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Identification of a DEF-type Docking Domain for Extracellular Signal-regulated Kinases 1/2 That Directs Phosphorylation and Turnover of the BH3-only Protein Bim_{EL}*

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The BH3-only protein, Bim, exists as three splice variants (Bim_S, Bim_L, and Bim_{EL}) of differing pro-apoptotic potency. Bim_{EL}, the least effective killer, is degraded by the proteasome in response to phosphorylation by extracellular signal-regulated kinases 1 and 2 (ERK1/2). ERK1/2-dependent phosphorylation correlates with the presence of a domain unique to the Bim_{EL} splice variant that includes the major ERK1/2 phosphorylation site Ser^{65}. However, efficient phosphorylation by ERK1/2, c-Jun N-terminal kinase, or p38 requires the presence in the substrate of a discrete kinase-docking domain as well as the phosphoacceptor site. Here we show that the region unique to Bim_{EL} (amino acids 41–97) harbors two potential DEF-type ERK1/2 kinase-docking domains, DEF1 and DEF2. Peptide competition assays revealed that the DEF2 peptide could act autonomously to bind active ERK1/2, whereas the DEF1 peptide did not. Truncation analysis identified a minimal region, residues 80–97, containing the DEF2 motif as sufficient for ERK1/2 binding. Mutation of key residues in the DEF2 motif abolished the interaction of ERK1/2 and Bim_{EL} and also abolished ERK1/2-dependent phosphorylation of Bim_{EL} *in vivo*, thereby stabilizing the protein and enhancing cytotoxicity. Our results identify a new physiologically relevant functional motif in Bim_{EL} that may account for the distinct biological properties of this splice variant.

The BH3-only protein Bim is a pro-apoptotic member of the Bcl-2 protein family that links stress-induced signals to the core apoptotic machinery (1, 2). Expression of the *Bim* gene is induced at the transcriptional level in response to withdrawal of cytokines and survival factors due to inactivation of protein kinase B (3) or the ERK1/2 pathway (4). In addition, the JNK[1] (c-Jun N-terminal kinase) pathway promotes c-Jun-dependent Bim expression in neurons following the withdrawal of nerve growth factor (5, 6). Alternative splicing of the *Bim* gene gives rise to the short, long, and extra-long Bim proteins (Bim_S, Bim_L, and Bim_{EL}) (7), thereby introducing additional levels of regulation that may account for their differences in pro-apoptotic potency. For example, Bim_S is the most effective killer and is the simplest form, consisting largely of the pro-death BH3 domain and a C-terminal membrane-tethering domain (7). Bim_L contains an additional domain through which it can interact with dynein light chain 1 (DLC1) with the result that in viable cells Bim_L is sequestered at microtubules and so is a less effective killer (8). Disruption of microtubules can cause the redistribution of Bim_L to the mitochondria, and this may be due to JNK-dependent phosphorylation of Bim_L at sites adjacent to the DLC1-binding site (9). Bim_{EL} also contains the DLC1-binding site but is the least effective killer, and this may be explained by the fact that Bim_{EL} protein stability is subject to post-translational regulation. Activation of the ERK1/2 pathway promotes the proteasomal degradation of Bim_{EL} (10), and this correlates with the presence of a unique domain encoded by exon 3 (11) that includes an ERK1/2 phosphorylation site required for Bim_{EL} turnover (12).

ERK1/2, JNK, and the related stress kinase, p38, all bind to their substrates directly at "docking domains," which are distinct from the phosphoacceptor site. Although the literature describing these regions has become increasingly complex, it is apparent that ERK1/2, JNK, and p38 can all bind to a common motif termed the D-domain, which contains limited sequence similarity but can be determined by the presence of basic amino acids followed by a L*X*L motif and/or a hydrophobic region (13). Co-crystallization studies of p38α and peptides from myocyte enhancer factor 2A have identified binding interactions between the L*X*L motif and hydrophobic residues in the conserved kinase core (14). A second docking domain, containing the consensus sequence F*X*F, has been termed the DEF domain (docking site for ERK, F*X*FP) (15). It has been found in several substrates including Elk1, SAP-1, and kinase suppressor of Ras (15), and although initially thought to be specific for ERK1/2, it may allow docking of active p38α to SAP-1 (16). In contrast to the constitutive binding of JNK to the D-domain in c-Jun, DEF domains can only bind to ERK2 when it is active (17).

We have recently shown that the specific phosphorylation of Bim_{EL} by ERK1/2 is due to the presence of an ERK1/2 phos-

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[1] The abbreviations used are: JNK, c-Jun N-terminal kinase; BH, Bcl-2 homology domain; Bim, Bcl-2-interacting modulator; Bim_{EL}, Bim extra long; Bim_L, Bim long; Bim_S, Bim short; D-domain, docking domain; DEF, docking for ERK, F*X*FP; DLC1, dyenin light chain 1; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GST, glutathione *S*-transferase; HEK, human embryonic kidney; MEK, mitogen-activated protein kinase or ERK kinase; ER, endoplasmic reticulum; MEKK, MEK kinase; HA, hemagglutinin; U0126, 1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene; 4-HT, 4-hydroxytamoxifen; EGFP, enhanced green fluorescent protein; SAP-1, SRF accessory protein 1.

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phoacceptor site within the domain unique to Bim_{EL} that is encoded by exon 3 (12). Here we have identified a docking domain in Bim_{EL}, also encoded by exon 3, that accounts for ERK1/2 binding, ERK1/2-dependent phosphorylation, and turnover of Bim_{EL}.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Invitrogen. U0126 was purchased from Promega. The following antibodies were used throughout this study. HA was provided by the Babraham Institute Monoclonal Antibody Facility. Phospho-ERK1/2 and total ERK1/2 were from Cell Signaling Technology/New England Biolabs. Bim was from Chemicon, and JNK was from Santa Cruz Biotechnology. Isoelectric focusing tube gels were purchased from Millipore Investigator Systems. All of the other chemicals were purchased from Sigma and were of the highest grade available unless otherwise stated in the text. Peptides corresponding to the DEF1 (SPLFIFVRR) or DEF2 domains (SGYFSFDTDR) of Bim_{EL} were synthesized by Dr. Ram Sharma (Southhampton Polypeptides Ltd.).

