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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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Rebecca Ley‡, Katherine E. Ewings§, Kathryn Hadfield, Elizabeth Howes, Kathryn Balmanno, and Simon J. Cook¶

From the Laboratory of Molecular Signalling, Signalling Programme, The Babraham Institute, Cambridge CB2 4AT, United Kingdom

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 Bim_{EL}, targeting it for degradation via the proteasome. However, the nature of the kinase responsible for Bim_{EL} phosphorylation remained unclear. We now show that Bim_{EL} is phosphorylated on at least three sites in response to activation of the ERK1/2 pathway. By using the peptidylprolyl isomerase, Pin1, as a probe for proline-directed phosphorylation, we show that ERK1/2-dependent phosphorylation of Bim_{EL} occurs at (S/T)P motifs. ERK1/2 phosphorylates Bim_{EL}, but not Bim_S or Bim_L, *in vitro*, and mutation of Ser^{65} to alanine blocks the phosphorylation of Bim_{EL} 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-Bim_{EL}, but not GST-Bim_L or GST-Bim_S, *in vitro*. ERK1/2 also binds to full-length Bim_{EL} *in vivo*, and we have localized a potential ERK1/2 "docking domain" lying within a 27-amino acid stretch of the Bim_{EL} protein. Our findings provide new insights into the post-translational regulation of Bim_{EL} 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-x_L, bind to and repress the multidomain pro-apoptotic proteins, Bax and Bak. BH3[1]-only proteins respond to stresses by binding to Bcl-2 or Bcl-x_L, 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), $p90^{RSK}$ (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-x_L 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: Bim_{EL}, Bim_L, and Bim_S (16–18). Both Bim_{EL} and Bim_L 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-x_L and may account for their weaker apoptotic potential relative to Bim_S.

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

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‡ To whom correspondence may be addressed. Tel.: 44-1223-496381; Fax: 44-1223-496043; E-mail: becky.ley@bbsrc.ac.uk.

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¶ Senior Cancer Research Fellow of Cancer Research UK. To whom correspondence may be addressed. Tel.: 44-1223-496453; Fax: 44-1223-496043; E-mail: simon.cook@bbsrc.ac.uk.

[1] 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; Bim_{EL}, Bim extra long; Bim_L, Bim long; Bim_S, 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; $p90^{RSK}$/RSK, ribosomal protein S6 kinase; PD184352, 2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropylmethoxy-3–4-difluorobenzamide; U0126, 1,4-diamino-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.

kinase responsible for Bim_{EL} phosphorylation was not identified. Here we show that activation of the ERK1/2 pathway promotes the phosphorylation of Bim_{EL} on at least three sites *in vivo*, some of which are proline-directed. ERK1/2 phosphorylates Bim_{EL} *in vitro* at serine 65; mutation of this site inhibits ERK1/2-dependent phosphorylation *in vivo* and stabilizes the Bim_{EL} protein. Finally, we have mapped the ERK1/2 docking domain of Bim_{EL} and shown it to be distinct from the phospho-acceptor site. Phosphorylation of Bim_{EL} 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, p38α were prepared in-house, and anti-HA was provided by the Babraham Institute Monoclonal Antibody Facility. Antibodies for P-ERK1/2 and ERK1/2 were from Cell Signaling Technology; Bim was from Chemicon; and Bcl-2, Bad, JNK, and rabbit anti-HA were 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 CCl39, 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 once in serum-free medium and then placed in fresh serum-free medium with the indicated dose of 4-HT, FBS, 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—Bim_{EL}, Bim_L, Bim_S, and fragments of Bim_{EL} were expressed as GST fusion proteins in pGEX-4T1 or as HA-tagged proteins in pCAN-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 Bim_{EL} cDNA sequence that was used in these studies. Potential phosphorylation sites were altered by PCR-based site-directed mutagenesis using PfuTurbo 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. Giannino 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 either mouse anti-HA antibodies conjugated to protein G-Sepharose beads or rabbit anti-HA antibodies conjugated to protein A-Sepharose. For *in vivo* $[^{32}P]P_i$ labeling studies cells were transfected (as above); after 18 h these were switched to phosphate-free and serum-free media with 2 mCi of $[^{32}P]P_i$, and relevant treatments were performed 3 h later.

Western Blot Analysis—Cells were lysed, fractionated by SDS-PAGE, transferred to polyvinylidene difluoride, and analyzed by immunoblotting as described previously (24–26). Two-dimensional electrophoresis was performed as described previously (22).

GST Fusion Proteins and "Pull-down" Assays—GST fusion proteins were expressed in BL21 bacterial cells purified by affinity chromatography using glutathione-Sepharose-agarose (GSH) beads (Amersham Biosciences), as described previously (28). The concentration of proteins was 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-Bim_{EL} 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 "Bim_{EL} kinase" activity pulled down with GST-Bim_{EL} 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-Bim_{EL} or pEGFP-Bim_{EL}S65A, 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

Bim_{EL} Is Phosphorylated in an ERK1/2-dependent Fashion in Vivo—When serum-starved CCl39 fibroblasts were restimulated with FBS for 10 min, Bim_{EL} 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. 1*A*), or the ERK1/2 pathway-specific inhibitor, PD184352 (24). To confirm that Bim_{EL} was a phosphoprotein, we ectopically expressed HA-tagged Bim_{EL} in HEK293 cells, metabolically labeled with $[^{32}P]P_i$ in serum-free medium; HA-Bim_{EL} 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 Bim_{EL} phosphorylation (Fig. 1*B*), probably because these transformed cells exhibit residual ERK1/2 activity. Restimulation of cells with FBS caused a further shift in mobility and incorporation of $[^{32}P]P_i$ into Bim_{EL}. The mobility shift was inhibited by PD184352, and we observed an even more striking reduction in the incorporation of $[^{32}P]P_i$ into Bim_{EL}, with PD184352 reducing the labeling to below the basal level (Fig. 1*B*). A small amount of a Bim-reactive band below Bim_{EL} was also expressed and may represent one of the alternatively spliced forms of Bim such as Bim_L or BimAD (16–18, 29); this protein incorporated little, if any, $[^{32}P]P_i$. These results confirm that Bim_{EL} is phosphorylated in an ERK1/2-dependent fashion *in vivo*.

In parallel, we examined the phosphorylation of exogenous Bim_{EL} by two-dimensional gel electrophoresis. In $[^{32}P]P_i$-labeled lymphocytes Bim_{EL} 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 $[^{32}P]P_i$ (22); the nature of the kinase responsible for this phosphorylation was not reported. In serum-starved HEK293 cells exogenous Bim_{EL} resolved as three spots: the phosphorylated spot 1 and spot 2 and the more basic non-phosphorylated form (Fig. 1*C*). Stimulation with FBS caused the basic form of Bim_{EL} to almost disappear, and this was accompanied by the appearance of the additional phosphorylated forms (*spot 3* and *spot 4*, Fig. 1*C*). 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 Bim_{EL}. These results indicate that the appearance of the hyperphosphorylated forms of Bim_{EL} (spots 2–4) requires the ERK1/2 pathway. They also confirm the basal level of Bim_{EL} 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 $[^{32}P]P_i$ incorporation into Bim_{EL} (Fig. 1*B*). 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 Bim_{EL} at up to three discrete sites.

