The Transcriptional ETS2 Repressor Factor Associates with Active and Inactive Erks through Distinct FXF Motifs*†‡§¶

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The transcriptional ETS2 repressor factor (ERF) is phosphorylated by Erks both in vivo and in vitro. This phosphorylation determines the subcellular localization and biological function of ERF. Here, we show that active and inactive Erk2 proteins bind ERF with high affinity through a hydrophobic pocket formed by the cα and cα helices and the activation loop of Erk2. We have identified two FXF motifs on ERF that mediate the specific interaction with Erks. One of these motifs is utilized only by active Erks, whereas the other mediates the association with inactive Erks but also contributes to interaction with active Erks. Mutation of the phenylalanines of these motifs to alanines resulted in decreased association and phosphorylation of ERF by Erks both in cells and in vitro. ERF proteins carrying these mutations exhibited increased nuclear accumulation and increased inhibition of cellular proliferation. Expression of ERF regions harboring these motifs could inhibit Erk activity in cells. Our data suggest that, in the proper context, FXF motifs can mediate a strong and specific interaction not only with active but also inactive Erks and that these interactions determine protein function in vivo.

This phosphorylation determines its subcellular localization. In serum-starved cells, ERF is not phosphorylated and located within the nucleus. Upon serum induction, ERF is phosphorylated and exported into the cytoplasm. The absolute correlation between Erk activity and ERF phosphorylation and cytoplasmic localization is reinforced by the sensitivity of ERF localization and phosphorylation to specific MEK inhibitors. Phosphorylation-deficient ERF proteins with phosphorylation sites mutated to alanine localize in the nucleus, arrest fibroblasts at the G0/G1 phase of the cell cycle in a retinoblastoma-dependent manner, and can suppress ras-induced tumorigenicity. This indicates that ERF is a bona fide effector of ras, mediating cell cycle progression-specific signals (2).

Phosphorylated ERF shuttles continuously between the nucleus and the cytoplasm. In fibroblasts, both phosphorylation and dephosphorylation of ERF occur in the nucleus. Whereas ERF nuclear import does not appear to be affected by phosphorylation, ERF nuclear export is dependent on Erk phosphorylation. The phosphorylation of ERF and its export from the nucleus closely follow the appearance of active Erk in the nucleus and may serve as a continuous monitor of Erk-mediated receptor tyrosine kinase signaling. Thus, ERF appears to be a very sensitive monitor of Erk activity that can affect cell cycle progression, providing another link between the Ras/Erk pathway and this fundamental cellular process (3). The region of ERF required for its specific interaction with Erks has been narrowed to the central part of the protein (amino acids 316–416) (2).

The MAPK family of serine/threonine protein kinases is a major kinase family involved in multiple cellular processes and thus is a major target for therapeutic intervention. There is a plethora of cellular targets reported to be phosphorylated by one or more members of this kinase family. Given the homology among MAPKs and the number of proteins reported to be phosphorylated by these kinases, there is considerable interest and effort in understanding the specific structural requirements for the kinase-substrate interaction that determine the specificity of a kinase for a given protein (4, 5). Kinases interact directly with their substrates or indirectly through scaffold proteins, forming tight complexes. These interactions involve specific sequences on the substrates known as docking sites (6). These docking sites are thought to favor substrate phosphorylation by increasing the local concentration of the kinase. The first docking motif that was identified was the c-Jun δ-domain.
(7). Similar to the 8-domain, D-domains were also identified in a number of transcription factors that are regulated by ERK, JNK, and/or p38 MAPKs (for review, see Refs. 8 and 9). They are characterized by the presence of an LXL motif adjacent to a basic region. In some instances, the LXL motif is replaced by a hydrophobic amino acid triplet. The pointed domain found in other ETS domain proteins also contains a characteristic LXL motif adjacent to a conserved phenylalanine (10). Another MAPK docking motif is the FXF motif first identified in the ETS domain protein LIN1 (11), which appears to be conserved in a number of MAPK substrates. The FXF domain protein LIN1 (11), which appears to be conserved in a number of transcription factors that are regulated by ERK, either with Lipofectamine (Invitrogen) according to the manufacturer's protocol or with calcium phosphate.

**ERF-Erk Interaction**

Several ERFs have been found to interact with Erk in vitro and in vivo (12). This system is believed to determine both kinase specificity and phosphaeceptor site determination. However, docking domains can facilitate association with all three MAPK subfamilies. Either these motifs are necessary but not sufficient to provide the specificity observed by MAPK subfamilies, or small differences within the motifs and the surrounding region may be critical but as yet uncharacterized specificity determinants.

In this study, we have identified the structural determinants on ERF that mediate a highly specific interaction with Erks. Our data suggest that ERF can physically associate not only with active but also inactive Erks, but not with members from other MAPK subfamilies, although it was also found to contribute to the phosphorylation of Sap1 by p38α (12). Finally, there is a characteristic acidic amino acid region in MAPKs (the CD-domain) that interacts with a basic region on the associated protein (13).

MAPK substrates often contain more than one docking motif, often of a different kind, forming a modular docking system (12). This system is believed to determine both kinase specificity and phosphoacceptor site determination. However, docking domains can facilitate association with all three MAPK subfamilies. Either these motifs are necessary but not sufficient to provide the specificity observed by MAPK subfamilies, or small differences within the motifs and the surrounding region may be critical but as yet uncharacterized specificity determinants.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Ref1 (rat embryo fibroblasts) and COS-7 cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. NIH-3T3 cells (Swiss mouse embryonic fibroblasts) were grown in Dulbecco’s modified Eagle’s medium supplemented with 8% calf serum. For low serum growth assays, NIH-3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 or 0.2% calf serum and 10 mM HEPES buffer (pH 7). Cells were transfected either with Lipofectamine (Invitrogen) according to the manufacturer’s protocol or with calcium phosphate.