Cell Culture—The culture of RM3 cells has been described previously (18). HEK293 cells were maintained under identical conditions. For serum starvation, 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-hydroxytamoxifen (4-HT), FBS, inhibitors, or the relevant vehicle control for the times indicated in the figure legends. 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. Cells were harvested at times indicated for further analysis.

Plasmids and Transfections—Bim_{EL} 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 multiple cloning sites). Amino acid numbering refers to the rat Bim_{EL} cDNA sequence that was used in these studies. Potential docking sites were altered by PCR-based site-directed mutagenesis using *Pfu*Turbo DNA polymerase (Promega). Regions of interest were amplified by PCR and cloned into pGEX-4T1 or pCAN-HA. All of the inserts were verified by ABI automated sequencing. The sequences of all of the oligonucleotides are available upon request. pEGFP-hBimα1 and pEGFP-hBimβ1 plasmids were provided by Dr. Toshiyuki Miyashita (National Research Institute for Child Health and Development, Tokyo, Japan) and subcloned into pCAN-HA. A pGEX plasmid encoding amino acids 310–428 of Elk-1 was kindly provided by Prof. Andrew Sharrocks, University of Manchester, Manchester, United Kingdom).

HEK293 cells were transfected by the calcium phosphate precipitation technique (19) and left for the time indicated in the 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.

Western Blot Analysis—Cells were lysed and analyzed by immunoblotting exactly as described previously (12, 18). For two-dimensional electrophoresis, the protocol previously described (20) was used with the exception that tube gels were not pre-focused. After, isoelectric focus samples were separated by SDS-PAGE.

GST Fusion Proteins and Pull-down Assays—GST fusion proteins were expressed in BL21 bacterial cells and purified on GSH beads as previously described (12, 21). The concentration of proteins was quantified by Bradford assay and from Coomassie Blue-stained SDS-PAGE gels by densitometry. These recombinant proteins 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 at least four times with ice-cold lysis buffer followed by separation on SDS-PAGE and immunoblotting with relevant antibodies. For peptide competition studies, pull-downs were performed as described above but in the presence of competing peptides as indicated in the figure legends.

Assay of Bim-induced Cell Death—HEK293 cells were transfected with 0.5 μg of empty pCAN-HA, pCAN-HA-BimEL, or pCAN-HA-ΔDEF2 together with 0.1 μg of pCMV-EGFP-spectrin. After 18 h, cells were fixed, stained with propidium iodide (4), and analyzed on a FACS Calibur flow cytometer (BD Biosciences). The percentage of EGFP-positive cells exhibiting sub-G_1 DNA was recorded.

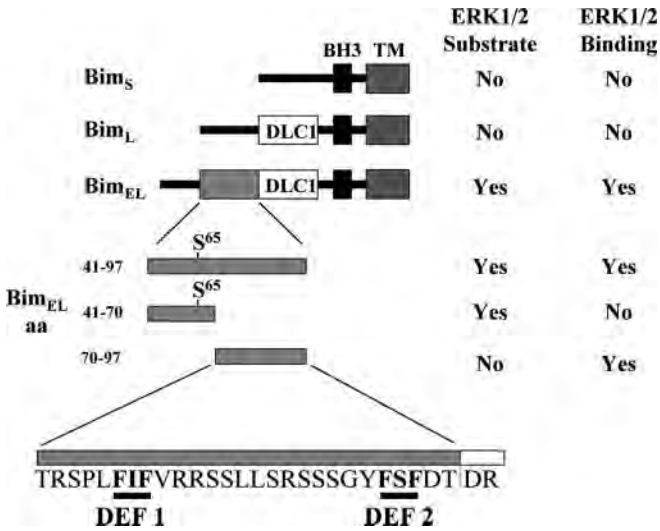

FIG. 1. **Putative ERK1/2-docking domains map to regions distinct from the phosphoacceptor site in the Bim_{EL} protein.** Schematic diagram summarizing our previously reported data (12) identifying the regions within the Bim_{EL} protein that contain the phosphoacceptor site and allow binding of ERK1/2. The three major isoforms Bim_{EL}, Bim_L, and Bim_S are depicted. The *black bar*, the *black box* (the BH3 domain), and the *gray box* (the transmembrane (*TM*) region) represent regions common to all three isoforms. The *white box* represents the DLC1-binding region common to Bim_L and Bim_{EL}, and the *hatched box* shows the region unique to Bim_{EL} encoded by exon 3. The distinct separable regions that contain the phosphoacceptor site, serine 65 (residues 41–70), and a putative ERK1/2-docking region (residues 70–97) are indicated with the amino acid sequence of the region previously identified as being sufficient for ERK1/2 binding shown underneath. The putative DEF domains are highlighted in *boldface* and are *underlined*.

RESULTS

The Minimal Region Required for ERK-docking Maps within Residues 80–97 of Bim_{EL}—We have previously shown that activated ERK1/2 can bind directly to Bim_{EL}, but not Bim_S or Bim_L, and have mapped this interaction to amino acid residues 70–97 within the region unique to Bim_{EL} (Fig. 1) (12). This region does not include the major ERK1/2 phosphoacceptor site at Ser^{65}. Within this region, we identified two F*X*F motifs (FIF at residues 76–78 and FSF at residues 93–95) that could be potential DEF domains. These were labeled DEF1 and DEF2, respectively (Fig. 1).

