Specificity Determinants in MAPK Signaling to Transcription Factors*

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One critical component in determining the specificity, fidelity, and efficiency of MAPK substrate phosphorylation is the presence of distinct docking domains in the substrate proteins. These docking domains are found in a range of substrates, including the transcription factors myocyte enhancer factor-2A and SAP-1. However, the sequences of these motifs differ, as do their targeting preferences by MAPKs, with SAP-1 being targeted by both the ERK and p38 isoforms, whereas myocyte enhancer factor-2A is targeted only by certain members of the p38 subfamily. Here, we have investigated the specificity determinants within these motifs and generated a model for how specificity is generated. We demonstrate that residues throughout the docking domains play important roles in the recognition process. However, residues located at different positions are important for discriminating between ERK and p38 MAPKs. Furthermore, the docking domains can be further subdivided into submotifs, which are differentially required for phosphorylation by ERK or p38 MAPKs. We have used loss- and gain-of-function mutagenesis to identify residues that discriminate between ERK and p38 MAPKs, residues that act to promote suboptimal interactions, and regions that are differentially required depending on the kinase involved. A model is proposed to explain how specificity is generated within these short docking domains.

Organisms as diverse as yeast and humans utilize MAPK pathways as a common route through which signals are transmitted into nuclear responses. Several parallel pathways exist in mammals, with the best studied pathways being the ERK, JNK, and p38 pathways (reviewed in Refs. 1 and 2). The MAPK pathways are regulated at multiple levels to ensure the fidelity and specificity of their action (reviewed in Ref. 3). A critical aspect of this regulation is the recognition and subsequent phosphorylation of the correct substrates for the terminal MAPKs. A rapidly emerging paradigm is that a large part of the recognition process involves the binding of MAPKs to docking sites in their substrates (reviewed in Refs. 4 and 5). These docking sites act to enhance the efficiency of phosphorylation, but also impart specificity, as each docking site is recognized by only a subset of MAPKs.

The docking sites found in transcription factors (referred to here as D-domains) are typically <20 amino acids long and show limited sequence similarity, but are characterized by a region rich in basic amino acids, followed by an LXL motif and/or a triplet of hydrophobic amino acids. In addition, the D-domains are highly portable and in many cases are sufficient to confer signaling specificity on a heterologous protein toward a particular pathway. In the case of the ETS domain proteins Elk-1 and SAP-1, these docking domains specify targeting and substrate phosphorylation by two different classes of MAPKs. A single D-domain is responsible for targeting of two different kinases to Elk-1 and SAP-1. Elk-1 is targeted by the ERK and JNK kinases (6, 7), whereas ERK and p38 kinases are targeted to SAP-1 (8). However, two different D-domains target the JNK and ERK MAPKs to the related protein SAP-2 (9). In contrast, the MADS box transcription factor MECP2 is targeted through its D-domain by just one class of MAPK, the p38 kinases p38α and p38β (10). In some substrates, an additional motif known as the FXF motif functionally cooperates with the docking domains to specify phosphorylation by members of the ERK and p38 subfamilies (8, 11, 12).

Recently, two regions have been identified on the surfaces of MAPKs that act as reciprocal docking sites for several substrates (13, 14). Both these regions are centered on negatively charged clusters of residues, named the CD and ED motifs. The first of these motifs (CD) is conserved among all MAPKs, whereas the second (ED) is specific to the p38 subclass and is thought to specify the recognition of the highly basic docking domains found in its substrates. However, it is currently unknown whether one or both of these motifs are important in determining transcription factor substrate recognition.

It is currently unclear how specificity is generated in the docking domains for particular subsets of MAPKs. In this study, we have focused on the docking domains found in the transcription factors MECP2, SAP-1, and Elk-1. The differing responses of these proteins to alternative MAPK cascades and similarities in their docking domain sequences make these ideal model systems. We demonstrate that the docking domains consist of a series of submotifs that are differentially important for phosphorylation by individual MAPKs. Significantly, we identify critical positions within the domains that

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The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEF2, myocyte enhancer factor-2; MBP, maltose-binding protein; GST, glutathione S-transferase; MKK, mitogen-activated protein kinase kinase; SAP-1, SRF accessory protein 1.
confer changes in specificity and also the strength of binding and hence phosphorylation efficiency.

