THE ROLE OF BAD PHOSPHORYLATION STATUS AND BINDING PARTNERS IN PROMOTING APOPTOSIS

By

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ABBREVIATIONS

3A	BAD S112A S136A S155A
4A	BAD S112A S128A S136A S155A
ATM	ataxia-telangiectasia, mutated
ATR	ataxia-telangiectasia, mutated (ATM) and Rad3-related
BH3-only	Bcl-2 homology 3-only
BMK1	big MAP kinase 1
c-IMDM	Complete IMDM
DNA	deoxyribonucleic acid
ERKs	extracellular signal-regulated kinases
FBM	3xFlag-BAD-myc construct
FBS	Fetal Bovine Serum
Glu	L-Glutamine
Hrs	hours
IL3	interleukin-3
IMDM	Iscoves Modified Dulbeccos Medium
IP	immunoprecipitating
JNK	c-jun N-terminal kinases
LB	Luria-Bertani Media
MAPK	Mitogen Activated Protein Kinase
NEB	New England Biolabs
PBS	Phosphate Buffered Saline

PCR	polymerase chain reaction
PKA	protein kinase A
PP2A	protein phosphatase 2A
P/S	Penicillin/Streptomycin
S	Serine
T	Threonine
TCA	Trichloroacetic acid
UVB	Ultra-Violet B
µl	microliter
WT	wild type

CHAPTER 1

INTRODUCTION

Bcl-2 Family Members

The Bcl-2 family of proteins are key regulators of caspase-dependent and caspase-independent apoptosis. Stressors such as the activation of the cell surface death receptor and DNA damage are known to activate caspases, which in turn stimulate cell death [1]. Classified according to function and structure, the Bcl-2 family of proteins have been divided into three categories: anti-apoptotic (i.e. Bcl-2, Bcl-xl), proapoptotic (with multiple domains—Bax, Bak), and Bcl-2 homology 3 (BH3)-only (i.e. Bid, Puma, Bad) [1].

Many BH3-only proteins have been extensively studied with regards to cell death signaling and cell cycle. Bid has been shown to affect the S phase checkpoint after DNA damage by ataxia-telangiectasia mutated (ATM) [2, 3]. BAD binds only selective subsets of antiapoptotic proteins, namely Bcl-2 and Bclx. Furthermore, BAD dephosphorylation at site S112 has been described as a "gatekeeper" for cell death activation [4, 5]. Recently, it was suggested that BH3-only proteins engage the antiapoptotic relatives which regulate multidomain proapoptotic proteins (Bax and Bak) to induce apoptosis [6] [7].

BAD Phosphorylation Status Regulates Function

The phosphorylation status of BAD is key to its activity [4]. Reported phosphorylation sites on murine BAD include S112, S128, S136, S155, S170, and T201[4, 8, 9]. BAD S128, S136, and T201 have all been proposed to be key regulators of apoptotic activity[4, 8]; some upstream kinases for these sites have been identified. Pim 3 has been reported to phosphorylate BAD predominantly at S136, but also S155 and S170, and least intensely S112 [10]. PKA is known to phosphorylate BAD at S112 and S155 to enhance cell survival [11]. AKT is an important kinase that phosphorylates BAD at S136 [12]. CDC2 is known to phosphorylate BAD at S128 [13], while another report identifies JNK to phosphorylate T201 on BAD [9]. While one report suggests that JNK phosphorylates BAD at S128 [14], another refutes that claim [15].

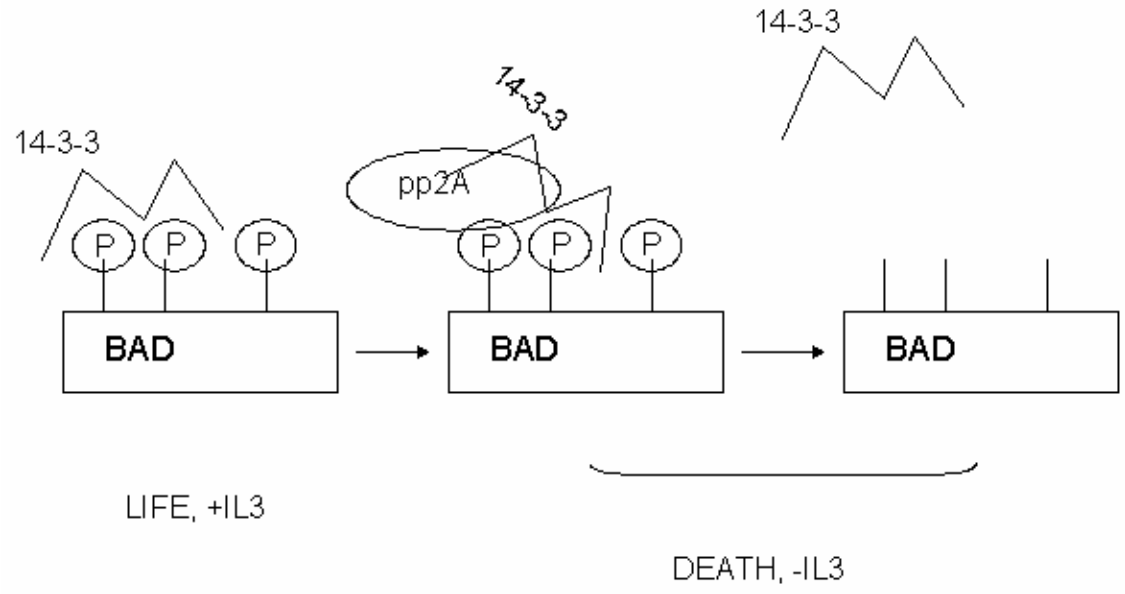

Figure 1: Model of BAD regulation [4]. Under +IL3 conditions, 14-3-3 caps the S112 and S136 phosphorylated BAD sites (shown also is phosphorylated S155). During induction of cell death, 14-3-3 loosens its tight binding to BAD, exposing the S112 site to PP2A. Upon S112 dephosphorylation, 14-3-3 dissociates, and S1136 and S155 are subsequently dephosphorylated under conditions of cell death.

The phosphorylation status of BAD impacts its binding partners; 14-3-3 is known to bind and protect BAD phosphorylation sites S112 and S136, sequestering BAD in the cytoplasm (Figure 1). However, under conditions of cell death, 14-3-3 becomes loose and exposes the phosphorylated S112 site to phosphatases. Upon PP2A dephosphorylation of S112, 14-3-3 detaches from BAD and S136 and S155 sequentially get dephosphorylated. The dephosphorylated BAD translocates to the mitochondria to bind Bcl-xL [4]. In one study conducted in neuronal cells, a cell-cycle dependent dissociation of BAD with 14-3-3 was attributed to BAD phosphorylation at S128 [16].

14-3-3 is a Regulatory Protein

14-3-3 proteins are a family of dimeric proteins that oversee eukaryotic cellular functioning in areas including protein kinase signaling pathways, apoptosis, and cell-cycle progression [17]. The dimeric structure of 14-3-3 facilitates its ability to act as a phosphoserine/phosphothreonine-binding protein [18, 19] [17]; the binding of 14-3-3 to a conserved phospho-motif site protects the phosphorylation sites and directs the bound protein's function. Additionally, 14-3-3 can function as an adaptor protein bridging two proteins [17]. This protein-protein interaction facilitates the activation of MAPK signaling pathways, prevents apoptosis, and maintains DNA damage checkpoints.

It is known that 14-3-3 can undergo post-translational modifications by phosphorylation [19]. Phosphorylation of 14-3-3 by JNK at S184 [20] releases 14-3-3 from BAD *in vitro*[21]. Furthermore, 14-3-3 monomers have been reported to be phosphorylated at S58, which prevents dimerization, but does not prevent binding to a downstream target [18].

Mitogen Activated Protein Kinase (MAPK) Family

The MAPK family plays prominent roles in transducing extracellular signals in processes such as cell growth, survival, and death [22]. At least three subgroups of the MAPK family have been described: the extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinases (JNK), and p38 MAPK [23]. While both JNK and p38 MAPK respond to stress such as ultraviolet radiation, heat, and inflammatory cytokines, ERK activation is centered on mitogenic stimuli including cytokines [22]. In order for ERK, JNK, or p38 to become activated, specific tyrosine and threonine motif residues must be phosphorylated [22].

While p38 has been loosely linked to promotion of cell growth in some cell lines, overwhelming evidence suggests that p38 is linked with apoptosis [23]. One study has suggested that p38 acts downstream of caspase activation [23]. In another, employing doxorubicin-induced endothelial cell death, p38 has been shown to inhibit BAD phosphorylation in a PI3K/Akt-dependent manner, thereby contributing to cell death [24].

JNK has also been linked with cell death, particularly modifying protein-protein interactions to yield a specific response. JNK has been reported to phosphorylate 14-3-3 to disociate it from its binding partner c-Abl, resulting in c-Abl translocation to the nucleus [25]. JNK has been reported to phosphorylate BAD at T201 thereby promoting cell survival [9]. A report has suggested that reactive oxygen species, which contribute to apoptosis signaling, is at least partially mediated by activated JNK [26].

Summary

Although the signaling mechanisms of the apoptotic pathway have been extensively studied, there is still much left unknown. Because key phosphorylation sites on BAD (and hence it's proapoptotic function) is under tight regulation by 14-3-3, this thesis examined the mechanism of the BAD-14-3-3 complex dissociation. It was hypothesized that a novel phosphorylation event occurring either on 14-3-3 or BAD, or the interaction of an unknown binding partner may aid in the dissociation of 14-3-3 to BAD under conditions of cell death. Methods used in these investigations included: Mass Spectrometry, kinase assays, pulldown experiments, and western blots. Several novel binding partners to BAD were identified in either a condition of cell life or cell death, including: p38, CDC2, Beclin-1**,** BAX, cytochrome c1 oxidase, and voltage-dependent anion-selective channel proteins 1, 2, and 3. ^{32}P kinase assays suggested that p38 phosphorylates a novel site on BAD, serine 6 (S6). The phosphorylation state of BAD S128 was observed 3-7 hours after cell death stimulation and was independent of p38 activation. Under cell life and death conditions, no new phosphorylation sites or monomeric changes on 14-3-3 were observed; p38 was concluded not to phosphorylate 14-3-3. Future studies examining the novel S6 BAD phosphorylation site may yield insights on the regulation and functional significance of this event in apoptosis.

CHAPTER II

MATERIALS AND METHODS

Cell Culture and Reagents

The hematopoietic pro-B cell line FL5.12 were grown in Iscoves Modified Dulbeccos Medium (IMDM, Sigma #i3390) media supplemented with 10% Fetal Bovine Serum (FBS, Gibco #10437-028), 1% Penicillin/Streptomycin (P/S) (BioWhittaker #17-602E), 1% L-Glutamine (Glu, BioWhittaker #17-605E), and Interleukin-3 (IL3). NIH 3T3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma #D5796) media supplemented with 10% calf serum (BioWhittaker, 14-401F), 1% P/S, and 1% Glutamine.