To investigate the role of these potential DEF domains, we used a series of GST-Bim_{EL} fusion proteins as bait to "pull down" active ERK1/2 from cell lysates (Fig. 2). In these experiments, we used Rat-1 cells that express the conditional protein kinase ΔMEKK3:ER* (so-called RM3 cells (18)). When activated by treatment with 4-HT, ΔMEKK3:ER stimulates the ERK1/2, JNK, and p38 pathways. Consequently, lysates prepared from these cells serve as an abundant source of active ERK1/2, JNK, and p38. Consistent with our previous study (12), a GST fusion protein containing residues 70–97, including both DEF1 and DEF2, was sufficient to pull down ERK1/2 (Fig. 2, *lane 1*), albeit less effectively than a protein containing residues 41–127 (Fig. 2, *lane 5*). A smaller GST fusion protein, residues 80–97 (Fig. 2, *lane 2*), which contained only the DEF2 F*X*F motif as a potential interacting site, was very effective at pulling down ERK1/2 from cell lysates and notably was more efficient than the larger GST-Bim_{EL}^{70-97} fusion protein. A fragment containing further C-terminal residues (GST-Bim_{EL}^{80-127}), including the entire DLC1-binding domain, was slightly less effective at binding ERK1/2 (Fig. 2, *lane 3* compared with *lane 2*). Equal amounts of each fusion protein were used in these pull-down assays, and we cannot dismiss the

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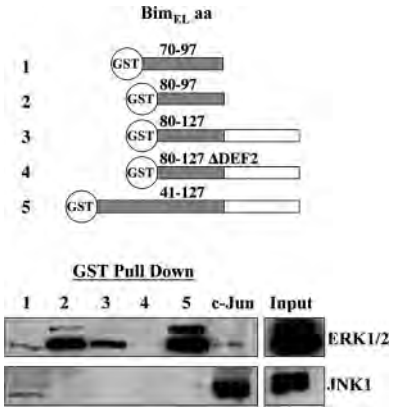

FIG. 2. **A region of Bim_{EL} from amino acids (*aa*) 80–97 is sufficient to precipitate ERK1/2 from cell lysates.** *A*, schematic diagram representing GST fusion proteins used in pull-down assays. *Numbers* above each schematic indicate the amino acid sequence of Rat Bim_{EL}. The *white box* represents the DLC1-binding region common to Bim_L and Bim_{EL}, and the *hatched box* shows the region unique to Bim_{EL} encoded by exon 3. ΔDEF2 denotes the disruption of the putative DEF2 domain (mutation of Phe^{95} to Ala and Phe^{97} to Ala). *B*, RM3 cells (Rat-1 cells expressing ΔMEKK3:ER*, for review see Ref. 18) were serum-starved for 18 h and stimulated with 100 nM 4-HT for 1 h to activate ERK, JNK, and p38. Equal quantities of fusion protein bound to beads were used in pull-down assays. Co-precipitates were subjected to SDS-PAGE and immunoblotted for ERK1/2 and JNK1. Cell lysates used as the assay "input" were immunoblotted as a control for the ERK1/2 or JNK1 put into the assay. GST-c-$Jun^{1–223}$ was used as a positive control for precipitation of JNK.

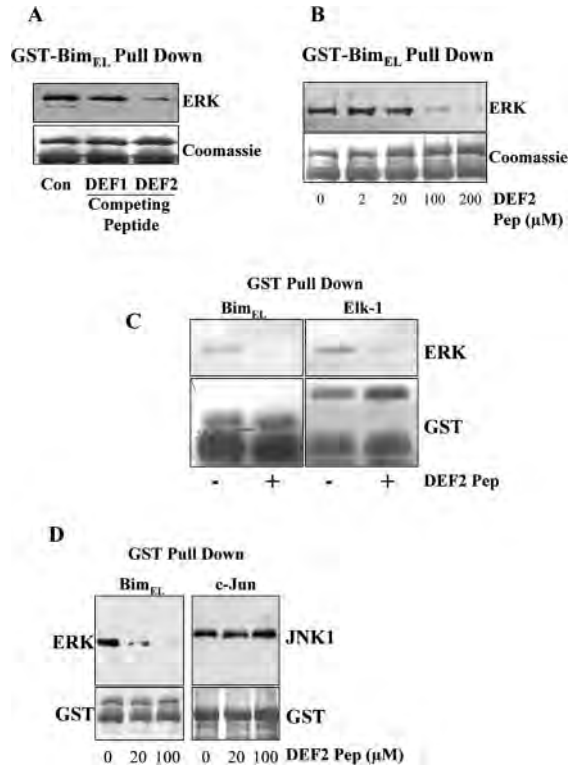

FIG. 3. **Peptides of the potential DEF2 domain block ERK binding to Bim_{EL} *in vitro*.** *A*, RM3 cells were serum-starved for 18 h and stimulated with 100 nM 4-HT for 1 h to activate ERK, JNK, and p38. 1 nmol of GST-$Bim_{EL}^{41–127}$ bound to beads was used to pull down ERK from these cell lysates in the absence (control, *Con*) or presence of competing peptides (200 μM), corresponding to the DEF1 or DEF2 motifs (*DEF1*, *DEF2*). Co-precipitates were resolved by SDS-PAGE and immunoblotted for ERK1/2. The blot was stained with Coomassie Blue to confirm equal loading of GST-$Bim_{EL}^{41–127}$. *B*, GST-$Bim_{EL}^{41–127}$ pull-downs were performed as above with the exception that increasing concentrations of the DEF2 peptide (*Pep*) were included. *C*, 0.1 nmol of either GST-$Bim_{EL}^{41–127}$ or GST-Elk-$1^{310–428}$ bound to beads were used to pull down ERK from RM3 cell lysates (as above) in the absence or presence of 500 μM DEF2 peptide. Precipitates were immunoblotted for ERK1/2 as above. The blot was stained with Coomassie Blue to confirm equal loading of full-length GST fusion proteins. *D*, GST-$Bim_{EL}^{41–127}$ or GST-c-$Jun^{1–223}$ were incubated with increasing concentrations of the DEF2 peptide and then used to pull down ERK1/2 or JNK, respectively, from RM3 cell lysates as above. The DEF2 peptide blocked precipitation of ERK by Bim but did not interfere with precipitation of JNK by c-Jun. The blot was stained with Coomassie Blue to confirm equal loading of GST fusion proteins.

possibility that differences in ERK1/2 binding might be due to differences in protein folding in bacteria. However, these results clearly show that a region of only 17 amino acids (residues 80–97) of Bim_{EL} is very effective at interacting with ERK1/2 *in vitro*. This fragment contains the DEF2 domain (FSF) but lacks the putative DEF1 domain (FIF) and the ERK1/2 phosphoacceptor site at Ser^{65}.