Binding to the Peptidylprolyl Isomerase, Pin1, Reveals That Bim_{EL} Is Subject to ERK-dependent Proline-directed Phosphorylation—The ability of PD184352 to block Bim_{EL} phosphoryl-

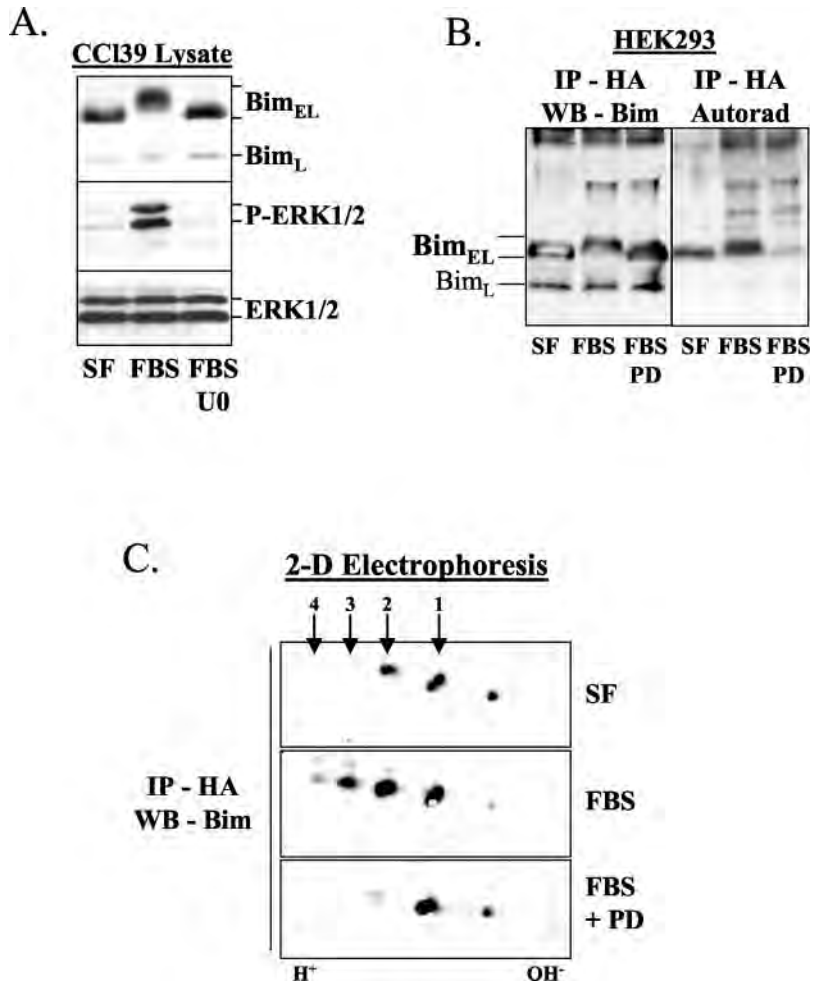

FIG. 1. **ERK1/2-dependent phosphorylation of Bim_{EL} *in vivo*.** *A*, CCl39 cells were serum-starved for 6 h. Cells were left untreated (*SF*) or stimulated with serum (FBS) in the absence or presence of U0126 (*FBS/U0*). Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. *B*, HEK293 cells were transfected with HA-Bim_{EL} in complete media. After 18 h cells were changed to serum- and phosphate-free media with [^{32}P]P_i, 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-Bim_{EL} 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.

ation might imply that ERK1/2 directly phosphorylates Bim_{EL}, but these results are also consistent with Bim_{EL} being phosphorylated by a kinase downstream of ERK1/2 such as $p90^{RSK}$, MAPK-interacting serine/threonine kinase 1/2, or mitogen- and stress-activated kinase 1/2. MAPKs, including ERK1/2, are proline-directed protein kinases that phosphorylate their substrates at serine or threonine residues immediately preceding a proline ((S/T)P). The peptidylprolyl isomerase Pin1 specifically binds to proteins that contain this (S/T)P motif but only when the Ser or Thr is phosphorylated (*i.e.* Ser(P)-Pro or Thr(P)-Pro) (30, 31). Binding is mediated by the N-terminal WW domain of Pin1 (Fig. 2*A*), and mutation of either the proline residue or the phospho-acceptor site (Ser/Thr) of the phosphoprotein abolishes Pin1 binding. Although most Pin1 targets have been shown to be substrates for cyclin-dependent kinases (CDKs) (32) or glycogen synthase kinase-3 (GSK-3) (33), binding of Pin1 to ERK1/2 or JNK substrates has been reported recently (34, 35). Accordingly, we postulated that Pin1 might serve as a probe for phosphorylation of (S/T)P motifs, and we tested this by examining whether recombinant Pin1 could interact with Bim_{EL}, which contains six (S/T)P motifs.

Plasmids encoding HA-Bim_S, HA-Bim_L, and HA-Bim_{EL} were expressed transiently in HEK293 cells, and cell lysates were subjected to a pull-down assay using GST-Pin1, followed by immunoblotting with anti-HA antibodies. Control experiments confirmed that GST alone did not precipitate any Bim, and so all subsequent experiments used GST as a pre-clearing step. GST-Pin1 was able to precipitate significant quantities of the Bim_{EL} isoform from cell lysates but little, if any, Bim_L and no Bim_S, although it was evident that all three isoforms expressed equally well (Fig. 2*B*). To determine whether GST-Pin1 could also interact with endogenous Bim_{EL}, 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 Bim_{EL} (Fig. 2*C*). GST-Pin1 was able to precipitate Bim_{EL} that had been phosphorylated following FBS stimulation, whereas there was no apparent binding of Bim_{EL} from lysates of serum-starved cells. In addition, U0126 prevented the activation of ERK1/2, inhibited the phosphorylation of Bim_{EL}, and completely blocked the binding of Bim_{EL} to GST-Pin1 (Fig. 2*C*). 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 immunereactive Bad (Fig. 2*C*).

The ability of Bim_{EL} to bind to GST-Pin1 absolutely required the WW domain because binding to wild type GST-Pin1 (*W*) was not replicated by GST-Pin1 with inactivating point mutations in the WW domain (Δ) (35, 36) (Fig. 2*D*). In addition, these results confirmed the phosphorylation dependence of the interaction between Pin1 and Bim_{EL}. For example, whereas lysates from serum-starved cells (SF) expressed the most Bim_{EL}, little of this was competent to bind to Pin1 in the pull-down assay. In contrast, although there was less Bim_{EL} in the extracts from FBS-stimulated cells (F) much more of this was competent to bind to Pin1 because it was phosphorylated (Fig. 2*D*).