The chemical inhibitors to p38 (SB203580) and CDC2 (Olomoucine, sc-3509) were from Santa Cruz biotechnology; the JNK inhibitor SP600125 was obtained from Alexis Biochemicals. Total BAD mouse antibody was from BD Transduction Laboratories (#610392), while 14-3-3β K-19 rabbit antibody was obtained from Santa Cruz Biotechnology (sc-629). Anti-Bad phospho-Serine 128 antibody was obtained from three sources: originally from the Bonni lab (Harvard University), BioSource (#44-523G), and Chemicon (AB3567). Total p38 (A-12) antibody was from Santa Cruz (sc-7972); active p38 was obtained from Cell Signaling (9211S). Commercial HisBAD (#14-281) and purified kinase p38 came from Upstate (#14-210), and purified CDC2 from New England Biolabs (NEB, #P6020S). PKA was a generous gift from Dr. Jackie

Corbin (Vanderbilt University). Isoelectric (pI) strips were from BioRad, while the colloidal blue kit was from Invitrogen. Glycine was obtained from MP BioMedicals.

Cell Death Assay

It is known that removing the cytokine IL3 from the media results in cell death; for experiments examining potential changes during cell death, the FL5.12 cells were washed three times with PBS, plated in –IL3 media (IMDM supplemented with 10% FBS, 1% P/S and 1% Glu), and grown in an incubator kept at 37°C with 5% CO_2. 3T3 cells were arrested as previously reported [27].

Generation of HisBAD mutant constructs

Wild type (pcDNA BAD WT) or 112, 136, 155 serine to alanine mutation (3A) (pMSCV IRES GFP BAD 3A) templates were used as polymerase chain reaction (PCR) template deoxyribonucleic acid (DNA). The following internal primers were used to introduce the 128A mutation (in bold, all written 5'->3): forward-ATGGAGGAGGAGCTT**GCC**CCTTTTCGAGGA; reverse-TCCTCGAAAAGG**GGC**AAGCTCCTCCTCCAT. These PRC reactions were carried out at 94° for 10 minutes, 20 cycles of 94°, 50°, and 72° each for one minute, extended at 72° for 5 minutes, and kept indefinitely at 4°. Once the BAD sequence contained the desired point mutations, the following primers were used to introduce desired restriction sites for pQE-30 subcloning: forward-AAAAAAGCATGC(Sph1)GGATCC(BamH1)ATGGGAACCCCAAAGCAGCCCTCG; reverse-

AAAAAAAAACTCGAC(Sal1)AAGCTT(Hind3)TCACTGGGAGGGGGTGGAGCCT CCTTT. Different primers were used to introduce other restriction enzymes into the pMSCV vector: forward (with 6xHis tag)- AAGAATTC(EcoR1)CTCGAG(Xho1)GCCGCCACC(Kozak)ATGCATCACCATCAC CATCAC(6XHis)ATGGGAACCCCAAAGCAGCCCTCG; forward (with no 6xHis tag): AAGAATTC(EcoR1)CTCGAG(Xho1)GCCGCCACC(Kozak) ATGGGAACCCCAAAGCAGCCCTCGCCCTCG; reverse- AACTCGAC(Sal1)GAATTC(EcoR1)TCACTGGGAGGGGGTGGAGCCTCCTTT. PCR conditions were carried out at 94° for 10 minutes, 35 cycles of 94°, 55°, and 72° each for one minute, extended at 72° for 5 minutes, and kept indefinitely at 4° for all constructs except the 4A or 128A creations. PCR products were then analyzed on ethidium bromide stained 1% agarose gels, gel extracted (Qiagen QIAquick gel extraction Kit #28704), ligated into the Topo vector (Invitrogen Topo TA Cloning kit #45-0640), transformed into competent DH5α cells, plated on X-gal treated ampicillin plates, and grown overnight at 37°. White colonies were picked and grown in Luria-Bertani Media (LB) for less than 16 hrs at 37° with 250 rpm shaking. Plasmid DNA was purified (Qiagen miniprep) and digested with either Xho1/EcoR1 for pMSCV-bound inserts or Sph1/Hind3 (from New England Biolabs, NEB) for pQE-30 inserts, and placed at 37° for 2 hrs or more. Completed digestions were then run on an ethidium bromide stained agarose gel; colonies that appeared to contain the correct sized insert were then sent to the Vanderbilt Sequencing Core facility for sequence verification. Once sequence verification was obtained, Topo colonies were digested with appropriate pairs of

endonucleases, gel extracted, and ligated into either pQE-30 or pMSCV for further use in bacteria or cells.

Construction of Cell Lines

FL5.12 cells over expressing 3xFlag-BAD-Myc (FBM) or different His tagged BAD mutations (4A, 128A, WT with no His tag, and WT) were created using retroviral infection as previously described [27]. The His-tagged BAD mutations were introduced into FL5.12 parental cells and cells already overexpressing BCL-xL. The FBM construct was kindly made by Dr. Kwong-woon Kim (Vanderbilt University). Briefly, 10^5 cells were combined with 500 µl purified virus and 1 µl polybrene. Complete IMDM (c-IMDM) media was added to this mixture to bring the total volume to 1 ml. The cell mixture was gently pipetted and spun at 11,000 rpm for 60 minutes at room temperature, then pipetted to resuspend the cell pellet, and added into 4 ml complete IMDM with 4 µl polybrene, and incubated in an incubator kept at 37°C with 5% CO_2. This process was repeated three times, grown overnight, and then selected with 2 µg/ml puromyocin. HisBAD selected cells were pooled for further use. A high expresser of FL5.12 FBM cells (M58) was further isolated by clonal selection.

Kinase Assays

To perform hot kinase assays, varying amount of substrate (0.2-1µg) was incubated in kinase buffer (containing an end concentration of 50mM Tris pH 7.4 and 10 mM magnesium chloride), cold ATP (final concentration 0.2µM), and either purified kinase (p38 or CDC2) or total cellular lysate, at room temperature for 10 minutes. Five

μCi ^{32}P-γ-ATP (Perkins Elmer, Blu 502 H250uc) was added to the mixture and incubated at 30°C for 30 minutes. If necessary, GST or His pull down was then performed, beads were washed three times in either isotonic buffer (containing 0.25% NP40, 10mMHepes pH7.5, 5mM $MgCl_2$, 132.5 mM KCl) or nickel wash buffer (containing 20-40mM imidizole, 300mM NaCl, 50mM sodium phosphate pH7.8), 4x Laemmli sample buffer added, loaded on a 12.5% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a Polyvinylidene fluoride (PVDF) membrane (MidSci #10-485-289) and visualized by autoradiography. To perform cold kinase assays, the same steps were followed, omitting the addition of radiolabeled ^{32}P ATP, and followed with western blotting with particular phospho-specific antibodies.

Pull Down Experiments

Cellular lysate was made by collecting cells, washing 3 times in PBS, adding isotonic buffer to the cell pellet, nutating the eppendorf at 4° for 20 minutes, performing a high speed (13k rpm) spin and collecting the supernate. For all pull down experiments (GSH, microcystin, Flag, nickel), this total lysate was precleared with 10% sepharose beads (Sigma #2B300-100ml) incubated with agitation for 2 hours at 4°, spun at 13,000rpm in a tabletop centrifuge, and supernate collected. This precleared total lysate was then used for subsequent pull down experiments.

GST-BAD pull downs were preformed on total lysate from FL5.12 cells over-expressing GSTBAD or GST alone. Ten percent the total lysate volume of GSH beads slurry (50/50 in isotonic buffer) was added to the pre-cleared cellular lysate and incubated with agitation for 2 hours at 4°. After a high speed spin to pellet the GSH

beads, the beads were washed with isotonic buffer three times, and eluted in 100mM glutathione, 100mM Tris pH 7.5, 150mM NaCl. Thrombin was a generous gift from members in Dr. Paul Bock's lab (Pathology Department, Vanderbilt University). Thrombin cleavage to remove the GST tag from BAD in the GSTBAD constructs was preformed in accordance with the method proposed in Current Protocols in Molecular Biology, Enzymatic and Chemical Cleavage of Fusion Proteins, Supplement 28, 16.4.8.

For microcystin pull downs, microcystin conjugated to beads (Upstate #16-147) added to pre-cleared cellular lysate, incubated with agination at 4° for 2 hrs, spun at 13,000rpm in a tabletop centrifuge, supernate aspirated, beads washed three times with isotonic buffer, and beads collected. Beads would then either be western blotted or pulled down again to determine the presence or absence of suspected binding partners.

Flag pull downs were performed on the high expressing FL5.12 FBM cell line (M58) or pMSCV vector alone (p11). Ten percent the total lysate volume of Flag bead (Sigma #A2220) slurry (50/50 in isotonic buffer) was added to the pre-cleared cellular lysate and incubated with agitation for 2 hours at 4°. After being spun at 13,000rpm in a tabletop centrifuge to pellet the flag beads, the beads were washed with isotonic buffer three times, and eluted in 0.1 mg/ml freshly made 3x flag peptide (Sigma #F4799).

A reconstituted nickel chelated bead (Pierce #89827) in isotonic buffer (resulting in 50/50 slurry) was used to pull down His-Bad constructs expressed in FL5.12 cells. Total lysate was incubated with agitation with 10% volume His beads at 4° for 15 minutes, pulse spun at 13,000rpm, washed three times with a 20-40mM imidazole buffer (optimized for experimental conditions), and aspirated with a 30G1 needle (BD #305128).

Immunoprecipitation (IP) of over-expressed or endogenous proteins was also used to extract a specific protein complex from total lysate. Bad C-20 (goat, Santa Cruz, sc943) was added to precleared cellular lysate at a concentration of 3µg antibody/200µg total lysate protein (approximately 1ml volume) and incubated with agitation at 4° for 2 hours. Ten percent protein A and G sepharose in isotonic buffer was then added to the total lysate/antibody slurry and incubated with agitation for another hour at 4°. Beads were then spun down, washed three times with isotonic buffer, sucked dry with a 30G1 needle, and prepared to be run on an SDS-PAGE gel with the addition of 4 x Laemmli sample buffer with 2-mercaptoethanol added to the dry beads.

Cross-linking Protocol

Aqueous soluble cross linkers (DTSSP and BS^3, Pierce) were employed to determine nearby proteins. Powdered cross linker was added directly to pre-cleared total lysate to a final concentration of 5mM, incubated with agitation for 2 hours at 4°, and then quenched with the addition of Tris-Cl pH 7.5 to a final concentration of 50mM for 15 minutes at room temperature. After quenching, a standard immunoprecipitation was carried out.