A Bim_{EL} DEF2 Domain Peptide Is Sufficient to Compete with $Bim_{EL}^{41–127}$ for ERK1/2 Binding—To further investigate the role of the DEF domains in ERK1/2 binding, we used peptide competition assays. We based this assay on Bardwell *et al.* (22) who showed that D-domain peptides (25–100 μM) could inhibit the binding of full-length MEK2 to GST-ERK2. Peptides corresponding to the DEF1 (SPLFIFVRR) or DEF2 domains (SGYFSFDTDR) were tested for their ability to compete with GST-$Bim_{EL}^{41–127}$ for ERK1/2 binding in a pull-down assay and revealed that the DEF2 domain peptide, but not DEF1, was able to compete for ERK1/2 binding (Fig. 3*A*). It did not prove possible to examine the effect of both peptides in combination in this assay, because this repeatedly caused a nonspecific aggregation and precipitation of proteins from the cell lysate.[2] The DEF2 peptide caused a dose-dependent decrease in the amount of ERK2 precipitated by GST-$Bim_{EL}^{41–127}$ (Fig. 3*B*). The inhibition of ERK binding to 2 μM GST-Bim_{EL} was apparent at 20 μM DEF2 peptide and was complete at 100 μM, indicating that a 10–50-fold excess of peptide was sufficient to inhibit ERK binding.

Because the DEF2 peptide could bind to ERK1/2, we reasoned that it should also inhibit the binding of ERK1/2 to other substrates. To test this possibility, we compared the effect of the Bim DEF2 peptide on binding of ERK1/2 to the E-twenty six-specific (Ets) domain transcription factor Elk-1 (15, 16). Inclusion of the Bim DEF2 peptide in a pull-down assay again inhibited the binding of ERK1/2 to GST-$Bim_{EL}^{41–127}$ and also inhibited the binding of ERK1/2 to GST-Elk-$1^{310–428}$ (Fig. 3*C*). The DEF2 peptide inhibited the Bim_{EL}-ERK interaction, but it failed to block the interaction between JNK and c-Jun (Fig. 3*D*). Because the JNK-c-Jun interaction is mediated by the D-domain of c-Jun, rather than a DEF domain, this confirms the specificity of the DEF2 peptide as a minimal ERK-binding motif. Taken together, these results confirm that the DEF2 peptide is sufficient for ERK1/2-specific binding.

Mutation of the DEF Domain Blocks ERK1/2 Binding to Residues 80–127 of Bim_{EL} in Vitro—The phenylalanine residues in DEF domains at position 1 and 3 are important determinants for ERK binding (15). Consequently, we mutated these two phenylalanine residues to alanine in the GST-$Bim_{EL}^{80–127}$ fusion protein (80–127 ΔDEF2) and compared this with the wild type fusion protein (residues 80–127) in pull-down experiments using lysates from RM3 cells treated with 4-HT. Compared with the wild type fusion protein, interaction with ERK1/2 was completely blocked by mutation of the two phenylalanine residues in the putative DEF2 motif (Fig. 2, compare *lanes 3* and *4*). Taken together with the preceding results, this observation suggests that the DEF2 F*X*F sequence at residues 93–97 in Bim_{EL} is indeed a DEF motif that, at least in the

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

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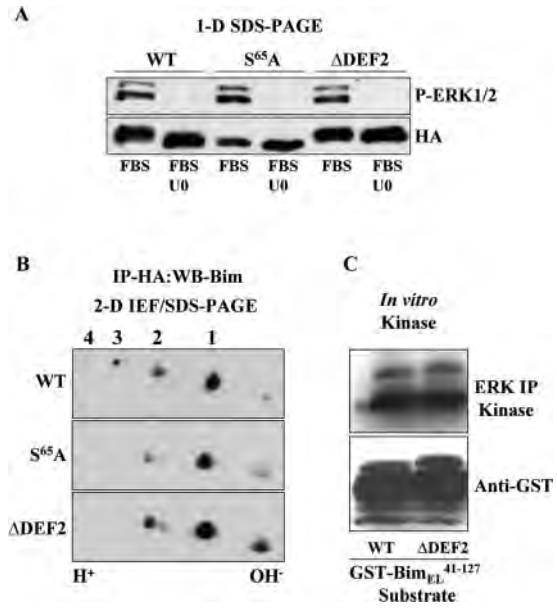

FIG. 4. **Mutation of the DEF2 ERK1/2-docking domain blocks phosphorylation of Bim$_{EL}$ *in vivo*.** *A*, HEK293 cells were transfected with HA-Bim$_{EL}$, HA-Bim$_{EL}$S65A, or HA-Bim$_{EL}$ ΔDEF2 in serum-free conditions. After 18 h, cells were stimulated with FBS in the absence or presence of the MEK inhibitor, U0126 (*U0*). Lysates were resolved by SDS-PAGE and immunoblotted with anti-HA and phospho-ERK1/2. *B*, HEK293 cells were transfected with HA-Bim$_{EL}$, HA-Bim$_{EL}$S65A, or HA-Bim$_{EL}$ΔDEF2 and treated as in *A*. HA-conjugated beads were used to precipitate the expressed proteins from cell lysates (*IP-HA*), and these were resolved by two-dimensional (*2-D*) electrophoresis and immunoblotted for Bim (*WB-Bim*). *H*$^+$, acidic; *OH*$^-$, basic. Similar results were obtained in an independent experiment. *C*, RM3 cells were serum-starved for 18 h and stimulated with 100 nM 4-HT for 1 h. ERK1 was immunoprecipitated from lysates and used in kinase reactions with equal quantities of either wild type (*WT*) or mutant (ΔDEF2) GST-BimEL$^{41-127}$ fusion protein eluted from beads as substrates. Equal loading of the kinase assays with substrate protein was confirmed by staining with anti-GST antibodies.

context of GST-Bim$_{EL}^{80-127}$, is absolutely required for direct binding to ERK1/2 *in vitro*.

