Extracellular Signal-regulated Kinases 1/2 Are Serum-stimulated "BimEL Kinases" That Bind to the BH3-only Protein BimEL Causing Its Phosphorylation and Turnover*

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Bim, a “BH3-only” protein, is expressed de novo following withdrawal of serum survival factors and promotes cell death. We have shown previously that activation of the ERK1/2 pathway promotes phosphorylation of BimEL, targeting it for degradation via the proteasome. However, the nature of the kinase responsible for BimEL phosphorylation remained unclear. We now show that BimEL is phosphorylated on at least three sites in response to activation of the ERK1/2 pathway. By using the peptidylprolyl isomerase, PnI, as a probe for proline-directed phosphorylation, we show that ERK1/2-dependent phosphorylation of BimEL occurs at (S/T)P motifs. ERK1/2 phosphorylates BimEL, but not BimS or BimL, in vitro, and mutation of Ser65 to alanine blocks the phosphorylation of BimEL by ERK1/2 in vitro and in vivo and prevents the degradation of the protein following activation of the ERK1/2 pathway. We also find that ERK1/2, but not JNK, can physically associate with GST-BimEL, but not GST-BimS or GST-BimL, in vitro. ERK1/2 also binds to full-length BimEL in vivo, and we have localized a potential ERK1/2 “docking domain” lying within a 27-amino acid stretch of the BimEL protein. Our findings provide new insights into the post-translational regulation of BimEL and the role of the ERK1/2 pathway in cell survival signaling.

The cell intrinsic or mitochondrial pathway of apoptosis is regulated by the Bcl-2 family of proteins (1). In viable cells the pro-survival proteins, Bcl-2 and Bcl-xL, bind to and repress the multidomain pro-apoptotic proteins, Bax and Bak. BH3-only proteins respond to stresses by binding to Bcl-2 or Bcl-xL, thereby neutralizing their anti-apoptotic effects. As a result Bax and Bak then undergo a conformational change, oligomerize and disrupt the outer mitochondrial membrane, promoting the release of apoptogenic factors that initiate caspase activation and lead to cell death (1). The BH3-only proteins link stress and survival signaling pathways to the decision-making machinery of the apoptotic pathway, and are regulated in a variety of ways (2). Some, such as Noxa and Puma, are transcriptionally up-regulated in response to DNA damage (3, 4), whereas others, such as Bid, are regulated by post-translational mechanisms (5).

Apoptosis following withdrawal of survival factors can be mimicked in cell culture by the withdrawal of serum. Both the Raf-MEK-ERK and phosphatidylinositol 3-kinase/PKB signaling pathways can protect cells from apoptosis following withdrawal of serum or defined survival factors (6, 7). The regulation of the BH3-only protein, Bad, by these signaling pathways may play a key role in survival. PKB (7), p90RSK (8), and protein kinase A (9) have been shown to phosphorylate Bad, promoting its physical sequestration by 14-3-3 proteins. Withdrawal of survival factors is thought to result in the de-phosphorylation of Bad, allowing it to bind to Bcl-xL, and so release Bax.

In many cells apoptosis resulting from withdrawal of survival factors requires de novo gene expression, and yet Bad expression is not inducible under these conditions. However, the BH3-only protein, Bim, is rapidly and substantially expressed de novo following withdrawal of survival factors, and this is likely to represent a major apoptotic signal (10–14). Bim mRNA levels are normally repressed by the PKB (10, 11, 14, 15) and ERK1/2 pathways (14) or induced by the JNK-c-Jun pathway (12, 13). In addition, Bim is regulated by post-translational mechanisms. There are three major isoforms of Bim created by alternative splicing: BimEL, BimL, and BimS (16–18). Both BimEL and BimL contain a region that allows them to interact with dynein light chain-1 (DLC1 or LC8) (19). This interaction sequesters them to microtubules away from Bcl-2 and Bcl-xL and may account for their weaker apoptotic potential relative to BimS.

BimEL is a phosphoprotein (11, 14, 20–23), and we have shown previously (24) that activation of the ERK1/2 pathway promotes BimEL phosphorylation, thereby targeting it for ubiquitination and degradation by the proteasome. However, the

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The abbreviations and trivial names used are: BH3, Bcl-2 homology domain 3; Bad, Bcl-2 antagonist of cell death; Bim, Bcl-2 interacting modulator; BimEL, Bim extra long; BimL, Bim long; BimS, Bim short; HEK, human embryonic kidney; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; PKB, protein kinase B; p90RSK/RSK, ribosomal protein S6 kinase; PD184352, 2-(2-chloro-4-iodo-phenylamino)-N-cyclopropylmethoxy-3,4-difluorobenzamide; U0126, 1,4-diamine-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; HA, hemagglutinin; FACS, fluorescence-activated cell sorter; 4-HT, 4-hydroxytamoxifen; MBP, myelin basic protein; EGFP, enhanced green fluorescent protein; WT, wild type.
ERK1/2 Binds to and Phosphorylates BimEL

kinase responsible for BimEL phosphorylation was not identified. Here we show that activation of the ERK1/2 pathway promotes the phosphorylation of BimEL on at least three sites in vivo, some of which are proline-directed. ERK1/2 phosphorylates BimEL in vitro at serine 65; mutation of this site inhibits its ERK1/2-dependent phosphorylation in vivo and stabilizes the BimEL protein. Finally, we have mapped the ERK1/2 docking domain of BimEL, and shown it to be distinct from the phospho-acceptor site. Phosphorylation of BimEL by ERK1/2 defines a new mechanism by which survival factors can prevent apoptosis.

