Selective Targeting of MAPKs to the ETS Domain Transcription Factor SAP-1*

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MAPK pathways play important roles in regulating the key cellular processes of proliferation, differentiation, and apoptosis. There are multiple MAPK pathways, which are subject to different regulatory cues. It is important that these pathways maintain specificity in signaling to elicit the activation of a specific program of gene expression. MAPK-docking domains in several transcription factors have been shown to play important roles in determining the specificity and efficiency of their phosphorylation by MAPKs. Here we investigate the mechanisms by which MAPKs are targeted to the ETS domain transcription factor SAP-1. We demonstrate that SAP-1 contains two different domains that are required for its efficient phosphorylation in vitro and activation in vivo by ERK2 and a subset of p38 MAPKs. The D-domain is closely related to other MAPK-docking domains, but exhibits a novel specificity and serves to promote selective targeting of ERK2, p38α, and p38β, to SAP-1. A second important region, the FXF motif, also plays an important role in directing MAPKs to phosphorylate SAP-1. The FXF motif promotes targeting by ERK2 and, to a lesser extent, p38α, but not p38β. Our data therefore demonstrate that a modular system of motifs is responsible for directing specific MAPK subtypes to SAP-1, but also point to important distinctions in the mechanism of action of the D-domain and FXF motif.

Stringent controls are required to permit the transmission of extracellular signals into a specific cellular response. Indeed, multiple mechanisms exist to ensure specificity in cellular signaling (reviewed in Ref. 1). The MAPK* pathways represent a common route through which signals are transmitted into nuclear responses. At least six parallel pathways exist in mammals (reviewed in Ref. 2), the best studied pathways being the ERK, JNK, and p38 pathways. The p38 pathways can themselves be further subdivided into different isoforms (p38α, p38β, p38γ, and p38δ) that are subject to different regulation and that have different substrate specificity (reviewed in Ref. 3; see Refs. 4–10). The MAPK pathways are subject to multiple tiers of regulation (reviewed in Ref. 11), with cytoplasmic scaffolds representing one mechanism by which particular cascades are assembled with the exclusion of components of other related pathways (reviewed in Refs. 12 and 13). Within these scaffolds, defined protein-protein interactions play key roles in specifying the interaction of individual components. Similarly, in the nucleus, the interactions of the kinases with their substrates are regulated by specific docking sites on transcription factors (14, 15). These docking motifs enhance the efficiency and efficacy of substrate phosphorylation by MAPKs.

MAPK-docking sites were initially identified in c-Jun (16, 17) and subsequently in a series of different transcription factors and cytoplasmic substrates (reviewed in Refs. 14 and 15). The docking sites found in transcription factors are typically <20 amino acids long and show limited sequence similarity, but are characterized by a region rich in basic amino acids, followed by either an LXX motif and/or a triplet of hydrophobic amino acids. These docking domains specify substrate phosphorylation by one (e.g. c-Jun-JNK, MEF2A-p38α/p38β) (16–19) or two (e.g. Elk-1-ERK/JNK) (20) different classes of MAPKs. In the case of Elk-1, for example, the D-domain specifies targeting by ERK and JNK MAPKs, but does not appear important for p38 MAPKs (20, 21). Docking domains do, however, exist in other proteins such as MEF2A and MEF2C, which specify targeting by p38 MAPKs (19).

The ETS domain transcription factor SAP-1 belongs to the ternary complex subfamily and is highly related to Elk-1 (reviewed in Ref. 22). SAP-1 contains a domain that exhibits strong sequence similarity to the Elk-1 D-domain. However, it is not known whether this domain functions in an analogous manner as a MAPK-docking domain. SAP-1 has been shown to be able to act as a target of the ERK, JNK, and p38 MAPK families (23–26). A direct comparison of Elk-1 and SAP-1 demonstrated that SAP-1 is preferentially phosphorylated by p38α (25), indicating that specificity determinants exist. As the Elk-1 docking domain does not appear to target p38 MAPKs (20), this difference in kinase selectivity might be determined by the SAP-1 D-domain.

More recently, a second type of MAPK-binding site was identified conforming to the consensus sequence FXF, which plays an important role in ERK-mediated substrate phosphorylation (27, 28). This motif is conserved between SAP-1 and Elk-1 and might also play a role in determining the proficiency of SAP-1 as a MAPK substrate.

