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

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The BH3-only protein, Bim, exists as three splice variants (BimS, BimL, and BimEL) of differing pro-apoptotic potency. BimEL, 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 BimEL splice variant that includes the major ERK1/2 phosphorylation site Ser65. 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 BimEL (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 BimEL and also abolished ERK1/2-dependent phosphorylation of BimEL in vivo, thereby stabilizing the protein and enhancing cytotoxicity. Our results identify a new physiologically relevant functional motif in BimEL 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 JNK1 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 (BimS, BimL, and BimEL) (7), thereby introducing additional levels of regulation that may account for their differences in pro-apoptotic potency. For example, BimS 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). BimL contains an additional domain through which it can interact with dynein light chain 1 (DLC1) with the result that in viable cells BimL is sequestered at microtubules and so is a less effective killer (8). Disruption of microtubules can cause the redistribution of BimL to the mitochondria, and this may be due to JNK-dependent phosphorylation of BimL at sites adjacent to the DLC1-binding site (9). BimEL also contains the DLC1-binding site but is the least effective killer, and this may be explained by the fact that BimEL protein stability is subject to post-translational regulation. Activation of the ERK1/2 pathway promotes the proteasomal degradation of BimEL (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 BimEL 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 LXXL 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 LXXL motif and hydrophobic residues in the conserved kinase core (14). A second docking domain, containing the consensus sequence FXF, has been termed the DEF domain (docking site for ERK, EXFP) (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 BimEL by ERK1/2 is due to the presence of an ERK1/2 phospho-mitogen-activated protein kinase isozyme ERK kinase; ER, endoplasmic reticulum; MEKK, MEK kinase; HA, hemagglutinin; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminoethyl)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 BimEL, that is encoded by exon 3 (12). Here we have identified a docking domain in BimEL, also encoded by exon 3, that accounts for ERK1/2 binding, ERK1/2-dependent phosphorylation, and turnover of BimEL.

**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 (SPLIFYFVR) or DEF2 domains (SGYFSFDTDR) of BimEL were synthesized by Dr. Ram Sharma (Southampton Polypeptides Ltd.).

**Cell Culture**—The culture of RM3 cells has been described previously (18). HEK293 cells were maintained under identical conditions. For serum deprivation, cells were judged to be 50–60% confluent and 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**—BimEL and fragments of BimEL 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 BimEL 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.

**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 to bind 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 by 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-G1 DNA was recorded.

**RESULTS**

**The Minimal Region Required for ERK-docking Maps within Residues 80–97 of BimEL**—We have previously shown that activated ERK1/2 can bind directly to BimEL but not BimS or BimL and have mapped this interaction to amino acid residues 70–97 within the region unique to BimEL (Fig. 1) (12). This region does not include the major ERK1/2/phosphoacceptor site at Ser65 (12). Within this region, we identified two F motif (FIF at residues 76–78 and FSF at residues 93–95) that could be the 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-BimEL 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 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-BimEL 70–97 fusion protein. A fragment containing further C-terminal residues (GST-BimEL 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
Identification of an ERK-docking Domain in BimEL

A Def2 Domain Peptide Is Sufficient to Compete with BimEL for ERK1/2 Binding—To further investigate the role of the DEF domains in ERK1/2 binding, we used peptide competition assays. We basWed 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-BimEL\textsubscript{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. 3A). 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.\textsuperscript{2}

The DEF2 peptide caused a dose-dependent decrease in the amount of ERK2 precipitated by GST-BimEL\textsubscript{41–127} (Fig. 3B). The inhibition of ERK binding to 2 μM GST-BimEL\textsubscript{41–127} 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 (Et2) 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-BimEL\textsubscript{41–127} and also inhibited the binding of ERK1/2 to GST-Elk-1\textsubscript{310–428} (Fig. 3C). The DEF2 peptide inhibited the BimEL-ERK interaction, but it failed to block the interaction between JNK and c-Jun (Fig. 3D). 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 BimEL 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-BimEL\textsubscript{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 FXF sequence at residues 93–97 in BimEL is indeed a DEF motif that, at least in the