EXPERIMENTAL PROCEDURES
Materials—Cell culture reagents were purchased from Invitrogen. U0126 was purchased from Promega. LY294002 was from Calbiochem. Antibodies to ERK1, JNK1, p38MAPK were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. Components for isoelectric focusing tube gels were purchased from Genomics Solutions and were resolved on the Millipore Investigator System. All other chemicals were purchased from Sigma, unless otherwise stated in the text, and were of the highest grade available.

Cell Culture—Culture of CC139, CR1-11, and CM3 cells has been described previously (14, 25, 26); HEK293 cells were maintained under identical conditions. Cells judged to be 50–60% confluent were washed twice in serum-free medium and then placed in fresh serum-free medium with the indicated dose of 4-HT, PBS, or inhibitors for the times indicated. For emetine chase experiments, cells were starved for 18 h and then treated with emetine (10 μM) for 30 min to block protein synthesis prior to further treatments.

Plasmids and Transfections—BimEL, BimL, BimAD, and fragments of BimEL were expressed as GST fusion proteins in pGEX-4T1 or as HA-transfected proteins in pCA-HA (a derivative of pCDNA3 that includes an ATG and in-frame HA tag at the 5′ end of the MCS). GST-Bim constructs were expressed as C-terminal truncation mutations that removed the last 18 amino acids to aid expression of soluble protein in bacteria. Amino acid numbering refers to the rat BimEL cDNA sequence that was used in these studies. Potential phosphorylation sites were altered by PCR-based site-directed mutagenesis using Phusion DNA polymerase (Promega). All inserts were verified by automated sequencing from Applied Biosystems. The pGEX-4T1-Pin1 plasmid, encoding a GST fusion protein of human Pin1, was kindly provided by Dr. Gianni Del Sal, Laboratorio Nazionale CIB, AREA Science Park, Padriciano 99, 34012 Trieste, Italy. Introduction of the S16E and W34A point mutations in the Pin1 WW domain was also by site-directed mutagenesis (as above). The sequences of all oligonucleotides are available upon request.

HEK293 cells were transfected by the calcium phosphate precipitation technique (27) and left for the time indicated in figure legends. HA-tagged Bim was immunoprecipitated from cell lysates using HA antibodies conjugated to protein A-Sepharose beads or GST-Bim EL pre-bound to glutathione-agarose (GSH) beads (Amersham Biosciences). GST fusion proteins were quantified by Bradford assay and from Coomassie Blue-stained SDS-PAGE gels by densitometry. Recombinant proteins were eluted from the beads to use as substrates in in vitro kinase assays or were used bound to beads in pull-down experiments. For co-precipitation/pull-down experiments, whole cell lysates were incubated with equivalent amounts of GST fusion protein-bound beads for 1–2 h at 4 °C. The beads were then washed four times with ice-cold lysis buffer followed by separation on SDS-PAGE and immunoblot with relevant antibodies.

In Vitro Kinase Reactions—Active kinases were immunoprecipitated from normalized cell extracts, using ERK1, JNK1, and p38α antibodies with protein A-Sepharose beads or GST-BimEL pre-bound to glutathione beads. The captured complexes were washed twice in lysis buffer. ERK1 was assayed using MBP as a substrate as described previously (25, 26). The “BimEL kinase” activity pulled down with GST-BimEL was also treated in this manner, without MBP. JNK1 and p38α samples were assayed as described previously (26). Kinase reactions were terminated by boiling the samples in 4× Laemmli SDS-PAGE sample buffer before being analyzed by SDS-PAGE and autoradiography.

Analysis of Cell Cycle Profiles and Apoptosis—HEK293 cells were transfected with pEGFP-BimEL or pEGFP-BimELS65A, and after 18 h EGFP-positive cells were sorted by FACS, fixed, and stained with propidium iodide and analyzed by flow cytometry as described previously (25).

RESULTS

BimEL Is Phosphorylated in an ERK1/2-dependent Fashion in Vivo—When serum-starved CC139 fibroblasts were restimulated with FBS for 10 min, BimEL exhibited reduced mobility on SDS-PAGE, but this was prevented when the ERK1/2 pathway was blocked by the ERK1/2 and ERK5 pathway inhibitor, U0126 (Fig. 1A), or the ERK1/2 pathway-specific inhibitor, PD184352 (24). To confirm that BimEL was a phosphoprotein, we ectopically expressed HA-tagged BimEL in HEK293 cells, metabolically labeled with [32P]Pi, in serum-free medium; HA-BimEL was immunoprecipitated and detected by Western blot with anti-Bim antibodies or subjected to autoradiography. Even in serum-starved HEK293 cells we observed a basal level of BimEL phosphorylation (Fig. 1B), probably because these transformed cells exhibit residual ERK1/2 activity. Restimulation of cells with FBS caused a further shift in mobility and incorporation of [32P]Pi into BimEL. The mobility shift was inhibited by PD184352, and we observed an even more striking reduction in the incorporation of [32P]Pi into BimEL, with PD184352 reducing the labeling to below the basal level (Fig. 1B). A small amount of a Bim-reactive band below BimEL was also expressed and may represent one of the alternatively spliced forms of Bim such as BimL or BimAD (16–18, 29); this protein incorporated little, if any, [32P]Pi. These results confirm that BimEL is phosphorylated in an ERK1/2-dependent fashion in vivo.