To further probe the mechanisms that establish specificity in MAPK signaling, we have analyzed the specificity determinants in SAP-1 that control its proficiency as a MAPK substrate. We demonstrate that the D-domain of SAP-1 acts as a classical docking domain that recruits ERK2 and a subset of...
p38 MAPKs. This domain constitutes part of a MAPK recognition module that also contains an FXF motif. This second binding motif promotes SAP-1 phosphorylation by both the ERK and p38α MAPKs. Our data therefore demonstrate that a complex modular system, consisting of the D-domain and FXF motif, directs specific MAPK subtypes to SAP-1.

**MATERIALS AND METHODS**

**Plasmid Constructions**—The following plasmids were constructed for expressing MBP and GST fusion proteins in *Escherichia coli*. pAS777 (encoding MBP-SAP-1/LD, SAP-1 amino acids 339–430) was derived from pAS1056 (encoding MBP-SAP-1, SAP-1 amino acids 303–431), pAS1056 (encoding MBP-Elk-1, Elk-1 amino acids 310–428), and pAS1057 (encoding MBP-Elk-1/LD, Elk-1 amino acids 330–428) were constructed by inserting a BamHI-HindIII-cleaved polymerase chain reaction-derived fragment into the same sites of pMAL-C2 (New England Biolabs Inc.). pAS1080 (encoding MBP-SAP-1/mF) and pAS1081 (encoding MBP-SAP-1/LD-mF) were derived from pAS1056 and pAS1057, respectively, with site-directed mutations Q399AF400A. pAS1096 (encoding MBP-Elk-1/mF) and pAS1097 (encoding MBP-Elk-1/LD-mF) were derived from pAS1056 and pAS1057, respectively, with site-directed mutations Q399AF397A. The plasmids pAS860 (encoding GST-MEF2A, MEF2A amino acids 266–413), pAS861 (encoding GST-MEF2A2/LD, MEF2A amino acids 283–413), pAS862 (encoding GST-MEF2A2D, MEF2A amino acids 283–413), pAS873 (encoding GST-EKD-MEF2A, Elk-1 amino acids 310–327 and MEF2A amino acids 283–413) were described previously (19). pAS1456 (encoding GST-ElkD-MEF2A-F), pAS1457 (encoding GST-ElkD-MEF2A2D-mF), and pAS1458 (encoding GST-ElkD-MEF2A2D-F) were generated by inserting a BamHI-XbaI-cleaved polymerase chain reaction-derived fragment into the same sites of pAS867, whereas pAS1460 (encoding GST-MEP2A2D-F1), pAS1492 (encoding GST-F-MEF2A2D), and pAS1461 (encoding GST-MEF2A2D-mF) were constructed by inserting a BamHI-XbaI-cleaved polymerase chain reaction-derived fragment into the same sites of pAS860.

The following plasmids were constructed for use in mammalian cell transfections. pG5Elb-luc contains five GAL4 DNA-binding sites cloned upstream of a minimal E1b promoter element and the firefly luciferase gene (29). pSG424 (pSG424-HA) encodes the GAL4 DNA-binding domain (30). pAS1068 (pSG424-New) was derived from pSG424 with the insertion of a new polylinker (oligonucleotides ADS673 and ADS674) to provide a BamHI site whose reading frame is compatible with the MBP system. The plasmids pCMV (pAS188), pCMV-F-p38α (31), pCDNA3-F-p38α, pCDNA3-F-p38β (9), pCDNA3-F-p38δ (8), pCMV-HA-ERK2 (22), pCDNA3-MKK6 (33), pAS900 (pCMV-GAL4-Elk-1), and pAS1351 (pCMV-GAL4-Elk-1D) (19) have been described previously. pAS1069 (GAL4-SAP-1), pAS1070 (GAL4-SAP-1D), pAS1092 (GAL4-SAP-1/mF), pAS1093 (GAL4-SAP-1/LD-mF), pAS1100 (GAL4-Elk-1), and pAS1451 (GAL4-Elk-1D-mF) were constructed by ligating the BamHI-XhoI fragments from pAS785, pAS777, pAS1068, pAS1071 (pCMV-GAL4-SAP-1), pAS1072 (pCMV-GAL4-SAP-1D), pAS1094 (pCMV-GAL4-SAP-1/mF), pAS1095 (pCMV-GAL4-SAP-1D-mF), pAS1452 (pCMV-GAL4-Elk-1-mF), and pAS1453 (pCMV-GAL4-Elk-1D-mF) were constructed by ligating the HindIII-XhoI fragments from pAS1069, pAS1070, pAS1092, pAS1093, pAS1100, and pAS1451, respectively, into the same sites of pCMV5. All plasmid constructs made by polymerase chain reaction were verified by automated dyeideo sequencing.

