C for 30 minutes prior to the addition of radiolabeled oligonucleotide. Nucleoprotein complexes were subjected to electrophoresis in native polyacrylamide gels, which were dried and exposed to film.

Determination of viral titer in infected organs.

Organs (intestine, liver, heart, and brain) from infected p50+/+ and p50-/- were placed into vials containing 1 ml gelatin saline, frozen (-20°C) and thawed once, and

sonicated for 20 seconds. Titers of virus present in organ homogenates were determined by plaque assay using L cell monolayers (205).

Histology, immunohistochemistry, and TUNEL staining

Litters of newborn p50+/+ and p50-/- mice were inoculated intracranially or perorally with either 10^4 PFU T3SA+ or PBS. Mice were euthanized, and brain and heart tissues were fixed in 10% buffered paraformaldehyde. Fixed organs were embedded in paraffin, and 6-μm histological sections were prepared. Sections were stained with H&E for evaluation of histopathologic changes, processed for immunohistochemical detection of reovirus antigen, assayed for DNA fragmentation using the TUNEL technique (201), or processed for the immunohistochemical detection of activated caspase 3 (12). Cells demonstrating TUNEL staining were quantitated separately in each parasagittal brain section in the following regions: cerebral cortex, hippocampus, basal ganglia, diencephalons, and brain stem. The mean number of positive cells per region was determined for each treatment group and time point. Observers were blinded to the identity of the mouse strain and the nature of the inoculum.

Echocardiography

Echocadiography was perfomed on conscious 10-day-old pups as previously described for adult mice (152) except that the total field depth was set to 1 cm (minimum possible), and external heating and rapid sample acquisition were used to prevent excessive heat loss. Electrocardiograms were digitally sampled and correspons to the usual surface lead I.

RNA isolation and real-time PCR

Two-day-old p50+/+ and p50-/- mice were inoculated perorally with either 10^4 PFU

T3SA+ or PBS. Mice were euthanized, and brain and heart tissues were homogenized using

a Dounce homogenizer. RNA was extracted from brain and heart homogenates using the

TRIZOL RNA extraction protocol (Invitrogen Corp.). RNA (3 μg) was used in a reverse

transcription reaction containing x10 buffer, 25 mM MgCl$_2$, 100 μM dithiothreitol, 1 U

RNasin (Promega), 10 mM dNTPs, 50 μM random hexamers, and 1 U AMV reverse

transcriptase (Promega). The reaction was incubated at 43°C for 1 h and then at 95°C for 10

min.

Real-time PCR reactions were carried out using the Bio-Rad iCycler and iQ

Supermix buffer containing DNA polymerase and SYBR Green (Bio-Rad Laboratories).

Two to 3 replicate amplification reactions were performed in 96-well plates (Bio-Rad

Laboratories). Each reaction contained 12.5 μl iQ Supermix buffer, 300 nM forward and

reverse primers, and 1 μl cDNA in a final volume of 25 μl. Primers for the reactions were as

follows: A) IFN-β forward, 5'-GGAGATGACGGAGAAGATGC-3', B) IFN-β reverse, 5'-

CCCAGTGCTGGAGAAATTGT-3', C) GAPDH forward, 5'-

CAACTACATGGTCTACATGTTC-3', D) GAPDH reverse, 5'-

CTCGCTCCTGGAAGATG-3'. Cycling conditions were as follows: 95°C for 10 min and

then 45 cycles at 95°C for 15 sec, 60°C for 30 sec, and 72°C for 15 sec.

Data were analyzed using Bio-Rad iCycler PCR detection and analysis software

version 3.0 (Bio-Rad Laboratories). DNA was quantitated using the standard curve method

with the background subtracted. Known concentrations of cDNA were used to obtain the

standard curve for each gene (concentrations between 0.0228 and 710 ng). A melting curve

was determined for each sample to detect primer dimers, in which case data were not used. Results are expressed as values for IFN-β cDNA divided by those for GAPDH cDNA.

IFN-β treatment of mice

Two-day-old p50-/- mice were inoculated intraperitoneally with either 5 x 10^4 U IFN-β (Calbiochem) suspended in PBS containing 0.1% BSA or PBS alone in a volume of 25 μl 1 day prior to peroral inoculation with 10^4 PFU T3SA+. Infected mice were treated daily for 9 days with either IFN-β or PBS. On day 10, following viral inoculation, animals were euthanized and organs were removed. Organs were processed for determination of viral titer and histopathological analysis.

Animal husbandry and experimental procedures were performed in accordance with NIH Public Health Service policy and approved by the Vanderbilt University School of Medicine Institutional Animal Care and Use Committee.

Primary cardiac myocyte generation

To generate primary cardiac myocyte cultures, 2- to 4-day-old neonates were euthanized and the apical two-thirds of the hearts were removed, minced, and trypsinized (13). Cells were plated at a density of 1.25 x 10^6 per well in six-well plates (Costar) and incubated for 1.5 to 2 h in order to isolate rapidly adhering cardiac fibroblasts. Cardiac myocytes were resuspended in DMEM (Gibco BRL) supplemented with 7% fetal calf serum (Gibco BRL), 0.06% thymidine (Sigma-Aldrich) and plated as indicated for each procedure. Myocyte cultures contained 5 to 20% fibroblasts, consistent with levels reported by others (65, 72, 113), and consistent with cell heterogeneity in the heart.

Statistical analysis

Mean values obtained in infectivity and apoptosis assays were compared using the unpaired Student's *t* test as applied with Microsoft Excel software. *P* values of less than 0.05 were considered to be statistically significant. The contribution of each of the reovirus gene segments to apoptosis induction was assessed by using the non-parametric Mann-Whitney test without adjusting for multiple comparisons and the panel of T1L x T3D reassortant viruses as previously described ((145)).

APPENDIX A

JAM-A-INDEPENDENT, ANTIBODY-MEDIATED UPTAKE OF REOVIRUS INTO

CELLS LEADS TO APOPTOSIS

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JAM-A-Independent, Antibody-Mediated Uptake of Reovirus into Cells Leads to Apoptosis

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Apoptosis plays a major role in the cytopathic effect induced by reovirus following infection of cultured cells and newborn mice. Strain-specific differences in the capacity of reovirus to induce apoptosis segregate with the S1 and M2 gene segments, which encode attachment protein σ1 and membrane penetration protein μ1, respectively. Virus strains that bind to both junctional adhesion molecule-A (JAM-A) and sialic acid are the most potent inducers of apoptosis. In addition to receptor binding, events in reovirus replication that occur during or after viral disassembly but prior to initiation of viral RNA synthesis also are required for reovirus-induced apoptosis. To determine whether reovirus infection initiated in the absence of JAM-A and sialic acid results in apoptosis, Chinese hamster ovary (CHO) cells engineered to express Fc receptors were infected with reovirus using antibodies directed against viral outer-capsid proteins. Fc-mediated infection of CHO cells induced apoptosis in a σ1-independent manner. Apoptosis following this uptake mechanism requires acid-dependent proteolytic disassembly, since treatment of cells with the weak base ammonium chloride diminished the apoptotic response. Analysis of T1L × T3D reassortant viruses revealed that the μ1-encoding M2 gene segment is the only viral determinant of the apoptosis-inducing capacity of reovirus when infection is initiated via Fc receptors. Additionally, a temperature-sensitive, membrane penetration-defective M2 mutant, *ts*A279.64, is an inefficient inducer of apoptosis. These data suggest that signaling pathways activated by binding of σ1 to JAM-A and sialic acid are dispensable for reovirus-mediated apoptosis and that the μ1 protein plays an essential role in stimulating proapoptotic signaling.

The mammalian reoviruses are prototype members of the *Orthoreovirus* genus within the *Reoviridae* family (70). These viruses are composed of two concentric icosahedral capsids enclosing a segmented, double-stranded RNA genome (46). Reoviruses are highly virulent in newborn mice and injure a variety of organs, including the brain, heart, and liver (70). Apoptosis induced as a consequence of reovirus infection plays an important role in the pathogenesis of both reovirus-induced encephalitis (48, 50, 57) and myocarditis (20, 21, 50). Reoviruses also induce apoptosis of cultured cells (19, 59, 71).

Reovirus infection is initiated by the attachment of virions to cell surface receptors via the σ1 protein (37, 74). The σ1 protein of all three reovirus serotypes engages junctional adhesion molecule-A (JAM-A) (5, 11, 27, 53). The σ1 protein also binds to cell surface carbohydrate; however, the type of carbohydrate bound varies with serotype (1, 23, 51, 52). After receptor binding, virions are internalized into cells by receptor-mediated endocytosis (7, 26). Virions undergo acid-dependent proteolytic disassembly within cellular endosomes, leading to the formation of infectious subvirion particles (ISVPs) (2, 8, 15, 24, 63, 66). ISVPs are characterized by the loss of outer-capsid protein σ3, a conformational change in attachment pro-

tein σ1, and cleavage of outer-capsid protein μ1 to form particle-associated fragments δ and φ (13, 44, 47). Subsequent to ISVP formation the σ1 protein is shed, and the μ1 cleavage fragments undergo conformational rearrangement, yielding ISVP*s (12, 14). ISVP*s are putative entry intermediates that penetrate endosomes and deliver transcriptionally active cores into the cytoplasm (45, 49).

Clues about mechanisms by which reoviruses induce apoptosis first emerged from studies of strain-specific differences in the efficiency of apoptosis induction. Reovirus strain type 3 Dearing (T3D) induces apoptosis in cultured cells more efficiently than strain type 1 Lang (T1L) (17, 59, 71). Studies using T1L × T3D reassortant viruses demonstrated that these strain-specific effects are determined by the viral S1 and M2 gene segments (59, 71, 72). The S1 gene encodes the attachment protein σ1 (37, 74), and the M2 gene encodes membrane penetration protein μ1 (38, 44, 49). Thus, these genetic experiments suggest critical functions for the σ1 and μ1 proteins in apoptosis induction by reovirus.

Analysis of reovirus strains T3/C44-SA− (T3SA−) and T3/C44MA-SA+ (T3SA+), which are isogenic at all loci except for a single amino acid polymorphism in σ1 (4), has pointed to an important role for sialic acid binding in reovirus-induced apoptosis (17). Sialic-acid-binding strain T3SA+ induces apoptosis significantly more efficiently than non-sialic-acid-binding strain T3SA−. Concordantly, removal of cell surface sialic acid with neuraminidase or blockade of virus binding to cell surface sialic acid using a soluble competitor, sialyllactose, abolishes the capacity of T3SA+ to induce apoptosis (17). However, engagement of sialic acid is not sufficient to induce apoptosis.

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Blockade of σ1 binding to JAM-A using either σ1- or JAM-A-specific monoclonal antibodies (MAbs) also diminishes the apoptosis-inducing capacity of sialic-acid-binding reoviruses (5, 71). Collectively, these data demonstrate that reovirus strains that bind to both JAM-A and sialic acid are the most potent inducers of apoptosis.

In addition to receptor binding, postattachment events also are required for reovirus-mediated apoptosis induction (18). Inhibition of viral disassembly using ammonium chloride (AC), a weak base that increases vacuolar pH (43), or E64, an inhibitor of cysteine proteases such as those contained in the endocytic compartment (3), abolishes reovirus-induced apoptosis. On the other hand, interference with steps in viral replication subsequent to ISVP formation and membrane penetration using ribavirin, an inhibitor of viral RNA synthesis (55), does not perturb apoptosis induced by reovirus (18). Thus, in addition to sialic acid- and JAM-A-mediated attachment of reovirus to cells, replication steps during or after viral disassembly that occur before the cytoplasmically delivered core becomes transcriptionally active also contribute to reovirus-induced apoptosis. Since the M2-encoded μ1 protein functions in virus-induced endosomal membrane penetration following disassembly but prior to viral RNA synthesis (38, 44, 49), the deleterious effects of reovirus disassembly inhibitors on apoptosis induction suggest a functional link between the M2 gene segment and differences in the efficiency of apoptosis exhibited by different reovirus strains (59, 71, 72).

In this study, we determined whether reovirus is capable of inducing apoptosis independent of JAM-A and sialic acid binding. We found that antibody-mediated uptake of reovirus into JAM-A-negative, Fc-receptor-expressing cells results in productive infection and leads to apoptosis in a σ1-independent fashion. Moreover, apoptosis induced following this uptake pathway also is dependent on viral disassembly. Analysis of reassortant viruses and an M2 mutant virus demonstrates that the μ1 protein influences the strength of proapoptotic signaling following reovirus infection. These data suggest that signaling induced as a result of σ1 interactions with JAM-A and sialic acid are not necessary for apoptosis induced by reovirus and that the μ1 protein is the viral factor that stimulates the cellular apoptotic machinery.

MATERIALS AND METHODS

Cells, viruses, and antibodies. HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented to contain 10% fetal bovine serum (FBS), 50 U per ml of penicillin, and 50 μg per ml streptomycin (Invitrogen, Carlsbad, CA). Chinese hamster ovary (CHO) cells were maintained in HAM's F12 medium supplemented to contain 10% FBS, 50 U per ml of penicillin, and 50 μg per ml streptomycin. For maintenance of stably transfected CHO cells, the medium was additionally supplemented to contain 1 mg per ml G418 (Invitrogen). CHO-B1 cells, which express the B1 isoform of murine Fc receptor II, were obtained from Ira Mellman (Yale University). Cells were maintained in MEM Alpha supplemented to contain 10% FBS, 50 U per ml of penicillin, 50 μg per ml streptomycin, and 10 μM methotrexate (EMD Biosciences, San Diego, CA) as previously described (34).

T1L and T3D are laboratory stocks. Isolation and characterization of T3SA− and T3SA+ have been previously described (4). Reovirus temperature-sensitive mutant tsA279.64 was obtained from Kevin Coombs (University of Manitoba) (30). Purified reovirus virions were generated by using second- or third-passage L-cell lysate stocks of twice-plaque-purified reovirus as previously described (28). Viral particles were freon-extracted from infected cell lysates, layered onto 1.2- to 1.4-g/cm^3 CsCl gradients, and centrifuged at 62,000 × g for 18 h. Bands corresponding to virions (1.36 g/cm^3) were collected and dialyzed in virion

storage buffer (150 mM NaCl, 15 mM MgCl$_2$, 10 mM Tris-HCl [pH 7.4]). Concentrations of reovirus virions in purified preparations were determined from an equivalence where an optical density at 260 nm of 1 equaled 2.1 × 10^{12} virions (64).

Reovirus T3D virions were inactivated using a UV cross-linker (Ultra-lum-UVC-508; Marsh Biomedical, Rochester, NY). Virus at a concentration of 10^9 PFU per ml was irradiated by using short-wave (254 nm) UV on ice at a distance of 10 cm for 5 min at 1,20,000 μJ/cm^2 in a 6-cm tissue culture dish (Costar, Cambridge, MA). These conditions are sufficient to reduce viral infectivity to levels that are undetectable by fluorescent focus assays using confluent L929 cell monolayers (data not shown).

Generation and characterization of MAbs 5C6, 9BG5, and 7F4, which are specific for the type 1 σ1, type 3 σ1, and λ2 proteins, respectively, have been previously described (10, 73). The immunoglobulin G (IgG) fractions of polyclonal rabbit antisera raised against T1L and T3D (76) were purified by using protein A-Sepharose as previously described (4). A mixture of these sera was capable of recognizing all strains of reovirus used in this study. Protein A-purified, JAM-A-specific MAb J10.4 was obtained from Charles Parkos (Emory University) (40). Human coxsackievirus and adenovirus receptor (hCAR)-specific MAb RmcB was provided by Jeffrey Bergelson (University of Pennsylvania). Anti-Myc MAb 9E10 and rat anti-Fc MAb were obtained from BD Biosciences (San Jose, CA). Fluorescent dye-conjugated secondary antibodies were obtained from Molecular Probes (Invitrogen).

Generation of CHO cells stably expressing JAM-A or JAM-AΔCT. Human JAM-A was subcloned into expression plasmid pcDNA3.1 (Invitrogen). Truncation mutant JAM-AΔCT was generated by PCR using full-length JAM-A cDNA as template. Sequences encoding amino acids 1 to 260 (Δ261 to 299) were cloned and appended with a stop codon using T7 primer and 5′-TACGGGATCCTCAGGCAAACCAGATGCC-3′ as forward and reverse primers, respectively. The gene-specific primer encompasses nucleotides 981 to 995 of the JAM-A cDNA. The PCR product was digested with BamHI and introduced into pcDNA3.1 using complementary restriction sites. Fidelity of cloning was confirmed by automated sequencing. CHO cells stably expressing empty vector alone, JAM-A, and JAM-AΔCT were generated by transfection of CHO-K1 cells with empty pcDNA3.1 vector, pcDNA3.1 encoding JAM-A, or pcDNA3.1 encoding JAM-AΔCT. Cells were grown to 50% confluence in 6-cm dishes and transfected with 4 μg of each plasmid by using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected by growth in the presence of 1 mg per ml G418. After the 10th passage in the presence of G418, cells expressing high levels of JAM-A as assessed by JAM-A-specific MAb J10.4 staining (mean fluorescence intensity, >5,000) were separated using a BD FACsort cell sorter (Becton-Dickinson, Palo Alto, CA).

Immunoblot for JAM-A. Cell extracts were prepared from CHO cells (1 × 10^6) transfected with empty vector, JAM-A, or JAM-AΔCT by sonication in phosphate-buffered saline (PBS) supplemented to contain protease inhibitor cocktail (Roche, Indianapolis, IN). Extracts were resolved by electrophoresis in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblots were performed by using JAM-A-specific MAb J10.4 followed by horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ), each diluted 1:1,000 in PBS containing 0.1% Tween 20 and 5% low-fat dry milk.

Flow cytometric analysis for JAM-A surface expression. CHO cells stably transfected with empty vector, JAM-A, or JAM-AΔCT were detached from plates using PBS containing 10 mM EDTA. Cells were washed twice with chilled PBS prior to incubation with 10 μg per ml of either JAM-A specific MAb J10.4 or control MAb (hCAR-specific MAb) at 4°C for 1 h. Cells were stained with a 1:1,000 dilution of phycoerythrin (PE)-labeled anti-mouse IgG at 4°C for 1 h and analyzed using a FACScan flow cytometer (Becton-Dickinson).

