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 LXL motif and/or a triplet of hydrophilic 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

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¶ The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MBP, maltose-binding protein; GST, glutathione S-transferase; MKK, MAPK/ERK kinase kinase; MEK, MAPK/ERK kinase.

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MAPK docking Domains in SAP-1

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) was cotransfected with vectors encoding various GAL4 DNA-binding domain fusion proteins as described previously (21). The activities of the GAL4 fusion proteins were measured in cotransfection assays in COS-7 cells using 1 μg of the reporter plasmid pG5E1b-luc and, where indicated, 250 ng of vectors encoding MAPK and constitutively activated MAPK. Luciferase assays were carried out using the Tropix dual light system, and transfection efficiencies were monitored as described previously (19).

Protein Kinase Assays—Recombinant active p38α, p38β, p38γ, and p38δ were prepared from transfected COS-7 cells as described above. Recombinant active ERK2 was obtained from New England Biolabs Inc. Reactions were performed as described previously (21). The phosphorylation of substrate proteins was examined after SDS-polyacrylamide gel electrophoresis and quantified by phosphorimaging (Fuji BAS1500 phosphorimager and Tina Version 2.08e software). Peptide competition assays were performed essentially as described previously (21). However, no preincubation of the peptides with the kinases was performed. Protein kinase assays and kinase binding assays were carried out as described previously (21).

Data Analysis and Presentation—Figures were generated electronically using Picture Publisher (Micrografx) or Adobe Photoshop Version 5.5 and Powerpoint Version 7.0 (Microsoft) software. Data from Western blots are computer-generated images (FluorS Max and Quantity One, Bio-Rad). Phosphorimager data from kinase assays were quantified using either Tina Version 2.08e software or Quantity One.

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 MAPK targeting as observed in Elk-1 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-1ΔD) (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, p38γ, and, to a lesser extent, p38α, was reduced upon deletion of the D-domain. Phosphorylation of SAP-1 by p38δ 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 phosphorylating efficiency as an ERK2, p38α, and p38δ 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 p38β is reflected in vivo, we analyzed the activation of a GAL4-SAP-1 fusion protein (Fig. 2). We examined the effect of p38 isoforms in vivo. p38δ (33-fold) and, to a lesser extent, p38α, 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 in vitro phosphorylation (Fig. 1) and the degree of activation 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 p38α and p38β₂, but had no effect on p38δ (Fig. 3B, lanes 2–4). Similarly, the MEFD peptide selectively inhibited SAP-1 phosphorylation by p38α and p38β₂ (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 p38a and p38β2 MAPK isozymes.

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 known to inhibit ERK (Fig. 4A, lanes 1 and 3). 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 motif. To verify this conclusion, we carried out kinase binding assays with various MBP-SAP-1 or MBP-Elk-1 fusion proteins bound to agarose beads. Both MBP-SAP-1 and MBP-Elk-1 fusion proteins were able to retain ERK2 on the beads (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 1 and 3). Collectively, these data demonstrate that, in addition to its role in binding to p38a 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. The reactions were performed for 30 min at 30 °C with equimolar concentrations of all proteins (5 pmol) as substrates. Quantification of in vitro phosphorylation data from B (left panel) and in vivo reporter gene activity (right panel). COS-7 cells were cotransfected with expression vectors encoding various GAL4-SAP-1 or GAL4-Elk-1 derivatives, a GAL4-driven luciferase reporter plasmid, and a constitutively active form of MEK and ERK2. The data presented were calculated as described in the legend to Fig. 2B, WT, wild type.

FIG. 4. The SAP-1 D-domain acts as a binding site for ERK2. A, diagrammatic representation of the substrates and alignment of the sequences of the peptides used as competitors in the kinase assays. The SAPD peptide corresponds to the D-domain of SAP-1, whereas the ElkD peptide encompasses the Elk-1 D-domain (20). Annotations to the peptides are as described in the legend to Fig. 3A. B, peptide competition assays for phosphorylation of MBP-SAP-1 and MBP-Elk-1 fusion proteins by ERK2. Phosphorylation of the proteins by ERK2 was performed in the absence (lane 1) or presence of competitor peptides (a 500–1000-fold excess over substrate) at 2.5 nmol (lanes 2, 4, and 6) and 5 nmol (lanes 3, 5, and 7). The reactions were performed as described in the legend to Fig. 3. ND represents assays that were not performed. C, kinase binding assays for ERK2 with MBP-SAP-1 and MBP-Elk-1 fusion proteins. The proteins (50 pmol) were incubated with ERK2 (25 units) in kinase binding buffer for 4 h at 4 °C. Following extensive washing, the remaining kinase-substrate complexes were used in kinase assays that were performed at 30 °C for 90 min.