**Plasmids**—The pGEX-4T-1 vector (GE Healthcare) was used to generate glutathione S-transferase (GST)-ERF chimeras with standard recombinant DNA techniques. Briefly, the pGST257–425 plasmid was constructed by inserting the 502-bp Sau3A1/Sau3AI ERF fragment (amino acids 257–425) into the BamHI site of the pGEX-4T-1 vector. The pGST257–293 plasmid (amino acids 257–293) was generated by replacing the Avai/EcoRI fragment of pGST257–425. The pGST257–311 plasmid (amino acids 257–311) was generated by replacing the 330-bp BglII/SalI fragment of pGST257–425. The pGST294–425 plasmid (amino acids 294–425) was generated by replacing the 110-bp Nael/EcoRI fragment of pGST257–425. The pGSTM50–425 plasmid (amino acids 350–425) was generated by removing the 290-bp BamHI/NcoI fragment of pGST257–425. The pGST294–385 plasmid (amino acids 294–385) was generated by replacing the 110-bp Nael/EcoRI fragment of pGST257–385. The pGST294–352 plasmid (amino acids 294–352) was generated by replacing the 210-bp Nael/SalI fragment of pGST257–425. The pGST313–425 plasmid (amino acids 313–425) was generated by replacing the 70-bp BamHI/SalI fragment of pGST257–425. The pGSTM50–425 plasmid (amino acids 350–425) was generated by removing the 290-bp BamHI/NcoI fragment of pGST257–425. The pGST294–385 plasmid (amino acids 294–385) was generated by replacing the 110-bp Nael/EcoRI fragment of pGST257–385. The pGST294–352 plasmid (amino acids 294–352) was generated by replacing the 210-bp Nael/SalI fragment of pGST294–425. The pGST313–385 plasmid (amino acids 313–385) was generated by replacing the 70-bp BamHI/SalI fragment of pGST257–385. The pGST313–352 plasmid (amino acids 313–352) was generated by replacing the 290-bp Nael/NcoI fragment of pGST294–352. The pGSTM50–385 plasmid (amino acids 350–385) was generated by removing the 290-bp BamHI/NcoI fragment of pGST257–385. The pGSTM66 expression plasmid (amino acids 257–425/295–312) was generated by inserting the 330-bp BamHI/Nhel fragment of pGST257–425 into the EcoRI/Sacl site of pGST257–293. The pGST257–425 expression plasmid, carrying mutations of Phe303 and Phe305 to alanines, and the AKA expression plasmid, carrying mutations of Phe375 and Phe377 to alanines, were generated using the GeneEditor system (Promega) on the pGST257–425 plasmid according to the manufacturer’s protocol. The AKA/ERF expression plasmid was generated by replacing the 200-bp NcoI/Nhel fragment of the AKA expression plasmid with the respective region of the AKA expression plasmid. The pGSTM50–385 plasmid (amino acids 350–385) was generated by replacing the 70-bp BamHI/SalI fragment of pGST257–385. The pGSTM66 expression plasmid, carrying mutations of Phe303 and Phe305 to alanines, and the AKA expression plasmid, carrying mutations of Phe375 and Phe377, were generated using the GeneEditor system (Promega) on the pGST257–425 plasmid according to the manufacturer’s protocol. The AKA/ERF expression plasmid was generated by replacing the 200-bp NcoI/Nhel fragment of the AKA expression plasmid with the respective region of the AKA expression plasmid.
the same 425-bp BstXI/Nhel fragment of the pCMV-HA-ERF plasmid. The pEBG294–385, pEBG257–352, pEBG257–385, and pEBG294–425 plasmids were constructed by cloning the corresponding BamHI/NotI fragments of pGST294–385, pGST257–352, pGST257–385, and pGST294–425 into the pEBG vector (15). pSG5-ERF (1), the Myc-Erk2 (16) and HA-Rsk2 (17) expression plasmids, and the 3xSRE-Luc reporter plasmid (18) were described previously.

**Fusion Protein Production, Purification, and Interaction Assays**—Bacterially expressed GST fusion proteins were purified on glutathione-Sepharose beads (GE Healthcare) according to the manufacturer’s protocol. Briefly, expression of the fusion protein was induced in bacteria by 0.1 mM isopropyl β-D-thiogalactopyranoside at room temperature for 2 h. Bacteria were lysed by sonication in phosphate-buffered saline, and Triton X-100 was added at a final concentration of 1% to facilitate solubilization of proteins. The extract was incubated overnight with glutathione-Sepharose at 4°C; the beads were washed extensively with phosphate-buffered saline; and when needed, the protein was eluted with 100 mM glutathione. The proteins were at least 99% pure as estimated by SDS electrophoresis. To determine protein-protein interactions, 3–5 μg of glutathione-Sepharose-bound GST fusion proteins were incubated with 100 ng of bacterially expressed purified Erk2 proteins in 50 μl of 20 mM Tris (pH 7.5), 100 mM NaCl, 0.1% Triton X-100, 100 ng/μl bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin for 30 min at room temperature. The beads were washed four times with the same buffer, and the remaining proteins were analyzed by Western blotting using anti-Erk or anti-p38 (New England Biolabs Inc.), anti-phospho-Erk (Sigma), or anti-GST (GE Healthcare) antibody.