Assessment of viral infectivity by indirect immunofluorescence. Monolayers of cells (2 × 10^5) in 24-well plates (Costar) were adsorbed with the indicated multiplicity of infection (MOI) of reovirus at room temperature for 1 h. Following removal of the inoculum, cells were washed with PBS and incubated in complete medium at 37°C for 18 h to permit completion of a single cycle of viral replication. Monolayers were fixed with 1 ml of methanol at −20°C for a minimum of 30 min, washed twice with PBS, blocked with 2.5% Ig-free bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS, and incubated at room temperature for 1 h with polyclonal rabbit anti-reovirus serum at a 1:1,000 dilution in PBS–0.5% Triton X-100. Monolayers were washed twice with PBS–0.5% Triton X-100 and incubated with a 1:1,000 dilution of Alexa546-labeled anti-rabbit IgG. Monolayers were washed with PBS, and infected cells were visualized by indirect immunofluorescence using an Axiovert 200 fluorescence microscope (Carl Zeiss, New York, NY). Infected cells were identified by the presence of intense cytoplasmic fluorescence that was excluded from the nucleus. No back-
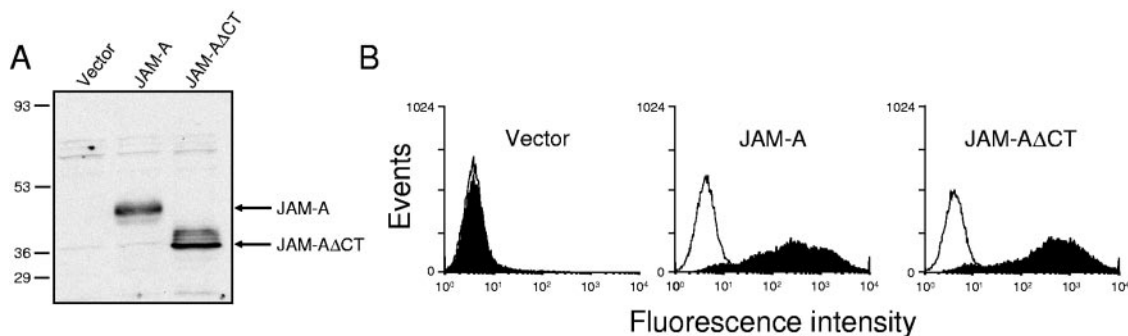
FIG. 1. Stable expression of JAM-A and JAM-AΔCT in CHO cells. (A) Whole-cell lysates (1×10^5 cell equivalents) were prepared from CHO cells stably transfected with empty vector, JAM-A, or JAM-AΔCT; resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; transferred to nitrocellulose; and immunoblotted using anti-JAM-A MAb J10.4. The positions of full-length JAM-A and truncated JAM-AΔCT are shown on the right. The positions of molecular mass standards (in kilodaltons) are shown on the left. (B) Stably transfected CHO cells (1×10^6) were incubated with either anti-JAM MAb J10.4 (filled histograms) or an isotype-matched control (anti-hCAR) MAb (open histograms) at 10 μg per ml, followed by incubation with PE-labeled anti-mouse Ig secondary antibody. The results are presented as fluorescence intensity.

ground staining of uninfected control monolayers was noted. Reovirus antigen-positive cells were quantified by counting fluorescent cells in at least three random fields of view in duplicate wells at a primary magnification of 20×.

Antibody-mediated infections. Reovirus virions (1×10^{11}) were incubated at 4°C overnight with various concentrations of MAbs in 1 ml PBS. Cells were adsorbed with virus at an MOI of either 100 or 500 PFU per ml at room temperature for 1 h. Following removal of the inoculum, cells were washed with PBS and incubated in complete medium at 37°C for various intervals. For inhibition of viral disassembly and RNA synthesis, cells were maintained in 20 mM AC or 200 μM ribavirin, respectively, throughout the course of infection. For inhibition of disassembly, cells also were pretreated for 1 h with medium containing 20 mM AC.

Quantitation of apoptosis by acridine orange (AO) staining. Cells (2×10^5) grown in 24-well tissue culture plates were adsorbed with reovirus virions or MAb-virion complexes at various MOIs. Following incubation at 37°C for 48 h, the percentage of apoptotic cells was determined by using AO staining as previously described (71). For each experiment, 200 to 300 cells were counted in three independent wells, and the percentage of cells exhibiting condensed chromatin was determined by epi-illumination fluorescence microscopy using a fluorescein filter set (Carl Zeiss).

Antibody-mediated cross-linking of Fc receptors. Cells (2×10^5) were chilled to 4°C and incubated with 1 μg per ml of rat anti-Fc MAb 2.4G2 at 4°C for 30 min. Unbound antibody was removed by washing with chilled PBS, and cells were incubated with 5 μg per ml of IgM specific for rat IgG at 4°C for 30 min. Following incubation at 37°C for 48 h, cells were scored for apoptosis.

Statistical analysis. Mean values obtained in infectivity and apoptosis assays were compared using the unpaired Student's t test as applied with Microsoft Excel software. P values of less than 0.05 were considered statistically significant. The contribution of each of the reovirus gene segments to apoptosis induction was assessed by using the nonparametric Mann-Whitney test without adjusting for multiple comparisons and a panel of T1L × T3D reassortant viruses as previously described (59).

RESULTS

A JAM-A truncation mutant lacking the cytoplasmic tail is expressed at the cell surface. Anti-JAM-A MAb J10.4, which inhibits reovirus binding to JAM-A, also blocks reovirus-induced apoptosis (5). These findings suggest that JAM-A binding is essential for reovirus-induced apoptosis. The JAM-A cytoplasmic tail is approximately 45 amino acids in length, contains 13 potential phosphorylation sites, and interacts with several PDZ domain-containing proteins, suggesting a role in ligand-induced cell signaling (6, 25). To determine whether the cytoplasmic tail of JAM-A contributes to reovirus-induced apoptosis by evoking proapoptotic signaling events, CHO cells were stably transfected with empty vector or vector encoding full-

length JAM-A or a C-terminally truncated form of JAM-A that lacks the cytoplasmic tail (JAM-AΔCT). CHO cells were selected for these experiments, since they are poorly permissive to reovirus infection (27); yields of reovirus following infection of CHO cells are 100- to 1,000-fold higher following ectopic expression of JAM-A (11, 27). Whole-cell extracts from stably expressing cells were analyzed for expression of JAM-A by immunoblotting (Fig. 1A). While no JAM-A-specific band was detected in the vector-transfected cells, both full-length JAM-A and the faster migrating JAM-AΔCT proteins were expressed to high levels in the cell lines tested. The surface expression of both JAM-A and JAM-AΔCT was assessed by flow cytometry using JAM-A-specific MAb J10.4 (Fig. 1B). Both wild-type and mutant JAM-A proteins displayed approximately equivalent surface expression, suggesting that removal of the C-terminal domain of JAM-A does not prevent transport of JAM-A to the cell surface. These stably transfected cells are therefore suitable for analysis of infection and apoptosis induction by reovirus.

Reovirus induces equivalent levels of apoptosis in CHO cells that express JAM-A and JAM-AΔCT. To determine whether CHO cells stably expressing JAM-AΔCT can support reovirus infection, cells were adsorbed with reovirus strain T3D at an MOI of 10 PFU per cell, and viral infectivity was assessed by using indirect immunofluorescence (Fig. 2A). CHO cells transfected with either empty vector or those engineered to stably express full-length JAM-A were used as controls. In contrast to vector-transfected cells, cells expressing either JAM-A or JAM-AΔCT were equivalently capable of supporting infection by T3D. These data indicate that although JAM-A expression is required for efficient infection of CHO cells, the JAM-A cytoplasmic tail is dispensable.

To determine whether the JAM-A cytoplasmic tail is required for apoptosis, stably transfected CHO cell lines were adsorbed with T3D at an MOI of 100 PFU per cell, and apoptosis was assessed by using AO staining (Fig. 2B). None of the cell lines tested showed significant apoptosis following mock infection (<5%). T3D infection of cells transfected with vector alone induced levels of apoptosis equivalent to those following mock infection of cells. In contrast, T3D infection of either the JAM-A- or JAM-AΔCT-expressing cell lines in-
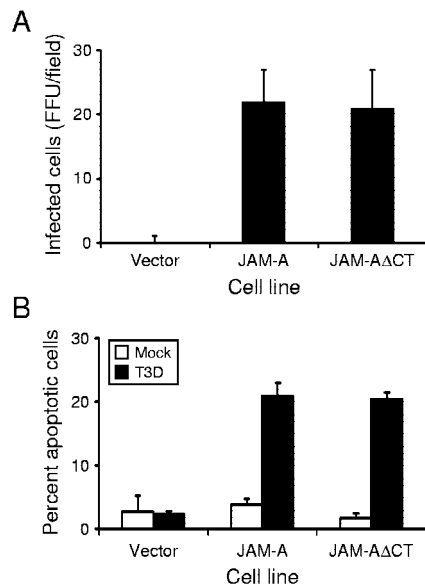
FIG. 2. Infection and apoptosis of JAM-A- and JAM-AΔCT-expressing CHO cells. (A) Cells were adsorbed with T3D at an MOI of 10 PFU per cell. After incubation at 37°C for 18 h, cells were fixed using methanol. Infected cells were visualized by immunostaining with polyclonal rabbit anti-reovirus sera, followed by incubation with Alexa546-labeled anti-rabbit IgG. Reovirus-infected cells were quantified by counting fluorescent focus units (FFU) per field. Error bars indicate standard deviations. (B) Cells were adsorbed with either PBS (mock) or T3D at an MOI of 100 PFU per cell. Cells were harvested at 48 h after infection and stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations.

duced an equivalent percentage of cells to undergo apoptosis (~22%). Therefore, analogous to our findings in the infectivity assays, the cytoplasmic tail of JAM-A is not required for reovirus-induced apoptosis.

Antibody-mediated uptake of reovirus into Fc receptor-expressing cells leads to infection and apoptosis. To determine whether the requirement for JAM-A during reovirus infection and apoptosis can be bypassed, we utilized antibody-mediated infection of Fc receptor-expressing CHO (CHO-B1) cells. These cells stably express the B1 isoform of the mouse Fc receptor II (34). Therefore, in the absence of JAM-A, these cells should allow internalization of antibody-reovirus complexes into cells via Fc receptors, resulting in efficient infection of normally nonpermissive cells. For these experiments, T3D virions were incubated with increasing concentrations of σ1-specific, neutralizing MAb 9BG5 (10) prior to infection of CHO-B1 cells. HeLa cells were used in parallel to confirm the neutralizing efficacy of MAb 9BG5 at the concentrations tested. For each cell line, the number of infected cells was assessed 18 h postinfection by indirect immunofluorescence. As anticipated, the efficiency of reovirus infection of HeLa cells decreased in proportion to antibody concentration, with little infection detected in the presence of 2.5 μg per ml of 9BG5 (Fig. 3A). We conclude that 9BG5 interferes with σ1-JAM-A interactions, which are critical for reovirus infection of HeLa cells. In contrast, the efficiency of infection of CHO-B1 cells by T3D increased in proportion to 9BG5 concentration,
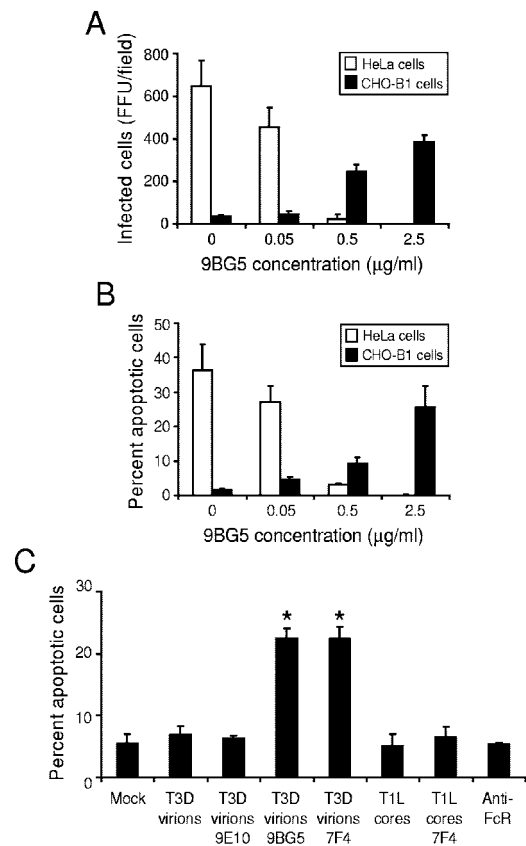
FIG. 3. Infection and apoptosis of HeLa cells and CHO-B1 cells in the presence of MAb 9BG5. (A) Reovirus T3D particles were incubated overnight with the indicated concentration of MAb 9BG5 and adsorbed to either HeLa cells or CHO-B1 cells at an MOI of 100 PFU per cell. After incubation at 37°C for 18 h, cells were fixed using methanol. Infected cells were visualized by immunostaining with polyclonal rabbit anti-reovirus sera, followed by Alexa546-labeled anti-rabbit IgG. Reovirus-infected cells were quantified by counting fluorescent cells. The results are presented as mean fluorescent focus units (FFU) per field. Error bars indicate standard deviations. (B) HeLa cells or CHO-B1 cells were adsorbed with 100 PFU per cell of either virus or virus-antibody complex, harvested at 48 h after infection, and stained with AO. (C) CHO-B1 cells were mock-infected, infected with T3D virions with or without 2.5 μg per ml of Myc-specific MAb 9E10 (antibody control), σ1-specific MAb 9BG5, or λ2-specific MAb 7F4 at an MOI of 100 PFU per cell or were infected with T1L core particles with or without 2.5 μg per ml of MAb 7F4 at an MOI of 10^4 particles per cell. CHO-B1 cells also were incubated with 1 μg per ml of anti-Fc receptor rat IgG MAb 2.4G2, followed by incubation with 5 μg per ml of IgM specific for rat IgG (anti-FcR). Cells were harvested at 48 h after infection and stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to T3D incubated with control MAb 9E10.

with maximal infection observed in the presence of a completely neutralizing concentration of 9BG5, 2.5 μg per ml (Fig. 3A). These findings demonstrate that reovirus infection can be established in a JAM-A-independent manner if an alternative high-affinity binding moiety is provided. These data corroborate a previous report of antibody-mediated enhancement of reovirus infection of a murine macrophage-like cell line (9).

To determine whether infection initiated in a JAM-A-inde-

pendent manner also triggers apoptosis, the capacity of reovirus-9BG5 complexes to induce apoptotic cell death of both HeLa cells and CHO-B1 cells was assessed by using AO staining (Fig. 3B). Approximately 35% of HeLa cells showed apoptotic nuclei at 48 h postinfection in the absence of antibody treatment of virions. Consistent with the decrease in the capacity of reovirus to infect HeLa cells in the presence of 9BG5, the percentage of cells undergoing apoptosis also decreased with increasing 9BG5 concentrations. However, in the absence of 9BG5, T3D induced minimal apoptosis in CHO-B1 cells in comparison to mock-infected cells. In concordance with the infectivity data, the percentage of apoptotic CHO-B1 cells increased with increasing concentrations of 9BG5, with maximal apoptosis seen following pretreatment with 2.5 μg per ml MAb (~25%). These findings demonstrate that reovirus infection initiated in the absence of JAM-A binding also leads to apoptosis. Therefore, σ1-JAM-A interactions or signaling pathways induced as a consequence of these interactions are dispensable for apoptosis induction by reovirus.

To exclude the possibility that binding of MAb to the Fc receptor contributes to apoptosis induction, we tested whether incubation of reovirus with irrelevant anti-Myc MAb 9E10 was capable of inducing apoptosis (Fig. 3C). While incubation of T3D with two different MAbs directed against the reovirus outer-capsid, 7F4 (λ2) and 9BG5 (σ1), induced 21% and 22% apoptosis, respectively, following infection of CHO-B1 cells, incubation of T3D with MAb 9E10 failed to induce apoptosis at levels higher than those of mock-infected cells. These findings suggest that only antibodies directed against reovirus outer-capsid proteins allow virus attachment to CHO-B1 cells and subsequent induction of apoptosis. To exclude the involvement of signaling induced as a result of Fc receptor cross-linking due to binding of reovirus-antibody complexes, Fc receptors were cross-linked using 2.4G2 (a rat anti-Fc receptor MAb) and an IgM antibody directed against rat IgG. This treatment did not induce apoptosis in CHO-B1 cells in comparison to mock-treated cells. As an additional control for the effect of cross-linking Fc receptors, T1L core particles, which lack outer-capsid proteins σ1, σ3, and μ1, were incubated with MAb 7F4 and added to CHO-B1 cells. Neither untreated cores nor 7F4-core complexes induced apoptosis of these cells. These data suggest that apoptosis does not result from proapoptotic signaling induced as a consequence of Fc-receptor cross-linking but requires binding of reovirus particles containing outer-capsid proteins.

Viral disassembly is required for apoptosis induced by Fc-mediated uptake of reovirus. To determine whether viral disassembly in cellular endosomes is required for apoptosis following Fc-mediated delivery of reovirus, CHO-B1 cells were treated with AC prior to infection by MAb-treated T3D virions. Treatment of cells with 20 mM AC, a concentration sufficient to block reovirus disassembly (66, 77), abolished the capacity of T3D to induce apoptosis (Fig. 4A). Thus, acid-dependent proteolytic disassembly is required for apoptosis induction by this uptake mechanism. To determine whether the apoptosis-inhibitory effect of AC is due to blockade of viral RNA synthesis, we tested ribavirin, an inhibitor of viral RNA synthesis (55), for the capacity to diminish apoptosis. In keeping with our previously published results (18), apoptosis induced by reovirus infection via Fc-mediated uptake was unaf-
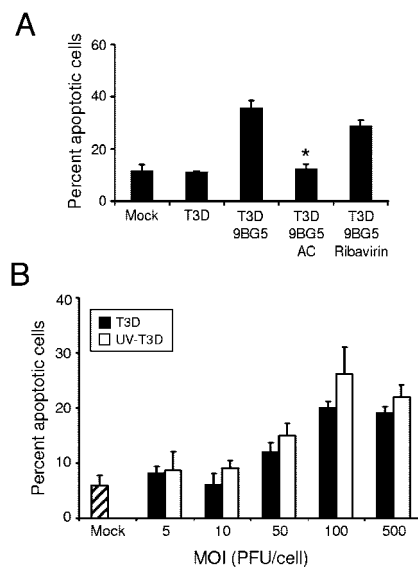

FIG. 4. Apoptosis following Fc receptor-mediated uptake of viable and UV-inactivated reovirus virions. (A) T3D alone or preincubated with 2.5 μg per ml of MAb 9BG5 was adsorbed to CHO-B1 cells at an MOI of 500 PFU per cell. (B) UV-inactivated T3D preincubated with 2.5 μg per ml of MAb 9BG5 was adsorbed to CHO-B1 cells at the indicated MOIs. After incubation at 37°C for 48 h in untreated medium or medium containing either 20 mM AC or 200 μM ribavirin, cells were stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations. ∗, P < 0.05 as determined by Student's t test in comparison to T3D incubated with MAb 9BG5.

fected by ribavirin (Fig. 4A). To corroborate these findings, we tested the apoptosis-inducing capacity of UV-inactivated reovirus virions, which are incapable of establishing productive infection (60 and data not shown). We found that UV-inactivated reovirus induced apoptosis following Fc receptor-mediated uptake of a high MOI of virus (Fig. 4B), consistent with our previously published observations (71). Collectively, these results demonstrate that steps in reovirus replication that occur after attachment but before transcription are required for induction of apoptosis, regardless of the type of receptor used to initiate infection (18). In addition, these results suggest that death signaling during reovirus infection may occur independently of receptor engagement.

Fc receptor-dependent infection abolishes σ1-related differences in the apoptosis-inducing capacity of reovirus. Differences in the capacity of some reovirus strains to induce apoptosis are linked to differences in the affinity for sialic acid (17). To determine whether reovirus strains that are incapable of binding to sialic acid can induce apoptosis when infection is initiated via Fc-dependent uptake, strains T1L and T3SA−, neither of which is capable of binding to sialic acid (4, 16, 23), were incubated with σ1-specific antibodies and adsorbed to CHO-B1 cells. Incubation of T1L virions with type 1 σ1-specific MAb 5C6 (73) and incubation of T3SA− virions with type 3 σ1-specific 9BG5 (10) resulted in significantly higher levels of apoptosis in CHO-B1 cells in comparison to levels observed following infection with untreated T1L and T3SA− virions (Fig. 5). The percentage of cells undergoing apoptosis following antibody-mediated infection of non-sialic-acid-binding
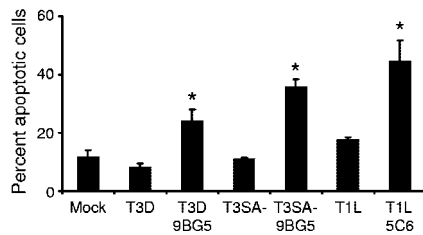
FIG. 5. Apoptosis induction in CHO-B1 cells following Fc-mediated infection by T1L and T3SA−. Cells were adsorbed with T3D, T3SA−, or T1L with or without 2.5 µg per ml of MAb 9BG5 (for type 3 strains) or MAb 5C6 (for T1L) at an MOI of 100 PFU per cell. After incubation at 37°C for 48 h, cells were harvested and stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations. ∗, $P < 0.05$ as determined by Student's t test in comparison to the same strain incubated without antibody.

reovirus strains was at least equal to that observed following infection with T3D. Therefore, σ1-related differences in apoptosis efficiency are overcome when infection is initiated via Fc-mediated uptake. These results make it unlikely that the σ1 protein is the viral effector of apoptosis induction.