MATERIALS AND METHODS

Plasmid Constructs—The following plasmids were used for expressing MBP and GST fusion proteins in Escherichia coli. GST-MEF2A-(266–413) fusion proteins with mutations S268A (pAS1664), R283A (pAS1665), R270A (pAS1666), P271A (pAS1667), D272A (pAS1668), L273A (pAS1669), L273E (pAS1670), and D272A (pAS1668) were constructed by inserting BamHI-EcoRI-cleaved PCR-derived fragments into the same sites of pGEX-3X (Amersham Biosciences, Inc.). GST-MEF2A fusion proteins with mutations R274A (pAS1670), V275A (pAS1671), L276A (pAS1672), I277A (pAS1673), and P279A (pAS1674) were constructed by inserting BglII/EcoRI-cleaved PCR-derived fragments into the same sites of pAS860. The GST-MEF2A (pGL) clone (pAS1680) was constructed using mutagenic primers with additional GOGCCCT nucleotides encoding glycine and leucine inserted after nucleotides encoding Leu273 and cloning the 293 cells grown in six-well plates and lysed in 1 ml of Triton lysis buffer (6) using 4 μl of anti-FLAG antibody prebound to 100 μl of 50% protein G beads (Amersham Biosciences, Inc.). Proteins were bound to the beads for 4 h at 4 °C and washed five times with Triton lysis buffer once with kinase buffer (25 mM HEPES (pH 7.5), 25 mM β-glycerophosphate, and 25 mM MgCl2). Purified kinases were eluted from protein G beads by competing with 0.1 mg/ml FLAG peptide.

Tissue Culture, Cell Transfection, and Reporter Assays—293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). Transfections were performed using Superfect transfection reagent (QiAGEN Inc.) as described previously (6, 7). For reporter gene assays, a luciferase reporter construct controlled by a GAL4-driven promoter (pG5E1bLUC; 1 μg) was cotransfected with pCMV promoter-driven vectors encoding various GAL4-MEF2A fusion proteins (50 ng), and where indicated, 250 ng of p38β2 and MKK6(E) were transfected.

Phospho-Protein Assay Efficiency—To normalize by cotransfecting the pCH110 plasmid (Amersham Biosciences, Inc.) in β-galactosidase activity was measured. Cell extracts were prepared, and luciferase and β-galactosidase assays were performed as described previously (6, 7).

Protein Kinase Assays—Recombinant active p38α, p38β2, p38γ, and p38ε were prepared from transfected COS-7 cells as described above. Recombinant active ERK2 was obtained from New England Biolabs, Inc. Protein kinase assays were carried out essentially as described previously (7) in 20-μl reaction volumes containing 5 pmol of GST and MBP fusion proteins as a substrate, 1–2 μl of kinase, 50 μM ATP, and 0.083 μM [γ-32P]ATP (6000 Ci/mmol) in kinase buffer for 15–30 min. The phosphorylation of substrate proteins was examined by autoradiography following SDS-PAGE and quantified by phosphorimaging (7). Kinase binding assays were carried out as described previously (8).

Western Blot Analysis—GAL4 fusion proteins were detected in total 293 cell extracts using anti-GAL4 antibody against the amino-terminal DNA-binding domain (Santa Cruz Biotechnology). Immunocomplexes were detected using horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence (Pierce).

Figure Generation and Data Quantification—Figures were generated electronically using Adobe Photodeluxe Business Edition Version 1.0, Adobe Photoshop Version 5.5, and Powerpoint Version 7.0 (Microsof). Data from western blots were quantitated using FluorS Max and Quantity One (Bio-Rad). PhosphorImager data from kinase assays were quantified either using NIH software (Version 2.08e, Fuji) or Quantity One (Bio-Rad).

RESULTS

Alanine Scanning Reveals Residues in the MEF2A D-domain Required for Targeting of p38 MAPK—MEF2A is specifically phosphorylated by a subset of p38 MAPKs and does not represent an ERK target (10, 21, 22). A docking domain consisting of residues 266–282 in MEF2A has previously been identified and shown to specify substrate phosphorylation by p38α and p38β2. Three sets of double alanine mutations were generated in this domain, and all caused a decrease in the proficiency of MEF2A as a p38α and p38β2 substrate (10).

To investigate the contributions of individual residues within the MEF2A docking domain to targeting by p38 MAPKs, a series of single alanine mutations were introduced at each position in the docking domain (Fig. 1A). These mutant proteins were first tested as substrates for phosphorylation in vitro by p38α (Fig. 1B) and p38β2 (Fig. 1C) MAPKs. The effects of individual mutations on phosphorylation by p38α and p38β2 were virtually identical (Fig. 1, compare B and C). None of the mutations caused reductions as severe as deleting the whole docking domain. However, decreases in the proficiency of phosphorylation were apparent at all positions, except Ser288 and Asp272, which exhibited >90% of wild-type levels. Interestingly, the D272A mutant was a consistently better substrate for p38β2 than the wild-type protein. Moderate decreases were observed upon mutations of basic residues toward the N-terminal end of the domain (Arg269 and Lys270), and more severe reductions were observed in two residues in a hydrophobic triplet located toward the C-terminal end of the domain (Val275 and Ile277). Reduced phosphorylation was also observed upon mutation of residues in the intervening region (Leu273 and Arg274). Thus, although both the basic and hydrophobic clus-
MAPK Docking Site Determinants

