IDENTIFICATION AND CHARACTERIZATION OF CELLULAR DETERMINANTS OF REOVIRUS INTERNALIZATION

By

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To my grandfather, Hugh P. Maginnis,
who taught me the best teaching method is example.

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LIST OF ABBREVIATIONS

AA	Amino acid
AC	Ammonium chloride
AP-2	Adaptor protein-2
ARH	Autosomal recessive hypercholesterolemia
BSA	Bovine serum albumin
ds	Double-stranded
Dab-2	Disabled protein 2
CAR	Coxsackievirus and adenovirus receptor
CEFs	Chicken embryo fibroblasts
CHO	Chinese hamster ovary
CCP	Clathrin-coated pit
CCV	Clathrin-coated vesicle
CNS	Central nervous system
D1	Domain 1 of Ig Superfamily member
D2	Domain 2 of Ig Superfamily member
DNA	Deoxyribonucleic acid
EIA	Early infectivity assay
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron microscopy
ER	Endoplasmic reticulum

FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FFU	Fluorescent focus unit
FITC	Fluorescein isothiocyanate
FMDV	Foot-and-Mouth Disease Virus
gp120	Glycoprotein 120
gp41	Glycoprotein 41
GST	Glutathione-S-transferase
h	Hour(s)
HIV	Human immunodeficiency virus
HCMV	Human cytomegalovirus
Ig	Immunoglobulin
ILK	Integrin linked kinase
IC	Intracranial
I domain	Inserted domain
IM	Intramuscular
IP	Intraperitineal
ISVP	Infectious subvirion particle
JAM-A	Junctional adhesion molecule-A
kDa	Kilodalton
KGE	Amino acid residues Lys-Gly-Glu
L	L929 cell
LDL	Low-density lipoprotein

MAb	Monoclonal antibody
M cell	Microfold cell
MIDAS	Metal-Ion-Dependent Adhesion Site
min	Minute(s)
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NPXY	Amino acid motif containing Asn-Pro-any residue-Tyr
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDZ	Post-synaptic density-95/Discs-large/Zonula occludens-1
PE	R-phycoerythrin
PIP2	Phosphatidylinosital (4,5)-bisphosphate
PO	Peroral
PPs	Peyer's patches
RGD	Amino acid residues Arg-Gly-Asp
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SA	Sialic acid
SDS	Sodium dodecyl sulfate
SH2	Src homology 2
ss	Single-stranded

SV40	Simian Virus 40
T1	Type 1
T3	Type 3
T1L	Type 1 Lang
T2J	Type 2 Jones
T3D	Type 3 Dearing
T3SA+	Type 3 sialic-acid binding strain
T3SA-	Type 3 non-sialic-acid binding strain
WNV	West Nile Virus
ZO-1	Zonula occludens-1

CHAPTER I

BACKGROUND

Introduction

Virus attachment and internalization into host cells are essential steps in the viral infectious cycle that enable viruses to engage target cells and initiate infection in the host. A virus must bind to cells and breach the host cell membrane to deliver the genetic payload to the site of viral replication. Hence, virus-receptor interactions serve as important determinants of viral tropism and pathogenesis as they are requisite for all subsequent steps in the viral life cycle. To ensure proper entry into cells, viruses have adapted complex and elegant strategies to recognize their respective cellular targets.

Mammalian reoviruses are nonenveloped double-stranded (ds) RNA viruses that infect a broad range of hosts in nature (248). Reoviruses are pathogenic in mice, infecting most organs including the central nervous system (CNS), heart, and liver (248). Reoviruses first attach to the cell surface by binding to carbohydrate (9, 52), which is α-linked sialic acid for serotype 3 strains (14, 52, 189, 196). All reovirus strains bind to proteinaceous receptor junctional adhesion molecule-A (JAM-A) to mediate high-affinity attachment (15, 43, 204).

Upon commencement of my dissertation research, it was not known whether JAM-A mediates reovirus internalization following attachment to host cells. Thus, the receptor responsible for reovirus cell entry and mechanisms responsible for this process were undetermined. Reovirus is reported to enter cells by receptor-mediated endocytosis

(32, 33, 239), which is most likely clathrin-dependent (85, 215). However, it was not known whether clathrin-dependent uptake serves as a functional route of reovirus entry resulting in productive infection. Reovirus requires disassembly steps in the endocytic compartment (72, 239), but the exact fate of reovirus virions in the endocytic pathway was unknown.

In my dissertation research, I sought to determine and characterize mechanisms of reovirus internalization. In Chapter II, I report that β1 integrin mediates reovirus internalization. In Chapter III, I show that tyrosine residues in the β1 integrin cytoplasmic domain are required for functional reovirus entry. This research provides a deeper understanding of how reovirus enters into host cells and is delivered to the endocytic pathway. Furthermore, these findings contribute to an understanding of how a nonenveloped virus utilizes cellular machinery to mediate the functions of attachment and cell entry and elucidates the physiologic function of β1 integrin in endocytosis.

Virus Attachment and Entry

Initial engagement of a host cell by a virus is mediated by an attachment protein on the outer shell of the virus and receptors on the surface of target cells. Interactions between the attachment protein and viral receptors serve to tether the virus to the cell surface. Viral receptors include cell-surface carbohydrates, which serve as a low-affinity, high-avidity attachment moiety, and in many cases, proteinaceous receptors, which mediate high-affinity interactions with viral proteins (161). Some viruses utilize one receptor to mediate both attachment and internalization. For example, echovirus, a nonenveloped virus, binds to α2β1 integrin, which mediates cell attachment (24) and

caveloae-dependent endocytic uptake (159). However, many viruses use independent receptors for attachment and entry.

There are two main types of viruses, enveloped and nonenveloped, which use distinctly different mechanisms to enter host cells. Entry of most enveloped viruses is mediated by fusion of the viral envelope with cell membranes. Nonenveloped virus entry is facilitated by receptor engagement and internalization through one of many endocytic pathways. In the case of human immunodeficiency virus type 1 (HIV-1), which is enveloped, the viral attachment glycoprotein, gp120 binds to CD4 on the surface of T cells or macrophages (67, 138). After initial binding, gp120 interacts with chemokine receptors (75), which induces a conformational change in gp120. This conformational change exposes a fusion peptide in gp41 that mediates fusion of the viral envelope and plasma membrane (92). In the case of the DNA tumor virus simian virus 40 (SV40), which is nonenveloped, binding to cell-surface major histocompatibility complex class I (36) triggers entry by caveolae-dependent endocytosis for delivery to the endoplasmic reticulum (ER) and finally to the nucleus (131).

There are a variety of mechanisms by which nonenveloped viruses can internalize into target cells. Most nonenveloped viruses use receptor-mediated endocytosis, a process by which cells internalize cargo into vesicles formed at the plasma membrane (161). Endocytic pathways employed by viruses include clathrin-mediated endocytosis (127, 194) and non-clathrin-dependent pathways such as caveolae-mediated endocytosis (159, 201), macropinocytosis (164), and non-clathrin/non-caveolae-mediated endocytosis (161, 219). The most common type of receptor-mediated endocytosis employed by viruses is clathrin-dependent endocytosis (161). Clathrin consists of heavy and light

chains that are capable of self-assembling into a lattice to form a clathrin cage (173). In clathrin-mediated endocytosis, clathrin is recruited to the plasma membrane by adaptor proteins, which serve many cellular trafficking functions. Some adaptor proteins recruit clathrin to the cell surface and link clathrin, the plasma membrane, and the receptor that has induced the endocytic signal. For example, the adaptor protein-2 (AP-2) complex consists of four subunits: α, β2, μ2, and σ (82). The μ2 subunit directly engages specific sorting signal motifs in the cytoplasmic domain of receptors to mediate clathrin-dependent endocytosis and cargo sorting (55, 116, 182, 186). The β2 subunit binds to clathrin through a specific sequence known as a clathrin-box motif (187); β2 also functions in cargo selection (82). The α subunit binds to the plasma membrane (96). The AP-2 complex leads to clathrin accumulation at the plasma membrane, where clathrin self-assembles to form a clathrin-coated pit (CCP) (173). Cargo is internalized into the CCP, and the nascent vesicle is excised from the plasma membrane by the activity of dynamin, a GTPase (69). The CCP then forms a clathrin-coated vesicle (CCV) that transports cargo to the proper endocytic compartment (173). Many nonenveloped viruses are targeted to early or late endosomes to undergo pH-dependent conformational changes to initiate viral disassembly or membrane fusion (161).

Reovirus Structure and Genome Organization

Mammalian reoviruses are in the orthoreovirus genus of the *Reoviridae* family. Reoviruses are nonenveloped, icosahedral viruses (76) that contain a segmented double-stranded (ds) RNA genome (178) (Fig. 1). Reovirus genes are arranged into three classes

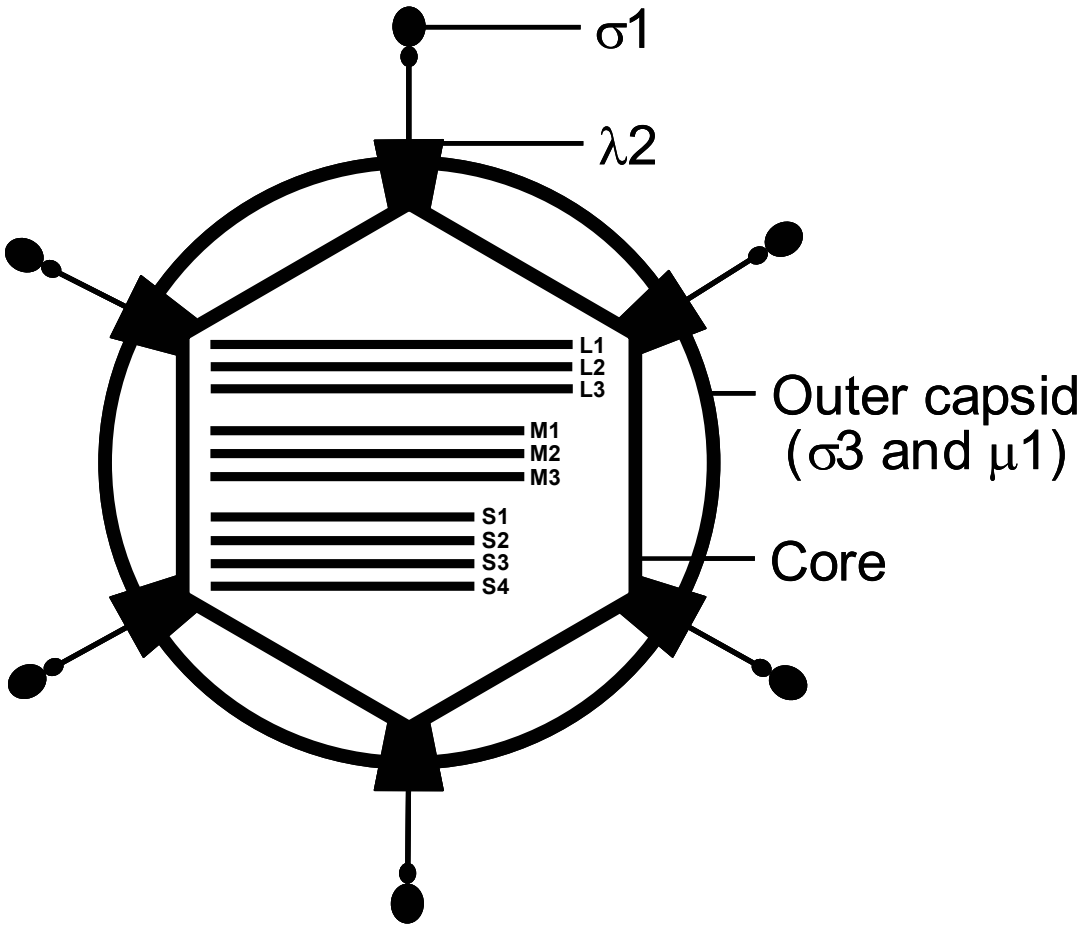


Fig. 1. Schematic Diagram of Reovirus Virion. Reoviruses form icosahedral virions with an outer capsid and an inner core. The outer capsid proteins σ1, σ3, μ1, and λ2 are indicated. The inner core encapsidates the viral genome, which is composed of 10 dsRNA segments.

based on size: three large (L1, L2, L3), three medium (M1, M2, M3), and four small (S1, S2, S3, S4) (178). Each gene segment encodes a single protein, with the exception of S1, which encodes two proteins (87, 125). Reovirus proteins are named with Greek letters λ, μ, and σ relating to the size class of the encoding gene. However, the gene segments and protein names do not always correspond. Reovirus encodes eight structural proteins (λ1, λ2, λ3, μ1, μ2, σ1, σ2, and σ3), which form the viral outer capsid and core (178), and three nonstructural proteins (μNS, σNS, σ1s), which are expressed in reovirus-infected cells (20, 21, 39, 45).

Reovirus virions form two concentric protein shells, the outer capsid and core (Fig. 1). The outer capsid serves to stabilize the virion in the environment, and the core contains the viral genome and is responsible for viral transcription and genome replication. The outer capsid consists of structural proteins σ1, σ3, μ1, and λ2 (76, 145). σ1 is the viral attachment protein and mediates binding to target cells (93, 145, 260). σ3 is a major component of the viral outer capsid and serves to protect the virion in the environment and regulate viral disassembly in cellular endosomes (104, 149). During viral disassembly, σ3 is removed from the virion particle and μ1 is exposed, which leads to virus escape from the endosomal compartment (46, 180). λ2 is a pentameric protein at the virion fivefold axes and serves as the structural foundation for σ1 (76). The aminoterminal domain of λ2 functions as a guanylyltransferase, which mediates the enzymatic activity of capping the 5' end of viral plus-strand transcripts (60, 155).

The structural proteins λ1, λ2, λ3, μ2, and σ2 form the reovirus core. λ1 is the main structural component of the core. σ2 assembles on to the λ1 shell and makes contacts with both λ1 and λ2 (208). λ2 forms turrets at the icosahedral vertices of the

core (76). λ3 is the catalytic subunit of the viral RNA-dependent RNA polymerase (234). μ2 is thought to interact with λ1, λ2, and λ3 and is hypothesized to be involved in RNA polymerase activity (178). Nonstructural proteins σNS and μNS induce the formation of viral inclusions in the perinuclear space that serve as sties of viral replication (20, 21, 38, 39). Both σ1 and σ1s are translated from the viral S1 gene (87, 125). σ1s is not required for viral replication (210), yet it has been linked to apoptosis and cell-cycle arrest (202) by a mechanism that remains unclear.

Reovirus Pathogenesis

Reoviruses were first isolated in the 1950s by Albert Sabin (216). The original isolates were collected from children who presented with diarrhea. The term reovirus (*r*espiratory *e*nteric *o*rphan virus) was coined based on the absence of disease in association with a virus isolated from the respiratory and enteric tracts of humans (216). Reovirus is spread from host to host by fecal-oral transmission. Generally, most individuals encounter the virus by five years of age and are asymptomatic (240, 248).

While reovirus is termed an orphan virus, reovirus infection can lead to disease in humans. Experimental reovirus infections in seronegative adult male prisoners produced malaise, headache, nasal congestion, and cough (212). Reovirus isolated from children has been associated with gastrointestinal symptoms including diarrhea (213) and respiratory symptoms (121) including runny nose, sore throat, and earache (213). Some studies have suggested that reovirus contributes to biliary artresia, a progressive inflammatory disease of the liver, in newborn children (251). In support of this association, reovirus produces biliary disease in mice (16, 267). Additionally, reovirus

has been isolated from children with CNS disease including encephalitis (129, 137) and meningitis (249). Therefore, while reovirus is not considered a significant human pathogen, reovirus infection can cause disease in humans, especially in neonates.

Reovirus Mouse Model

Reovirus is highly virulent in neonatal mice (242), which are the preferred experimental system for studies of reovirus pathogenesis (257). Mice can be infected with reovirus by peroral (PO), intracranial (IC), intraperitoneal (IP), or footpad inoculation. After PO inoculation of mice, reovirus attaches to and enters intestinal microfold (M) cells (269) and subsequently undergoes primary replication in lymphoid cells of the Peyer's patches (PPs) (17, 214). After primary replication in the small intestine, reovirus spreads and infects distant organs including the kidney (192), liver (191), heart (110), and brain (126, 158, 200). Reoviruses can cause a variety of disease phenotypes in mice including myocarditis (110, 227) and biliary inflammation (16, 190). Reoviruses are most noted for the capacity to cause disease in the CNS (260) in which viral infection can lead to hydrocephalus (259) or a lethal encephalitis (242, 259), depending on the strain.

Reovirus serotypes differ in host-cell tropism and disease outcomes. There are three reovirus serotypes (T1, T2, and T3). For each serotype there exists a commonly used laboratory prototype strain, type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D) (248). After PO inoculation of neonatal mice, T1 reovirus spreads hematogenously (132), infects ependymal cells in the CNS (243, 250), and causes hydrocephalus (259). T3 reovirus spreads neurally through the vagus nerve (171), infects

neurons (171, 248, 260), and causes lethal encephalitis (242, 259). The striking differences in viral tropism observed between T1 and T3 reovirus strains in the CNS segregate with the σ1-encoding S1 gene segment (243, 260), suggesting an important function for receptor engagement in tropism determination (250, 260).

The σ1 protein engages two receptors on the cell surface, carbohydrate and JAM-A. T3 reovirus strains bind to α-linked sialic acid (14, 52, 189, 196), while T1 strains bind to an unidentified carbohydrate. Differences in reovirus tropism by T1 and T3 strains cannot be explained by differential utilization of the only known σ1 head receptor, JAM-A. Cell culture and biochemical studies show that JAM-A is a serotype-independent receptor (43). Moreover, JAM-A-null mice are susceptible to reovirus infection (Antar, A.A.R. and Dermody, T.S., unpublished results). In contrast, carbohydrate binding serves an important function in reovirus pathogenesis and disease outcome. Sialic-acid binding strain T3SA+ and non-sialic acid binding strain T3SA-, which differ only by a single point mutation that confers binding to sialic acid (14), produce equivalent viral titers in the murine intestine after PO inoculation (16). However, T3SA+ spreads more rapidly from the intestine to sites of secondary infection and produces higher viral titers in several organs including the liver and brain (16). Furthermore, mice infected with T3SA+ develop inflammation of intrahepatic bile ducts and steatorrhea, similar to the findings associated with biliary atresia in humans (16). These observations indicate that virus coreceptor utilization can influence viral tropism and disease outcome.

The σ1 protein is not the sole viral determinant of reovirus disease outcome. The reovirus λ2-encoding L2 gene segment is linked to viral shedding from the intestine and

spread to littermates in pathogenesis studies in mice (134). The L2 gene also modulates reovirus infection in the intestine after PO inoculation (28). Therefore, other components of the viral outer capsid influence reovirus tissue tropism and disease outcome.

Reovirus Attachment and Entry

Reovirus attachment protein σ1 is a long filamentous protein that extends away from the virion surface and initiates contact with target cells (93). σ1 has a globular head and a long fibrous tail. The structure of the C-terminal half of σ1 reveals a homotrimer (53) that bears striking homology to the adenovirus attachment protein, fiber (255) (Fig. 2) . The head domain folds into a compact eight-stranded β-barrel motif (53) and is the portion of the protein that binds to JAM-A (223). σ1 contains a second receptor-binding domain below the head in the tail region of the molecule, which is known to bind carbohydrate (51, 52). T3 reovirus strains bind sialic acid (14, 52, 189, 196), while T1 strains bind to an unidentified carbohydrate. Carbohydrate binding is thought to tether the virus to the cell surface prior to high-affinity binding to JAM-A in an adhesion-strengthening manner (16).

JAM-A is an immunoglobulin (Ig) superfamily member, with two Ig-like domains, D1 and D2, a short transmembrane region, and a short cytoplasmic tail (162). The cytoplasmic region of JAM-A contains multiple phosphoacceptor sites and a PDZ (Post-synaptic density-95/Discs-large/Zonula occludens-1)-binding motif, which could serve as a binding site for PDZ-containing proteins, such as zonula occludens-1 (ZO-1) (79) and multiple-PDZ-domain-protein (MUPP1) (81). JAM-A localizes to tight junctions in polarized epithelial cells (162) and homodimerizes with other JAM-A

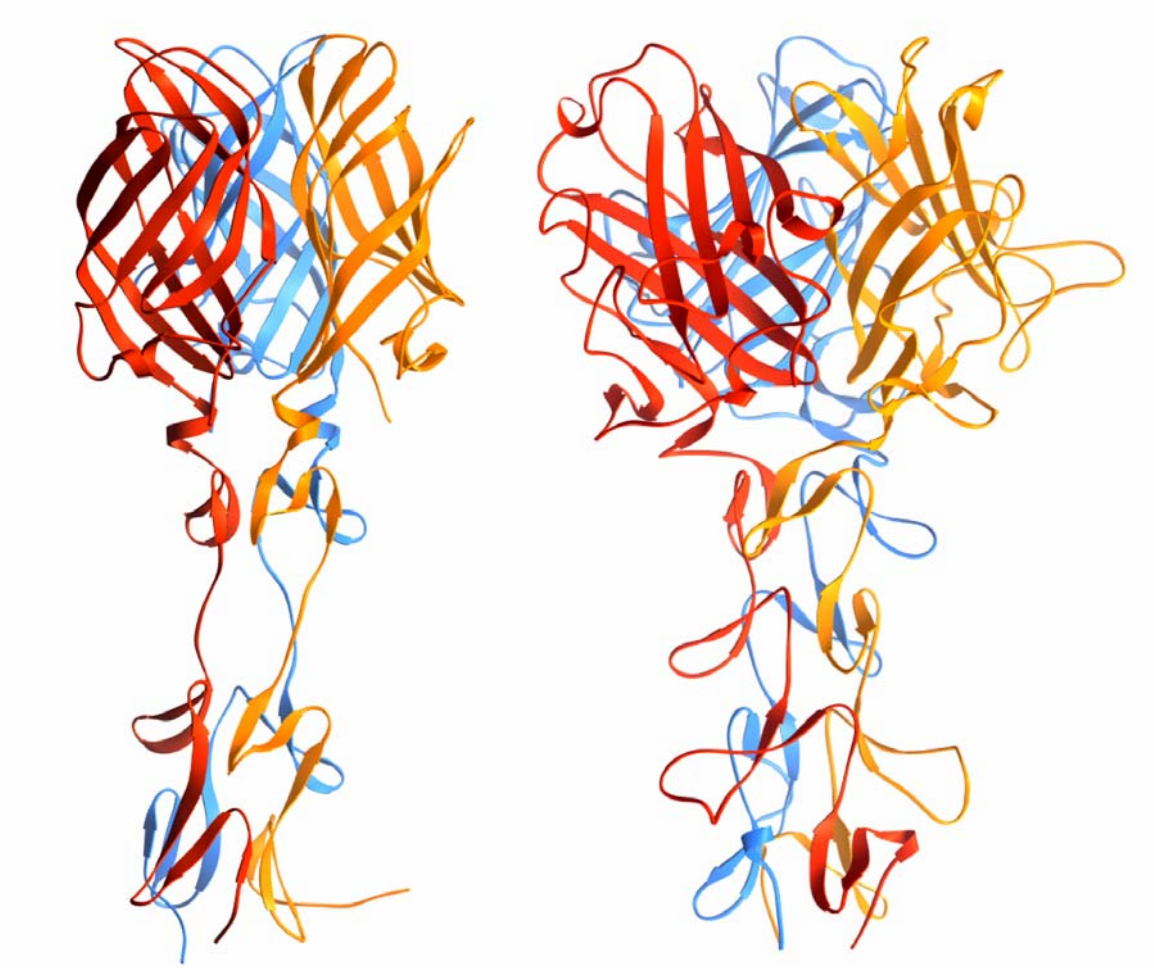


Fig. 2. Structural Similarities Between the Reovirus σ1 and Adenovirus Fiber. Shown are ribbon tracings of the crystallized portions of reovirus σ1 (left) and adenovirus fiber (right). Both proteins are timers shown as three monomers in red, orange, and blue. σ1 and fiber have large globular head domains formed by an eight-stranded β-barrel and long fibrous tails containing a triple β-spiral motif. These proteins are homologous in structure and function. The σ1 structure image was reproduced from The EMBO Journal, 2002, 21:1-11. Copyright © 2002 Nature Publishing Group. The fiber structure image was reproduced from Nature, 1999, 401:935-938. Copyright © 1999 Nature Publishing Group.

molecules either on the same or opposing cells (18). JAM-A serves as a receptor for prototype and field-isolate strains of all three reovirus serotypes (43). σ1 binds to the D1 domain of JAM-A with a K_D of ~10^{-8}M (15, 223).

The crystal structure of JAM-A reveals a dimer formed by interactions of the GFCC' faces of opposing D1 domains (136, 204). JAM-A bears striking homology to the coxsackievirus and adenovirus receptor (CAR) (27, 204) (Fig. 3). Reovirus σ1 engages JAM-A monomers using sequences in β-strands on C and C' of the GFCC' face of the JAM-A D1 domain, which mediate dimer interactions (Guglielmi, K.M. and Dermody, T.S., submitted). Since σ1 engages JAM-A using contacts that stabilize JAM-A dimers, it is possible that σ1 disrupts the dimer to bind monomeric forms of the receptor (Guglielmi, K.M. and Dermody, T.S, submitted).

Reovirus enters cells by receptor-mediated endocytosis (32, 33, 239), which is thought to be dependent on clathrin (85, 215) (Fig. 4). Reovirus is delivered to an endocytic compartment where steps in viral disassembly occur to form infectious subvirion particles (ISVPs) (33, 49, 228, 239). During this process, σ3 is removed and μ1 is cleaved to form two particle-associated fragments, δ and ϕ (176, 239). The σ1 protein undergoes a conformational change and extends away from the surface of the virion (93). ISVPs also can be generated in vitro by digesting virions with protease (11). ISVPs generated in vitro bind to cell-surface receptors (15) but directly penetrate the plasma membrane, thus bypassing a requirement for disassembly in the endocytic compartment (11, 115, 239). The ISVP is subject to further conformational change to yield the ISVP*, in which the μ1 protein has undergone a conformational change to μ1* (46, 47). This conformational change exposes hydrophobic residues in the μ1 cleavage

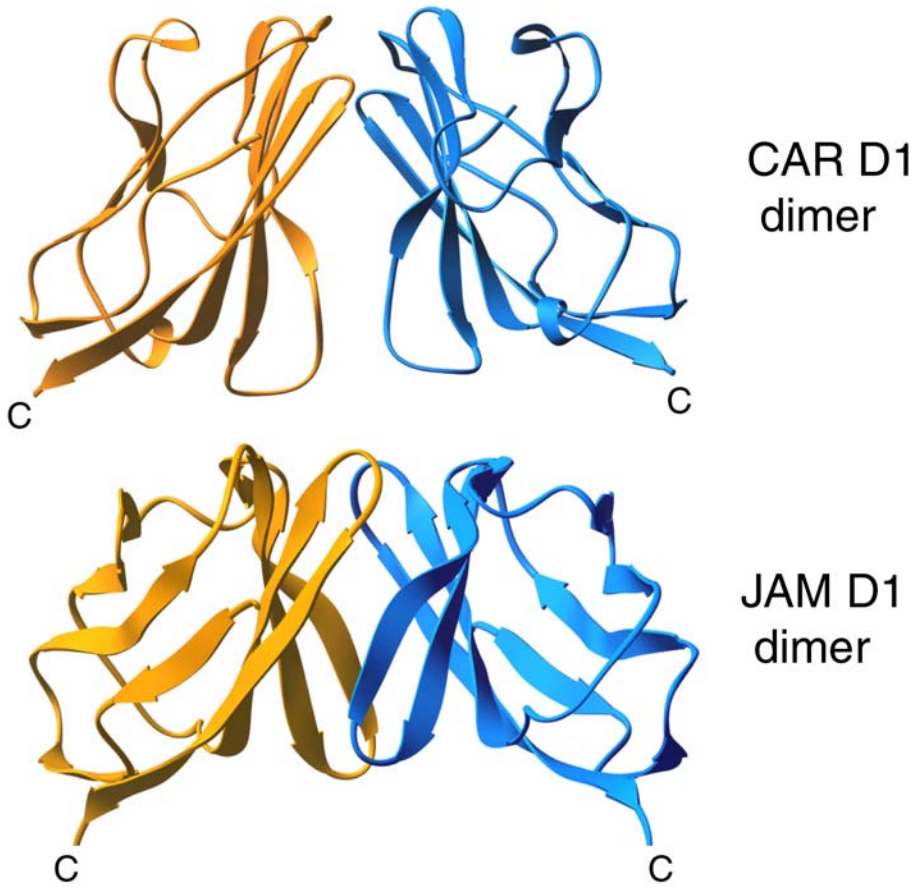


Fig. 3. Structural Similarities Between the Crystal Structure of JAM-A and CAR. Shown are the ribbon tracings of the crystallized D1 domains of reovirus receptor JAM-A (bottom) and adenovirus receptor CAR (top). Both proteins are dimers shown as two monomers in blue and gold. JAM-A and CAR are Ig-superfamily members, and both localize to tight junctions of polarized epithelial cells. The JAM-A structure image was reproduced from PNAS, 2003, 100: 5366-5371. Copyright, © 1993-2005, The National Academy of Sciences of the United States of America. The CAR structure image was reprinted from Structure, 8, van Raaij, M.J., Chouin, E., van der Zandt, H., Bergelson, J.M., Cusack, S. Dimeric Structure of the Coxsackievirus and Adenovirus Receptor D1 Domain at 1.7 A Resolution.,1147-1155, Copyright © 2000, with permission from Elsevier.

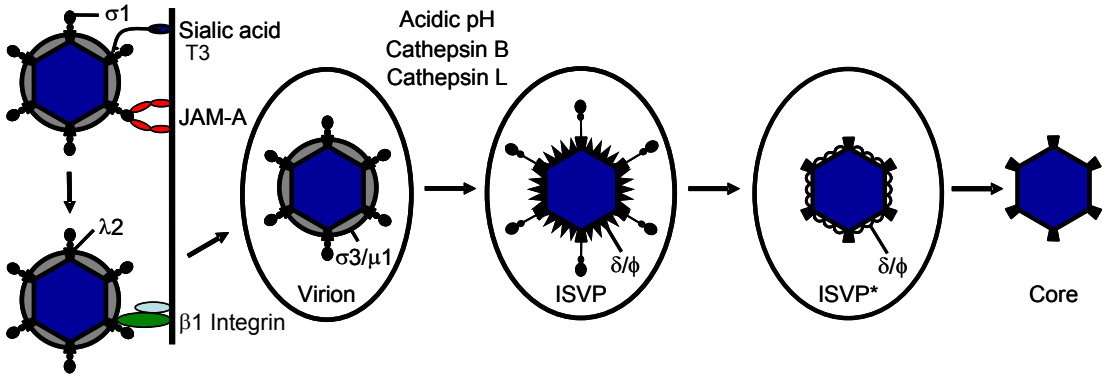


Fig. 4. Reovirus Entry and Disassembly. Reovirus attachment to cells is mediated via σ1 binding to carbohydrate, sialic acid for type 3 strains, and JAM-A for all reovirus serotypes. After binding to receptors, the virus enters cells by receptor-mediated endocytosis, which is thought to be clathrin-dependent. Virions are delivered to an endocytic compartment where they undergo disassembly to an infectious subvirion particle (ISVP), in which the σ3 protein is removed and the μ1 protein is cleaved to particle-associated fragments, δ and ϕ. The ISVP undergoes further conformational rearrangement to form an ISVP*, in which σ1 is lost and μ1 undergoes a conformational change to μ1*, which can penetrate the endosomal membrane and release the transcriptionally active core into the cytoplasm.

fragments and renders the ISVP* capable of penetrating endosomal membranes (2, 46, 47). During the ISVP-to-ISVP* transition, conformational changes also occur in λ2 (76) (Fig. 5), leading to loss of σ1 from the λ2 turrets. ISVP*s penetrate the endosomal membrane and release the transcriptionally active core into the cytoplasm (180).

Reovirus disassembly requires an acidic compartment, as treatment of cells with ammonium chloride, a weak base, blocks infection by reovirus virions but not by ISVPs (72, 239). Sensitivity to ammonium chloride is linked to the σ3-encoding S4 gene segment, and specific residues in σ3 regulate acid-dependent reovirus disassembly (58, 78, 268). Reovirus disassembly is catalyzed by the activity of cathepsins, a class of endosomal proteases (11, 77). In fibroblasts, cathepsins B and L are capable of mediating σ3 removal and μ1 cleavage. Cathepsin L is more efficient, but cathepsin B can catalyze reovirus disassembly in the absence of cathepsin L (77). Thus, reovirus disassembly is a tightly regulated process during which viral and cellular components act to remove the viral outer capsid.