In summary, these results show that ectopically expressed

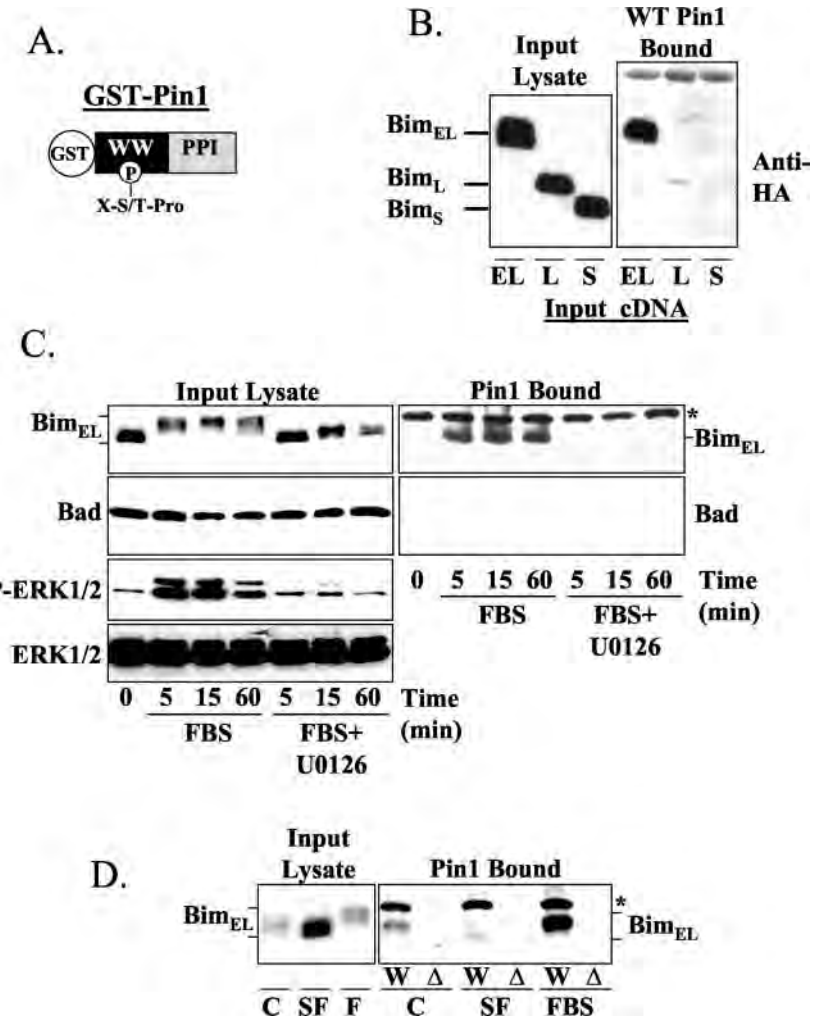

FIG. 2. **Exogenous and endogenous Bim$_{EL}$ interact with GST-Pin1 in an ERK-dependent fashion.** *A*, schematic representation of GST-Pin1 showing the WW domain that specifically interacts with Ser(P)-Pro or Thr(P)-Pro motifs, as a *black box*, and the peptidylprolyl isomerase (*PPI*) catalytic domain, as a *shaded box*. *B*, HEK293 cells were transfected with HA-Bim$_{EL}$ (*EL*), HA-Bim$_L$ (*L*), and HA-Bim$_S$ (*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 ERK1/2. *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.

Bim$_{EL}$ in HEK293 cells or endogenous Bim$_{EL}$ in Rat-1 and CCl39 cells can bind to GST-Pin1. Activation of the ERK1/2 pathway promotes Bim$_{EL}$ 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 Bim$_{EL}$ at (S/T)P motifs *in vivo*.

Bim$_{EL}$ Is Phosphorylated by ERK1 in Vitro and Associates with a MEK1/2-dependent Kinase in Cell Extracts—To investigate the phosphorylation of Bim$_{EL}$, we performed *in vitro* kinase assays. CM3 cells are CCl39 cells that express the conditional protein kinase ΔMEKK3: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-Bim$_{EL}$, 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-Bim$_{EL}$ was phosphorylated *in vitro* by ERK1, JNK1 and p38α (Fig. 3*A*). However, we reproducibly noted a clear order of preference; ERK1 always phosphorylated GST-Bim$_{EL}$ more effectively than JNK, which in turn was more effective than p38α. Indeed, in many cases ERK1 phosphorylated GST-Bim$_{EL}$ as effectively as it did MBP (see also Fig. 4*A*).

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-Bim$_{EL}$ was phosphorylated *in vitro* by ERK1/2, JNK, and p38 and yet Bim$_{EL}$ hyper-phosphorylation *in vivo* correlated with activation of the ERK1/2 pathway, we sought to determine whether specificity might be determined by Bim$_{EL}$ binding to the relevant kinase. We incubated cell lysates with bead-immobilized GST-Bim$_{EL}$ 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-Bim$_{EL}$ (Fig. 3*B*). The kinetics of this Bim$_{EL}$-associated auto-kinase closely matched that of the phosphorylation-induced mobility shift of the endogenous Bim$_{EL}$ protein when both were assayed in parallel from the same cell lysates (Fig. 3*B*). In contrast, GST did not precipitate an FBS-stimulated auto-kinase activity. The ERK1/2 pathway inhibitor, PD184352, prevented the activation of ERK1 (Fig. 3*C*) and also prevented the activation of the Bim$_{EL}$ kinase assayed in parallel by the pull-down assay from the same lysates (Fig. 3*C*). Together these data strongly implicate ERK1/2 as the kinase responsible for the FBS-stimulated phosphorylation of Bim$_{EL}$.

Bim$_{EL}$ Interacts with Active ERK1/2 in Vitro and in Vivo—To determine whether ERK1/2 could associate with Bim$_{EL}$, serum-starved CCl39 cells were stimulated with FBS for 5 min to activate ERK1/2, and cell lysates were subjected to precipi-