Differences in apoptosis induction following Fc receptor-dependent infection of T1L × T3D reassortant viruses are linked to the M2 gene segment. We have demonstrated previously that the viral S1 and M2 gene segments segregate with differences in the apoptosis-inducing capacity of T1L and T3D (59, 71). To ascertain whether any strain-specific differences exist when the effect of the S1 gene segment is circumvented by Fc receptor-dependent uptake, CHO-B1 cells were adsorbed with T1L × T3D reassortant viruses after incubation with σ1-specific antibodies. Type 1 and type 3 strains were incubated with σ1-specific MAbs 5C6 and 9BG5, respectively, prior to infection. Each of the reassortant viruses tested produced a similar number of infected cells as assessed by indirect immunofluorescence (data not shown), suggesting equivalent efficiency of antibody-mediated uptake and infection. The percentage of cells undergoing apoptosis as a result of antibody-mediated infection of cells was assessed at 48 h after infection. The reassortant viruses were ranked from highest to lowest by apoptosis-inducing capacity (Table 1). Although the reassortants did not cluster distinctly into two groups with high and low apoptotic potential, six of the seven strains with the highest levels of apoptosis had an M2 gene segment derived from T3D. Conversely, seven of the eight strains with the lowest levels of apoptosis had an M2 gene segment derived from T1L. Analysis of the data using the Mann-Whitney test showed that only the M2 gene segregated at a statistically significant level with the capacity of these strains to induce apoptosis ($P = 0.02$). There was not a statistically significant association between apoptosis and the S1 gene ($P = 0.28$), which is the primary apoptosis determinant following infection of JAM-A-expressing cells (17, 59, 71, 72), when infection was initiated in an Fc-dependent fashion. These data suggest that the M2-encoding µ1 protein, which functions in penetration of cell membranes (38, 44, 49), is the primary viral determinant of strain-specific differences in apoptosis induction following infection by Fc-mediated uptake.

Reovirus mutant *ts*A279 is inefficient in apoptosis induction. To determine whether a mutant virus with a defective µ1

TABLE 1. Apoptosis induction by T1L × T3D reassortant viruses in CHO-B1 cells

Virus isolate	Origin of gene segments[a]										% Apoptosis[b]	SD
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4		
Reassortant												
EB138	D	L	L	D	D	L	D	D	L	L	80.5	3.84
KC150	D	L	L	L	D	L	D	D	L	D	62.8	2.23
KC9	D	D	L	D	D	D	L	D	D	D	58.6	3.28
EB97	D	D	L	D	D	D	D	D	D	L	52.3	3.89
EB68	L	D	L	L	D	L	L	L	D	D	41.2	4.98
1HA.3	L	L	L	L	L	L	D	L	L	L	40.6	7.15
EB144	L	L	L	L	D	D	L	D	L	L	38.2	3.99
EB98	L	D	L	L	L	L	L	D	L	D	32.6	3.17
EB121	D	D	L	D	L	D	L	D	D	D	28.9	8.13
EB113	L	L	L	D	L	L	L	L	D	L	26.6	3.85
G16	L	L	L	D	L	L	L	D	L	L	24.4	3.62
G2	L	D	L	L	L	L	D	L	L	L	23.1	4.90
EB143	D	L	L	L	L	L	D	L	L	L	23.0	4.05
EB145	D	D	D	D	D	L	L	D	D	D	16.2	3.40
EB120	D	D	D	L	L	D	D	D	L	L	14.5	3.33
Parental												
T3D	D	D	D	D	D	D	D	D	D	D	23.0	1.81
T1L	L	L	L	L	L	L	L	L	L	L	33.6	2.34

[a] Parental origin of each gene segment: L, gene segment derived from T1L; D, gene segment derived from T3D.
[b] CHO-B1 cells (2×10^5) were adsorbed with virus strains at an MOI of 100 PFU per cell. After 1 h, the inoculum was removed, fresh medium was added, and cells were incubated at 37°C for 48 h and stained with AO to assess apoptosis. Shown are the mean percentage and standard deviations of cells undergoing apoptosis for three independent experiments. The M2 gene was the only gene associated with the efficiency of apoptosis as determined by using the nonparametric Mann-Whitney test, without adjusting for multiple comparisons ($P = 0.02$).

protein is altered in the capacity to trigger apoptosis, we used reovirus strain *ts*A279.64, which contains a temperature-sensitive mutation that maps to the M2 gene segment (30). This virus was derived from a coinfection of T1L and *ts*A279, which contains temperature-sensitive mutations in both the M2 and L2 gene segments. The *ts*A279.64 virus contains the mutant M2 gene segment but not the mutant L2 gene segment, thus facilitating analysis of the contribution of the M2 gene segment to apoptosis induction. When assembled at nonpermissive temperature, virions containing the mutant M2 gene segment cannot penetrate membranes due to a misfolded µ1 protein (30). To examine whether µ1-mediated membrane penetration is required for apoptosis induction, HeLa cells were adsorbed with increasing MOIs of *ts*A279.64 virions assembled under permissive and nonpermissive conditions. The percentage of cells with apoptotic nuclei was assessed by using AO staining 48 h after infection at nonpermissive temperature (Fig. 6). At all MOIs tested, particles assembled at nonpermissive temperature induced less apoptosis than particles assembled at permissive temperature. These data demonstrate that virions containing a µ1 protein that is inefficient in membrane penetration are less potent inducers of apoptosis, which further highlights a key role for the µ1 protein in apoptosis induction.

DISCUSSION

We have previously shown that binding of reovirus to JAM-A and sialic acid is required for efficient induction of apoptosis (5, 17). In addition, we have reported that viral disassembly in cel-
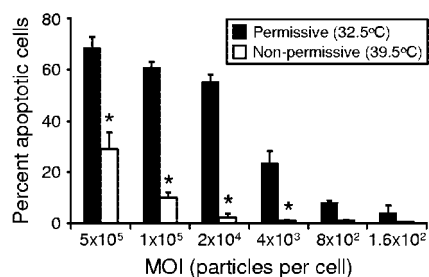
FIG. 6. Apoptosis induced by μ1 temperature-sensitive mutant *ts*A279.64. HeLa cells were adsorbed with *ts*A279.64 grown at permissive or nonpermissive temperatures at the MOIs shown. After incubation at 37°C for 48 h, cells were harvested and stained with AO. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's *t* test in comparison to virions grown at permissive temperature at an equivalent MOI.

lular endosomes is also necessary for apoptosis induction by reovirus (18). However, since receptor binding is a prerequisite for virus disassembly, these findings do not resolve the question of whether reovirus attachment and disassembly provide two distinct signals, both of which are required for apoptosis induction, or whether the viral disassembly events are sufficient for proapoptotic signaling. To address this question, we uncoupled reovirus attachment to JAM-A and sialic acid from viral disassembly by providing an alternative means of viral entry. We report here that antibody-mediated uptake of reovirus into Fc receptor-expressing CHO cells independent of binding to JAM-A and sialic acid leads to productive infection (9) and apoptosis. Furthermore, we demonstrate that antibody-directed binding of reovirus to Fc receptors expressed on CHO cells is not sufficient for reovirus-induced apoptosis. Analogous to JAM-A- and sialic acid-dependent infection, viral replication steps during or after disassembly in endosomes but prior to RNA synthesis also are required for reovirus-induced apoptosis when infection is initiated by Fc-mediated uptake. Analysis of apoptosis induction by T1L × T3D reassortant viruses following Fc-mediated uptake showed that differences in the efficiency of apoptosis exhibited by type 1 and type 3 strains segregate with the μ1-encoding M2 gene segment. Neither core particles that lack the μ1 protein and are therefore incapable of penetrating endosomal membranes nor the thermosensitive M2 mutant virus *ts*A279.64, which contains a misfolded, penetration-defective μ1 protein, can efficiently induce apoptosis. These findings suggest that reovirus membrane penetration protein μ1 induces proapoptotic signaling during or after endosomal membrane penetration.

In our reassortant analysis, the viral M2 gene segment was the only viral gene segment that segregated at a statistically significant level with apoptosis-inducing capacity. Although levels of apoptosis induced by reassortant viruses containing a T3D M2 gene segment were generally higher than those induced by reassortant viruses containing a T1L M2 gene segment, the percentage of cells undergoing apoptosis formed a continuum of values rather than two distinct clusters. Since our data suggest that apoptosis is induced during or after endosomal membrane penetration mediated by the μ1 protein, steps in viral replication preceding membrane penetration, such as

attachment, internalization, and disassembly, also may influence the magnitude of the apoptotic response. Thus, viral gene products that mediate these steps also may contribute to the observed differences in levels of apoptosis. Minor strain-specific effects attributed to other viral gene segments also may explain our finding that antibody-mediated uptake of parental strain T1L into Fc receptor-expressing cells induced higher levels of apoptosis than those induced by T3D (Fig. 5, Table 1). Alternatively, the type 1 and type 3 σ1-specific MAbs used in our experiments may differ in either affinity for σ1 or efficiency in internalization via Fc receptors. However, we think that this explanation is unlikely, since no association between the σ1-encoding S1 gene and apoptosis-inducing potential was observed. Therefore, although it is likely that there are multiple viral genetic determinants of reovirus-induced apoptosis, our statistical analysis of T1L × T3D reassortant viruses, in addition to our findings using M2 temperature-sensitive mutant *ts*A279.64, lead us to conclude that the M2-encoded μ1 protein is the primary mediator of proapoptotic signaling.

In addition to the findings here, three independent lines of evidence support a crucial role for μ1 in reovirus-induced apoptosis. First, differences in apoptosis efficiency displayed by strains T1L and T3D following infection of JAM-A-expressing cells are linked in part to the μ1-encoding M2 gene (59, 71, 72). Second, studies using pharmacologic inhibitors of reovirus replication place the apoptosis-inducing events subsequent to viral disassembly but prior to RNA synthesis (18), which coincides with μ1-mediated membrane penetration (12, 38, 44, 49). Third, transient transfection of a plasmid encoding T3D μ1 is sufficient to induce apoptosis in CHO cells (C. M. Coffey, L. J. Anguish, A. Sheh, I. S. Kim, K. Chandran, M. L. Nibert, and J. S. Parker, Abstr. Am. Soc. Virol. Annu. Meet., abstr. W41-4, 2005). Interestingly, although plasmid-mediated expression of μ1 neither mimics normal delivery of μ1 via endosomal rupture during viral membrane penetration nor de novo expression of μ1 in infected cells (since μ1 is mostly found associated with its protector protein σ3 [61, 62, 67]), it leads to the same consequence.

It is not known how the viral disassembly events culminating in μ1-mediated membrane penetration elicit proapoptotic signaling. We envision two possibilities. First, endosomal disruption by μ1 may lead to release of hydrolytic enzymes such as cathepsins, which in turn damage mitochondria and stimulate death signaling (22, 29, 58). Interestingly, mitochondrial injury has been reported as early as 4 h following reovirus adsorption (35, 36), suggesting the involvement of an early viral replication event. It is also possible that release of these enzymes causes apoptosis via their action on death regulators such as Bid (65). Of note, Bid cleavage has been observed during reovirus infection and has been hypothesized to play a role in apoptosis induction (35). Second, fragments of μ1 produced during proteolytic viral disassembly are known to gain access to the cytoplasm (14). These fragments may activate other cellular sensors of viral infection or directly injure mitochondria to induce apoptosis. Concordantly, the μ1 protein localizes to mitochondria during infection or when expressed from plasmids in transfected cells (C. M. Coffey, L. J. Anguish, A. Sheh, I. S. Kim, K. Chandran, M. L. Nibert, and J. S. Parker, Abstr. Am. Soc. Virol. Annu. Meet., abstr. W41-4, 2005), suggesting a postendosomal site of action. Interestingly, a 30-residue C-termi-

nal fragment of μ1 is sufficient to localize to mitochondria and induce apoptosis (C. M. Coffey, L. J. Anguish, A. Sheh, I. S. Kim, K. Chandran, M. L. Nibert, and J. S. Parker, Abstr. Am. Soc. Virol. Annu. Meet., abstr. W41-4, 2005).

Although our findings point to μ1 as a key viral regulator of proapoptotic signaling, this work does not explain the previously established unequivocal association between the S1-encoded σ1 protein and the efficiency of apoptosis induction by reovirus (59, 71, 72). Strains encoding a σ1 protein capable of binding to JAM-A and sialic acid are the most potent inducers of apoptosis (17). We did not observe efficient infection of CHO-B1 cells in the absence of MAb pretreatment at the MOIs used. Therefore, we think that these cells do not express sufficient quantities of sialic acid or JAM-A on the cell surface to effect productive infection. Thus, infection of these cells appears to be dependent only on the presence of a high-affinity receptor such as the Fc receptor. Since the efficiency of antibody-mediated uptake and delivery of both sialic-acid-binding and non-sialic-acid-binding strains of reovirus via Fc receptors is essentially equivalent in CHO-B1 cells, σ1-related differences are negated. An alternative explanation for our findings is that antibody-mediated attachment of virions to Fc receptors stimulates a signaling cascade in cells that mimics signaling induced as a consequence of σ1 binding to JAM-A and sialic acid and that Fc receptor-mediated signaling acts in concert with the viral disassembly events to elicit apoptosis. However, given the marked differences in functional properties displayed by JAM-A and Fc receptors, this explanation seems less likely. Therefore, our current and previous studies suggest that the linkage of σ1 to apoptosis efficiency is not related to σ1-mediated stimulation of the cellular proapoptotic machinery but rather the capacity of σ1 to efficiently deliver virions into endosomes for disassembly. We hypothesize that, as opposed to viral infection, which requires penetration by just one infectious virion, efficient apoptosis induction by reovirus requires endosomal penetration by multiple virions. Since sialic acid allows more avid binding of virions to cells (4), we think that this entry route may deliver virions more efficiently into endosomal compartments for uncoating and subsequent membrane penetration, leading to higher levels of apoptosis (17).

This study highlights a new role for the viral membrane penetration protein μ1 in apoptosis induction. Other viruses, such as coronavirus, Sindbis virus, and vaccinia virus, also have been reported to require postattachment cell entry events in endosomes to induce apoptosis (33, 39, 54). However, the viral determinants of apoptosis by these viruses are unknown. Interestingly, entry of Sindbis virus into endosomes induces apoptosis through activation of sphingomyelinases and release of the proapoptotic second messenger, ceramide (32). Although we anticipate that interactions between the enveloped Sindbis virus with endosomes differ from those with μ1, it is possible that a lipid second messenger also plays a role in reovirus-induced apoptosis. Collectively, these studies point to cellular endosomes as sites from which proapoptotic signaling events are initiated and imply a conserved mechanism by which host cells detect the presence of invading pathogens. Pathogen detection during the entry phase may allow host cells to more efficiently limit the spread of infection by initiating cell death. Alternatively, induction of apoptosis by a virus early in its replication cycle may prevent or attenuate the development of

an inflammatory response, thereby allowing the virus to better evade host defenses. A fascinating similarity exists between the properties of reovirus protein μ1 and several toxins elaborated by bacteria and viruses. Analogous to the capacity of μ1 to mediate membrane permeabilization (12, 31, 41, 69), α toxin of *Staphylococcus aureus*, lysteriolysin O of *Listeria monocytogenes*, and killer toxins (K1 and K2) of the yeast L-A virus also can form pores in host cell membranes (42, 68, 75, 78). Interestingly, each of these toxins also has the capacity to induce apoptotic cell death (56, 75). Our ongoing studies are focused on understanding the precise mechanism by which μ1 induces apoptosis during reovirus infection. Through these studies we hope to gain broader insight into events at the pathogen-host interface that evoke death signaling and cause disease.

ACKNOWLEDGMENTS

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APPENDIX B

IκB KINASE SUBUNITS α AND γ ARE REQUIRED FOR ACTIVATION OF NF-κB

AND INDUCTION OF APOPTOSIS BY MAMMALIAN REOVIRUS

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IκB Kinase Subunits α and γ Are Required for Activation of NF-κB and Induction of Apoptosis by Mammalian Reovirus

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Running title: IKK activation and reovirus apoptosis

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ABSTRACT

Reoviruses induce apoptosis both in cultured cells and in vivo. Apoptosis plays a major role in the pathogenesis of reovirus encephalitis and myocarditis in infected animals. Reovirus-induced apoptosis is dependent on activation of transcription factor NF-κB and downstream cellular genes. To better understand the mechanism of NF-κB activation by reovirus, NF-κB signaling intermediates under reovirus control were investigated at the level of Rel, IκB, and IκB kinase (IKK) proteins. We found that reovirus infection leads initially to nuclear translocation of p50 and RelA, followed by delayed mobilization of c-Rel and p52. This biphasic pattern of Rel protein activation is associated with degradation of the NF-κB inhibitor IκBα, but not the structurally-related inhibitors IκBβ or IκBε. Using cells deficient in individual IKK subunits, we demonstrate that IKKα but not IKKβ is required for reovirus-induced NF-κB activation and apoptosis. Despite the preferential usage of IKKα, both NF-κB activation and apoptosis were attenuated in cells lacking IKKγ/Nemo, an essential regulatory subunit of IKKβ. Moreover, deletion of the gene encoding NF-κB-inducing kinase (NIK), which is known to modulate IKKα function, had no inhibitory effect on either response in reovirus-infected cells. Collectively, these findings indicate a novel pathway of NF-κB/Rel activation involving IKKα and IKKγ/Nemo, which together mediate the expression of downstream proapoptotic genes in reovirus-infected cells.

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INTRODUCTION

Mammalian reoviruses are nonenveloped viruses that contain a genome of 10 segments of double-stranded RNA (43). Following infection of newborn mice, reovirus disseminates systemically, causing injury to the central nervous system (CNS), heart, and liver (65). Apoptosis induced by reovirus appears to be the primary mechanism for virus-induced encephalitis (44, 45, 48) and myocarditis (19, 20, 45). Disassembly of internalized virus in the endocytic pathway provides the viral trigger for stimulating the signaling pathways that elicit an apoptotic response (16, 18).

Transcription factor NF-κB plays an important regulatory role in apoptosis evoked by reovirus in cultured cells (17) and in vivo (45). Inducible members of the NF-κB family are sequestered in the cytoplasm by inhibitory IκB proteins, including IκBα, IκBβ, IκBε, and p100 (2, 28, 57, 68, 71). In response to a wide variety of NF-κB inducers, IκB proteins are phosphorylated at specific serine residues, earmarking these molecules for destruction by the ubiquitin-proteasome pathway (6, 10, 28, 46, 64, 71). Phosphorylation of IκB proteins is mediated by cytokine-inducible IκB kinases (IKKs) IKKα and IKKβ (39, 67), which can form higher order complexes containing a regulatory subunit called IKKγ/Nemo (23, 41, 51, 75, 78). A primary function of IKKβ is to modulate the inhibitory interaction of IκBα with the prototypical form of NF-κB containing p50/RelA dimers (22, 23, 41, 47, 58). This regulatory circuit, termed the classical pathway of NF-κB activation, is strictly dependent on the presence of IKKγ/Nemo (53, 54, 75). In sharp contrast, IKKα functions in an alternative IKKγ-independent pathway of NF-κB activation that leads to proteolytic processing of p100/NF-κB2 and production of a fully functional p52 Rel subunit (13, 55, 60). Unlike the classical, IKKβ-directed pathway of NF-κB activation, the alternative pathway involving IKKα is dependent on prior phosphorylation of this IKK by NF-κB-inducing kinase (NIK) (36, 55, 73).