Reovirus Replication and Assembly

Following attachment, internalization, and disassembly, the reovirus core is released into the cytoplasm. The core is transcriptionally active (147, 226, 229) and synthesizes 10 capped viral mRNAs (94, 95), which are released from the core through the five-fold channels formed by the λ2 pentamer (13, 76, 154) (Fig. 6). Viral transcripts serve as templates for synthesis of viral proteins (279) or are packaged into nascent particles and serve as templates for minus-strand synthesis to generate the dsRNA segments (8, 224, 277, 278). Additional transcripts are synthesized by newly formed

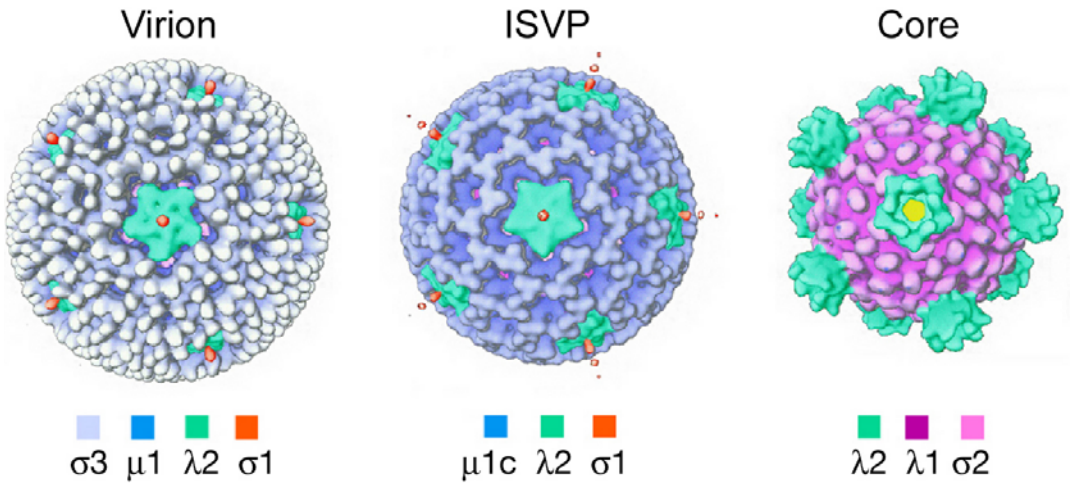


Fig. 5. Conformational Changes in the Reovirus Virion, ISVP, and Core. Cyro-electron micrograph (EM) reconstructions demonstrate the transition of reovirus from a virion to core. The reovirus virion shows icosahedral symmetry with λ2 shown at the five-fold axes of symmetry. The outer capsid proteins σ3 and μ1 project from the surface of the virion. σ1 is not visualized by cryo-EM. In the ISVP, σ3 has been removed. In the core particle, outer capsid proteins have been completely removed, and λ2 has undergone conformational changes that open the turret. Reproduced from Journal of cell Biology, 1993, 122: 1023-1041. Copyright 1993 Rockefeller University Press.

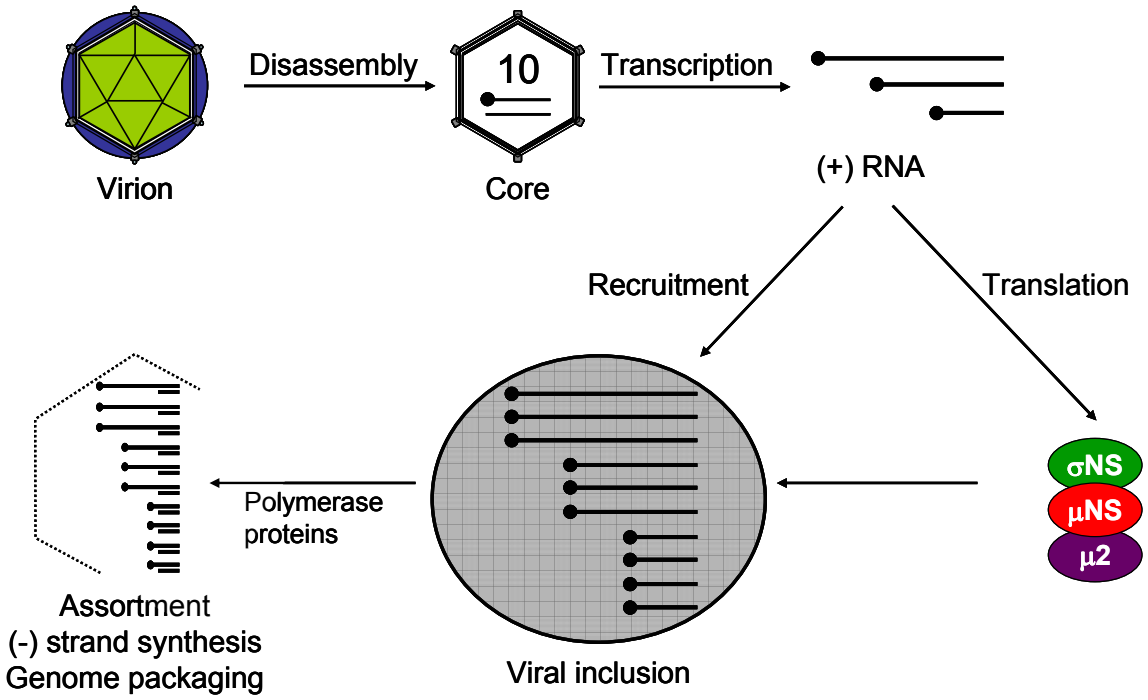


Fig. 6. Reovirus Replication and Assembly. Following attachment, endocytosis, and disassembly in endosomes, the transcriptionally active reovirus core is delivered into the cytoplasm. Viral transcripts are released from the viral core and serve as template for translation of viral proteins or are packaged into particles and serves as template for minus-stand synthesis of the dsRNA genome. Transcripts released from newly formed cores serve as template for secondary rounds of transcription and translation. Structural proteins encapsidate the core particle containing the viral genome, and virions are released from cells.

particles for secondary rounds of protein synthesis (230, 275, 278). Reovirus genome packaging is a highly regulated process that ensures that the 10 unique gene segments are packaged into cores (76, 109), but the mechanism by which this occurs is unknown. The transcriptionally active core particles containing viral genomic dsRNA are encapsidated with outer-capsid proteins to form mature viral progeny (48, 62, 111, 270), which then egress from the cell.

Integrins

Integrins are cell-surface adhesion molecules that have broad physiologic expression in invertebrates and vertebrates. They mediate adhesion to the extracellular matrix (ECM) and link the ECM to the cytoskeleton (117). Integrins function in many cellular processes including cell-cell contacts, intracellular signaling, endocytosis, and cell spreading (117, 153). Integrins play a major role in normal physiology and disease. Integrins function in development (10, 237, 272, 273), immunity (84, 238), cancer metastases (5, 34), and tissue homeostasis (117). Integrins also serve as receptors for many pathogens including viruses and bacteria (117, 120).

There are 18 α and 8 β integrin subunits that assemble to form 24 types of αβ heterodimer pairs (153). Integrin αβ pairs differ in ligand recognition, links to cytoplasmic proteins, and signaling properties and, thus, these molecules perform largely non-redundant functions (117). The α and β subunits are type I transmembrane glycoproteins with globular extracellular domains, transmembrane regions, and short cytoplasmic domains less than 50 amino acids (153, 241) (Fig. 7). The α subunits are

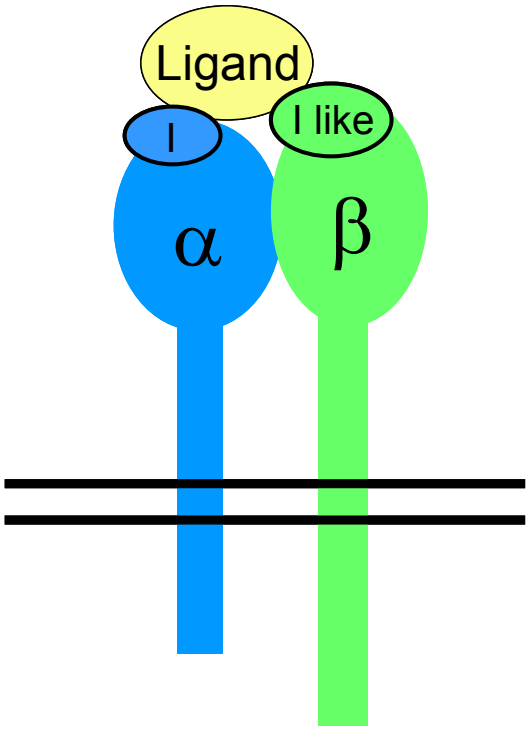


Fig. 7. Schematic Diagram of Integrins. Integrins are cell adhesion receptors comprised of an α and β subunit. Each subunit is comprised of an extracellular domain, a transmembrane region, and a short cytoplasmic tail. Some α subunits contain an I (inserted) domain, which serves as a ligand-binding domain. β subunits contain an I-like domain, which can also serve as a ligand-binding domain.

approximately 120-180 kilo Daltons (kDa), while the β subunits are approximately 90-110 kDa (241). The amino termini of the α and β subunits form globular heads that associate on the cell surface. Divalent cations mediate association of some α and β subunits and are required for ligand specificity and binding. Half of the known α integrins have an extra, "inserted" (I) domain of approximately 200 amino acids, which contains a unique metal ion coordination site known as a metal-ion-dependent adhesion site (MIDAS) that binds to negatively charged residues of ligands (153, 241). The I domain is the ligand-binding domain for integrins that incorporate this feature (70, 74, 133). The integrin β subunit contains a domain that is similar to the α integrin I domain, which is approximately 240 amino acids and known as an I-like domain (241). The β integrin I-like domain contains residues that resemble the MIDAS sequence in the α subunit (143). The β integrin I-like domain can function as a ligand-binding domain for integrins that lack an α I domain (205, 246) and also cooperates in ligand binding in integrin heterodimers that engage ligands using an α I domain (241). Ligands also can bind integrins via a pocket formed by the α and β subunits (117).

Many integrins recognize ligands that contain a short peptide motif that incorporates an acidic amino acid. The most commonly recognized integrin-binding motif is the Arg-Gly-Asp (RGD) sequence in ECM proteins fibronectin and vitronectin (117). α4, α5, α8, α_{IIb}, and αv integrins engage ECM ligands using an RGD-dependent interaction (241). Other integrin-binding motifs have been reported including Asp/Lys-Gly-Glu (117). α2β1 integrin binds to a DGEA sequence in collagen I (233). Although there are many examples of integrin binding mediated by specific sequence motifs, this is

not the only mechanism of integrin adhesion. In fact, some RGD-binding integrins can engage ligands in an RGD-independent fashion (86, 117).

Integrins stimulate bidirectional signaling events upon ligand binding that regulate cell function, proliferation, and survival (117). Following integrin-ligand engagement and integrin clustering, the integrin cytoplasmic domains recruit signaling proteins and induce assembly and remodeling of actin filaments forming focal adhesions (FA) (35). The β integrin subunit recruits several cytoplasmic kinases including focal adhesion kinase (FAK) (148), integrin-linked kinase (ILK) (107), and Src-family kinases (139). For example, many β integrin cytoplasmic domains activate the FAK signaling pathway (195). FAK binds either directly to the β integrin cytoplasmic domain (148) or indirectly via adaptor proteins, such as talin (42) or paxillin (222). For example, β1 integrin clustering leads to autophosphorylation of FAK through tyrosine residues in the Asn-Pro-any residue-Tyr (NPXY) motifs in the cytoplasmic domain (195). Autophosphorylation of FAK creates a binding site for the src homology 2 (SH2) domain of Src kinase (221), which can phosphorylate cytoplasmic proteins including paxillin (22).

Integrin-linked signaling proteins bind to the actin cytoskeleton directly or via actin-binding proteins that regulate actin cytoskeleton formation and remodeling (35). For example, talin is capable of binding directly to β integrin cytoplasmic domains (271) and actin (113). Biochemical linkage of talin and actin is important for cell adhesion, spreading, and formation of FAs and stress fibers (203). Talin also links other cytoplasmic proteins to the actin cytoskeleton. For example, the binding of phosphatidylinosital (4,5)-bisphosphate (PIP2) to the cytoskeletal protein vinculin reveals

a talin-binding site, and talin binding to vinculin and leads to association with actin (100). PIP2 and vinculin bind to the actin-related protein (Arp) 2/3 complex, which leads to actin polymerization and filament branching, thus mediating movement of the plasma membrane (71).

Integrins and Pathogens

Given the ubiquitous nature of integrin expression, it is not surprising that a number of viruses and bacteria utilize integrins as receptors to facilitate attachment or entry into target cells (120, 244). Integrins serve as receptors for enveloped viruses that are internalized into target cells by fusion mechanisms. West Nile virus (WNV), an enveloped ssRNA virus of the *Flaviviridae* family uses αvβ3 as a receptor (57). WNV is a mosquito-borne pathogen that causes West Nile fever, which is associated with encephalitis and death in the elderly (105). The WNV envelope protein engages αvβ3 in an RGD-independent fashion (144), leading to activation of FAK and viral internalization (57). Human cytomegalovirus (HCMV), an enveloped dsDNA virus of the *Herpesviridae* family, utilizes α2β1, α6β1, and αvβ3 integrins to mediate viral entry via fusion and signal transduction (89). The HCMV envelope glycoprotein B binds to integrins using a conserved integrin-binding disintegrin-like domain, $RX_{5-7}DLXXF/L$ (88, 89). HCMV engagement of β1 integrins leads to phosphorylation of β1 integrin on threonine residues 788 and 789 and phosphorylation of FAK to mediate HCMV entry (89).

Integrins are utilized by a number of nonenveloped viruses to mediate endocytosis. Rotavirus, a member of the *Reoviridae* family that causes infantile diarrhea

(193), engages a variety of integrins for attachment and cell entry. Rotavirus strains RRV, SA11, and Wa bind to the I domain of α2β1 integrin via a DGE integrin-binding motif in the VP4 spike protein to effect viral attachment (102). Interactions of rotavirus outer-capsid protein VP7 with integrins αxβ2 and αvβ3 can mediate viral entry (102, 274). Integrin α4β1 also can serve as a receptor for rotavirus strain SA11, which contains α4β1 integrin-binding sequences Leu - Asp - Val in VP7 and Ile - Asp - Ala in VP4 (114). The entry pathway of rotavirus is not well understood, but it is thought that rotavirus enters cells by receptor-mediated endocytosis that requires dynamin and cholesterol (219). Foot-and-mouth disease virus (FMDV), a ssRNA virus of the *Picornaviridae* family that causes disease in animals including livestock, uses a number of αv integrins for attachment and entry. αvβ1 (123), αvβ3 (25), αvβ6 (124), and αvβ8 (122) all serve as receptors for FMDV. The viral capsid protein, VP1, contains an RGD motif on a surface exposed loop, which is thought to bind αv integrins (123). FMDV is internalized into cells by clathrin-dependent endocytosis and requires acidification in endosomes (26). Confocal microscopy experiments demonstrate FMDV delivery to early and recycling endosomes along with αvβ6 integrin, suggesting that αvβ6 is required for both viral attachment and delivery of the virus to the endocytic pathway (26).

Integrins also serve as receptors for both Gram-positive and Gram-negative bacteria. *Staphylococcus aureus* is a Gram-positive bacterium that most commonly causes dermal infections, abscesses, pneumonia, and bone and joint infections (152). *S. aureus* strains express extracellular matrix proteins in the cell wall including fibronectin-binding proteins A and B, which mediate attachment to fibronectin. As *S. aureus* interacts with fibronectin in an RGD-independent fashion, the fibronectin receptor α5β1

expressed on eukaryotic cells is still capable of interacting with fibronectin that is bound by *S. aureus* (1). Thus, *S. aureus* acts as a link between fibronectin and host cell α5β1, which leads to internalization of the organism (1). β1 integrin-mediated uptake of *S. aureus* requires β1 integrin-induced Src family phosphotyrosine kinase signaling events and actin remodeling (1).

Yersinia pseudotuberculosis is a Gram-negative bacterium that causes tuberculosis-like symptoms in animals and fever and abdominal pain in humans after ingesting contaminated food products (198). In mice, *Yersinia* can cause localized infection in the lymph nodes and fatal systemic disease. *Yersinia* infects the intestine by binding to the apical surface of intestinal M cells. Once bound, *Y. pseudotuberculosis* is internalized into M cells and translocated to underlying Peyer's patches (160). Bacterial translocation and attachment to epithelial cells is mediated by the bacterial membrane protein, invasin (197), which binds to β1 integrin to promote bacterial internalization (119). Invasin binds to β1 integrin paired with a number of α subunits including α3, α4, α5, α6, and αv (119). Binding and entry of *Yersnia pseudotuberculosis* into mouse intestinal M cells are dependent on invasin binding to β1 integrins expressed on the apical surface (59, 160). Additionally, the β1 integrin cytoplasmic domain NPXY motifs are required for efficient bacterial uptake (106, 254) via a mechanism independent of tyrosine phosphorylation (106). Therefore, β1 integrin is required for both binding and signaling events to enhance internalization of *Y. pseudotuberculosis*.

Significance of the Research

Viral entry into cells is a complex, multi-step process that initiates infection in the host. Cell entry steps are poorly understood for most nonenveloped viruses including reovirus. Internalization mechanisms used by nonenveloped viruses are likely to be conserved. Thus, the use of reovirus as a model system to study viral attachment and internalization will provide a deeper understanding of virus entry for nonenveloped viruses. Since viral attachment and cell entry play key roles in disease tropism, these studies also may enhance an understanding of how receptors function as host-cell determinants of viral pathogenesis and provide a framework for the development of antiviral therapies. Moreover, information gained from work on virus-receptor interactions will allow a greater appreciation of the functions of these receptor molecules, including β1 integrin and JAM-A. Finally, studies of integrin-mediated virus entry will contribute new information about the function of integrins in endocytosis and signaling and enhance knowledge of integrin-pathogen interactions. My dissertation research has focused on identification and characterization of cell-surface receptors required for reovirus internalization. This research has led to the identification of β1 integrin as a reovirus internalization receptor and elucidated the importance of the NPXY motifs in the β1 integrin cytoplasmic domain in functional reovirus entry.

CHAPTER II

β1 INTEGRIN MEDIATES INTERNALIZATION OF MAMMALIAN REOVIRUS

Introduction

Viral attachment and cell entry are key determinants of target-cell selection in the infected host and thus play important roles in pathogenesis. Many viruses interact with multiple cell-surface molecules to mediate the processes of attachment and internalization (231). Receptors that serve as initial binding sites have been identified for many viruses (73). However, little is known about the post-attachment events that lead to nonenveloped virus internalization and delivery into the endocytic pathway. In particular the cellular determinants of reovirus receptor-mediated internalization following attachment and preceding uncoating are poorly defined.

We previously identified JAM-A as a serotype-independent receptor for reovirus (15, 43, 91). JAM-A is a type 1 transmembrane protein expressed in a variety of cell types, including polarized endothelial and epithelial cells and circulating leukocytes (150, 162, 266). JAM-A interacts with several scaffolding proteins and cytoplasmic adaptor molecules (19, 79, 80) and is hypothesized to play an important role in maintaining barrier function of epithelial junctions (142, 150, 162, 188). JAM-A is phosphorylated during platelet activation and required for mitogen-activated protein kinase activation following treatment of endothelial cells with basic fibroblast growth factor (174). These data indicate that JAM-A is intimately associated with cytoskeletal and signaling

machinery, which raises the possibility that reovirus binding to JAM-A mediates cytoskeletal rearrangement or signaling events to facilitate virus internalization.

The attachment mechanisms of reovirus and adenovirus are remarkably similar (235, 236). The trimeric attachment proteins of both viruses, σ1 and fiber, respectively, are structural homologues and fold using a highly unusual triple β-spiral motif (27, 53, 276). The globular head domains of these molecules are formed from eight-stranded β-barrels with identical interstrand connectivity (235). The receptors for σ1 and fiber, JAM-A (15) and CAR (23), respectively, are two-domain, immunoglobulin superfamily proteins that form homodimers using analogous molecular surfaces (236). Both JAM-A and CAR localize to tight junctions in polarized epithelial cells (61, 150, 162, 188). Remarkably, reovirus and adenovirus engage their respective receptors by thermodynamically favored disruption of receptor homodimers (91, 151).

Despite mediating high-affinity attachment of adenovirus to cells, engagement of CAR does not permit efficient adenovirus internalization. Instead, adenovirus entry is enhanced by high-avidity interactions of the viral penton base complex with integrins, including αvβ3 and αvβ5 (265). Integrins function to mediate cellular adhesion to the extracellular matrix, regulate cellular trafficking, and transduce both outside-in and inside-out signaling events (117). In addition to adenovirus, several other pathogenic microorganisms have usurped the adhesion and signaling properties of integrins to bind or enter host cells (4, 24, 89, 102, 103, 114, 123, 146).

To define the molecular basis of reovirus internalization, we first tested the capacity of a JAM-A mutant lacking a cytoplasmic tail to support reovirus attachment and infection. We found that, while JAM-A is necessary for efficient attachment to cells,

the JAM-A cytoplasmic tail is not required for reovirus infection. Given the mechanistic conservation of reovirus and adenovirus attachment strategies, and the observation that reovirus outer-capsid protein λ2 contains conserved integrin-binding sequences RGD and KGE, we tested the role of integrins in reovirus internalization. I found that infection by reovirus virions is inhibited by antibodies specific for β1 integrin. In addition, cells deficient in β1 integrin have a diminished susceptibility to reovirus infection due to a post-attachment block to viral entry. Together, these data indicate that following attachment to JAM-A, β1 integrin facilitates internalization of reovirus into cells. These findings further demonstrate that two seemingly unrelated viruses utilize distinct cellular molecules to mediate attachment and internalization in a remarkably similar manner.

When I began my dissertation research in the Dermody laboratory, Craig Forrest, a graduate student, had made the initial observation that the JAM-A cytoplasmic tail was not required for reovirus infection, and he hypothesized that integrins were required for reovirus internalization. I acknowledge Craig for intellectual contributions, for generation of the JAM-A and JAM-A-ΔCT constructs, and for Figure 13. I acknowledge Sarah Kopecky-Bromberg for assistance with confocal microscopy in Figure 14.

Results

The JAM-A Cytoplasmic Tail Is Dispensable for Reovirus Infection

JAM-A is a serotype-independent reovirus receptor with a cytoplasmic tail known to interact with a variety of cytoplasmic proteins (19, 79, 80). To determine whether the JAM-A cytoplasmic tail is required for reovirus entry, we generated a JAM-A cytoplasmic-tail deletion mutant (JAM-A-ΔCT) and tested its capacity to support

reovirus infection following transfection of Chinese hamster ovary (CHO) cells. CHO cells do not express detectable levels of JAM-A (162, 188) and are poorly permissive for reovirus infection (43, 91). Cells were transiently transfected with plasmids encoding full-length JAM-A, JAM-A-ΔCT, or empty vector as a control. Equivalent cell-surface expression of transfected constructs was confirmed by flow cytometry (data not shown).

The capacity of reovirus to infect CHO cells following transfection with the JAM-A constructs was tested using reovirus fluorescent focus assays. Following transient transfection of CHO cells with empty vector, JAM-A, or JAM-A-ΔCT, cells were adsorbed with reovirus strains T1L or T3D and scored for infection by indirect immunofluorescence at 20 h post-infection (Fig. 8). Expression of either full-length or truncated JAM-A was sufficient to allow reovirus infection of CHO cells, permitting viral protein production of both type 1 and type 3 reovirus strains. These results indicate that the JAM-A cytoplasmic tail is not required for efficient reovirus attachment and infection.

Reovirus Outer-Capsid Proteins Contain Integrin-Binding Sequences

Structural and functional analyses indicate that reovirus and adenovirus share remarkably similar mechanisms of attachment (235, 236). To determine whether reovirus outer-capsid proteins contain sequences that could potentially engage integrins, I performed a search for integrin-binding motifs in the σ1, σ3, μ1, and λ2 proteins, which form the reovirus outer capsid (76). I identified two common integrin-binding motifs, RGD and KGE, in the deduced amino acid sequence of the λ2 protein (Fig. 9). The RGD motif is conserved in all reovirus strains for which sequence information is available (37,

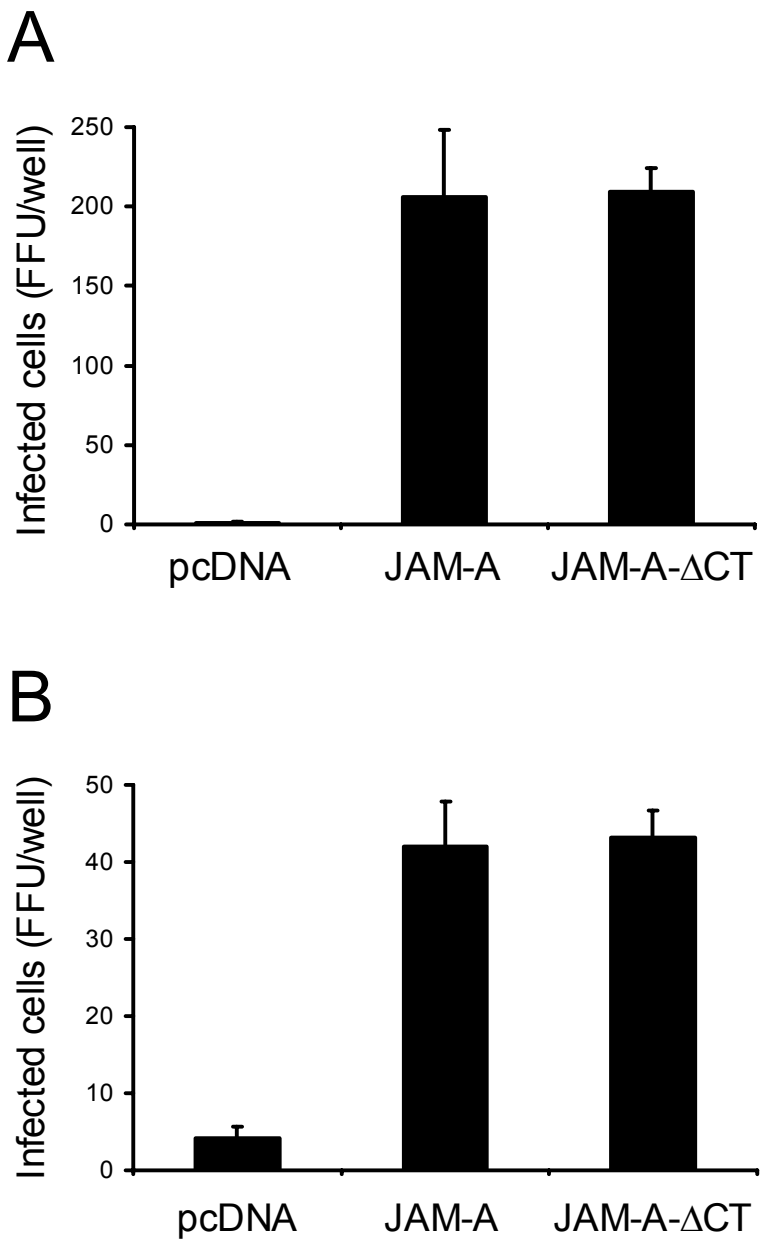


Fig. 8. The JAM-A Cytoplasmic Tail Is Not Required for Reovirus Infection. CHO cells were transiently transfected with empty vector or plasmids encoding JAM-A or JAM-A-ΔCT. Following incubation for 24 h to permit receptor expression, cells were adsorbed with reovirus strains **(A)** T1L or **(B)** T3D at an MOI of 0.1 or 1 FFU per cell, respectively, at room temperature for 1 h. Cells were washed with PBS, incubated in complete medium at 37°C for 20 h, and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in entire wells for triplicate experiments. The results are expressed as the mean fluorescent focus units (FFU) per well for triplicate samples. Error bars indicate standard deviations.

225); the KGE motif is conserved in all of those strains except T2J (37, 225). The λ2 protein is a component of the reovirus outer capsid and core (76). It is structurally arranged as a pentamer at the virion fivefold axes of symmetry and forms the base for attachment protein σ1 (76, 208) (Fig. 5). The presence of conserved integrin-binding motifs in the reovirus λ2 protein led us to test whether reovirus utilizes integrins to mediate internalization.

An Antibody Specific for β1 Integrin Inhibits Reovirus Infection of HeLa Cells

To determine whether integrins are required for reovirus infection, I first used flow cytometry to analyze integrin expression on the surface of HeLa cells. HeLa cells were incubated with integrin-specific monoclonal antibodies (MAbs) and a PE-labeled secondary antibody (Table 1). RGD-binding integrin subunits α3, α5, αv, and β1 and KGE-binding integrin subunits α1, α2, α6, and β1 (118) were detected on HeLa cells at levels above control antibody-treated cells. RGD-binding integrin heterodimer αvβ5 also was detected at levels above control, while there was low-level expression of αvβ3. Thus, HeLa cells express both RGD- and KGE-binding integrins.

To assess a role for integrins in reovirus replication, I tested antibodies specific for the RGD- and KGE-binding integrins expressed on HeLa cells for the capacity to block reovirus infection. HeLa cells were preincubated with integrin-specific and control antibodies prior to adsorption with reovirus virions. Viral infection was detected by indirect immunofluorescence (Fig. 10A). I found that β1-specific MAb DE9 resulted in a

Table 1. Surface expression of integrins on HeLa cells

Antibody	Specificity	Mean fluorescence intensity[a]
MAB1997 (IgG control)	Murine β1 integrin	2.65
MAB1973	Human α1 integrin	76.09
MAB6F1	Human α2 integrin	84.47
MAB2057	Human α3 integrin	73.31
MABBIIG2	Human α5 integrin	76.68
MAB1378	Human α6 integrin	81.86
MAB1980	Human αv integrin	78.75
MAB2253Z	Human β1 integrin	61.14
MAB1976	Human αvβ3 integrin	5.46
MAB1961Z	Human αvβ5 integrin	42.10

[a] Results are expressed as mean fluorescence intensity for an average of 14,000 gated events as assessed by flow cytometry.

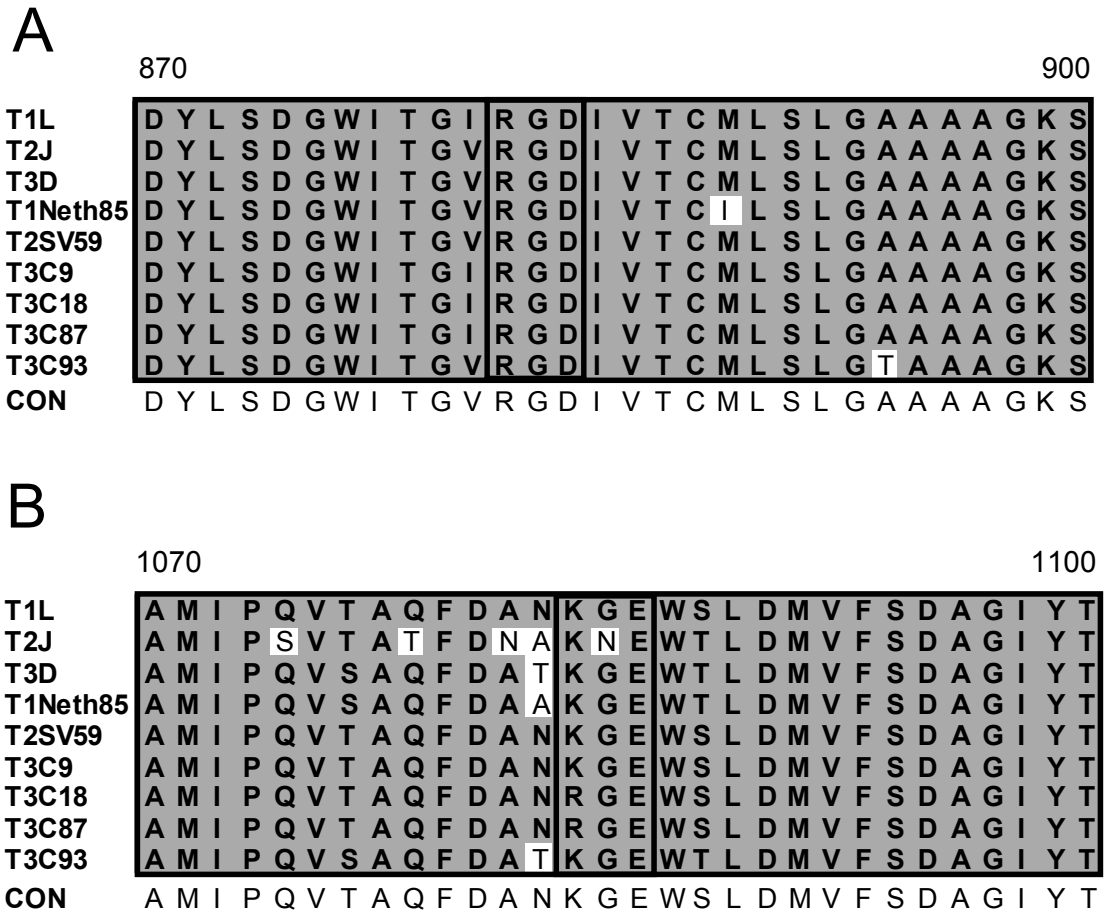


Fig. 9. Reovirus Outer-Capsid Protein λ2 Contains Integrin-Binding Sequences. Alignment of deduced amino acid sequences of the reovirus λ2 protein for the indicated strains. Amino acid residues are designated by the single-letter code. Amino acid positions are indicated above the first and last letter. Integrin-binding **(A)** RGD and **(B)** KGE motifs are highlighted by a black box. Non-conserved sequences are shown in unshaded boxes. CON, consensus sequence.