Immunoblot Analysis

One-dimensional SDS PAGE gels were run as previously reported [4]. Silver staining was performed following a protocol received from Dr. David Friedman [28]. Native gels were run following the same protocol as SDS PAGE gels, except all SDS was

removed from the reagents making the lower, upper, Laemmli loading buffer, and running buffers.

Proteomics Analysis

The Mass Spectrometry Proteomics Core was essential for carrying out and analyzing many technically advanced methods including the following: coating of SDS-PAGE glass plates with Bind-Silane for subsequent Sypho Ruby picking, cy2/3/5 imaging, extraction of protein gel bands, trypsin and chymotrypsin protein digestion, MS/MS and LC/MS running and analysis of samples, amino acid sequence analysis, phosphor-peptide identification, and focusing GSTBAD eluate samples on isoelectric strips for the commencement of a 2D gel. All of these procedures were followed according to their in-house protocols.

Ultra-Violet B (UVB) Radiation Experiments

UVB experiments were performed by resuspending FL5.12 cells in 200µl complete IMDM media, plating these cells in 5 cm plates, exposing the uncovered dished to 60 µJ UVB radiation using a UV Stratalinker 1800 machine (Moses lab, Vanderbilt University), adding 4.8 ml c-IMDM to the plate under sterile conditions, and growing DNA damaged cells at 37°C with 5% CO_2 for set time points.

CHAPTER III

RESULTS

Techniques examining BAD localization and GSTBAD complex binding partners

The dissociation of 14-3-3 from BAD under conditions of cell death regulates BAD's proapoptotic activity. The dissociation of the BAD-14-3-3 binding event under conditions of cell death was hypothesized to involve the interaction of an unknown binding partner. In this section, we examined and identified novel binding proteins to BAD under conditions of cell death and cell life.

To examine the localization of BAD under conditions of cell death when protein phosphatase 2A (PP2A) has been reported to be bound, a PP2A inhibitor vector (pARP) was used. After transient transfection with plasmids expressing pARP, which is localized either to the mitochondria or cytosol, NIH 3T3 cells were washed with PBS and placed in DMEM media without serum. The cells were then collected at defined times, lysed, run on a SDS-PAGE gel, and western blotted for BAD dephosphorylation. While cell death was observed using trypan blue hemocytometer counting, during the serum withdraw time BAD dephosphorylation (112, 136, 155) was not detected via western blot. Because a reliable model for cell death in 3T3 cells could not be established, we could not reasonably pursue our original question of BAD localization while PP2A was unbound.

In order to identify candidate proteins binding to BAD, we used GST-BAD fusion protein pull downs. Aqueous-soluble cross linkers (DTSSP and BS^3) were individually added to the GSTBAD total lysates to a final concentration of 5mM, run on a SDS PAGE

gel, western blotted for known binding partners and/or silver stained. While western blot analysis detected the absence of 14-3-3 under cell death conditions, as expected, silver stained results did not visually yield notable differences between conditions. Thinking that perhaps novel binding proteins may exist but were just not visible by silver stain, a shotgun approach was employed to determine potential binding partners. With the help of the Mass Spectrometry Proteomics Core, a one-dimension SDS-PAGE gel of GSTBAD eluate samples from + and –IL3 conditions were stained with colloidal blue (Figure 2A). Bands that appeared in one condition or the other were excised and identified using MS/MS. The results yielded an extremely large overlap between the two conditions with only one protein, Rab1A (an ER localization protein involved with trafficking to and from the lysozome), emerging unique to in the –IL3 sample. However, because two other Rab family members were identified in both experimental conditions (Rab11B and Rab7), and the unlikely co-localization of Rab 1A and BAD, these results were not pursued.

Thinking that perhaps employing a difference of isoelectric points may help yield novel binding partners, a two dimensional approach was also attempted (Figure 2B) to examine bound proteins that may change under cell life and death conditions. Eluates from GSTBAD expressing cells under cell life (+IL3) and death (-IL3 5hrs) conditions were used. Samples were focused on a 3-10 pI strip, resolved in the second dimension on a 12.5% SDS-PAGE gel, and then stained for total proteins with fluorescent dyes (cy2/cy3/cy5). However, again, there were no novel differences between the two conditions. Using the DIGE 2D method to examine binding partners to BAD did not produce any candidate proteins.

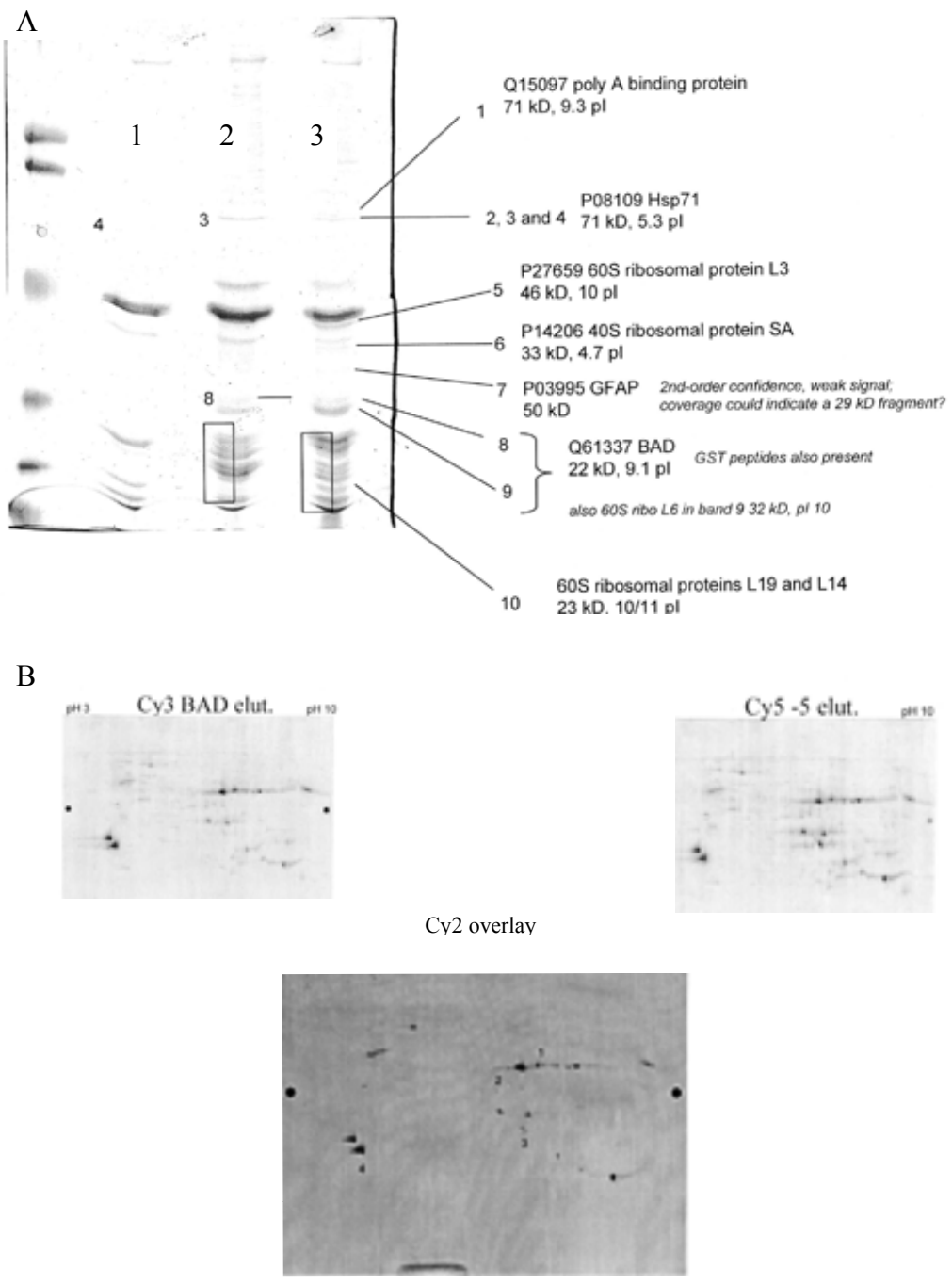

Figure 2: Detection of different binding partners to GSTBAD under cell life and death conditions. A) Colloidal blue stain of GSTBAD eluate under + and – IL3 conditions. Indicated areas were picked and/or digested and sent for peptide analysis. 1=GST; 2=GSTBAD +IL3; 3=GSTBAD –IL3 4hrs. B) No difference observed by 2D cy3/5 experiment between cell life or death (-IL3 5hrs) conditions. GSTBAD was pulled down from cells grown under the indicated conditions, and eluted. The eluate was then run on a pI3-10 strip in the first dimension and on a 12.5% SDS-PAGE gel in the 2nd dimension, and imaged fluorescently.

As a large amount of non-specific binding was thought to occur on the GST tag of the fusion protein, and hence when eluting off the GSH beads these nonspecific proteins would contaminate the BAD complex results, we then tried to release BAD from its GST tag using a specific protease, thrombin. Thrombin was added to pulled-down GSTBAD; however, thrombin cleavage was inefficient in cutting only the GST tag from GSTBAD, and resulted in a highly degraded BAD protein.

Using a double pull down—starting with a GSTBAD pull down, eluting, then following up with a BAD IP on the GSTBAD complex, and acid eluting off the GSTBAD complex—was the first attempt to clean up the GSTBAD eluate. While this approach was attempted at three different temperatures (4°, 25°, and 34°C), all were unsuccessful, as shown in Figure 3. As there were problems in getting the GSTBAD protein complex to acid elute off, other strategies were ultimately devised to twice-purify the BAD complex.