**Protein Expression and Purification**—The following procedure was used to isolate MBP fusion proteins from 50-mL cultures. Inoculation was carried out according to the manufacturer’s instructions (New England Biolabs Inc.). Cells were harvested, and the pellet was resuspended in 4 ml of column buffer (10 mM Tris-Cl, 200 mM NaCl, and 1 mM EDTA) including 200 µl of 100 mM phenylmethylsulfonyl fluoride (Sigma). Then, the sample was sonicated (3 × 10-s bursts at 14 °C) and spun down at 18,000 × g for 10 min at 4 °C. The supernatant was transferred to a fresh tube containing 400 µl of 50% reduced amyllose resin beads (New England Biolabs Inc.). The sample was gently mixed for 30 min at 4 °C and then pelleted briefly at 1000 × g. The pelleted beads were washed three times with 10 ml of column buffer and resuspended in an equimolar amount of column buffer (200 µl). The MBP fusion protein was eluted by adding 10 µl maltose (Sigma) to the purified protein, followed by incubation for 30 min at 4 °C and removal of the beads by brief centrifugation at 1000 × g. This process was repeated to elute more protein, and the samples were pooled with 20% glycerol and stored at −80 °C. The concentrations of proteins were determined after SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue (Sigma) and by comparison with bovine serum albumin as a standard. Purification of GST fusion proteins was performed as described previously (21).

**RESULTS**

**The SAP-1 D-domain Is Important for Phosphorylation by Specific p38 Isoforms**—SAP-1 is highly related to Elk-1 and exhibits a high degree of sequence similarity within the conserved D-domain (see Fig. 4A). We therefore tested whether the D-domain of SAP-1 plays a role in targeting SAP-1 in vivo and whether any specificity in targeting occurs.

Initially, we constructed fusion proteins consisting of the MBP fused to either the entire C terminus of SAP-1 (MBP-SAP-1) or the same region lacking the D-domain (MBP-SAP-1D) (Fig. 1A). These fusion proteins were subsequently tested as in vitro substrates for ERK and p38 MAPKs (Fig. 1B), and the data were quantified (Fig. 1C). p38δ could efficiently phosphorylate SAP-1 with similar kinetics, irrespective of the presence of the D-domain. In contrast, the efficiency of substrate phosphorylation by ERK2, p38δ, and, to a lesser extent, p38α, was reduced upon deletion of the D-domain. Phosphorylation of SAP-1 by p38γ was inefficient and occurred independently of the presence of the D-domain (data not shown). These data therefore demonstrate that the SAP-1 D-domain plays an important role in determining its proficiency as an ERK2, p38δ, and p38γ substrate, but that it does not play a major role in determining its efficiency of phosphorylation by p38δ in vitro.

**SAP-1 Is Selectively Activated by Specific p38 Isoforms in Vivo**—One consequence of targeting MAPKs to substrates is thought to be the promotion of their specificity of action in vivo. To establish whether the selective targeting of p38α and p38δ substrates reflected by the data on selectivity, we analyzed the activation of a GALA-SAP-1 fusion protein (Fig. 2). p38δ could efficiently activate SAP-1, whereas in comparison, p38γ (0.4-fold) and p38δ (1.8-fold) were poor activators (Fig. 2B). We also compared the activation of SAP-1 by the p38δ, and ERK2 pathways (Fig. 2C). Similar levels of activation of SAP-1 were obtained, although absolute comparisons are difficult, as differences in the activities of the
constitutively active upstream kinases cannot be controlled. However, a good correlation exists between the importance of the D-domain in \textit{in vitro} phosphorylation (Fig. 1) of SAP-1 and the degree of activation \textit{in vivo} by ERK2 and distinct p38 subfamily members.