To better understand the mechanism of NF-κB activation by reovirus, we conducted experiments to define the NF-κB/Rel, IκB, and IKK proteins that are under reovirus control. These studies revealed that NF-κB/Rel proteins are mobilized to the nuclear compartment with biphasic kinetics following reovirus infection. Reovirus-induced activation of NF-κB/Rel proteins is accompanied by selective degradation of IκBα, suggesting a role for IKKβ. However, subsequent studies with IKK subunit-deficient cells clearly demonstrate that IKKα rather than IKKβ plays an essential role in the mechanism by which reovirus activates NF-κB and downstream apoptotic genes. We also assembled evidence indicating that the reovirus/IKKα axis is intact in cells lacking NIK, an upstream activator of IKKα, but not in cells lacking the IKKβ regulatory subunit IKKγ/Nemo. Taken together, these data suggest that reovirus activates the IKKα pathway of the NF-κB signaling apparatus downstream of NIK, perhaps via direct interactions with the regulatory subunit IKKγ/Nemo.

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MATERIALS AND METHODS

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        Cells, viruses, and reagents. HeLa cells, 293T cells, and murine embryo fibroblasts (MEFs) were maintained in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/ml streptomycin, and 25 ng/ml of amphotericin B (Invitrogen, Carlsbad, CA). MEFs deficient in IKKα (29, 33), IKKβ

145 (34, 61), IKKγ (53), and NIK (76) have been described previously.

        Reovirus type 3 Dearing (T3D) is a laboratory stock. Purified reovirus virions were generated by using second- or third-passage L-cell lysate stocks of twice-plaque-purified reovirus as described previously (24). Viral particles were Freon-extracted from

150 infected cell lysates, layered onto 1.2- to 1.4-g/cm^3 CsCl gradients, and centrifuged at 62,000 x g for 18 h. Bands corresponding to virions (1.36 g/cm^3) were collected and dialyzed in virion storage buffer (150 mM NaCl, 15 mM MgCl$_2$, 10 mM Tris-HCl [pH 7.4]). Concentrations of reovirus virions in purified preparations were determined from an equivalence of one optical density unit at 260 nm equals 2.1 x 10^{12} virions (59). Viral

155 titer was determined by plaque assay using L cells (69).

        Antisera specific for IκBα, IκBβ, IκBε, p50, p65/RelA, RelB, c-Rel, IKKγ, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody antiserum for p100/p52 was purchased from Upstate Biotechnology (Lake Placid, NY).

160 The agonistic lymphotoxin-β receptor antiserum was purchased from BD Biosciences (San Jose, CA). The IKK inhibitor BAY 65-1942 was obtained from Dr. K. Ziegelbauer (Bayer Health Care AG, Leverkusen, Germany).

        Electrophoretic mobility shift assay (EMSA). Cells (3 x 10^6) grown in 100 mm

165 tissue-culture dishes (Costar, Cambridge, MA) were treated with 20 ng/ml of TNF-α (Sigma-Aldrich, St. Louis, MO), adsorbed with T3D at a multiplicity of infection (MOI) of 100 plaque forming units (PFU)/cell, or treated with gel saline (mock-infection). After incubation at 37°C for various intervals, nuclear extracts (10 µg total protein) were assayed for NF-κB activation by an EMSA using a ^{32}P-labeled oligonucleotide consisting

170 of the NF-κB consensus-binding sequence (Santa Cruz Biotechnology) as previously described (17). For supershift assays, 2 µg of rabbit polyclonal antiserum specific for p50, p52, RelA, RelB, or c-Rel was added to the binding-reaction mixtures and incubated at 4°C for 30 min prior to the addition of radiolabeled oligonucleotide. Nucleoprotein complexes were subjected to electrophoresis in native 5% polyacrylamide gels at 180 V

175 for 90 min, dried under vacuum, and exposed to Biomax MR film (Kodak, Rochester, NY). Band intensity was quantified by determining photostimulus luminescence (PSL) units using a Fuji2000 phosphor imager and the Multi Gauge software (Fuji Medical Systems, Inc., Stamford, CT).

180   **Immunoblot assay.** Cells (8 x 10^5) were treated with 20 ng/ml of TNF-α, adsorbed with T3D at an MOI of 100 PFU/cell, or mock-infected. Whole-cell extracts

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were generated by incubation with lysis buffer (10 mM HEPES [pH7.4], 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1% Igepal, 1 mM $Na_4O_7P_2$, 1 mM NaF, 1 mM $NaVO_3$, and 1 µM microsystin). Extracts (10-50 µg total protein) were resolved by electrophoresis in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked at 4°C overnight in blocking buffer (PBS containing 0.1% Tween-20 and 5% BSA). Immunoblots were performed by incubating the membranes with primary antibodies diluted 1:500 to 1:2000 in blocking buffer at room temperature for 1 h. Membranes were washed three times for 10 min each with washing buffer (PBS containing 0.1 Tween-20) and incubated with horseradish peroxidase-conjugated goat anti-rabbit (Amersham Biosciences, Piscataway, NJ) and bovine anti-goat antibodies (Santa Cruz Biotechnology) diluted 1:2000 and 1:3000, respectively. Following three washes, membranes were incubated for 1 min with chemiluminescent peroxidase substrate (Amersham Biosciences) and exposed to film. Band intensity was quantified using the Image J program (NIH, Bethesda, MD).

Kinase assay. Cells (8 x 10^5) were treated with 20 ng/ml of TNFα, adsorbed with T3D at an MOI of 100 PFU/cell, or mock-infected. Whole-cell extracts were incubated with an IKKγ-specific antiserum in the presence of ELB buffer (50 mM HEPES [pH 7.4], 250 mM NaCl, 5 mM EDTA, 0.1% Igepal). Immunoprecipitates were equilibrated in kinase buffer (10 mM HEPES [pH 7.4], 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 12.5 mM β-glycerophosphate, 50 µM Na_3VO_4, 2 mM NaF) and incubated with 10 µM ATP, 5 µCi of [γ-^{32}P]ATP (Perkin Elmer), and 1 µg of recombinant GST protein fused to amino acids 1-54 of IκBα (GST-IκBα) at 30°C for 30 minutes (11). Kinase reactions were terminated by heat denaturation in the presence of 1% SDS. Radiolabeled products were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and visualized by autoradiography.

Caspase 3 activity assay. Cells (3 x 10^3) grown in clear-bottom, black-walled, 96-well tissue-culture plates (Costar) were inoculated with 10 ng/ml of TNFα, a combination of 10 ng/ml of TNFα and 10 µg/ml of cycloheximide (Sigma-Aldrich), T3D at an MOI or 1000 PFU/cell, or mock-infected. Following incubation at 37°C for 24 and 12 h with respect to T3D- or TNFα-treatment, caspase 3 activity was quantified by using the Caspase-Glo 3/7 Assay (Promega). Luminescence was detected by using a Topcount NXT luminometer (Packard Biosciences Co., Meriden, CT).

Trypan blue exclusion assay. Cells (4 x 10^4) grown in 6-well tissue-culture plates were inoculated with 10 ng/ml of TNFα, a combination of 10 ng/ml of TNFα and 10 µg/ml of cycloheximide, T3D at an MOI of 1000 PFU/cell, or mock-infected. Following incubation at 37°C for 48 and 24 h with respect to T3D- or TNFα-treatment, cells were collected and washed with PBS. The cell pellet was resuspended in 50 ml of PBS and stained using 100 ml of a solution containing 0.4% trypan blue (Kodak) in PBS. For each experiment, 200 to 300 cells were counted and the percentage of cell death was determined by light microscopy (Axiovert 200; Zeiss, Oberköchen, Germany).

Statistical analysis. Mean values obtained in EMSA, immunoblot, and apoptosis assays were compared using the unpaired Student's *t* test as applied with Microsoft Excel software (Microsoft, Redmond, WA). *P* values of less than 0.05 were considered to be statistically significant.

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RESULTS

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  Reovirus infection results in the biphasic activation of NF-κB/Rel DNA-binding proteins. In prior studies, we found that reovirus activates the functional expression of p50/RelA complexes, suggesting the involvement of classical NF-κB signaling (17). However, it remained unclear whether NF-κB/Rel proteins linked to the

280 alternative pathway of the NF-κB pathway are activated during reovirus infection. To more completely define the composition of NF-κB complexes activated by reovirus, we used nuclear extracts from reovirus-infected HeLa cells and Rel-specific antibodies to monitor the composition of DNA-binding complexes formed in EMSAs. NF-κB/Rel DNA-binding activity was readily detected over background levels (mock treatment)

285 within 2 hours after infection with reovirus strain T3D (Fig. 1A), which potently induces apoptosis in cultured cells (17, 49, 66) and the murine CNS (44). Peak levels of NF-κB/Rel DNA-binding activity were observed at 4-8 h post-infection. Supershift analysis of extracts obtained at 4, 6, and 8 h post-infection revealed the presence of DNA/protein complexes containing p50 and RelA, but neither p52 nor RelB (Fig. 1B), suggesting

290 preferential usage of the classical versus alternative NF-κB pathway by reovirus. Complexes containing c-Rel were apparent in supershift assays only after 8 h of infection (Fig. 1B). Thus, reovirus induces a biphasic pattern of NF-κB/Rel activation featuring the initial nuclear translocation of complexes consisting of p50 and RelA, followed by those containing p50, RelA, and c-Rel. Given that the cellular gene encoding c-Rel contains

295 functional NF-κB binding sites (26), this expression pattern may reflect de novo synthesis of c-Rel rather than its mobilization from a latent cytoplasmic pool.

  These initial experiments conducted over an 8 h timecourse provided no evidence for the capacity of reovirus to stimulate the nuclear expression of p52, a signature Rel

300 protein involved in the alternative pathway of NF-κB signaling. To further investigate whether reovirus interfaces with the alternative NF-κB pathway, we extended the timecourse of T3D infection to 24 h and monitored extracts for processing of p100 to p52 (55). Levels of p100 were significantly reduced between 16 and 24 h post-infection (Fig. 2). Consistent with a precursor/product relationship, diminution in p100 protein levels

305 were accompanied by a significant increase in the steady-state levels of p52 (Fig. 2, panels A and C). Taken together with the NF-κB/Rel profiling data shown in Fig. 1B, this finding suggests that reovirus engages not only the classical pathway of NF-κB signaling but also the alternative pathway, albeit at much later times of infection.

310   **Reovirus infection leads to the selective degradation of IκBα.** Activation of the classical NF-κB pathway by physiologic agonists is primarily dependent on degradation of IκBα (reviewed in (3, 25, 27)), an inhibitor that sequesters p50/RelA complexes in the cytoplasmic compartment (2). We have previously shown that degradation-resistant forms of IκBα attenuate reovirus-induced apoptosis, which is critically dependent on NF-

315 κB activation (17). However, mammalian cells express other labile inhibitors that are structurally similar to IκBα, such as IκBβ (37) and IκBε (71). Indeed, prior studies suggest a potential role for signal-dependent degradation of IκBβ (63) and IκBε (71) in the inducible nuclear entry of c-Rel. To determine whether any of these inhibitors is

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under reovirus control, we monitored their levels in T3D-infected cells in immunoblotting studies using IκB-specific antibodies. The cellular pool of IκBα was significantly reduced within 4 h after infection with T3D (Fig. 3, A and B). In contrast, levels of IκBβ and IκBε were maintained under the same stimulatory conditions over the entire 8 h timecourse (Fig. 2, C-F). We conclude that IκBα is a primary cellular target of reovirus, which is fully consistent with its capacity to stimulate nuclear translocation of NF-κB p50/RelA.

IKKα and IKKγ are required for reovirus-induced NF-κB activation. We next investigated the mechanism by which reovirus destabilizes IκB proteins. Cytokine-induced degradation of NF-κB inhibitors is dependent on their phosphorylation at specific serine residues by IKKs such as IKKα and IKKβ (6, 23, 78). These structurally-related enzymes can interact and form higher order complexes with other cellular proteins (reviewed in (25, 27)). Integration of the regulatory protein IKKγ/Nemo into such complexes is required for activation of IKKβ (53, 54, 75) but not IKKα (13, 21, 55). The most well-characterized substrate of IKKβ is IκBα (30, 41), whereas IKKα catalyzes phosphorylation of p100/NF-κB2 (55, 73).

To determine whether either IKKα or IKKβ is required for reovirus-induced activation of NF-κB, cellular IKK complexes were immunopurified from HeLa cells either before or after infection with T3D and monitored for their capacity to phosphorylate IκBα in vitro. In keeping with the kinetics of IκBα degradation (Fig. 3A) and NF-κB (Fig. 1A) activation, IκB kinase activity exceeding basal levels in uninfected cells was readily detected within 4 h after exposure to T3D and sustained for at least an additional 4 h (Fig. 4A). These data suggest that IKKs are critically involved in the mechanism by which reovirus diminishes the cellular pool of IκBα (Fig. 3A).

To determine whether IKK activation is required for the nuclear translocation of NF-κB by reovirus, cells were treated with escalating doses of the IKK inhibitor BAY 65-1942 prior to infection with T3D. Importantly, BAY 65-1942 inhibits IKKβ more efficiently than IKKα (79). As demonstrated in EMSAs, treatment of cells with BAY 65-1942 suppressed NF-κB signaling induced by reovirus (Fig. 4, B and C), although incompletely, perhaps reflecting incomplete blockade of IKKα. Immunoblotting studies of nuclear extracts from the same panel of infected cells indicated that BAY 65-1943 had a profound inhibitory effect on reovirus-induced nuclear translocation of p65/RelA (Figs. 4, D and E), which is primarily under the control of IKKβ. These pharmacological data suggest that reovirus activates NF-κB via a mechanism involving either IKKα, IKKβ, or both of these IKKs.

To identify the IKK subunits responsible for NF-κB activation by reovirus, NF-κB DNA-binding activity was assessed in MEFs deficient for IKKα, IKKβ, or the IKKβ regulatory subunit IKKγ/Nemo. In initial experiments, EMSAs were conducted with nuclear extracts from wild-type MEFs following infection with T3D for 8 h, which corresponds to peak levels of NF-κB DNA-binding activity (Fig. 1A). Reovirus induced NF-κB DNA binding activity to levels comparable to or exceeding those observed in

control experiments with wild-type MEFs treated with the cytokine TNFα (Fig. 5A), a potent agonist of IKK. Similar results were obtained with nuclear extracts from reovirus-infected MEFs lacking IKKβ (Fig. 5A). However, the capacity of reovirus to activate NF-κB was completely disrupted in MEFs lacking IKKα (Fig. 5A), indicating preferential usage of this IKK relative to IKKβ. MEFs lacking the regulatory subunit IKKγ/Nemo (Fig. 5A) also were incapable of reovirus-mediated NF-κB signaling. Immunoblotting studies of nuclear extracts from the same panel of infected cells confirmed these results (Fig. 5C). Nuclear translocation of p65/RelA was detected in wild-type MEFs and IKKβ-deficient MEFs, but not in MEFs lacking IKKα or IKKγ. Differences in reovirus-mediated signal transduction in IKK-deficient MEFs could not be attributed to differences in viral infection or growth (data not shown). Thus, these findings suggest that IKKα and IKKγ are required for reovirus-induced NF-κB activation.

NIK is capable of phosphorylating and activating IKKα in response to some agonists of the alternative NF-κB pathway, such as lymphotoxin β (36, 40). To determine whether NIK is required for NF-κB activation in response to reovirus, NIK-deficient MEFs were used to probe for NF-κB induction by EMSA and immunoblotting in response to reovirus T3D infection (Fig. 6). Reovirus infection resulted in NF-κB activation in both wild-type and NIK-deficient MEFs, indicating that NIK is dispensable for reovirus-induced NF-κB activation. Taken together, these data confirm a requirement for endogenous IKK in the mechanism by which reovirus activates NF-κB and strongly suggest that this virus selectively utilizes the IKKα arm of the NF-κB signaling pathway. Surprisingly, IKKγ/Nemo, which is known to regulate IKKβ rather than IKKα, is also required for NF-κB activation by reovirus, but NIK is dispensable.

IKKα and IKKγ are required for reovirus-induced apoptosis. Since IKKα and IKKγ are required for NF-κB activation following reovirus infection (Fig. 5), we examined whether IKK stimulation by reovirus leads to apoptotic cell death. IKK-deficient MEFs were infected with reovirus T3D, and apoptosis was assessed by quantitation of caspase 3 activity (Fig. 7A). Levels of activated caspase 3 following infection of wild-type and IKKβ-deficient MEFs were substantially greater than those following infection of MEFs deficient in either IKKα or IKKγ. To corroborate these results, we tested wild-type and IKK-null MEFs for viability following infection with T3D (Fig. 7B). In comparison to wild-type and IKKβ-deficient MEFs, a significantly greater percentage of IKKα- and IKKγ-deficient MEFs remained viable during a time course of reovirus infection.

To determine whether NIK is required for apoptosis induction following reovirus infection, NIK-deficient MEFs were infected with reovirus T3D, and apoptosis was assessed by quantification of caspase 3 activity (Fig. 7C). Levels of caspase 3 activity in MEFs deficient in NIK were equivalent to those in wild-type cells following infection with T3D. In parallel with these results, we observed no significant difference in the viability of wild-type and NIK-deficient MEFs following T3D infection (Fig. 7D). Together, these functional data with IKK- and NIK-deficient MEFs strongly correlate with the capacity of reovirus to modulate IκBα and NF-κB during infection (Figs. 1-6).

Our findings suggest that IKKα and IKKγ are required for the induction of NF-κB activation leading to apoptosis in response to reovirus. In keeping with the activation of NF-κB by reovirus, apoptosis is completely independent of the IKKα inducer NIK, indicating a different mechanism of IKKα activation in response to reovirus.

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DISCUSSION

Apoptosis is a genetically programmed form of cell death that plays an important regulatory role in many biological processes. Many viruses are capable of inducing apoptosis of infected cells (52). In some cases, apoptosis triggered by viral infection may serve as a component of host defense to limit viral replication or spread. In other instances, apoptosis may enhance viral infection by facilitating viral dissemination or allowing virus to evade host inflammatory responses (4, 5, 52). Apoptosis has been shown to play an important role in reovirus-induced disease (19, 20, 44, 45). Although we previously uncovered an essential function for NF-κB activation in reovirus-induced apoptosis (17), it was not known how reovirus activates this signal-transduction mechanism. Results reported here identify constituents of the NF-κB signaling apparatus induced by reovirus and provide evidence that NF-κB activation during reovirus infection requires integral components of both the classical and alternative pathways.

Using MEFs deficient in the expression of individual IKK subunits, we demonstrate that reovirus-infected cells lacking IKKα are impaired for NF-κB activation (Fig. 5) and apoptotic programming (Fig. 7), whereas both of these processes are operative in cells lacking IKKβ. Despite its preferential usage of IKKα, reovirus retains the capacity to elicit both NF-κB activation and apoptosis in the absence of NIK (Fig. 6 and 7), a known activator of IKKα in cytokine-treated cells (36, 55). Furthermore, targeted disruption of the gene encoding IKKγ/Nemo, which is dispensable for cytokine-induced signaling of IKKα (13, 21), significantly attenuates reovirus-induced NF-κB activation and apoptosis (Fig. 5 and 7). In light of these findings with NIK and IKKγ, the precise mechanism of reovirus action on IKKα remains unclear. The simplest interpretation of these results is that reovirus accesses the cellular NF-κB machinery by directly interfacing with IKKα/IKKγ complexes, with IKKγ serving as an adaptor that docks one or more reovirus gene products. In keeping with this possibility, IKKγ tethers the HTLV1 Tax protein to IKK complexes, resulting in persistent activation of IKKβ and NF-κB (7, 12). In what may be another related finding, IKKγ also is required for Tax-induced activation of IKKα (72). Although data emerging from studies of IKKβ-deficient mice suggest the presence of functional IKKα/IKKγ complexes (34, 35, 50, 61), direct evidence for the existence of IKKα/IKKγ complexes in wild-type animals has not been reported. Notwithstanding, our results clearly establish that reovirus activates NF-κB and downstream proapoptotic genes via a mechanism involving IKKα but not IKKβ.