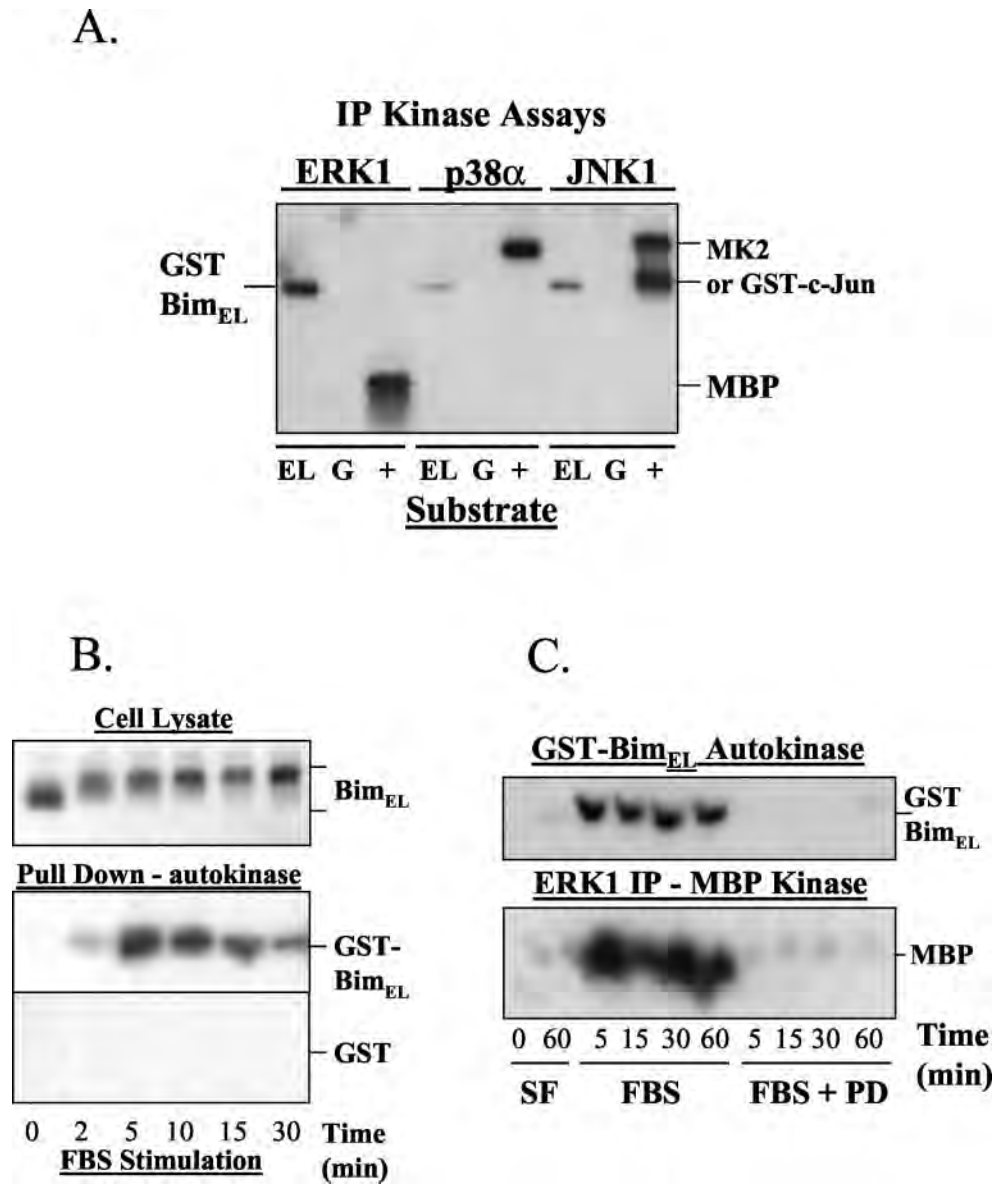

FIG. 3. **Bim$_{EL}$ is phosphorylated by ERK1 *in vitro* and associates with a MEK1/2-dependent Bim$_{EL}$ kinase in serum-stimulated cells.** *A*, CM3 cells (CCl39 cells expressing MEKK3:ER (25)) were treated with 100 nM 4-HT for 1 h, and active ERK1, JNK1, or p38α kinases were isolated by immunoprecipitation (*IP*). Each kinase was then incubated with either recombinant GST-Bim$_{EL}$ (*EL*), GST (*G*), or an appropriate positive control (+) as a substrate (MBP for ERK1, His-MAPKAP-K2 for p38α, and GST-c-Jun-(1–223) for JNK1) in kinase reactions with [γ-^{32}P]ATP. After SDS-PAGE, samples were subjected to autoradiography. The positions of GST-Bim$_{EL}$, 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 Ser^{63} and Ser^{73}. *B*, serum-starved CCl39 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-Bim$_{EL}$ or GST followed by SDS-PAGE and autoradiography. *C*, serum-starved CCl39 cells were left untreated (*SF*) or stimulated with serum in the absence (*FBS*) or presence of PD184352 (*FBS+PD*). Cell lysates were used to assay ERK1 by immune complex kinase assay (*upper panel*) or the Bim$_{EL}$ kinase activity by pull-down auto-kinase assay (*lower panel*). Similar results were obtained in three independent experiments.

tation with GST beads, GST-Bim$_{EL}$ beads, or anti-ERK1 antiserum followed by kinase assays. GST was unable to precipitate kinase activity, whereas GST-Bim$_{EL}$ again precipitated an FBS-stimulated auto-kinase (Fig. 4*A*). Immunoblotting revealed that GST-Bim$_{EL}$ was able to selectively precipitate active, but not inactive, ERK1/2 from lysates of FBS-stimulated cells (Fig. 4*A*). The immunoprecipitation of active ERK1, assayed with MBP as the substrate, served as a positive control. Because these assays required the use of GST-Bim$_{EL}$ (which lacks the C terminus), we also examined the interaction of full-length Bim$_{EL}$ with ERK1/2. When HA-Bim$_{EL}$ was expressed in HEK293 cells and immunoprecipitated with anti-HA antibodies, we detected ERK1/2 co-precipitating with Bim$_{EL}$, and this association was abolished by PD184352 (Fig. 4*B*).

To determine whether Bim$_{EL}$ could interact with JNK, we again used CM3 cells, expressing ΔMEKK3:ER*. Lysates from CM3 cells that had been stimulated with 4-HT to activate ERK, JNK, or p38 were incubated with GST-Bim$_{EL}$ beads, and the resulting complexes were subjected to immunoblotting with the relevant antibodies. GST-Bim$_{EL}$ was again able to precipitate ERK1/2 from CM3 cell lysates (Fig. 4*C*). As a positive control GST-c-Jun-(1–223) was clearly able to precipitate significant quantities of JNK1 from CM3 cell lysates, indicating that JNK had been activated by the 4-HT treatment; despite this, GST-Bim$_{EL}$ was not able to precipitate JNK (Fig. 4*C*). Similarly, no