50% reduction in infection ($P < 0.05$), while antibodies specific for the other integrin subunits expressed on HeLa cells had no effect. Control antibodies produced anticipated effects; JAM-A-specific MAb J10.4 inhibited infection, whereas ICAM-specific MAb (data not shown) or control mouse ascites (Fig. 10A) did not. The effect of MAb DE9 was dose-dependent (Fig. 10B), providing further evidence that the inhibition of infection resulted from integrin blockade.

To determine whether particular α subunits pair with β1 integrin to facilitate reovirus infection, I tested whether treatment with α integrin-specific antibodies was capable of enhancing the inhibitory effect of β1 integrin-specific MAb DE9 on reovirus infection. I also tested whether antibodies specific for other β integrin subunits expressed on HeLa cells, β3 and β5, were capable of infection blockade. HeLa cells were treated with MAb DE9 in combination with other integrin-specific antibodies prior to adsorption with reovirus virions (Fig. 10C). While treatment of HeLa cells with MAb DE9 resulted in a 50% reduction in reovirus infection, none of the other integrin-specific antibodies tested reduced reovirus infection greater than that resulting from treatment with DE9 alone. These results suggest that the integrin epitope bound by reovirus is blocked by β1-specific MAb DE9 and not by the other MAbs used in these experiments.

JAM-A MAb J10.4 blocks reovirus infection ~90% (Fig. 10D). To determine whether the residual level of infection in the presence of MAb J10.4 was dependent on reovirus interactions with β1 integrin, I treated HeLa cells with JAM-A-specific MAb J10.4 in combination with MAb DE9 prior to adsorption with reovirus virions (Fig. 10D). Treatment of HeLa cells with MAb J10.4 and MAb DE9 completely abrogated reovirus infection, indicating that the effect of JAM-A blockade is enhanced when β1 integrin is

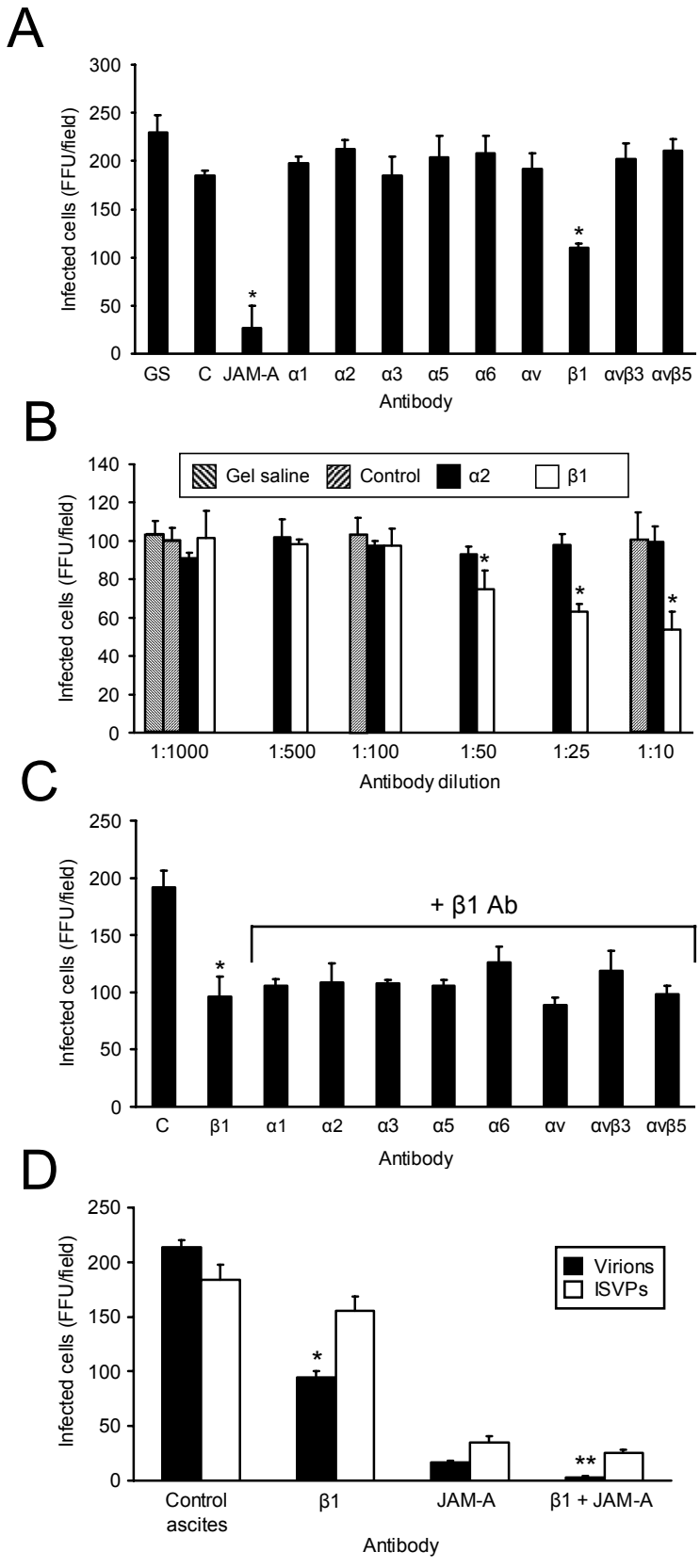

A
Infected cells (FFU/field)
300
250
200
150
100
50
0
GS
C
JAM-A
α1
α2
α3
α5
α6
αv
β1
αvβ3
αvβ5
Antibody
B
Gel saline
Control
α2
β1
Infected cells (FFU/field)
140
120
100
80
60
40
20
0
1:1000
1:500
1:100
1:50
1:25
1:10
Antibody dilution
C
Infected cells (FFU/field)
250
200
150
100
50
0
+ β1 Ab
C
β1
α1
α2
α3
α5
α6
αv
αvβ3
αvβ5
Antibody
D
Infected cells (FFU/field)
250
200
150
100
50
0
Virions
ISVPs
Control ascites
β1
JAM-A
β1 + JAM-A
Antibody

Fig. 10. β1 Integrin Antibody Reduces Reovirus Infection of HeLa Cells. HeLa cells were treated with **(A)**,gel saline (GS), control ascites (C), JAM-A-specific MAb J10.4, or antibodies specific for the α and β integrins shown (20 μg per ml or as diluted ascites), **(B)** gel saline, control ascites (Control), α2-specific MAb AA10, or β1-specific MAb DE9 (at the indicated dilutions), **(C)** antibodies specific for the α and β integrins shown in the presence of β1-specific MAb DE9 (1:10), control ascites, β1-specific MAb DE9, JAM-A-specific MAb J10.4, or **(D)** JAM-A-specific MAb J10.4 in combination with β1-specific MAb DE9 and incubated at room temperature for 1 h. Antibody-treated cells were infected with virions or ISVPs of T1L at an MOI of 0.1 FFU per cell at 4°C for 30 min. Cells were washed with PBS, incubated in complete medium at 37°C for 16 h, and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in three fields of view for triplicate samples. The results are expressed as the mean FFU per field for triplicate experiments. Error bars indicate standard deviations. *, $P < 0.05$ in comparison to control; **, $P < 0.05$ in comparison to HeLa cells treated with JAM-A-specific MAb J10.4 alone.

not available for interactions with reovirus. Treatment with MAb DE9 did not significantly inhibit infection by ISVPs (Fig. 10D), suggesting that viral attachment is not affected by β1 integrin blockade. Taken together, these results support the conclusion that a β1 integrin-specific antibody blocks reovirus infection at a step subsequent to attachment, but prior to uncoating, thereby implicating β1 integrin in reovirus internalization.

Transient Transfection of Integrin cDNAs Allows Reovirus Infection of JAM-A-Expressing CEFs

Ectopic expression of JAM-A in chicken embryo fibroblasts (CEFs) rescues infection by reovirus ISVPs but not by virions (15), suggesting that these cells exhibit a cell-specific block at the entry or uncoating phases of reovirus infection. To test the capacity of integrins to confer infection of CEFs by reovirus virions, CEFs were transiently transfected with a JAM-A-encoding plasmid in the presence or absence of murine αv, α2, or β1 integrin-encoding plasmids singly or in αβ pairs. Transfected cells were infected with reovirus virions or ISVPs, and infection was assessed by indirect immunofluorescence (Fig. 11). Expression of β1 integrin, paired with either of the murine α integrin subunits, provided an approximately four-fold enhancement of infection by reovirus virions in comparison to cells transfected with JAM-A alone. These data suggest that β1 integrin complements a reovirus cell-entry defect in CEFs and provide further support for the involvement of β1 integrin in reovirus internalization.

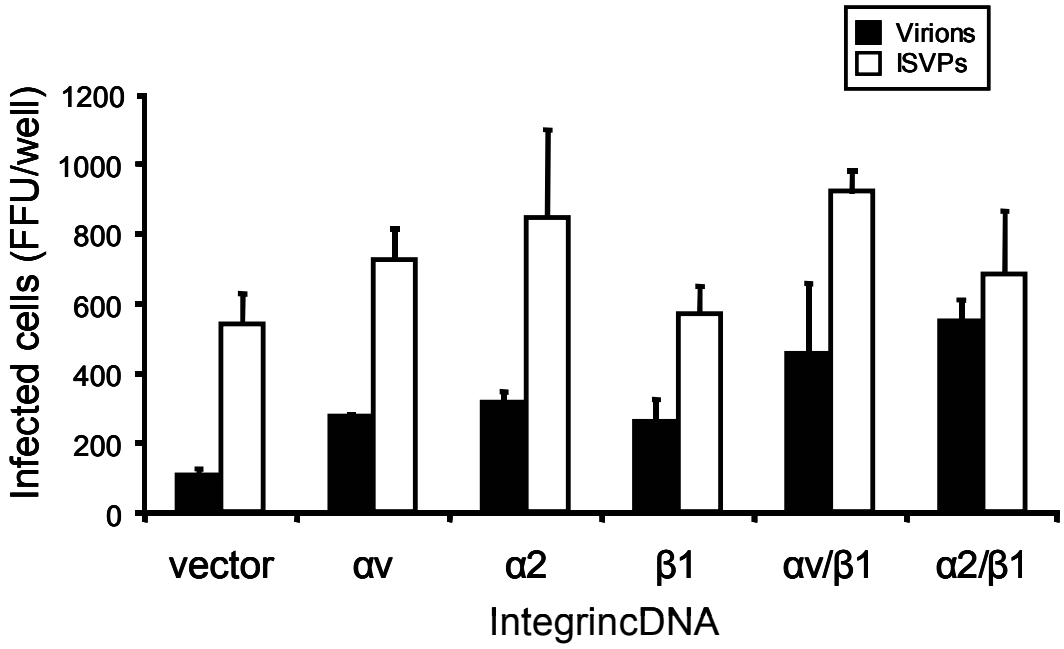


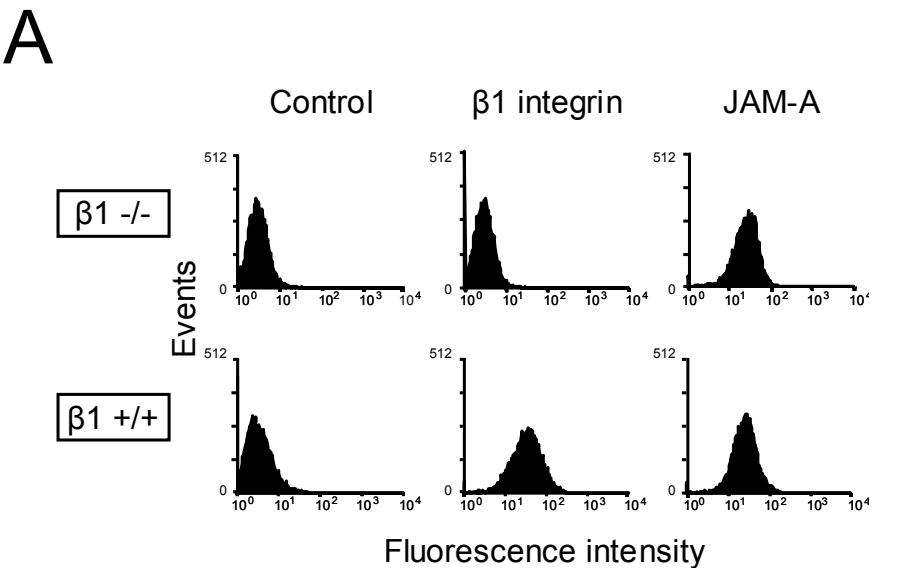
Fig. 11. β1 Integrin Expression Enhances Reovirus Infection of CEFs. CEFs were transiently transfected with JAM-A-encoding plasmid alone (vector) or in combination with plasmids encoding the integrin subunits shown. Following 24 h to allow receptor expression, transfected cells were adsorbed with T1L virions or ISVPs at an MOI of 1 FFU per cell at room temperature for 1 h. Cells were washed with PBS, incubated in complete medium at 37°C for 20 h, and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in entire wells for duplicate samples. The results are expressed as the mean FFU per well for duplicate experiments. Error bars indicate the range of data. Shown is a representative experiment of three independent experiments performed.

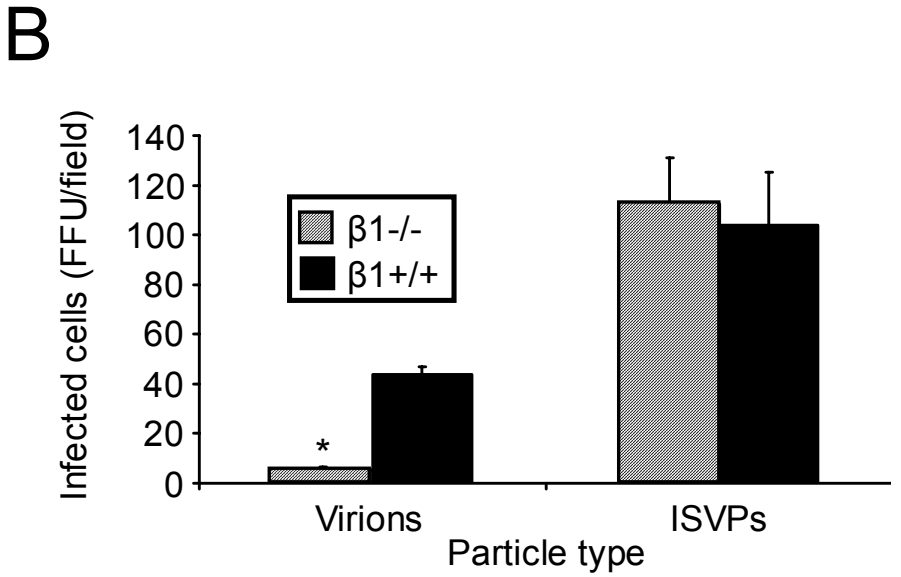
Cells Deficient in β1 integrin Have a Decreased Capacity to Support Reovirus Infection

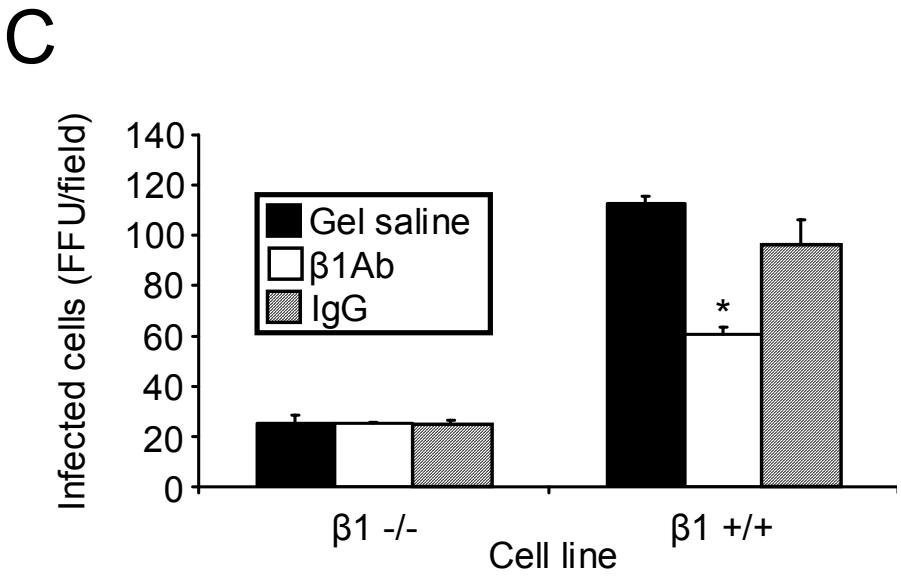
To further assess a role for β1 integrin in reovirus infection, I tested the capacity of reovirus to infect cells deficient in the β1-integrin subunit. GD25 cells are murine embryonic stem cells derived from β1-null embryos (263). GD25β1A cells are GD25 cells that have been engineered to stably express β1 integrin and thus serve as an isogenic control for GD25 cells. Flow cytometric analysis confirmed that while both cells express JAM-A, only GD25β1A cells express β1 integrin (Fig. 12A). GD25 cells (β1-/-) and GD25β1A cells (β1+/+) (263) were adsorbed with reovirus virions or ISVPs, and infection was scored by indirect immunofluorescence (Fig. 12B). In comparison to β1+/+ cells, β1-/- cells were substantially less susceptible to infection by virions, while infection by ISVPs was equivalent in both cell types. Importantly, pre-incubation of β1+/+ cells with murine β1 integrin-specific MAb CD29 reduced infection in β1+/+ cells (Fig. 12C), indicating that enhancement of infection is due to expression of β1 integrin. Therefore, β1 integrin is required for efficient reovirus infection.

Reovirus Binding to β1-/- and β1+/+ Cells Is Equivalent

Equivalent infection of β1-/- and β1+/+ cells by ISVPs (Fig. 12B) suggests that reovirus is capable of efficiently binding to both cell types. To directly test this hypothesis, β1-/- and β1+/+ cells were mock-treated or incubated with FITC-labeled virions and binding was assessed by flow-cytometry (Fig. 13). In these experiments, we found that reovirus binds equivalently to β1-/- and β1+/+ cells. These data demonstrate a function for β1 integrin in reovirus infection at a step subsequent to viral attachment.


A
Control
β1 integrin
JAM-A
β1 -/-
β1 +/+
Events
512
0
10^0 10^1 10^2 10^3 10^4
Fluorescence intensity


B
Infected cells (FFU/field)
140
120
100
80
60
40
20
0
β1-/-
β1+/+
*
Virions
ISVPs
Particle type


C
Infected cells (FFU/field)
140
120
100
80
60
40
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0
Gel saline
β1Ab
IgG
*
β1 -/-
β1 +/+
Cell line

Fig. 12. Cells Deficient in β1 Integrin Are Less Permissive for Reovirus Infection. (A) GD25 (β1 -/-) and GD25β1A (β1 +/+) cells were detached from plates with 20 mM EDTA, washed, and incubated with antibodies specific for either murine β1 integrin or murine JAM-A. Cell-surface expression of these molecules was detected by flow cytometry. Data are expressed as fluorescence intensity. β1-/- and β1+/+ cells were **(B)** untreated or **(C)** pretreated with β1-specific MAb CD29 (β1 Ab) or a hamster isotype-matched control MAb (IgG) at room temperature for 1 h, adsorbed with virions or ISVPs of T1L at an MOI of 0.1 FFU per cell, and incubated at 4°C for 30 min. Cells were washed with PBS, incubated in complete medium at 37°C for 20 h, and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in five fields of view for triplicate samples. The results are expressed as the mean FFU per field for triplicate experiments. *, $P < 0.05$ in comparison to control.

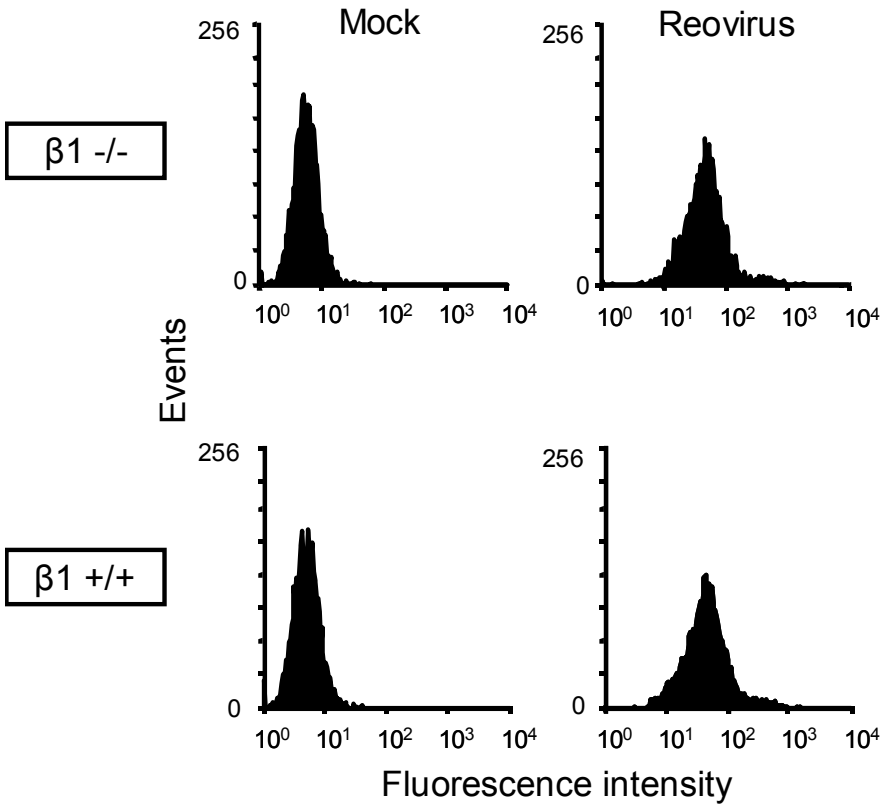


Fig. 13. Reovirus Exhibits Equivalent Binding to β1 -/- and β1 +/+ Cells. β1 -/- and β1 +/+ cells were incubated with either PBS (mock) or 2 x 10^{11} FITC-labeled T1L virions at 4°C for 1 h and analyzed by flow cytometry to assess reovirus binding to the cell surface. The results are expressed as fluorescence intensity.

β1 Integrin Enhances the Efficiency of Reovirus Internalization

To directly assess the role of β1 integrin in reovirus internalization, β1-/- and β1+/+ cells were infected at 4°C and then warmed to 37°C over a time-course concurrent with reovirus entry (11, 239). At 10-min intervals, cells were fixed, stained for reovirus using indirect immunofluorescence, and examined by confocal microscopy. Representative confocal micrographic images of reovirus-infected β1-/- and β1+/+ cells are shown in Fig. 14. Immediately after viral adsorption, both cell types exhibited reovirus staining at the cell periphery. At 10 min post-adsorption, some reovirus staining was observed at the cell periphery, yet intracellular staining in β1+/+ cells also was observed. At 20 and 30 min post-adsorption, the majority of virions had entered the β1+/+ cells and had a perinuclear location. In contrast, viral entry was markedly delayed in β1-/- cells, with the majority of reovirus virions remaining at the cell periphery throughout the time-course. At later time-points (30 min post-adsorption), some virions were present within the cytoplasm, but these were the minority. These findings suggest that expression of β1 integrin enhances reovirus entry.

To quantify reovirus internalization into β1-/- and β1+/+ cells, we determined the number of internalized fluorescent particles as a percentage of the total number of fluorescent particles per cell at various times post-adsorption (Fig. 15). At 0 and 10 min post-adsorption, the percentage of particles internalized into β1-/- and β1+/+ cells was equivalent, ~10% and ~30%, respectively. However, at 20 and 30 min post-adsorption, the percentage of reovirus particles internalized into β1+/+ cells was ~50%, while the percentage of particles internalized into β1-/- cells was only ~30% ($P < 0.05$) (Fig. 15).

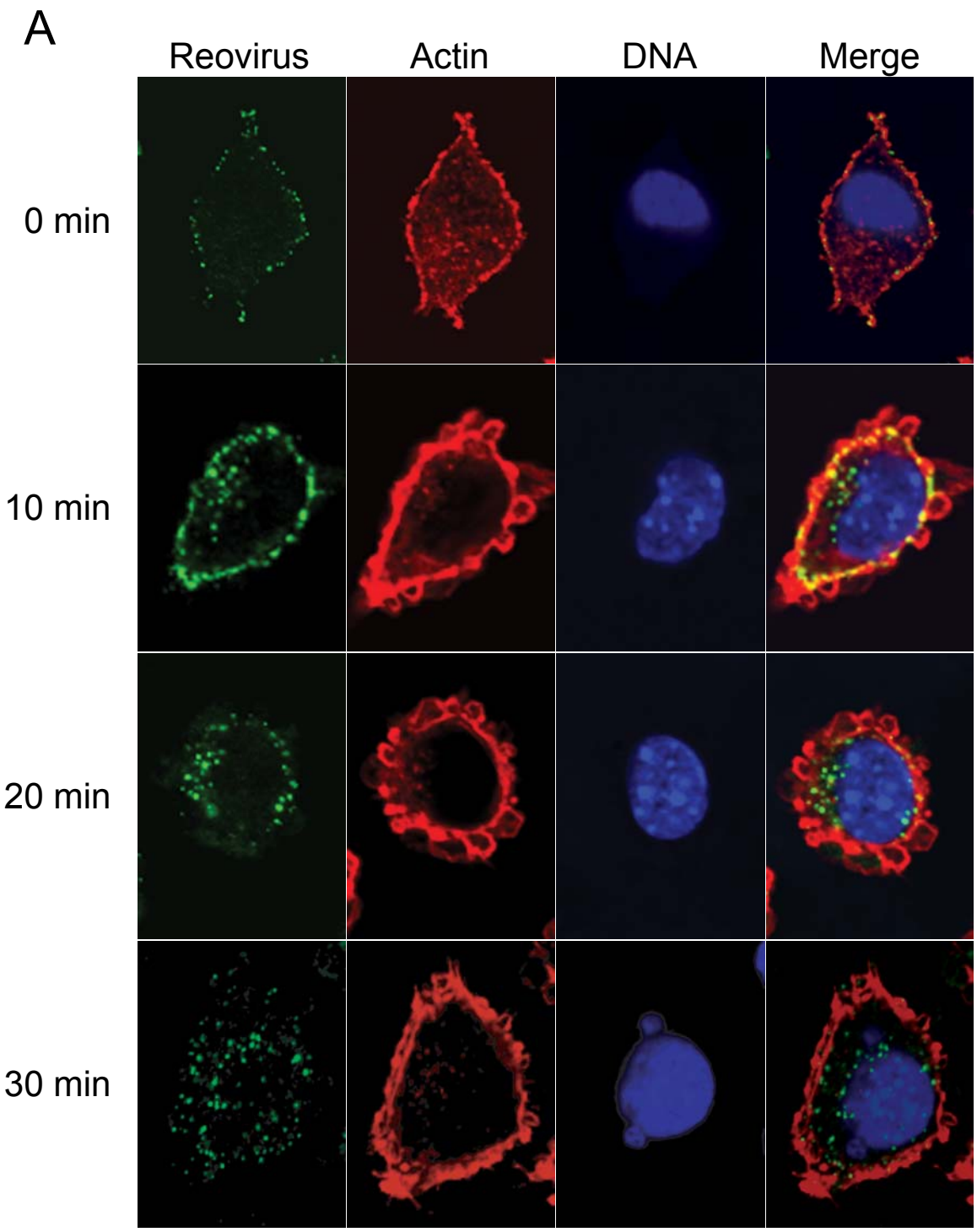


Fig. 14. β1 Integrin Enhances Reovirus Entry into Cells. (A) β1 +/+ and **(B)** β1 -/- cells were chilled, adsorbed with T1L virions, and incubated at 4°C for 1 h. Non-adherent virus was removed, warm medium was added, and cells were incubated at 37°C for the times shown. Cells were fixed, stained for reovirus (green), actin (red), and DNA (blue), and imaged using confocal immunofluorescence microscopy. Representative digital fluorescence images of the same field are shown in each row.

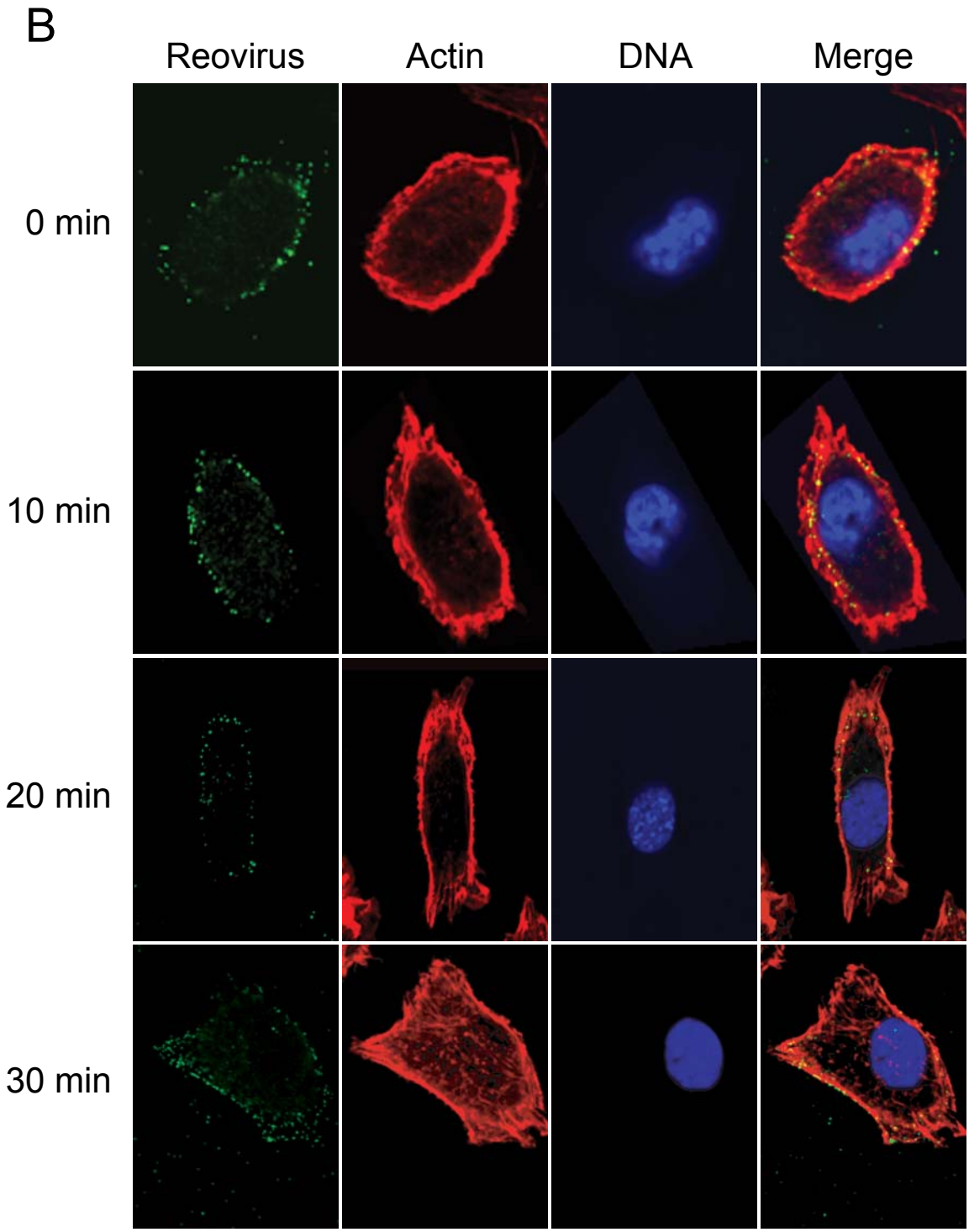


Fig. 14. - continued.