For interaction with Erk proteins from cellular extracts, cells were lysed in 20 mM Tris (pH 7.5), 100 mM NaCl, 1% Triton X-100, 100 ng/μl bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml microcystin. The tagged proteins were immunoprecipitated with 1 μg of rat anti-HA monoclonal antibody (Roche Applied Science) or 1 μg of mouse anti-Myc monoclonal antibody (Sigma). The precipitated complexes were washed three times and analyzed by electrophoresis. The presence of ERF, Erk, and phospho-Erk in the complexes was detected by immunoblotting using antibody S17S (1), rabbit anti-Erk antibody (New England Biolabs Inc.), and mouse anti-phospho-Erk monoclonal antibody YT (Sigma), respectively.

Subcellular localization of phosphorylated ERF was determined by immunofluorescence using anti-phospho-ERF antibody P3–4 as described previously (3). For enhanced phospho-ERF signal in the simultaneous detection of phospho-ERF and total ERF, immunofluorescence was performed as described previously (3), but the P3–4 antibody was used at a dilution of 1:20, whereas the S17S antibody was used at 1:150. The localization of green fluorescent protein (GFP)-ERF fusion proteins was determined either by direct GFP fluorescence or using anti-GFP monoclonal antibody (Clontech)–ERF fusion proteins were determined by direct GFP fluorescence or using anti-GFP monoclonal antibody (Clontech) at 1:50 dilution on formaldehyde-fixed cells. Samples were scanned on the xy plane with a Leica TCS SP confocal microscope with a ×63 oil submersion objective. Images were electronically merged with Leica LCS Version 2.5 confocal software.

BrdUrd incorporation assays were performed as described previously (2). Briefly, the cells were transfected with an ERF expression plasmid and a GFP expression plasmid to mark the transfected cells. 24 or 48 h after transfection, BrdUrd was added at a final concentration of 50 μM for 4, 8, or 16 h. Cells were fixed with 3.5% formaldehyde and stained with mouse anti-BrdUrd monoclonal antibody (Sigma) at 1:100 dilution. GFP-positive cells were scored with rabbit anti-GFP polyclonal antibody (Clontech) at 1:250 dilution.

**RESULTS**

We have shown previously that ERF specifically interacts with and is phosphorylated by Erks in cells and in vitro in a cell-free system (2). However, it was not clear whether this specificity was mediated by direct interaction of the two proteins and, if so, the nature of the structural determinants of this interaction. Thus, we first determined regions on Erk that may

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mediate ERF-specific phosphorylation. We utilized Erk2 chimeras with other MAPKs and determined their ability to phosphorylate ERF produced in a cell-free system. Activated Erk2 readily phosphorylated ERF, whereas 10–20-fold higher amounts of activated p38 MAPK were required to achieve comparable phosphorylation under these conditions (Fig. 1A, compare 250 ng of p38 with 25 and 12.5 ng of Erk2). The Erk2-p38 chimeras PIVECTP and EIIPIVE (19), which have domains I–IV and CT and domains II–IV of p38, respectively, could bind to Erk2 substrates specifically (25) and could also readily phosphorylate ERF. In contrast, the Erk2/5 (22) and Erk2/3 (23) hybrids, which have domains I–IV from Erk2 and the substrate recognition domains of Erk5 and Erk3, respectively, were unable to phosphorylate ERF. This was also true for the kinase-dead K52R mutant of Erk2 and the Erk2 deletion mutant Δ241–271, which fails to interact with MEK1 (21), further suggesting a direct interaction with and phosphorylation by Erk2 (Fig. 1B).

The basic ΦXΦ (8) and FXF (11) motifs have been identified previously to mediate specific substrate interaction with Erks. The ERF sequence harbors four FXF motifs and an inverted basic ΦXΦ motif adjacent to Erk phosphorylation sites (Fig. 2A). However, neither the two optimal FXFP motifs found in the N-terminal portion of the protein nor the inverted basic ΦXΦ motif at the C terminus is within the region spanning amino acids 316–416, previously identified to mediate Erk-ERF interaction (2). Utilizing segments of the ERF protein expressed as GST fusion proteins in bacteria, we determined their interaction with bacterially expressed activated Erk2 (Fig. 1A) before the final purification. PIVECTP and EIIPIVE are Erk2-p38 hybrids containing domains I–IV and CT and only the catalytic center and domains II–IV of p38, respectively. Erk2 D242/272 has a deletion of the corresponding amino acids of Erk2. Erk2 K52R has a lysine to arginine mutation in the catalytic center. Erk5/2 and Erk3/2 have domains I–V from Erk5 and Erk3, respectively, whereas Erk2/5 is Erk5 with domains I–V from Erk2. C lane, control.

FIGURE 1. ERF is specifically phosphorylated by Erks in vitro. A, 35S-labeled ERF produced in a cell-free system was phosphorylated by the addition of the indicated amounts of bacterially expressed activated and purified Erk2 or p38 kinase. Phosphorylation was determined after SDS electrophoresis and autoradiography by the slower mobility of the phosphorylated ERF bands. B, 35S-labeled ERF produced in a cell-free system was phosphorylated by the addition of 100 ng of the indicated bacterially expressed purified kinase or kinase hybrids and mutations. P at the end of the kinase name indicates that the kinase was also activated in vitro before the final purification. PIVECTP and EIIPIVE are Erk2-p38 hybrids containing domains I–IV and CT and only the catalytic center and domains II–IV of p38, respectively. Erk2 D242/272 has a deletion of the corresponding amino acids of Erk2. Erk2 K52R has a lysine to arginine mutation in the catalytic center. Erk5/2 and Erk3/2 have domains I–V from Erk5 and Erk3, respectively, whereas Erk2/5 is Erk5 with domains I–V from Erk2. C lane, control.