The SAP-1 D-domain Acts as a MAPK-docking Domain—Peptide competition assays were used to probe the potential roles of short protein sequences as binding sites for protein kinases. The principle behind these assays is that peptides that correspond to docking motifs will competitively bind to the MAPKs and thereby block interactions with docking sites on substrates and hence reduce the efficiency of substrate phosphorylation by the kinase (e.g. Ref. 20). We therefore analyzed the ability of a peptide corresponding to the SAP-1 D-domain (SAPD) (Fig. 3A) to inhibit phosphorylation of SAP-1 by p38 MAPKs. In comparison, we analyzed a peptide corresponding to a known p38-binding motif in MEF2A and a mutant version of this peptide that can no longer inhibit p38-mediated phosphorylation (MEFD and MEFmD, respectively) (19).

Increasing concentrations of the SAPD peptide led to a dose-dependent decrease in phosphorylation of SAP-1 by p38\(_{\alpha}\) and p38\(_{\beta}\)_2, but had no effect on p38\(_{\delta}\) (Fig. 3B, lanes 2–4). Similarly, the MEFD peptide selectively inhibited SAP-1 phosphorylation by p38\(_{\alpha}\) and p38\(_{\beta}\)_2 (Fig. 3B, lanes 5–7), whereas a mutant form of this peptide (MEFmD) did not inhibit phosphorylation by these kinases (lanes 8–10). Thus, like the docking site in...
MEF2, the SAP-1 D-domain acts specifically to bind to p38α and p38β2 MAPK isoforms.

As SAP-1 can also be phosphorylated by ERK MAPK, we tested whether the SAPD peptide acts as an inhibitor of ERK-mediated phosphorylation. The SAPD peptide was compared with the ElkD peptide, which was derived from the same region of Elk-1 and is a known ERK inhibitor (Fig. 4A) (20). Increasing concentrations of the SAPD peptide resulted in a decrease in the ability of ERK2 to phosphorylate SAP-1 (Fig. 4B, lanes 2 and 3). Similarly, the ElkD peptide, but not a mutant version (ElkmD), led to reduced SAP-1 phosphorylation by ERK2 (Fig. 4B, lanes 4–7). These results suggest that the SAP-1 D-domain acts as an ERK2-binding site. To verify this conclusion, we carried out kinase binding assays with either MBP-SAP-1 or MBP-Elk-1 fusion proteins bound to agarose beads. Both MBP-SAP-1 and MBP-Elk-1 fusion proteins bound to agarose beads. Both MBP-Elk-1 and Elk-1 (Fig. 4C, lanes 1 and 3). However, in contrast, ERK2 binding was significantly decreased upon deletion of the D-domain of SAP-1 or Elk-1 (Fig. 4C, lanes 2 and 4). Collectively, these data demonstrate that, in addition to its role in binding to p38α and p38β2 MAPKs, the SAP-1 D-domain also recruits ERK MAPK.

The “FXF Motif” Is Required for Efficient SAP-1 Phosphorylation and Activation by ERK2 and Specific p38 MAPKs—Recently, a novel motif was identified that is required for efficient substrate phosphorylation by MAPKs (27). This motif, which we have termed the FXF motif, is also functionally conserved in Elk-1 and at the sequence level (FQFP) in SAP-1. We therefore investigated whether the FXF motif also plays a role in mediating SAP-1 phosphorylation by ERK2 in vitro. First, we created a series of GAL4 and MBP fusion proteins containing either the intact C termini of Elk-1 and SAP-1 or mutant forms either lacking the D-domain or containing point mutations in the FXF motif (Fig. 5A). These proteins were then tested for their proficiency as in vitro substrates for ERK2 (Fig. 5B) and for activation by the ERK pathway in vivo (Fig. 5C).

In Elk-1, deletion of the D-domain or mutation of the FXF motif led to a decrease in phosphorylation by ERK2 in vitro (Fig. 5B, lanes 2 and 3). Furthermore, the simultaneous disruption of both motifs led to a further drop in ERK2-mediated Elk-1 phosphorylation (Fig. 5B, lane 4). Similarly, individually deleting the D-domain and FXF motif of SAP-1 led to decreases in in vitro phosphorylation (Fig. 5B, lanes 6 and 7), and their
simultaneous deletion led to a further drop in phosphorylation by ERK2 (lane 8).