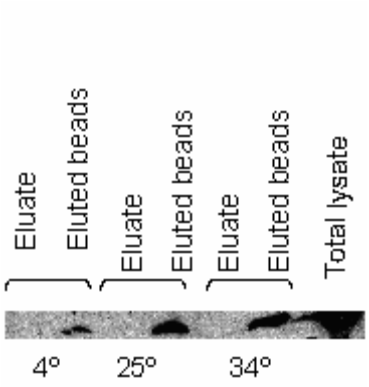

Figure 3: Unsuccessful GSTBAD acid elution. GSTBAD pulled down on GSH beads was subjected to 100mM glycine pH1.8 at the designated temperatures for 30', vortexing every 5', and western blotted for BAD.

p38 and CDC2 identified as binding partners of BAD

FL5.12 cells expressing a double tagged BAD construct (3X Flag-BAD-Myc) were then employed. While the flag pull down and elution were successful (Figure 4A),

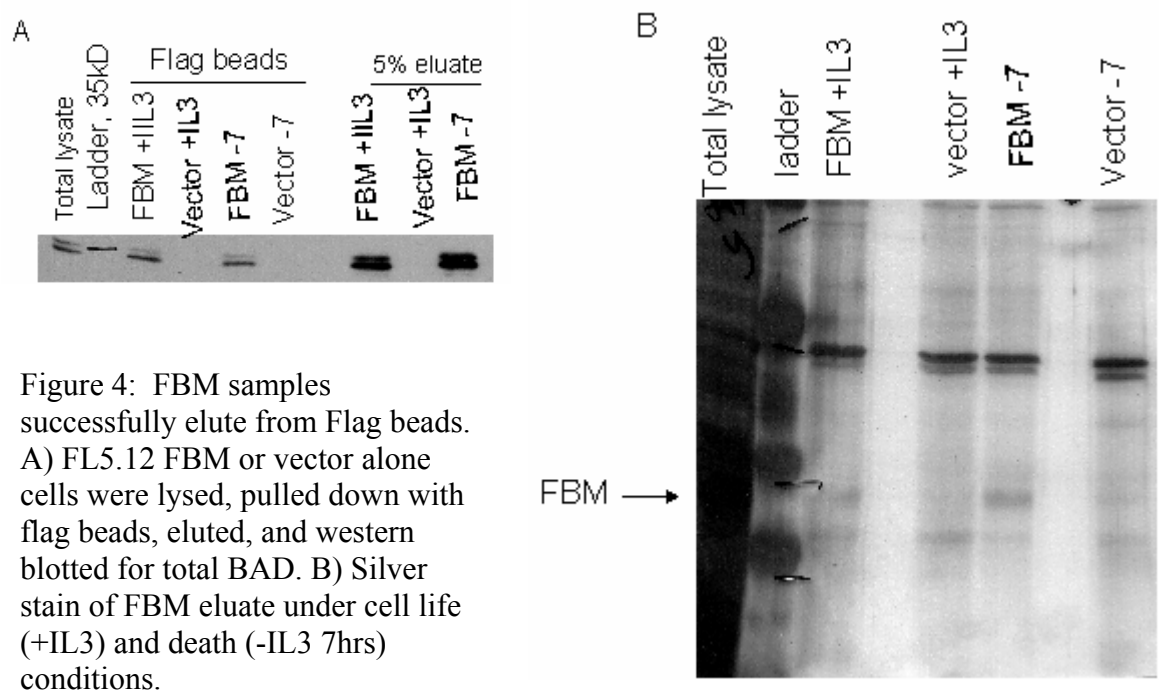

Figure 4: FBM samples successfully elute from Flag beads. A) FL5.12 FBM or vector alone cells were lysed, pulled down with flag beads, eluted, and western blotted for total BAD. B) Silver stain of FBM eluate under cell life (+IL3) and death (-IL3 7hrs) conditions.

Table 1: Proteins identified via Mass Spectrometry analysis of FBM complex. Cells were grown under + or -IL3 (7hrs) conditions, collected, lysed, precleared, flag-pulled down, 3xflag peptide eluted, and sent to Mass Spectrometry on 3 different occasions.

+	-7
Adseverin	BAX alpha
Cdc2	Beclin-1
Cullin associated NEDD8	Coronin-1B
Cytochrome c1 oxidase	Filamin A
Dynamin-2	GRIP1-associated coactivator 63
FK506-binding protein 4	Inducible 6-phosphofructo-2-kinase
Flightless	Profilin 1
Importin	Protein FAM3C precursor
Serine/threonine protein kinase PLK1	Psmd1 protein
Serine/threonine protein kinase 38	Ran-binding protein 2
Serine/threonine protein kinase SNF-1 like kinase 2	Reticulon protein 3
Serine/threonine protein kinase VRK2	Ribose-phosphate Pyrophosphokinase I
Voltage-dependent anion-selective channel proteins 1, 2, and 3	Transgelin-3

eluting off the subsequent myc pull down was consistently unsuccessful. Given that the myc beads may introduce another host of non-specific binding partners, a flag pull down and elution sample (a representative silver stain may be seen in Figure 4B) was sent to the Vanderbilt Proteomics Core for Mass Spectrum analysis for identification of binding partners on three different occasions. While there were not overt visual differences from the silver stain eluate beside the presence or absence of the ~35kD FBM protein, the proteomics analysis yielded a spectrum of differences; Table 1 shows a list of screened results. Based on suggestions in the literature, two kinases, CDC2 and p38, were then targeted as candidate proteins and followed up. CDC2 has been reported to phosphorylate BAD at serine S128 [13].

To validate the interaction of CDC2 and p38 with BAD in cells, overexpressed BAD, GSTBAD, or HisBAD was immunoprecipitated, pulled down with GSH or nickel beads from total lysate (under survival and death conditions) and immunoblot for the candidate p38 and CDC2 proteins. As the results suggested no binding between BAD and p38 under either condition, we then employed crosslinker (DTSSP) to the total lysate before IPing BAD for western blot analysis. Even using the crosslinker, there was no evidence of p38 interacting with BAD. Conversely, p38 was IPed from total lysate and underwent western blot analysis for BAD, again showing no support for BAD-p38 binding under cell life or cell death conditions. While p38 was present on the HisBAD nickel pull downs, the binding was concluded to be nonspecific as the negative control (nickel beads incubated with total lysate that contained no His-tagged proteins) also contained the p38 band. While p38 was identified in a mass spectrometry screen for BAD binding partners, there is no western blot evidence that p38 binds to BAD. As many

kinase substrates can be transient, it is reasonable to conclude from these data that the p38-BAD interaction is very transient.

p38 phosphorylates a novel site on BAD

As key phosphorylation events on BAD dictate the protein's function, we then examined if any of the novel binding partners identified in Table 1 may phosphorylate BAD. Starting *in vitro*, we then pursued the possibility of p38 being a novel kinase of BAD. A hot kinase assay was performed using purified GSTBAD and p38 kinase. The results (Figure 5A) suggested that while there was a modest phosphorylation of wild-type BAD and a GSTBAD 128A mutant by p38, when 112, 136, and 155 serine sites were all mutated to alanine (3A), p38 greatly phosphorylated BAD. This membrane was then western blotted for the phosphorylation of S128 BAD and total BAD. Figure 5A suggested that while CDC2 does phosphorylate BAD at S128 as reported [13], p38 phosphorylated BAD on a site that is not 128. While this hot kinase assay result was repeated numerous times, and at all times there has been a strong signal of p38 phosphorylating GSTBAD 3A, the WT and 128A proteins were not always phosphorylated by p38. A HisBAD 4A (112, 128, 136, 155) construct was then made and also underwent a hot kinase assay with p38; due to subcloning difficulties the HisBAD 3A was unavailable for this experiment, so the GSTBAD 3A was used. As seen in Figure 5B, the 4A mutation was not phosphorylated by p38. However, as neither was the wild type HisBAD in this experiment, the result is difficult to interpret. Combined, these data suggested that p38 may phosphorylate a novel site on BAD, one that is not serine 112, 128, 136, or 155.

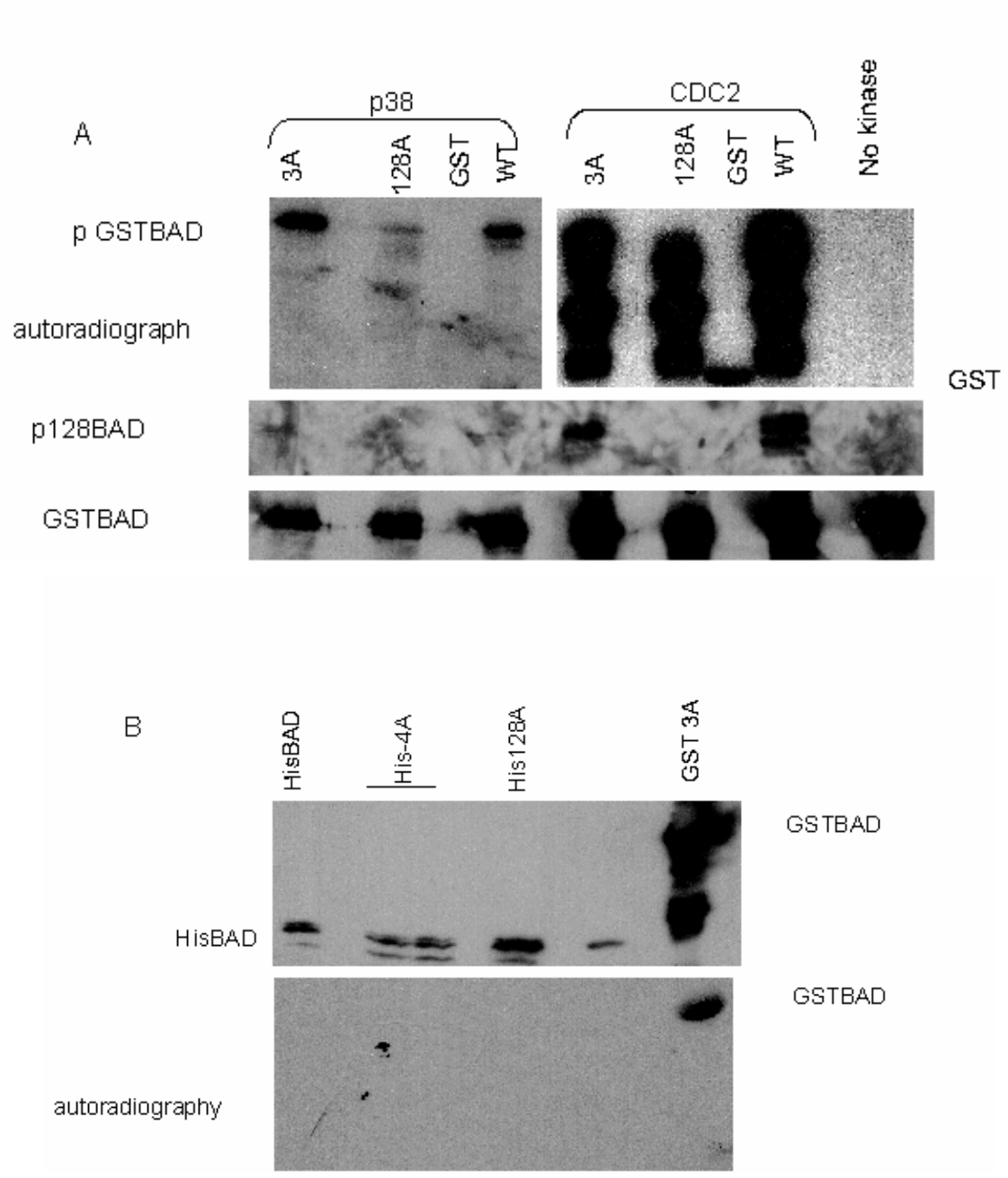

Figure 5: p38 phosphorylates a novel site on BAD. A) A hot kinase assay using purified p38 or CDC2 with GSTBAD mutants. B) Bacterially expressed and purified HisBAD WT and mutants (4A, 128A) and GSTBAD3A (all on beads) underwent a hot kinase assay with p38, was autoradiographed, and then probed for total BAD.