FIGURE 2. ERF physically interacts with Erks in vitro. A, shown is a schematic representation of the ERF protein. DBD, ETS DNA-binding domain; RD, repression domain. The numbers below indicate the residues at the boundaries of these domains. Closed triangles indicate FXF motifs, and the open triangle included the inverted basic ΦXΦ motif. Circles indicate the PXS/TTP putative MAPK phosphorylation sites. Closed circles indicate sites found phosphorylated in vivo. B, the indicated bacterially expressed and purified GST-ERF proteins were phosphorylated in vitro using recombinant activated Erk2, analyzed by SDS electrophoresis, and visualized by Coomassie Brilliant Blue staining. Phosphorylation was determined by the slower mobility of the phosphorylated bands. C, 5 μg of bacterially expressed GST-ERF proteins were incubated with 100 ng of recombinant purified Erk2 or activated Erk2 (Erk2-P) in the presence or absence of ATP as indicated. GST-associated complexes were precipitated with glutathione-Sepharose and washed, and the presence of Erk2 in the complexes was analyzed after SDS electrophoresis and Western blotting with rabbit anti-Erk antibody. D, the recombinant activated Erk2-p38 hybrid proteins EIIPIVE and PIVECTP (containing the catalytic center and domains II–IV and domains I–IV and CT of p38, respectively) and the recombinant activated (P) and inactive (p38) proteins were subjected to GST pulldown assays using the GST257–425 hybrid protein as in C. The total amount of the respective kinase added to the reaction and the amount of the kinase associated with GST257–425 (Bound lanes) were visualized after SDS electrophoresis and Western blotting with anti-Erk2 and anti-p38 antibodies as indicated.
proteins as evidenced by the mobility shift of the proteins (Fig. 2B), consistent with the distribution of the phosphorylation sites on ERF and the distribution of putative interaction motifs. However, neither the 256-amino acid N-terminal segment (data not shown) nor the 78-amino acid C-terminal segment (GST-RD) (Fig. 2C) could form stable complexes with Erk2 regardless of phosphorylation state (Fig. 2B). In contrast, the middle portion of the protein from amino acids 257 to 424 could effectively associate with Erk2 in a phosphorylation state-independent manner. Interestingly, the association with activated Erk2 appeared to be stronger than that with inactive Erk2 (Fig. 2C, GST257–425 lanes). The Erk2-p38 hybrids PIVECTP and ELIPIVE, which have the catalytic center of p38 MAPK, could also phosphorylate ERF (Fig. 1B) and could physically interact with the same region of ERF. In contrast, wild-type p38 could not associate with ERF (Fig. 2D), suggesting that the Erk2 specificity is mediated by interactions outside the catalytic center.

To further define the region mediating ERF-Erk2 interaction, we generated a number of ERF deletion mutations and tested them by GST pulldown assays. Our data suggest that the region spanning amino acids 294–385 is the minimal region on ERF required for ERF-Erk interaction (Fig. 3A). Interestingly, although both active and inactive Erk2 could interact with this region, they appear to have distinct requirements. Thus, the region between amino acids 294 and 313 affected the interaction with activated Erk2, but not with non-phosphorylated inactive Erk2 (compare deletions Δ257–425 and D66, Δ294–425 and Δ313–425, and Δ294–385 and Δ313–385) (Fig. 3A, upper panel). The region between amino acids 352 and 385 affected primarily interaction with Erk2 (compare deletions Δ257–385 and Δ257–352, Δ294–385 and Δ294–352, and Δ313–385 and Δ313–352) (Fig. 3A, middle panel, and supplemental Fig. S1).

In addition to the differences in structural requirements, our data suggest that activated Erk2 had a higher affinity for ERF than did inactive Erk2 (Fig. 2C) (2). We confirmed this using a series of ERF concentrations with a constant amount of active or inactive Erk2. Consistent with our previous data, activated Erk2 had a >4-fold higher apparent affinity for ERF than did inactive Erk2 (Fig. 3B, compare 0.6 μg ERF+p-Erk and 2.5 μg ERF+Erk lanes).

Regions 294–313 and 352–385 identified above contain FXF motifs. An FSF motif resides at amino acids 303–305, and an FKF motif at amino acids 374–376. We mutated the phenylalanine residues to alanines in both of these motifs for ASA and AKA, respectively, and tested the ability of these proteins to interact with Erk2. Consistent with our deletion analysis data, it appeared that mutation of the FSF motif to ASA affected interaction with activated Erk2, whereas mutation of the FKF motif to AKA affected interaction with non-phosphorylated Erk2. Mutation of both sites further decreased interaction with activated Erk2 compared with the ASA mutation, but not with inactive Erk2 compared with the AKA mutation (Fig. 4A). This was also apparent when the same GST-ERF fusion proteins were used to pull down Erks from cellular extract (Fig. 4B). Although in limited amounts in extracts from exponentially growing cells, phosphorylated Erk2 was preferentially precipitated by wild-type ERF and the AKA mutant, to a lesser extent by the D66 deletion and ASA mutants, and to negligible levels by the double ASA/KA mutant. In contrast, the highly abundant Erk protein was precipitated by wild-type ERF as well as the D66 deletion and ASA mutants, but not by the AKA or double ASA/KA mutant. Comparison of the amounts of phosphorylated and total Erk proteins in the cellular extracts (Fig. 4B, C lane) and in the ERF-associated fraction also indicated

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that phosphorylated Erk had a higher affinity for ERF. This was also the case in cells as estimated by the ability of transfected epitope-tagged wild-type and mutant ERF proteins to associate with activated cellular Erk kinases (Fig. 4C, lower panel). In addition, as indicated by the slower mobility bands (Fig. 4C, upper panel), ERF phosphorylation was decreased in the association-deficient mutants.