Transcriptional activation in vivo following activation of the ERK pathway was monitored for each of the mutant proteins. In both Elk-1 and SAP-1, deletion of the D-domain or FXF motif led to a substantial drop in activation by the ERK pathway, and simultaneous disruption of these two motifs resulted in a further reduction (Fig. 5C, right panel, black and white bars, respectively). These results essentially mirror the in vitro situation (Fig. 5C, compare with left panel) and demonstrate the importance of the D-domain and FXF motif in ERK2 targeting to SAP-1 in vivo.

Previously, the FXF motif was concluded to be specifically involved in targeting ERK MAPKs to substrates (27). However, we tested whether the FXF motif is also important in determining the proficiency of SAP-1 as a p38 substrate in vitro and in vivo. In vitro kinase assays demonstrated that, whereas deletion of the D-domain led to a large decrease in SAP-1 phosphorylation by p38α and p38β2, mutation of the FXF motif alone had either a small or virtually no effect on its phosphorylation in vitro by p38α and p38β2, respectively (Fig. 6B). Simultaneous disruption of the D-domain and FXF motif led to a further decrease in the proficiency of SAP-1 as a p38α substrate (Fig. 6B, lane 4). In contrast, phosphorylation of SAP-1 by p38δ was barely affected by disruption of these two regions. These data therefore demonstrate that, in vitro, the FXF motif of SAP-1 plays a selective role in determining its phosphorylation by the p38α isoform, although the D-domain appears to be more important.

We also analyzed the response of the mutant SAP-1 fusion protein to activation by the p38 MAPK pathways in vivo (Fig. 6C). Deletion of the D-domain led to a large decrease in p38α- and p38β2-mediated transactivation. This is consistent with the decreases in in vitro phosphorylation observed with this mutant protein (Figs. 1B and 6B). Mutation of the FXF motif also led to a large decrease in p38α-mediated transactivation, but barely affected p38β2-mediated transactivation (Fig. 6C). Simultaneous disruption of the D-domain and FXF motif in GAL4-SAP-1 ΔD-mF led to a further decrease in p38α-mediated transactivation, but did not lead to any further effects on p38β2-mediated transactivation compared with deletion of the D-domain alone (Fig. 6C). Again, these in vivo effects are fully consistent with the in vitro phosphorylation data (Fig. 6B). Finally, as a control, we tested the response of the SAP-1 derivatives to activation by p38δ in vivo, as neither motif appears to be important for targeting of this kinase in vitro (Fig. 6B). As predicted from this in vitro data, neither deletion of the D-domain nor mutation of the FXF motif, either individually or in combination, led to a significant decrease in p38δ-mediated transactivation (Fig. 6C). In contrast, the activity of the fusion proteins was actually increased in the absence of the D-domain (see “Discussion”).

The D-domain of SAP-1 therefore plays a major role in promoting its phosphorylation in vitro and activation in vivo by p38α and p38β2. However, the FXF motif plays a lesser role, which is only apparent for p38α. Further specificity is implied by the observation that p38δ is not targeted to SAP-1 by these motifs.

The FXF Motif Acts as a Binding Site for Subsets of MAPKs—
The peptide competition assay was used to investigate whether the FXF motif of SAP-1 acts as a MAPK-binding site. First, we tested the ability of a peptide encompassing the SAP-1 FXF motif (SAPF) (Fig. 7A) to inhibit its phosphorylation by ERK2 and p38 isoforms. The SAPF peptide inhibited SAP-1 phosphorylation by both ERK2 and p38α, but did not affect phosphorylation by p38β2 and p38δ (Fig. 7B, lanes 2 and 3). A mutant peptide containing two changes in the conserved phenylalanine residues (Fig. 7A) was unable to inhibit the activity of any of the ERK or p38 MAPKs (Fig. 7B, lanes 4 and 5). Thus, the SAP-1 FXF motif acts as a selective binding site for subsets of MAPKs.