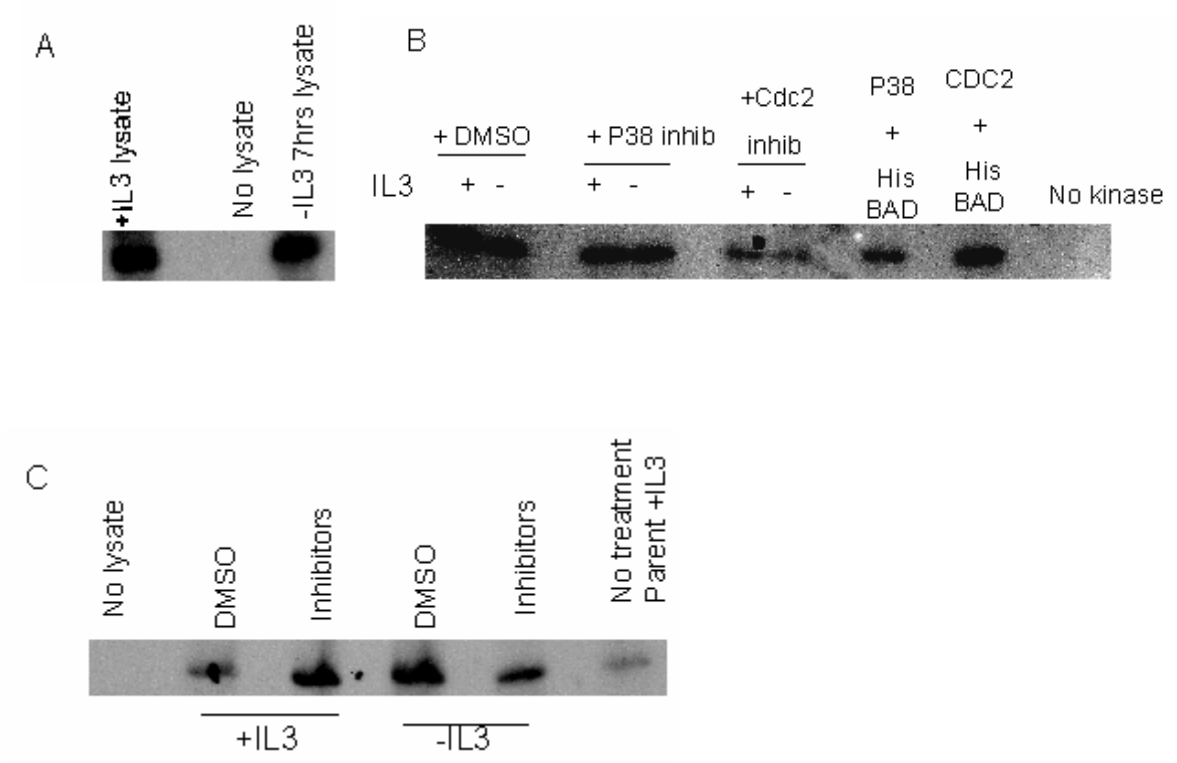

Figure 6: BAD is phosphorylated *in vitro* under conditions of cell death A) 100µg total lysate of FL5.12 parental cells were added to HisBAD in a hot kinase assay and autoradiographed B) FL5.12 parental cells were pretreated with a specific inhibitor to p38 (10 µM) or CDC2 (20 µM), washed three times with PBS, put in + or –IL3 media, grown for 7hrs, then lysed, added to commercial HisBAD in a hot kinase assay, and autoradiographed C) both p38 (10 µM) and CDC2 (20 µM) inhibitors were simultaneously added to FL5.12 cells under conditions of growth or death, lysed, applied to HisBAD in a hot kinase assay, and audioradiographed.

With our encouraging *in vitro* results, we then examined the phosphorylation status of BAD in growing cells. FL5.12 parental cells growing under healthy (+IL3) or cell death conditions (-IL3 for 7hrs), were lysed, and added to a HisBAD substrate in a hot kinase assay. Figure 6A suggested there were kinases still active under cell death conditions that are able to act upon BAD. In panel B, FL5.12 parental cells were pretreated for 30 minutes with a specific inhibitor to either p38 (SB203580 at 10µM) or CDC2 (olomoucine at 20µM), washed three times with PBS, placed in either survival or

death media, and inhibitors to either p38 (10μM) or CDC2 (20μM) reintroduced. Growing cells were then collected after 7 hours, lysed, and added to commercial HisBAD in a hot kinase assay. There was no difference in the phosphorylation of BAD with or without p38 inhibitor, in growing or cell death conditions. BAD was phosphorylated by cellular kinases that were not p38 or CDC2 even under cell death conditions. When both p38 and CDC2 inhibitors were added to growing or dying cells, Figure 6C again suggested that BAD was still phosphorylated when both p38 and CDC2 were presumably inactive. Given the many kinases known to phosphorylate BAD, these results are not surprising.

We attempted to preserve BAD's novel phosphorylation site(s) by using trichloroacetic acid (TCA) precipitation from cellular lysate, performed as previously reported [29]. However, despite increasing the amount of resolubization time and percentage of TCA, I was unable to resolubilize BAD into in the urea resolubilization buffer following TCA protein precipitation. Because of technical problems, the TCA approach was aborted.

In order to determine if there was a difference in cell survival due to BAD mutation, HisBAD constructs (4A, 3A, 128A, and WT) were introduced in FL5.12 Bclx cells by retroviral infection. Cells were then washed 3 times with PBS, plated in –IL3 media, and live and dead cells counted using trypan blue dye and a hemocytometer. Figure 7 indicates that the different HisBAD mutations did not have a different effect on cell survival. However, the expression levels of the constructs were not equivalent, so this experiment needs to be repeated normalizing construct expression levels before a conclusion can be drawn.

In order to determine where p38 phosphorylates wild type BAD, a cold kinase assay of commercial HisBAD and p38 was conducted, run on a SDS-PAGE gel, colloidal blue stained (Figure 8A), bands excised, and sent to the Mass Spectrometry Proteomics Core for phospho-peptide analysis. A small scale hot kinase assay was done in parallel to confirm that the p38/HisBAD sample sent was phosphorylated (Figure 8B). There was good coverage of the HisBAD protein sent to the proteomics core with the trypsin and chymotrypsin digestions. Phosphopeptide analysis implicated serine six on BAD (a site that has not been previously reported to be modified) as a possible p38 phosphorylation. While this serine 6 site does not contain the conserved MAPK consensus motif P-X(1-2)-T/S-P [30, 31], possessing instead $_3$KQPSLAP$_9$, it may prove to be a novel phosphorylation site.

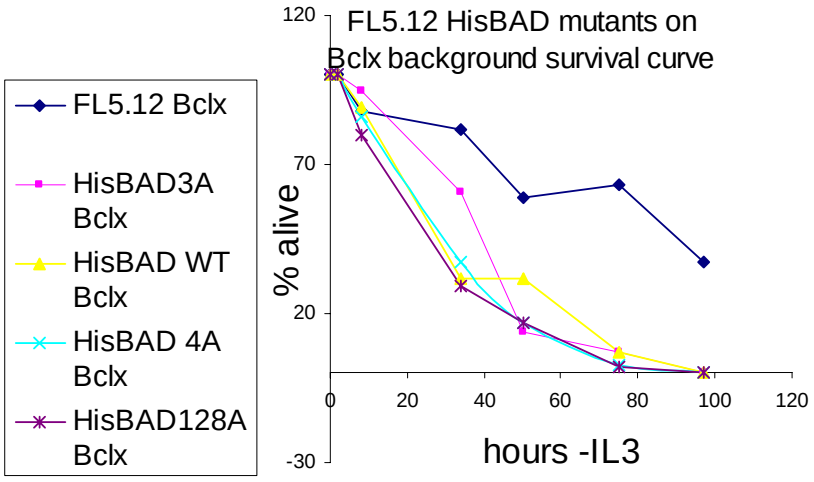

Figure 7: Survival curve of FL5.12 Bclx HisBAD mutant cells. Cells were washed 3x with PBS, put in –IL3 media, and counted at indicated times for live and dead cells using trypan blue on a hemocytometer. Percent alive calculated by dividing the number of alive cells by the total number of cells (alive and dead) counted.

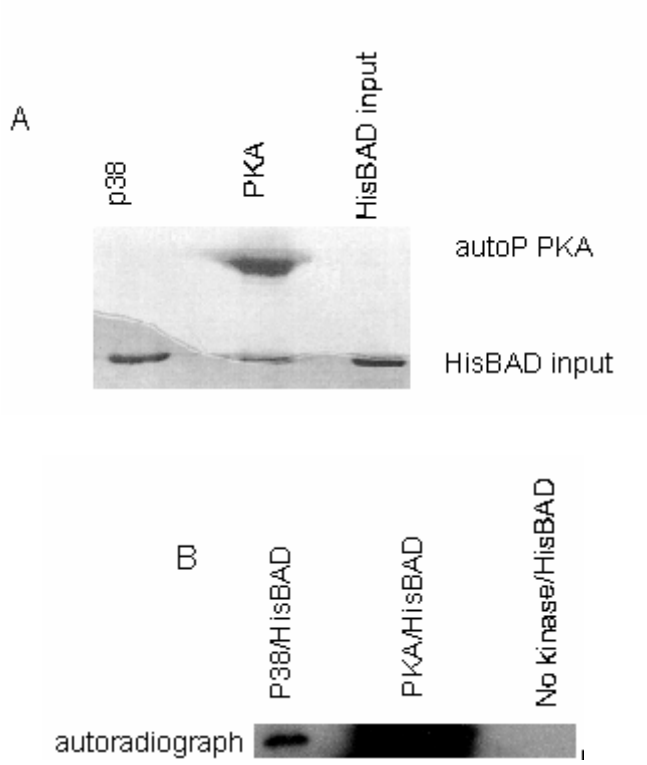

Figure 8: Submission for Mass Spectrometry Phosphopeptide analysis. A) Colloidal blue stain on cold kinase assay using either purified p38 or PKA on substrate HisBAD. HisBAD bands were then excised, digested with trypsin and chymotrypsin, and analyzed for changes in mass. B) Hot kinase assay of p38 and PKA kinases on HisBAD.

p38 may be activated under UVB radiation

As p38 emerged as an *in vitro* kinase of BAD, I then pursued establishing a model in which p38 activation could be experimentally controlled; I first examined conditions in which p38 is active. Since the literature suggested that p38 is active in cells under conditions of arrest [32], this model was first examined. However, p38 appeared active in both cycling and arrested 3T3 cells in my hands (Figure 9A). This model was not pursued.