To determine whether the apparent strength of the interaction between phospho-Erk2 and ERF determines the efficiency of ERF phosphorylation by Erk2, we performed in vitro kinase assays. Both wild-type and AKA mutant ERF proteins were phosphorylated in vitro by activated Erk2 at low nanomolar concentrations (Fig. 4D). The D66 deletion and ASA ERF mutants required a 3–5-fold higher Erk2 concentration, whereas the ASA/AKA mutant required an ~10-fold higher Erk2 concentration to achieve comparable phosphorylation. These data suggest both that the mutations do not affect the ERF phosphorylation sites and that the highly specific phosphorylation of ERF by Erk2 is mediated by high affinity interactions.

To determine the region of Erk2 mediating the interaction with ERF, we carried out pulldown assays using recombinant Erk2 proteins with amino acid substitutions within the region that has been reported to mediate interaction with FXF motifs (26). Interaction of inactive Erk2 with ERF appeared to be affected to a similar extent by all of the mutations tested, including the single Tyr261 mutation to alanine (Fig. 5, upper panel). The interaction of activated Erk2 with ERF was also severely affected by the double Met197/Leu198 and triple Met197/Leu198/Tyr261 mutations to alanines. The effect of the triple Tyr231/Leu232/Tyr261 mutation to alanines also affected the interaction, but less than the above two mutations. In contrast, the effect of the single Tyr261 mutation to alanine was marginal (Fig. 5, middle panel). These data suggest that the same region adjacent to the activation loop is utilized for the interaction with ERF by both active and inactive Erk2. However, the exact intermolecular interface appears to be distinct for active and inactive Erk2, as suggested by both the mutations used and the extensive structural changes following Erk activation (27, 28).

We have shown that ERF subcellular localization depends on Erk activity levels. ERF is phosphorylated in the nucleus by Erks and exits the nucleus as a phosphoprotein. During exponential cell growth, phospho-ERF is constantly shuttling between the cytoplasm and the nucleus and exhibits a mostly cytoplasmic localization because of increased export kinetics (3). We transiently transfected GFP-ERF fusion protein expression plasmids into Ref1 cells to determine whether ERF mutants that
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Exponent NoSerum Induced

wt
M1-7
D66
ASA
AKA
ASA/AKA

FIGURE 6. Erk-association-deficient ERF mutations affect subcellular localization. A, REF1 cells were transfected with plasmids expressing either wild-type (wt) or mutant GFP-ERF fusion proteins. The localization of the fusion proteins was determined by the GFP fluorescence under UV light during exponential growth (Exponent), after 4 h of serum starvation (No Serum), and after 4 h of serum starvation and induction with 20% fetal calf serum (Induced). M1–7 has all seven optimal MAPK phosphorylation sites mutated to alanines. D66 contains an internal deletion between amino acids 294 and 313. ASA has Phe303 and Phe305 mutated to alanines; AKA has Phe373 and Phe377 mutated to alanines; and ASA/AKA has all four phenylalanines mutated to alanines. Representative cell images are shown. B, the localization of the GFP-ERF fusion proteins described for A was quantified in multiple independent experiments under the growth condition described above. A minimum of 100 transfected cells were counted for each plasmid and growth condition in at least three independent experiments. The percentage of cells with predominantly cytoplasmic, nuclear, or ubiquitous distribution of GFP fluorescence under each condition is shown.

exhibited decreased association and phosphorylation by Erk2 had altered subcellular localization. The localization of the AKA mutant paralleled that of wild-type ERF, with the exception of a small decrease in the “ubiquitous” fraction (omnipresent in transient transfection experiments) (Fig. 6B). In contrast the D66, ASA, and ASA/AKA mutants exhibited increased nuclear and decreased cytoplasmic localization under all conditions. However, all of the mutants could enter the nucleus during serum starvation, and they were effectively exported from the nucleus during serum stimulation (Fig. 6). Finally, the subcellular distribution of the mutants in several experiments and under different conditions indicated that the double ASA/AKA mutant exhibited increased nuclear localization compared with the ASA mutant, further suggesting the utilization of the FKF site by activated Erks (Fig. 6B, upper panel).

To determine whether the increased nuclear localization was due to limited ERF phosphorylation, we investigated the phosphorylation state of the nuclear and cytoplasmic proteins by direct immunofluorescence utilizing an anti-phospho-ERF antibody that recognizes phospho-Ser246 and phospho-Ser251. Our data suggest that the increased nuclear accumulation observed with interaction-deficient ERF mutants was due to their decreased phosphorylation (Fig. 7A and supplemental Fig. S2). Thus, decreased association of ERF and Erk leads to decreased phosphorylation in vivo. However, the FXF motifs have also been implicated in the nuclear import-export machinery interactions through the FTFG nucleoporin repeats (for review, see Ref. 29). If the FXF motifs are also involved in ERF shutting, it is possible that these mutations could affect export kinetics rather than phosphorylation. To determine the possible contribution of the mutations in export kinetics, we analyzed the localization of the mutant proteins during serum induction. All of the mutants were exported from the nucleus and released in the cytoplasm at similar rates (Fig. 7B), suggesting that the association with export machinery components is not affected. This would be consistent with our previous findings indicating that ERF is exported from the nucleus via an adapter protein.