The principle in vivo substrate of IKKβ is IκBα (23, 41, 47). This cytoplasmic inhibitor tightly controls the nuclear translocation of p50/RelA dimers (2), effectors of the classical NF-κB pathway (reviewed in (3, 25, 27)). The principle in vivo substrate of IKKα is p100/NF-κB2 (55). An integral inhibitor in the alternative NF-κB pathway, p100 assembles with the transactivator protein RelB (55, 60). Following IKKα-mediated phosphorylation, p100 is processed to p52 via a proteasome-dependent mechanism, permitting the nuclear entry of p52/RelB complexes (55, 60). Given these distinct mechanisms, our findings with reovirus-infected cells suggest an unconventional function for IKKα in substrate targeting. Specifically, we were unable to detect either p52 or RelB

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500　DNA-binding activity in nuclear extracts from cells following 4 to 8 h of reovirus infection (Fig. 1B). Instead, at these early timepoints the predominant Rel species detected were p50 and RelA (Fig. 1B), which are primarily under IκBα control (1, 2). Consistent with this Rel profile, IκBα protein levels were significantly reduced by 4 h post-infection (Fig. 3). Although p100 processing to p52 was observed at late time points
505　during reovirus infection (16 h), it seems unlikely that this delayed response contributes to the more rapidly evolving signals required for apoptosis (16, 17, 49). Accordingly, we propose that IKKα rather than IKKβ targets IκBα for proteolytic destruction and regulates the nuclear translocation of p50/RelA complexes in reovirus-infected cells. This working model is fully concordant with the phenotype of cell lines deficient for either
510　IKKα, p50, or p65/RelA, all of which are impaired for reovirus-induced NF-κB activation and proapoptotic signaling (Figs. 4, 5, and (17)). In agreement with this model, prior studies with recombinant proteins indicate that IKKα can efficiently phosphorylate NF-κB-bound forms of IκBα in vitro (77).

515　　　　　Both IKKα and IKKβ contain regulatory serine phosphoacceptors in their so-called "T loop" domains (22, 41). Signal-dependent phosphorylation of the T loop serines in IKKα and IKKβ is a prerequisite for their catalytic activation (22). Based on in vitro studies with recombinant proteins, IKKα and IKKβ can autophosphorylate at these T loop serines (22, 54, 62). Physiologic agonists of the alternative NF-κB pathway
520　stimulate T loop phosphorylation and activation of IKKα via the upstream kinase NIK (36, 55, 73). However, NIK is dispensable in the mechanism by which reovirus induces NF-κB activation and apoptosis (Fig. 6 and 7). What signal transducers couple reovirus to IKK? Experiments using pharmacological inhibitors suggest that NF-κB-dependent apoptotic signaling is triggered by viral replication steps that occur after disassembly but
525　prior to RNA synthesis (16). Strain-specific differences in the capacity of reovirus to induce apoptosis segregate with viral genes encoding the σ1 and μ1 proteins (15, 49, 66), which play important roles in viral attachment (32, 70) and membrane penetration (8, 9, 38), respectively. Importantly, transient expression of μ1 is sufficient to induce apoptosis in cell culture (14), implicating this protein in the reovirus/NF-κB signaling axis. We
530　envision three potential mechanisms by which μ1, or perhaps another viral gene product, initiates NF-κB signal transduction during reovirus infection. First, μ1 may activate viral sensors that mediate the recruitment of the adaptor protein interferon-β promoter stimulator (IPS-1), which mediates the activation of NF-κB in response to viral infections by recruiting TNF-associated factor 6 to the signaling complex (31, 42, 56, 74). Second,
535　μ1 may activate a novel cellular kinase that phosphorylates the T loop of IKKα. Third, μ1 may interact with IKK complexes directly, leading to conformational changes that stimulate oligomerization and trigger autophosphorylation of the T loop in IKKα {Poyet, 2000 #5490; Inohara, 2000 #4965; . Studies to test these models for reovirus-induced activation of IKKα are currently underway.
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　　　　　Reovirus infection leads to NF-κB activation in numerous cell types {Connolly, 2000 #2472; Clarke, 2003 #5019; O'Donnell, 2005 #5195}. However, the functional consequences of NF-κB activation in vivo differ depending on the infected tissue. NF-κB activation in the CNS leads to high levels of neuronal apoptosis and encephalitis (45). In

545   contrast, NF-κB activation in the heart leads to interferon-β production, which limits viral replication and protects against apoptosis (45). In the absence of NF-κB signaling, reovirus infection induces widespread apoptotic damage to cardiac myocytes, resulting in myocarditis. Mechanisms underlying the divergent cellular fates following NF-κB activation by reovirus in vivo remain unclear. Considering the results presented in this

550   study, these mechanisms may involve tissue-specific activation of different IKK subunits, which in turn may influence the composition of nuclear NF-κB complexes. Experiments using tissue-specific IKKα- or IKKβ-deficient mice should clarify the function of IKK in reovirus-induced disease.

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ACKNOWLEDGEMENTS

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FIGURE LEGENDS

910

FIG. 1. Biphasic activation of NF-κB/Rel proteins in reovirus-infected cells. (A) Nuclear extracts were prepared from uninfected HeLa cells (0 h), mock-infected cells (Mock), or cells infected with T3D at an MOI of 100 PFU/cell for the times shown. Cells also were treated with 20 ng/ml of TNFα for 30 min as a positive control. Extracts were

915 incubated with a radiolabeled NF-κB consensus oligonucleotide, and resulting protein-oligonucleotide complexes were resolved by acrylamide gel electrophoresis, dried, and exposed to film. (B) Nuclear extracts prepared at 4, 6, and 8 h post infection were incubated with antisera specific for p50, p52, RelA, RelB, or c-Rel prior to the addition of a radiolabeled NF-κB consensus oligonucleotide. NF-κB-containing complexes are

920 indicated.

FIG. 2. Processing of p100 to p52 during reovirus infection. (A) Whole-cell extracts were prepared from uninfected HeLa cells (0 h), mock-infected cells (Mock), or cells infected with reovirus T3D at an MOI of 100 PFU/cell for the times shown. Cells

925 also were treated with 2 μg/ml of an agonistic lymphotoxin-β receptor antiserum for 8 h as a positive control. Extracts were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted by using an antiserum specific for p100/p52. Band intensity was quantified by using the Image J program. The results are presented as the mean ratio of (B) p100/actin or (C) p52/p100 for three independent experiments. Error

930 bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to untreated cells (0 h).

FIG. 3. Reovirus infection leads to degradation of IκBα but not IκBβ or IκBε. Cytoplasmic extracts were prepared from uninfected HeLa cells (0 h), mock-infected

935 cells (Mock), or cells infected with reovirus T3D at an MOI of 100 PFU/cell for the times shown. Cells also were treated with 20 ng/ml of TNFα for 10 min as a positive control. Extracts were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted by using antisera specific for (A) IκBα, (C) IκBβ, or (E) IκBε. An actin-specific antiserum was used to detect levels of actin as a loading control. Band intensity

940 corresponding to levels of (B) IκBα, (D) IκBβ, and (F) IκBε was quantified by using the Image J program. The results are presented as the mean ratio of IκB/actin for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to untreated cells (0 h).

945 FIG. 4. Involvement of IKKs in reovirus-induced NF-κB activation. (A) Whole-cell extracts were prepared from uninfected HeLa cells (0 h), mock-infected cells (Mock), or cells infected with reovirus T3D at an MOI of 100 PFU/cell for the times shown. Cells also were treated with 20 ng/ml of TNFα for the times shown as a positive control. The IKK complex was immunoprecipitated by using an IKKγ-specific antiserum prior to

950 incubation with a GST-IκBα substrate in the presence of [γ-^{32}P]ATP. Kinase reactions were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. (B) HeLa cells were pretreated with IKK inhibitor BAY 65-1942 for 1 h at the concentrations shown and uninfected (Untreated), mock-infected (Mock), or infected with reovirus T3D at an MOI of 100 PFU/cell for the times shown. Nuclear

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955   extracts were incubated with a radiolabeled NF-κB consensus oligonucleotide, and resulting protein-oligonucleotide complexes were resolved by acrylamide gel electrophoresis, dried, and exposed to film. (C) Band intensity was quantified by determining PSL units relative to uninfected cells for four independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in

960   comparison to untreated cells (0 μM). (D) Nuclear extracts from the experiment shown in panel B were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted by using a RelA-specific antiserum. (E) Band intensity was quantified relative to uninfected cells by using the Image J program. The results are presented as the mean RelA band intensity for three independent experiments. Error bars indicate standard

965   deviations. *, $P < 0.05$ as determined by Student's t test in comparison to untreated cells (0 μM).

       Fig. 5. IKKα and IKKγ are required for reovirus-induced activation of NF-κB. (A) Wild-type MEFs or MEFs deficient in IKKα, IKKβ, or IKKγ were uninfected (0 h),

970   mock-infected (Mock), infected with reovirus T3D at an MOI of 100 PFU/cell for 8 h, or treated with 20 ng/ml of TNFα for 1 h. Nuclear extracts were incubated with a radiolabeled NF-κB consensus oligonucleotide. Resulting protein-oligonucleotide complexes were resolved by acrylamide gel electrophoresis, dried, and exposed to film. NF-κB-containing complexes are indicated. (B) Band intensity was quantified by

975   determining PSL units relative to uninfected cells for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to mock-treated cells (0 h). (C) Nuclear extracts from the experiment shown in panel A were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted by using a RelA-specific antiserum.
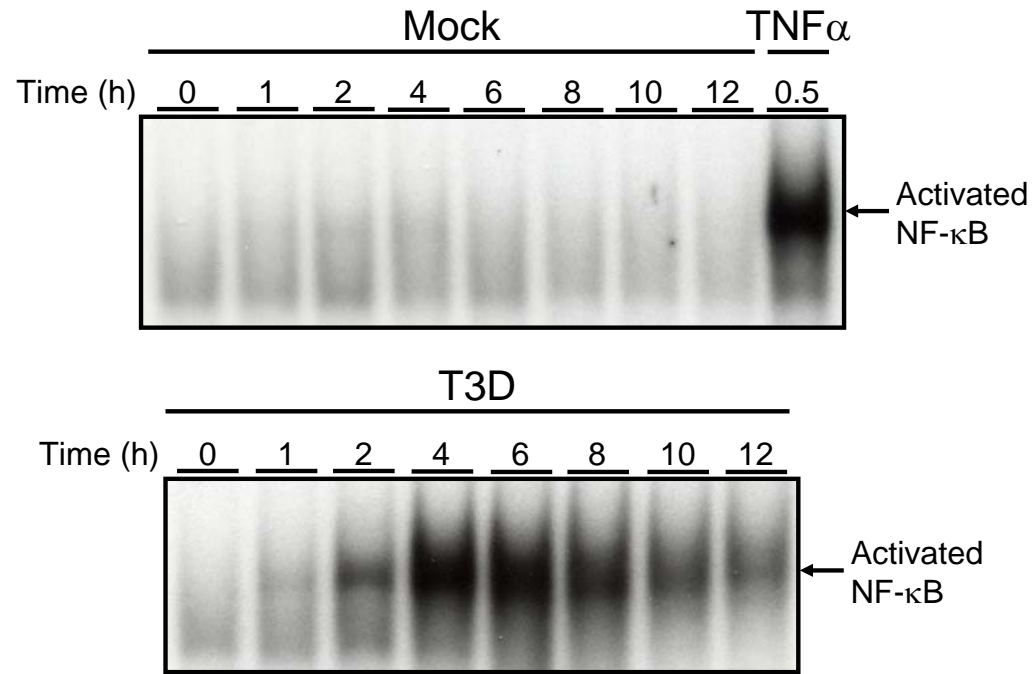
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       FIG. 6. Reovirus-induced activation of NF-κB in NIK-deficient cells. (A) Wild-type MEFs or NIK-deficient MEFs were uninfected (0 h), mock-infected (Mock), infected with reovirus T3D at an MOI of 1000 PFU/cell for 8 h, or treated with 20 ng/ml of TNFα for 30 min. Nuclear extracts were incubated with a radiolabeled NF-κB

985   consensus oligonucleotide. Resulting protein-oligonucleotide complexes were resolved by acrylamide gel electrophoresis, dried, and exposed to film. NF-κB-containing complexes are indicated. (B) Nuclear extracts from the experiment shown in panel A were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted by using a RelA-specific antiserum.

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       FIG. 7. IKKα and IKKγ are required for reovirus-induced apoptosis. Wild-type MEFs or (A) MEFs deficient in IKKα, IKKβ, IKKγ, or (C) NIK were mock-infected, infected with reovirus T3D at an MOI of 1000 PFU/cell for 24 h, treated with 10 ng/ml of TNFα for 12 h, or treated with 10 ng/ml of TNFα and 10 μg/ml of cycloheximide for 12

995   h. Caspase 3/7 activity was quantified by using a luminescent substrate. The results are expressed as the mean caspase activity relative to mock-infected cells for three independent experiments. Wild-type MEFs or (B) IKK-deficient MEFs or (D) NIK-deficient MEFs were mock-infected, infected with reovirus T3D at an MOI of 1000 PFU/cell for 48 h, treated with 10 ng/ml of TNFα for 24 h, or treated with 10 ng/ml of

1000   TNFα and 10 μg/ml of cycloheximide for 24 h. Cell viability was quantified by trypan blue exclusion. The results are expressed as the mean percentage of cell death for three independent experiments. Error bars indicate standard deviations. *, $P < 0.05$ as determined by Student's t test in comparison to mock-infected cells.
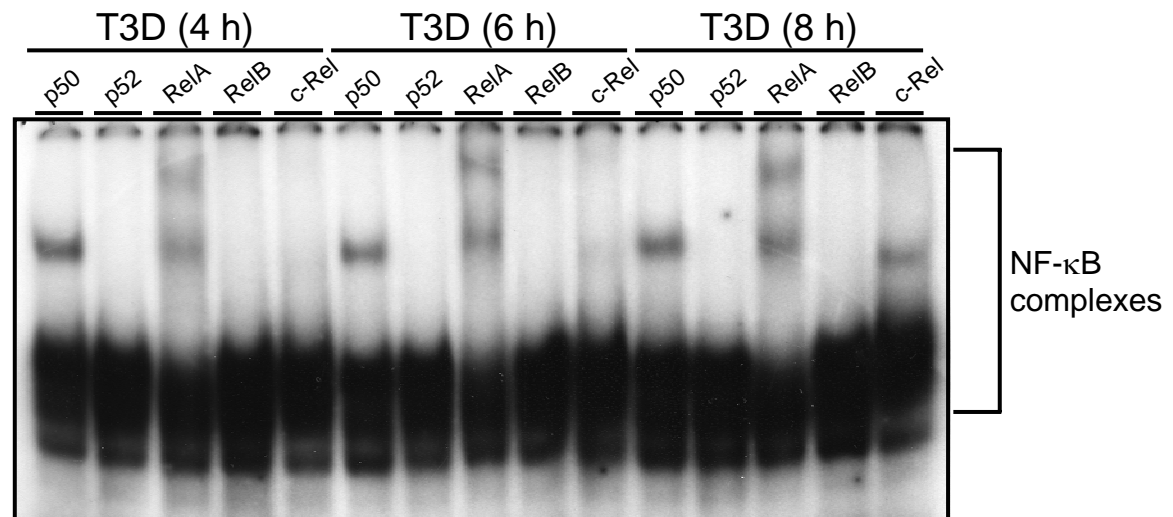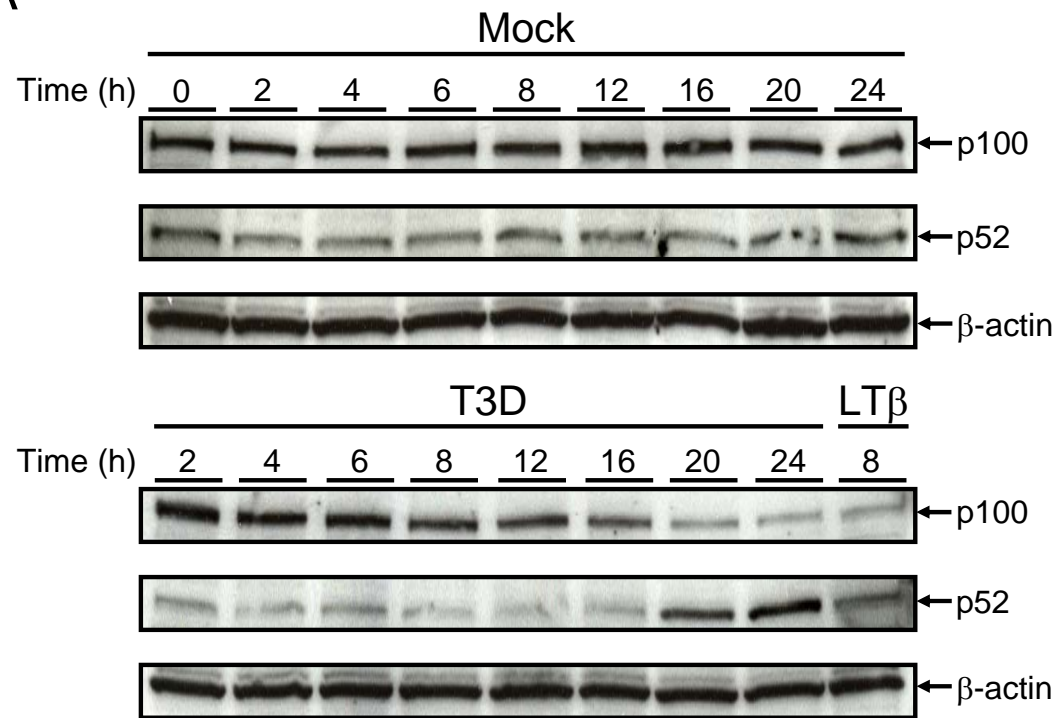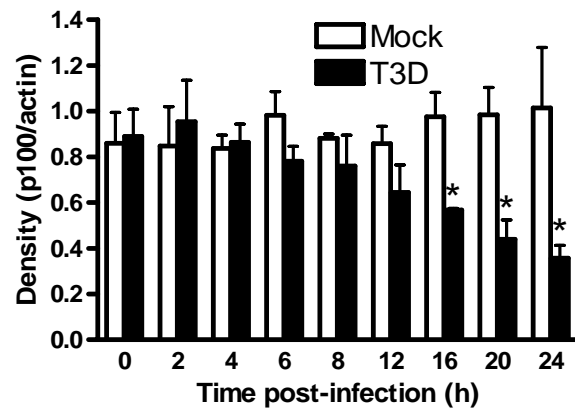
Figure 1

A

Mock   TNFα

Time (h)   0   1   2   4   6   8   10   12   0.5

← Activated NF-κB

T3D

Time (h)   0   1   2   4   6   8   10   12

← Activated NF-κB

B

T3D (4 h)   T3D (6 h)   T3D (8 h)

p50 p52 RelA RelB c-Rel p50 p52 RelA RelB c-Rel p50 p52 RelA RelB c-Rel

NF-κB complexes

Figure 2

A

Mock

Time (h)   0   2   4   6   8   12   16   20   24

← p100

← p52

← β-actin

T3D   LTβ

Time (h)   2   4   6   8   12   16   20   24   8

← p100

← p52

← β-actin

B

C
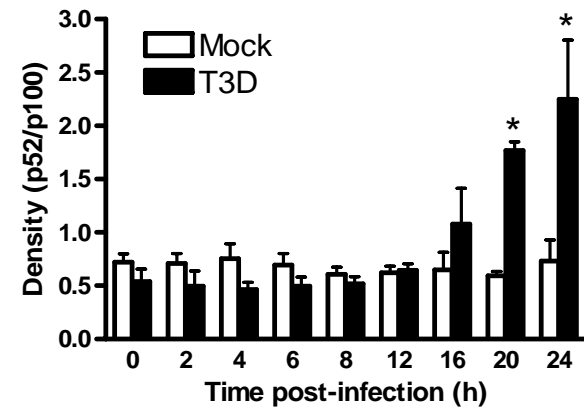
Figure 3

Figure 4

Figure 5

A

Wild-type

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

← Activated NF-κB

IKKα -/-

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

IKKβ -/-

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

← Activated NF-κB

IKKγ -/-

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

B

C

Wild-type

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

IKKα -/-

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

← RelA

IKKβ -/-

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

IKKγ -/-

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

← RelA

Figure 6

A

Wild-type

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

NIK -/-

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

← Activated NF-κB

B

Wild-type

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

← RelA

NIK -/-

	Mock	T3D	TNFα
0 h	8 h	8 h	0.5 h

← RelA

Figure 7

Data not shown

A

Wild-type

IKKα -/-

IKKβ -/-

IKKγ -/-

B

Data not shown. Cells deficient in IKK subunits are permissive for reovirus infection and growth. (A) Wild-type MEFs or MEFs deficient in IKKα, IKKβ, or IKKγ were infected with T3D at an MOI of 1000 PFU/cell. After 24 h incubation, cells were fixed and incubated with reovirus-specific antiserum. Infected cells were identified by using indirect immunofluorescence. (B) Cells and medium were infected with reovirus T3D at an MOI of 1 PFU/cell and incubated for the times shown. Cells and medium were frozen and thawed twice, and viral titers were determined by plaque assay. The results are expressed as the mean viral titers for three independent experiments. Error bars indicate standard deviations.