As p38 emerged as a potentially interesting kinase for BAD, we attempted to determine when p38 is active in FL5.12 cells. IL3 was withdrawn from FL5.12 cells for

up to 3 hours and then, in one sample, IL3 was added back and cells grown for 30 minutes (Figure 9B). As a change in active p38 was not observable under these conditions, other stressors were tried to activate p38. FL5.12 cells were exposed to heat shock or varying microjoules (μJ) of UVB radiation, collected over a time course, lysed, and western blotted for active and total p38. While the heat shock had no effect on p38 activation, there was some weak evidence that 26 hours after 60 μJ UVB radiation, cells

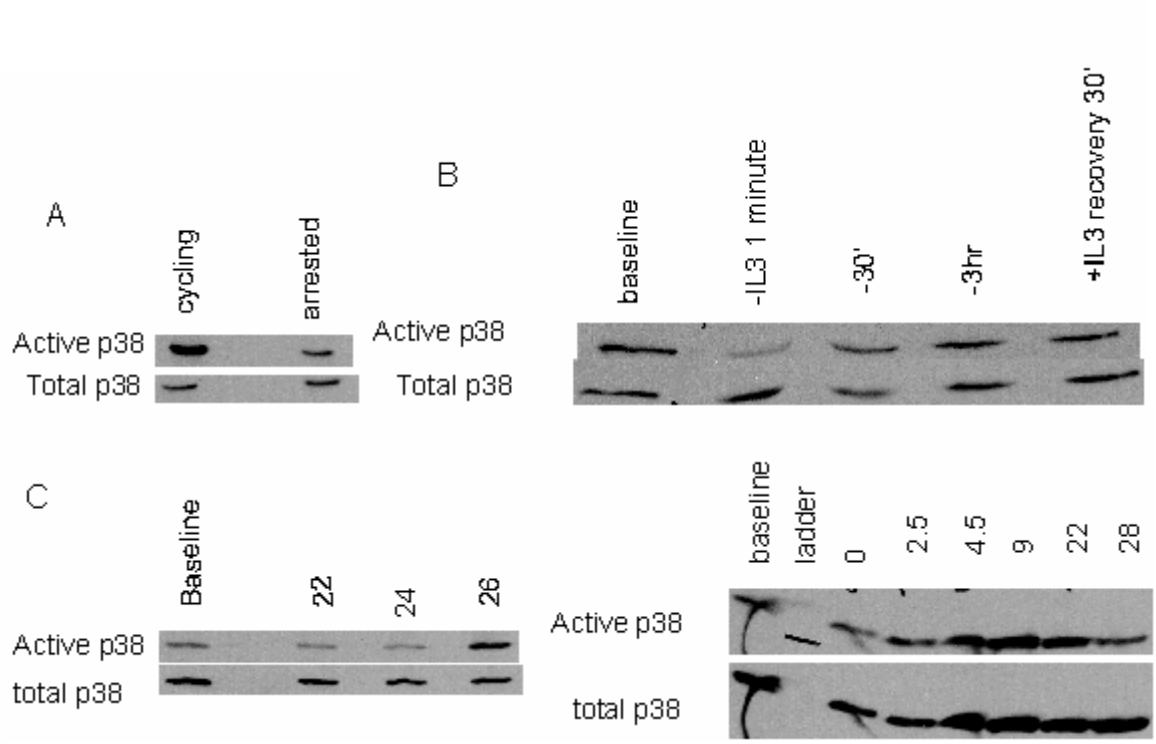

Figure 9: Examining when p38 is active in 3T3 and FL5.12 cells. A) 3T3 cells arrested or cycling both have active p38. Contrary to reports in the literature, cycling cells seems to have more active p38. B) IL3 was withdrawn from FL5.12 cells for the indicated times, and then reconstituted. C) FL5.12 cells were exposed to 60 uJ UVB radiation, grown in c-IMDM for indicated times (in hours) before collection, lysed, and subjected to western blotting.

had elevated levels of active p38 (Figure 9C). However, as this single time point showing p38 activation was immediately preceded by two inactive time points (22 and 24hrs) and, in a separate experiment, a 28 hour time period also had low levels of p38 activation, one must interpret these data cautiously. The experiment needs to be repeated to include hourly time points between 22 and 28 hours post UVB radiation in order to draw conclusions about the timing of p38 activation after exposure to UVB radiation.

BAD modification studies

As BAD is known to have several sites of phosphorylation events that are central to its regulation and function, we then pursued the question of there were other, undiscovered modifications on BAD under conditions of cell death. To examine if BAD underwent novel post translational modifications under conditions of cell death, we employed a 2 dimensional gel and looked for a spot shift representing the gain or loss of a phosphate group. Running GSTBAD eluate on a 3-10 pI strip and examining the results yielded no conclusive evidence for a novel BAD phosphorylation site under healthy or cell death conditions (Figure 10). As there were no points of reference (ex: actin) to line up the blots, the results were difficult to compare to each other. Advice from Dr. David Friedman suggested that overloading and/or using too broad an isoelectric strip may have contributed to the suboptimal focusing observed here.

BAD serine 128 is activated under conditions of cell death

As the phosphorylation of BAD at serine 128 has been implemented as a key site that dissociates BAD from 14-3-3 when undergoing the G_2/M phase in growing lymphoid

cells, and in inducing cell death when deprived of growth factors and being in the G_2/M phase [16], we then examined if this site may have a role in cell death independent of cell cycle. Total lysate was made from FL5.12 cells over expressing BAD and BCL-xL during a time course of IL3 withdraw and western blotted with an antibody for the phosphorylation of S128 BAD (Figure 11A). The phosphorylation of BAD 128 is low under cell growth conditions and under minus IL3 conditions for up to half an hour. However, when cells have been in media without IL3 for 3 hours or more, there is an increase of phosphorylation at BAD S128. The phosphorylation of S128 was identified during induction of cell death. In a different cell line over expressing 3xFlag-BAD-Myc, a similar effect of BAD 128 phosphorylation under –IL3 conditions for 7 hours was also observed (Figure 11B). While this experiment was highly reproducible with one working solution of the p128 antibody (from Bonnie), subsequent dilutions of both commercial and homemade (Bonnie) antibodies have not suggested any phosphorylation of BAD at 128 under conditions of IL3 withdraw. It is conceivable that the polyclonal commercial antibody batches were not as sensitive and had a lower concentration of antigens specific to the p128 site as did the original p128 (Bonnie) antibody. Furthermore, it is possible that the homemade (Bonnie) antibody, stored at 4°, degraded over time and lost its ability to bind the phosphorylated 128 BAD epitope.

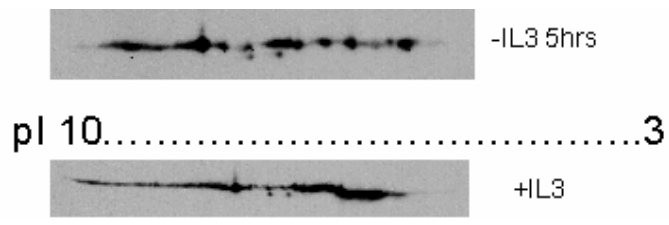

Figure 10: No clear evidence for GSTBAD eluate post translational modification using 2D gels. GSTBAD eluate from cell life (+IL3) and death (-IL3 5hrs) conditions run on a 2D gel (3-10 pI strip). Examining the dot shifts yielded no conclusive evidence for a novel BAD phosphorylation site under healthy or cell death conditions.

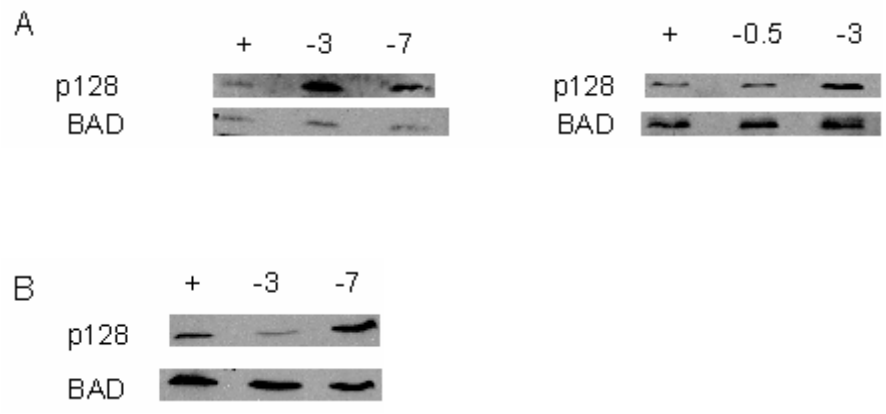

Figure 11: Phosphorylation at BAD S128 is observed under conditions of cell death. A) FL5.12 cells overexpressing BAD and Bcl-x (WTN1) were subjected to –IL3 conditions for indicated times; total lysate was then run on a SDS-PAGE gel and immunoblotted. B) FL5.12 cells expressing FBM construct was subjected to cell death conditions for indicated times, lysed, and western blotted.

ATM/ATR does not affect BAD or 14-3-3 Phosphorylation

ATM/ATR kinases are responsive to DNA damage and are known to have a TQ and SQ consensus sequence [33]; as murine BAD has both TQ and SQ sites near the end of its sequence, it was deemed possible that ATM/ATR may phosphorylate BAD to induce a modification. ATM/ATR kinases were examined as potential agents acting upon BAD using hot kinase assays (using purified ATM/ATR, their kinase dead or inhibited equivalent, and HisBAD). There was no evidence of a ^{32}P band corresponding at the size of HisBAD (~36kD) or His14-3-3 (~42kD) with either ATR or with ATR's inhibitor caffeine added; a similar lack of difference was also observed with the ATM hot kinase assays. I found no evidence for ATM/ATR phosphorylation of BAD or 14-3-3.

Modification of 14-3-3 studies

14-3-3 proteins are usually found as functional dimers in cells; however, there was a report of 14-3-3 monomers existing and retaining the ability to bind downstream targets [18]. In order to determine if 14-3-3 may monomerize during conditions of cell life or death, cells with or without IL3 survival factor (and with or without R18, a competitive peptide to 14-3-3) were lysed, 14-3-3 was IPed from total lysate, and then run on SDS or native gels. One would expect the dimer to be approximately twice the size of the monomer on native gels. I found no shift in the molecular weight of 14-3-3 from dimer to monomer under conditions of cell death (Figure 12). There was no evidence that monomerization was a mechanism of 14-3-3 dissociation from BAD under IL3 withdrawal.