FIG. 1. Identification of residues in the MEF2A D-domain required for targeting of p38 MAPK. A, shown is a diagram illustrating the domain structure of full-length MEF2A and the truncated MEF2A protein (MEF2A-(266–413)) fused to GST. The DNA-binding domain (DBD), kinase docking domain (black box), minimal transcriptional activation domain (TAD) (gray box), and p38 phosphorylation sites (Thr204 and Thr211) are indicated. The sequence of the D-domain and the numbers of N- and C-terminal amino acids of each domain are shown. Residues composing the basic and hydrophobic submotifs are bracketed. B and C, kinase assays of wild-type MEF2A (WT) and a series of GST-MEF2A alanine mutant fusion proteins using p38a MAPK (B) and p38β MAPK (C) were performed for 15 min at 30 °C with 5 pmol of GST-MEF2A as a substrate. Shown are graphic representations of phosphorylation levels (means ± S.E., n = 3). The residues mutated to alanines are indicated. The intensity of phosphorylation was standardized to that of the wild-type protein (100%), which is represented by the upper dashed line. The level of phosphorylation of MEF2AΔD (GST-MEF2A-(283–413)) is shown by the lower dashed line. Representative kinase assays are shown below the graphs.

Mutation Analysis of the D-domain reveals that the hydrophobic region of the D-domain is critical for targeting p38 MAPK. The D-domain of MEF2A is required for p38-mediated phosphorylation of MEF2A, other residues are clearly also important.

Next, we investigated the functional consequences of introducing mutations into the MEF2A D-domain by testing the response of GAL4 fusions with the MEF2A transcriptional activation domain to activation of the p38 MAPK pathway in vivo (Fig. 2, A and B). Little difference was observed in the expression levels of each of the GAL-MEF2A fusion proteins (Fig. 2C). In the presence of active p38β, a 14-fold stimulation of wild-type MEF2A was observed, whereas deletion of the docking domain reduced this stimulation to ~3-fold. The effect of individual alanine mutations on p38β-mediated transcriptional activation closely mirrored that observed in in vitro phosphorylation reactions (compare Figs. 2B and 1C). Again, the D272A mutant exhibited enhanced inducibility. The rest of the mutations, including residues from the basic and hydrophobic clusters and the intervening region, resulted in decreases in p38β-mediated activation. It appears, however, that these mutations are generally more severe in vivo, with inducibility being reduced to around the level of complete D-domain deletion, e.g., Lys270, Leu273, Val275, and Ile277). Consistent with the in vitro data, Val275 and Ile277 showed the largest decreases in response to p38β.

Collectively, these data point to residues throughout the MEF2A D-domain as being important for p38 MAPK targeting in vitro and in vivo. Particularly important residues appear to be located within a hydrophobic triplet at the C-terminal end of the domain.

Alanine Scanning Reveals Residues in the SAP-1 D-domain Required for Targeting of p38 and ERK MAPKs—SAP-1 is specifically phosphorylated by a subset of p38 MAPKs and, in contrast to MEF2A, also represents an ERK target (8). Thus, differences between the docking domains of MEF2A and SAP-1 must determine that MEF2A is an ERK substrate or confer extra properties on SAP-1 that permit it to be an ERK substrate.

To investigate the contributions of individual residues within the SAP-1 docking domain to targeting by p38 and ERK MAPKs, a series of single alanine mutations were introduced at each position in the docking domain (Fig. 3A). These mutant proteins were then tested as substrates for phosphorylation in vitro. The typical properties on SAP-1 that permit it to be an ERK substrate.ERS.