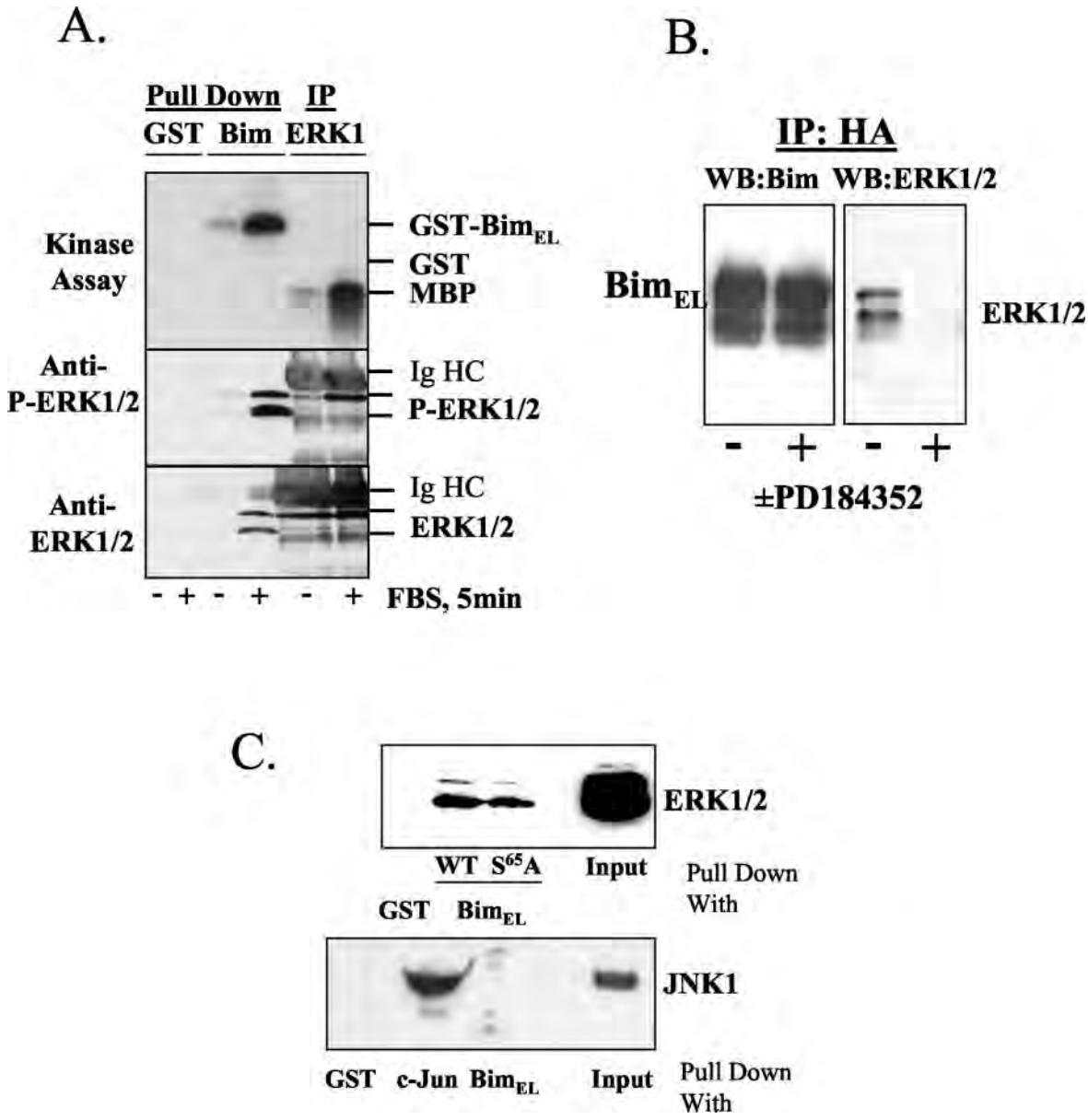

FIG. 4. **Bim$_{EL}$ physically interacts with ERK1/2 but not JNK.** *A*, CCl39 cells were serum-starved for 18 h and then left untreated (−) or serum-stimulated (+) for 5 min. Cell lysates were incubated with GST beads (negative control), GST-Bim$_{EL}$ beads, or anti-ERK1 antibodies (positive control) and then subjected to an auto-kinase reaction (GST or GST-Bim$_{EL}$) 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-Bim$_{EL}$ 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-Bim$_{EL}$, and these were subjected to SDS-PAGE and immunoblotted (*WB*) for HA and ERK1/2. *C*, CM3 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-Bim$_{EL}$ GST-Bim$_{EL}$S65A 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.

interaction was detected between activated p38 and GST-Bim$_{EL}$.[2] Taken together, these results reveal that ERK1/2 are FBS-stimulated Bim$_{EL}$ kinases that can physically associate with Bim$_{EL}$ *in vitro* and *in vivo*. In contrast, whereas JNK and p38 can weakly phosphorylate GST-Bim$_{EL}$ *in vitro*, their inability to bind to Bim$_{EL}$ makes it unlikely that they are FBS-stimulated Bim$_{EL}$ kinases *in vivo*.

Bim$_{EL}$ Is Phosphorylated at Ser^{65} by ERK1/2—We next compared the ability of ERK1 to phosphorylate the three common splice variants, Bim$_S$, Bim$_L$, and Bim$_{EL}$, in an *in vitro* kinase assay. This revealed that ERK1 could phosphorylate GST-Bim$_{EL}$ but not GST-Bim$_S$ or GST-Bim$_L$ (Fig. 5*A*). Bim$_{EL}$ contains six potential MAPK phosphorylation sites as defined by the minimal consensus of (S/T)P (Fig. 5*B*). Three of these sites fall within the region unique to Bim$_{EL}$ (assigned 1, 2, and 3) whereas the other three fall within the region shared by Bim$_L$ and Bim$_{EL}$ (assigned 4, 5, and 6). To define which sites were phosphorylated by ERK1 *in vitro,* we mutated each site individually to non-phosphorylatable alanine residues, and each mutant was expressed as a GST fusion. For this analysis we excluded regions of Bim encoding Bim$_S$, because Bim$_S$ was not a substrate in *in vitro* assays, does not contain any (S/T)P motifs, and is not a phosphoprotein[2]; instead, we focused on the regions found in Bim$_L$ and Bim$_{EL}$ (referred to as Bim$_{EL+L}$-

[2] R. Ley and S. Cook, unpublished observations.

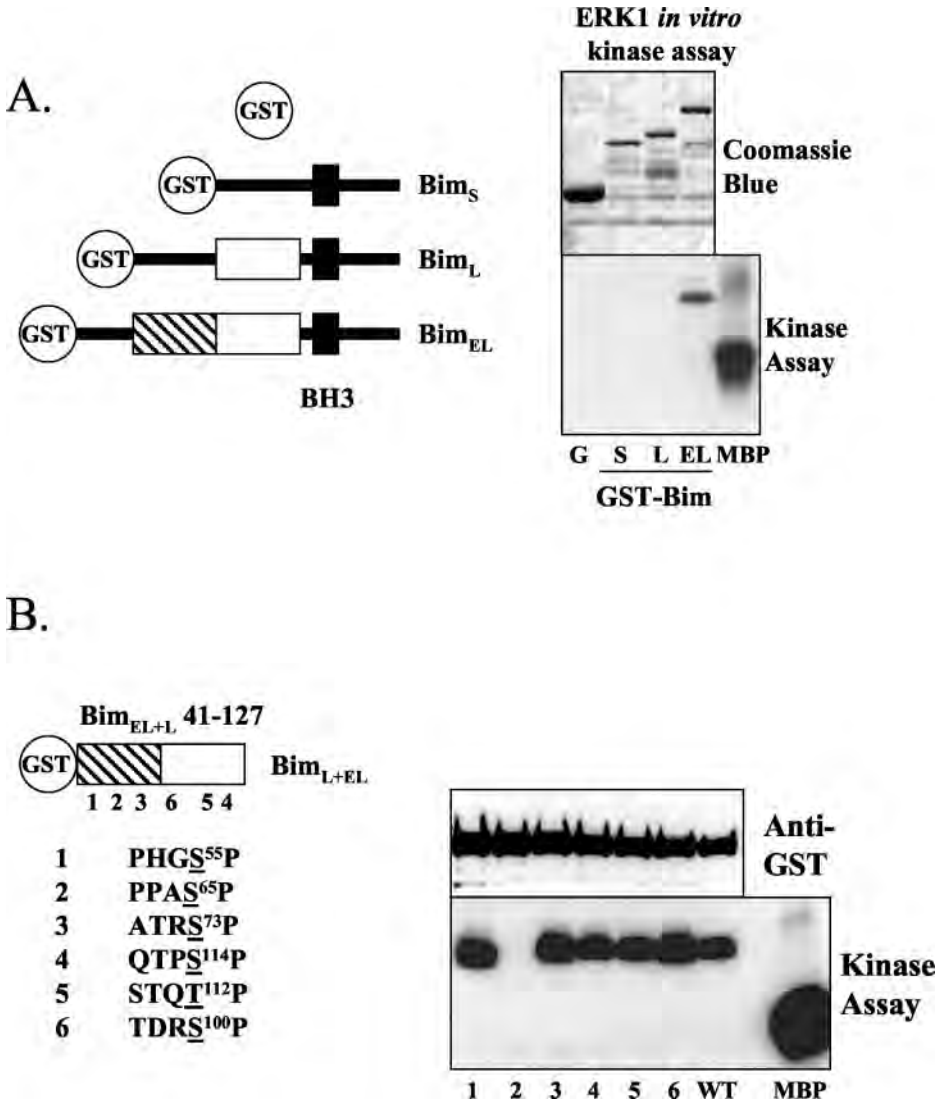

FIG. 5. **ERK1 phosphorylates Bim_{EL} but not Bim_L or Bim_S *in vitro*, and Ser^{65} is the major phospho-acceptor site.** *A*, *left panel*, diagram depicting Bim isoforms fused to GST. The *black bar* indicates the region common to short (*S*), long (*L*), and extra long (*EL*) isoforms. The *white box* indicates region common to long (*L*) and extra long (*EL*) isoforms. The *hatched box* indicates region unique to the extra long (*EL*) isoform. *Right panel*, CCl39 cells were serum-starved for 18 h and restimulated with FBS for 10 min. ERK1 was immunoprecipitated from cell lysates and used in a kinase assay with GST (*G*), GST-Bim_S (*S*), GST-Bim_L (*L*), GST-Bim_{EL} (*EL*), or MBP as substrates. The reactions were resolved by SDS-PAGE, stained with Coomassie Blue to confirm equal substrate loading of the kinase reactions (*upper panel*), and subjected to autoradiography (*lower panel*). *B*, *left panel,* diagram representing the regions fused to GST (key as for *A*) showing the potential MAPK phospho-acceptor sites 1–6, with the residues (*underlined*) 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 with mutants 1–6 fused to GST as substrates. Wild type GST-Bim_{L+EL} and MBP were included as positive controls. Equivalent amounts of the substrate proteins were resolved by SDS-PAGE and immunoblotted with anti-GST antibodies to confirm equal loading of the kinase reactions with substrate proteins (*upper panel*). Similar results were obtained in three independent experiments.