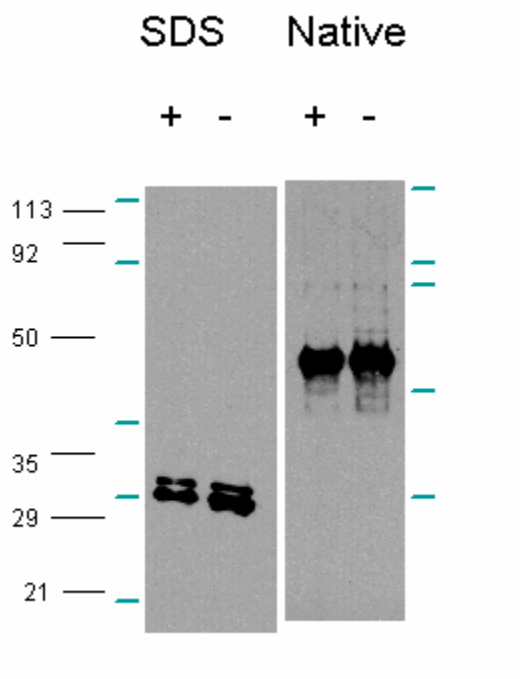

Figure 12: 14-3-3 does not monomerize under conditions of cell death. FL5.12 parental cells were grown under +IL3 (+) or –IL3 (-) conditions for 7 hours, lysed, run on SDS or native gels, and western blotted for 14-3-3. Kaleidoscope markers (indicated by the tick marks) were run on both gels in order to estimate the native protein size.

While 14-3-3 is a key regulator that acts as a phosphoserine/phosphothreonine-binding protein [18, 19] [17], it is also subject to modifications that alter 14-3-3's ability

to bind to targets. We then examined if 14-3-3 may undergo a modification/phosphorylation under conditions of cell death that encourages its dissociation from BAD.

A phosphorylation change of 14-3-3 (which has also been reported [25, 34]) will result in a different isoelectric point. We used 2D gel electrophoresis to examine both 14-3-3 that bound GSTBAD and unbound 14-3-3 under cell death conditions (Figure 13). Figure 13A shows a 14-3-3 western blot of a 2D gel. Since focusing was suboptimal, this protocol was repeated using a narrower isoelectric strip (pI 4-7) more attuned to the pI of 14-3-3 (pI 4.5); the results are shown in Figure 13B. Because there was not an obvious new spot to suggest a new phosphorylation event, I decided not to pursue this line of experimentation. This experiment may have been suboptimal; technical improvements on performing a 2D gel include loading less protein and having a common reference point in all three blots in order to accurately line up the developed blots.

To look more globally for 14-3-3 modifications under conditions of cell life and death, a 2D experiment was conducted with a narrower pI strip (3-5.5) to allow for even better focusing. After total lysate from growing and cell death conditions was focused on the pI 3-5 strips, samples were run in the second dimension on a SDS PAGE gel and compared using cy3/5 fluorescent staining. The overlay (Figure 14) identified a single spot which was different between life and death conditions. This spot was later identified as translation initiation factor 5A. No 14-3-3 modification spot was detected under conditions of cell life and cell death using DIGE 2D.

One study has suggested that 14-3-3 phosphorylation altered its structure (becoming a monomer) [18], while another report has implicated JNK in phosphorylating

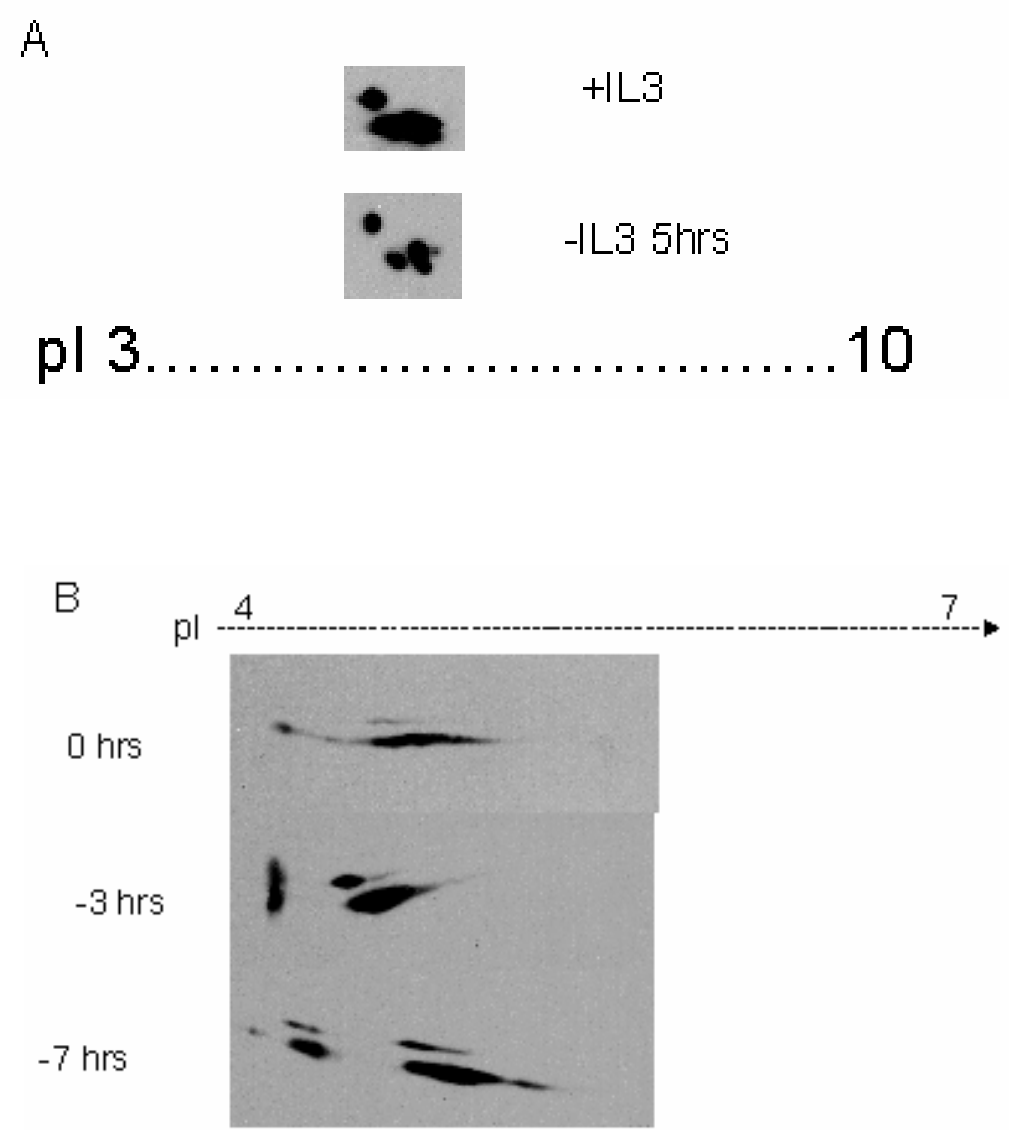

Figure 13: 14-3-3 modifications can not be clearly detected on a 2D gel. A) GSTBAD eluate run on 2D gel, pI strip 3-10 in the first dimension, 12.5% SDS Page gel in the second dimension. Western blotted for 14-3-3. B) Total lysate was analyzed on a 2D gel (pI 4-7) under indicated cell death conditions and western blotted for 14-3-3.

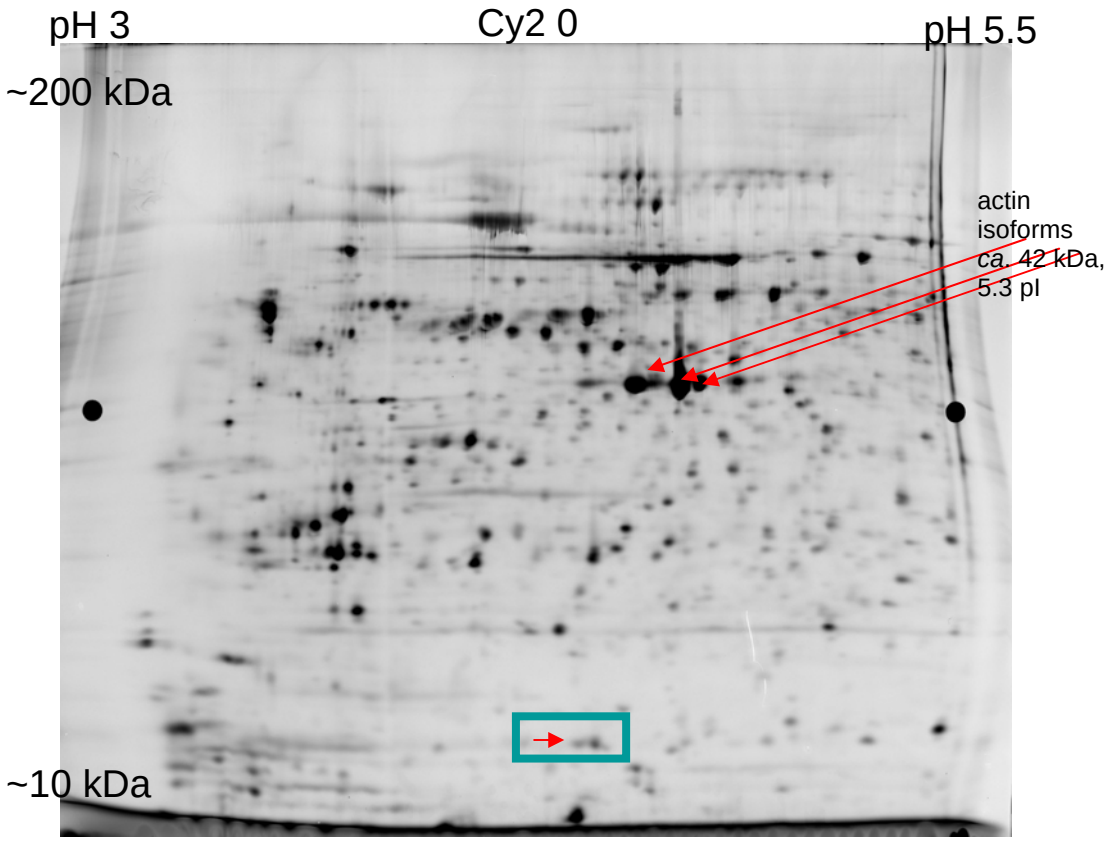

Figure 14: No new 14-3-3 modification was detected under cell death induction. 2D analysis of total lysate from cell life and death conditions (-IL3, 7hrs) displays a phosphorylation shift in translation initiation factor 5A

14-3-3 to release it from binding Bcr-Abl [25]. We explored the possibility that JNK may phosphorylate 14-3-3 to release it from BAD. We hypothesized that if JNK phosphorylated 14-3-3 to aid in its dissociation from BAD, then preventing this phosphorylation event would lead 14-3-3 to remain associated with BAD even in –IL3 conditions and render BAD more phosphorylated at key sites protected by the bound 14-3-3. Using a JNK specific chemical inhibitor (SP600125, 10μM), total lysates were collected, BAD immunoprecipitated, and western blotted for BAD-14-3-3 binding and BAD phosphorylation status. Figure 15 demonstrates that BAD was successfully

immunoprecipiated, as indicated by the presence of BAD on the (protein A/G) beads. However, the positive control (beads under +IL3 conditions) did not demonstrate what was expected (the presence of BAD). Therefore, this experiment is difficult to interpret. During the time these experiments were conducted, a report was published confirming that JNK phosphorylates 14-3-3 to allow the release of BAD [21].