Mutation of the DEF Domain (FSF to ASA) Prevents Phosphorylation by ERK1/2 in Vivo—To test the role of the DEF2 domain in Bim$_{EL}$ phosphorylation *in vivo*, we introduced the mutation disrupting the DEF2 domain (ΔDEF2) into a construct that allowed expression of the full-length HA-tagged protein in mammalian cells. We transiently transfected HEK293 cells with DNA expressing wild type Bim$_{EL}$, Bim$_{EL}$S65A, or Bim$_{EL}$ΔDEF2. Bim$_{EL}$S65A was included as a positive control since we have previously shown that this mutation causes the loss of at least two ERK-dependent phosphorylation sites *in vivo* (12). The following day, cells were stimulated with 10% FBS in the absence or presence of the MEK1/2 inhibitor, U0126. As seen previously (10, 12), FBS stimulation caused the characteristic gel retardation of wild type Bim$_{EL}$ that was inhibited by U0126, whereas Bim$_{EL}$S65A failed to undergo gel retardation and its mobility on one-dimensional SDS-PAGE gels was not affected by U0126 (Fig. 4*A*). The Bim$_{EL}$ΔDEF2 protein migrated slightly more slowly on one-dimensional SDS-PAGE gels compared with the wild type protein, probably reflecting a change in its electrophoretic properties due to the two Phe→Ala mutations. However, treatment with U0126 had little if any effect on the migration of the Bim$_{EL}$ΔDEF2 protein (Fig. 4*A*), indicating that the loss of the DEF2 domain prevented MEK1/2-dependent (*i.e.* ERK-dependent) phosphorylation of Bim$_{EL}$ *in vivo*.

These results were confirmed by parallel two-dimensional electrophoresis. Upon serum stimulation, HA-Bim$_{EL}$ resolves as a basic, non-phosphorylated spot and up to four additional acidic spots representing phosphorylated forms of the protein (Fig. 4*B*) (12, 20). Mutation of Ser^{65}, the major site of ERK1/2 phosphorylation *in vitro*, causes the disappearance of the two most acidic spots (spots 3 and 4), indicating that they contain ERK1/2-dependent phosphorylation sites *in vivo* (Fig. 4*B*) (12). When we transiently expressed the Bim$_{EL}$ΔDEF2 protein in parallel, we also saw a reduction in the number of phosphoprotein spots to just spot 1 and spot 2 on two-dimensional gels (Fig. 4*B*). Thus, the mutation of the DEF2 domain alone is sufficient to block ERK1/2-dependent phosphorylation *in vivo*. A trivial explanation for this observation was that the DEF2 mutation disrupted protein structure at the phosphoacceptor site and so prevented phosphorylation nonspecifically. However, the GST-Bim$_{EL}^{41-127}$ΔDEF2 fusion protein was phosphorylated as efficiently as wild type GST-Bim$_{EL}^{41-127}$ when added to an *in vitro* ERK1/2 kinase reaction (Fig. 4*C*). In such assays, the components are present in excess so that phosphorylation requires intact phosphoacceptor sites but not the presence of a docking domain. Consequently, the DEF2 mutation did not disrupt the integrity of the phosphoacceptor sites. Thus, the simplest conclusion from these results is that the DEF2 motif is an important determinant allowing ERK1/2 to bind to and phosphorylate Bim$_{EL}$ *in vivo*.

Mutation of the DEF Domain Blocks Serum-stimulated Bim$_{EL}$ Turnover and Enhances Cell Death—ERK1/2 phosphorylates Bim$_{EL}$ *in vitro* at Ser^{65}, which is also an ERK1/2-dependent phosphorylation site *in vivo* (12, 23, 24). Activation of ERK1/2 upon serum stimulation targets Bim$_{EL}$ for degradation (10), and mutation of Ser^{65} to a non-phosphorylatable alanine prevents the turnover of Bim$_{EL}$ (12, 24). We postulated that mutation of the DEF2-docking domain of Bim$_{EL}$ should also reduce Bim$_{EL}$ turnover by preventing ERK-dependent phosphorylation. To test this possibility, we expressed wild type HA-Bim$_{EL}$, HA-Bim$_{EL}$S65A, or HA-Bim$_{EL}$ΔDEF2 in HEK293 cells in serum-free conditions. After 18 h, cells were stimulated with 10% FBS for 7 h (to activate ERK1/2) in the presence of emetine (to inhibit new protein synthesis) and expression of the HA-tagged Bim proteins was analyzed by Western blot. Data from a single representative experiment is shown in Fig. 5*A* and is quantified in Fig. 5*B*. These results revealed both HA-Bim$_{EL}$S65A and HA-Bim$_{EL}$ΔDEF2 were expressed at a higher level than wild type Bim$_{EL}$ in the basal state and that serum stimulation caused a 76% reduction in the amount of wild type HA-Bim$_{EL}$, whereas both HA-Bim$_{EL}$S65A and HA-Bim$_{EL}$ΔDEF2 were less sensitive to serum-stimulated turnover. The simplest explanation for this result is that the loss of the phosphoacceptor site (S65A) or the ERK1/2-docking site (ΔDEF2) prevents ERK1/2-dependent phosphorylation and turnover of Bim$_{EL}$ *in vivo*.