FIG. 5. Differential requirement of the D-domain and FXF motif for SAP-1 phosphorylation in vitro and transcriptional activation in vivo by ERK2. A, schematic illustration of truncated SAP-1 proteins fused to either MBP or the GAL4 DNA-binding domain. Domains in SAP-1 (see Fig. 1) and the alanine substitutions within the FXF motif of the proteins SAP-1-mF and SAP-1-D-mF are indicated. B, phosphorylation of the indicated MBP-Elk-1 (lanes 1–4) and MBP-SAP-1 (lanes 5–8) fusion proteins in vitro by ERK2. Kinase assays were performed for 30 min at 30 °C with equimolar concentration of all proteins (5 pmol) as substrates. C, quantification of in vitro phosphorylation data from B (left panel) and in vivo reporter gene activity (right panel). COS-7 cells were cotransfected with expression vectors encoding various GAL4-SAP-1 or GAL4-Elk-1 derivatives, a GAL4-driven luciferase reporter plasmid, and a constitutively active form of MEK and ERK2. The data presented were calculated as described in the legend to Fig. 2B, WT, wild type.
MAPK-docking Domains in SAP-1

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 p38β 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-D-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 p38β in vivo, as neither motif appears to be important for targeting of this kinase in vivo (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 p38β2-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 p38β 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-
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 protein SAP-1-mF are also indicated. The amino acids altered in the mutant peptide (SAPmF) are indicated in the FxF motif and the D-domain of SAP-1, respectively. The serum competitors are presented. The SAPF and SAPD peptides correspond to protein SAP-1-mF are also indicated. The sequences of the peptide

Collectively, these results demonstrate that the D-domain and FxF motif play important roles in mediating selective substrate phosphorylation by MAPKs. However, peptide competition assays indicate that these motifs might function differently.

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 "Discussion"). This hypothesis predicts that these peptides will inhibit phosphorylation of other substrates that lack FxF motifs. Indeed, both the SAPF and ElkF peptides inhibited phosphorylation of myelin basic protein by ERK2 with a similar potency to their ability to inhibit Elk-1 phosphorylation (Fig. 8B). Furthermore, as the ElkF peptide was unable to inhibit the activity of either the JNK or p38β MAPKs (Fig. 8B), their inhibitory effect appears to be kinase-specific. Finally, the SAPF peptide could inhibit phosphorylation of MEF2C by p38α, despite the lack of an FxF motif in this substrate (Fig. 8B). Thus, peptides containing FxF motifs show selectivity in their inhibition of protein kinases (ERK2 and p38α), but act in a substrate-independent manner.

The FxF Motif Promotes Kinase Binding and Substrate Phosphorylation in Heterologous Contexts—The D-domains of Elk-1 and MEF2A/C can act in heterologous contexts to enhance substrate phosphorylation when fused to different proteins (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 p38α isoforms.

MEF2A is a p38α and p38β2 substrate (19), and its D-domain was required for efficient phosphorylation (Fig. 9B, compare...
lanes 8 and 15 and lanes 11 and 18). 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 p38β 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 p38β.

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 p38β. 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 p38β 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 motif (19). Finally, to confirm the specificity of the effects we observed, phosphorylation of each of the chimeric MEF2A proteins by p38δ was compared. All the chimeric proteins were phosphorylated to similar extents by p38δ (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 p38δ-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
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 MEF2AΔΔ-F, in which the FXF motif is located downstream from the phosphoacceptor motifs. Whereas MEF2AΔΔ-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 rule in determining phosphorylation by ERK2, p38α, and p38αβ. 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 p38β2. 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 an 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 juxtaposition 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 (Fig. 11A) composed of at least three determinants: a D-domain, a transcriptional activation domain that contains the phosphoacceptor motifs, and an FXF motif. A similar module has previously been proposed for LIN-1 and Elk-1 (27). A comparison of Elk-1 and SAP-1 demonstrates how the combination of domains can determine their efficiency as MAPK substrates (Fig. 11B). For Elk-1, the D-domain alone promotes phosphorylation by JNK, whereas ERK phosphorylation is promoted by a combination of the D-domain and FXF motif. Similarly, phosphorylation of SAP-1 by ERK is promoted by a combination of the D-domain and FXF motif. However, whereas p38α is influenced by the FXF motif, the D-domain is sufficient to permit phosphorylation by p38β. Thus, by using a combination of motifs and by altering the sequence of the D-domains, differences in the specificity of MAPK phosphorylation can be elicited.

It is clear that the D-domain and FXF motif can act in concert to promote substrate phosphorylation with high specificity. However, mechanistically, they may act differently. For example, in proteins that contain composite regulatory modules, the D-domains might initially recruit the kinases to the substrate, and the FXF motifs then act as a further filter to determine whether the kinase becomes locked onto the phosphoacceptor motifs or not. Other proteins that lack additional docking domains might require these to be provided in trans for the initial recruitment phase. Further work is required, however, to substantiate this model and to determine the precise functions of the D-domains and FXF motifs.

In summary, we have identified 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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