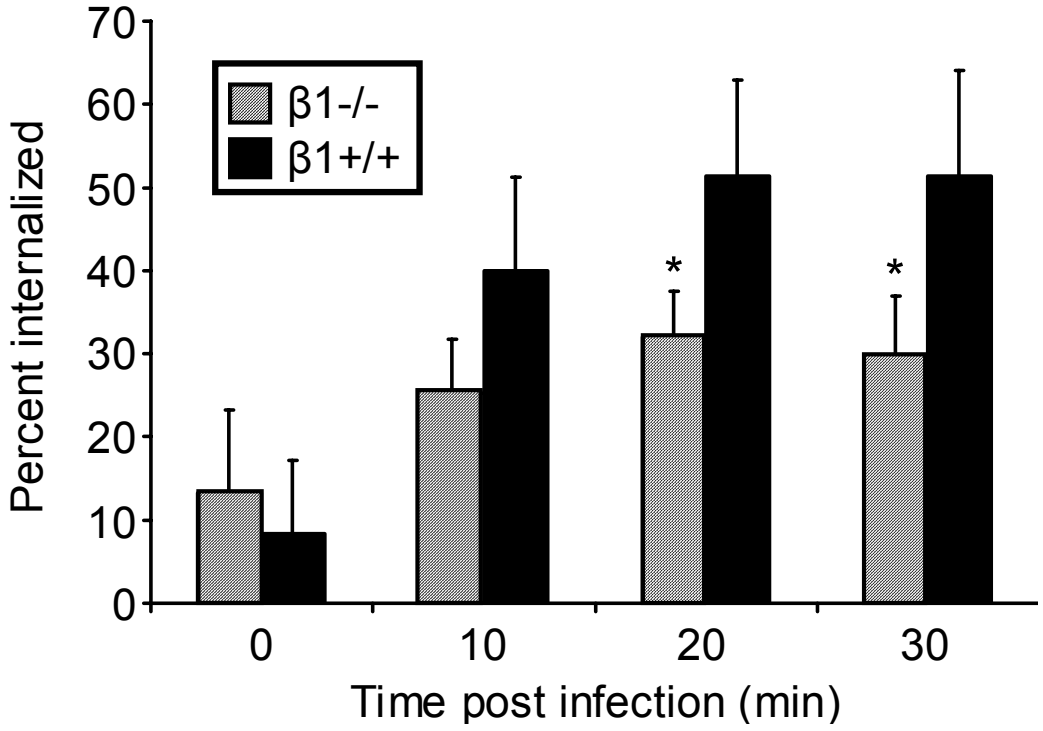


Fig. 15. Quantification of Reovirus Internalization into β1 -/- and β1 +/+ Cells. Viral internalization was quantitated by enumerating fluorescent particles localized at the cell periphery and particles internalized into the cytoplasm to determine the total number of fluorescent particles per cell. The results are expressed as mean percent internalization (internalized fluorescent particles divided by the total number of fluorescent particles per cell) for 10 cells for each time point. *, $P < 0.05$ in comparison to β1 +/+ cells.

These data indicate that β1 integrin enhances reovirus entry at early times post-adsorption, suggesting a direct role for β1 integrin as a reovirus internalization receptor.

Discussion

In this study, we performed experiments to define molecular determinants of reovirus internalization. Antibodies specific for β1 integrin inhibit reovirus infection at a post-attachment step. Expression of β1 integrin promotes infection by reovirus virions in cells with a block to viral internalization, and viral entry is substantially diminished in cells deficient in β1 integrin expression. Together, these data provide strong evidence that β1 integrin serves as a coreceptor to mediate reovirus internalization. These findings engender a new model for attachment and cell entry of reovirus (Fig. 16). In this model, I propose that reovirus initially interacts with cells via low-affinity binding to carbohydrate. These interactions are followed by high-affinity engagement of JAM-A, which positions the virus on the cell surface for subsequent interactions with β1 integrin to trigger viral endocytosis. Furthermore, the identification of β1 integrin as a reovirus internalization receptor suggests that the conservation of attachment strategies used by reovirus and adenovirus (235, 236) extends to mechanisms of internalization.

Although the specific reovirus protein required for integrin binding is not apparent from our studies, the λ2 protein is a promising candidate. The λ2 protein forms a pentameric turret at the virion fivefold symmetry axes and serves as the insertion site for trimers of attachment protein σ1 (76). Thus, λ2 is the reovirus analogue of the adenovirus penton base protein, which mediates the engagement of integrins by

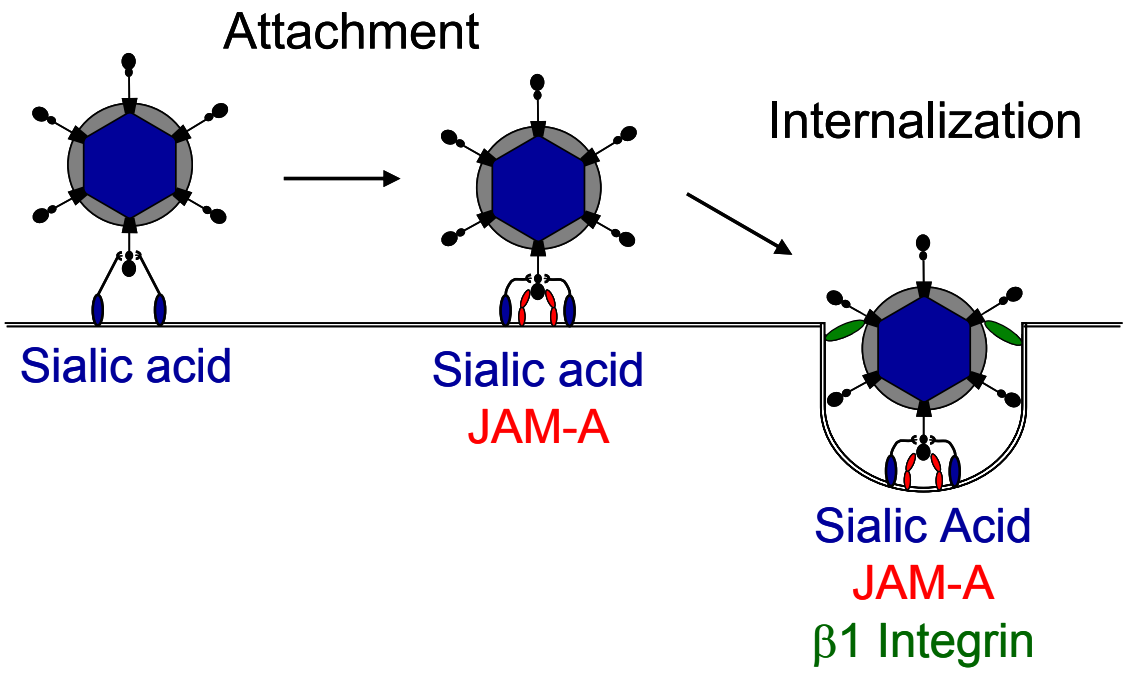


Fig. 16. Model of Reovirus Attachment and Cell Entry. Reovirus initially engages cells by low-affinity interactions with carbohydrate. For type 3 reovirus strains, this carbohydrate is sialic acid. Reovirus-carbohydrate interactions are followed by high-affinity binding to JAM-A, which positions the virus on the cell surface for subsequent interactions with β1 integrin to trigger viral endocytosis.

adenovirus (56, 265). Interestingly, λ2 also contains conserved RGD and KGE motifs (37), the preferred interaction motifs for several β1 integrin heterodimers (118). Structural information for λ2 is available in the context of the reovirus core but not for the intact virion (208). In the core, the KGE motif is exposed on the top of the λ2 turret, where it would be accessible to a receptor (208). The RGD motif is also surface exposed, but it appears to be less accessible. However, the λ2 structure in the core may not be identical to that in the virion, as the protein undergoes major conformational changes during virion-to-core disassembly (76) (Fig. 5). Therefore, it is possible that both the RGD and KGE motifs are accessible to interactions with β1 integrin during engagement of the cell surface by the virus.

A human β1 integrin-specific antibody (DE9) reduced reovirus infection of HeLa cells by 50% (Fig. 10). Similarly, a murine β1 integrin-specific antibody (CD29) blocked infection of β1-expressing mouse embryonic stem cells by ~50% (Fig. 12). Interestingly, MAb DE9 also blocks infection of echovirus (24) and cytomegalovirus (89), suggesting that an epitope in β1 integrin recognized by MAb DE9 may be a preferred binding site for multiple viruses. It is possible that the residual level of reovirus infection following β1 integrin antibody treatment is attributable to other internalization receptors on the cell surface that may be integrin or non-integrin molecules. However, it is noteworthy that treatment of HeLa cells with both MAb DE9 and JAM-A-specific MAb J10.4 completely abolishes reovirus infection (Fig. 10D). This finding suggests that the residual infection in J10.4-treated HeLa cells is due to reovirus interactions with β1 integrin. Thus, it appears that blockade of reovirus infection by integrin-specific antibodies is inefficient

because complete inhibition of virus-integrin interactions is not possible if the virus is tightly adhered to the cell surface by JAM-A.

Since antibodies specific for β3 and β5 integrins did not inhibit reovirus infection, it is likely that only β1 integrin can serve a reovirus internalization function. Antibodies specific for the α integrin subunits expressed on HeLa cells did not further reduce reovirus infection following treatment with a β1 integrin-specific antibody (Fig. 10C). I envision three possible explanations for this result. First, reovirus may directly engage the ligand-binding I-like domain of β1 integrin. Second, reovirus may utilize β1 integrin when paired with numerous α subunits that serve redundant functions. However, treatment of HeLa cells with a β1 integrin-specific antibody and a mixture of antibodies specific for α1, α2, α3, α5, α6, and αv integrins did not diminish reovirus infection in comparison to cells treated with a β1 integrin-specific antibody alone (data not shown). Third, reovirus may engage an epitope of an integrin α subunit that is not recognized by the antibodies used in these experiments. Further studies are required to define the biophysical basis of reovirus-integrin interactions.

JAM-A is required for high-affinity reovirus attachment to numerous cell types (15, 43, 91, 165). However, the JAM-A cytoplasmic tail is not necessary for viral endocytosis (Fig. 8). JAM-A likely tethers the virus to the cell surface to facilitate secondary interactions with β1 integrin (Fig. 16). This model is analogous to the mechanism of lymphocyte homing, in which adhesion molecules such as JAM-A provide initial cellular contacts to facilitate subsequent interactions with integrins for diapedesis or signaling (217). An interesting possibility is that JAM-A may be associated with β1 integrin on the host-cell plasma membrane. If such were the case, initial JAM-A

engagement might facilitate integrin binding, clustering, and viral endocytosis. In support of this hypothesis, JAM-A has been shown to regulate β1 integrin expression and localization (157).

Identification of β1 integrin as a receptor that triggers reovirus entry raises the possibility that coreceptor binding influences reovirus tropism and disease. Reovirus serotypes differ in mechanisms of spread, tropism for cells in the central nervous system, and disease outcome in the infected host (248). Previous studies using reassortant genetics and comparative sequence analysis demonstrate that these phenotypes most strongly segregate with viral attachment protein σ1, suggesting that reovirus serotypes bind to different receptors (250, 259, 260). However, the σ1-encoding S1 gene is not the sole determinant of reovirus growth at some sites within the host. For example, the λ2-encoding L2 gene influences viral growth in the intestine (28) and spread to new hosts (134). Moreover, JAM-A functions as a receptor for all three reovirus serotypes (43); therefore, JAM-A cannot explain serotype-dependent differences in reovirus pathogenesis. The presence or absence of particular integrins at distinct physiologic sites may critically influence the course of reovirus infection. In support of a role for coreceptor utilization in reovirus growth, reovirus infection can occur in the absence of σ1 (48) or JAM-A (15), albeit at greatly reduced efficiency. These findings highlight the complex nature of reovirus attachment and entry and suggest that reovirus tropism and pathogenesis are not dictated by primary receptor interactions alone. It is possible that tropism and pathogenesis are determined by the concerted action of attachment and internalization receptors, perhaps not all of which have been discovered.

CHAPTER III

TYROSINE-BASED SIGNALING MOTIFS IN THE β1 INTEGRIN CYTOPLASMIC TAIL ARE REQUIRED FOR FUNCTIONAL REOVIRUS ENTRY

Introduction

Many viruses utilize distinct cellular molecules to mediate attachment and internalization (161). After reovirus binds to JAM-A (15, 43), internalization is facilitated by β1 integrin (156). Although the precise mechanism of reovirus entry is not known, available evidence suggests that reovirus enters cells by receptor-mediated endocytosis (32, 33, 239). Thin-section electron micrographs demonstrate reovirus particles internalized into electron-dense invaginations that resemble clathrin-coated pits and clathrin-coated vesicles (215). In keeping with these data, experiments using video fluorescence microscopy indicate that reovirus colocalizes with clathrin during viral entry (85). These findings suggest that reovirus internalization is clathrin-dependent, but it is not known whether clathrin is required for the uptake pathway that leads to productive infection.

Reovirus undergoes proteolytic disassembly of the viral outer capsid in the endocytic pathway (33, 49, 228, 239). Studies monitoring reovirus entry in real-time using fluorescently-labeled virions suggest that reovirus collects in endocytic vesicles (99). Treatment of L cells with ammonium chloride, which raises the intracellular pH of endosomes (163) and lysosomes (181), blocks infection by virions but not by ISVPs (239), suggesting that viral disassembly is dependent on acidic pH. Reovirus disassembly in fibroblasts requires acid-dependent cysteine-containing proteases

cathepsins B and L, which mediate removal of σ3 and cleavage of μ1 (77). Cathepsins B and L generally reside in late endosomes or lysosomes (247), providing support for the contention that reovirus disassembly occurs in these endocytic compartments.

Many receptors that mediate uptake of cargo contain amino acid sequences in their cytoplasmic domains that recruit components of the endocytic machinery and regulate sorting to endocytic compartments. Endocytic sorting signals include tyrosine-based motifs such as tyrosine-any residue-any residue-bulky hydrophobic residue (YXX∅) and NPXY or dileucine-based motifs such as aspartic acid-any residue-any residue-leucine-leucine (DXXLL) (31). These linear sequences are recognized by adaptor proteins and clathrin (29, 30, 166, 168, 182). The importance of NPXY motifs was discovered by Brown and Goldstein, who found that a naturally occurring mutation of the tyrosine residue in the NPXY motif of the low-density lipoprotein (LDL) receptor results in decreased internalization of LDL cholesterol. This mutation causes familial hypercholesterolemia, a disorder in which LDL cholesterol levels are elevated leading to premature atherosclerosis (68). The β1 integrin cytoplasmic domain contains two NPXY motifs (209) with tyrosine residues at amino acid positions 783 and 795 (209).

In this study, I performed experiments to determine whether the β1 integrin NPXY motifs are involved in reovirus internalization. I also investigated the transport pathway used by reovirus to reach the compartment used for disassembly. Using β1-/- cells stably expressing β1 integrin with altered NPXY motifs, I found that the β1 integrin NPXY motifs are required for reovirus infection but not for attachment or internalization. Furthermore, I found that reovirus particles enter β1+/+ cells using a clathrin-dependent mechanism and are delivered to vesicles that morphologically resemble early and late

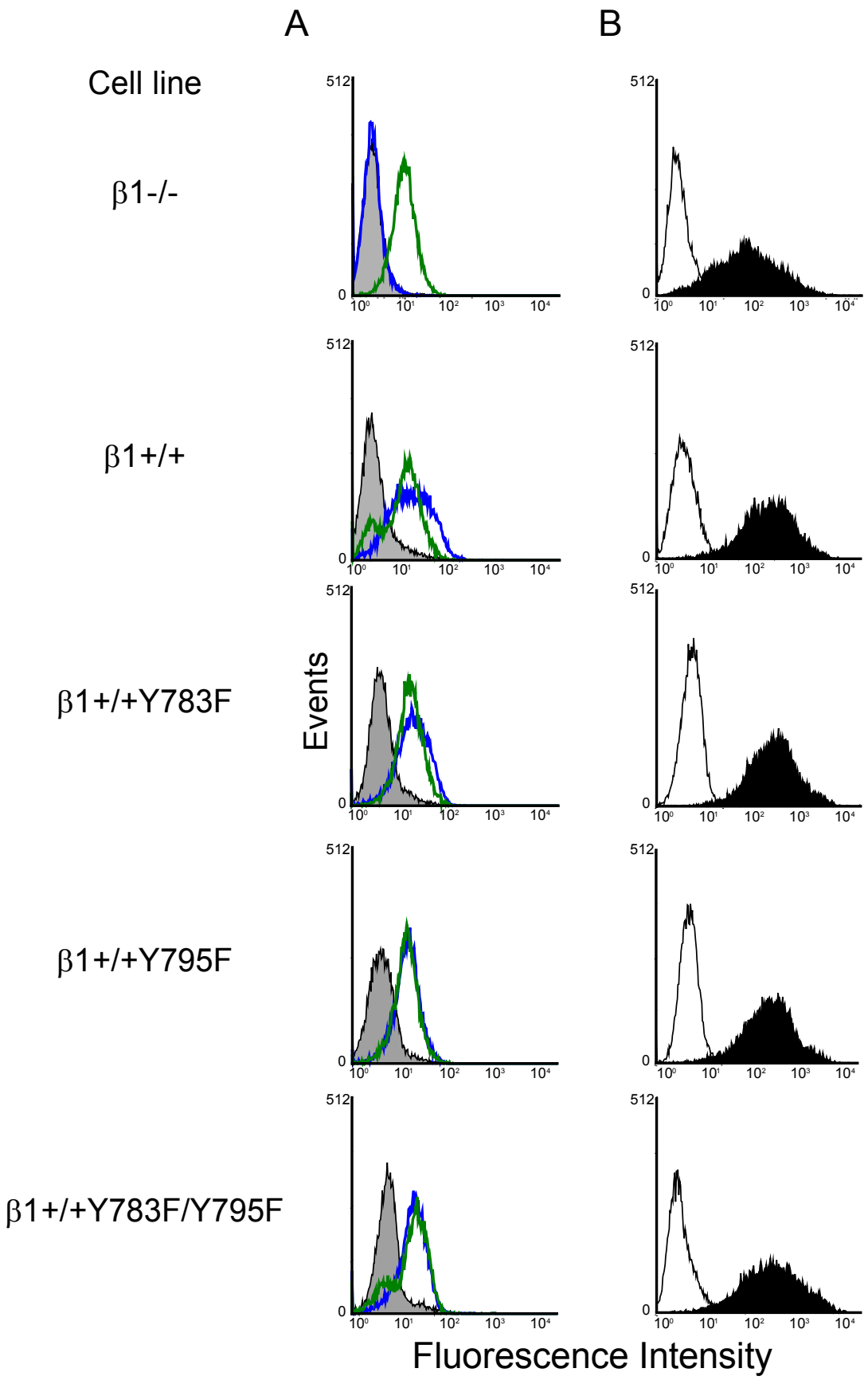
endosomes. In contrast, particles are internalized into cells with altered NPXY motifs using a clathrin-independent process and have an altered intracellular localization. These findings suggest that the β1 integrin NPXY motifs are required for functional reovirus entry and infection.

I acknowledge Elvin Woodruff, Magnify, Inc., Vanderbilt University, for processing and acquisition of electron micrographs (Fig. 23).

Results

Reovirus Infection Is Diminished in Cells with Tyrosine-to-Phenylalanine Mutations in the β1 Integrin NPXY Motifs

To determine whether the NPXY motifs in the cytoplasmic domain of β1 integrin are required for functional reovirus entry, I tested the capacity of reovirus to infect cells with mutations in the β1 integrin NPXY motifs. GD25 (β1-/-) cells are embryonic stem cells derived from β1-null embryos. GD25β1A (β1+/+) cells stably express full-length, wild-type β1 integrin (263). GD25β1AY783F (β1+/+Y783F), GD25β1AY795F (β1+/+Y795F), and GD25β1AY783F/Y795F (β1+/+Y783F/Y795F) are GD25 cells engineered to stably express β1 integrin in which the tyrosine residues of the NPXY motifs at amino acid positions 783 and 795 have been substituted with phenylalanine (262, 263). I first confirmed the cell-surface expression of β1 integrin and JAM-A by β1-/-, β1+/+, β1+/+Y783F, β1+/+Y795F, and β1+/+Y783F/Y795F cells using flow cytometry (Fig. 17A). Each of the cell types tested expressed equivalent levels of β1 integrin, with the exception of β1-/- cells, and all cell types expressed JAM-A. To directly assess reovirus attachment, β1-/-, β1+/+, β1+/+Y783F, β1+/+Y795F, and


A
B
Cell line
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β1+/+
β1+/+Y783F
β1+/+Y795F
β1+/+Y783F/Y795F
Events
Fluorescence Intensity
512
0
10⁰
10¹
10²
10³
10⁴

Fig. 17. Reovirus Exhibits Equivalent Binding to β1-/-, β1+/+, and β1+/+ Cells with Altered NPXY Motifs. (A) GD25 (β1 -/-), GD25β1A (β1 +/+), GD25β1AY783F (β1+/+Y783F), GD25β1AY795F (β1+/+Y795F), and GD25β1AY783F/Y795F (β1+/+Y783F/Y795F) cells were detached from plates with 20 mM EDTA, washed, and incubated with antibodies specific for either murine β1 integrin or murine JAM-A. Cell-surface expression of these molecules was detected by flow cytometry. Data are expressed as fluorescence intensity. **(B)** β1 -/-, β1 +/+, β1+/+Y783F, β1+/+Y795F, and β1+/+Y783F/Y795F cells were detached from plates with 20 mM EDTA, washed, and incubated with 1 x 10^4 particles per cell of reovirus strain T1L at 4°C for 1 h to allow attachment. Cells were washed and then incubated with reovirus specific antiserum or an IgG control antibody. Reovirus binding was detected by flow cytometry. Data are expressed as fluorescence intensity.

β1+/+Y783F/Y795F cells were incubated with reovirus particles, and binding was detected using flow cytometry (Fig. 17B). Reovirus bound equivalently to all cell types, suggesting that reovirus attachment is not affected by mutation of the β1 integrin NPXY motifs to NPXF.

To determine whether mutations in the β1 integrin NPXY motifs alter reovirus infection, β1-/-, β1+/+, β1+/+Y783F, β1+/+Y795F, and β1+/+Y783F/Y795F cells were adsorbed with reovirus virions or ISVPs, and infectivity was scored by indirect immunofluorescence (Fig. 18). In comparison to β1+/+ cells, β1-/-, β1+/+Y783F, β1+/+Y795F, and β1+/+Y783F/Y795F cells were less susceptible to infection by reovirus virions (Fig 18). While reovirus infection was significantly reduced in β1+/+Y783F and β1+/+Y795F cells, infection was most dramatically reduced in β1+/+Y783F/Y795F cells, to an extent even greater than in β1-/- cells (Fig. 18). ISVPs, which bind to JAM-A (15) but bypass a requirement for endocytosis and disassembly (11, 239), were capable of infection in all cell types. Equivalent infection by ISVPs suggests that the block to reovirus infection in cells expressing mutant β1 integrin is at a step subsequent to attachment but preceding disassembly.

Reovirus Virions Are Internalized into the Cytoplasm of β1+/+Y783F/Y795F Cells

To determine whether the block to reovirus infection in β1+/+Y783F/Y795F cells is due to a defect in internalization, β1-/-, β1+/+, and β1+/+Y783F/Y795F cells were adsorbed with reovirus particles at 4°C for 1 h to allow virus binding and then warmed to 37°C to allow internalization over a time course concurrent with reovirus entry. At 15-min intervals, cells were fixed, stained using indirect immunofluorescence, and examined

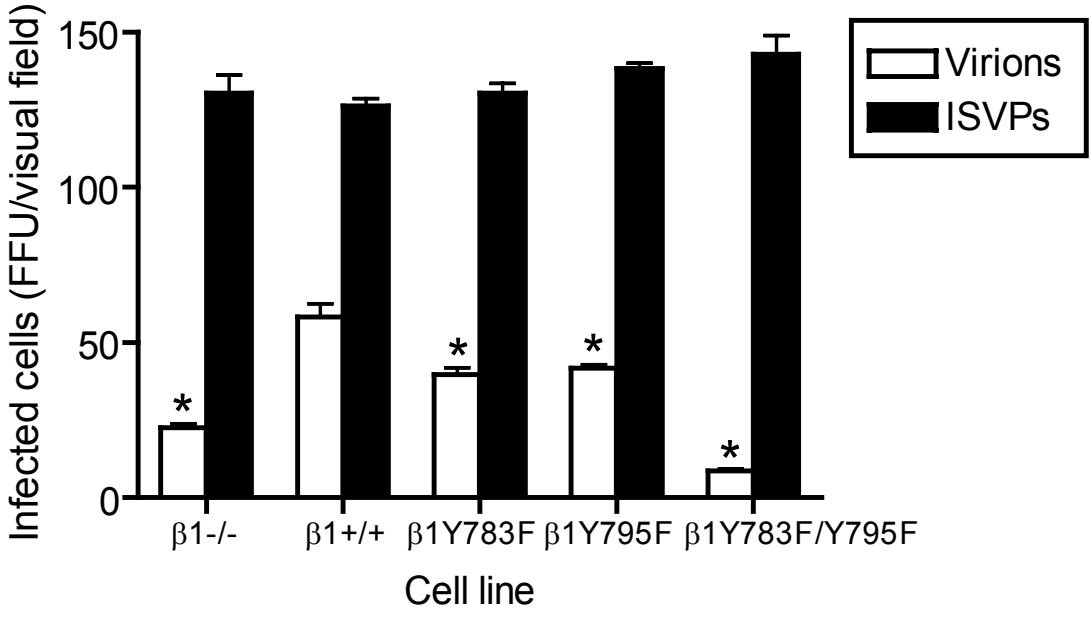

Fig. 18. Reovirus Infection is Diminished in Cells with Altered β1 Integrin NPXY Motifs. β1-/-, β1+/+, β1+/+Y783F, β1+/+Y795F, and β1+/+Y783F/Y795F cells were adsorbed with reovirus virions or ISVPs of strain T1L at an MOI of 1 FFU per cell at 4°C for 1 h. Cells were washed with PBS, incubated in complete medium at 37°C for 20 h, and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in entire wells for triplicate experiments. The results are expressed as the mean FFU per well for triplicate samples. Error bars indicate standard deviations. *, $P < 0.05$ in comparison to β1+/+ cells.

by confocal microscopy. Representative confocal micrographs of β1-/-, β1+/+, and β1+/+Y783F/Y795F cells infected with reovirus and fixed at 30 min post-adsorption are shown in Fig. 19A. Reovirus particles were observed in the cytoplasm of all cell types, with a notable decrease in β1-/- cells, as observed previously (Chapter II). Surprisingly, reovirus particles were internalized into both β1+/+ and β1+/+Y783F/Y795F cells (Fig. 19A). To determine whether the reovirus particles in β1+/+Y783F/Y795F cells were uniformly distributed, I collected multiple confocal micrographs in the Z-plane and examined them using Z-stack analysis and 3D image reconstructions (data not shown). This experiment indicated that reovirus particles were distributed throughout the non-nuclear compartment of β1+/+Y783F/Y795F cells in a pattern similar to β1+/+ cells (data not shown). To directly quantify the fluorescence intensity of internalized reovirus particles, confocal micrographs of images collected over a time course were analyzed using Metamorph software (Fig. 19B). The average pixel intensity representing reovirus particles was equivalent in β1+/+ and β1+/+Y783F/Y795F cells, while pixel intensity was significantly less in β1-/- cells, suggesting that an equivalent number of particles are internalized into β1+/+ and β1+/+Y783F/Y795F cells.

Viral Particles Internalized into β1+/+Y783F/Y795F Cells Are Not Functional

Although reovirus particles are internalized into β1+/+Y783F/Y795F (Fig. 19), these cells do not become infected (Fig. 18). This finding suggests that productive reovirus internalization and endocytic trafficking requires intact NPXY motifs. To determine whether input particles are capable of initiating transcription, cells were adsorbed with reovirus virions and incubated for 3.5 h. RNA was purified from infected

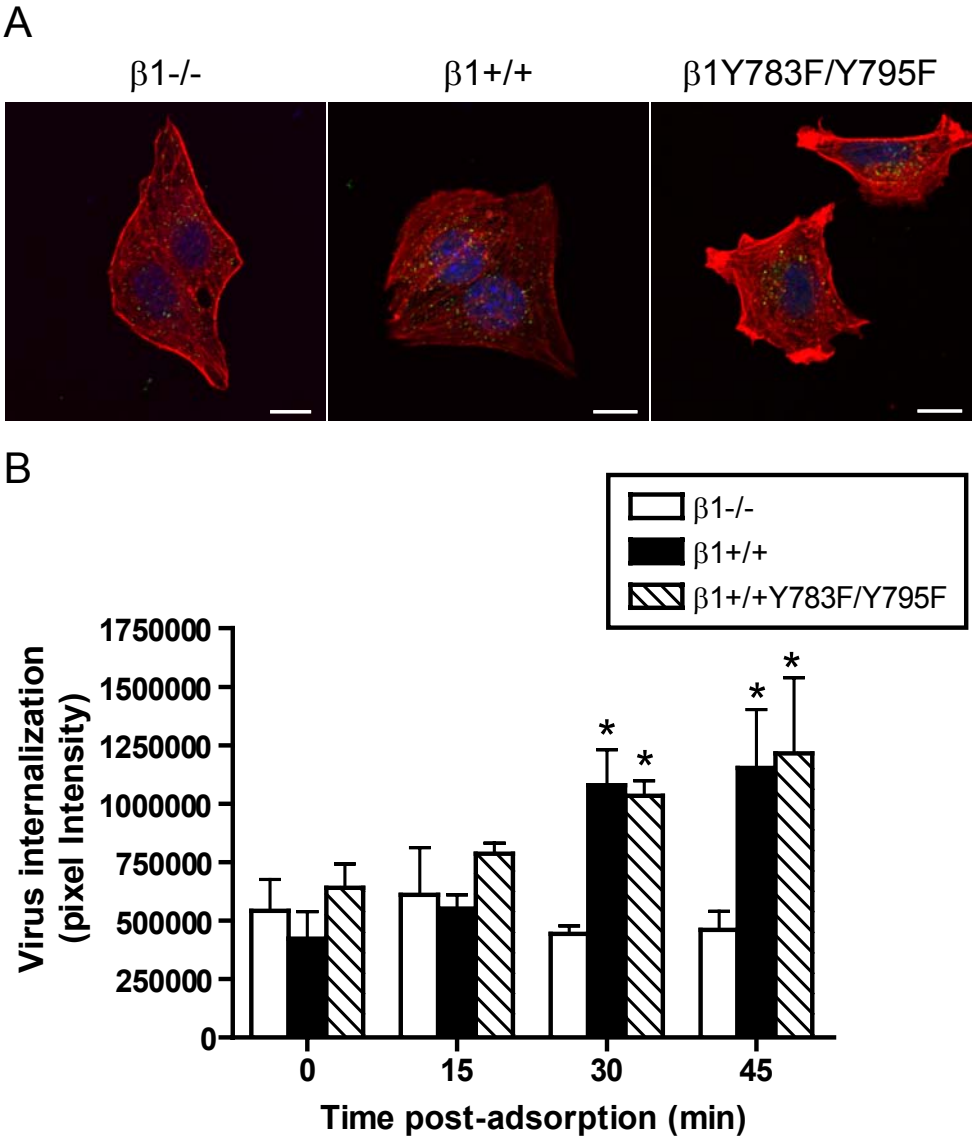

Fig. 19. Reovirus Is Internalized into β1+/+ and β1+/+Y783F/Y795F Cells. β1-/-, β1+/+, β1+/+Y783F/Y795F cells were adsorbed with T1L virions, and incubated at 4°C for 1 h. Non-adherent virus was removed, warm medium was added, and cells were incubated at 37°C. Cells were fixed, stained for reovirus (green), actin (red), and DNA (blue), and imaged using confocal immunofluorescence microscopy. Images were collected on a Zeiss LSM510 microscope with Meta software. **(A)** Representative digital fluorescence images of cells fixed at 30 min post adsorption are shown. Scale bars, 10μm. **(B)** Reovirus internalization was quantitated by Metamorph software. Fluorescent particles localized at the cell periphery were excluded from analysis and particles internalized into the cytoplasm were analyzed to determine the average pixel intensity of fluorescent particles per cell and multiplied by cell area for total pixel intensity. The results are expressed as pixel intensity for 5 cells at each time point. *, $P < 0.05$ in comparison to β1 -/- cells.

cells and used as template for reverse transcriptase-polymerase chain reaction (RT-PCR) with primers specific for β-actin (control) or the reovirus L1 gene. Resultant RT-PCR products were subjected to agarose gel electrophoresis and visualized following ethidium bromide staining. In comparison to β1-/- cells, amplicon band intensity was greater in both β1+/+ and β1+/+Y783F/Y795F cells (Fig. 20). To determine whether the viral RNA detected by RT-PCR represents viral genomic dsRNA or newly synthesized viral mRNA, cells were infected in the presence and absence of ribavirin, which blocks viral transcription (206) (Fig. 20). Reovirus RNA levels were diminished in ribavirin-treated β1+/+ cells, suggesting that the RNA detected is newly synthesized viral RNA. However, ribavirin treatment did not diminish reovirus RNA levels in β1+/+Y783F/Y795F cells, suggesting that the RNA detected represents an accumulation of genomic dsRNA from input viral particles that do not undergo disassembly. These data suggest that decreased reovirus infectivity in β1+/+Y783F/Y795F cells results from a block in the replication cycle prior to viral transcription.