We next compared the ability of peptides encompassing the FXF motif and D-domain to inhibit substrate phosphorylation by ERK2. Again, competition assays were used, but two differ-
acids altered in the mutant peptide (SAPmF) are indicated in
the F motif and the D-domain of SAP-1, respectively. The amino
competitors are presented. The SAPF and SAPD peptides correspond to
protein SAP-1-mF are also indicated. The sequences of the peptide
X 1. The numbers of the altered amino acids within the F
protein fused to MBP. Annotations are as described in the legend to Fig.
lanes 2
the indicated MAPKs for 30 min at 30 °C in the absence ( ) or
presence of competitor peptides (a 500–1000-fold excess over substrate)
B.

MAPK-docking Domains in SAP-1

The above results indicate that peptides containing
F motifs of Elk-1 and
PEptides Containing the FXF Motif Act as Selective MAPK Inhibitors—The above results indicate that peptides containing
the FXF motif act as inhibitors of ERK2 and p38α, but do so in
a substrate-independent manner. One way in which they might
do this is by binding to the kinase and blocking its catalytic
activity, either by a steric or allosteric mechanism (see “Dis-
ussion”). This hypothesis predicts that these peptides will
inhibit phosphorylation of other substrates that lack FXF mo-

The D-domains of
Phosphorylation in Heterologous Contexts—The D-domains of
Elk-1 and MEF2A are p38β substrates (19, 20). To probe whether the FXF motifs of Elk-1 and
SAP-1 can function in an analogous manner, we created a
series of fusions with MEF2A (Fig. 9A) and tested them as
substrates for ERK2 and p38 isoforms.

MEF2A is a p38α and p38β substrate (19), and its D-domain
was required for efficient phosphorylation (Fig. 9B, compare

A

FIG. 7. The FXF motif peptides function as inhibitors of ERK2
and p38α activity. A, diagram showing truncated forms of SAP-1
protein fused to MBP. Annotations are as described in the legend to Fig.
1. The numbers of the altered amino acids within the FXF motif of the
protein SAP-1-mF are also indicated. The sequences of the peptide
competitors are presented. The SAPF and SAPD peptides correspond to
the FXF motif and the D-domain of SAP-1, respectively. The amino
acids altered in the mutant peptide (SAPmF) are indicated in boldface.
B, peptide competition assays for phosphorylation of MBP-SAP-1 fusion
protein by the indicated MAPKs. The protein was phosphorylated by
the indicated MAPKs for 30 min at 30 °C in the absence (lane 1) or
presence of competitor peptides (a 500–1000-fold excess over substrate)
at 2.5 nmol (lanes 2 and 4) and 5 nmol (lanes 3 and 5). C, competition
assays were performed as described for B using 5 pmol of SAP-1, D
and SAP-1-D-mF as substrates. The proteins were phosphorylated by
ERK2 in the absence (lanes 1, 4, 7, and 10) or presence of the SAPD
(upper panel) or SAPF (lower panel) competitor peptide (a 500–1000-
fold excess over substrate) at 2.5 nmol (lanes 2, 5, 8, and 11) and 5 nmol
(lanes 3, 6, 9, and 12). Increases in the concentration of added peptides
are indicated schematically above each set of lanes.

motif (Fig. 7C, lanes 7–12).

Collectively, these results demonstrate that the D-domain
and FXF motif play important roles in mediating selective
substrate phosphorylation by MAPKs. However, peptide com-
petition assays indicate that these motifs might function
differently.
lanes 8 and 15). In contrast, MEF2A was a poor ERK2 substrate (Fig. 9B, lane 1), possibly reflecting the absence of an FXF motif. However, upon fusion of an FXF motif to MEF2A, MEF2A became a good ERK2 substrate. In contrast, fusion of a mutated FXF motif did not promote MEF2A phosphorylation by ERK2 (Fig. 9B, compare lanes 2 and 3). Phosphorylation by p38α and p38β2 was virtually unaffected by the presence of the FXF motif (Fig. 9B, lanes 9, 10, 16, and 17), presumably reflecting the dominance of the MEF2A D-domain in promoting phosphorylation by these kinases. The effect of adding the FXF motif to MEF2A in the absence of its own D-domain was therefore tested (Fig. 9B, lanes 5, 12, 19, and 26). The presence of the FXF motif strongly enhanced MEF2A phosphorylation by ERK2 and slightly enhanced phosphorylation by p38α, but did not affect its phosphorylation by p38β2.