We have shown previously that phosphorylation-deficient mutants of ERF in which the phosphoacceptor serine and threonine sites are mutated to alanines localize to the nucleus and can arrest cell cycle progression (3) and revert Ras-induced transformation of NIH-3T3 cells (2). We tested the Erk association-deficient deletion mutation of ERF (D66) to determine whether it can also block cell cycle progression. Indeed, proliferation of NIH-3T3 cells expressing this mutation was inhibited at levels comparable with the m1–7 phosphorylation-deficient mutation (Fig. 8A). This indicates that ERF-Erk association is required for effective in vivo phosphorylation. We also established clones over-expressing the D66 mutation in Ras-transformed NIH-3T3 cells. Only a small percentage (~10%) of the neomycin-resistant clones expressed D66. This is consistent with our previous findings that phosphorylation-deficient ERF mutations are detrimental to the growth of Ras-transformed NIH-3T3 cells (2). We tested the proliferation potential of two clones expressing D66. Clone 2 expressed low levels of D66 (~4-fold over endogenous ERF), whereas clone 6 expressed high levels of D66 (~15-fold over endogenous ERF) (supplemental Fig. S3A). Under normal growth conditions in medium supplemented with 8% or 2% serum, all clones proliferated at similar rates with the exception of clone 6, which exhibited decreased proliferation (Fig. 8B). With a minimal amount of serum (0.2%), both high and low expressing clones and NIH-3T3 cells died after 2 weeks in culture, whereas the parental Ras/NIH-3T3 cells, after the initial crisis, continued to proliferate (Fig. 8B). Cell morphology was also consistent with the inhibition of the Ras-transformed phenotype (supplemental Fig. S3B). These data suggest that, even in a system in which there is continuous and elevated Erk activity, decreased ERF-Erk association leads to phenotypes reminiscent of non-phosphorylated ERF.

Our data suggest that the FXF motifs within the central part of the ERF protein make a major contribution to the specific interaction with Erks. However, mutations of these motifs did
not eliminate this interaction but decreased the affinity by more than an order of magnitude, indicating that these motifs are necessary but not the only interaction determinants. Consistent with this, ERF-derived peptides harboring the FxF motifs (amino acids 297–311 and 371–385) inhibited the in vitro ERF-Erk interaction by no more than 50%, even at a 100-fold molar excess. This minimal inhibition appeared to be independent of the phenylalanine residues because peptides carrying alanine mutations gave comparable competition. This minimal inhibition did not change when the peptides were used alone or in combination or when they were attached on a flexible peptido-

mimetic backbone (data not shown). To determine the region of ERF that may be required to effectively block ERF-Erk interaction in vivo, we transferred regions of ERF into eukaryotic expression vectors and tested their ability to block ERF-Erk interaction as evidenced by the enhanced nuclear localization of wild-type ERF (Fig. 9A). Indeed, peptides derived from residues 257–424 of ERF had a variable effect on ERF localization, ranging from limited (pEBG294–385) to significant (pEBG294–425). The minimal ERF region harboring both FxF motifs (amino acids 294–385, pEBG294–385) increased the nuclear localization of ERF (Fig. 9A). However, when only one

FIGURE 7. FxF mutations affect in vivo phosphorylation rather than transport through nuclear pores. A, Ref1 cells were transfected with plasmids expressing either GFP-fused wild-type ERF or D66 mutant ERF (containing an internal deletion between amino acids 294 and 313). The localization of the total and phosphorylated GFP-ERF proteins was determined by indirect immunofluorescence and confocal microscopy during exponential growth using anti-GFP (green) and anti-phospho-Ser246/Ser247 ERF (red) antibodies, respectively. Antibody concentrations and confocal settings were adjusted to eliminate signal with anti-phospho-ERF antibody from endogenous ERF. B, Ref1 cells were transfected with the same plasmids as described in the legend to Fig. 6. Cells were serum-starved for 4 h (w/o) and then induced with 20% fetal calf serum. 10, 20, 30, and 40 min after serum addition, the cells were fixed with 4% formaldehyde, and the localization of the GFP-ERF fusion proteins was determined by the GFP fluorescence under UV light. A minimum of 50 transfected cells were counted for each plasmid and time point in at least three independent experiments.

FIGURE 8. Decreased ERF-Erk association affects ERF function. A, NIH-3T3 cells were cotransfected with empty pSG5 vector or with wild-type (wt) ERF-, D66-, m1–7-, or GFP-expressing vector as a marker for the transfected cells. 24 h after transfection, cells were labeled for 8 h with BrdUrd (BrdU), stained, and scored under a fluorescence microscope. At least 100 GFP-positive cells were scored in each sample, and a minimum of three independent experiments were performed. B, NIH-3T3 cells ( ), Ras/NH-3T3 cells ( ■), D66/Ras/NH-3T3 clone 2 ( ■), and D66/Ras/NH-3T3 clone 6 ( ■) were grown in 35-mm plates with 2% (upper panel) or 0.2% (lower panel) calf serum, and the cell numbers were counted at the indicated days. 30,000 cells were seeded in all cases except for the NIH-3T3 cells in 0.2% serum, where 100,000 cells were seeded. Duplicate samples in three independent experiments were assayed.
ERF has been shown to interact with and be phosphorylated at multiple sites by Erks both in vivo and in vitro, and it exhibits a high degree of specificity and sensitivity for Erks (Fig. 2) (2). We have shown that the specific ERF-Erk interactions occurring with both active and inactive Erks are mediated by two FXF motifs at positions 303–305 and 375–377 of the human ERF protein. Active Erks utilize predominantly the FXF motif (positions 303–305), whereas inactive Erks utilize exclusively the FXF motif (positions 375–377). Mutation of these phenylalanine residues to alanines results in decreased association with and phosphorylation by Erks both in vivo and in vitro. ERF proteins carrying these mutations exhibit increased nuclear accumulation and increased inhibition of cellular proliferation and suppress Ras-induced transformation. These functions are associated with the non-phosphorylated form of ERF. Expression of ERF regions harboring these motifs can inhibit in vivo ERF phosphorylation by Erks, without inhibition of Erk activation. These data verify that ERF is a direct and specific target for Erk kinases and may also provide a unique model for Erk-substrate interactions.