APPENDIX C

ORGAN-SPECIFIC ROLES FOR TRANSCRIPTION FACTOR NF-κB IN REOVIRUS-

INDUCED APOPTOSIS AND DISEASE

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Organ-specific roles for transcription factor NF-κB in reovirus-induced apoptosis and disease

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Reovirus induces apoptosis in cultured cells and in vivo. In cell culture models, apoptosis is contingent upon a mechanism involving reovirus-induced activation of transcription factor NF-κB complexes containing p50 and p65/RelA subunits. To explore the in vivo role of NF-κB in this process, we tested the capacity of reovirus to induce apoptosis in mice lacking a functional *nfkb1/p50* gene. The genetic defect had no apparent effect on reovirus replication in the intestine or dissemination to secondary sites of infection. In comparison to what was observed in wild-type controls, apoptosis was significantly diminished in the CNS of p50-null mice following reovirus infection. In sharp contrast, the loss of p50 was associated with massive reovirus-induced apoptosis and uncontrolled reovirus replication in the heart. Levels of IFN-β mRNA were markedly increased in the hearts of wild-type animals but not p50-null animals infected with reovirus. Treatment of p50-null mice with IFN-β substantially diminished reovirus replication and apoptosis, which suggests that IFN-β induction by NF-κB protects against reovirus-induced myocarditis. These findings reveal an organ-specific role for NF-κB in the regulation of reovirus-induced apoptosis, which modulates encephalitis and myocarditis associated with reovirus infection.

Introduction

Mechanisms of viral disease involve complex interactions of pathogen virulence factors and host responses. Perhaps the best-understood basis of organ-specific viral pathology is the availability of cell-surface molecules required for viral attachment and entry. Rarely, however, is viral disease ascribable solely to receptor recognition. More commonly, additional virus-host interactions determine the outcome of infection (1), and these pivotal steps are of much interest in studies of viral pathogenesis. Factors expected to modulate viral growth and virulence in an organ-dependent manner include the capacity of virus to efficiently utilize the host translational apparatus, including strategies to circumvent antiviral effects of IFN; availability of cellular proteins to facilitate viral replication and gene expression; and changes in the intracellular signaling dynamic induced by viral infection.

Mammalian orthoreoviruses (simply called reoviruses here) have served as highly tractable models for studies of viral pathogenesis. Reoviruses are nonenveloped, icosahedral viruses with a genome consisting of 10 double-stranded RNA segments (2). After infection of newborn mice, reoviruses disseminate systemically, producing injury to a variety of organs, including the CNS, heart, and liver (3). Strain-specific differences in receptor utilization influence some types of reovirus disease (4, 5); however, disease pathogenesis at other sites is more complex (6, 7).

The NF-κB family of transcription factors plays a key role in the regulation of cell growth, activation, differentiation, and survival. Following exposure of cells to a variety of stimuli, NF-κB is activated and translocated to the nucleus (8), where it serves as a transcriptional regulator (9, 10). In systems in which NF-κB is activated during apoptosis, NF-κB can either prevent (10–13) or potentiate (14–17) cell death signaling. Following reovirus infection of cultured cells, the heterodimeric NF-κB complex p50/p65 translocates to the nucleus and activates proapoptotic gene expression (18). When NF-κB activation is inhibited using proteasome inhibitors or dominant-negative forms of IκBα, reovirus-induced apoptosis is blocked (18). Moreover, cell lines deficient in either of the p50 or p65 NF-κB subunits do not undergo apoptotic cell death following reovirus infection. These findings indicate that activation of NF-κB in cell culture is required for reovirus-induced apoptosis.

In the CNS (19) and heart (20) of newborn mice, reovirus induces the morphological and biochemical features of apoptosis. This cell-death response in brain and heart tissue is associated with reovirus-induced disease. Inhibitors of apoptosis ameliorate heart disease (20), which indicates a causal relationship between programmed cell death and reovirus-induced myocarditis. However, the molecular basis of CNS and cardiac pathology during reovirus infection has not been fully elucidated.

We now demonstrate that the p50 subunit of NF-κB plays an essential role in the development of encephalitis and myocarditis in reovirus-infected mice. Although reovirus infects the intestine and disseminates systemically following peroral inoculation of mice lacking the NF-κB p50 subunit, apoptosis is diminished in the brain yet strikingly enhanced in the heart. These findings sug-

gest a novel role for NF-κB in the pathogenesis of viral infection; it serves a proapoptotic function in the CNS, while mediating a prosurvival function in the myocardium.

Results

Reovirus activates NF-κB in vivo. To determine whether reovirus is capable of NF-κB activation in the intact host, we performed in vivo luciferase assays using transgenic mice engineered to express luciferase under control of an HIV long-terminal repeat promoter that contains NF-κB consensus binding sites (21). These mice were inoculated perorally with either PBS (mock-infected) or 10⁴ PFU reovirus strain T3SA⁺, which was chosen for these studies because of its capacity to activate NF-κB and induce a potent apoptotic response in cultured cells (22). Seven days after inoculation, the mice were imaged for luciferase activity as a marker for NF-κB activation (Figure 1, A and B). Little luciferase activity was detected in the mock-infected mice (Figure 1A). In contrast, reovirus-infected animals exhibited systemic luciferase activity (Figure 1B), which indicated that reovirus is capable of NF-κB activation in vivo.

Reovirus-induced activation of NF-κB in the murine CNS and heart is dependent on p50. To determine whether reovirus activates NF-κB in the murine CNS and heart, we performed electrophoretic mobility shift assays (EMSAs) using brain and heart extracts prepared from reovirus-infected or mock-infected wild-type and p50-null mice. Newborn p50⁺/⁺ and p50⁻/⁻ mice were inoculated with either PBS or 10⁴ PFU reovirus T3SA⁺. Cell extracts were prepared from brain and heart tissue 12 days after inoculation, incubated with a radiolabeled oligonucleotide consisting of the NF-κB consensus binding sequence, and resolved by PAGE using nondenaturing conditions (Figure 2, A and D). NF-κB DNA-binding activity was detected in extracts from the brain of reovirus-infected p50⁺/⁺ but not p50⁻/⁻ mice (Figure 2A). Similarly, NF-κB DNA-binding activity was detected in the heart of p50⁺/⁺ mice infected with reovirus but not p50⁻/⁻ animals (Figure 2D). These findings indicate that reovirus infection in

the murine CNS and heart induces nuclear translocation of NF-κB, which is contingent on the expression of the NF-κB p50 subunit.

To confirm the specificity of NF-κB DNA-binding activity in these experiments, we incubated cell extracts from reovirus-infected p50⁺/⁺ mouse brain and heart with a ³²P-labeled NF-κB consensus oligonucleotide in the presence of excess unlabeled consensus oligonucleotide (Figure 2, B and E). Binding of the radiolabeled probe was competed with that of unlabeled consensus oligonucleotide, which suggests that the gel-shift activity detected following reovirus infection is specific for sequences that are bound by NF-κB.

In cell culture, reovirus infection results in the nuclear translocation of NF-κB complexes containing subunits p50 and p65 (18). As an additional specificity control in these experiments for the activation of NF-κB, nuclear extracts were prepared from reovirus-infected p50⁺/⁺ mouse brain or heart and incubated with an antiserum specific to p65 prior to the addition of the NF-κB–specific oligonucleotide (Figure 2, C and F). Addition of the p65-specific antiserum resulted in bands of higher relative molecular mass, which verified that p65 is present in the NF-κB complexes activated following reovirus infection. These findings provide strong evidence that reovirus infection of the murine CNS induces the nuclear translocation of NF-κB and this effect is abolished in mice lacking p50.

NF-κB subunit p50 is not required for efficient reovirus replication or dissemination in the murine host. To determine whether p50 plays a role in reovirus growth in vivo, we inoculated p50⁺/⁺ and p50⁻/⁻ mice intracranially or perorally with 10⁴ PFU reovirus T3SA⁺. Viral titers in the brain were determined by plaque assay 2, 4, and 6 days after intracranial inoculation (Figure 3A) and in the intestine, liver, brain, and heart 4, 6, 8, 10, and 12 days after peroral inoculation (Figure 3B). Following intracranial inoculation, viral titers in p50⁺/⁺ and p50⁻/⁻ mice were equivalent at all time points tested. After peroral inoculation, virus replicated efficiently in the intestines of both p50⁺/⁺ and p50⁻/⁻ mice and disseminated to the liver, brain, and heart. Viral titers in the intestine, liver, and brain did not differ between p50⁺/⁺ and p50⁻/⁻ mice. In sharp contrast, viral titers in the hearts of p50⁻/⁻ mice were more than 1,000-fold higher than those in the hearts of p50⁺/⁺ animals. These findings suggest that p50 is dispensable for reovirus growth in vivo and that the absence of p50 in the heart, but not in other tissues tested, allows for increased reovirus replication.

NF-κB subunit p50 is required for efficient induction of apoptosis in the CNS following reovirus infection. To assess reovirus-induced pathologic changes in the CNS of p50⁺/⁺ and p50⁻/⁻ mice, we prepared brain sections from mice euthanized at 12 days following peroral inoculation with reovirus T3SA⁺ and examined them after staining with H&E (Figure 4, A and B and data not shown). Brain sections from reovirus-infected p50⁺/⁺ and p50⁻/⁻ mice exhibited evidence of meningoencephalitis. Inflammatory infiltrates were detected primarily in the cerebral cortex, hippocampus, diencephalon, and brain stem. Morphologically, inflammatory cells were mostly lymphocytes and macrophages/microglia with some plasma cells and neutrophils. Inflammatory changes were more extensive in p50⁺/⁺ mice (Figure 4A) than in p50⁻/⁻ mice (Figure 4B),
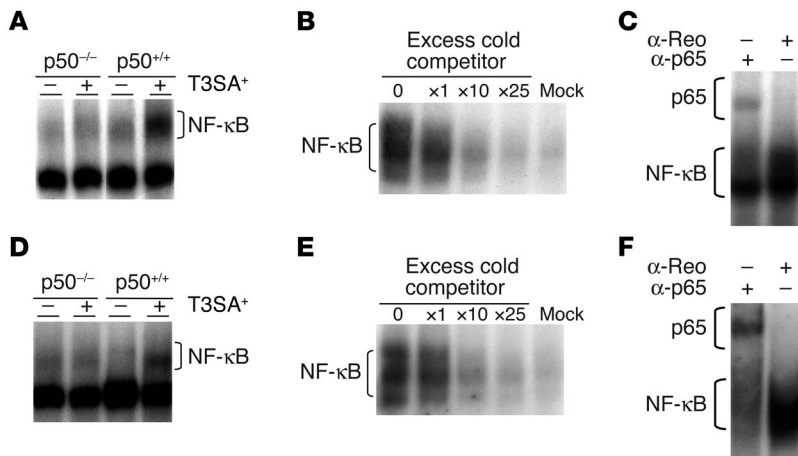
Figure 2
Reovirus-induced NF-κB gel-shift activity following infection of p50^{+/+} and p50^{–/–} mice. (**A** and **D**) Newborn p50^{+/+} and p50^{–/–} mice were inoculated perorally with either 10[4] PFU reovirus T3SA[+] or PBS. Brains (**A**) and hearts (**D**) were resected 12 days after inoculation, and cell extracts were prepared. Extracts were incubated with a ^{32}P-labeled NF-κB consensus oligonucleotide and resolved by nondenaturing PAGE. Activated NF-κB complexes are indicated. Shown is a representative experiment of 4 performed. (**B** and **E**) Extracts were prepared from either brains (**B**) or hearts (**E**) of reovirus-infected p50^{+/+} mice and incubated with ^{32}P-labeled NF-κB consensus oligonucleotide in the presence of unlabeled NF-κB consensus probe (cold competitor) at the molar concentrations shown. Extracts prepared from either brains or hearts of uninfected p50^{+/+} mice were incubated with ^{32}P-labeled NF-κB consensus oligonucleotide and electrophoresed in the lane labeled "Mock." NF-κB complexes are indicated. (**C** and **F**) Extracts were prepared from either brains (**C**) or hearts (**F**) of reovirus-infected p50^{+/+} mice, and prior to the addition of the ^{32}P-labeled oligonucleotide probe, extracts were incubated with either a control antibody specific to reovirus protein σ3 (α-Reo) or an antibody specific to NF-κB subunit p65 (α-p65). Supershifted complexes containing p65 are indicated.

which suggests that the neurovirulence of reovirus is attenuated in mice lacking an intact NF-κB signaling apparatus.

To assess the distribution of reovirus protein expression in the CNS of p50^{+/+} and p50^{–/–} mice, we prepared brain sections from mice euthanized 12 days following peroral inoculation and stained them using a reovirus-specific antiserum (Figure 4, A and B and data not shown). Immunohistochemical staining for reovirus protein demonstrated the presence of immunoreactive neurons in brains of both p50^{+/+} and p50^{–/–} mice (Figure 4, A and B). Antigen-positive neurons were detected in a pattern recapitulating the inflammatory changes; the cerebral cortex, hippocampus, diencephalon, and brain stem were primarily involved. The number of reovirus-infected cells and their distribution was similar in p50^{+/+} and p50^{–/–} mice (Figure 4, A and B). These results suggest that the lack of p50 does not alter reovirus tropism for specific neural regions.

To determine whether p50 is required for apoptosis in the murine CNS, we prepared brain sections from reovirus-infected p50^{+/+} and p50^{–/–} mice 12 days following peroral inoculation (Figure 4, A and B) or 6 days following intracranial inoculation (Figure 4C) and assayed them for fragmented DNA using the TUNEL technique. Apoptotic cells were quantitated by counting all TUNEL-positive cells in cortex, hippocampus, basal ganglia, diencephalon, and brain stem of each section obtained from mice inoculated intracranially (Figure 5). Numbers of TUNEL-positive cells in the brains of infected p50^{+/+} mice were significantly greater than those in the brains of infected p50^{–/–} mice. These findings were the same following both peroral

and intracranial inoculation (Figure 4). Thus, reovirus-induced apoptosis in the murine CNS is dependent on the p50 subunit of NF-κB.

Activation of caspase-3 is a highly specific biomarker of apoptotic cell death (23). To confirm that DNA fragmentation observed in the brains of reovirus-infected p50^{+/+} mice is due to apoptosis, we stained brain sections with an antiserum specific to the activated form of caspase-3 (Figure 4, A and B). Activated caspase-3 was detected in regions of the brain in which TUNEL-positive staining also was observed. Moreover, cells immunoreactive for caspase-3 were detected at a much higher frequency in the brains of p50^{+/+} mice. Morphologically, cells immunoreactive for caspase-3 were primarily neurons, and most immunoreactive neurons also exhibited morphologic evidence of apoptosis. These results provide additional evidence that expression of NF-κB subunit p50 is required for efficient induction of apoptosis during reovirus infection in the murine CNS.

Absence of NF-κB subunit p50 leads to enhanced pathology and massive apoptosis in the murine heart following reovirus infection. Since viral titers in the hearts of p50^{–/–} mice were more than 1,000-fold higher than in those of p50^{+/+} mice (Figure 3B), we examined heart tissue for evidence of inflammation and tissue injury. Newborn p50^{+/+} and p50^{–/–} mice were inoculated perorally with either 10[4] PFU reovirus T3SA[+] or PBS and weighed daily. Mice were euthanized at various time points following inoculation, and hearts were removed and weighed. There were no significant differences in the heart weights of mock-infected p50^{+/+} and p50^{–/–} mice (Figure 6A). Surprisingly, heart weights of reovirus-infected p50^{–/–} mice were significantly greater than those of p50^{+/+} mice (Figure 6B). Differences in the percent heart weight (heart weight relative to total body weight) of infected p50^{+/+} and p50^{–/–} mice became detectable at 8 days after inoculation and continued to increase with time, while there was no significant increase in the percent heart weight of infected p50^{+/+} mice (Figure 6B). Dramatic differences were observed in the gross appearance of hearts dissected from p50^{+/+} and p50^{–/–} animals following infection with reovirus (Figure 6C). Hearts from reovirus-infected p50^{–/–} mice had a blanched appearance with diffuse surface irregularities corresponding to confluence of purulent lesions, consistent with overt myocarditis. In contrast, hearts from mock-infected p50^{–/–} or p50^{+/+} mice or reovirus-infected p50^{+/+} mice displayed no overt abnormalities.