\textsuperscript{2} R. Ley and S. Cook, unpublished observations.
Identification of an ERK-docking Domain in BimEL

context of GST-BimEL80–127, 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 BimEL 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 BimEL, BimELΔS65A, or BimELΔDEF2. BimELΔS65A 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 MEK inhibitor, U0126 (U0). Lysates were resolved by SDS-PAGE and immunoblotted with anti-HA and phospho-ERK1/2. These were resolved by two-dimensional (2-D) electrophoresis and immunoprecipitated from lysates and used for kinase reactions with HA-BimEL, HA-BimELS65A, or HA-BimELΔDEF2 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-BimEL81–127 fusion protein eluted from beads as substrates. Equal loading of the kinase assays with substrate protein was confirmed by staining with anti-GST antibodies.

as a basic, non-phosphorylated spot and up to four additional acidic spots representing phosphorylated forms of the protein (Fig. 4B) (12, 20). Mutation of Ser65, 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. 4B) (12). When we transiently expressed the BimELΔDEF2 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. 4B). 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-BimELΔDEF2 fusion protein was phosphorylated as efficiently as wild type GST-BimEL41–127 when added to an in vitro ERK1/2 kinase reaction (Fig. 4C). 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 BimEL in vivo.

 Mutation of the DEF Domain Blocks Serum-stimulated BimEL Turnover and Enhances Cell Death—ERK1/2 phosphorylates BimEL in vitro at Ser65, which is also an ERK1/2-dependent phosphorylation site in vivo (12, 23, 24). Activation of ERK1/2 upon serum stimulation targets BimEL for degradation (10), and mutation of Ser65 to a non-phosphorylatable alanine prevents the turnover of BimEL (12, 24). We postulated that mutation of the DEF2-docking domain of BimEL should also reduce BimEL turnover by preventing ERK1/2-dependent phosphorylation. To test this possibility, we expressed wild type HA-BimEL, HA-BimELΔS65A, or HA-BimELΔDEF2 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. 5A and is quantitated in Fig. 5B. These results revealed both HA-BimEL and HA-BimELΔS65A were expressed at a higher level than wild type BimEL in the basal state and that serum stimulation caused a 76% reduction in the amount of wild type HA-BimEL, whereas both HA-BimELΔS65A and HA-BimELΔDEF2 were less sensitive to serum-stimulated turnover. The simplest explanation for this result is that the loss of the phosphoacceptor site (S65A) or the DEF2-docking site (ΔDEF2) prevents ERK1/2-dependent phosphorylation and turnover of BimEL in vivo.

Since the loss of DEF2 prevented turnover of BimEL, we postulated that the DEF2 mutant would elicit greater cell death than wild type BimEL. Indeed, when we transiently transfected these constructs into HEK293 cells, we again found that BimELΔDEF2 was expressed at higher levels than wild type BimEL and caused significantly greater cell death (Fig. 5C), 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-
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 BimEL at a Site Distinct from DEF2**—Whereas several groups have shown that ERK1/2 phosphorylate BimEL at Ser\(^65\) in vitro and in vivo (12, 23, 24), two recent studies (27, 28) have suggested that BimEL is phosphorylated at Ser\(^65\) by JNK. Although we could observe phosphorylation by both ERK1/2 and JNK in vitro using recombinant GST-BimEL 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-BimEL\(^{70–97}\) 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-BimEL\(^{70–97}\) 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-BimEL\(^{51–97}\) caused the complete loss of this weak JNK binding but substantially enhanced the binding of ERK1/2 to GST-BimEL\(^{70–97}\) (Fig. 2, lane 1 compared with lane 2). These data confirm our original observation that BimEL 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 BimEL. Indeed, we have previously shown that when we activate \(\Delta MEKK3\) or U0126 treatment (which inhibits the ERK pathway but not JNK) abolishes phosphorylation of BimEL. Furthermore, the weak binding of JNK to GST-BimEL\(^{70–97}\), but not to GST-BimEL\(^{80–97}\), 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 BimEL protein increases within 1–2 h, preceding increases in Bim\(_1\) 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 BimEL (10, 12, 23, 24). Thus, the rapid increase in BimEL expression following serum withdrawal most probably reflects rapid protein stabilization as well as de novo transcription. In contrast, isoforms such as Bim\(_{1}\) or Bim\(_{1}\) 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 BimEL appears to be the ability of ERK1/2 to phosphorylate BimEL at Ser\(^65\) within the region unique to BimEL in vitro and in vivo (12, 23, 24), because mutation of this site stabilizes BimEL 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-like docking domain within the region of BimEL encoded by exon 3 and shown that it is required for ERK1/2-dependent phosphorylation and turnover of BimEL in vivo.