Our current understanding of MAPK substrate specificity is that specificity is mediated by docking sites (8) and/or scaffolding proteins (31) in addition to inherent enzymatic specificity. The D-domain or DEJL domain (docking site for ERK and JNK, LXL) has been shown to facilitate interaction with all three subfamilies of MAPKs, utilizing a groove formed by the β7–β8 and αD–αE regions of the MAPKs (32). The FXF motif or DEF domain (docking site for ERK, FXTP) is believed to be relatively more specific for Erk docking (33) and binds to Erk2 near the MAPK insert (26). ERF has four FXF motifs and an inverted D-domain sequence, which can serve as docking site (32). These motifs, with the exception of the FXF motif at residues 9–11, are adjacent to phosphorylation sites (2). However, only the FXF motifs at residues 303–305 and 375–377 support high affinity association with Erks. It is not clear if this interaction directs phosphorylation of all of the phosphoacceptor sites identified throughout ERF. These two central motifs are adjacent to amino acid stretches that could provide sufficient structural flexibility to allow phosphorylation of all of the sites identified on ERF. Another equally plausible hypothesis is that the association through the centrally located FXF motifs increases local Erk concentration, allowing the association with the other putative docking sites and the phosphorylation of the adjacent phosphoacceptor sites. In either case, disruption of the primary docking sites would result in decreased phosphorylation of all of the phosphoacceptor sites.

Interaction with active and inactive Erks is within the range of the physiological Erk concentration. This increased associa-
tion with activated Erk could result from the higher affinity of activated Erks for the FSF rather than the FKF motif of ErF or from some other event such as Erk dimerization upon activation (34). The additional contribution of the FKF motif also to the association with active Erks would be consistent with the Erk dimerization hypothesis. However, it is evident that the affinity of both active and inactive Erk2 for the FKF motif is lower that that of active Erk2 for the FSF motif. Thus, the increased association of activated Erk with ErF may be due to both Erk dimerization and increased affinity for the FSF motif.

It is not clear if ErF also interacts with inactive Erks in vivo. Diphosphorylated ErF is nuclear, whereas depending on the cell type and stimulation conditions, Erks are distributed in both compartments (35–37). It is conceivable that inactive Erks associate with ErF in vivo and are brought close to their chromatin targets. Erks have been reported to phosphorylate a variety of chromatin-associated molecules (38–40) and transcription factors, and ErF can serve as a tether to the region of interest. In addition, the association of ErF with the inactive kinase could facilitate the sequestration of Erk in the nucleus (41, 42). Finally, the association with inactive Erk could explain the almost instantaneous phosphorylation of ErF upon Erk activating signals (3).

A recent study (26) with hydrogen exchange spectrometry and modeling indicated that the hydrophobic clef of Erk2 mediating association with FXF motifs involves the kinase activation loop. Thus, the association through these motifs occurs preferentially with the activated kinase (26, 43). Indeed, the interaction of activated Erk2 with ErF was decreased by mutations in this region of the kinase. Mutations of Met197/Leu198 and Tyr231/Leu232 severely decreased ErF-Erk association. Mutation of Tyr261 also affected the interaction. Thus, it is possible that all five mutated residues may participate in the interaction interface, as suggested by others (26, 43). However, in the ERF-Erk association, the same region of inactive Erk2 appears to mediate the interaction with the FXF motif (amino acids 375–377) of ERF. All of the mutations within the region tested dramatically decreased ERF-Erk interaction. The decreased contribution of the FKF motif of ERF to the interaction with activated Erk may be the consequence of the structural changes upon kinase activation, indicating that the interaction may involve the same region on active and inactive Erks. This is consistent with the ability of both active and inactive Erk2 to interact with nucleoporins that contain FXF motifs (44, 45). Thus, our data suggest that the region around the αF and αG helices and the activation loop can mediate specific interactions of inactive Erks with certain FXF motifs. Structural analysis will be necessary to determine the exact nature of this novel interaction.

However, not every Erk2 mutation affected ERF-Erk association. Alanine mutations of Tyr314 and Tyr315 at the opposite site of the Erk2 protein, which have been shown to mediate interaction with MEK1 (46), and the mutation of Phe226 had no effect on the interaction of active or inactive Erk2 with ERF.4 FXF motifs are regarded as selective interaction motifs for Erk1/2, although an FXF motif has been reported to affect phosphorylation of Sap1 by p38α (12). However, in the case of ErF, the FXF motifs were incapable of supporting association with p38 (Fig. 1). This is consistent with the lack of any indication that p38 and JNK activators or inhibitors affect ERF phosphorylation in vivo (1, 2). Thus, our findings support the hypothesis that FXF motifs are Erk-specific interaction determinants. However, it is clear that the overall structure of the protein harboring the FXF motifs is crucial for proper interaction. This is indicated not only by the inability of the other two FXF motifs of ERF to support high affinity interactions with Erk, but also by peptide inhibition experiments involving the interaction-mediating FXF motifs. Indeed, peptides derived from ErF residues 299–310 and 371–382 did not inhibit ERF-Erk interaction even at a molar excess of 2 orders of magnitude. It is conceivable that these peptides do not assume the proper conformation that would allow their association with Erk. However, our deletion analysis (Fig. 3) suggests considerable contribution of sequences adjacent to the FXF motifs. These regions may influence the overall structure of the protein or provide additional contact surfaces. This was also evident in the in vivo blockade of ERF phosphorylation, in which a 10-fold molar excess of the interaction domains was sufficient to eliminate ERF phosphorylation and to induce ERF nuclear localization (Fig. 9). However, only constructs that included sequences adjacent to the FXF motifs were capable of effectively inhibiting ERF phosphorylation.