In parallel, we examined the phosphorylation of exogenous BimEL by two-dimensional gel electrophoresis. In [32P]Pi-labeled lymphocytes BimEL resolves as a series of spots on two-dimensional gels including a basic, non-phosphorylated form and a series of more acidic forms (spots 1–4) that incorporate [32P]Pi (22); the nature of the kinase responsible for this phosphorylation was not reported. In serum-starved HEK293 cells exogenous BimEL resolved as three spots: the phosphorylated spot 1 and spot 2 and the more basic non-phosphorylated form (Fig. 1C). Stimulation with FBS caused the basic form of BimEL to almost disappear, and this was accompanied by the appearance of the additional phosphorylated forms (spot 3 and spot 4, Fig. 1C). Pretreatment with PD184352 prior to FBS had no effect on spot 1 but abolished the appearance of spot 3 and spot 4, caused the almost complete loss of spot 2, and the reappearance of the basic non-phosphorylated form of BimEL. These results indicate that the appearance of the hyperphosphorylated forms of BimEL (spots 2–4) requires the ERK1/2 pathway. They also confirm the basal level of BimEL phosphorylation even in serum-starved cells (spots 1 and 2). One of these basal forms (spot 2) is inhibited by PD184352 and probably accounts for the ability of PD184352 to reduce the basal level of [32P]Pi incorporation into BimEL (Fig. 1B). Spot 1 is phosphorylated in the basal state and is refractory to PD184352. These results suggest that activation of the ERK1/2 pathway promotes the phosphorylation of BimEL at up to three discrete sites.

Binding to the Peptidylprolyl Isomerase, Pin1, Reveals That BimEL Is Subject to ERK-dependent Proline-directed Phosphorylation—The ability of PD184352 to block BimEL phosphorylation...
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**A.** CC39 cells were serum-starved for 6 h. Cells were left untreated (SF) or stimulated with serum (FBS) in the absence or presence of U0126 (FBS/U0). Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. BimEL isoform from cell lysates was not precipitated by GST-Pin1 with inactivating point mutations, whereas BimS, although it was evident that all three isoforms expressed equally well (Fig. 2B). To determine whether GST-Pin1 could also interact with endogenous BimEL, Rat-1 cells were serum-starved for 6 h, to allow for induction of Bim protein, and then stimulated with FBS in the absence or presence of the MEK inhibitor, U0126; these lysates were then used for a GST-Pin1 pull-down assay. Immunoblot analysis of cell lysates confirmed that FBS stimulation promoted activation of ERK1/2 and the phosphorylation of BimEL (Fig. 2C). GST-Pin1 was able to precipitate BimEL that had been phosphorylated following FBS stimulation, whereas there was no apparent binding of BimEL from lysates of serum-starved cells. In addition, U0126 prevented the activation of ERK1/2, inhibited the phosphorylation of BimEL, and completely blocked the binding of GST-Pin1 to BimEL (Fig. 2C). As a control we found that Bad, which is also regulated by phosphorylation but has not been reported to be a substrate of ERK1/2 in vivo, failed to bind to GST-Pin1 even when the same cell lysates contained plenty of immune-reactive Bad (Fig. 2C).

**B.** HEK293 cells were transfected with HA-BimS, HA-BimL, and HA-BimEL. After 18 h cells were left untreated (SF) or serum-stimulated (FBS) in the absence or presence of PD184352 (PD). HA-conjugated beads were used to immunoprecipitate protein from cell lysates, and these were subjected to two-dimensional (2-D) electrophoresis and immunoblotted for Bim. H+, acidic; OH+, basic. Similar results were obtained in three independent experiments.

**C.** 2-D Electrophoresis

**D.** IP - HA

**E.** WB - Bim

**F.** Autorad

**Fig. 1.** ERK1/2-dependent phosphorylation of BimEL in vivo. A, CC39 cells were serum-starved for 6 h. Cells were left untreated (SF) or stimulated with serum (FBS) in the absence or presence of U0126 (FBS/U0). Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. B, HEK293 cells were transfected with HA-BimEL in complete media. After 18 h cells were changed to serum- and phosphate-free media with [32P]Pi, left for 3 h, then left untreated (SF) or stimulated with serum (FBS) in the absence or presence of the MEK inhibitor PD184352 (PD) for 5 min. Immunoprecipitates (IP) were prepared from cell lysates using HA-conjugated beads, subjected to SDS-PAGE, and immunoblotted (WB) for Bim or subjected to autoradiography. C, HEK293 cells were transfected with HA-BimEL in complete media. After 18 h cells were left untreated (SF) or serum-stimulated (FBS) in the absence or presence of PD184352 (FBS+PD). HA-conjugated beads were used to immunoprecipitate protein from cell lysates, and these were subjected to two-dimensional (2-D) electrophoresis and immunoblotted for Bim. H+, acidic; OH+, basic. Similar results were obtained in three independent experiments.
Fig. 2. Exogenous and endogenous BimEL interact with GST-Pin1 in an ERK-dependent fashion. A, a schematic representation of GST-Pin1 showing the WW domain that specifically interacts with Ser(P)-Pro or Thr(P)-Pro motifs, as a black box, and the peptidylprolyl isomerase (PPI) catalytic domain, as a shaded box. B, HEK293 cells were transfected with HA-BimEL (EL), HA-BimS (EL), and HA-BimS (S) in complete media. Cells were lysed and were either subjected to SDS-PAGE directly (Input Lysates) or used in pull-down assays with GST-Pin1; precipitates were then resolved by SDS-PAGE (WT Pin1 Bound), and both gels were immunoblotted for HA. C, Rat-1 cells were serum-starved for 6 h and then either left untreated (0) or stimulated with serum (FBS) for the indicated times in the absence or presence of the MEK inhibitor, U0126 (FBS+U0). Cell lysates were either subjected to SDS-PAGE directly (Input Lysate) or used for GST-Pin1 pull-down assays prior to SDS-PAGE. Samples were then immunoblotted with antibodies for Bim, Bad, P-ERK1/2, and Input Lysate for HA. D, CCl39 cells were either left cycling in 10% FBS (C), serum-starved for 6 h (SF), or serum-starved and then re-stimulated with 10% FBS for 1 h (F or FBS). Cell lysates were either subjected to SDS-PAGE directly (Input Lysate) or used in pull-down assays with WT GST-Pin1 (W) or a Pin1 mutant with an inactivated WW domain (Δ). Precipitates were resolved by SDS-PAGE and immunoblotted for Bim. The asterisk indicates a cross-reactive band which is not Bim but served as useful loading control as it interacted with GST-Pin1 in an ERK-independent fashion. Similar results were obtained in five independent experiments.