Since the loss of DEF2 prevented turnover of Bim$_{EL}$, we postulated that the DEF2 mutant would elicit greater cell death than wild type Bim$_{EL}$. Indeed, when we transfected these constructs into HEK293 cells, we again found that Bim$_{EL}$ΔDEF2 was expressed at higher levels than wild type Bim$_{EL}$ and caused significantly greater cell death (Fig. 5*C*), confirming the physiological relevance of the DEF2 site.

The Novel Splice Variants Bimα1 and Bimβ1 Possess the DEF2 Domain and Are Phosphorylated in an ERK1/2-dependent Fashion in Vivo—The ERK1/2 phosphoacceptor site and the DEF2 domain are both encoded by exon 3 (11). To investigate whether exon 3 was sufficient to confer phosphorylation by ERK1/2, we examined additional Bim splice variants. Recent studies have reported the identification of at least six additional splice variants of Bim (25, 26). Of these, Bimα1, Bimβ1, and Bimβ2 include exon 3. Based on our analysis, we postu-

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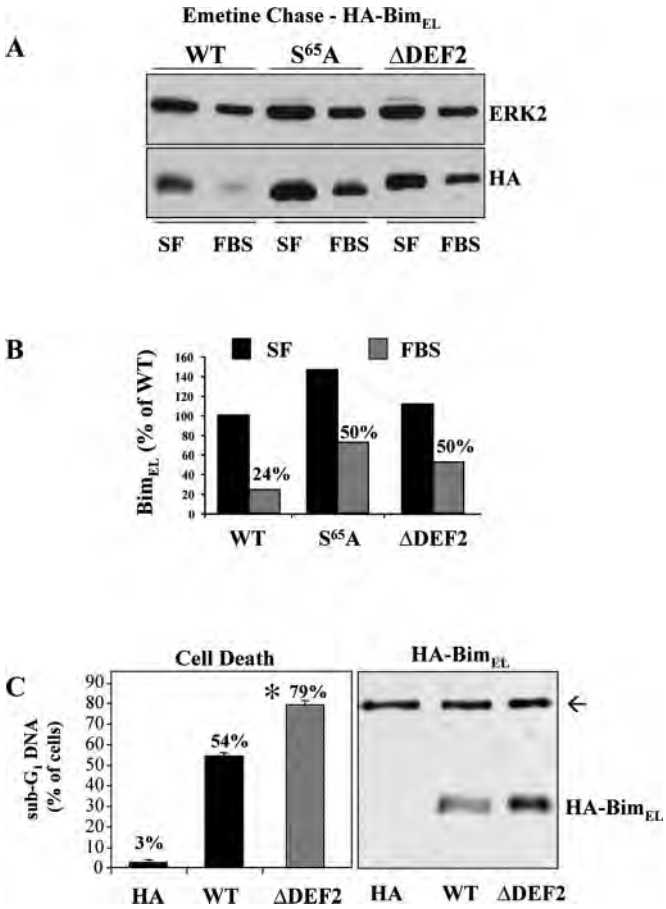

FIG. 5. **Mutation of the DEF2 domain inhibits FBS-stimulated turnover of Bim$_{EL}$ and enhances Bim-induced cell death.** *A*, HEK293 cells were transfected with HA-Bim$_{EL}$, HA-Bim$_{EL}$S65A, or HA-Bim$_{EL}$ΔDEF in serum-free conditions. After 18 h, cell were treated with emetine (10 μM) and left serum free (*SF*) or stimulated with 10% FBS for 7 h. Lysates were resolved by SDS-PAGE and immunoblotted with anti-HA and total ERK. *WT*, wild type. *B*, the expression of the HA-Bim$_{EL}$ proteins was quantified by densitometry and expressed as a percentage of that for the wild type protein at $t = 0$ (SF). The values represent the percent reduction in Bim$_{EL}$ levels following the emetine chase relative to the control at $t = 0$ (SF). *C*, HEK293 cells were transfected with equal quantities of empty HA vector (*HA*), HA-Bim$_{EL}$ (*WT*), or HA-Bim$_{EL}$ΔDEF2 (Δ*DEF2*) together with EGFP-spectrin to mark transfected cells. Cell death in the green cell population was analyzed by flow cytometry by quantifying the percentage of green cells exhibiting sub-G$_1$ DNA after staining with propidium iodide. The data represent the mean ± S.D. of triplicate determinations, and the *asterisk* indicates that cell death induced by ΔDEF2 was significantly greater than that with WT by *t* test ($p < 0.05$). In the *right panel*, parallel transfectants were assayed for expression of wild type or mutant HA-Bim$_{EL}$. The *arrow* indicates a nonspecific band, which served as a loading control.

lated that these splice variants would be subject to ERK-dependent phosphorylation *in vivo* because they contained the phosphoacceptor site (11, 23, 24) and the DEF2 domain (this study). To test this directly, we expressed two of these splice variants, HA-Bimα1 and HA-Bimβ1, in HEK293 cells and compared them with HA-Bim$_L$ and HA-Bim$_{EL}$. Prior to lysis, cells were serum-starved and then re-stimulated with FBS to activate ERK1/2 with or without U0126. Phosphorylation was monitored by the characteristic ERK1/2-dependent gel retardation on SDS-PAGE gels (4, 12, 23, 24). These studies revealed that HA-Bim$_L$ failed to exhibit a U0126-dependent change in mobility, whereas HA-Bimα1, HA-Bimβ1, and HA-Bim$_{EL}$ all resolved as broad bands in FBS-stimulated cells and this mobility shift was reduced by treatment with U0126. Thus, the presence of exon 3 in Bim$_{EL}$, Bimα1, and Bimβ1, including the DEF2-docking domain and a major ERK1/2 phosphorylation site, appears to be the minimum requirement for ERK1/2-dependent phosphorylation *in vivo*.