To determine whether reovirus-induced myocardial injury in p50^{–/–} mice is associated with contractile dysfunction, we performed echocardiography on 10-day-old mice after peroral inoculation with either reovirus T3SA[+] or PBS. Fractional shortening, assessed by 2-dimensional, directed M-mode measurements, was substantially decreased in reovirus-infected p50^{–/–} mice (~10%; Figure 6D), while it was preserved in mock-infected p50^{–/–} mice (>40%; Figure 6E) and reovirus-infected p50^{+/+} mice (>40%; Figure 6F). Heart size was also increased in reovirus-infected p50^{–/–} mice compared with mock-infected p50^{–/–} mice and reovirus-infected p50^{+/+} mice. Intact atrioventricular conduction was observed in all mice, which suggests that the pathologic process was not specifically
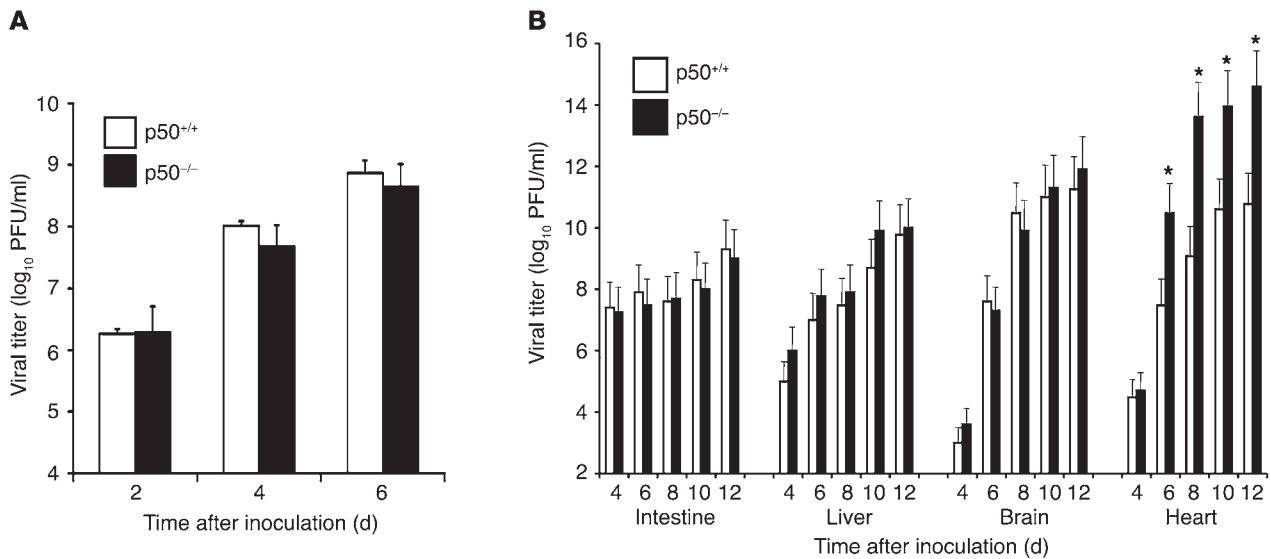
Figure 3
Growth of reovirus in p50^{+/+} and p50^{–/–} mice. (**A**) Titers of reovirus in brain after intracranial inoculation of p50^{+/+} and p50^{–/–} mice. Newborn mice were inoculated with 10^4 PFU reovirus T3SA[+]. At days 2, 4, and 6 after inoculation, mice were euthanized, brains were harvested, and viral titers were determined by plaque assay. (**B**) Titers of reovirus in intestine, liver, brain, and heart after peroral inoculation of p50^{+/+} and p50^{–/–} mice. Newborn mice were inoculated with 10^4 PFU T3SA[+]. At days 4, 6, 8, 10, and 12 after inoculation, mice were euthanized, organs were harvested, and viral titers were determined by plaque assay. The results are expressed as the mean viral titers for 2–4 (**A**) or 4–8 (**B**) animals for each time point. Error bars indicate SDs. *$P < 0.05$ by Student's t test.

targeted to the conduction system. These results suggest that the myocardial pathology associated with reovirus infection of p50^{–/–} mice is associated with diminished contractility.

On a microscopic level, hearts of p50^{–/–} animals displayed extensive myocyte destruction with features of apoptotic and necrotic cell death. Affected areas were notable for cell fragments, granular debris, and scattered calcifications. Thorough sectioning of the organ block revealed that pathology was not limited to any particular region of the heart. Hearts from reovirus-infected p50^{+/+} mice and mock-infected p50^{–/–} and p50^{+/+} mice demonstrated no significant microscopic pathology. We conclude that reovirus is more pathogenic in the heart in the absence of NF-κB subunit p50.

To assess the extent and location of reovirus infection in the murine myocardium in the presence and absence of p50, we performed reovirus antigen staining on heart sections from p50^{+/+} and p50^{–/–} mice euthanized 12 days following peroral inoculation with reovirus T3SA[+] (Figure 7, A and B). Immunohistochemical staining for reovirus protein demonstrated immunoreactive myocytes in heart sections prepared from both p50^{+/+} and p50^{–/–} mice (Figure 7, A and B). However, the number of reovirus-infected cells differed substantially between p50^{+/+} and p50^{–/–} mice, consistent with the significant difference in viral titer in the hearts of these animals.

To determine whether expression of p50 influences apoptosis in the murine heart, we inoculated p50^{+/+} and p50^{–/–} mice perorally with reovirus and assessed them for apoptosis using TUNEL staining (Figure 7, A and B). There were rare TUNEL-positive cells in the hearts of p50^{+/+} mice following reovirus infection (Figure 7A), whereas numerous foci of apoptosis were present in the hearts of p50^{–/–} mice (Figure 7B). Interestingly, foci of apoptotic cells in the hearts of p50^{–/–} mice coincided with areas of intense staining for reovirus antigen, which suggests a link between reovirus replication and apoptosis in cardiomyocytes.

To confirm that the absence of p50 leads to enhanced apoptosis in the heart during reovirus infection, we prepared heart sections from p50^{+/+} and p50^{–/–} mice 12 days following peroral inoculation with reovirus and stained them for activated caspase-3 (Figure 7, A and B). Caspase-3 staining revealed numerous positive myocytes in the same areas of the heart that also were positive for reovirus antigen and TUNEL staining. These results suggest that, in contrast to its effects in the murine CNS, the NF-κB p50 subunit protects against apoptosis induced by reovirus infection in the murine myocardium.

IFN-β is induced in the heart of wild-type mice following reovirus infection. Results presented thus far demonstrate that enhanced reovirus growth in the heart of p50^{–/–} mice is associated with massive apoptosis. We thought it possible that the absence of NF-κB–mediated activation of innate immune responses might lead to increased viral replication and resultant pathology in the heart. To test this hypothesis, we inoculated p50^{+/+} and p50^{–/–} mice perorally with reovirus T3SA[+] or PBS. Twelve days after inoculation, heart and brain were removed, and levels of IFN-β mRNA were determined using real-time PCR (Figure 8). Using GAPDH mRNA as a standardization control, little IFN-β mRNA was induced in the brain of either p50^{–/–} or p50^{+/+} mice in the presence or absence of reovirus infection (Figure 8). In contrast, IFN-β mRNA levels were substantially increased in the heart of reovirus-infected wild-type mice compared with p50^{–/–} animals (Figure 8). These results indicate that IFN-β induction by reovirus in the murine heart is dependent on NF-κB and suggest that IFN-β protects the heart from reovirus-induced apoptosis and disease.

IFN-β treatment of p50-null mice attenuates reovirus-induced myocarditis. To determine whether NF-κB–mediated expression of IFN-β plays a direct role in protection of the heart against apoptosis and disease caused by reovirus, we tested the effect of IFN-β treatment on reovirus infection of p50^{–/–} mice. Newborn p50^{–/–} mice were inoculated intraperitoneally with either IFN-β or PBS 1 day prior
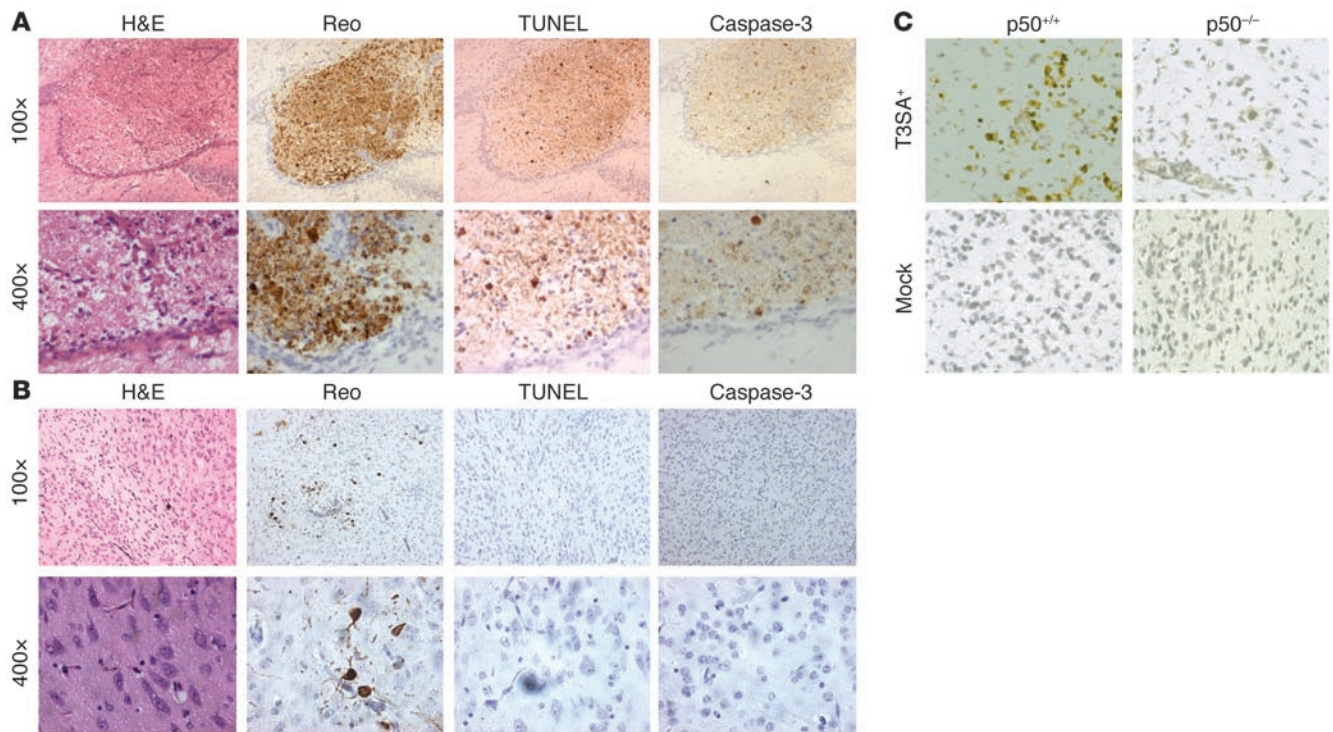
Figure 4
Inflammation, reovirus protein expression, TUNEL staining, and immunohistochemical detection of activated caspase-3 in the brain of reovirus-infected p50^{+/+} (**A**) and p50^{–/–} (**B**) mice. Newborn mice were inoculated perorally with 10[4] PFU reovirus T3SA[+]. At 12 days after inoculation, brains were harvested, paraffin embedded, sectioned, and stained with H&E, polyclonal reovirus-specific antiserum (Reo), TUNEL, or activated caspase-3–specific antiserum as indicated. Shown are consecutive sections of diencephalon. Original magnification, ×100 (top panels) and ×400 (bottom panels). (**C**) Newborn mice were inoculated intracranially with 10[4] PFU T3SA[+] or gelatin saline (Mock). At 6 days after inoculation, mice were euthanized, and brain sections were stained using a TUNEL assay. Shown are sections of the upper brain stem. Original magnification, ×200. Brown staining indicates reovirus protein, fragmented DNA, or activated caspase-3.

to peroral inoculation with reovirus T3SA[+] and treated daily for 9 days thereafter. On day 10, the animals were euthanized, and brain and heart were removed for determination of viral titer and histopathology (Figure 9). IFN-β treatment significantly decreased viral titer in both brain and heart (Figure 9A). In p50^{–/–} mice treated with IFN-β, viral titers reached only 10[2] PFU in the brain and were less than 10[2] PFU in the heart (Figure 9A). In parallel with these results, apoptosis in the heart of IFN-β–treated p50^{–/–} mice was substantially diminished (Figure 9B). Thus, a critical component of the underlying mechanism of NF-κB–mediated protection against reovirus-induced myocardial injury is contingent on IFN-β.

Discussion

Here we report organ-specific roles for NF-κB in the pathogenesis of viral disease, which is a heretofore unknown property of this signaling molecule. The key finding is that marked differences in the pathogenesis of reovirus infection in the CNS and heart are dependent on the action of NF-κB. Following reovirus infection in the CNS, p50^{+/+} mice exhibited significant neuronal apoptosis, while p50^{–/–} mice displayed a minimal apoptotic response. In sharp contrast, reovirus induced little apoptosis in the heart of p50^{+/+} mice, whereas extensive apoptosis occurred in the heart of p50^{–/–} mice. These findings indicate that NF-κB subunit p50 plays 2 distinctly different roles in reovirus pathogenesis, serving a proapoptotic function in the brain, while mediating a prosurvival function in the heart.

The NF-κB family of transcription factors is composed of p50/p105, p52/p100, p65 (RelA), c-Rel, and RelB. Studies using mice with targeted disruptions of specific NF-κB subunits have shown that NF-κB serves important functions in the development and function of innate and adaptive immunity (24–26). Mice lacking p50 have no apparent developmental defects (24), and immune cells mature normally. However, p50^{–/–} mice display defects in B cell activation, isotype switching, and antibody production (24). These defects render p50^{–/–} mice more susceptible to infection by the Gram-positive bacterial pathogen *Streptococcus pneumoniae*, but they remain capable of efficiently clearing infection by the Gram-negative pathogens *Escherichia coli* and *Haemophilus influenzae* (24). When p50^{–/–} mice are infected with encephalomyocarditis virus, they are actually more resistant to infection than controls. This difference is thought to be due to an increase in apoptosis that leads to a decrease in viral growth (24). These findings stand in stark contrast to what occurs in the CNS and heart of reovirus-infected mice.

In experiments comparing reovirus infection of p50^{+/+} and p50^{–/–} mice, we found that the presence or absence of p50 did not alter primary viral replication in intestinal tissue or dissemination of virus to the liver, brain, or heart. Although viral replication in the brain after intracranial inoculation also was independent of p50, replication in the heart was increased in p50^{–/–} mice by approximately 1,000-fold. What might explain the enhancement of reovirus replication in the heart of p50^{–/–} mice? Reovirus strains have
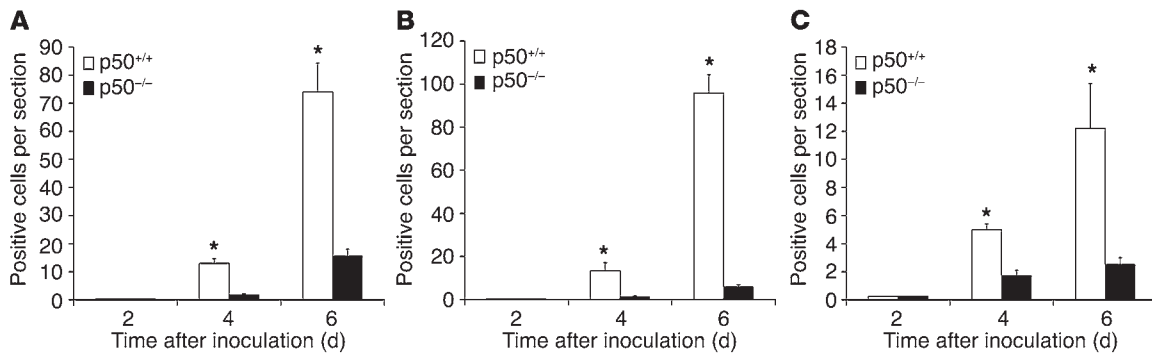
Figure 5
Quantitation of TUNEL staining in cortex and hippocampus (**A**), basal ganglia and diencephalon (**B**), and brain stem (**C**) of reovirus-infected p50+/+ and p50–/– mice. TUNEL staining was performed using tissue sections prepared 2, 4, and 6 days following intracranial inoculation of p50+/+ and p50–/– mice with 10^4 PFU reovirus T3SA+. For each brain region, all positive cells in a single parasagittal section were counted for 4–8 animals. The results are expressed as the mean number of apoptotic cells per region. Error bars indicate SDs. *$P < 0.05$ by Student's t test.

been characterized previously as having the capacity to grow in the murine heart and produce cardiac disease (27, 28). In primary cardiomyocytes, nonmyocarditic reovirus strains induce more IFN-β and are more sensitive to the antiviral effects of this cytokine than myocarditic reovirus strains (29). Furthermore, normally nonmyocarditic strains are capable of producing myocarditis in infected IFN-α/β–/– mice (29). Thus, it appears that type I IFNs restrict viral replication in the heart and attenuate cardiac disease.

NF-κB is known to induce the expression of several mediators of innate immune responses including type I IFNs (30–32). Therefore, absence of p50 may allow reovirus to achieve much higher titers and cause myocarditis. We tested this hypothesis by determining brain and heart levels of IFN-β mRNA in response to reovirus infection of p50+/+ and p50–/– mice (Figure 8) and by treating reovirus-infected p50–/– mice with IFN-β (Figure 9). In these experiments, we found a dramatic increase in IFN-β expression in the hearts of wild-type mice but only a minimal IFN-β response in the hearts of p50-null animals. Moreover, reconstitution of p50–/– mice with IFN-β substantially diminished reovirus replication and apoptosis, which resulted in diminished myocardial injury. These results indicate that IFN-β is a necessary component of the NF-κB–mediated protective response against reovirus in the heart. However, it is likely that other components of innate immunity are involved in this effect. Preliminary data from our laboratory suggest that in addition to IFN-β, IL-6, MIF, and TNF are expressed at higher levels in the heart of p50+/+ mice than p50–/– mice (S.M. O'Donnell and T.S. Dermody, unpublished observation). These findings suggest that following reovirus infection of the heart, NF-κB is activated and leads to induction of potent innate immune responses, which in turn attenuate viral replication at that site, resulting in diminished apoptosis and disease.

The enhanced growth of reovirus in the heart of p50–/– mice compared with p50+/+ mice was associated with extensive myocarditis and resultant tissue injury and dysfunction. This result was confirmed by histopathological studies, echocardiography, and physical examination revealing signs of heart failure. The pathology observed in the heart of p50–/– animals was characterized by extensive tissue damage and little inflammatory infiltrate, similar to findings made in previous studies of reovirus myocarditis (27). Therefore, our results suggest that apoptosis is the primary mechanism of cardiac damage in reovirus-induced myocarditis, as reported previously (20). Damage to cardiomyocytes during reovirus infection occurs in

the complete absence of adaptive components of host defense (33). It is possible that a similar mechanism occurs in humans, which would explain why some patients with acute myocarditis develop heart failure in the setting of sustained viremia (34).

In contrast to the enhanced growth of reovirus in the heart of p50–/– mice, viral growth in the CNS of p50+/+ and p50–/– mice was equivalent. However, we observed dramatic differences in the number of apoptotic cells in the 2 mouse strains as indicated by TUNEL and caspase-3 staining. Therefore, the efficiency of viral growth is not strictly correlated with the extent of the apoptotic response. Nonetheless, despite these p50-dependent differences in viral growth, our results suggest that apoptosis is an important mechanism of reovirus-induced disease in both the CNS and heart. In the CNS of p50–/– mice, apoptosis and inflammation following reovirus infection were diminished. However, in the hearts of these animals, apoptosis and tissue injury were enhanced. This correlation between apoptosis and pathology lends support to the hypothesis that therapies directed at blocking programmed cell death might attenuate viral virulence, consistent with results from previous studies of reovirus-induced myocarditis (20). However, our findings suggest that pharmacologic inhibition of NF-κB activation may reduce pathologic injury at some sites and exacerbate disease at others, depending on the nature of the NF-κB agonist.

The precise cell types responsible for the p50-dependent effects on apoptosis in response to reovirus infection in mice are not apparent from our study. It is possible that expression of p50 in neurons is required for apoptosis of these cells and expression of p50 in cardiomyocytes mediates protection of these cells against apoptotic injury. However, it is also possible that p50-dependent immune responses contribute to the observed differences in cell fate. For example, NF-κB–mediated release of cytokines such as TNF-α from immune cells might contribute to the neuronal apoptosis that occurs during reovirus infection of the CNS, whereas NF-κB–mediated release of type I IFNs from immune cells might mediate a protective effect in the heart. Since adoptive transfer of immune cells is not technically feasible in the newborn mice required for studies of reovirus pathogenesis, discrimination between these possibilities awaits the development of mice with tissue-specific ablation of NF-κB activity.