BimEL in HEK293 cells or endogenous BimEL in Rat-1 and CCl39 cells can bind to GST-Pin1. Activation of the ERK1/2 pathway promotes BimEL phosphorylation, making it competent to bind to GST-Pin1, and this binding requires the intact WW domain of Pin1. Given the known specificity of ERK1/2 for Ser-Pro or Thr-Pro motifs and the specificity of the Pin1 WW domain for Ser(P)-Pro or Thr(P)-Pro motifs, these results strongly suggest that ERK1/2 directly phosphorylates BimEL at (S/T)P motifs in vitro.

BimEL Is Phosphorylated by ERK1 in Vitro and Associates with a MEK1/2-dependent Kinase in Cell Extracts—To investigate the phosphorylation of BimEL, we performed in vitro kinase assays. CM3 cells are CCl39 cells that express the conditional protein kinase MEKK3/ER*, which when treated with 4-hydroxytamoxifen (4-HT) strongly activates the ERK1/2, JNK, and p38 pathways (24, 25). CM3 cells were stimulated with 4-HT for 1 h, and cell lysates were used to isolate active ERK1, JNK1, and p38α by immunoprecipitation. Each kinase was then incubated with either recombinant GST, GST-BimEL, or an appropriate positive control substrate (MBP for ERK1, His-MAPKAP-K2, for p38α and GST-c-Jun-(1–223) for JNK1). GST was not phosphorylated, whereas GST-BimEL was phosphorylated in vitro by ERK1, JNK1 and p38α (Fig. 3A). However, we reproducibly noted a clear order of preference; ERK1 always phosphorylated GST-BimEL more effectively than JNK, which in turn was more effective than p38α. Indeed, in many cases ERK1 phosphorylated GST-BimEL as effectively as it did MBP (see also Fig. 4A).

Although many substrates can be phosphorylated by multiple MAPks in vitro, specificity in vivo is determined by the ability of MAPks to interact physically with their substrates through docking domains. For example, c-Jun can bind JNK (37), whereas Elk-1 can bind to ERK1/2 and JNK at a common site distinct from p38 (38). Because GST-BimEL was phosphorylated in vitro by ERK1/2, JNK, and p38 and yet BimEL hyper-phosphorylation in vivo correlated with activation of the ERK1/2 pathway, we sought to determine whether specificity might be determined by BimEL binding to the relevant kinase. We incubated cell lysates with bead-immobilized GST-BimEL and subjected these Bim precipitates to an auto-kinase assay.

FBS stimulation of serum-starved CCl39 cells caused the time-dependent appearance of a kinase activity that could bind to and phosphorylate GST-BimEL (Fig. 3B). The kinetics of this BimEL-associated auto-kinase closely matched that of the phosphorylation-induced mobility shift of the endogenous BimEL protein when both were assayed in parallel from the same cell lysates (Fig. 3B). In contrast, GST did not precipitate an FBS-stimulated auto-kinase activity. The ERK1/2 pathway inhibitor, PD184352, prevented the activation of ERK1 (Fig. 3C) and also prevented the activation of the BimEL kinase assayed in parallel by the pull-down assay from the same lysates (Fig. 3C). Together these data strongly implicate ERK1/2 as the kinase responsible for the FBS-stimulated phosphorylation of BimEL.

BimEL Interacts with Active ERK1/2 in Vitro and in Vivo—To determine whether ERK1/2 could associate with BimEL, serum-starved CCl39 cells were stimulated with FBS for 5 min to activate ERK1/2, and cell lysates were subjected to precipi-
tation with GST beads, GST-BimEL beads, or anti-ERK1 antibodies followed by kinase assays. GST was unable to precipitate kinase activity, whereas GST-BimEL again precipitated an FBS-stimulated auto-kinase (Fig. 4A). Immunoblotting revealed that GST-BimEL was able to selectively precipitate active, but not inactive, ERK1/2 from lysates of FBS-stimulated cells (Fig. 4A). Immunoprecipitation of active ERK1, assayed with MBP as the substrate, served as a positive control. Because these assays required the use of GST-BimEL (which lacks the C terminus), we also examined the interaction of full-length BimEL with ERK1/2. When HA-BimEL was expressed in HEK293 cells and immunoprecipitated with anti-HA antibodies, we detected ERK1/2 co-precipitating with BimEL, and this association was abolished by PD184352 (Fig. 4B).