(41–127). The individual mutants were assessed for their ability to act as substrates in ERK1 *in vitro* kinase assays. Strikingly the mutation of Ser^{65} to Ala (site 2 in Fig. 5*B*) completely abolished the ability of the GST-Bim_{EL+L} protein to be phosphorylated by ERK1 *in vitro*. This residue lies in the region of Bim unique to Bim_{EL} 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.* P*X*(S/T)P). Thus Ser^{65} is the major site of ERK1/2 phosphorylation *in vitro*.

Ser^{65} Is a Major Site of ERK Phosphorylation in Vivo—To confirm these data *in vivo*, HA-Bim_{EL} and HA-Bim_{EL}S65A were transiently expressed in HEK293 cells maintained in FBS and resolved by standard SDS-PAGE. Under these conditions we always observed that Bim_{EL} resolved as at least two bands, whereas Bim_{EL}S65A resolved as a monomer with enhanced migration on SDS-PAGE (Fig. 6*A*). When samples were resolved by two-dimensional electrophoresis, we again observed loss of the most acidic spots of Bim_{EL} (spot 4, spot 3, and most of spot 2) upon treatment of cells with PD184352 (Fig. 6*B*). Furthermore, the introduction of a single mutation at S65A effectively abolished the two most acidic spots (spot 4 and spot 3). These results confirm that Ser^{65}, an ERK1 phosphorylation site *in vitro*, is also required for optimal FBS-induced, ERK1/2-dependent phosphorylation of Bim_{EL} *in vivo*. They also indicate that phosphorylation at this single residue may also be required for at least one other phosphorylation event in Bim_{EL} *in vivo* (Fig. 6*B*).

Mutation of Ser^{65} Stabilizes Bim_{EL} and Enhances Its Cell Killing Activity—We have reported previously that ERK1/2-dependent phosphorylation of Bim_{EL} targets it for degradation by the proteasome (24). Because Ser^{65} 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-Bim_{EL} or Bim_{EL}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 Bim_{EL} turned over in the presence of FBS so that by 4 h the amount of HA-Bim_{EL} was reduced by nearly 50% (Fig. 7*A*). In contrast, the HA-Bim_{EL}S65A was expressed at a 50% higher level than wild type HA-Bim_{EL} (also see Fig. 6*A*) and turned over only very slowly so that by 4 h it had only reduced by 11% (Fig. 7*A*). Thus, introduction of the S65A mutation blocked degradation and stabilized the Bim_{EL} protein.

The stabilization of Bim_{EL}S65A was also reflected in an enhanced apoptotic effect. For example, when HEK293 cells were transfected with EGFP-Bim_{EL} or EGFP-Bim_{EL}S65A, sorted for EGFP positives, and stained with propidium iodide, we observed that the percentage of EGFP-positive cells exhibiting sub-G_1 DNA content rose from 34 ± 10% for EGFP-Bim_{EL}

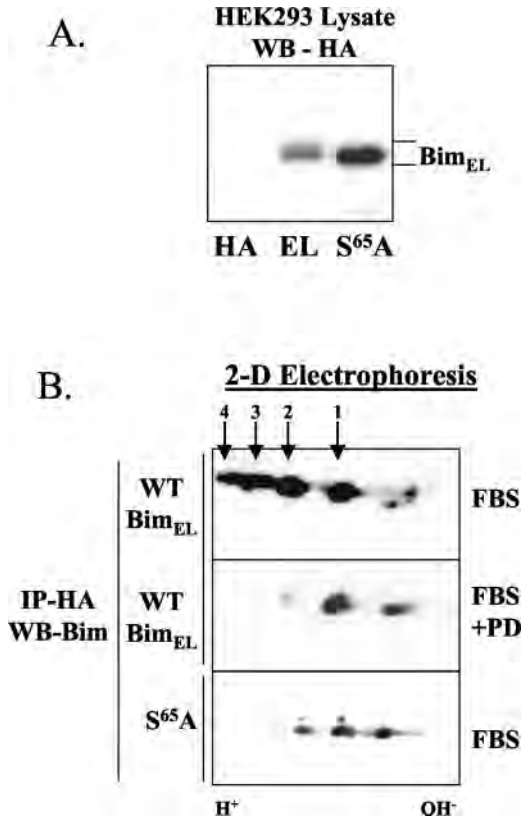

FIG. 6. **Ser^{65} is an FBS- and ERK1/2-dependent phosphorylation site *in vivo*.** *A*, HEK293 cells were transfected with HA (empty vector), HA-Bim$_{EL}$, or HA-Bim$_{EL}$S65A in complete media. Lysates were resolved by SDS-PAGE and immunoblotted (*WB*) with anti-HA. *B*, HEK293 cells were transfected with HA-Bim$_{EL}$ or HA-Bim$_{EL}$S65A in serum-free conditions. After 18 h cells were stimulated with serum (*FBS*). As a control cells expressing HA-Bim$_{EL}$ were treated with PD184352 (*FBS+PD*). HA-conjugated beads were used to immunoprecipitate (*IP*) HA-Bim$_{EL}$ or HA-Bim$_{EL}$S65A from cell lysates, and these were resolved by two-dimensional (*2-D*) electrophoresis and immunoblotted for Bim. *H*$^+$, acidic. *OH*$^-$, basic. Similar results were obtained in three independent experiments.

to 53 ± 3% for EGFP-Bim$_{EL}$S65A (Fig. 7*B*). Thus, stabilization of Bim$_{EL}$S65A causes it to accumulate at higher levels and thereby induce more cell death.

The ERK Docking Domain Maps within Residues 70–97 of Bim$_{EL}$—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-Bim$_S$, GST-Bim$_L$, and GST-Bim$_{EL}$ to interact with ERK1/2 in pull-down assays. We used CR1-11 cells, expressing the conditional kinase ΔRaf-1:ER*, 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-Bim$_{EL}$ was able to precipitate ERK1/2 from cell lysates (Fig. 8*A*), suggesting that ERK1/2 binding requires sequences unique to Bim$_{EL}$.