JNK Binds Poorly to Bim$_{EL}$ at a Site Distinct from DEF2—Whereas several groups have shown that ERK1/2 phosphorylate Bim$_{EL}$ at Ser^{65} *in vitro* and *in vivo* (12, 23, 24), two recent studies (27, 28) have suggested that Bim$_{EL}$ is phosphorylated at Ser^{65} by JNK. Although we could observe phosphorylation by both ERK1/2 and JNK *in vitro* using recombinant GST-Bim$_{EL}$ as a substrate, ERK1/2 were always significantly more effective than JNK under such conditions (12). However, the identity of the kinase responsible for phosphorylation *in vivo* will be determined by the presence of docking domains within the substrate that confer specificity for one or other kinases. To look again at this issue, we examined the binding of JNK in parallel with that of ERK1/2 in the assays described in this study.

In the course of our experiments, we observed that, as well as ERK1/2, GST-Bim$_{EL}^{70-97}$ was able to pull down a small amount of JNK1 from RM3 cell lysates (Fig. 2, *lane 1*). We never observed any interaction between p38 and GST-Bim proteins. However, the ability of GST-Bim$_{EL}^{70-97}$ to precipitate JNK was very poor when compared with that of c-Jun, a *bona fide* JNK substrate (Fig. 2, compare *lanes 1* and *c-Jun*). We also noted that truncation of amino acids 70–79 to yield GST-Bim$_{EL}^{80-97}$ caused the complete loss of this weak JNK binding but substantially enhanced the binding of ERK1/2 to GST-Bim$_{EL}^{80-97}$ (Fig. 2, *lane 1* compared with *lane 2*). These data confirm our original observation that Bim$_{EL}$ binds JNK very poorly, so that even under conditions when both ERK1/2 and JNK are active, ERK1/2 are likely to be the predominant kinases responsible for phosphorylation of Bim$_{EL}$. Indeed, we have previously shown that when we activate ΔMEKK3:ER*, U0126 treatment (which inhibits the ERK pathway but not JNK) abolishes phosphorylation of Bim$_{EL}$. Furthermore, the weak binding of JNK to GST-Bim$_{EL}^{70-97}$, but not to GST-Bim$_{EL}^{80-97}$, suggests that JNK may interact at a site distinct from ERK1/2, consistent with the observation that JNK does not bind to DEF motifs (15).

DISCUSSION

Following withdrawal of serum or survival factors, the level of the Bim$_{EL}$ protein increases within 1–2 h, preceding increases in Bim$_L$ protein (4), suggesting that this splice variant is subject to a discrete mode of regulation. Indeed, the activation of ERK1/2 promotes the phosphorylation and proteasomal degradation of Bim$_{EL}$ (10, 12, 23, 24). Thus, the rapid increase in Bim$_{EL}$ expression following serum withdrawal most probably reflects rapid protein stabilization as well as *de novo* transcription. In contrast, isoforms such as Bim$_S$ or Bim$_L$ are not phosphorylated by ERK1/2 (12) and their expression is likely to be regulated primarily at the transcriptional level. The molecular basis for this specific regulation of Bim$_{EL}$ appears to be the ability of ERK1/2 to phosphorylate Bim$_{EL}$ at Ser^{65} within the region unique to Bim$_{EL}$ *in vitro* and *in vivo* (12, 23, 24), because mutation of this site stabilizes Bim$_{EL}$ against ERK1/2-dependent turnover (12, 24). Efficient phosphorylation by ERK1/2 requires an appropriate phosphoacceptor site and a discrete kinase-docking domain. Here we have identified a novel DEF-type docking domain within the region of Bim$_{EL}$ encoded by exon 3 and shown that it is required for ERK1/2-dependent phosphorylation and turnover of Bim$_{EL}$ *in vivo*.

Characterization of a DEF-type ERK1/2-specific Docking Domain in Bim$_{EL}$—Truncation analysis and peptide competition studies indicated that, of the two potential DEF domains encoded by exon 3, only the DEF2 domain was necessary and sufficient for strong ERK1/2 binding. Furthermore, our results

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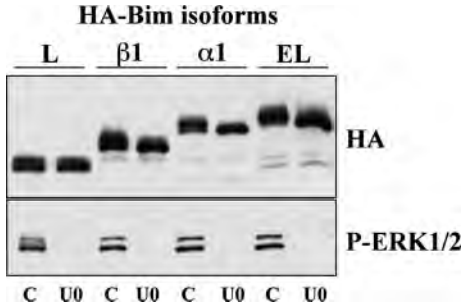

FIG. 6. **Isoforms of Bim that contain exon 3 are phosphorylated in an ERK-dependent manner.** HEK293 cells were transfected with HA-Bim$_L$, HA-hBimβ1, HA-hBimα1, or HA-Bim$_{EL}$ in serum-free conditions. After 18 h, cells were treated with 10% FBS in the absence (*C*) or presence (*U0*) of the MEK inhibitor, UO126. Lysates were resolved by SDS-PAGE and immunoblotted with anti-HA or phospho-ERK1/2.

agree with the analysis of DEF domains in other ERK substrates in four key respects. First, and in common with the DEF domain of SAP-1 (16), the DEF2 domain alone was sufficient for ERK1/2 binding (Figs. 2 and 3). Second, DEF domains are invariably situated downstream from the phosphoacceptor site (16) and this was also the case for the DEF2 domain in Bim$_{EL}$. Third, as with other DEF domains, the two Phe residues in the Bim$_{EL}$ DEF2 domain were critical for ERK1/2 binding (15). In contrast, the identity of the neighboring residues may be less important. For example, although many DEF domains exhibit conservation of a Pro residue at position 4 (*e.g.* FQFP in LIN-1), the aspartate in the Bim$_{EL}$ DEF2 domain (FSFD) is more reminiscent of the acidic glutamate residue in the DEF domain of the cyclic nucleotide phosphodiesterase PDE4D (29). In addition, the amino acid found at position 2 of this motif is most frequently a proline residue but has also been reported to be a serine, glutamine, leucine, or asparagine (Ref. 30 and references therein). Finally, we only observed association of Bim$_{EL}$ with phosphorylated, active ERK1/2. Unlike the D-domain of c-Jun, which can even bind inactive JNK, DEF domains only bind to activated ERK1/2. Presumably, phosphorylation by MEK allows a conformational change in ERK1/2 that is required for access of the DEF motif (17).