To determine whether BimEL could interact with JNK, we used CM3 cells, expressing MEKK3:ER(C25). Lysates from CM3 cells that had been stimulated with 4-HT to activate ERK, JNK, or p38 were incubated with either recombinant GST-BimEL (EL), GST (G), or an appropriate positive control (H9251) as a substrate (MBP for ERK, His-MAPKAP-K2 for p38α, and GST-c-Jun-(1–223) for JNK) in kinase reactions with [γ-32P]ATP. After SDS-PAGE, samples were subjected to autoradiography. The positions of GST-BimEL, MBP, MAPKAP-K2, and GST-Jun-(1–223) are indicated. The doublet of phosphorylated bands with GST-Jun-(1–223) is due to partial degradation of the recombinant substrate to a smaller form that includes Ser63 and Ser73. B, serum-starved CM3 cells were restimulated with FBS for 2–30 min. Cell lysates were prepared and either subjected to SDS-PAGE and immunoblotted for Bim or subjected to a pull-down auto-kinase assay with GST-BimEL or GST followed by SDS-PAGE and autoradiography. C, serum-starved CM3 cells were left untreated (SF) or stimulated with serum in the presence of FBS or presence of PD184352 (FBS+PD). Cell lysates were used to assay ERK1 by immune complex kinase assay (upper panel) or the BimEL kinase activity by pull-down auto-kinase assay (lower panel). Similar results were obtained in three independent experiments.

Fig. 3. BimEL is phosphorylated by ERK1 in vitro and associates with a MEK1/2-dependent BimEL kinase in serum-stimulated cells. A, CM3 cells (CC39 cells expressing MEKK3:ER(C25)) were treated with 100 nM 4-HT for 1 h, and active ERK1, JNK1, or p38α kinases were isolated by immunoprecipitation (IP). Each kinase was then incubated with either recombinant GST-BimEL (EL), GST (G), or an appropriate positive control (H9251) as a substrate (MBP for ERK, His-MAPKAP-K2 for p38α, and GST-c-Jun-(1–223) for JNK) in kinase reactions with [γ-32P]ATP. After SDS-PAGE, samples were subjected to autoradiography. The positions of GST-BimEL, MBP, MAPKAP-K2, and GST-Jun-(1–223) are indicated. The doublet of phosphorylated bands with GST-Jun-(1–223) is due to partial degradation of the recombinant substrate to a smaller form that includes Ser63 and Ser73. B, serum-starved CM3 cells were restimulated with FBS for 2–30 min. Cell lysates were prepared and either subjected to SDS-PAGE and immunoblotted for Bim or subjected to a pull-down auto-kinase assay with GST-BimEL or GST followed by SDS-PAGE and autoradiography. C, serum-starved CM3 cells were left untreated (SF) or stimulated with serum in the presence of FBS or presence of PD184352 (FBS+PD). Cell lysates were used to assay ERK1 by immune complex kinase assay (upper panel) or the BimEL kinase activity by pull-down auto-kinase assay (lower panel). Similar results were obtained in three independent experiments.
interaction was detected between activated p38 and GST-
BimEL.2 Taken together, these results reveal that ERK1/2 are
FBS-stimulated BimEL kinases that can physically associate
in vitro and in vivo. In contrast, whereas JNK and
p38 can weakly phosphorylate GST-BimEL in vitro, their
ability to bind to BimEL makes it unlikely that they are FBS-
stimulated BimEL kinases in vivo.

BimEL Is Phosphorylated at Ser65 by ERK1/2—We next com-
pared the ability of ERK1 to phosphorylate the three common
splice variants, BimS, BimL, and BimEL, in an in vitro kinase
assay. This revealed that ERK1 could phosphorylate GST-
BimEL, but not GST-BimS or GST-BimL (Fig. 5A). BimEL con-
tains six potential MAPK phosphorylation sites as defined by
the minimal consensus of (S/T)P (Fig. 5B). Three of these sites
fall within the region unique to BimEL (assigned 1, 2, and 3)
whereas the other three fall within the region shared by BimL
and BimEL (assigned 4, 5, and 6). To define which sites were
phosphorylated by ERK1 in vitro, we mutated each site indi-
vidually to non-phosphorylatable alanine residues, and each
mutant was expressed as a GST fusion. For this analysis we
excluded regions of Bim encoding BimS, because BimS was not
a substrate in vitro assays, does not contain any (S/T)P
motifs, and is not a phosphoprotein2; instead, we focused on
the regions found in BimL and BimEL (referred to as BimEL-1.7

References:

1. Ley and S. Cook, unpublished observations.

Fig. 4. BimEL physically interacts with ERK1/2 but not JNK. A, CC39 cells were serum-starved for 18 h and then left untreated (–) or serum-stimulated (+) for 5 min. Cell lysates were incubated with GST beads (negative control), GST-BimEL beads, or anti-ERK1 antibodies (positive control) and then subjected to an auto-kinase reaction (GST or GST-BimEL) or an MBP kinase reaction (ERK1). Following SDS-PAGE, samples were subjected to autoradiography (upper panel) or immunoblotted for phospho-ERK (Anti-P-ERK1/2, middle panel) and total ERK (anti-ERK1/2, bottom panel). B, HEK293 cells were transfected with HA-BimEL in serum-free conditions. After 18 h cells were stimulated with FBS in the absence (–) or presence (+) of PD184352. HA-conjugated beads were used to immunoprecipitate (IP) HA-BimEL, and these were subjected to SDS-PAGE and immunoblotted (WB) for HA and ERK1/2. C, C39 cells (expressing ΔMEKK3:ER*, which activates ERK1/2, JNK or p38 (25)) were starved for 18 h and then treated with 100 nM 4-HT. GST, GST-BimEL, GST-BimELS65A or GST-c-Jun(1–223) bound to beads were used to pull-down protein from these lysates, and precipitates were resolved by SDS-PAGE and immunoblotted for ERK (upper panel) or JNK1 (lower panel). Cell lysates were immunoblotted as a control for the ERK1/2 or JNK1 in the input lysate. Similar results were obtained in three independent experiments.
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(41–127). The individual mutants were assessed for their ability to act as substrates in ERK1 in vitro kinase assays. Strikingly, the mutation of Ser65 to Ala (site 2 in Fig. 5B) completely abolished the ability of the GST-BimEL-L protein to be phosphorylated by ERK1 in vitro. This residue lies in the region of Bim unique to BimEL and also exhibits a proline at the −2 position with respect to the phospho-acceptor site. It has been suggested previously (39) that ERK1/2 exhibits a secondary preference for Pro at this position (i.e. PX(S/T)/P). Thus Ser65 is the major site of ERK1/2 phosphorylation in vitro.

Ser65 Is a Major Site of ERK Phosphorylation in Vivo—To confirm these data in vivo, HA-BimEL and HA-BimEL-S65A were transiently expressed in HEK293 cells maintained in FBS and resolved by standard SDS-PAGE. Under these conditions we always observed that BimEL resolved as at least two bands, whereas BimEL-S65A resolved as a monomer with enhanced migration on SDS-PAGE (Fig. 6A). When samples were resolved by two-dimensional electrophoresis, we again observed loss of the most acidic spots of BimEL (spot 4, spot 3, and most of spot 2) upon treatment of cells with PD184352 (Fig. 6B). Furthermore, the introduction of a single mutation at S65A effectively abolished the two most acidic spots and the positions that were mutated to alanine shown. Right panel, cells were treated as above, and ERK1 was immunoprecipitated and used in kinase reactions (upper panel), and subjected to autoradiography (lower panel).

To confirm these data in vivo, WE have reported previously that ERK1/2-dependent phosphorylation of BimEL targets it for degradation by the proteasome (24). Because Ser65 appeared to be a major site of ERK-catalyzed phosphorylation of Bim in vitro and in vivo, we hypothesized that a mutation at this residue, to prevent phosphorylation, would stabilize the protein. HA-BimEL or BimEL-S65A were transiently expressed in HEK293 cells under serum-free conditions. Cells were then treated with emetine, stimulated with FBS, and chased for a further 2 or 4 h. As we observed previously (24), wild type BimEL, is also required for optimal FBS-induced, ERK1/1-dependent phosphorylation of BimEL in vitro. We have reported previously (24) that BimEL-S65A was reduced by nearly 50% (Fig. 7A). In contrast, the HA-BimEL-S65A was expressed at a 50% higher level than wild type HA-BimEL (also see Fig. 6A) and turned over very slowly so that by 4 h it had only reduced by 11% (Fig. 7A). Thus, introduction of the S65A mutation blocked degradation and stabilized the BimEL protein.

The stabilization of BimEL-S65A was also reflected in an enhanced apoptotic effect. For example, when HEK293 cells were transfected with EGFP-BimEL or EGFP-BimEL-S65A, sorted for EGFP positives, and stained with propidium iodide, we observed that the percentage of EGFP-positive cells exhibiting sub-G1 DNA content rose from 34 ± 10% for EGFP-BimEL.
of Bim EL-(41–127) causes it to accumulate at higher levels and thereby induce more cell death.

The ERK Docking Domain Maps within Residues 70–97 of BimEL—Studies on a number of ERK, JNK, or p38 substrates have defined discrete docking domains, distinct from the phospho-acceptor site, which are necessary and sufficient for interaction with their relevant kinase (40–42). To define further the ERK1/2 docking domain, we analyzed the ability of GST-BimEL, GST-BimL, and GST-BimELS65A to interact with ERK1/2 in pull-down assays. We used CR1-11 cells, expressing the conditional kinase 

\[ \Delta R a f-1: E R K \] 

which selectively stimulates the ERK1/2 pathway when it is activated by 4-HT (14). Serum-starved CR1-11 cells were stimulated with 4-HT for 2 h, and lysates were incubated with GST fusion proteins. Only GST-BimEL was able to precipitate ERK1/2 from cell lysates (Fig. 8A), suggesting that ERK1/2 binding requires sequences unique to BimEL.