The decreased phosphorylation of Erk substrates by increased amounts of the ERF EID indicated that expression of a high affinity domain can regulate Erk function in vivo. However, it did not appear to affect the Erk activation state because phospho-Erk2 was increased 2–3-fold in the presence of the ERF interaction domains (Fig. 9B).4 It is not clear if this increase is a direct effect of the ERF–Erk association to induce conformational changes in Erk or is a result of feedback mechanisms controlling the amount of phospho-Erk (46, 47). The inhibition of substrate phosphorylation appears to be specific to FXF motif-containing Erk substrate because it extends to ternary complex factors. However, Rsk that associate with Erks via D-domain motifs (17) are also inhibited by the overexpression of the ERF EID. It is not clear if this inhibition is due to the block of the D-domain-dependent Rsk-Erk interaction. Rsk are phosphorylated by Erks at multiple sites, and phosphorylation of Thr273 and Ser363 is critical for Rsk activation (48). Interestingly, Ser363 is adjacent to a conserved FSF motif at positions 379–381 and an FYF motif at positions 348–351 that could direct phosphorylation at Ser363. Thus, it is conceivable that the ERF EID-mediated inhibition of Rsk activation involves inhibition of Rsk-Erk association through FXF motifs. In either case, our data suggest that the EID of ERF can serve as a potent inhibitor of the association and phosphorylation of proteins by Erks.

The ERF-Erk interaction that we have identified appears to determine proper Erk regulation by Erks in vivo. Mutants that exhibited decreased association with Erks also exhibited decreased phosphorylation and increased nuclear localization without affecting Erk activation or ErF nuclear export. These mutants induced the ability of ErF to arrest cell cycle progression and to inhibit growth of Ras-transformed NIH-3T3 cells, consistent with its nuclear accumulation. These data suggest that proper association of the two proteins in vivo is required for effective ERF phosphorylation. However, disruption of docking motifs or deletion of a small region did not totally abol-
ish ERF phosphorylation. This was evident by the nuclear export of the proteins during serum induction and the decreased ability of the mutants to inhibit ras transformation of NIH-3T3 cells compared with the ERF m1–7 phosphorylation-deficient mutant (2). These data indicate that the kinase-substrate docking provides quantitative changes rather than absolute regulation of substrate phosphorylation and that the phosphoacceptor sites are still accessible in the absence of proper docking sites. The decreased phosphorylation of the ERF mutants as a result of decreased ERF-Erk association provides a paradigm for the effect that the amplitude of kinase activity may have on regulation. Within a cell, assuming that factors such as subcellular localization, scaffolding proteins, and component concentrations remain constant, the affinity of the kinase for a substrate determines substrate phosphorylation. Thus, the relative affinity of Erk for its different substrates may provide an additional level of regulation. Different levels of Erk activation in the same cell would result in the regulation of a different set of substrates and consequently could induce distinct biological outcomes.

In conclusion, our data indicate that ERF-Erk association determines ERF regulation and function in vivo and suggest that ERF may represent a new class of Erk substrates that can interact specifically but differentially with both active and inactive Erks through FXF motifs. The ERF EID can be used to specifically block Erk activity for a subset of its substrates in vivo and thus provides a new interface for fine-tuning Erk regulation.

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REFERENCES

1. Sgouras, D. N., Athanasiou, M. A., Beal, G. J., Jr., Fisher, R. J., Blair, D. G., and Mavrothalassitis, G. J. (1995) EMBO J. 14, 4781–4793
2. Le Gallic, L., Sgouras, D., Beal, G., Jr., and Mavrothalassitis, G. (1999) Mol. Cell. Biol. 19, 4121–4133
3. Le Gallic, L., Virgilio, L., Cohen, P., Biteau, B., and Mavrothalassitis, G. J. (1995) Cell. Biol. 176, 24613–24621
4. Lee, T., Hoofnagle, A. N., Kabuyama, Y., Stroud, J., Min, X., Goldsmith, E. J., Chen, L., Resing, K. A., and Ahn, N. G. (2004) Mol. Cell 14, 43–55
5. Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, E. J. (1994) Nature 367, 704–711
6. Adachi, M., Fukuda, M., and Nishida, E. (1999) EMBO J. 18, 5347–5358
7. Fukuda, M., Gotoh, I., Adachi, M., Gotoh, Y., and Nishida, E. (1997) J. Biol. Chem. 272, 32642–32648
8. Mol. Cell. Biol. 19, 1319–1324
9. Jacobs, D., Glossip, D., Xing, H., Muslin, A. J., and Kornfeld, K. (1999) Genetics 149, 1809–1822
10. Jacobs, D., Beitel, G. J., Clark, S. G., Horvitz, H. R., and Kornfeld, K. (1998) Nature 392, 605–615
11. Whitehurst, A., Cobb, M. H., and Goldsmith, E. J. (1999) Cell 97, 859–869
12. Stewart, M. (2000) Cell Struct. Funct. 25, 217–225
13. Jones, A., Poteet-Smith, C. E., Malarkey, K., and Sturgill, T. W. (1999) J. Biol. Chem. 274, 2893–2898
14. Volmat, V., Camps, M., Arkinstall, S., Pouyssegur, J., Shaw, J. P., and Arkinstall, S. (2000) J. Biol. Chem. 275, 4613–4621
15. Lee, T., Hoofnagle, A. N., Kabuyama, Y., Stroud, J., Min, X., Goldsmith, E. J., Chen, L., Resing, K. A., and Ahn, N. G. (2004) Mol. Cell 14, 43–55
16. Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, E. J. (1994) Nature 367, 704–711

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