Reovirus Uptake into β1+/+ Cells is Clathrin-Dependent

To determine whether clathrin-mediated endocytosis is required for functional reovirus entry, β1+/+ cells were treated with chlorpromazine, a pharmacologic agent that inhibits clathrin assembly at the cell surface (258), and infected with reovirus virions or ISVPs (Fig. 21). Chlorpromazine treatment blocked infection by virions but not by ISVPs, suggesting that chlorpromazine affects a step in reovirus infection that occurs after attachment but before disassembly. Failure of chlorpromazine to inhibit infection by ISVPs also indicates that the concentration of chlorpromazine does not affect

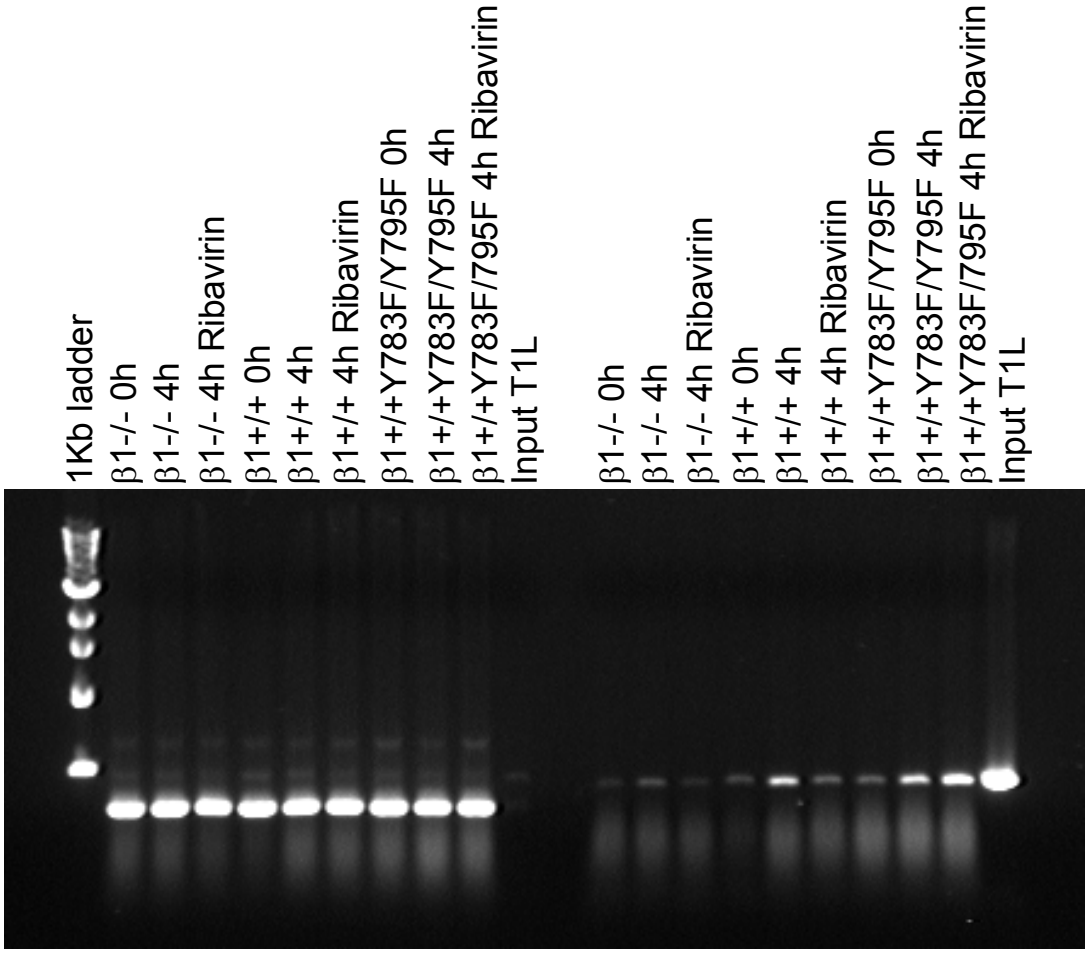

Fig. 20. Virus Particles Internalized into β1+/+Y783F/Y795F Cells Are Not Functional.

β1-/-, β1+/+, β1+/+Y783F/Y795F cells were plated in 60 mm dishes, infected with reovirus virions of strain T1L, incubated at 4°C for 1 h, washed, and then complete medium with or without ribavirin (200μM) was added and cells were incubated at 37°C for 0 or 3.5 h. Cells were washed, harvested, and pelleted. RNA was isolated from cell pellets and used as template for RT-PCR using primers specific for β-actin (control) (left) or reovirus L1 gene (right). RT-PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide. A representative agarose gel is shown. β-actin transcripts are shown on the left and reovirus transcripts are shown on the right.

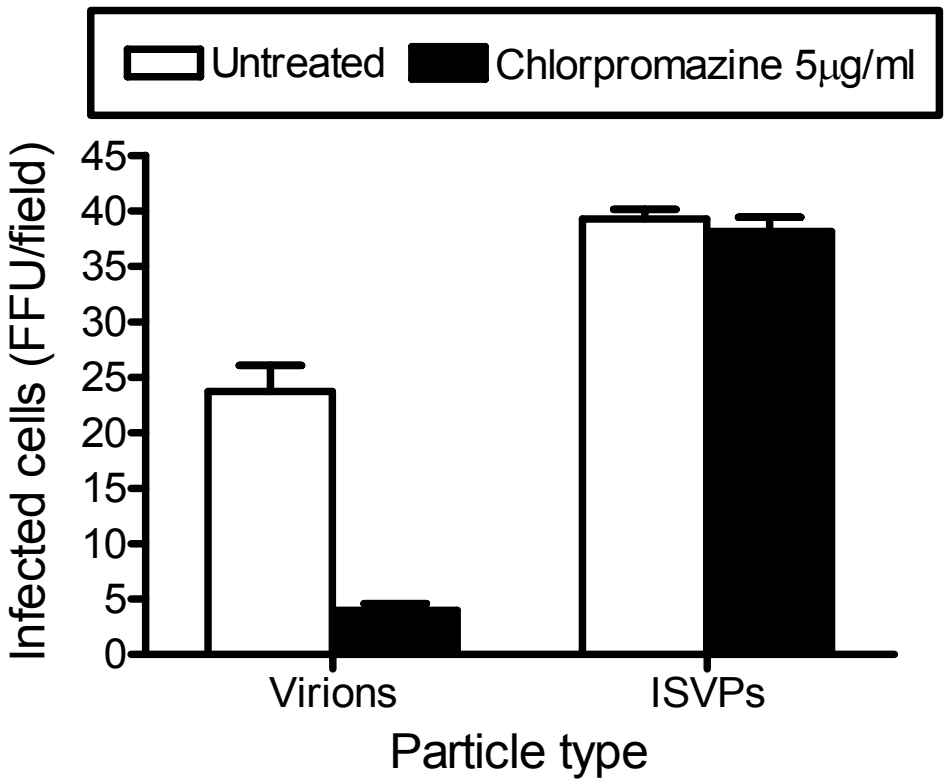

Fig. 21. Reovirus Uptake into β1+/+ Cells Is Clathrin Dependent. β1 +/+ cells were pre-treated with 5μg/mL of chlorpromazine for 3 h, infected with reovirus virions or ISVPs of strain T1L and incubated at 4°C for 1 h, washed, and complete medium with or without chlorpromazine was added, and cells were incubated at 37°C for 20 h. Cells were fixed and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in entire wells for triplicate experiments. The results are expressed as the mean FFU per well for triplicate samples. Error bars indicate standard deviations. *, $P < 0.05$ in comparison to untreated cells.

the capacity of cells to support the reovirus replication program.

The β1 Integrin NPXY Motifs Are Required for Functional Reovirus Uptake

To determine the role of the β1 integrin NPXY motifs in clathrin-dependent reovirus endocytosis, β1+/+ and β1+/+Y783F/Y795F cells were either treated with chlorpromazine or left untreated, infected with reovirus particles at 4°C for 1 h to allow attachment, and then warmed to 37°C to allow internalization. At 0 and 20 min post-adsorption, cells were fixed, stained using indirect immunofluorescence, and examined by confocal microscopy. Representative confocal micrographs of β1+/+ and β1+/+Y783F/Y795F cells at 20 min post-infection are shown in Fig. 22A. The fluorescence intensity of reovirus particles internalized into β1+/+ and β1+/+Y783F/Y795F cells was quantified by averaging the pixel intensity for representative confocal micrograph images using Metamorph software (Fig. 22B). In β1+/+ cells treated with chlorpromazine, the average pixel intensity representing reovirus particles was significantly diminished in comparison to untreated β1+/+ cells (Fig. 22B), consistent with the infectivity data. However, chlorpromazine treatment did not diminish the pixel intensity in β1+/+Y783F/Y795F cells in comparison to untreated cells, indicating that reovirus internalization into β1+/+Y783F/Y795F cells is not affected by chlorpromazine (Fig. 22B). However, chlorpromazine treatment altered the intracellular distribution of reovirus particles in β1+/+Y783F/Y795F cells (Fig. 22A). Thus, the reovirus uptake pathway in β1+/+ cells is chlorpromazine-sensitive, most likely clathrin-dependent endocytosis, while reovirus internalization into β1+/+Y783F/Y795F cells is

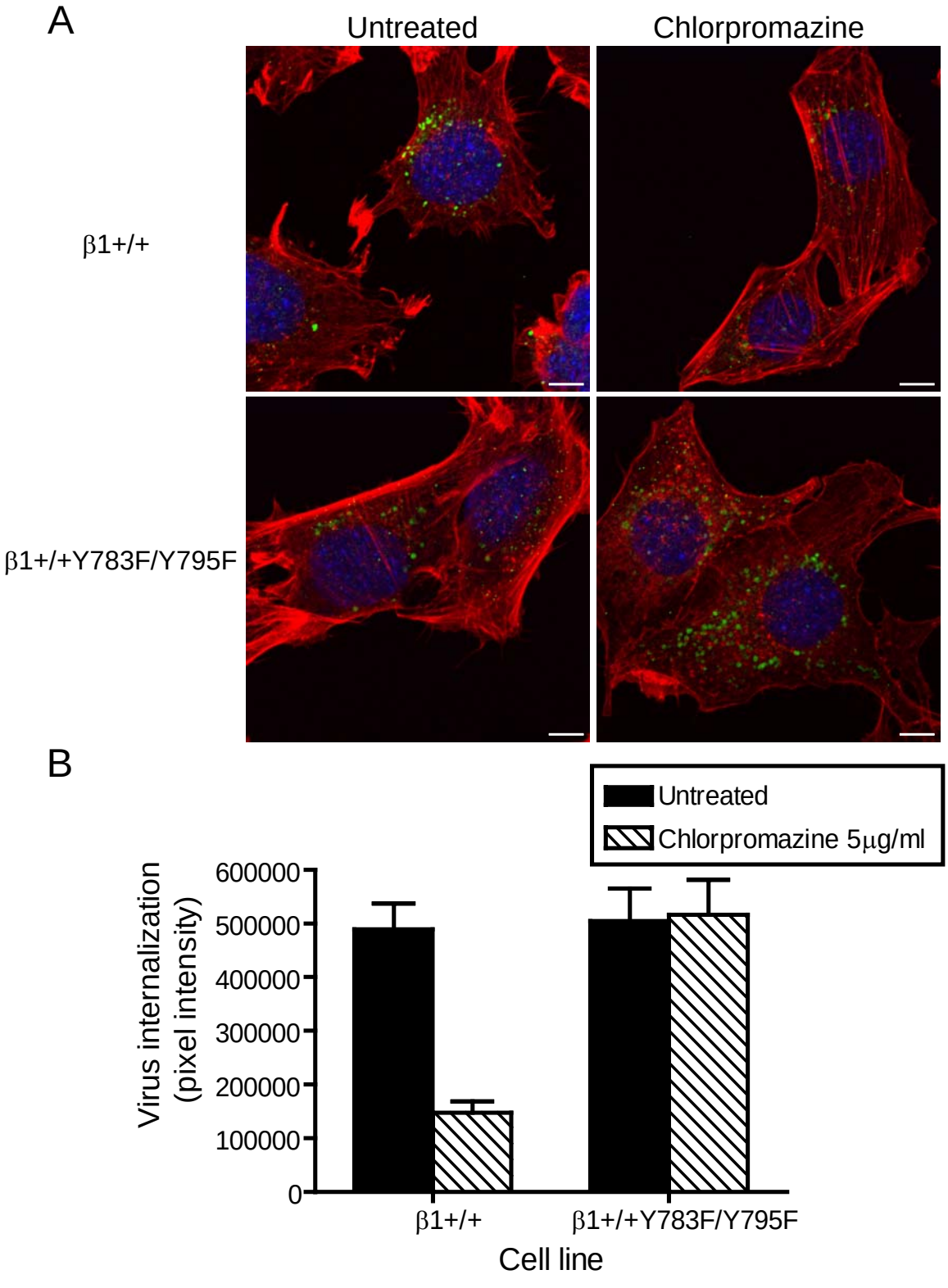
A
Untreated
Chlorpromazine
β1+/+
β1+/+Y783F/Y795F
B
Untreated
Chlorpromazine 5μg/ml
Virus internalization (pixel intensity)
600000
500000
400000
300000
200000
100000
0
β1+/+
β1+/+Y783F/Y795F
Cell line

Fig. 22. β1 Integrin NPXY Motifs Are Required for Functional Reovirus Internalization. β1 +/+ and β1+/+Y783F/Y795F cells were pre-treated with 5μg/mL of chlorpromazine for 3 h, infected with reovirus virions of strain T1L and incubated at 4°C for 1 h, washed, and complete medium with or without chlorpromazine was added, and cells were incubated at 37°C for 0 or 20 min. Cells were fixed and stained by indirect immunofluorescence and analyzed by confocal microscopy. Images were collected on a Zeiss LSM510 microscope with Meta software. **(A)** Representative confocal micrographs of untreated and chlorpromazine-treated cells at 20 min post adsorption are shown. Scale bars, 10μm. **(B)** Reovirus internalization into untreated and chlorpromazine-treated cells at 20 min post adsorption was quantitated by Metamorph software. Fluorescent particles localized at the cell periphery were excluded from analysis and particles internalized into the cytoplasm were analyzed to determine the average pixel intensity of fluorescent particles per cell and multiplied by cell area for total pixel intensity. The results are expressed as pixel intensity for 10 cells at each time point. *, $P < 0.05$ in comparison to untreated cells.

mediated by an alternative uptake mechanism that does not give rise to infectious progeny.

Intracellular Trafficking Pathway of Reovirus Virions

To define the fate of reovirus virions in the endocytic compartment during internalization and disassembly, I performed an ultrastructural analysis of reovirus-infected cells. β1-/-, β1+/+, and β1+/+Y783F/Y795F cells were infected with reovirus, incubated at 37°C for 10-min intervals for 30 min, and processed for electron microscopy. Fewer reovirus particles appeared within β1-/- cells in comparison to β1+/+ and β1+/+Y783F/Y795F cells. In β1+/+ cells, and to a much lesser extent in β1-/- cells, a few reovirus particles were localized in coated-pit structures, while most particles appeared in structures that morphologically resemble early and late endosomes and lysosomes (Fig. 23). In β1+/+Y783F/Y795F cells, reovirus particles were observed in coated-pit structures, yet few particles were observed in structures that resemble endosomes. Instead, the majority of reovirus particles in β1+/+Y783F/Y795F cells appeared to coalesce in dark vesicular structures of unknown origin (Fig. 23). These structures were found in all cell types, yet there appeared to be a greater number of these structures in the β1+/+Y783F/Y795F cells (Fig. 23). While the nature of these cellular organelles is unclear, they do not appear to support reovirus disassembly.

Discussion

The goal of this study was to gain a better understanding of mechanisms by which β1 integrin mediates reovirus internalization. The data demonstrate a function for the β1

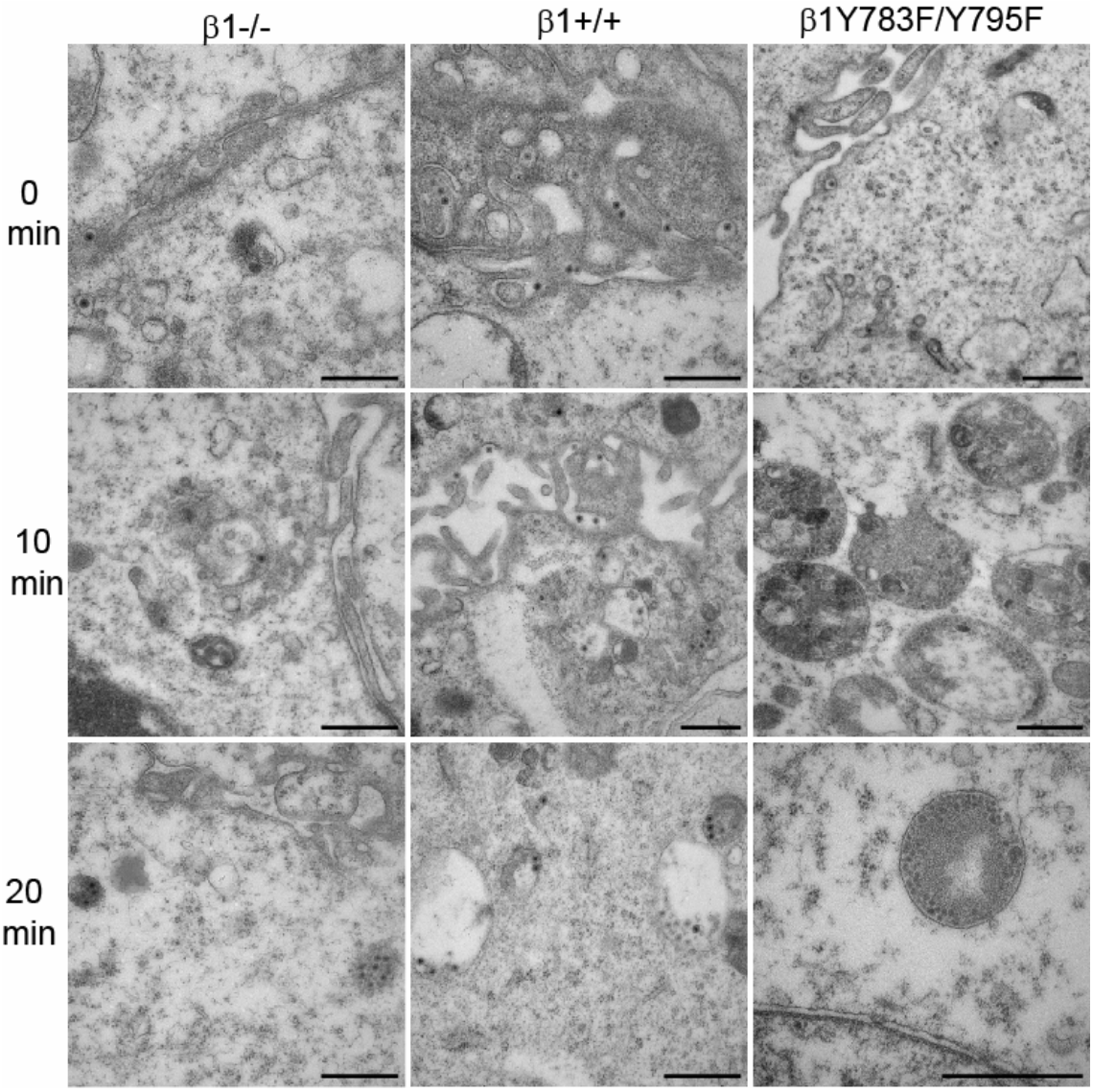

Fig. 23. Intracellular Trafficking of Reovirus Particles Is Altered in β1+/+Y783F/Y795F Cells. β1-/-, β1+/+ and β1+/+Y783F/Y795F cells were infected with $1x10^5$ particles of reovirus T1L per cell at 4°C for 1 h, washed, and fixed or incubated in complete medium at 37°C. At 10-min intervals cells were washed, pelleted, and fixed. Cells were processed for electron microscopy and images were captured on a Phillips CM10 Transmission Electron Microscope equipped with an AMT 2 mega pixel camera. Representative EM images at 0, 10, and 20 min post infection are shown. Reovirus Particles are ~90nm, with electron dense centers. Scale bars, 500nm.

integrin NPXY motifs in reovirus endocytosis and endocytic transport. Tyrosine-to-phenylalanine mutations of the β1 integrin NPXY motifs lead to an aberrant pathway of reovirus internalization that does not yield infectious particles. Using EM analysis, I found that reovirus virions are internalized into β1+/+Y783F/Y795F cells yet localize in an undefined cellular compartment, rather than in endosomes and lysosomes as they do in β1+/+ cells. Reovirus internalization into β1+/+ cells is mediated by a chlorpromazine-sensitive pathway, most likely clathrin-dependent endocytosis, yet particles internalize into β1+/+Y783F/Y795F cells by a different route. Collectively, these data indicate that β1 integrin serves to deliver reovirus to the proper endocytic compartment required for subsequent steps in the viral life cycle. A model of β1 integrin-mediated reovirus internalization is shown in Fig. 24.

NPXY motifs in the cytoplasmic domain of the LDL receptor (55, 68) and β integrin subunits (31) function in clathrin-dependent endocytosis. NPXY motifs recruit clathrin or adaptor proteins such as AP-2 (30) or Dab2 (168, 183) to the cell surface, which leads to accumulation and assembly of clathrin at the plasma membrane (183). NPXY motifs can recruit AP-2 by directly interacting with the AP-2 μ2 subunit, which selects proper cargo for endocytosis (30). The AP-2 β2 subunit binds clathrin subunits (187) and initiates clathrin assembly at the plasma membrane, resulting in clathrin-mediated endocytosis. NPXY motifs also can recruit Dab2 (168, 183), which directly interacts with NPXY motifs via the Dab2 PTB domain (166). Dab2 can induce clathrin-mediated endocytosis by either directly binding to clathrin (166) or binding to AP-2 (169). Therefore, it is possible that mutation of the β1 integrin NPXY motifs results in

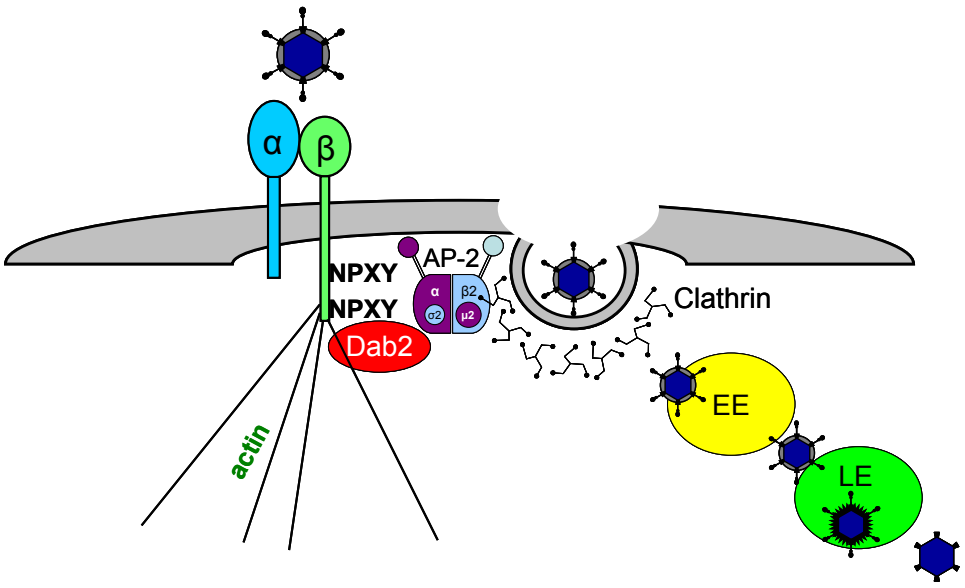

Fig. 24. Model of β1 Integrin-Mediated Reovirus Internalization. Reovirus activates β1 integrin, which contains NPXY motifs in the cytoplasmic domain. The NPXY motifs are required for functional reovirus infection. NPXY motifs could mediate reovirus internalization by interaction with adaptor proteins, AP-2 or Dab2, which could recruit clathrin the plasma membrane, leading to reovirus endocytosis. NPXY motifs are also required for reovirus trafficking in the endocytic pathway to endosomes and lysosomes where reovirus disassembly is thought to occur.

failure of the β1 integrin cytoplasmic domain to recruit the proper endocytic machinery for clathrin-dependent reovirus uptake.

NPXY motifs also serve as cargo recognition motifs, which direct the delivery of cargo to endosomes and lysosomes (31, 166). Thus, mutation of the β1 integrin NPXY motifs also may lead to improper delivery of reovirus to a functional endocytic compartment independent of an alteration in clathrin-dependent uptake. In support of this possibility, Dab2 recognizes non-phosphorylated tyrosines of the NPXY motif (166, 168). Thus, in β1+/+Y783F/Y795F-infected cells, Dab2 could potentially engage β1 integrin with NPXY-to-F mutations and mediate reovirus internalization but direct particles to a non-functional endocytic compartment. Reovirus infection requires acid-dependent proteolytic disassembly (11, 77, 239). Thus, delivery to an intracellular site other than an endosomal compartment containing the appropriate pH (239) and enzymes (77) could result in failure of reovirus particles to uncoat.

In addition to the role of NPXY motifs in diseases such as familial hypercholesterolemia, naturally occurring mutations in adaptor proteins that engage NPXY motifs also have been associated with certain pathological states. For example, autosomal recessive hypercholesterolemia (ARH) is a rare disorder in which LDL is not effectively cleared from the bloodstream (108). LDL is normally removed by the liver via binding to LDL receptors. Bound LDL is rapidly internalized by clathrin-dependent endocytosis (6, 7, 40, 101) through interactions of Dab2 with the LDL NPXY motif, which leads to recruitment of AP-2 (168). Although symptoms of ARH parallel those in familial hypercholesterolemia, the LDL receptors in ARH patients are normal (108). Instead, ARH is caused by mutations in a gene encoding an adaptor protein called ARH

(97). ARH incorporates a PTB domain that directly engages the NPXY motif in the LDL receptor cytoplasmic domain and binds to the AP-2 β2 subunit and clathrin heavy chain to induce clathrin-mediated endocytosis of the LDL receptor following binding by LDL (98, 112, 167). Mutations in ARH that affect binding to the NPXY motif of the LDL receptor or interactions with AP-2 or clathrin result in inefficient clearance of LDL and elevated serum cholesterol (98).

Substitution of the β1 integrin NPXY-motif tyrosines with phenylalanines, which cannot be phosphorylated, results in reovirus delivery to a cellular compartment that does not support infection. This finding raises the possibility that reovirus interactions with β1 integrin lead to tyrosine phosphorylation of the NPXY motifs. While it has been challenging to demonstrate β1 integrin tyrosine phosphorylation, v-src transformation of β1+/+ but not β1+/+Y783F/Y795F cells results in phosphorylation of β1 integrin (218), suggesting that NPXY tyrosines can serve as targets for phosphorylation. It is possible that phosphorylation of the β1 integrin NPXY motifs is required for downstream signaling events required for reovirus endocytosis. For example, phosphorylation of the NPXY tyrosine residues is required for autophosphorylation of FAK (261). Thus, it is possible that reovirus-β1 integrin interactions induce phosphorylation and activation of integrin-linked signaling pathways.

Studies presented in this chapter identify residues in the β1 integrin cytoplasmic domain that function in reovirus endocytosis and delivery of internalized virions to the endocytic compartment where disassembly occurs. Furthermore, these findings describe how a nonenveloped virus usurps the normal physiologic function of integrins to mediate internalization. Integrins are required for internalization of many pathogenic

microorganisms (24, 89, 114, 119, 175, 211, 265). Therefore, understanding mechanisms by which integrins engage endocytic machinery to mediate endocytosis of microbes provides a framework for the rational design of antiviral drugs that target pathogen internalization via integrin-linked pathways.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Introduction

The virus life cycle begins with viral attachment to cellular receptors and internalization into host cells. Virus attachment and entry mechanisms can be cell type specific and, thus, these early replication events can influence viral tissue tropism and pathogenesis. Although progress has been made in understanding virus receptor selection and engagement, little is known about mechanisms by which nonenveloped viruses enter cells. An understanding of virus cell entry is important for development of molecular models of virus tropism and design of antiviral therapeutics to inhibit viral entry. The goal of my dissertation research was to identify the receptor responsible for mediating reovirus internalization and to define mechanisms by which reovirus enters cells. Findings presented in this thesis provide evidence that β1 integrin mediates reovirus internalization (Chapter II) and that β1 integrin cytoplasmic domain NPXY motifs are required for internalization of reovirus to a functional endosomal compartment (Chapter III). This chapter summarizes the findings presented herein and provides insights into future directions for this research.

The Role of β1 Integrin in Reovirus Internalization

Data presented in Chapter II provide evidence that reovirus utilizes β1 integrin to mediate internalization. However, it remains to be determined how β1 integrin serves

this function. The presence of integrin-binding motifs RGD and KGE in the reovirus λ2 protein suggests that reovirus directly engages integrins. I envision that following reovirus binding to JAM-A on the cell surface, the virus undergoes conformational changes that allow engagement of β1 integrins. In support of this model, the σ1 attachment protein binds to the D1 domain of JAM-A with a K_D of 10^{-8}M (15, Guglielmi, K.M. and Dermody, T.S., submitted). The σ1 protein contains a "hinge" region in the fibrous tail below the head that introduces substantial flexibility between head and tail (53). After σ1 binds to JAM-A, conformational changes in σ1 may allow movement of the virion about the surface of the cell. Such movement may position the virus so that the λ2 protein, which contains surface-exposed RGD and KGE motifs (208), could engage β1 integrins, presumably via the I-like domain (205, 246). Future directions described here could be used to determine whether and how reovirus engages β1 integrin.

To test whether λ2 RGD and KGE motifs are involved in integrin interactions, the newly-developed, plasmid-based, reverse genetics system (135) should be employed to generate mutant viruses with alterations in these sequences. These viruses should be compared to wild-type viruses for cell attachment by flow cytometry, entry by confocal microscopy, and infectivity by FFU assay. Furthermore, ISVPs of both wild-type and mutant viruses, generated by treatment of virions with protease in vitro, should be used as positive controls for infection. I anticipate that altering the RGD or KGE motifs in λ2 will reduce entry and infection by virions but not by ISVPs. Although the presence of RGD and KGE motifs in λ2 provide rationale for studies of reovirus-integrin interactions, it is important to consider the possibility that reovirus engages integrins in an RGD- and KGE-independent manner.

It is reasonable to hypothesize that reovirus directly engages the β1 integrin I-like domain. To test this hypothesis, virions should be tested for the capacity to bind to recombinant β1 integrin using surface plasmon resonance (SPR). Recombinant integrin heterodimers, α5β1 and α3β1 are commercially available and functional (R&D Systems Inc., Minneapolis, MN). Integrin heterodimers should be captured on the surface of a biosensor chip (15), and purified virions of wild-type or RGD and KGE mutants, in a range of concentrations, should be injected across the biosensor surface to measure association and dissociation rates. These experiments will provide a detailed assessment of interaction kinetics and affinity and permit direct comparison of reovirus-β1 integrin binding interactions between wild-type and mutant virus strains. I anticipate that wild-type virions will directly bind to integrin heterodimers, and mutant viruses may not bind to integrins or bind with diminished affinity. If reovirus binding to β1 integrin heterodimers is detected, then studies should be performed to determine if reovirus directly engages the β1 integrin I-like domain. Recombinant β1 integrin I-like domain should be expressed, purified, and subjected to site-directed mutagenesis to determine the residues required for reovirus binding. The capacity of reovirus to bind mutant β1 integrin I-like domains can be tested using SPR, and infectivity can be tested using β1-/- cells expressing wild-type β1 and β1 integrin with altered I-like domain sequences. These experiments will identify residues required for reovirus-β1 integrin engagement.

While I find it most likely that reovirus directly engages β1 integrin, it is entirely possible that reovirus engages an α subunit. There are a number of explanations for the finding that α integrin-specific antibodies did not inhibit reovirus infection, most of which are described in Chapter II. I did not test a complete panel of α integrin-specific

antibodies. Therefore, I think a very careful characterization of a role for an integrin α subunit in reovirus infection should be performed using antibodies to additional α subunits capable of pairing with β1, as well as antibodies that recognize different epitopes in integrin α subunits.

Although direct engagement of integrins by reovirus seems to be the most likely scenario, it remains possible that reovirus does not directly bind to integrins. Instead, reovirus binding to JAM-A may trigger interactions between JAM-A and integrins to mediate internalization. In support of this hypothesis, various JAM family members can interact with integrins in a number of cellular processes (174, 184, 220). Interestingly, JAM-A has been reported to regulate the cell-surface expression of β1 integrin (157). To test whether β1 integrin and JAM-A directly interact during reovirus entry, cells should be infected, harvested over a time course of reovirus entry, and subjected to immunoprecipitation for JAM-A, followed by immunoblotting for β1 integrin. To determine whether JAM-A and β1 integrin interact through extracellular, transmembrane, or cytoplasmic regions, chimeric molecules of JAM-A and CAR should be generated and expressed in CHO cells (204) with wild-type β1 integrin or chimeric molecules comprised of β1 integrin with another β integrin subunit. These studies would provide evidence that β1 integrin mediates reovirus entry by a mechanism involving JAM-A.

The Role of the β1 Integrin Cytoplasmic Domain in Reovirus Internalization

The NPXY motifs of the β1 integrin cytoplasmic domain are required for reovirus infection. Cells expressing NPXY-to-NPXF β1 mutants do not support infection by reovirus virions but do so by ISVPs (Chapter III). Reovirus is internalized into cells

expressing β1 integrin NPXF mutants, but the particles are delivered to a compartment that is non-functional for reovirus infection (Chapter III). This finding indicates that β1 integrin NPXY motifs regulate reovirus delivery via a clathrin-dependent endocytic pathway.