It had been suggested that the presence of a DEF motif in a substrate serves largely to increase its affinity for ERK1/2 (*e.g.* SAP-1 (15)). However several ERK1/2 substrates, including c-Fos (31), contain a DEF motif but no D-domain, suggesting that the DEF motif is the only site of interaction. We believe the DEF domain is critical to allow ERK1/2 docking to Bim$_{EL}$, because the FSFD→ASAD mutation introduced into full-length Bim$_{EL}$ inhibited ERK1/2-dependent phosphorylation, prevented turnover of the protein, and thereby increased cell death. Taken together, these results identify the DEF2 motif as a physiologically relevant docking domain in Bim$_{EL}$ that directs ERK1/2 binding, ERK-dependent phosphorylation, and turnover of Bim$_{EL}$ as a cytoprotective mechanism.

The presence of exon 3 in Bim$_{EL}$, Bimα1, and Bimβ1 (but not in Bim$_L$ or Bim$_S$) correlates precisely with their ERK-dependent phosphorylation (or lack thereof) *in vivo* (Fig. 6), but the physiological relevance of Bimα1 and Bimβ1 remains unclear. For example, Bimα1 lacks the C-terminal hydrophobic region and so exhibits reduced activity in cell death assays, whereas Bimβ1 lacks both the hydrophobic region and the BH3 domain and so is completely defective in cell death assays. However, the fact that both are still subject to ERK1/2-dependent phosphorylation *in vivo* does provide us with some important information. First, it suggests that neither the hydrophobic region nor the BH3 domain is required for phosphorylation, suggesting that Bim$_{EL}$ does not need to be at the mitochondria or engaged with Bcl-2 proteins to be phosphorylated by ERK1/2. Second, it strongly suggests that exon 3 represents the minimal ERK1/2-binding and phosphorylation cassette.

Does Bim$_{EL}$ Also Contain an Additional D-type Docking Domain for JNK?—As with our previous analysis (12), GST-Bim$_{EL}^{70-97}$ bound JNK1 very poorly. This weak binding was abolished by the loss of amino acids 70–79 with the result that GST-Bim$_{EL}^{80-97}$ bound ERK1/2 much more effectively. This result suggests that there may be a weak JNK-docking domain that at least partially overlaps with amino acids 70–79. The general mitogen-activated protein kinase-docking motif or D-domain contains basic amino acid(s) followed by an L*X*L motif and/or a triplet of hydrophobic amino acids (13). The sequence of amino acids LFIF (residues 75–78) located downstream of a basic residue (Arg^{72}) is perhaps reminiscent of a D-domain with LFI representing the L*X*L motif (see Fig. 1). However, whereas the D-domains of Elk-1 and SAP-1 possess four basic residues, Bim$_{EL}$ contains only a single Arg residue, perhaps explaining why JNK can only bind weakly. Furthermore, the hydrophobic stretch of such a putative D-domain is adjacent to the DEF2 domain, suggesting that the binding of kinases to these sites may be mutually exclusive. This could explain why truncation of amino acids 70–79 abolishes the weak JNK binding but enhances access of ERK1/2 to the DEF2 domain. If these kinases do compete for binding, it might be expected that inhibition of the ERK1/2 pathway would prevent ERK1/2 binding to DEF2 and thereby enhance binding of JNK. However, when we completely inhibit the ERK1/2 pathway *in vivo* with U0126, this fails to facilitate JNK binding.[2] Thus, the loss of JNK binding may enhance ERK1/2 binding but not *vice versa*, suggesting that the binding of JNK to Bim$_{EL}$ is inherently weak.

The ability of JNK to phosphorylate Bim$_{EL}$ is subject to some controversy. JNK has been shown to phosphorylate Bim$_L$ at residues Thr^{56} and either Ser^{44} or Ser^{58} of Bim$_L$ (corresponding to residues Thr^{112}, Ser^{100}, and Ser^{114} in Bim$_{EL}$), and this may regulate the interaction between Bim$_L$ and DLC1 (9). Although we can observe phosphorylation of recombinant GST-Bim$_{EL}$ at Ser^{65} by JNK (and indeed p38) *in vitro*, we have no evidence at all for phosphorylation of Ser^{65} by JNK *in vivo* and this presumably reflects the fact that JNK binds poorly to Bim$_{EL}$ (Ref. 12 and this study). However, the ability of JNK to phosphorylate Bim$_{EL}$ at Ser^{65} has only been observed in neuronal cells (27). All of the other reports have been in fibroblast, epithelial, and hematopoietic cell lines (13, 23, 24) and have implicated ERK1/2 as the kinase responsible. Perhaps the regulation of Bim$_{EL}$ by JNK is a property unique to neuronal cells. Further work is required to determine the relevance, if any, of the weak JNK-Bim$_{EL}$ interaction.

In summary, we have identified and characterized a DEF-type, ERK1/2-specific docking domain in Bim$_{EL}$ downstream of the major ERK1/2 phosphoacceptor site in the domain encoded by exon 3. This domain is found in Bim$_{EL}$, Bimα1, and Bimβ1, which are all ERK-dependent phosphoproteins, but not in Bim$_S$ and Bim$_L$. We propose that exon 3 confers ERK1/2 binding and ERK1/2-dependent phosphorylation, thereby promoting turnover of Bim$_{EL}$ as a cytoprotective mechanism. ERK1/2-dependent phosphorylation may also disrupt interactions with Bax, providing another mechanism by which ERK1/2 can blunt the pro-apoptotic activity of Bim$_{EL}$ (32).

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Mechanisms of Signal Transduction:

Identification of a DEF-type Docking Domain for Extracellular Signal-regulated Kinases 1/2 That Directs Phosphorylation and Turnover of the BH3-only Protein Bim EL

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