To define the docking domain in greater detail, we used a series of deletion mutants of GST-Bim$_{EL+L}$-(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-Bim$_{EL+L}$ was very effective at precipitating ERK1/2 from cell lysates (Fig. 8*B*, *lane 5*); indeed, in parallel analysis it precipitated ERK1/2 as well as GST-Bim$_{EL}$.[2] A GST fusion protein containing residues 70–97, a region specific to Bim$_{EL}$, was able to pull down ERK1/2 from cell lysates (Fig. 8*B*, *lane3*) albeit with reduced efficiency compared with Bim$_{EL+L}$ (Fig. 8*B, lane 5*). Because the relative amounts of GST-Bim-(70–97) and GST-Bim$_{EL+L}$-(41–127) were the same, the poorer binding of GST-Bim-(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 Bim$_{EL}$ and Bim$_L$ proteins, GST-Bim-(98–127), failed to pull down ERK1/2 from cell lysates (Fig. 8*B*, *lane 4*) as did other regions unique to Bim$_{EL}$ such as Bim$_{EL}$–(41–60) and Bim$_{EL}$–(41–70) (Fig. 8*B*, *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 Bim$_{EL}$. Once again, none of these fragments of Bim was able to precipitate JNK or p38 from lysates (Fig. 8*B*) 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. 8*B*. 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-Bim$_{EL+L}$–(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-Bim$_{EL}$ and GST-Bim$_{EL}$S65A were equally effective at precipitating ERK1/2 from cell lysates (Fig. 4*C*). Thus the phospho-acceptor site maps outside the minimal ERK1/2 docking domain and is not required for Bim$_{EL}$ to bind ERK1/2.

DISCUSSION

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

Bim$_{EL}$ exhibited a basal level of phosphorylation in HEK293 cells whether assayed by [^{32}P]P$_i$ 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 Bim$_{EL}$ 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-Pin1 to selectively precipitate Bim$_{EL}$ in an ERK1/2-dependent fashion indicated that Bim$_{EL}$ was phosphorylated at Ser-Pro or Thr-Pro sites *in vivo*, suggesting that ERK1/2 were the kinases responsible. Indeed, whereas ERK1, JNK1, and p38α all phosphorylated GST-Bim$_{EL}$ *in vitro*, ERK1 was clearly the most effective. Furthermore, GST-Bim$_{EL}$ could precipitate an FBS-stimulated Bim$_{EL}$ kinase activity from extracts of serum-stimulated cells (Fig. 3), and this was abolished by PD184352. Finally, GST-Bim$_{EL}$ *in vitro* and HA-Bim$_{EL}$ *in vivo* were able to precipitate active but not inactive ERK1/2 (Fig. 4), indicating that ERK1/2 are not constitutively bound to Bim$_{EL}$ but only interact when they become activated. Taken

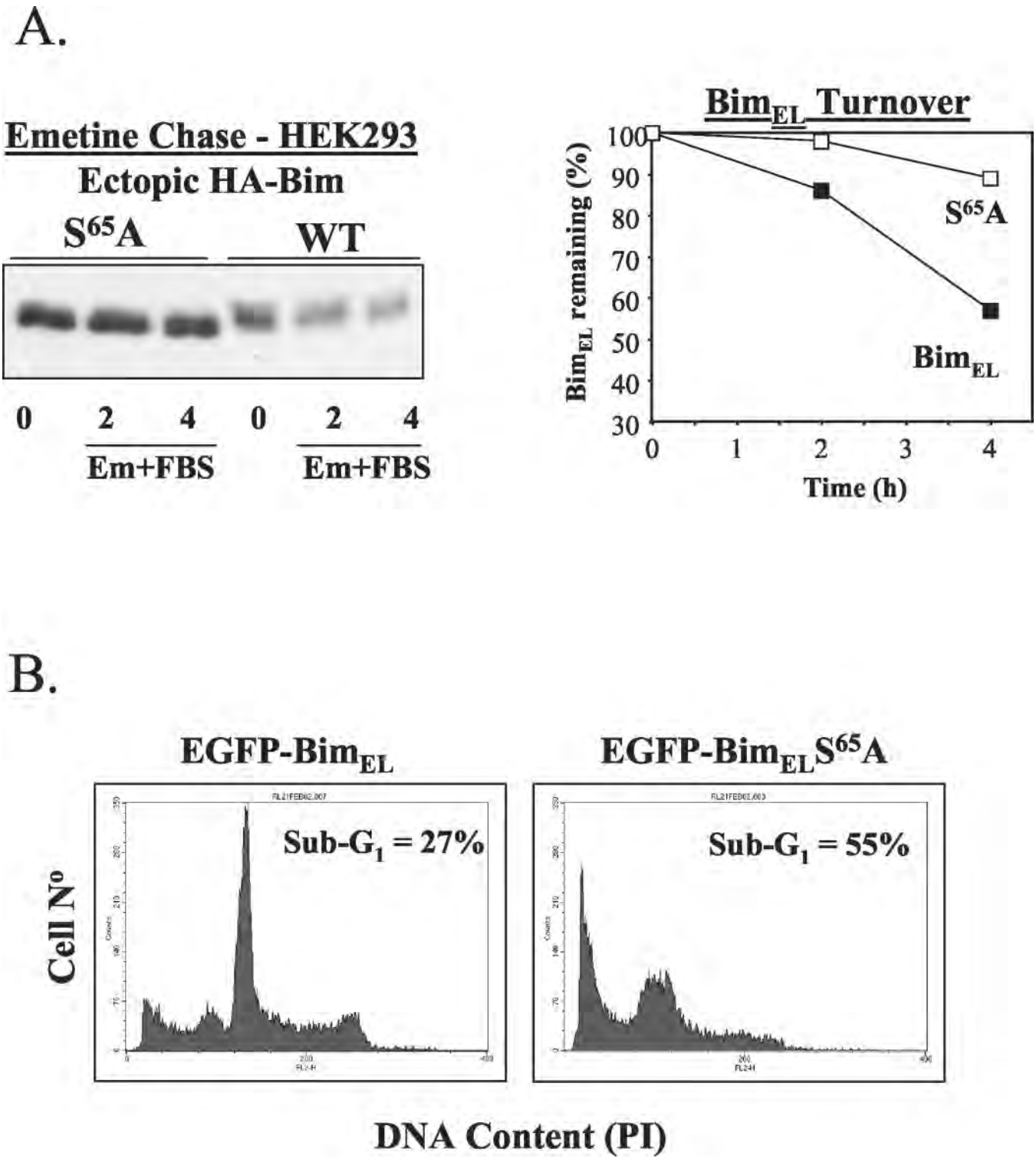

FIG. 7. **Mutation of Ser^{65} inhibits FBS-stimulated turnover of Bim_{EL} and potentiates its cell killing activity.** *A*, *left panel*, HEK293 cells were transfected with HA-Bim_{EL} or HA-Bim_{EL}S65A in serum-free media for 18 h ($t = 0$). Cells were then treated with emetine (*Em*) (10 μM) and restimulated with serum for the indicated times. Lysates were resolved by SDS-PAGE and immunoblotted with anti-HA. *Right panel*, expression of HA-Bim_{EL} and HA-Bim_{EL}S65A was quantified by densitometry and expressed as a percentage of the expression at $t = 0$. Similar results were obtained in three independent experiments. *B*, EGFP-Bim_{EL} or EGFP-Bim_{EL}S65A were transfected into HEK293 cells. After 18 h EGFP-positive cells were sorted by FACS, stained with propidium iodide, and analyzed for the accumulation of cells with sub-G_1 DNA content by flow cytometry. Data were taken from a single representative experiment, and the mean values from pooled experiments are cited in the text.

together these results indicate that ERK1/2 are FBS-stimulated Bim_{EL} kinases that physically interact with Bim_{EL} *in vitro* and *in vivo*.