The role of NF-κB in response to a variety of cellular stresses has been studied extensively using cultured cells (35). However, little is known about the contributions of specific NF-κB subunits in vivo.
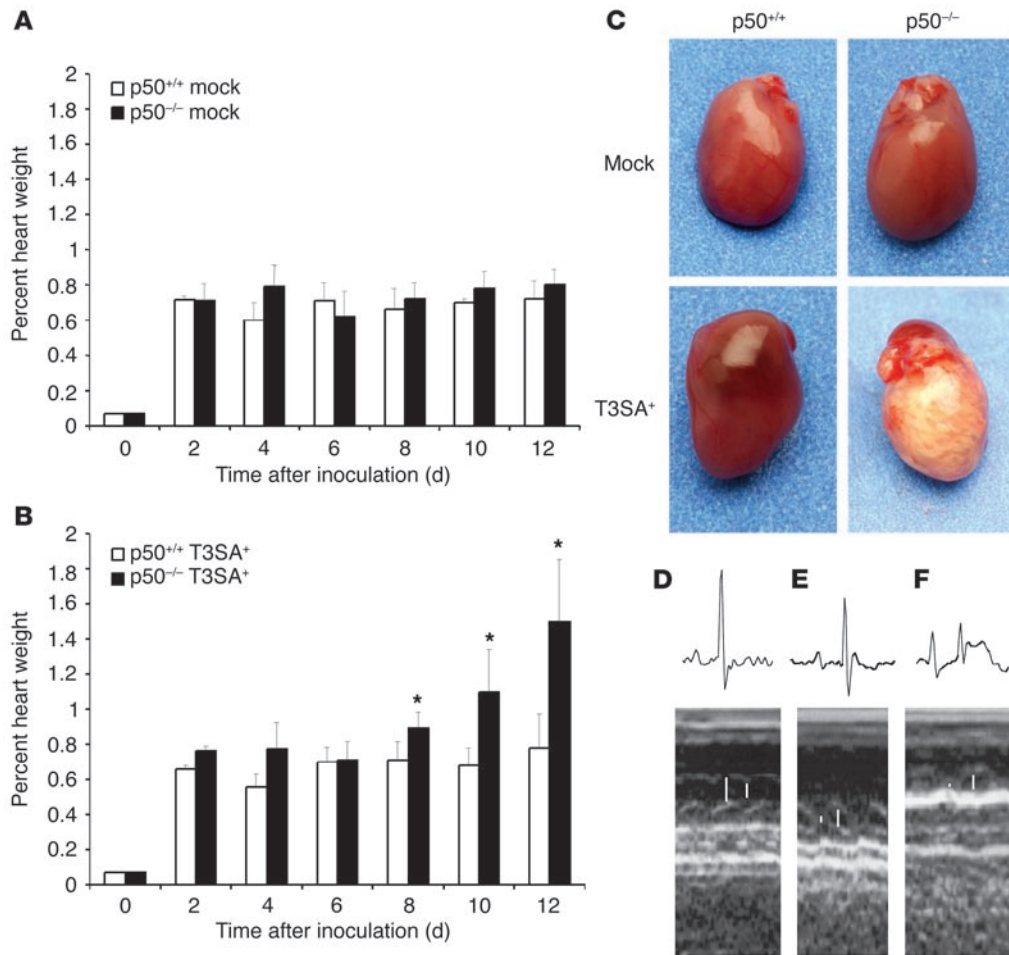
Figure 6
Heart pathology following reovirus infection of p50^{+/+} and p50^{–/–} mice. (**A** and **B**) Newborn p50^{+/+} and p50^{–/–} mice were inoculated perorally with either PBS (mock) (**A**) or 10^4 PFU reovirus T3SA[+] (**B**), and heart size was monitored at 2-day intervals. Percent heart weight was calculated as heart weight divided by body weight. The results are expressed as the mean heart weights of at least 4 animals for each time point. Error bars indicate SDs. *$P < 0.05$ by Student's *t* test. (**C**) Hearts from mice euthanized 12 days following peroral inoculation with reovirus T3SA[+] or gelatin saline (Mock). (**D**, **E**, and **F**) Electrocardiography and echocardiography of reovirus-infected p50^{–/–} (**D**), mock-infected p50^{–/–} (**E**), and reovirus-infected (**F**) p50^{+/+} mice. Newborn mice were inoculated perorally with 10^4 PFU T3SA[+], and tests were performed 10 days after inoculation. A P-wave/QRS ECG complex is displayed above the corresponding echocardiographic image. Systolic and diastolic LV cavity dimensions are indicated by bars superimposed on the M-mode images.

reveal new layers of control of NF-κB responses and extend understanding of how viruses cause tissue-specific injury.

Methods

Cells, virus, and antibodies. Spinner-adapted murine L929 cells (L cells) were grown in suspension or monolayer culture and maintained as described previously (18). Reovirus strain T3SA[+] was generated by reassortment of reovirus strains type 1 Lang (T1L) and type 3 clone 44-MA (5). Virus was purified after growth in L cells by cesium chloride gradient centrifugation (38). Rabbit polyclonal anti-reovirus serum raised against strain T1L was obtained as described previously (22). Rabbit polyclonal antiserum specific to the activated form of caspase-3 (anti–caspase-3/Asp 175) was obtained from Cell Signaling Technology.

Mice and inoculations. HIV long-terminal repeat luciferase (HLL) mice were generated as described previously (39). Control p50^{+/+} mice (B6129PF1/J-A[W-J]/A[W]) and p50^{–/–} mice (B6129P-Nfkb1[tm1Bal]) (24) were obtained from The Jackson Laboratory. Newborn mice weighing 2.0–2.5 g (2–4 days old) were inoculated either intracranially or perorally with purified T3SA[+] diluted in PBS. Intracranial inoculations were delivered to the right and left cerebral hemispheres (5 µl each) using a Hamilton syringe (BD Bio-

sciences) and a 30-gauge needle (40). Peroral inoculations were delivered into the stomach (50 µl) by passage of a polyethylene catheter 0.61 mm in diameter (BD) through the esophagus (41). The inoculum contained 0.5% (vol/vol) green food coloring so that accuracy of delivery could be judged. For determination of NF-κB activation, viral titer, and immunohistochemical staining, mice were euthanized at various intervals following inoculation, and organs were collected.

Assessment of NF-κB activation by in vivo luciferase activity. Two-day-old HLL mice were inoculated perorally with either 10^4 PFU T3SA[+] or PBS. Mice were anesthetized with isoflurane before imaging and immobilized for the duration of the integration time of photon counting (3 minutes). Luciferin (0.75 g/mouse in 0.2 ml isotonic saline) was inoculated intraperitoneally, and mice were imaged using an intensified charge-coupled devise camera (C2400-32; Hamamatsu Corp.). For the duration of photon counting, mice were placed inside a light-tight box. Light

The extensive array of NF-κB inducers and target genes (36) suggests that numerous mechanisms exist to direct transcription of appropriate NF-κB–dependent genes in response to specific stimuli. One such regulatory mechanism is likely to be the activation of specific NF-κB complexes (e.g., p50/p65 heterodimers) for each inducing signal. Individual homodimeric and heterodimeric NF-κB complexes exhibit different affinities for target DNA sequences (37), and this provides a potential mechanism by which NF-κB–inducing stimuli regulate transcriptional activity of specific subsets of cellular genes. We showed previously that reovirus requires p50/p65 for efficient apoptosis in cell culture (18). However, we found in the current study that p50 plays organ-specific roles in disease pathogenesis in vivo. These findings emphasize that NF-κB subunits can have different functions following activation with the same stimulus depending on the cellular environment. Continuing studies in this area may
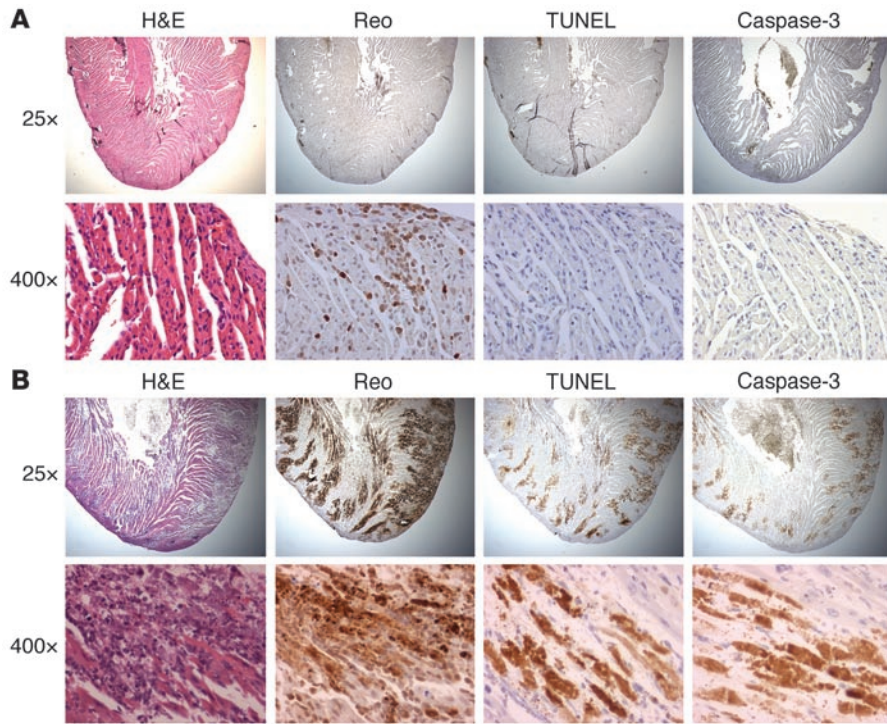
Figure 7
Inflammation, reovirus protein expression, TUNEL staining, and immunohistochemical detection of activated caspase-3 in the heart of reovirus-infected p50$^{+/+}$ (**A**) and p50$^{-/-}$ mice (**B**). Newborn mice were inoculated perorally with 10^4 PFU reovirus T3SA$^+$. At 12 days after inoculation, hearts were harvested, paraffin embedded, sectioned, and stained with H&E, polyclonal reovirus-specific antiserum, TUNEL, or activated caspase-3–specific antiserum as indicated. Original magnification, ×25 (top panels) and ×400 (bottom panels). Brown staining indicates reovirus protein, fragmented DNA, or activated caspase-3.

emission from each mouse was detected as photon counts, and a digital false-color photon emission image of the mouse was generated.

EMSAs. Two-day-old p50$^{+/+}$ and p50$^{-/-}$ mice were inoculated perorally with either 10^4 PFU T3SA$^+$ or PBS. Mice were euthanized 12 days after inoculation. Brains and hearts were aseptically removed, snap frozen on dry ice, and stored at –70°C. Organs were weighed, placed in a mortar with liquid nitrogen, and ground into a powder. Lysis buffer (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) was added in a ratio of 1 ml per 200 mg of tissue. Samples were frozen and thawed 3 times and centrifuged at 12,000 *g* for 10 minutes. The supernatant was used as the whole-cell extract.

Whole-cell extracts (10 μg total protein) were assayed for NF-κB activation by EMSA using a ^{32}P-labeled oligonucleotide (1.0 ng) consisting of the NF-κB consensus binding sequence (Santa Cruz Biotechnology Inc.) as described previously (18). For competition experiments, unlabeled consensus oligonucleotide at various concentrations was added to the reaction mixtures along with radiolabeled oligonucleotide. For supershift experiments, 1 μl of a rabbit polyclonal antiserum specific to p65 (250 μg/ml; Santa Cruz Biotechnology Inc.) was added to the binding reaction mixtures and incubated at 4°C for 30 minutes prior to the addition of radiolabeled oligonucleotide. Nucleoprotein complexes were subjected to electrophoresis in native polyacrylamide gels, which were dried and exposed to film.

Determination of viral titer in infected organs. Organs (intestine, liver, heart, and brain) from infected p50$^{+/+}$ and p50$^{-/-}$ mice were placed into vials containing 1 ml gelatin saline, frozen (–20°C) and thawed once, and sonicated for 20 seconds. Titers of virus present in organ homogenates were determined by plaque assay using L cell monolayers (42).

Histology, immunohistochemistry, and TUNEL staining. Litters of newborn p50$^{+/+}$ and p50$^{-/-}$ mice were inoculated intracranially or perorally with either 10^4 PFU T3SA$^+$ or PBS. Mice were euthanized, and brain and heart tissues were fixed in 10% buffered paraformaldehyde. Fixed organs were embedded in paraffin, and 6-μm histological sections were prepared. Sections were stained with H&E for evaluation of histopathologic changes, processed for immunohistochemical

detection of reovirus protein, assayed for DNA fragmentation using the TUNEL technique (43), or processed for the immunohistochemical detection of activated caspase-3 (5). Cells demonstrating TUNEL staining were quantitated separately in each parasagittal brain section in the following regions: cerebral cortex, hippocampus, basal ganglia, diencephalon, and brain stem. The mean number of positive cells per region was determined for each treatment group and time point. Observers were blinded to the identity of the mouse strain and the nature of the inoculum.

Echocardiography. Echocardiography was performed on conscious 10-day-old pups as previously described for adult mice (44) except that the total field depth was set to 1 cm (minimum possible), and external heating and rapid
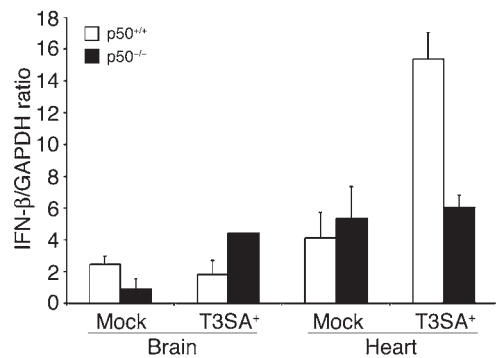
Figure 8
Levels of IFN-β mRNA in brain and heart of p50$^{+/+}$ and p50$^{-/-}$ mice. Newborn mice were inoculated perorally with either PBS (Mock) or 10^4 PFU reovirus T3SA$^+$. At 12 days after inoculation, brains and hearts were resected, and whole-organ RNA was isolated and used as a template to generate cDNA. Levels of IFN-β and GAPDH cDNA were assessed by real-time PCR. The results are expressed as the mean ratio of IFN-β cDNA to that of GAPDH for 2 animals. Error bars indicate SDs.
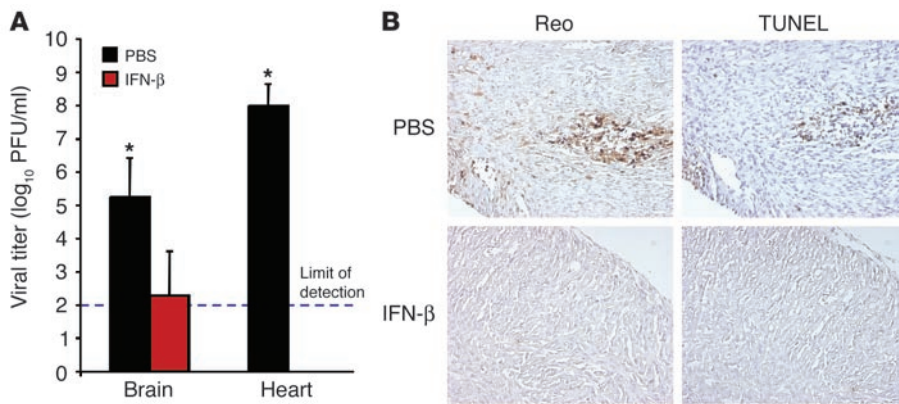
Figure 9
Reovirus replication and apoptosis in infected p50^{−/−} mice following treatment with IFN-β. Newborn mice were inoculated intraperitoneally with either IFN-β or PBS 1 day prior to peroral inoculation with 10^4 PFU reovirus T3SA[+]. Animals were treated with either IFN-β or PBS for an additional 9 days, and brains and hearts were resected. (**A**) Viral titers in the brain and heart. Organs were homogenized, and viral titers were determined by plaque assay. The results are expressed as the mean viral titers for 3 animals. Error bars indicate SDs. *$P < 0.05$ by Student's t test. (**B**) Histopathology of reovirus infection in the heart. Hearts of the reovirus-infected p50^{−/−} animals represented in **A** were paraffin embedded, sectioned, and stained with polyclonal reovirus-specific antiserum or processed for TUNEL analysis. Original magnification, ×100. Brown staining indicates reovirus protein or fragmented DNA.

were euthanized, and organs were removed. Organs were processed for determination of viral titer and histopathological analysis.

Animal husbandry and experimental procedures were performed in accordance with NIH Public Health Service policy and approved by the Vanderbilt University School of Medicine Institutional Animal Care and Use Committee.

Data analysis. Results are expressed as the mean ± SD. Differences between mean values were compared using unpaired Student's t tests as applied with Microsoft Excel software. P values less than 0.05 were considered statistically significant.

sample acquisition were used to prevent excessive heat loss. Electrocardiograms were digitally sampled and correspond to the usual surface lead I.

RNA isolation and real-time PCR. Two-day-old p50^{+/+} and p50^{−/−} mice were inoculated perorally with either 10^4 PFU T3SA[+] or PBS. Mice were euthanized, and brain and heart tissues were homogenized using a Dounce homogenizer. RNA was extracted from brain and heart homogenates using the TRIZOL RNA extraction protocol (Invitrogen Corp.). Three micrograms of RNA was used in a reverse-transcription reaction containing ×10 buffer, 25 mM $MgCl_2$, 100 μM dithiothreitol, and 1 U RNasin (Promega), 10 mM dNTPs, 50 μM random hexamers, and 1 U AMV reverse transcriptase (Promega). The reaction was incubated at 43°C for 1 hour and then at 95°C for 10 minutes.

Real-time PCR reactions were carried out using the Bio-Rad iCycler and iQ Supermix buffer containing DNA polymerase and SYBR Green (Bio-Rad Laboratories). Two to 3 replicate amplification reactions were performed in 96-well plates (Bio-Rad Laboratories). Each reaction contained 12.5 μl iQ Supermix buffer, 300 nM forward and reverse primers, and 1 μl cDNA in a final volume of 25 μl. Primers for the reactions were as follows: (a) IFN-β forward, 5′-GGAGATGACGGAGAAGATGC-3′, (b) IFN-β reverse, 5′-CCCAGT-GCTGGAGAAATTGT-3′, (c) GAPDH forward, 5′-CAACTACATGGTCTA-CATGTTC-3′, and (d) GAPDH reverse, 5′-CTCGCTCCTGGAAGATG-3′. Cycling conditions were as follows: 95°C for 10 minutes and then 45 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 15 seconds.

Data were analyzed using Bio-Rad iCycler PCR detection and analysis software version 3.0 (Bio-Rad Laboratories). DNA was quantitated using the standard curve method with the background subtracted. Known concentrations of cDNA were used to obtain the standard curve for each gene (concentrations between 0.0228 and 710 ng). A melting curve was determined for each sample to detect primer dimers, in which case data were not used. Results are expressed as values for IFN-β cDNA divided by those for GAPDH cDNA.

IFN-β treatment of mice. Two-day-old p50^{−/−} mice were inoculated intraperitoneally with either $5 × 10^4$ U IFN-β (Calbiochem) suspended in PBS containing 0.1% BSA or PBS alone in a volume of 25 μl 1 day prior to peroral inoculation with 10^4 PFU T3SA[+]. Infected mice were treated daily for 9 days with either IFN-β or PBS. On day 10 following viral inoculation, animals

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