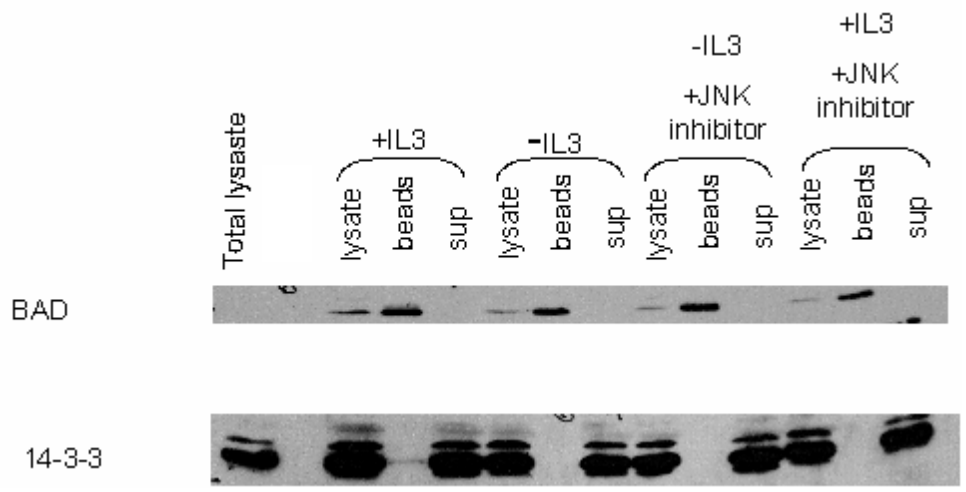

Figure 15: No conclusive evidence JNK has an effect on 14-3-3 binding BAD. FL5.12 cells overexpressing Bclx and BAD (WTN1) were treated with JNK inhibitor (SP600125, 10μM) under the indicated conditions, IPed with BAD goat C-10, and subject to western blotting.

From the candidate proteins identified from the BAD pull down, p38 and CDC2 emerged as potential candidate kinases that may interact with the BAD complex, including 14-3-3. For this reason, we performed a hot kinase assay to see if 14-3-3 was modified by either p38 or CDC2 kinases as well as using total lysate from different death conditions (+IL3, -IL3 3hrs, -IL3 7 hrs) (Figure 16). Unfortunately, the GST tag was discovered to be phosphorylated by CDC2 (see Figure 5), and there was no evidence that

p38 phosphorylated 14-3-3. It appeared that under +IL3 or –IL3 conditions there are cellular kinases which can phosphorylate 14-3-3.

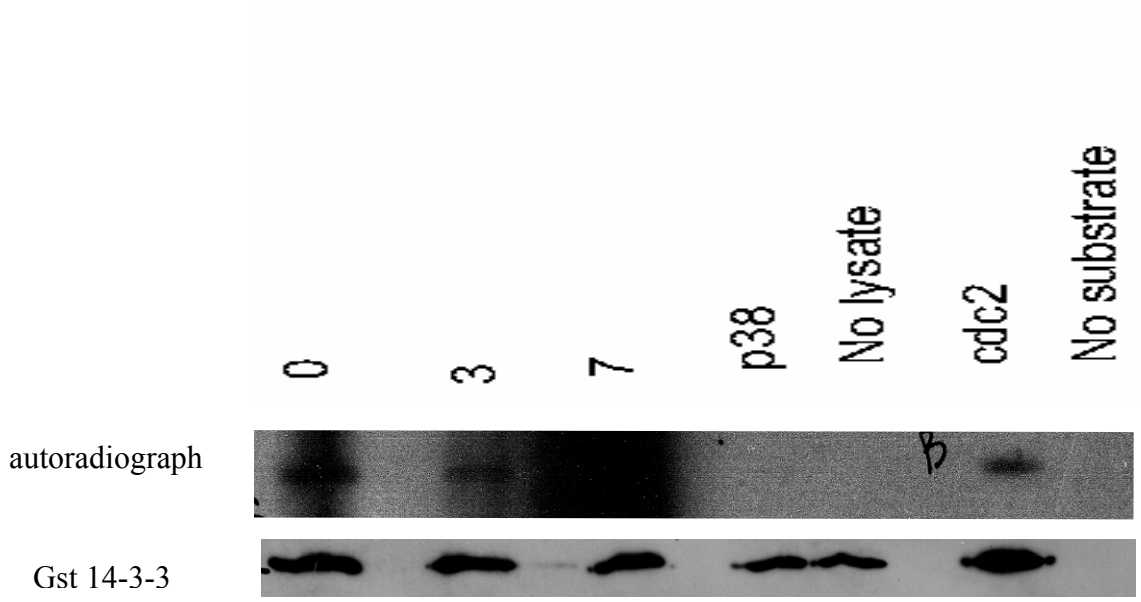

Figure 16: No evidence p38 or CDC2 phosphorylates 14-3-3. FL5.12 parental cells total lysate under 0, -3, or -7 hours of IL3 deprivation was one source of kinases. Additionally, purified p38 and CDC2 were also used as kinases against the substrate GST 14-3-3 in a hot kinase assay.

Results Summary

Several novel binding partners to BAD were identified that existed in either a condition of cell life or cell death; this list included: p38, CDC2, Beclin-1**,** BAX, cytochrome c1 oxidase, voltage-dependent anion-selective channel proteins 1, 2, and 3, FK506-binding partner 4, and kinases PLK1, SNF-1 like kinase 2, and VRK2. While p38 and CDC2 were followed up, other binding partners identified here may warrant deeper investigations.

Hot kinase assays suggested that p38 phosphorylates a novel site on BAD, perhaps serine 6. Since p38 activation was not observed during times BAD 128 has

shown to be active, it is unlikely that p38 phosphorylates BAD S128. Using *in vitro* techniques, BAD was shown to be phosphorylated under both +IL3 and –IL3 conditions from cellular kinases. The phosphorylation state of BAD S128 was observed after 3-7 hours of –IL3. No evidence was observed for ATM/ATR phosphorylation of BAD or 14-3-3.

Under +IL3 and –IL3 conditions, no new phosphorylation sites or monomeric changes on 14-3-3 were observed. Additionally, p38 was concluded not to phosphorylate 14-3-3, although under both +IL3 and –IL3 conditions, there was evidence that 14-3-3 was phosphorylated by cellular kinases.

CHAPTER IV

DISCUSSION

In this study, we demonstrate the possibility of a novel phosphorylation site on BAD. *In vitro* data suggest BAD S6 may be phosphorylated by p38. There are a number of follow up experiments that could be conducted to support or refute this finding. In order to confirm p38 phosphorylates BAD S6, a cold *in vitro* kinase assay using a clean recombinant WT or S6A could be conducted, then sent to Mass Spectrometry for phosphopeptide analysis. Additionally, to examine if the phosphorylation state at other sites on BAD affects p38's ability to phosphorylate S6, other BAD mutants (112A, 128A, 136A, 155A, and all combinations) could also be used *in vitro* kinase assays. Although the biological significance of this novel serine 6 site is currently unknown, it may act as another regulatory phosphorylation event on BAD, perhaps linking the p38 MAPK signaling pathway with the apoptotic machinery.

To verify that p38 does phosphorylate BAD in FL5.12 cells, a hot *in vivo* cellular experiment could be conducted with and without a dominant negative p38. Different HisBAD mutants (3A, 4A, WT, S112A, S128A, S6A) could be pulled out of the total lysate, and immunoblotted for specific phosphorylation events. Additionally, culturing cells expressing a BAD WT, S6A, 3A, and 4A mutation under conditions that activate p38 may also aid in identifying a p38 phosphorylation site.

A previous study has suggested that in tumor necrosis factor α (TNF)-induced apoptosis in endothelial cells, p38 regulates phosphorylation of BAD S112, in addition to

coimmunoprecipitating with PP2A [22], a known phosphatase of the S112 site of BAD [4]. While this study did not examine other known phosphorylation sites on BAD, it is possible that TNF induced apoptosis stimulates other known and novel phosphorylation sites on BAD, such as S6.

There is much known about the phosphorylation of BAD and how that regulates binding partners, cell cycle under growth factor deprivation/starvation conditions, and apoptosis [8, 13, 14, 16]; however, the significance for the phosphorylation of S128 BAD on survival in the FL5.12 cell model deserves elucidation. Since the original experiments in which phosphorylation of 128 was observed did not use arrested or synchronized cells, it is unlikely that this phosphorylation event was cell cycle dependent (as has been suggested by another report [16]). Immunoprecipitating BAD when it is known to be phosphorylated at S128 and looking for different binding partners may warrant follow up.

One may want to reevaluate the binding partner data to pursuing other potentially interesting proteins from this list. Cytochrome c1 oxidase, the voltage-dependent anion-selective channel proteins, BAX, and beclin-1 are all proteins yielded from a Mass Spectrometry binding analysis (Table 1) that are known to be involved in the apoptosis or autophagosome pathways, and warrant follow up study. Furthermore, the Rab1A protein (identified in analyzing an one-dimension SDS-PAGE gel of GSTBAD eluate samples from cell life and death conditions, Figure 2A) may also be reexamined in light of the autophagosome pathway. Studies looking at the localization patterns of candidate Rab proteins and BAD under conditions of cell death or autophagy may uncover a connection between the Rab family to the cell death machinery pathway, as other members of the Bcl-2 family have been indirectly linked with autophagy [35].

The literature has provided several examples of 14-3-3 modification and cleavage to facilitate dissociation from target proteins [18, 20, 25, 34]; there has even been a particular report of caspace-3 cleavage of 14-3-3 at D238 which facilitates the dissociation of BAD in human 293T cells [34]. The data collected here using IL-3 deprivation as the death stimulus has not produced any observable modification of 14-3-3. Performing a double pull down—first on 14-3-3 and then BAD—may enrich for other interacting proteins in the 14-3-3 BAD complex.

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