As the Elk-1 D-domain can also target ERK2 to substrates (21, 27), we also tested whether it can lead to enhanced MEF2A phosphorylation by ERK2. However, in contrast to the effect of the FXF motif, little enhancement of phosphorylation by ERK2 was observed upon fusion of the Elk-1 D-domain to MEF2A (Fig. 9B, lane 6) (21). Similarly, inclusion of the Elk-1 D-domain did not strongly enhance MEF2A phosphorylation by p38α and p38β2. In combination with the FXF motif, no further enhancement of MEF2A phosphorylation by ERK2 was observed (Fig. 9B, compare lanes 5 and 7), and MEF2A remained a poor substrate for p38α and p38β2 when its D-domain was replaced with that of Elk-1. Together, these results are consistent with the observation that the Elk-1 D-domain does not represent a p38-binding motif and therefore cannot functionally replace the p38-binding motif in MEF2A. This is in contrast to the observation that the SAP-1 D-domain can fulfill this function as a p38-binding site (19). Finally, to confirm the specificity of the effects we observed, phosphorylation of each of the chimeric MEF2A proteins by p38d was compared. All the chimeric proteins were phosphorylated to similar extents by p38d (Fig. 9B, lanes 22–28), in keeping with the observation that the Elk-1 and MEF2A D-domains and the FXF motif do not represent p38β2-binding sites.

To assess whether the FXF motif is sufficient for ERK2 binding in these heterologous contexts, we carried out kinase binding assays. In these assays, MBP-SAP-1 was added as a substrate following the binding reaction with GST-MEF2 derivatives. MEF2A was unable to bind to ERK2 (Fig. 9C, lane 1). However, the introduction of an FXF motif promoted binding of ERK2, whereas a mutant version of this motif was unable to impart this activity (Fig. 9C, lanes 2 and 3). Thus, the FXF motif is sufficient to promote ERK2 binding to a heterologous substrate.

Collectively, these data show that the FXF motif can act in
an independent manner as a portable motif to impart differences in the proficiency of heterologous proteins as ERK2 substrates. By contrast, the Elk-1 D-domain is insufficient to promote MEF2A phosphorylation by ERK2, although it can promote its phosphorylation by other MAPKs (19).

The FXF Motif Acts in a Position-dependent Manner—The FXF motif in LIN-1, Elk-1, and SAP-1 is located downstream from the key phosphoacceptor motifs, suggesting that this spatial arrangement might be critical. We therefore introduced an FXF motif into MEF2A upstream from the phosphoacceptor motifs (F-MEF2AΔD) (Fig. 10A) and compared its proficiency as an ERK substrate with MEF2AΔD-F, in which the FXF motif is located downstream from the phosphoacceptor motifs. Whereas MEF2AΔD-F represented a good ERK2 substrate, F-MEF2AΔD was a poor substrate (Fig. 10B, lanes 2 and 3). Thus, although the FXF motif is sufficient for recruiting ERK2 to substrates, this is not sufficient to promote their phosphorylation, and the correct spatial arrangement with the phosphoacceptor motifs is required.

DISCUSSION

Specificity in cellular signaling is maintained by multiple mechanisms that permit specific responses to be elicited in response to activation of individual pathways (reviewed in Ref. 1). The MAPK signaling pathways are subject to multiple levels of regulation. One such regulatory event occurs in the nucleus, where the interactions of the MAPKs with their substrates are regulated by specific docking sites on transcription factors (reviewed in Refs. 14 and 15). These docking motifs enhance the efficiency and efficacy of substrate phosphorylation by MAPKs. Here we have identified a module, composed of two different motifs, that determines the proficiency of SAP-1 as a substrate for specific MAPK subtypes. One component of this module, the D-domain, plays a key role in determining phosphorylation by ERK2, p38α, and p38β. A second component, the FXF motif, also plays a role in directing phosphorylation by ERK2 and, to a lesser extent, p38α, although this motif appears to be unimportant for p38β. However, the relative contribution of each motif to determining the specificity of SAP-1 as a MAPK substrate differs, and their mechanisms of action also appear to differ.