Adaptor proteins function in clathrin-dependent endocytosis to recognize the cytoplasmic tails of receptors and mediate clathrin assembly at the plasma membrane. While there are multiple adaptor proteins that may function in reovirus internalization, it is reasonable to hypothesize that AP-2 or Dab-2 mediate reovirus internalization based on their known interactions with NPXY motifs (166, 168). To determine whether β1 integrin is linked to clathrin and whether adaptor proteins are required for reovirus uptake, β1+/+ and β1+/+Y783F/Y795F cells should be treated with siRNAs corresponding to murine AP-2, Dab2, or clathrin (Dharmacon, Chicago, IL) to inhibit their endogenous expression. siRNA-treated cells should be infected with reovirus virions and ISVPs, and reovirus entry should be analyzed by confocal microscopy and infectivity by FFU assay (156). I anticipate that cells treated with AP-2, Dab2, or clathrin siRNA will exhibit a decrease in internalized reovirus particles. To determine whether β1 integrin colocalizes with clathrin and adaptor proteins during reovirus internalization, confocal microscopic analysis should be performed. β1+/+ and β1+/+Y783F/Y795F cells should be infected with reovirus, fixed over a time course of reovirus entry, and stained with antibodies specific for reovirus, β1 integrin, AP-2, Dab2, and clathrin. Images should be analyzed for the internalization of reovirus particles and colocalization of reovirus and β1 integrin with AP-2, Dab2, and clathrin using Metamorph software. To determine whether reovirus uptake leads to direct association of β1 integrin with

endocytic adaptor proteins, biochemical techniques should be employed. Cells should be infected with reovirus, harvested over a time course, and subjected to immunoprecipitation using a β1 integrin-specific antibody. Resultant complexes should be resolved by SDS-PAGE and immunoblotted using AP-2- and Dab2-specific antibodies. These experiments, with the utilization of the β1+/+Y783F/Y795F cells as a control, will define the function of the NPXY motifs in the recruitment of cellular machinery required for clathrin-mediated reovirus uptake.

Tyrosine-to-phenylalanine mutations of the β1 integrin NPXY motifs have unfavorable effects on reovirus infection. This finding suggests that tyrosine phosphorylation of β1 integrin or downstream integrin-linked tyrosine kinases function in reovirus entry. To determine whether tyrosine phosphorylation is required for reovirus uptake and endocytic transport, β1+/+ and β1+/+Y783F/Y795F cells should be pretreated with the tyrosine phosphorylation inhibitors, genistein (3) and herbimycin A (130), and infected with reovirus virions and ISVPs. Viral entry should be analyzed by confocal microscopy and infection by indirect immunofluorescence (156). Based on my preliminary results (data not shown), I expect that there will be a decrease in reovirus internalization in the presence of tyrosine phosphorylation inhibitors. To determine whether β1 integrin is phosphorylated during reovirus entry, β1+/+ and β1+/+Y783F/Y795F cells should be incubated with [^{32}P] orthophosphate, infected with virions and ISVPs, and lysed over a time course. Lysates should be used for immunoprecipitation with a β1 integrin-specific antibody and subjected to SDS-PAGE (44). These experiments will define whether tyrosine residues of β1 integrin NPXY motifs are phosphorylated during reovirus entry. If tyrosine phosphorylation inhibitors

lead to a decrease in reovirus infection, or if β1 integrin is phosphorylated, experiments should be performed to test whether integrin-linked proteins expressed in focal adhesions, such as α-actinin (185) and talin (199), and signaling molecules, such as paxillin, FAK (222) and ILK (107) are activated during reovirus entry. β1+/+ and β1+/+Y783F/Y795F cells should be infected, harvested over a time course, subjected to immunoprecipitation with a β1 integrin-specific antibody, resolved by SDS-PAGE, and immunoblotted with antibodies specific for α-actinin, talin, paxillin, FAK, or ILK.

Alternatively, alteration of the β1 integrin NPXY motifs may affect reovirus infection indirectly. The NPXY motifs are separated only by eight residues, and the structure of NPXY results in a β turn in solution (12, 252), which can serve as docking sites for PTBs (245). Furthermore, integrin-associated proteins that bind to NPXY motifs including talin (42) and integrin cytoplasmic domain-associated protein (ICAP)1-α (50) may play a role in reovirus infection. In addition, two of the eight intervening residues are threonines at positions 788 and 789, and T788 is essential for integrin-ligand engagement and activation (179). Therefore, it is possible that mutation of the NPXY motifs results in structural constraints that affect the β1 cytoplasmic tail and therefore fail to support reovirus infection.

Based on data presented in Chapter II (Fig. 14) and Chapter III (Fig. 19), it is possible that reovirus entry induces actin remodeling. Many viruses stimulate actin remodeling to allow viral entry, transport, or egress from cells (65). For example, vaccinia virus causes actin cytoskeleton rearrangement during entry and actin polymerization for cell-to-cell spread (63, 64). Additionally, human cytomegalovirus, which utilizes α2β1 and α6β1 integrins to mediate viral entry (89), induces actin

depolymerization at 10-20 min post-infection, which is required for viral entry and infectivity (128). These findings suggest that integrin-engaging viruses take advantage of the capacity of integrins to induce cytoskeletal rearrangement for viral internalization and infection of target cells.

To determine whether actin remodeling is required for reovirus entry, β1+/+ and β1+/+Y783F/Y795F cells should be treated with latrunculin A, which disrupts microfilament-mediated processes (172), and jasplakinolide, which stabilizes actin polymers (41), and analyzed for reovirus entry by confocal microscopy. Preliminary data suggest that treatment of cells with latrunculin A leads to a decrease in reovirus infectivity as measured by EIA (data not shown). Furthermore, to determine whether reovirus travels along the actin cytoskeleton to reach cellular endosomes, β1+/+ and β1+/+Y783F/Y795F cells should be transfected with a plasmid expressing EGFP-actin and infected with Alexa 546-labeled reovirus virions. Reovirus entry should be monitored in real time by confocal microscopy. Collectively, these studies should elucidate a requirement for β1-induced signaling and actin remodeling in reovirus entry and provide a better understanding of the role of β1 integrin in endocytosis.

The Role of β1 Integrin in Reovirus Pathogenesis

Receptor engagement plays a key role in viral pathogenesis. Differences in reovirus tropism in the CNS segregate with the σ1-encoding S1 gene segment (250, 260). However, differences in reovirus tropism cannot be fully explained by utilization of JAM-A, which is a serotype-independent receptor (43). Furthermore, JAM-A-/- mice are susceptible to reovirus infection (Antar, A.A.R. and Dermody T.S., unpublished results).

The reovirus RGD- and KGE-motif containing λ2-encoding L2 gene segment has been linked to viral shedding and spread to littermates in pathogenesis studies in mice (134). Therefore, it is possible that integrins influence tissue tropism and disease outcome in reovirus-infected animals.

To test the function of β1 integrin in reovirus pathogenesis, the most logical approach would be to utilize β1 integrin knockout mice. However, β1 integrin-null mice are not viable (237). Instead, transgenic mice in which the β1 integrin NPXY motifs are mutated to NPXF are viable, fertile, and do not exhibit phenotypic abnormalities (66). Thus, these mice should be used to determine whether mutation of the β1 integrin NPXY motifs affects reovirus virulence and disease. These experiments will test the hypothesis that β1 integrin NPXY-mediated recruitment of endocytic machinery and delivery of reovirus to the proper endocytic compartment *in vivo* can influence pathogenesis.

NPXY-to-F mice and wild-type mice should be infected with a wild-type LD_{50} dose of reovirus, and intestine, brain, liver, lung, and spleen should be harvested at days 4, 8, and 12 after peroral inoculation. Tissues should be homogenized by sonication, and viral titers should be determined by plaque assay (256). Titer in the intestine will indicate the extent of primary replication, while titer in other organs will reflect systemic spread and growth at secondary sites. To determine kinetics of viral growth in the CNS, newborn mice should be inoculated intracranially with an LD_{50} dose of virus, and mice should be euthanized on days 2, 4, 6, and 8 post-infection. Viral titers in brain-tissue homogenates should be determined by plaque assay. These experiments will determine whether integrin expression is required for growth at a primary site of inoculation, systemic dissemination, or growth at a site of secondary replication. I anticipate that β1

integrin utilization will affect reovirus pathogenesis, most likely at the level of tissue tropism. It will be especially interesting to investigate whether β1 integrin influences tropism in the CNS.

The role of β1 integrin as the primary attachment and entry receptor *in vivo* should be investigated. Similarities in reovirus and *Yersinia pseudotuberculosis* attachment and entry in cell culture and *in vivo* are remarkable. Both reovirus and *Yersinia* utilize β1 integrin to mediate internalization into target cells (119, 156). *Yersinia* engages β1 integrin directly (253) when paired with multiple α subunits (119), which also is possible for reovirus. Additionally, β1 integrin NPXY motifs mediate the AP-2-clathrin-dependent uptake mechanism of *Yersinia* (106, 254). Reovirus and *Yersinia* both initially bind to intestinal M cells to initiate infection in mice (59, 215) and then transcytose to infect underlying Peyer's patches (17, 214). Binding and internalization of *Yersinia* into M cells is dependent on interactions of the binding protein invasin with β1 integrin (59). By analogy to *Yersinia*, reovirus may initially engage β1 integrin on the apical surface of M cells to mediate attachment and uptake into host cells. To test this possibility, mice should be infected with reovirus by peroral inoculation, and intestinal sections should be analyzed over a time course immediately following infection for both M cell-specific markers (59) and reovirus (170). For these studies, wild-type and RGD- and KGE-mutant viruses should be used to further elucidate the role of reovirus-integrin engagement *in vivo*. This study would provide new information about the infectivity of reovirus in the intestinal lumen and may provide interesting insights into common mechanisms of infection by unrelated intestinal pathogens.

Conclusions

My thesis provides a framework to investigate mechanisms underlying reovirus internalization into target cells both in vitro and *in vivo*. The future studies described in this chapter will enhance an understanding of how reovirus engages its internalization receptor on the cell surface. Additionally, these studies will elucidate mechanisms of β1 integrin-mediated endocytosis, and may uncover novel components of the β1 integrin endocytic pathway. These studies also may identify signaling pathways elicited by β1 integrin in response to reovirus. This work will improve our understanding of nonenveloped virus cell entry and may serve as a platform for development of antiviral therapies that inhibit virus entry into target cells.

CHAPTER V

MATERIALS AND METHODS

Cells, Viruses, and Antibodies

Spinner-adapted murine L929 (L) cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, CA) supplemented to contain 5% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 U of streptomycin per ml, and 0.25 mg amphotericin per ml (Gibco Invitrogen Corp., Grand Island, NY). Chinese hamster ovary (CHO) cells were maintained in Ham's F12 medium (Irvine Scientific) supplemented to contain 10% fetal bovine serum, 100 U of penicillin per ml, and 100 U of streptomycin per ml. HeLa cells were maintained in Dulbecco's minimal essential medium (Gibco Invitrogen Corp.) and supplemented as described for CHO cells. Primary cultures of chick embryo fibroblasts (CEFs) were obtained from Paul Spearman (Vanderbilt University) and maintained in Medium 199 with Earle's salts and 2.2 mg sodium bicarbonate per ml (Gibco Invitrogen Corp.) supplemented to contain 5% fetal bovine serum, 10% tryptose phosphate broth, 1% chicken serum (Gibco Invitrogen Corp.), and antibiotics as described for CHO cells. GD25, GD25β1A GD25β1AY783F, GD25β1AY795F, and GD25β1AY783/F,Y795F cells were obtained from Deane Mosher (University of Wisconsin, Madison) (263) and maintained as described for HeLa cells. Medium for GD25β1A, GD25β1AY783F, GD25β1AY795F, and

GD25β1AY783F/Y795F cells was supplemented to contain 10 μg of puromycin (Sigma-Aldrich, St. Louis, MO) per ml to maintain β1 integrin expression.

Reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) are laboratory stocks. Working stocks of virus were prepared by plaque purification and passage in L cells (256). Purified virions were generated from second-passage L-cell lysate virus stocks. Virus was purified from infected cell lysates by freon extraction and CsCl gradient centrifugation as described (93). Bands corresponding to the density of reovirus particles (1.36 g/cm^3) were collected and dialyzed against virion storage buffer (150 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.4]). Reovirus particle concentration was determined from the equivalence of 1 OD_{260} unit = 2.1 x 10^{12} particles (232). Viral infectivity titers were determined by either plaque assay (256) or fluorescent focus assay (14). ISVPs were generated by treatment of 2 x 10^{11} virion particles per ml with 2 mg of α-chymotrypsin (Sigma-Aldrich) per ml in a volume of 100 μl virion storage buffer at 37°C for 30 min (11). Reactions were terminated by the addition of phenylmethylsulphonylfluoride to a final concentration of 1.0 mM. Purified T1L virions in carbonate-bicarbonate buffer (Sigma-Aldrich) were fluoresceinated by incubation in 50 μg fluorescein (FITC) (Pierce, Rockford, IL) per ml at room temperature for 1 h (99). Excess FITC was removed by exhaustive dialysis against PBS.

IgG fractions of rabbit antisera raised against T1L and T3D (264) were purified by protein A sepharose as previously described (14). Fluorescently conjugated secondary Alexa antibodies were obtained from Molecular Probes (Invitrogen, San Diego, CA). The hJAM-A-specific monoclonal antibody (MAb) J10.4 and control mouse ascites were provided by Charles Parkos (Emory University School of Medicine) (150), and the

mJAM-A-specific MAb H202-106-7-4 was provided by Beat Imof (University of Geneva). The human α2-specific MAb AA10 (IgM) (24) and human β1-specific MAb DE9 (IgG1) (24) were used as diluted ascites. Human integrin-specific MAbs MAB1980 (αv), MAB1973 (α1), MAB2057 (α3), MAB1378 (α6), MAB1976 (αvβ3), and MAB1961Z (αvβ5) were purchased from Chemicon International (Temecula, CA). Antibody BIIG2 (α5) (Developmental Hybridoma Studies Bank, University of Iowa, Iowa City, IA) was provided by John Williams (Vanderbilt University). Function-blocking human α2-specific MAb 6F1 was provided by Richard Bankert (State University of New York at Buffalo). Function-blocking murine β1 MAb CD29 (IgM) and hamster IgM isotype control were purchased from BD Biosciences Pharmingen (San Jose, CA). Murine β1-specific MAb MAB1997 (Chemicon) and human β1-specific MAb MAB2253Z (Chemicon) were used to assess expression of β1 integrin on GD25, GD25β1A, GD25β1AY783F, GD25β1AY795F, and GD25β1AY783F/Y795F cells and HeLa cells, respectively, by flow cytometry. ICAM-1-specific MAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies used for flow cytometric analysis of HeLa cells are shown in Table 1.

Sequence Analysis

Sequences of the reovirus λ2-encoding L2 gene from strains T1L (NC_004259), type 2 Jones (T2J) (NC_004260), T3D (NC_004275), T1Neth85 (AF378004), T2SV59 (AF378006), T3C9 (AF378007), T3C18 (AF378008), T3C87 (AF378009), and T3C93 (AF378010) were aligned using the protein sequence alignment algorithm in MacVector, version 8.0.1 (Accelrys, San Diego, CA).

Plasmid Constructs

Human JAM-A was subcloned into expression plasmid pcDNA3.1+ (Invitrogen) (91). Truncation mutant JAM-A-ΔCT was generated by PCR using full-length JAM-A cDNA as template. 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, respectively. The gene-specific primer encompasses nucleotides 981-995 of the JAM-A cDNA. The PCR product was digested with BamHI (recognition site underlined in the reverse primer sequence) and subcloned into complementary restriction sites of pcDNA3.1+. Fidelity of cloning was confirmed by automated sequencing. Plasmid constructs encoding murine integrin αv (90) and α2 (83) were previously described. A cDNA encoding murine β1 integrin cloned into the EcoR1 site of pGEM1 was obtained from Richard Hynes (Massachusetts Institute of Technology) (D. W. DeSimone, V. Patel, and R. O. Hynes, unpublished). Integrin cDNAs were subcloned into the expression plasmid pcDNA3.1+.

Transfections

Monolayers of cells in a 24-well plate (Costar, Cambridge, MA) were transfected with empty vector or plasmids encoding receptor constructs by using Lipofectamine PLUS reagent (Invitrogen). Cells were incubated for 24 h to allow for receptor expression prior to adsorption with either reovirus virions or ISVPs for infectivity studies.

Flow Cytometric Analysis: Expression and Binding

Surface expression of integrin subunits and JAM-A was determined by flow cytometry. Cells were detached from plates by using PBS-EDTA (20 mM EDTA). Cells were washed and centrifuged at 2,000 x g to form a pellet, resuspended with integrin-specific or control antibodies in PBS-BSA (Sigma-Aldrich) (5% BSA), and incubated at 4°C for 1 h. Cells were washed twice and incubated with an appropriate secondary antibody conjugated to R-phycoerythrin (PE) (BD Biosciences Pharmingen) at 4°C for 1 h. Cells were washed, resuspended in PBS, and analyzed by flow cytometry. Results were analyzed using Windows Multiple Document 2.8 Flow Cytometry Application (The Scripps Research Institute, La Jolla, CA). The fluorescence intensity was measured for an average of 14,000 gated events for cells treated with control antibodies, integrin-specific, or JAM-A-specific antibodies. Events were gated relative to cells stained with an appropriate secondary antibody conjugated to PE.

Chapter II: Reovirus binding to β1-/- and β1+/+ cells was analyzed by adsorbing cells with 2 x 10^{11} FITC-labeled particles of strain T1L at 4°C for 1 h. Cells were washed and analyzed by flow cytometry.

Chapter III: Reovirus binding to GD25, GD25β1A, GD25β1AY783F, GD25β1AY785F, and GD25β1AY783F/Y795F cells was analyzed by adsorbing cells with 10,000 particles per cell of strain T1L at 4°C for 1 h. Cells were washed and then stained with reovirus polyclonal antisera and an appropriate secondary antibody conjugated to PE (BD Biosciences Pharmingen) and analyzed by flow cytometry.

Fluorescent-Focus Assay of Viral Infection

Cells were plated in 24-well or 96-well plates (Costar) and adsorbed with virus at various MOIs at either 4°C or room temperature for 30 to 60 min. Inocula were removed, cells were washed, and complete medium was added. Infected cells were incubated at 37°C for 16 to 24 h to allow a single cycle of viral replication. Cells were fixed with methanol at -20°C for at least 30 min. Fixed cells were incubated with PBS-BSA (5% BSA) for at least 15 min, followed by incubation with reovirus-specific polyclonal antiserum (1:500) in PBS-Triton X-100 (0.5% TX-100) at room temperature for 1 h. Cells were washed twice and incubated with an Alexa 488- or 546-labeled anti-rabbit IgG (1:1000) in PBS-TX-100 (0.5% TX-100) at room temperature for 1 h. Cells were washed twice and visualized by indirect immunofluorescence at a magnification of 20X using Axiovert 200 fluorescence microscope (Carl Zeiss, New York, NY). Infected cells (fluorescent focus units [FFU]) were identified by diffuse cytoplasmic fluorescence staining that was excluded from the nucleus. Reovirus-infected cells were quantified by counting random fields of view of equivalently confluent monolayers for 3 to 5 fields of view for triplicate wells or by counting the entire well for triplicate wells (14).

Confocal Imaging of Reovirus Internalization

Cells were plated on coverslips in 24-well plates. Chapter II: Cells were chilled at 4°C for 45 min prior to infection, washed with PBS, adsorbed with 8 x 10^5 particles per cell of T1L virions in gelatin saline, and returned to 4°C for 1 h. The MOI used was the minimum number of particles required to detect signal by confocal immunofluorescence microscopy at early time-points post-infection. Chapter III: Cells were untreated or pre-

treated with 5μg/ml chlorpromazine (Alexis Biochemicals, San Diego, CA) for 3 h. Cells were adsorbed with 5 x 10^4 particles per cell of T1L virions in incomplete medium at 4°C for 1 h. Cells were either washed and fixed or non-adherent reovirus was aspirated and replaced with warm DMEM and returned to 37°C. At 10-min intervals, cells were washed with PBS and fixed with 4% formaldehyde for 20 min. Excess formaldehyde was quenched with an equal amount of 0.1 M glycine, followed by washing with PBS. Cells were treated with 1% TX-100 for 5 min and incubated with PBS-BGT (PBS, 0.5% BSA, 0.1% glycine, and 0.05% Tween-20) for 10 min. Cells were incubated with reovirus-specific polyclonal antiserum (1:500) in PBS-BGT for 1 h and washed with PBS-BGT. Cells were stained with donkey anti-rabbit immunoglobulin conjugated to Alexa Fluor 488 (Molecular Probes) (1:500) to visualize reovirus, phalloidin conjugated to Alexa Fluor 546 (Molecular Probes) (1:100) to visualize actin, and TO-PRO 3 conjugated to Alexa Fluor 642 (Molecular Probes) (1:1000) to visualize DNA. Cells were incubated for 1 h with secondary antibodies and fluorescent probes in PBS-BGT and washed with PBS-BGT. Coverslips were removed from wells and placed on slides using Prolong Anti-Fade mounting media (Molecular Probes). Images were captured on a Zeiss LSM 510 Laser-Scanning Confocal Microscope using LSM 510 software.

Chapter II: Virus internalization was quantitated by enumerating fluorescent particles localized at the cell periphery and particles internalized into the cytoplasm to determine the total number of fluorescent particles per cell. Ten cells were analyzed for each time point. The number of internalized particles was measured as a percentage of the total number of particles per cell.

Chapter III: Viral internalization was quanitated as a function of pixel intensity using Metamorph software. Confocal images were analyzed for reovirus particles by defining the intracellular space using the trace tool to exclude the plasma membrane, which was identified by intense actin staining. The region measurement function was used to measure the intensity of the green pixels in the trace region. Since cell size varies, the average pixel intensity was multiplied by the total area of the cell to determine the total pixel intensity per cell.

Electron Microscopy

$1x10^6$ cells were plated in 60 mm dishes (Costar) and incubated overnight at 37°C. Medium was removed and cells were infected with reovirus strain T1L at an MOI of 1×10^5 particles per cell in incomplete medium or incubated in incomplete medium alone. Virus was adsorbed at 4°C for 1 h to allow viral attachment. Cells were washed 2 times with PBS and then cells were harvested or complete medium was added and cells were incubated at 37°C for 10-min intervals for 30 min. Cells were harvested on ice in 1ml of PBS using a cell lifter (Costar), and transferred to a 1.5 ml microcentrifuge tube. Cells were pelleted at 2.5 x g for 5 min. PBS was aspirated and 1ml of 2% gluteraldehyde was placed over the cell pellet. Cells were incubated at room temperature for 45 min – 1.5 h. Gluteraldehyde was then aspirated and replaced with fresh 2% gluteraldehyde for overnight incubation. After overnight fixation samples were washed three times in PBS, transferred to 1% osmium textroxide in diH_2O for 1h, and washed three times in diH_2O. Preparations were then stained *en bloc* in 1% aqueous uranyl acetate for 1 hr, washed three times in diH_2O and dehydrated in a series of EtOH (30%-

100%). Samples were passed through propylene oxide, transferred to a 1:1 araldite:propylene oxide mixture, and removed and embedded in Araldite embedding media. Ultra-thin serial sections (50-60 nm) were obtained on a Leica UCT Ultracut microtome (Leica Microsystems, Vienna, Austria), transferred to formvar-coated grids, and examined on a Phillips CM10 TEM (FEI Company, Hillsboro, OR) equipped with an Advantage Plus Digital CCD System for CM10 TEM. (Advanced Microscopy Techniques, Danvers, MA). Chemical supplies were purchased from Electron Microscopy Sciences (Fort Washington, PA).

Early Infectivity Assay

$1x10^6$ cells were plated in 60 mm dishes (Costar) and incubated overnight at 37°C. Cells were infected with 1 x 10^4 particles per cell of T1L virions in incomplete medium at 4°C for 1 h. Cells were washed 3 times with PBS and then complete medium or media containing 200μM ribavirin (Sigma) was added and cells were incubated at 37°C for 3.5 h. Cells were harvested by scraping cells in 1 ml of PBS and then centrifuged at 3,000 x g to form a pellet. PBS was aspirated and pellets were frozen. RNA was extracted using a RNeasy mini RNA extraction kit (Qiagen, Valencia, CA). Samples were subjected to RT-PCR using a one-step RT-PCR kit (Qiagen) with reovirus L1-specific primers 5'CTGCATCCATTGTAAATGACGAGTC 3' and 5' GCTATGTCATATTTCCATCCGAATTC 3' (140, 141) and mouse β-actin-specific primers (Invitrogen). RT-PCR reactions were resolved by agarose gel electrophoresis and stained using ethidium bromide. For detailed protocol information see Appendix C.

Statistical Analysis

Means of triplicate samples were compared by using an unpaired Students' t-test (Microsoft Excel, Redmond, WA). P values < 0.05 were considered to be statistically significant.

APPENDIX A

β1 INTEGRIN MEDIATES INTERNALIZATION OF MAMMALIAN REOVIRUS

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β1 Integrin Mediates Internalization of Mammalian Reovirus

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Reovirus infection is initiated by interactions between the attachment protein σ1 and cell surface carbohydrate and junctional adhesion molecule A (JAM-A). Expression of a JAM-A mutant lacking a cytoplasmic tail in nonpermissive cells conferred full susceptibility to reovirus infection, suggesting that cell surface molecules other than JAM-A mediate viral internalization following attachment. The presence of integrin-binding sequences in reovirus outer capsid protein λ2, which serves as the structural base for σ1, suggests that integrins mediate reovirus endocytosis. A β1 integrin-specific antibody, but not antibodies specific for other integrin subunits, inhibited reovirus infection of HeLa cells. Expression of a β1 integrin cDNA, along with a cDNA encoding JAM-A, in nonpermissive chicken embryo fibroblasts conferred susceptibility to reovirus infection. Infectivity of reovirus was significantly reduced in β1-deficient mouse embryonic stem cells in comparison to isogenic cells expressing β1. However, reovirus bound equivalently to cells that differed in levels of β1 expression, suggesting that β1 integrins are involved in a postattachment entry step. Concordantly, uptake of reovirus virions into β1-deficient cells was substantially diminished in comparison to viral uptake into β1-expressing cells. These data provide evidence that β1 integrin facilitates reovirus internalization and suggest that viral entry occurs by interactions of reovirus virions with independent attachment and entry receptors on the cell surface.

Viral attachment and cell entry are key determinants of target cell selection in the infected host and thus play important roles in pathogenesis. Many viruses interact with multiple cell surface molecules to mediate the processes of attachment and internalization (68). For example, human immunodeficiency virus uses CD4 to bind the cell surface and chemokine receptors to facilitate the conformational alterations in envelope glycoproteins that culminate in fusion of the viral envelope and cell membrane (35). Receptors that serve as initial binding sites have been identified for many viruses (25). However, little is known about the postattachment events that lead to nonenveloped virus internalization, in particular those that mediate virus uptake into the endocytic pathway.

Mammalian reoviruses are large, nonenveloped, double-stranded RNA-containing viruses that infect a variety of mammalian species. Following attachment to target cells, reoviruses are internalized by receptor-mediated endocytosis (3, 13, 14, 72), which is mostly likely to be clathrin dependent (31). Proteolytic disassembly in endosomes leads to removal of outer capsid protein σ3 and cleavage of outer capsid protein μ1 (3, 14, 27, 72). The resultant disassembly intermediate formed by these events, the infectious subvirion particle (ISVP), is capable of penetrating endosomal membranes in a μ1-dependent manner to release the transcriptionally active viral core particle into the cytoplasm (17, 18, 58), where viral replication takes place. Cellular determinants of reovirus receptor-mediated internalization following attachment and preceding uncoating are poorly defined.

We previously identified junctional adhesion molecule A (JAM-A) as a serotype-independent receptor for reovirus (5, 16, 34). JAM-A is a type 1 transmembrane protein expressed in a variety of cell types, including polarized endothelial and epithelial cells and circulating leukocytes (52, 55, 81). JAM-A interacts with several scaffolding proteins and cytoplasmic adaptor molecules (6, 28, 29) and is hypothesized to play an important role in maintaining the barrier function of epithelial junctions (48, 52, 55, 60). JAM-A is phosphorylated during platelet activation and required for mitogen-activated protein kinase activation following treatment of endothelial cells with basic fibroblast growth factor (57). These data indicate that JAM-A is intimately associated with cytoskeletal and signaling machinery, which raises the possibility that reovirus binding to JAM-A mediates cytoskeletal rearrangement or signaling events to facilitate virus internalization.

The attachment mechanisms of reovirus and adenovirus are remarkably similar (70, 71). The trimeric attachment proteins of both viruses, σ1 and fiber, respectively, are structural homologues and fold using a highly unusual triple β-spiral motif (10, 20, 83). The globular head domains of these molecules are formed from eight-stranded β-barrels with identical interstrand connectivity (70). The receptors for σ1 and fiber, JAM-A (5) and coxsackievirus and adenovirus receptor (CAR)

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(7), respectively, are two-domain, immunoglobulin superfamily proteins that form homodimers using analogous molecular surfaces (71). Also, both JAM-A and CAR localize to tight junctions in polarized epithelial cells (23, 52, 55, 60). Remarkably, reovirus and adenovirus engage their respective receptors by thermodynamically favored disruption of receptor homodimers (34, 53).

Despite mediating high-affinity attachment of adenovirus to cells, engagement of CAR does not permit efficient adenovirus internalization. Instead, adenovirus entry is enhanced by high-avidity interactions of the viral penton base complex with integrins, including αvβ3 and αvβ5 (80). Integrins are heterodimeric cell surface molecules that consist of α and β subunits (43). Integrins function to mediate cellular adhesion to the extracellular matrix, regulate cellular trafficking, and transduce both outside-in and inside-out signaling events (42). In addition to adenovirus, several other pathogenic microorganisms have usurped the adhesion and signaling properties of integrins to bind or enter host cells (1, 8, 32, 39–41, 44, 49).

To define the molecular basis of reovirus internalization, we first tested the capacity of a JAM-A mutant lacking a cytoplasmic tail to support reovirus attachment and infection. We found that while JAM-A is necessary for efficient attachment to cells, the JAM-A cytoplasmic tail is not required for reovirus infection. Given the mechanistic conservation of reovirus and adenovirus attachment strategies and the observation that reovirus outer capsid protein λ2 contains the conserved integrin-binding sequences Arg-Gly-Asp (RGD) and Lys-Gly-Glu (KGE), we tested the role of integrins in reovirus internalization. We found that infection by reovirus virions is inhibited by antibodies specific for β1 integrin. In addition, cells deficient in β1 integrin have a diminished susceptibility to reovirus infection due to a postattachment block to viral entry. Together, these data indicate that, following attachment to JAM-A, β1 integrin facilitates internalization of reovirus into cells. Our findings further demonstrate that two seemingly unrelated viruses utilize distinct cellular molecules to mediate attachment and internalization in a remarkably similar manner.

MATERIALS AND METHODS

Cells, viruses, and antibodies. Spinner-adapted murine L929 (L) cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, CA) supplemented to contain 5% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 U of streptomycin per ml, and 0.25 mg amphotericin per ml (Gibco Invitrogen Corp., Grand Island, NY). Chinese hamster ovary (CHO) cells were maintained in Ham's F12 medium (Irvine Scientific) supplemented to contain 10% fetal bovine serum, 100 U of penicillin per ml, and 100 U of streptomycin per ml. HeLa cells were maintained in Dulbecco's modified Eagle's medium (Gibco Invitrogen Corp.) and supplemented as described for CHO cells. Primary cultures of chicken embryo fibroblasts (CEFs) were obtained from Paul Spearman (Vanderbilt University) and maintained in medium 199 with Earle's salts and 2.2 mg sodium bicarbonate per ml (Gibco Invitrogen Corp.) supplemented to contain 5% fetal bovine serum, 10% tryptose phosphate broth, 1% chicken serum (Gibco Invitrogen Corp.), and antibiotics as described for CHO cells. GD25 and GD25β1A cells were obtained from Deane Mosher (University of Wisconsin, Madison) (78) and maintained as described for HeLa cells. Medium for GD25β1A cells was supplemented to contain 10 μg of puromycin (Sigma-Aldrich, St. Louis, MO) per ml to maintain β1 integrin expression.

Reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) are laboratory stocks. Working stocks of virus were prepared by plaque purification and passage in L cells (75). Purified virions were generated from second-passage L-cell lysate virus stocks. Virus was purified from infected cell lysates by Freon extraction and CsCl gradient centrifugation as described (36). Bands corresponding to the density of reovirus particles (1.36 g/cm^3) were collected and dialyzed against virion storage buffer (150 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.4]). Reovirus particle concentration was determined by the equivalence of 1 unit of optical density at 260 nm to 2.1×10^{12} particles (69).

Viral infectivity titers were determined by either plaque assay (75) or fluorescent focus assay (4). ISVPs were generated by treatment of 2×10^{11} virion particles per ml with 2 mg of α-chymotrypsin (Sigma-Aldrich) per ml in a volume of 100 μl virion storage buffer at 37°C for 30 min (2). Reactions were terminated by the addition of phenylmethylsulfonyl fluoride to a final concentration of 1.0 mM. Purified T1L virions in carbonate-bicarbonate buffer (Sigma-Aldrich) were fluoresceinated by incubation with 50 μg fluorescein isothiocyanate (FITC) (Pierce, Rockford, IL) per ml at room temperature for 1 h (38). Excess FITC was removed by exhaustive dialysis against phosphate-buffered saline (PBS).