**Characterization of a DEF-type ERK1/2-specific Docking Domain in BimEL**—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
Identification of an ERK-docking Domain in BimEL

Fig. 6. Isoforms of Bim that contain exon 3 are phosphorylated in an ERK-dependent manner. HEK293 cells were transfected with HA-BimL, HA-hBimβ1, HA-hBimα1, or HA-BimEL in serum-free conditions. After 18 h, cells were treated with 10% FBS in the absence (C) or presence (U0) of the MEK inhibitor, U0126. 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 BimEL. Third, as with other DEF domains, the two Phe residues in the BimEL 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., FQPP in LIN-1), the aspartate in the BimEL 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 BimEL 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 BimEL, because the FSFD→ASAD mutation introduced into full-length BimEL 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 BimEL, downstream of the major ERK1/2 phosphoacceptor site in the domain encoded by exon 3. This domain is found in BimEL, Bimα1, and Bimβ1, which are all ERK-dependent phosphoproteins, but not in BimS and BimS. We propose that exon 3 confers ERK1/2 binding and ERK1/2-dependent phosphorylation, thereby promoting turnover of BimEL 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 BimEL (32).

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REFERENCES

1. Cory, S., and Adams, J. M. (2002) Nat. Rev. Cancer 2, 647–656
2. Pathakalakath, H., and Strasser, A. (2002) Cell Death Differ. 9, 505–512

Second, it strongly suggests that exon 3 represents the minimal ERK1/2-binding and phosphorylation cassette.

Does BimEL Also Contain an Additional D-type Docking Domain for JNK?—As with our previous analysis (12), GST-BimEL bound JNK1 very poorly. This weak binding was abolished by the loss of amino acids 70–79 with the result that GST-BimEL 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 LXL 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<sup>72</sup>) is perhaps reminiscent of a D-domain with LFI representing the LXL motif (see Fig. 1). However, whereas the D-domains of Elk-1 and SAP-1 possess four basic residues, BimEL 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.<sup>2</sup> Thus, the loss of JNK binding may enhance ERK1/2 binding but not versa versa, suggesting that the binding of JNK to BimEL is inherently weak.

The ability of JNK to phosphorylate BimEL is subject to some controversy. JNK has been shown to phosphorylate Bim<sub>S</sub> at residues Thr<sup>26</sup> and either Ser<sup>44</sup> or Ser<sup>58</sup> of Bim<sub>L</sub> (corresponding to residues Thr<sup>112</sup>, Ser<sup>100</sup>, and Ser<sup>114</sup> in BimEL), and this may regulate the interaction between Bim<sub>L</sub> and DLC1 (9). Although we can observe phosphorylation of recombinant GST-BimEL at Ser<sup>65</sup> by JNK (and indeed p38) in vitro, we have no evidence at all for phosphorylation of Ser<sup>65</sup> by JNK in vivo and this presumably reflects the fact that JNK binds poorly to BimEL (Ref. 12 and this study). However, the ability of JNK to phosphorylate BimEL at Ser<sup>65</sup> 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 BimEL by JNK is a property unique to neuronal cells. Further work is required to determine the relevance, if any, of the weak JNK-BimEL interaction.