To define the docking domain in greater detail, we used a series of deletion mutants of GST-BimELS65A-(41–127) in the pull-down assay. In these experiments CM3 cells were stimulated with 4-HT to activate the ERK1/2, JNK, and p38 pathways, and the various GST fusion proteins, spanning amino acids 41–127, were used to pull-down kinases from the cell lysates. GST-BimELS65A-(41–127) was very effective at precipitating ERK1/2 from cell lysates (Fig. 8B, lane 5); indeed, in parallel analysis it precipitated ERK1/2 as well as GST-BimELS65A-(41–127). A GST fusion protein containing residues 70–97, a region specific to BimEL, was able to pull down ERK1/2 from cell lysates (Fig. 8B, lane 3) albeit with reduced efficiency compared with BimEL-(41–127) (Fig. 8B, lane 5). Because the relative amounts of GST-BimEL-(70–97) and GST-BimELS65A-(41–127) were the same, the poorer binding of GST-BimEL-(70–97) may simply be because of the smaller fragments not being able to fold correctly in bacteria. A GST fusion protein encompassing the region common to both BimEL and BimL proteins, GST-Bim-(98–127), failed to pull down ERK1/2 from cell lysates (Fig. 8B, lane 4) as did other regions unique to BimL, such as BimL-(41–60) and BimL-(41–70) (Fig. 8B, lanes 1 and 2). These results indicate that the minimal region of Bim required for interaction with ERK1/2 maps to amino acids 70–97, within the region unique to BimEL. Once again, none of these fragments of Bim was able to precipitate JNK or p38 from lysates (Fig. 8D) despite the fact that these kinases are strongly activated by treatment with 4-HT (25).

JNK binds to the δ-domain of c-Jun (37). This site is distinct from the phospho-acceptor sites that are not required for c-Jun-JNK binding (43). To investigate the requirement for the phospho-acceptor site in ERK-Bim interactions, we first compared the ability of ERK to phosphorylate the GST-Bim fragments used in the pull-down assay in Fig. 8D. Equal amounts of each GST-Bim fragment were used as a substrate in an in vitro kinase assay with active ERK1. Under these conditions only GST-Bim-(41–70) and GST-BimEL-(41–127), both of which contain the phospho-acceptor site Ser\(^{65}\), were phosphorylated by ERK1. Because GST-Bim-(41–70) was unable to precipitate ERK1/2 from cell lysates, these results effectively separated the phospho-acceptor site from the ERK1/2 docking domain. This was supported by the observation that GST-BimEL and GST-BimELS65A were equally effective at precipitating ERK1/2 from cell lysates (Fig. 4C). Thus the phospho-acceptor site maps outside the minimal ERK1/2 docking domain and is not required for BimEL to bind ERK1/2.

**DISCUSSION**

**ERK1/2 Are Serum-stimulated BimEL Kinases That Specifically Associate with BimEL in Vitro and in Vivo—**Several groups (11, 14, 20–23) have reported that BimEL is a phospho-protein, and we have shown that activation of the ERK1/2 pathway promotes the phosphorylation and proteasomal degradation of BimEL (24). Here we have shown that ERK1/2 are FBS-stimulated BimEL kinases.

BimEL exhibited a basal level of phosphorylation in HEK293 cells whether assayed by \(^{32}P\)P\(_T\) labeling or two-dimensional electrophoresis; this increased upon FBS stimulation, but both the basal and stimulated phosphorylation were reduced by PD184352. By using two-dimensional electrophoresis (22), we found that at least three of the BimEL phosphorylation sites (represented by spots 2–4) required the ERK1/2 pathway; the resistance of spot 1 to PD184352 suggests that at least one phosphorylation site is independent of the ERK1/2 pathway. The ability of GST-Pi1 to selectively precipitate BimEL in an ERK1/2-dependent fashion indicated that BimEL was phosphorylated at Ser-Pro or Thr-Pro sites in vivo, suggesting that ERK1/2 were the kinases responsible. Indeed, whereas ERK1, JNK1, and p38a all phosphorylated GST-BimEL in vitro, ERK1 was clearly the most effective. Furthermore, GST-BimEL could precipitate an FBS-stimulated BimEL kinase activity from extracts of serum-stimulated cells (Fig. 3), and this was abolished by PD184352. Finally, GST-BimEL in vitro and HA-BimEL in vivo were able to precipitate active but not inactive ERK1/2 (Fig. 4), indicating that ERK1/2 are not constitutively bound to BimEL but only interact when they become activated. Taken
together these results indicate that ERK1/2 are FBS-stimulated kinases that physically interact with BimEL in vitro and in vivo.

Ser<sup>65</sup> Is a Major Site of ERK1/2-catalyzed BimEL Phosphorylation in Vitro and in Vivo and Is Required for Serum-stimulated Degradation of BimEL—In contrast to GST-BimEL, ERK1 was unable to phosphorylate GST-BimL or GST-BimS in an in vitro kinase assay (Fig. 5A), and mutagenesis of all six potential (S/T)P motifs in BimEL revealed that only Ser<sup>65</sup>, lying in the region unique to BimEL, was required for in vitro phosphorylation. Indeed, loss of Ser<sup>65</sup> abolished in vitro phosphorylation of GST-BimEL by ERK1; this site exhibits a proline at...
ERK1/2 Binds to and Phosphorylates Bim_{EL}.

A. Diagram depicting isoforms of Bim protein used as GST fusion proteins. Right panel, CC39 cells were serum-starved for 18 h and restimulated for 10 min with FBS. Equal quantities of GST (G), GST-Bim_{S} (S), GST-Bim_{L} (L), and GST-Bim_{EL} (EL) bound to beads were used to precipitate proteins from cell lysates in a pull-down assay. The proteins were resolved by SDS-PAGE and immunoblotted for GST and total ERK1/2. Note that all three GST-Bim proteins were partially degraded; consequently, after Bradford assay the amount of GST fusion proteins used in the assay was also adjusted according to densitometry of the intact full-length species. B, left panel, diagram to show the GST-Bim_{EL,L}–(41–127) deletion fragments (lanes 1–5) used in pull-down and kinase assays. Right panel, CM3 cells (expressing ΔMEKK3:ER*, which activates ERK1/2, JNK, or p38 (25)) were serum-starved for 18 h and treated with 100 nM 4-HT for 1 h. The indicated GST fusion proteins were bound to beads and used to precipitate proteins from cell lysates, which were then immunoblotted for ERK1/2, JNK1, or p85α; cell lysates were immunoblotted as a control for the ERK1/2, JNK1, or 38α in the input lysate. In parallel, the same GST fusion proteins were added as substrates to an ERK1 immune complex kinase assay and subjected to autoradiography after SDS-PAGE. Similar results were obtained in three independent experiments.