Immunoglobulin G (IgG) fractions of rabbit antisera raised against T1L and T3D (79) were purified by protein A-Sepharose as previously described (4). Fluorescently conjugated secondary Alexa antibodies were obtained from Molecular Probes (Invitrogen, San Diego, CA). The human JAM-A (hJAM-A)-specific monoclonal antibody (MAb) J10.4 and control mouse ascites were provided by Charles Parkos (Emory University School of Medicine) (52), and the murine JAM-A (mJAM-A)-specific MAb H202-106-7-4 was provided by Beat Imof (University of Geneva). The human α2-specific MAb AA10 (IgM) (8) and human β1-specific MAb DE9 (IgG1) (8) were used as diluted ascites. Human integrin-specific MAbs MAB1980 (αv), MAB1973 (α1), MAB2057 (α3), MAB1378 (α6), MAB1976 (αvβ3), and MAB1961Z (αvβ5) were purchased from Chemicon International (Temecula, CA). Antibody BIIG2 (α5) (Developmental Hybridoma Studies Bank, University of Iowa, Iowa City, IA) was provided by John Williams (Vanderbilt University). Function-blocking human α2-specific MAb 6F1 was provided by Richard Bankert (State University of New York at Buffalo). Function-blocking murine β1 MAb CD29 (IgM) and hamster IgM isotype control were purchased from BD Biosciences Pharmingen (San Jose, CA). Murine β1-specific MAb MAB1997 (Chemicon) and human β1-specific MAb MAB2253Z (Chemicon) were used to assess expression of β1 integrin on GD25 and GD25β1A cells and HeLa cells, respectively, by flow cytometry. ICAM-1-specific MAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies used for flow cytometric analysis of HeLa cells are shown in Table 1.

TABLE 1. Surface expression of integrins on HeLa cells

Antibody	Specificity	Mean fluorescence intensity[a]
MAB1997 (IgG control)	Murine β1 integrin	2.65
MAB1973	Human α1 integrin	76.09
MAB6F1	Human α2 integrin	84.47
MAB2057	Human α3 integrin	73.31
MABBIIG2	Human α5 integrin	76.68
MAB1378	Human α6 integrin	81.86
MAB1980	Human αv integrin	78.75
MAB2253Z	Human β1 integrin	61.14
MAB1976	Human αvβ3 integrin	5.46
MAB1961Z	Human αvβ5 integrin	42.10

[a] Results are expressed as mean fluorescence intensity for an average of 14,000 gated events as assessed by flow cytometry.

Sequence analysis. The sequences of the reovirus λ2-encoding L2 gene from strains T1L (NC_004259), type 2 Jones (T2J) (NC_004260), T3D (NC_004275), T1Neth85 (AF378004), T2SV59 (AF378006), T3C9 (AF378007), T3C18 (AF378008), T3C87 (AF378009), and T3C93 (AF378010) were aligned using the protein sequence alignment algorithm in MacVector, version 8.0.1 (Accelrys, San Diego, CA).

Plasmid constructs. Human JAM-A was subcloned into expression plasmid pcDNA3.1+ (Invitrogen) (34). Truncation mutant JAM-A-ΔCT was generated by PCR using full-length JAM-A cDNA as the template. Amino acids 1 to 260 (Δ261–299) were cloned and appended with a stop codon using T7 primer and 5′-TACGGGATCCTCAGGCAAACCAGATGCC-3′ as the 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 (recognition site underlined in the reverse primer sequence) and subcloned into the complementary restriction sites of pcDNA3.1+. Fidelity of cloning was confirmed by automated sequencing. Plasmid constructs encoding murine integrin αv (33) and α2 (30) were previously described. A cDNA encoding murine β1 integrin cloned into the EcoRI site of pGEM1 was obtained from Richard Hynes (Massachusetts Institute of Technology) (D. W. DeSimone, V. Patel, and R. O.

Hynes, unpublished). Integrin cDNAs were subcloned into the expression plasmid pcDNA3.1+.

Transient transfection of CHOs and CEFs. Monolayers of cells in a 24-well plate (Costar, Cambridge, MA) were transfected with empty vector or plasmids encoding receptor constructs by using Lipofectamine Plus reagent (Invitrogen). Cells were incubated for 24 h to allow receptor expression prior to adsorption with either reovirus virions or ISVPs for infectivity studies.

Flow cytometric analysis. Surface expression of integrin subunits on HeLa cells was determined by flow cytometry. Cells were detached from plates by using PBS-EDTA (20 mM EDTA). Cells were washed and centrifuged at 2,000 × *g* to form a pellet, resuspended with integrin-specific or control antibodies in PBS-bovine serum albumin (BSA) (Sigma-Aldrich) (5% BSA), and incubated at 4°C for 1 h. Cells were washed twice and incubated with an appropriate secondary antibody conjugated to R-phycoerythrin (BD Biosciences Pharmingen) at 4°C for 1 h. Cells were washed, resuspended in PBS, and analyzed by flow cytometry. Results were analyzed using the Windows Multiple Document 2.8 flow cytometry application (The Scripps Research Institute, La Jolla, CA).

The mean fluorescence intensity was measured for an average of 14,000 gated events for cells treated with control or integrin-specific antibodies. Events were gated relative to cells stained with an appropriate secondary antibody conjugated to phycoerythrin. Reovirus binding to GD25 and GD25β1A cells was analyzed by adsorbing cells with 2×10^{11} FITC-labeled particles of strain T1L at 4°C for 1 h. Cells were washed and analyzed by flow cytometry.

Fluorescent focus assay of viral infection. Cells were plated in 24-well or 96-well plates (Costar) and adsorbed with virus at various multiplicities of infection (MOIs) at either 4°C or room temperature for 30 to 60 min. Inocula were removed, cells were washed, and complete medium was added. Infected cells were incubated at 37°C for 16 to 24 h to allow a single cycle of viral replication. Cells were fixed with methanol at −20°C for at least 30 min. Fixed cells were incubated with PBS-BSA (5% BSA) for at least 15 min, followed by incubation with reovirus-specific polyclonal antiserum (1:500) in PBS–Triton X-100 (0.5% Triton X-100) at room temperature for 1 h. Cells were washed twice and incubated with an Alexa 488- or 546-labeled anti-rabbit IgG (1:1,000) in PBS-Triton X-100 (0.5% Triton X-100) at room temperature for 1 h.

Cells were washed twice and visualized by indirect immunofluorescence at a magnification of 20× using an Axiovert 200 fluorescence microscope (Carl Zeiss, New York, NY). Infected cells (fluorescent focus units [FFU]) were identified by diffuse cytoplasmic fluorescence staining that was excluded from the nucleus. Reovirus-infected cells were quantified by counting random fields of view of equivalently confluent monolayers for three to five fields of view for triplicate wells or by counting the entire well for triplicate wells (4).

Confocal imaging of reovirus internalization. GD25 and GD25β1A cells were plated on coverslips in 24-well plates. Cells were chilled at 4°C for 45 min prior to infection, washed with PBS, adsorbed with 8×10^5 particles per cell of T1L virions in gelatin saline, and returned to 4°C for 1 h. The MOI used was the minimum number of particles required to detect signal by confocal immunofluorescence microscopy at early time points postinfection. Cells were either washed and fixed or nonadherent reovirus was aspirated and replaced with warm Dulbecco's modified Eagle's medium and returned to 37°C. At 10-min intervals, cells were washed with PBS and fixed with 4% formaldehyde for 20 min. Excess formaldehyde was quenched with an equal amount of 0.1 M glycine, followed by washing with PBS. Cells were treated with 1% Triton X-100 for 5 min and incubated with PBS-BGT (PBS, 0.5% BSA, 0.1% glycine, and 0.05% Tween 20) for 10 min. Cells were incubated with reovirus-specific polyclonal antiserum (1:500) in PBS-BGT for 1 h and washed with PBS-BGT. Cells were stained with donkey anti-rabbit immunoglobulin conjugated to Alexa Fluor 488 (Molecular Probes) (1:500) to visualize reovirus, phalloidin conjugated to Alexa Fluor 546 (Molecular Probes) (1:100) to visualize actin, and TO-PRO 3 conjugated to Alexa Fluor 642 (Molecular Probes) (1:1,000) to visualize DNA. Cells were incubated for 1 h with secondary antibodies and fluorescent probes in PBS-BGT and washed with PBS-BGT. Coverslips were removed from wells and placed on slides using Prolong Anti-Fade mounting medium (Molecular Probes). Images were captured on a Zeiss LSM 510 laser-scanning confocal microscope using LSM 510 software.

Virus internalization was quantified by enumerating fluorescent particles localized at the cell periphery and particles internalized into the cytoplasm to determine the total number of fluorescent particles per cell. Ten cells were analyzed for each time point. The number of internalized particles was measured as a percentage of the total number of particles per cell.

Statistical analysis. Means of triplicate samples were compared by using an unpaired Students' *t* test (Microsoft Excel, Redmond, WA). *P* values of <0.05 were considered statistically significant.

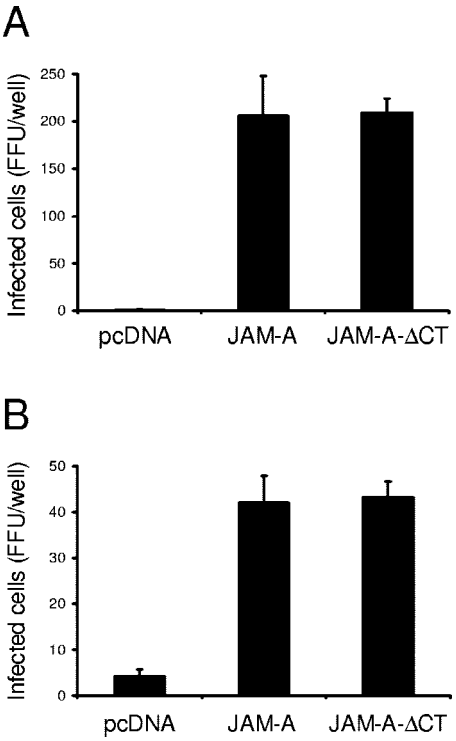

FIG. 1. JAM-A cytoplasmic tail is not required for reovirus infection. CHO cells were transiently transfected with empty vector or plasmids encoding JAM-A or JAM-A-ΔCT. Following incubation for 24 h to permit receptor expression, cells were adsorbed with reovirus strains T1L (A) or T3D (B) at an MOI of 0.1 FFU per cell at room temperature for 1 h. Cells were washed with PBS, incubated in complete medium at 37°C for 20 h, and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in entire wells for triplicate experiments. The results are expressed as the mean FFU per well for triplicate samples. Error bars indicate standard deviations. CHO cells support a low level of infection by type 3 reovirus in the absence of JAM-A, likely attributable to the expression of sialic acid (16, 34).

RESULTS

The JAM-A cytoplasmic tail is dispensable for reovirus infection. JAM-A is a serotype-independent reovirus receptor with a cytoplasmic tail known to interact with a variety of proteins (6, 28, 29). To determine whether the JAM-A cytoplasmic tail is required for reovirus entry, we generated a JAM-A cytoplasmic tail deletion mutant (JAM-A-ΔCT) and tested its capacity to support reovirus infection following transfection of CHO cells. CHO cells do not express detectable levels of JAM-A (55, 60) and are poorly permissive for reovirus infection (34). Cells were transiently transfected with plasmids encoding full-length JAM-A, JAM-A-ΔCT, or empty vector as a control. Equivalent cell surface expression of transfected constructs was confirmed by flow cytometry (data not shown).

The capacity of reovirus to infect CHO cells following transfection with the JAM-A constructs was tested using reovirus fluorescent focus assays. Following transient transfection of CHO cells with empty vector, JAM-A, or JAM-A-ΔCT, cells were adsorbed with reovirus strains T1L and T3D and scored for infection by indirect immunofluorescence at 20 h postinfection (Fig. 1). Expression of either full-length or truncated

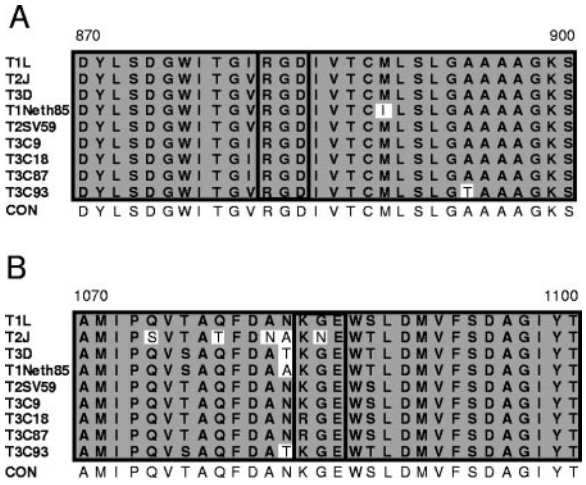

FIG. 2. Reovirus outer capsid protein λ2 contains integrin-binding sequences. Alignment of deduced amino acid sequences of the reovirus λ2 protein for the indicated strains. Amino acid residues are designated by the single-letter code. Amino acid positions are indicated above the first and last letters. Integrin-binding RGD (A) and KGE (B) motifs are highlighted by a black box. Nonconserved sequences are shown in unshaded boxes. CON, consensus sequence.

JAM-A was sufficient to allow reovirus infection of CHO cells, permitting viral protein production of both type 1 and type 3 reovirus strains. These results indicate that the JAM-A cytoplasmic tail is not required for efficient reovirus attachment and infection.

Reovirus outer capsid proteins contain integrin-binding sequences. Structural and functional analyses indicate that reovirus and adenovirus share remarkably similar mechanisms of attachment (70, 71). To determine whether reovirus outer capsid proteins contain sequences that could potentially engage integrins, we performed a search for integrin-binding motifs in the σ1, σ3, μ1, and λ2 proteins, which form the reovirus outer capsid (26). We identified two common integrin-binding motifs, RGD and KGE, in the deduced amino acid sequence of the λ2 protein (Fig. 2). The RGD motif is conserved in all reovirus strains for which sequence information is available (15, 67); the KGE motif is conserved in all of those strains except T2J (15, 67). The λ2 protein is a component of the reovirus outer capsid and core (26). It is structurally arranged as a pentamer at the virion fivefold axes of symmetry and forms the base for attachment protein σ1 (26, 63). The presence of conserved integrin-binding motifs in the reovirus λ2 protein led us to test whether reovirus utilizes integrins to mediate internalization.

An antibody specific for β1 integrin inhibits reovirus infection of HeLa cells. To determine whether integrins are required for reovirus infection, we first used flow cytometry to analyze integrin expression on the surface of HeLa cells. HeLa cells were incubated with integrin-specific MAbs and a phycoerythrin-labeled secondary antibody (Table 1). RGD-binding integrin subunits α3, α5, αv, and β1 and KGE-binding integrin subunits α1, α2, α6, and β1 (43) were detected on HeLa cells at levels above those in control antibody-treated cells. RGD-binding integrin heterodimer αvβ5 also was detected at levels above that of the control, while there was low-level expression of αvβ3. Thus, HeLa cells express both RGD- and KGE-binding integrins.

To assess a role for integrins in reovirus replication, we tested antibodies specific for the RGD- and KGE-binding integrins expressed on HeLa cells for the capacity to block reovirus infection. HeLa cells were incubated with integrin-specific and control antibodies prior to adsorption with reovirus virions. Viral infection was detected by indirect immunofluorescence (Fig. 3A). We found that β1-specific MAb DE9 resulted in a 50% reduction in infection ($P < 0.05$), while antibodies specific for the other integrin subunits expressed on HeLa cells had no effect. Control antibodies produced anticipated effects; JAM-A-specific MAb J10.4 inhibited infection, whereas ICAM-specific MAb (data not shown) or control mouse ascites (Fig. 3A) did not. The effect of MAb DE9 was dose dependent (Fig. 3B), providing further evidence that the inhibition of infection was dependent on integrin blockade.

To determine whether particular α subunits pair with β1 integrin to facilitate reovirus infection, we tested whether treatment with α integrin-specific antibodies was capable of enhancing the inhibitory effect of β1 integrin-specific MAb DE9 on reovirus infection. We also tested whether antibodies specific for other β integrin subunits expressed on HeLa cells, β3 and β5, were capable of infection blockade. HeLa cells were treated with MAb DE9 in combination with other integrin-specific antibodies prior to adsorption with reovirus virions (Fig. 3C). While treatment of HeLa cells with MAb DE9 resulted in a 50% reduction in reovirus infection, none of the other integrin-specific antibodies tested reduced reovirus infection to a greater extent than that resulting from treatment with DE9 alone. These results suggest that the integrin epitope bound by reovirus is blocked by β1-specific MAb DE9 and not by the other MAbs used in these experiments.

JAM-A MAb J10.4 blocks reovirus infection ~90% (Fig. 3A). To determine whether the residual level of infection in the presence of MAb J10.4 was dependent on reovirus interactions with β1 integrin, we treated HeLa cells with JAM-A-specific MAb J10.4 in combination with MAb DE9 prior to adsorption with reovirus virions (Fig. 3D). Treatment of HeLa cells with MAb J10.4 and MAb DE9 completely abrogated reovirus infection, indicating that the effect of JAM-A blockade is enhanced when β1 integrin is not available for interactions with reovirus. Treatment with MAb DE9 did not significantly inhibit infection by ISVPs (Fig. 3D), suggesting that viral attachment is not affected by β1 integrin blockade. Taken together, these results support the conclusion that a β1-specific antibody blocks reovirus infection at a step subsequent to attachment but prior to uncoating, implicating β1 integrin in reovirus internalization.

Transient transfection of integrin cDNAs allows reovirus infection of JAM-A-expressing CEFs. Ectopic expression of JAM-A in CEFs rescues infection by reovirus ISVPs but not by virions (5), suggesting that these cells exhibit a cell-specific block at the entry or uncoating phases of reovirus infection. To test the capacity of integrins to confer infection of CEFs by reovirus virions, CEFs were transiently transfected with a JAM-A-encoding plasmid in the presence or absence of murine αv, α2, or β1 integrin-encoding plasmids singly or in αβ pairs. Transfected cells were infected with reovirus virions or ISVPs, and infection was assessed by indirect immunofluores-

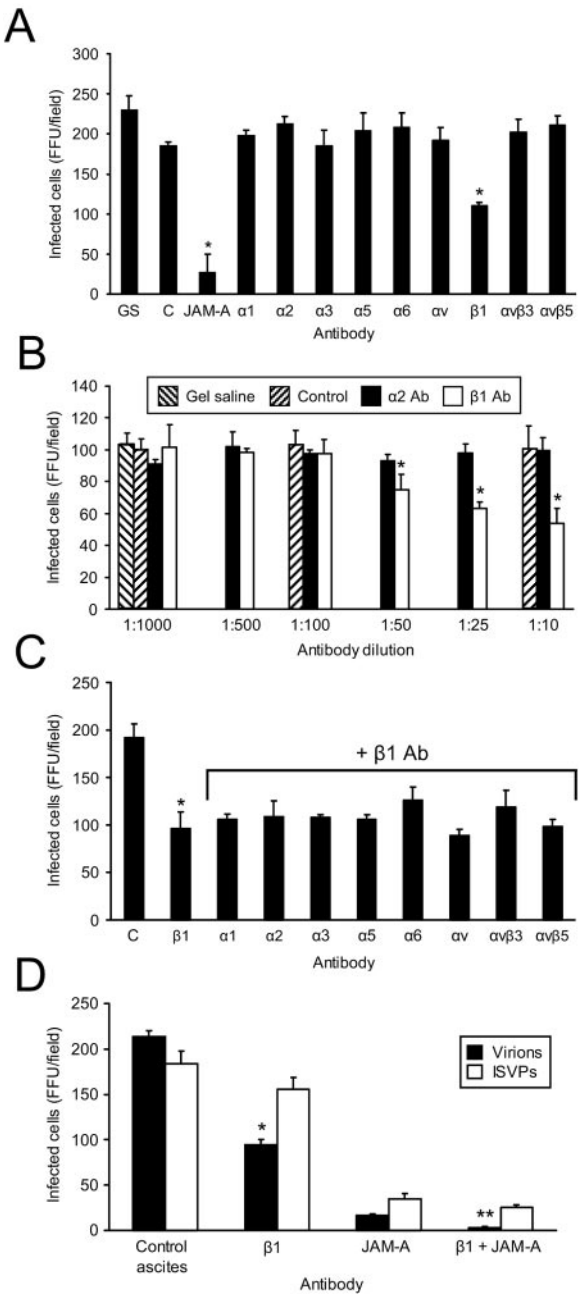

FIG. 3. β1 integrin antibody reduces reovirus infection of HeLa cells. HeLa cells were treated with gel saline (GS), control ascites (C), JAM-A-specific MAb J10.4, or antibodies specific for the α and β integrins shown (20 μg per ml or as diluted ascites) (A), gel saline, control ascites (Control), α2-specific MAb AA10, or β1-specific MAb DE9 (at the indicated dilutions) (B), antibodies specific for the α and β integrins shown in the presence of β1-specific MAb DE9 (1:10) (C), control ascites, β1-specific MAb DE9, JAM-A-specific MAb J10.4, or JAM-A-specific MAb J10.4 in combination with β1-specific MAb DE9 (D), and incubated at room temperature for 1 h. Antibody-treated cells were infected with virions or ISVPs of T1L at an MOI of 0.1 FFU per cell at 4°C for 30 min. Cells were washed with PBS, incubated in complete medium at 37°C for 16 h, and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in three fields of view for triplicate samples. The results are expressed as the mean FFU per field for triplicate experiments. Error bars indicate standard deviations. *, $P < 0.05$ in comparison to the control; **, $P < 0.05$ in comparison to HeLa cells treated with JAM-A-specific MAb J10.4 alone.

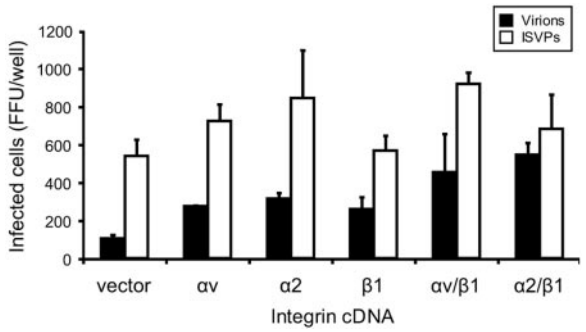

FIG. 4. β1 integrin expression enhances reovirus infection of CEFs. CEFs were transiently transfected with JAM-A-encoding plasmid alone (vector) or in combination with plasmids encoding the integrin subunits shown. Following 24 h to allow receptor expression, transfected cells were adsorbed with T1L virions or ISVPs at an MOI of 1 FFU per cell at room temperature for 1 h. Cells were washed with PBS, incubated in complete medium at 37°C for 20 h, and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in entire wells for duplicate samples. The results are expressed as the mean FFU per well for duplicate experiments. Error bars indicate the range of data. Shown is a representative experiment of three independent experiments performed.

cence (Fig. 4). Expression of β1 integrin paired with either of the murine α integrin subunits provided an approximately fourfold enhancement of infection by reovirus virions in comparison to that in cells transfected with JAM-A alone. These data suggest that β1 integrin expression complements a reovirus cell entry defect in CEFs and provide further support for the involvement of β1 integrin in reovirus internalization.

Cells deficient in β1 integrin have a decreased capacity to support reovirus infection. To further assess a role for β1 integrin in reovirus infection, we tested the capacity of reovirus to infect cells deficient in the β1-integrin subunit. GD25 cells are murine embryonic stem cells derived from β1-null embryos (78). GD25β1A cells are GD25 cells that have been engineered to stably express β1 integrin and thus serve as an isogenic control for GD25 cells. Flow cytometric analysis confirmed that while both cells express JAM-A, only GD25β1A cells express β1 integrin (Fig. 5A). GD25 cells ($\beta1^{-/-}$) and GD25β1A cells ($\beta1^{+/+}$) (78) were adsorbed with reovirus virions or ISVPs, and infection was scored by indirect immunofluorescence (Fig. 5B). In comparison to $\beta1^{+/+}$ cells, $\beta1^{-/-}$ cells were substantially less susceptible to infection by virions, while infection by ISVPs was equivalent in both cell types. Importantly, preincubation of $\beta1^{+/+}$ cells with murine β1 integrin-specific MAb CD29 reduced infection in $\beta1^{+/+}$ cells (Fig. 5C), indicating that enhancement of infection is due to expression of β1 integrin. Therefore, β1 integrin is required for efficient reovirus infection.

Reovirus binding to $\beta1^{-/-}$ and $\beta1^{+/+}$ cells is equivalent. Equivalent infection of $\beta1^{-/-}$ and $\beta1^{+/+}$ cells by ISVPs (Fig. 5B) suggests that reovirus is capable of efficiently binding to both cell types. To directly test this hypothesis, $\beta1^{-/-}$ and $\beta1^{+/+}$ cells were mock treated or incubated with FITC-labeled virions and binding was assessed by flow cytometry (Fig. 6). In these experiments, we found that reovirus binds equivalently to $\beta1^{-/-}$ and $\beta1^{+/+}$ cells. These data demonstrate a function for

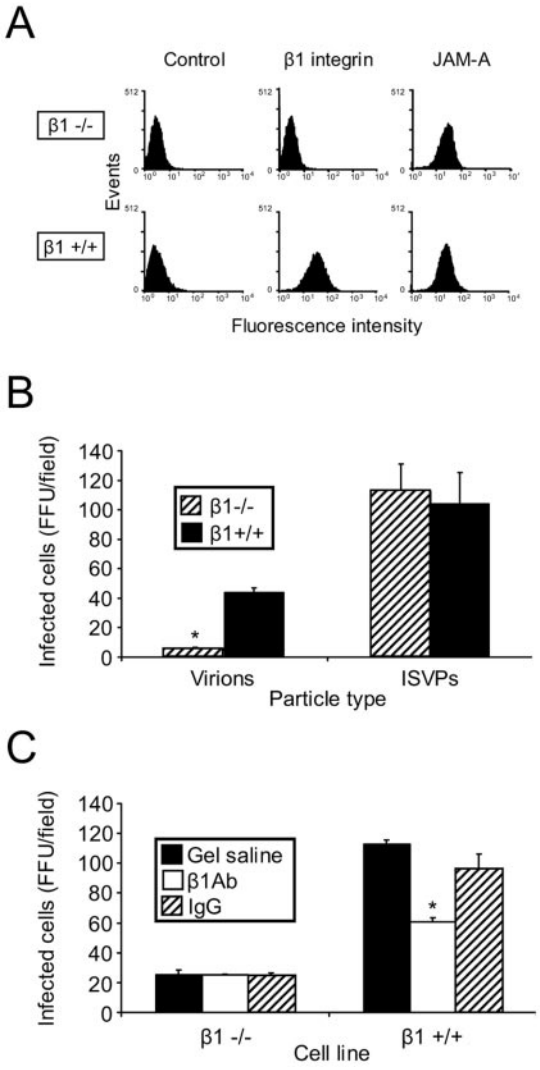

FIG. 5. Cells deficient in β1 integrin are less permissive for reovirus infection. (A) GD25 ($\beta1^{-/-}$) and GD25β1A ($\beta1^{+/+}$) cells were detached from plates with 20 mM EDTA, washed, and incubated with antibodies specific for either murine β1 integrin or murine JAM-A. Cell surface expression of these molecules was detected by flow cytometry. Data are expressed as fluorescence intensity. GD25 and GD25β1A cells were untreated (B) or pretreated with β1-specific MAb CD29 (β1 Ab) or a hamster isotype-matched control MAb (IgG) at room temperature for 1 h (C), adsorbed with virions or ISVPs of T1L at an MOI of 0.1 FFU per cell, and incubated at 4°C for 30 min. Cells were washed with PBS, incubated in complete medium at 37°C for 20 h, and stained by indirect immunofluorescence. Infected cells were quantified by counting cells exhibiting cytoplasmic staining in five fields of view for duplicate samples. The results are expressed as the mean FFU per field for triplicate experiments. *, $P < 0.05$ in comparison to the control.

β1 integrin in reovirus infection at a step subsequent to viral attachment.

β1 integrin enhances the efficiency of reovirus internalization. To directly assess the role of β1 integrin in reovirus internalization, $\beta1^{-/-}$ and $\beta1^{+/+}$ cells were infected at 4°C and then warmed to 37°C over a time course concurrent with reovirus entry (2, 72). At 10-min intervals, cells were fixed, stained for indirect immunofluorescence, and examined by confocal

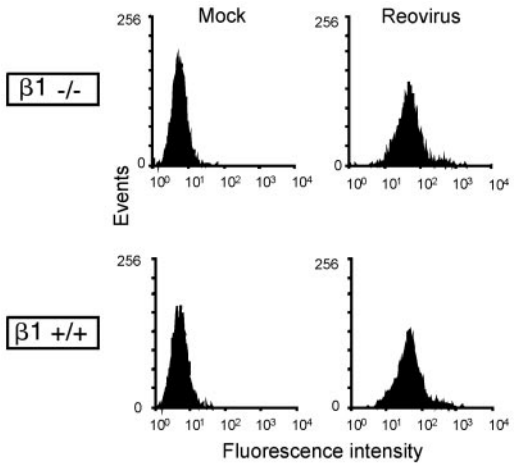

FIG. 6. Reovirus exhibits equivalent binding to $\beta1^{-/-}$ and $\beta1^{+/+}$ cells. GD25 ($\beta1^{-/-}$) and GD25β1A ($\beta1^{+/+}$) cells were incubated with either PBS (mock) or 2×10^{11} FITC-labeled T1L virions at 4°C for 1 h and analyzed by flow cytometry to assess reovirus binding to the cell surface. The results are expressed as fluorescence intensity.

microscopy. Representative confocal micrographic images of reovirus-infected $\beta1^{-/-}$ and $\beta1^{+/+}$ cells are shown in Fig. 7. Immediately after viral adsorption, both cell types exhibited reovirus staining at the cell periphery. At 10 min postadsorption, some reovirus staining was observed at the cell periphery, yet intracellular staining in $\beta1^{+/+}$ cells was also observed. At 20 and 30 min postadsorption, the majority of virions had entered the $\beta1^{+/+}$ cells and had a perinuclear location. In sharp contrast to the findings made using $\beta1^{+/+}$ cells, viral entry was markedly delayed in $\beta1^{-/-}$ cells, with the majority of reovirus virions remaining at the cell periphery throughout the time course. At later time points (30 min postadsorption), some virions were present within the cytoplasm, but these were the minority. These findings suggest that expression of β1 integrin enhances reovirus entry.

To quantify reovirus internalization into $\beta1^{-/-}$ and $\beta1^{+/+}$ cells, we determined the number of internalized fluorescent particles as a percentage of the total number of fluorescent particles per cell at various times postadsorption (Fig. 8). At 0 and 10 min postadsorption, the percentage of particles internalized into $\beta1^{-/-}$ and $\beta1^{+/+}$ cells was equivalent, ~10 and ~30%, respectively. However, at 20 and 30 min postadsorption, the percentage of reovirus particles internalized into $\beta1^{+/+}$ cells was ~50%, while the percentage of particles internalized into $\beta1^{-/-}$ cells was only ~30% ($P < 0.05$) (Fig. 8). These data indicate that β1 integrin enhances reovirus entry at early times postadsorption, suggesting a direct role for β1 integrin as a reovirus internalization receptor.