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

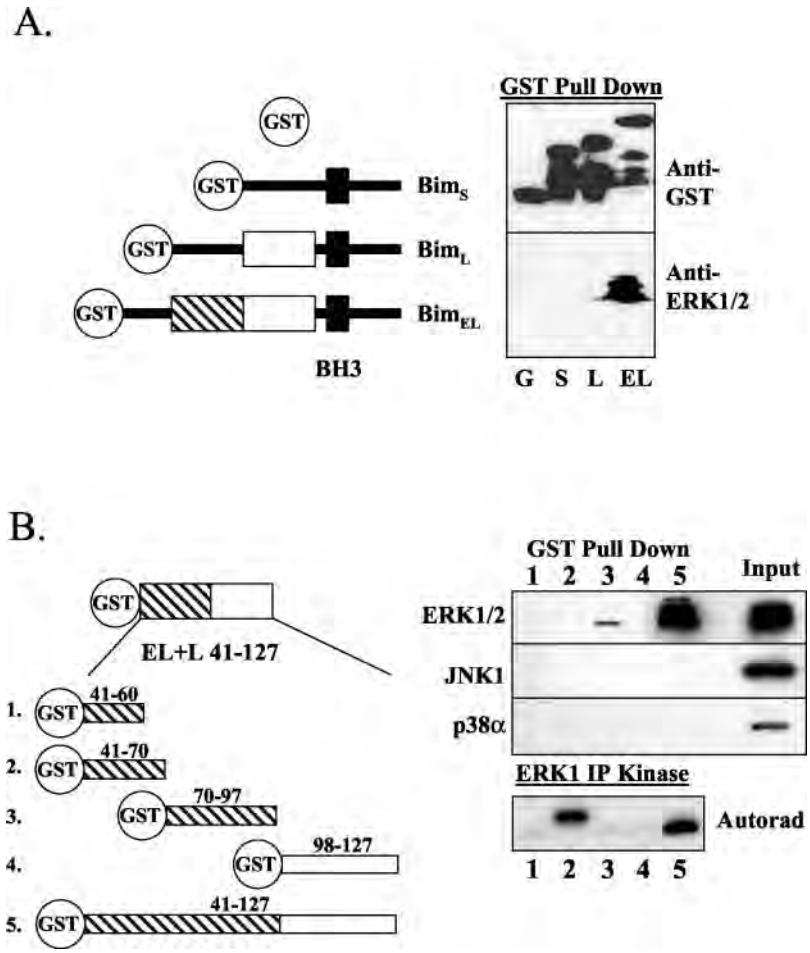

FIG. 8. **The ERK1/2 docking domain includes residues 70–97, within the region unique to Bim$_{EL}$.** *A*, *left panel*, diagram depicting isoforms of Bim protein used as GST fusion proteins. *Right panel*, CCl39 cells were serum-starved for 18 h and restimulated for 10 min with FBS. Equal quantities 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 p38α; 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.

position −2 relative to the phospho-acceptor residue (Pro-Ala-Ser^{65}-Pro), matching the preferred consensus phosphorylation site for ERK1/2 (P*X*(S/T)P) (39). When HA-Bim$_{EL}$S65A was overexpressed in HEK293 cells, it resolved with an increased mobility on SDS-PAGE compared with wild type HA-Bim$_{EL}$ (Fig. 6*A*), suggesting that it was also a phosphorylation site *in vivo*. Two-dimensional electrophoresis revealed that the single mutation of Ser^{65} caused the loss of both acidic phosphoprotein spots 3 and 4 that are also lost by PD184352 treatment. This result suggests that ERK1/2-dependent phosphorylation of Bim$_{EL}$ may proceed in a hierarchical fashion in which Ser^{65} phosphorylation is required for phosphorylation of at least one other site. Whether ERK is responsible for phosphorylating both sites is currently unclear, and the identification of the other sites of Bim$_{EL}$ phosphorylation is clearly a priority.

ERK1/2-dependent phosphorylation of Bim$_{EL}$ targets the protein for degradation via the proteasome (24), and emetine chase experiments in HEK293 cells revealed that mutation of Ser^{65} → Ala stabilized Bim$_{EL}$ (Fig. 7*A*). Indeed, we frequently observed that HA-Bim$_{EL}$S65A was expressed at higher levels than wild type HA-Bim$_{EL}$ in complete media, and this probably reflects the fact that it turns over only very slowly. This higher level of expression was biologically relevant because EGFP-Bim$_{EL}$S65A was twice as effective at killing cells as wild type EGFP-Bim$_{EL}$ (Fig. 7*B*). Together these observations support our hypothesis that ERK1/2 phosphorylation occurs on residue Ser^{65} *in vivo* and that although Bim$_{EL}$ is subject to multiple ERK1/2-dependent phosphorylation events *in vivo*, phosphorylation of Ser^{65} is absolutely required for Bim$_{EL}$ degradation. Thus ERK1/2-dependent phosphorylation serves as a means of neutralizing Bim$_{EL}$ by stimulating its turnover.

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$_{EL+L}$-(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 also 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 vivo* 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$_L$ at Thr^{56} and either Ser^{44} or Ser^{58}, thereby disrupting the Bim$_L$-DLC1 interaction and releasing Bim$_L$ to initiate cell death. It is notable that the ERK1/2 phosphorylation site

mapped here (Ser^{65} in the region unique to Bim_{EL}) is distinct from the JNK sites identified in Bim_L (21) and which are shared in Bim_{EL} (Thr^{112} and Ser^{100} or Ser^{114} using Bim_{EL} numbering); indeed, Bim_L lacks Ser^{65}. Although we have not examined Bim_L phosphorylation, we did not observe JNK binding to Bim_{EL} in cell extracts under any conditions. It is possible that ERK1/2 binding to Bim_{EL} precludes JNK binding. Because Bim_L lacks an ERK1/2-binding site (Fig. 8), this might allow JNK to bind to a putative JNK docking domain in Bim_L and phosphorylate the DLC1 binding domain. In such a scenario ERK1/2 binding to Bim_{EL} might allow phosphorylation of Ser^{65} and also prevent binding of JNK, thereby preserving the Bim_{EL}-DLC1 interaction. Such a model would require that ERK1/2 and JNK both phosphorylate Bim_{EL} but under different conditions, because even when both ERK1/2 and JNK were activated, we could only observe ERK1/2 binding to Bim_{EL} (Fig. 4*C*). It remains to be determined whether any of the JNK phosphorylation sites mapped in Bim_L (21) contribute to the multiple phosphorylation sites we see in Bim_{EL}, 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 Bim_{EL} in nerve growth factor-deprived neurons (23) is more difficult to reconcile with our results. Although JNK did weakly phosphorylate recombinant GST-Bim_{EL} *in vitro* (Fig. 3), we repeatedly failed to demonstrate an interaction between JNK and Bim_{EL} in cells with active JNK, even when a JNK-c-Jun interaction was readily detected. This disparity probably reflects the expression of a tissue-specific adaptor or scaffold protein that may facilitate the JNK-Bim_{EL} interaction in neurons, and which is absent in the fibroblasts and HEK293 cells studied here. In addition, JNK is unlikely to be the Bim_{EL} kinase that we have studied here because FBS stimulation does not activate JNK or p38 and the FBS-stimulated Bim_{EL} kinase was blocked by PD184352, which does not prevent JNK activation.

In summary, we have shown that ERK1/2 are FBS-stimulated Bim_{EL} kinases that bind to Bim_{EL} via a discrete docking domain, causing its phosphorylation at Ser^{65}; this is required for serum-dependent degradation of Bim_{EL}. These findings provide new insights into the post-translational regulation of Bim_{EL}, 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

Rebecca Ley, Katherine E. Ewings, Kathryn Hadfield, Elizabeth Howes, Kathryn Balmanno and Simon J. Cook

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