Identification of an ERK1/2 Docking Domain in Bim_{EL}—Bim_{EL} could physically interact with active ERK1/2 in vitro and in vivo (Fig. 4). The ERK1/2 docking domain of Bim_{EL} was discrete from, and independent of, the phospho-acceptor site (Ser^{65}), in agreement with similar studies of the JNK–C-Jun interaction (37, 43). Our analysis revealed that GST-Bim–(70–97) (part of the region unique to Bim_{EL}) could interact with active ERK1/2, although this short fragment was less effective at binding ERK1/2 than GST-Bim–(127–41)–(41–127). This may reflect incorrect folding of small fragments in bacteria or may suggest that other additional contacts outside Bim–(70–97) increase the efficiency of the interaction. Future studies should aim to fully map this ERK docking domain, but this is the first report that any form of Bim can physically interact with its cognate kinase.

The ability of ERK1/2 to phosphorylate GST-Bim–(41–70) in vitro, despite the lack of the docking domain, presumably reflects the fact that the in vitro immune complex kinase assay contains all components at a considerable excess. This may also account for the ability of JNK and p38 to weakly phosphorylate GST-Bim_{EL} in vitro. Indeed, mutation of Ser^{65} –> Ala abolished the weak JNK and p38-mediated in vitro phosphorylation of GST-Bim_{EL}^{2} suggesting that all three kinases target the same phospho-acceptor site in vitro. However, specificity in vitro will be determined by the physical interaction between Bim_{EL} and its kinase, and in this case only ERK1/2 could interact with GST-Bim_{EL} in pull-down assays and with full-length Bim_{EL} in vivo, even when JNK and p38 were active in the same lysates.

A recent report (21) showed that JNK could phosphorylate Bim at Thr^{56} and either Ser^{44} or Ser^{58}, thereby disrupting the Bim_{EL}-DLC1 interaction and releasing Bim{L} to initiate cell death. It is notable that the ERK1/2 phosphorylation site...
mapped here (Ser65 in the region unique to BimEL) is distinct from the JNK sites identified in BimL (21) and which are shared in BimEL (Thr112 and Ser116); indeed, BimL lacks Ser65. Although we have not examined BimL phosphorylation, we did not observe JNK binding to BimEL in cell extracts under any conditions. It is possible that ERK1/2 binding to BimEL precludes JNK binding. Because BimL lacks an ERK1/2-binding site (Fig. 8), this might allow JNK to bind to a putative JNK docking domain in BimL and phosphorylate the DLC1 binding domain. In such a scenario ERK1/2 binding to BimEL might allow phosphorylation of Ser65 and also prevent binding of JNK, thereby preserving the BimEL-DLC1 interaction. Such a model would require that ERK1/2 and JNK both phosphorylate BimEL, but under different conditions, because even when both ERK1/2 and JNK were activated, we could only observe ERK1/2 binding to BimEL (Fig. 4C). It remains to be determined whether any of the JNK phosphorylation sites mapped in BimEL (21) contribute to the multiple phosphorylation sites we see in BimEL, but we did observe that the site represented by spot 1 on two-dimensional gels was refractory to the ERK1/2 pathway. Whether this site is a JNK target will require further characterization.

The ability of JNK to phosphorylate BimEL in nerve growth factor-deprived neurons (23) is more difficult to reconcile with JNK being the BimEL kinase that we have studied in fibroblasts and HEK293 cells studied here. In addition, the JNK-BimEL interaction in neurons, and which is absent in tissue-specific adaptor or scaffold protein that may facilitate ERK1/2 binding to BimEL might allow phosphorylation of Ser65 and prevent binding of JNK, thereby preserving the BimEL-DLC1 interaction. Such a model would require that ERK1/2 and JNK both phosphorylate BimEL, but under different conditions, because even when both ERK1/2 and JNK were activated, we could only observe ERK1/2 binding to BimEL (Fig. 4C). It remains to be determined whether any of the JNK phosphorylation sites mapped in BimEL (21) contribute to the multiple phosphorylation sites we see in BimEL, but we did observe that the site represented by spot 1 on two-dimensional gels was refractory to the ERK1/2 pathway. Whether this site is a JNK target will require further characterization.

In summary, we have shown that ERK1/2 are FBS-stimulated BimEL kinases that bind to BimEL via a discrete docking domain, causing its phosphorylation at Ser65; this is required for serum-dependent degradation of BimEL. These findings provide new insights into the post-translational regulation of BimEL, underscore the complexity of the different modes of regulation of even this one splice variant of Bim, and provide a novel role of ERK1/2 in cell survival signaling.

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Mechanisms of Signal Transduction: Extracellular Signal-regulated Kinases 1/2 Are Serum-stimulated Bim EL Kinases That Bind to the BH3-only Protein Bim EL Causing Its Phosphorylation and Turnover

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