DISCUSSION

In this study, we performed experiments to define the molecular determinants of reovirus internalization. We show that antibodies specific for β1 integrin inhibit reovirus infection at a postattachment step. We provide evidence that expression of β1 integrin promotes infection by reovirus virions in cells with a block to viral internalization and that viral entry is substantially

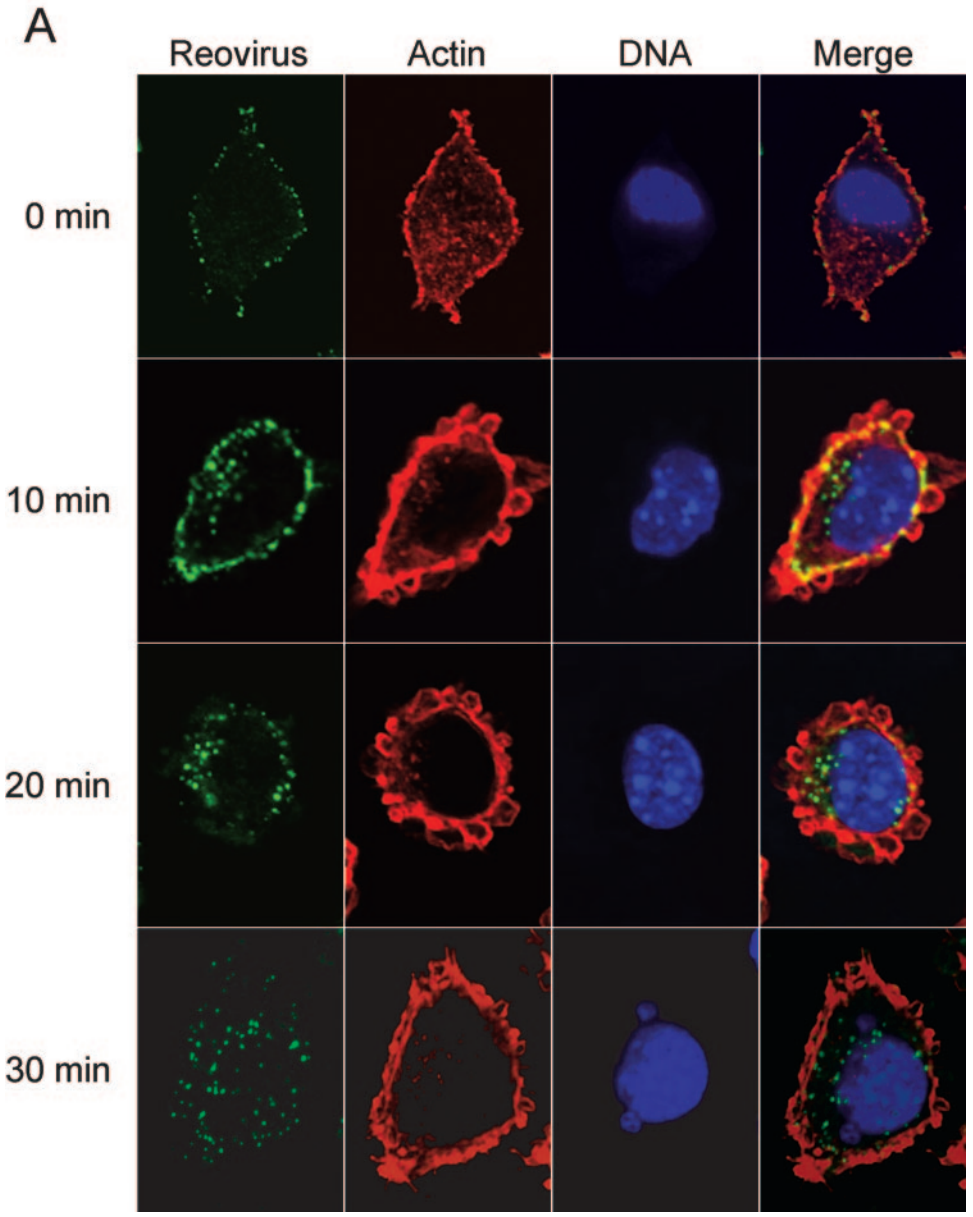

FIG. 7. β1 integrin enhances reovirus entry into cells. (A) GD25β1A ($\beta1^{+/+}$) and (B) GD25 ($\beta1^{-/-}$) cells were chilled, adsorbed with T1L virions, and incubated at 4°C for 1 h. Nonadherent virus was removed, warm medium was added, and cells were incubated at 37°C for the times shown. Cells were fixed, stained for reovirus (green), actin (red), and DNA (blue), and imaged using confocal immunofluorescence microscopy. Representative digital fluorescence images of the same field are shown in each row.

diminished in cells deficient in β1 integrin expression. Together, these data provide strong evidence that β1 integrin serves as a coreceptor to mediate reovirus internalization. These findings suggest a new model for attachment and cell entry of reovirus (Fig. 9). In this model, we propose that reovirus initially interacts with cells via low-affinity binding to carbohydrate. These interactions are followed by high-affinity engagement of JAM-A, which positions the virus on the cell surface for subsequent interactions with β1 integrin to trigger viral endocytosis.

Integrins have been identified as attachment and entry receptors for several viruses, including echovirus (α2β1) (8), foot-and-mouth disease virus (αvβ1, αvβ3, and αvβ6) (9, 44, 45), hantaviruses NY-1 and Sin Nombre virus (β3 integrins) (37), Kaposi sarcoma herpesvirus (α3β1) (1), and cytomegalovirus (α2β1, α6β1, and αvβ3) (32). The *Reoviridae* family member rotavirus also engages a variety of integrins for attachment and cell entry. Rotavirus strains RRV, SA11, and Wa bind to the I (inserted) domain of α2β1 integrin via an Asp-Gly-Glu integrin-binding motif in the VP4 spike protein to effect viral attachment (39). The interactions of rotavirus outer capsid protein VP7 with integrins αxβ2 and αvβ3 can mediate viral entry (39, 82). Integrin α4β1 also can serve as a receptor for rotavirus strain SA11, which contains α4β1 integrin-binding sequences Leu-Asp-Val in VP7 and Ile-Asp-Ala in VP4

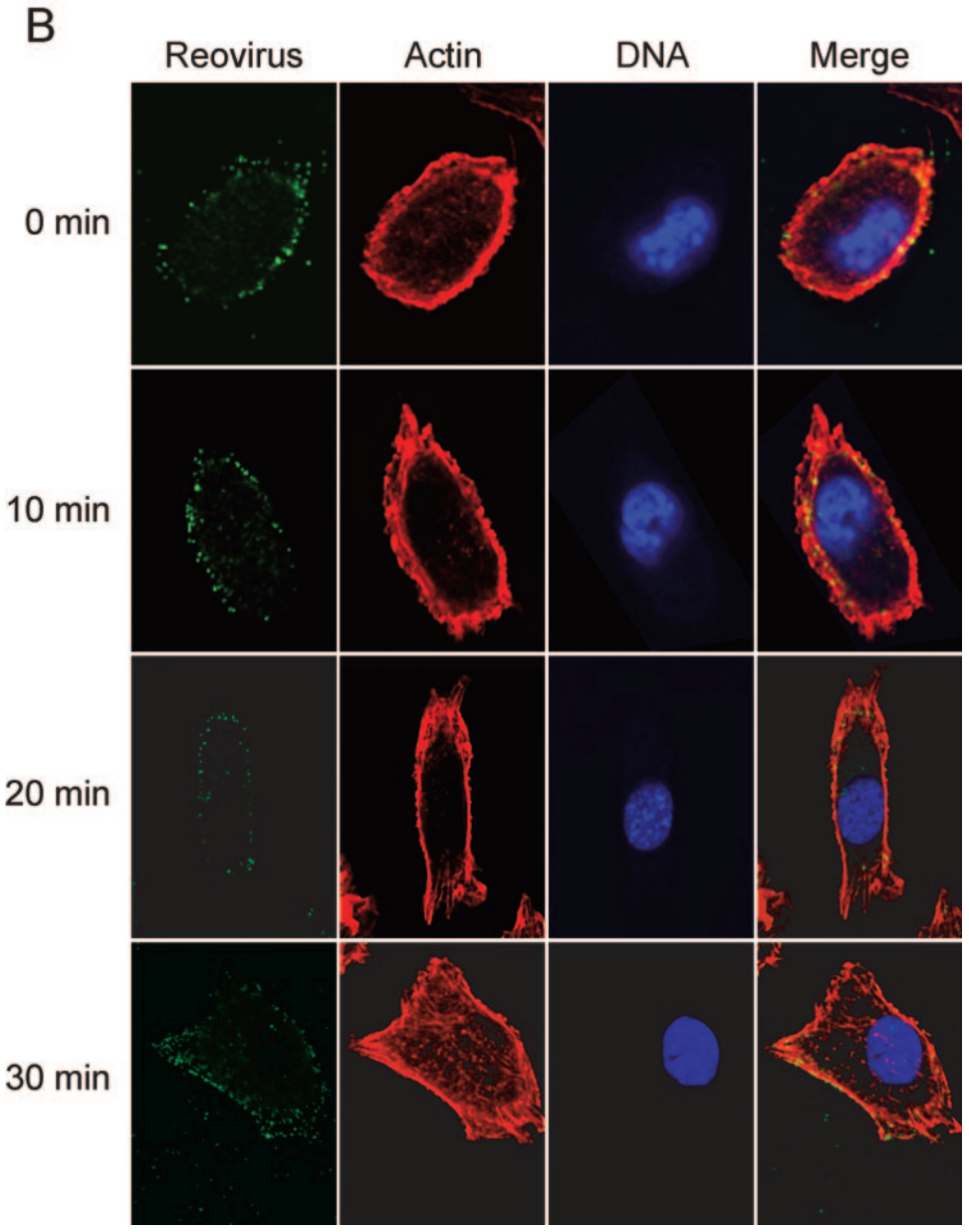

FIG. 7—*Continued.*

(41). Interestingly, like reovirus, adenovirus engages a specific cell surface protein, CAR, prior to interactions with integrins, which function subsequent to viral attachment to mediate viral endocytosis (50, 80). Therefore, the identification of β1 integrin as a reovirus internalization receptor suggests that the conservation of attachment strategies used by reovirus and adenovirus (70, 71) extends to mechanisms of internalization.

Although the specific reovirus protein required for integrin binding is not apparent from our studies, the λ2 protein is a promising candidate. The λ2 protein forms a pentameric turret at the virion fivefold symmetry axes and serves as the insertion site for trimers of attachment protein σ1 (26). Thus, λ2 is the reovirus analogue of the adenovirus penton base protein, which mediates the engagement of integrins by adenovirus (22, 80). Interestingly, λ2 also contains conserved RGD and KGE motifs (15), the preferred interaction motifs for several β1 integrin heterodimers (43).

Structural information for λ2 is available in the context of the reovirus core but not for the intact virion. In the core, the KGE motif is exposed on the top of the λ2 turret, where it would be accessible to a receptor. The RGD motif is also surface exposed, but it appears to be less accessible. However, the λ2 structure in the core may not be identical to that in the virion, as the protein undergoes major conformational changes during virion-to-core disassembly (26). Therefore, it is possible that both the RGD and KGE motifs are accessible to interactions with β1 integrin during engagement of the cell surface by the virus.

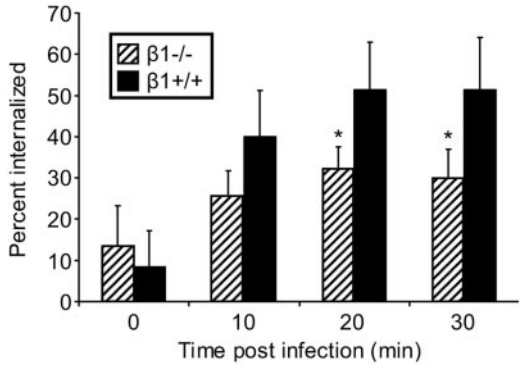

FIG. 8. Quantification of reovirus internalization into $\beta1^{-/-}$ and $\beta1^{+/+}$ cells. Viral internalization was quantitated by enumerating fluorescent particles localized at the cell periphery and particles internalized into the cytoplasm to determine the total number of fluorescent particles per cell. The results are expressed as mean percent internalization (internalized fluorescent particles/total number of fluorescent particles per cell) for 10 cells for each time point. *, $P < 0.05$ in comparison to $\beta1^{+/+}$ cells.

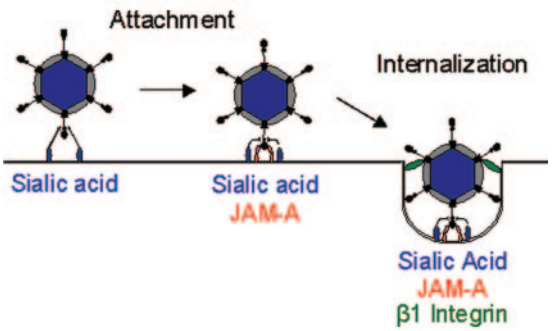

FIG. 9. Receptors for reovirus attachment and cell entry. Reovirus initially engages cells by low-affinity interactions with carbohydrate. For type 3 reovirus strains, this carbohydrate is sialic acid. Reovirus-carbohydrate interactions are followed by high-affinity binding to JAM-A, which positions the virus on the cell surface for subsequent interactions with β1 integrin to trigger viral endocytosis.

A human β1 integrin-specific antibody (DE9) reduced reovirus infection of HeLa cells by 50% (Fig. 3). Similarly, a murine β1 integrin-specific antibody (CD29) blocked infection of β1-expressing mouse embryonic stem cells by ~50% (Fig. 4). Interestingly, MAb DE9 also blocks infection of echovirus (8) and cytomegalovirus (32), suggesting that an epitope in β1 integrin recognized by MAb DE9 may be a preferred binding site for multiple viruses. It is possible that the residual level of reovirus infection following β1 integrin antibody treatment is attributable to other internalization receptors on the cell surface that may be integrin or nonintegrin molecules. However, it is noteworthy that treatment of HeLa cells with both MAb DE9 and JAM-A-specific MAb J10.4 completely abolishes reovirus growth (Fig. 3D). This finding suggests that the residual infection in J10.4-treated HeLa cells is due to reovirus interactions with β1 integrin. Thus, it appears that blockade of reovirus infection by integrin-specific antibodies is inefficient because complete inhibition of virus-integrin interactions is not possible if the virus is tightly adhered to the cell surface by JAM-A.

Since antibodies specific for β3 and β5 integrins did not inhibit reovirus infection, it is likely that only β1 integrin can serve a reovirus internalization function. Antibodies specific for the α integrin subunits expressed on HeLa cells did not further reduce reovirus infection following treatment with a β1 integrin-specific antibody (Fig. 3C). We envision three possible explanations for this result. First, reovirus may directly engage a ligand-binding domain of β1 integrin. Second, reovirus may utilize β1 integrin when paired with numerous α subunits that have redundant functions. However, treatment of HeLa cells with a β1 integrin-specific antibody and a mixture of antibodies specific for α1, α2, α3, α5, α6, and αv integrins did not diminish reovirus infection in comparison to cells treated with a β1 integrin-specific antibody alone (data not shown). Third, reovirus may engage an epitope of an integrin α subunit that is not recognized by the antibodies used in our experiments. Further studies are required to define the biophysical basis of reovirus-integrin interactions.

JAM-A is required for high-affinity reovirus attachment to numerous cell types (5, 16, 34, 56). However, the JAM-A cytoplasmic tail is not necessary for viral endocytosis (Fig. 1). JAM-A likely tethers the virus to the cell surface to facilitate secondary interactions with β1 integrin (Fig. 9). This model is analogous to the mechanism of lymphocyte homing, in which adhesion molecules such as JAM-A provide initial cellular contacts to facilitate subsequent interactions with integrins for diapedesis or signaling (65). An interesting possibility is that JAM-A may be associated with β1 integrin on the host cell plasma membrane. If such were the case, initial JAM-A engagement might facilitate integrin binding, clustering, and viral endocytosis. In support of this hypothesis, JAM-A has been shown to regulate β1 integrin expression and localization (54).

The cytoplasmic domains of integrin subunits are involved in a number of signaling pathways (42). The β1 integrin cytoplasmic domain is linked to cytoskeletal proteins, including talin (62) and α-actinin (59), and signaling molecules, including paxillin and focal adhesion kinase (66). In addition, the β1 integrin cytoplasmic domain contains two Asn-Pro-any residue-Tyr (NPXY) motifs (64), which are common sequence motifs in the cytoplasmic domains of many receptors and serve as recognition sites for the cellular endocytic machinery (21, 24). NPXY motifs interact with the μ2 subunit of the adaptor protein 2 complex (12, 61), which can recruit clathrin and trigger clathrin-mediated endocytosis (47).

Since clathrin-dependent mechanisms have been implicated in reovirus cell entry (31), it seems plausible that reovirus engagement of β1 integrin leads to clathrin-mediated endocytosis through signaling regulated by the β1 integrin cytoplasmic domain. It is noteworthy that Kaposi's sarcoma-related herpesvirus binding to α3β1 integrin (1) and cytomegalovirus binding to β1 integrin (32) activate focal adhesion kinase. In addition, adenovirus engagement of αv integrins induces activation of phosphoinositide-3-OH kinase, which is required for adenovirus endocytosis (51).

Identification of β1 integrin as a receptor that triggers reovirus entry raises the possibility that coreceptor binding influences reovirus tropism and disease. Reovirus serotypes differ in mechanisms of spread, tropism for cells in the central nervous system, and disease outcome in the infected host (73).

Previous studies using reassortant genetics and comparative sequence analysis demonstrated that these phenotypes segregate most strongly with viral attachment protein σ1, suggesting that reovirus serotypes bind to different receptors (74, 76, 77). However, the σ1-encoding S1 gene is not the sole determinant of reovirus growth at some sites within the host. For example, the λ2-encoding L2 gene influences viral growth in the intestine (11) and spread to new hosts (46). Moreover, JAM-A functions as a receptor for all three reovirus serotypes (16); therefore, JAM-A cannot explain serotype-dependent differences in reovirus pathogenesis. The presence or absence of particular integrins at distinct physiologic sites may critically influence the course of reovirus infection. In support of a role for coreceptor utilization in reovirus growth, reovirus infection can occur in the absence of σ1 (19) or JAM-A (5), albeit at greatly reduced efficiency. These findings highlight the complex nature of reovirus attachment and entry and suggest that reovirus tropism and pathogenesis are not dictated by primary receptor interactions alone. It is possible that tropism and pathogenesis are determined by the concerted action of attachment and internalization receptors, perhaps not all of which have been discovered.

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APPENDIX B

REOVIRUS INFECTION OF WILD-TYPE AND α2 INTEGRIN KNOCKOUT MICE

Introduction

Virus-receptor interactions are essential for initiation of viral infection, yet the significance of these events in reovirus pathogenesis is not known. Type 1 and type 3 reovirus strains exhibit striking differences in viral tropism in the CNS (260). These phenotypic differences segregate with the σ1-encoding S1 gene segment, suggesting an important function for receptor engagement in tropism determination (250, 260). However, these differences cannot be explained by differential receptor engagement alone, as the only known σ1 head receptor, JAM-A, is a serotype-independent receptor (43). Furthermore, reovirus pathogenesis is not solely determined by the S1 gene. The reovirus λ2-encoding L2 gene segment segregates with viral shedding and spread to littermates (134). Since integrin-binding motifs RGD and KGE are expressed in the reovirus λ2 protein (37, 156), it is possible that integrin-based components of the cell entry apparatus cooperate with JAM-A to influence tissue tropism and disease outcome.

Preliminary evidence from our laboratory suggested that α2β1 integrin is involved in reovirus infection. An α2-specific MAb, AA10 (24), diminished reovirus infection in some experiments (data not shown). Additional evidence suggested that reovirus binds directly to the α2 I domain of a glutathione-S-transferase (GST)-α2 I domain construct by enzyme-linked immunosorbent assay (ELISA) (data not shown). I sought to determine whether integrins influence reovirus pathogenesis *in vivo*. β1 integrin KO mice are embryonically lethal (237). However, α2-null mice are viable, fertile, and do not exhibit phenotypic abnormalities (54). α2-null mice were made available to our laboratory by Dr. Mary Zutter. The goal of experiments described here was to use well-characterized viruses and mice that differ in α2 integrin expression to

determine whether integrin utilization is required for reovirus pathogenesis and test the hypothesis that a viral receptor contributes to reovirus tropism, spread, and disease outcome.

Results

To determine whether α2 integrin contributes to reovirus pathogenesis, neonatal wild-type and α2-null mice were infected perorally with reovirus strain T3SA+ (14, 177). T3SA+ is a sialic-acid binding strain that is capable of growth in the intestine and spread to distant sites of replication (16). Mice were inoculated perorally with reovirus strain T3SA+ at doses ranging from 10^2 to 10^8 PFU/ml or PBS as a control. Inoculum titer was confirmed by plaque assay (256). Mice were monitored daily for 21 days post-infection for signs of reovirus disease and weight changes. Virulence was determined by defining the dose at which 50% of the infected mice become moribund (LD_{50}) (207). The dose of T3SA+ that resulted in 50% mortality in the mice was between 10^5 – 10^6 PFU/ml for both wild-type and α2-null mice (Fig. 1). These data suggest that α2 integrin expression does not influence reovirus virulence in mice. Both wild-type and α2-null mice developed signs of illness including weight loss, ruffled fur, and neurological effects such as hind-limb paralysis.

Conclusions

These data suggest that α2 integrin is not required for reovirus virulence and disease. LD_{50} analysis is a gross assessment of virulence with fatal illness as an end result. Therefore, it is possible that integrins contribute to reovirus pathogenesis at a

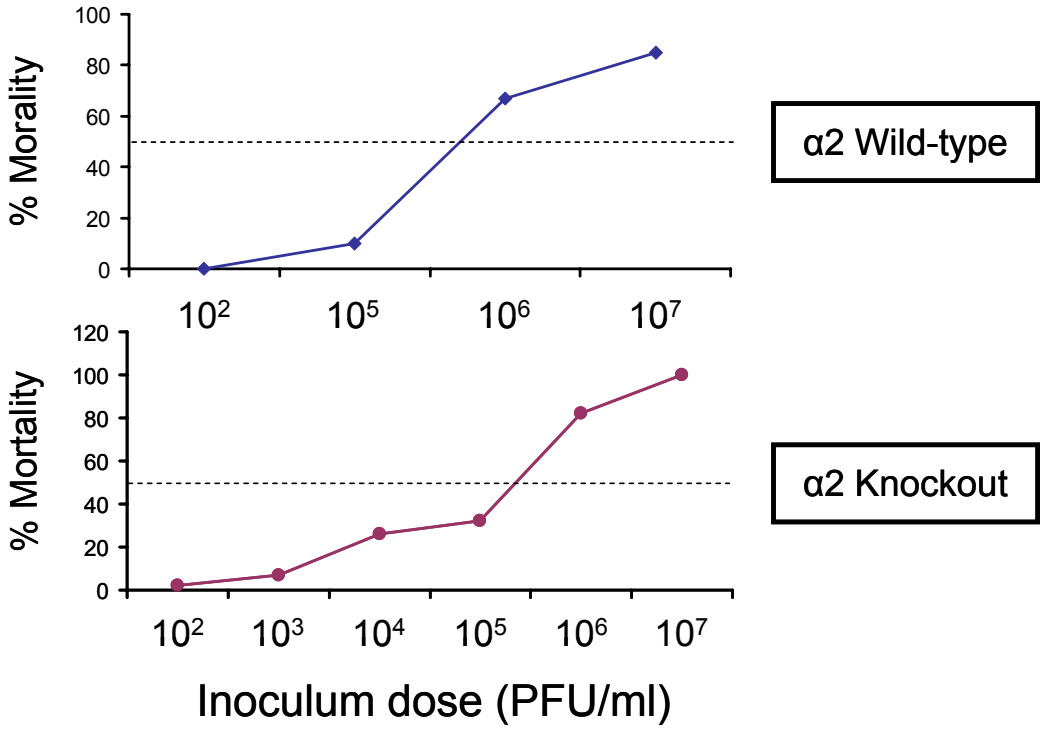

Fig. 1. The T3SA+ LD_{50} Dose is Equivalent in α2-null and Wild-type Mice. Neonatal α2-null and wild-type mice were inoculated PO with T3SA+ at a range of doses, 10^2 – 10^7 PFU/ml. Mice were monitored daily for weight changes and signs of illness. Mice that were moribund were euthanized. Data represent the % mortality of infected mice. The dotted line represents 50%. Each time point represents at least n≥4 mice.

tissue-specific level. Simultaneously, I performed further studies in the laboratory to determine whether α2β1 integrin is involved in reovirus infection. Studies using a larger panel of α2-specific antibodies failed to demonstrate a function for α2 integrin in reovirus infection (data not shown). It is possible that MAb AA10 has non-specific inhibitory effects on reovirus infection as the antibody is of the IgM isotype (24). Additionally, I performed extensive studies using ELISA and GST-precipitation assays to test for a direct interaction between the α2 I domain and reovirus. While there was modest binding of reovirus virions to the α2 I domain, binding was not inhibited by neutralizing reovirus antibodies or function-blocking α2 antibodies (data not shown), suggesting that the interaction was not specific. Furthermore, a preparation of L cells that were processed as a mock viral purification also were capable of modest binding to α2 I domain (data not shown), suggesting that a component of the viral purification may mediate binding to the α2 I domain, perhaps collagen. These data do not exclude the possibility that α2 integrin is required for reovirus infection. However, subsequent studies revealed that reovirus internalization is mediated by β1 integrin. Studies using mice altered in β1 integrin expression should be performed to determine whether integrins contribute to reovirus pathogenesis.

APPENDIX C

DEVELOPMENT OF ASSAYS TO DETECT REOVIRUS CELL ENTRY

Introduction

During my dissertation research I developed new methods to quantitate reovirus internalization and measure transcription as a marker for functional reovirus entry. The methods commonly used to assess reovirus infection are FFU assay and plaque assay. These assays are usually processed at 16-24 h or 24-48 h post-infection, respectively. However, I wanted to employ assays that would allow me to study viral entry events at earlier times. Studies using inhibitors of viral disassembly suggest that reovirus disassembly is complete within 30-45 mins post-adsorption (239), suggesting that internalization occurs rapidly after viral adsorption. Thus, I developed two techniques to study reovirus internalization. The first approach is a microscopy-based assay, which was developed with intellectual contributions from Aaron Derdowski and technical assistance from Sarah Kopecky-Bromberg. The second is an RT-PCR-based early infectivity assay (EIA), which was developed with intellectual contributions from Pranav Danthi and the technical assistance of Wesley Skelton.

Microscopy-Based Internalization Assay

A microscopy-based internalization assay was designed to quantify internalization of reovirus particles at early-times post adsorption. This method employs indirect immunofluorescence to assess internalization of reovirus particles into cells. Cells are plated onto glass coverslips and incubated overnight. Then cells are infected with reovirus virions and incubated at 4°C for 1h. Cells are washed with PBS and either fixed with 10% formalin or complete medium is added and cells are incubated over a time course of reovirus entry (0-45 min). Cells are fixed in 10% formalin for 20 mins, then

washed with PBS, and stored in PBS. Cells are incubated with 1% TX-100 for 5 mins, PBS-BGT for 10 mins, and then incubated with primary antibody, reovirus polyclonal antiserum (1:500) diluted in PBS-BGT at room temperature for 1 h. Cells are washed extensively with PBS-BGT, 3 times for 15 min, with rocking. Cells are then incubated with a fluorescently-conjugated secondary antibody (goat-anti-rabbit Alexa 488 or 546 [Molecular Probes]) in PBS-BGT at room temperature for 1 h, and then washed extensively. Coverslips are mounted onto slides using anti-fade polymount media and stored in the dark. Cells are analyzed by confocal microscopy using an LSM510 confocal microscope with Meta software.

Particle intracellular localization can be determined by obtaining confocal micrographs of Z-sections and evaluated using Z-stack analysis and 3D-image reconstruction. Pixel intensity can be measured for an individual cell using Metamorph software, providing a quantitative method to assess reovirus internalization. In the absence of Metamorph software, fluorescent particles can be enumerated manually. Since this assay does not detect new reovirus protein synthesis as in an FFU assay, it is necessary to use a high MOI to detect signal. I have performed the majority of these experiments using 50,000 particles per cell. This assay allows detection of reovirus particles internalized into the cell during very early times post-infection. However, this is not a functional assessment of reovirus infection, and thus should be paired with other methods to analyze infectivity. Examples of this assay can be seen in Chapters II (Fig. 14) and III (Fig. 17, 18).

Early Infectivity Assay (EIA)

To study early events in the reovirus life cycle by a method that would allow functional assessment of viral infection, I developed an RT-PCR-based protocol to quantify viral transcription. This assay was adapted from methods developed by Leary *et al.* to detect reovirus RNA using RT-PCR (140, 141). While this assay should be adjusted based on individual usage for each cell line, virus strain, and time point, the general principle should apply to studies of early events in the reovirus life cycle.

HeLa cells (10^6) are plated in a 60 mm dish and infected with virions or ISVPs of reovirus strain T1L at an MOI of 1,000 particles per cell. Cells are adsorbed with virus at 4°C for 1 h. Cells are then washed with PBS and either harvested or complete medium is added and cells are incubated at 37°C for 3-4 h. Cells are harvested by scraping in 1 ml of PBS and pelleted in a microfuge at 3,000 x g. PBS is aspirated and pellets are stored at -20 or -70°C until all time points are harvested. RNA is isolated from cell pellets using an RNeasy Mini Kit (Qiagen) and eluted in 30μl. Extracted RNA (5 μl) is utilized for a one-step RT-PCR (Qiagen) with β-actin-specific primers and reovirus λ3-encoding L1-specific primers. Resultant RT-PCR products are resolved by agarose gel electrophoresis in a 1% agarose gel and stained with ethidium bromide (Fig. 1). This method measures new viral RNA synthesis, as ribavirin, which inhibits viral transcription (206), inhibits RNA detected from cells infected with virions (Fig. 1). Ribavirin does not inhibit RNA detected from cells infected with ISVPs (Fig. 1). The lack of inhibition may be a result of the intensity of the amplicons in this experiment. However, since ISVPs enter cells more efficiently than do virions, the concentration of ribavirin used may not be sufficient to inhibit transcription by ISVPs. Additionally, treatment of cells with AC results in

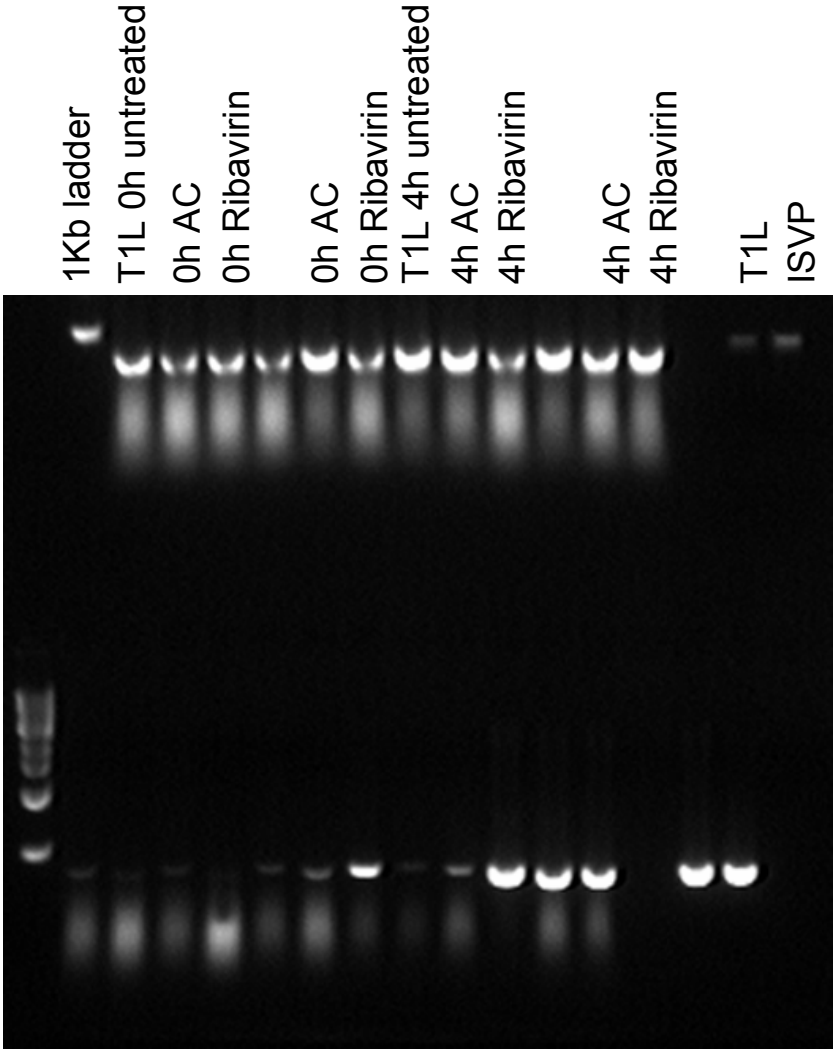

Fig. 1. Reovirus Early Infectivity Assay. $1x10^6$ HeLa cells were untreated or pretreated with 40mM AC at 37°C for 1 h. Cells were infected with virions or ISVPs of T1L at 4°C for 1 h, washed, and harvested (0 h) or complete medium with or without 200μM ribavirin or AC was added and cells were incubated at 37°C for 4 h. Cells were washed, scraped in PBS, and pelleted at 2,000 x g for 5 mins. PBS was aspirated, and RNA was isolated from cells and used as template for RT-PCR using β-actin- (control) (top) and reovirus L1-specific primers (bottom). 15μl of RT-PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide. T1L and ISVP represent input viral particles prepared as described as positive controls.

diminished detection of RNA by virions but not by ISVPs (Fig. 1). However, a slight amount of RNA can be detected at 0 h, especially with higher MOIs or when using ISVPs, suggesting that this assay is sufficiently sensitive to detect genomic RNA when a high MOI is used. This approach provides a new technique to detect early events in the reovirus life cycle. This assay should be adapted for quantitative PCR. The RT-PCR conditions and primer sequences are listed below.

RT-PCR conditions:
Preheat block to 50ºC
50ºC 30 min
95ºC 15 min
94ºC 1 min -----I
55ºC 1 min I 35 cycles
72ºC 1 min -----I
72ºC 10 min
4ºC ∞

L1 primers
L1.rv5m: 5' CTGCATCCATTGTAAATGACGAGTC 3'
L1.rv4m: 5' GCTATGTCATATTTCCATCCGAATTC 3'

Human B-actin
B-actin L AGAAAATCTGGCACCACACC
B-actin R CCATCTCTTGCTCGAAGTCC

Conclusions

The microscopy-based internalization assay provides a method to study reovirus internalization. The RT-PCR-based EIA provides a method to study functional reovirus entry, as viral transcription is assessed, as early as 3 h post-infection. These assays establish new ways to study early events in reovirus entry and can be adapted for future studies.

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