In summary, we have identified and characterized a DEF-type, ERK1/2-specific docking domain in BimEL, downstream of the major ERK1/2 phosphoacceptor site in the domain encoded by exon 3. This domain is found in BimEL, Bimα1, and Bimβ1, but not in Bim<sub>S</sub> or Bim<sub>α</sub>). This correlation 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<sub>EL</sub> does not need to be at the mitochondria or engaged with Bcl-2 proteins to be phosphorylated by ERK1/2.

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REFERENCES

1. Cory, S., and Adams, J. M. (2002) Nat. Rev. Cancer 2, 647–656
2. Pathakalakath, H., and Strasser, A. (2002) Cell Death Differ. 9, 505–512
Identification of an ERK-docking Domain in BimEL

3. Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenerman, L., and Coffer, P. J. (2000) Curr. Biol. 10, 1201–1204
4. Weston, C., Balmanno, K., Chalmers, C., Hadfield, K., Molton, S., Ley, R., Wagner, E. F., and Cook, S. J. (2003) Oncogene 22, 1281–1293
5. Whitfield, J., Neame, S. J., Paquet, L., Bernard, O., and Ham, J. (2001) Neuron 29, 629–643
6. Putcha, G. V., Moulder, K. L., Golden, J. P., Bouillet, P., Adams, J. A., Strasser, A., and Johnson, E. M., Jr. (2001) Neuron 29, 615–628
7. O’Connor, L., Strasser, A., O’Reilly, L. A., Hausmann, G., Adams, J. M., Cory S., and Huang, D. C. (1998) EMBO J. 17, 384–395
8. Puthalakath, H., Huang, D. C., O’Reilly, L. A., King, S. M., and Strasser, A. (1999) Mol. Cell. 3, 287–296
9. Levy, R., Balmanno, K., Hadfield, K., Weston, C., and Cook, S. J. (2003) J. Biol. Chem. 278, 18811–18816
10. Bouillet, P., Zhang, L. C., Huang, D. C., Webb, G. C., Bottema, C. D., Shore, P., Eyre, H. J., Sutherland, G. R., and Adams, J. M. (2001) Mamm. Genome 12, 163–168
11. Levy, R., Eyings, K. E., Hadfield, K., Howes, E., Balmano, K., and Cook, S. J. (2004) J. Biol. Chem. 279, 38477–38487
12. Sharrock, A. D., Yang, S. H., and Galasis, A. (2000) Trends Biochem. Sci. 25, 448–452
13. Chang, C. I., Xu, B. E., Akella, R., Cobb, M. H., and Goldsmith, E. J. (2002) Mol. Cell. 9, 1241–1249
14. Jacobs, D., Glassop, D., Xing, H., Muslin, A. J., and Kornfeld, K. (1999) Genes Dev. 13, 163–175
15. Galanis, A., Yang, S. H., and Sharrock, A. D. (2001) J. Biol. Chem. 276, 965–973
16. Lee, T., Hoonfagle, A. N., Kabuyama, Y., Stroud, J., Min, X., Elizabeth, J., Goldsmith, E. J., Chen, L., Resing, K. A., and Ahn, N. G. (2004) Mol. Cell 14, 43–55
17. Garner, A. P., Weston, C. R., Todd, D. E., Balmano, K., and Cook, S. J. (2002) Oncogene 21, 8089–8104
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Seward, R. J., van Haller, P. D., Aebersold, R., and Huber, B. T. (2003) Mol. Immunol. 39, 983–993
20. Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 67, 31–40
21. Bardwell, A. J., Abdollahi, M., and Bardwell, L. (2003) Biochem. J. 370, 1077–1085
22. Marani, M., Hancock, D., Lopes, R., Tenev, T., Downward, J., and Lemoine, N. R. (2003) Oncogene 22, 2431–2441
23. Luciano, F., Jacquel, A., Colosetti, P., Harrant, M., Cagnol, S., Pages, G., and Auberger, P. (2003) Oncogene 22, 6785–6793
24. Harada, H., Quearry, B., Ruiz-Vela, A., and Korsmeyer, S. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 15313–15317
Mechanisms of Signal Transduction:
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