MAPK-docking domains have been identified that specifically direct substrate phosphorylation by one or more MAPKs. For example, the δ-domain of c-Jun specifically binds to the JNK MAPKs (16, 17), whereas the docking domain of Elk-1 binds both the JNK and ERK MAPKs (20). Here we demonstrate that the D-domain of SAP-1 exhibits a novel specificity for MAPKs and is important for phosphorylation by ERK and a subset of p38 MAPKs. Interestingly, the SAP-1 and Elk-1 D-domains are highly conserved (see Fig. 4A), which is consistent with their ability to act as ERK-docking sites. However, differences in the amino acid sequences of these domains must be responsible for determining the ability of the SAP-1 D-domain, but not the Elk-1 D-domain, to act as a p38-binding site. Other MAPKs such as p38δ are not targeted by this motif. Indeed, deletion of the D-domain of SAP-1 leads to enhanced transactivation by p38δ in vivo. This might reflect that the D-domain is also the target of a negatively acting factor in vivo, whose effect is relieved upon deletion of this domain. One attractive candidate for such an inhibitory protein would be a protein phosphatase.

The FXF motif is highly conserved between SAP-1 and Elk-1 (see Fig. 8A) and in the Caenorhabditis elegans protein LIN-1 (27). Previously, it was thought that the FXF motif acted specifically to promote substrate phosphorylation by ERK MAPKs, hence the name DEF (docking site for ERK, FXF) for this motif (27). However, here we demonstrate that the FXF motif also plays a role in promoting substrate phosphorylation by p38α MAPKs. Importantly though, this motif does not appear to be able to function on its own with respect to p38 MAPKs and needs the presence of an additional docking domain to promote kinase binding and substrate phosphorylation. With ERK, however, the FXF motif is sufficient to promote substrate phosphorylation (Fig. 9). Indeed, these studies with chimeric proteins demonstrate that, like the D-domain, the FXF motif is...
portable and sufficient to promote phosphorylation of heterologous substrates. It appears, however, that the FXF motif exhibits quite stringent kinase selectivity, as it is unable to promote substrate phosphorylation by JNK and other p38 isoforms, and peptides containing FXF motifs inhibit only a subset of MAPKs.

An important question is whether the FXF motif and D-domains are functionally interchangeable. Although it is clear that both the D-domains and FXF motif can bind to MAPKs, the results of peptide competition assays suggest that they may function in a different manner. Whereas the D-domain acts as a classical competitive inhibitor for binding to substrates containing a D-domain, peptides containing the FXF motif inhibit substrate phosphorylation, irrespective of the presence of the FXF motif in the substrate (Figs. 7 and 8) (27). This suggests that the two motifs bind to different parts of the kinases. Indeed, due to the lack of sequence similarity, it is unlikely that they bind to the same region of the protein kinases. Thus, at least two regions exist on kinases for substrate binding, one of which has recently been identified that apparently binds to the basic regions of docking sites related to the D-domains (34). The FXF motif might lie adjacent to this or, alternatively, be completely separate. Interestingly, inhibitory peptides bind to a hydrophobic groove in protein kinase A, which is located adjacent to its catalytic site and permits insertion of a extended region containing the phosphoacceptor motif into the active site (35). It is tempting to speculate that the FXF motifs might bind to the MAPKs in an analogous manner to substrate binding to protein kinase A. As FXF motifs are hydrophobic in nature and are located close to the phosphoacceptor motifs, this mode of action is a distinct possibility. Such a mode of action would explain the inhibitory effects of the FXF motif, as binding of the kinase by the peptide would likely obstruct access to the active site. Furthermore, in support of this hypothesis, although the FXF motif is sufficient to promote substrate phosphorylation, it does so only in a position-dependent manner (Fig. 10), suggesting that the correct juxtapositioning of this motif relative to the phosphoacceptor sites is required. Future structural and mutagenic studies are required, however, to permit the identification of this binding site on MAPKs.

Our results point to the existence of a complex MAPK recognition module in SAP-1. This module exhibits several novel features in comparison with known modules and demonstrates how individual components can act either alone or in concert to determine specificity in substrate phosphorylation by MAPKs.

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