MAPK Docking Site Determinants

FIG. 2. p38β- MAPK-inducible transcriptional activation by wild-type MEF2A and GAL4-MEF2A mutant fusion proteins. A, shown is a diagram illustrating MEF2A proteins fused to the GAL4 DNA-binding domain. The D-domain structure and phosphorylation sites are indicated. TAD, transcriptional activation domain. Annotation is as described in the legend to Fig. 1. B, 293 cells were cotransfected with expression vectors encoding GAL4 fusions to either wild-type MEF2A (WT) or MEF2A D-domain mutant derivatives, vectors encoding MKK6(E) and p38β, and GAL4-driven luciferase reporter plasmid. The luciferase activities were standardized to β-galactosidase activity and are presented as -fold increases in transcriptional activity when p38β MAPK was cotransfected relative to the levels without cotransfected p38β (means ± S.E., n = 3). Lower and upper dashed lines indicate -fold increases in transcriptional activity by wild-type MEF2A-(266–413) and MEF2A-(283–413) (MEF2AΔD) mutants, respectively. C, expression levels of the GAL4 fusion proteins in the absence and presence of transfected p38β and MKK6(E) were examined by Western blotting with anti-GAL4 antibody. *, the levels of the Pro271 and Pro278 mutants were analyzed in a separate experiment and are equivalent to those of the wild-type protein.
phosphorylation of the intervening residues Pro\textsuperscript{329} and Thr\textsuperscript{330} was severely affected. Moderate effects on phosphorylation by p38\textsubscript{β2} and ERK2 were observed upon mutation of most of the basic residues. Ser\textsuperscript{319}, Gly\textsuperscript{324}, Gly\textsuperscript{326}, and Thr\textsuperscript{334} showed consistently low (or zero) decreases in phosphorylation by both kinases. However, there were a couple of key differences observed; in comparison with other basic residues, R318A was more severely affected in response to ERK2 phosphorylation, whereas L331A did not affect phosphorylation by ERK2, but severely affected phosphorylation by p38\textsubscript{β2}. The latter residue constitutes part of the hydrophobic triplet.

In summary, residues throughout the SAP-1 docking domain play important roles in directing phosphorylation by both the p38 and ERK2 MAPKs. Particularly important contributions come from the LXL motif and the hydrophobic triplet. However, a few key residues are clearly more important for attracting p38\textsubscript{β2} compared with ERK2.

Model for the Structure of MAPK Docking Domains and Specificity Determinants—Alignment of the MEF2A and SAP-1 D-domains shows several conserved blocks of residues and recognizable submotifs (Fig. 4A). These include the basic region, LXL motif, and hydrophobic triplet and have been recognized in more extensive alignments of MAPK substrates (5, 12). Here, our mutagenic analysis has demonstrated the importance of these submotifs in differentially directing ERK and p38 MAPKs to MEF2A and SAP-1. A simple model can be derived from our observations for how the docking domains are organized to specify recognition by these MAPKs (Fig. 4B). In the case of p38\textsubscript{β2} substrates, two types of domains are used that contain both a basic region and a hydrophobic triplet, but that differ in that an intervening LXL motif is either required (as in SAP-1) or dispensable (as in MEF2A). In contrast, ERK substrates appear to require contributions from all three submotifs, although the hydrophobic triplet appears less import-

\section*{MAPK Docking Site Determinants}

\begin{figure}[h]
\centering\includegraphics[width=\textwidth]{mapk_dockingDomains}
\caption{Model for the structures of the MEF2A and SAP-1 D-domain motifs important for selective p38 and ERK2 targeting. A, alignment of the MEF2A and SAP-1 D-domains. Identical and highly similar residues are highlighted. Residues composing the basic, LXL, and hydrophobic submotifs are indicated. \textbf{Numbers} indicate positions of N- and C-terminal amino acids. B, model derived from mutational analysis of the SAP-1, MEF2A, and Elk-1 D-domains to illustrate the differential importance of motifs present within the D-domain for interactions with MAPKs. p38 substrates have a basic region, a hydrophobic region (Φ), and a differential requirement for an LXL motif. ERK substrates possess basic and LXL motifs, but have a differential requirement for the hydrophobic region. Motifs that are absent or degenerate in p38 or ERK substrates are indicated (#).}
\end{figure}
FIG. 5. Mapping of D-domain motifs responsible for p38 targeting. A, sequences of the wild-type SAP-1 (italic type), Elk-1 (Roman type), and chimeric D-domains. The C-terminal extent of residues contained in the chimeric constructs is shown. The vertical dotted line represents the fusion point in the chimeras. B, kinetic analysis of phosphorylation of MEF2A fused to wild-type Elk-1- and SAP-1-derived D-domains by p38. Assays were carried out as described in the legend to Fig. 1 for the indicated times. C, phosphorylation of MEF2A linked to different D-domains. Diagrammatic illustrations of GST fusion proteins of MEF2A derivatives are shown on the left with the D-domains of MEF2A (gray box), Elk-1 (white box), and SAP-1 (black box) proteins and chimeric D-domains (white and black box). A graphical representation of the phosphorylation of these MEF2A proteins by p38 is shown on the right. Kinase assays were performed as described in the legend to Fig. 1. Phosphorylation levels relative to wild-type GST-MEF2A (means ± S.E., n = 3) are shown.

tant. The latter conclusion is supported by our mutagenic studies on SAP-1 (Fig. 3C) and also by the observation that this region is poorly conserved in the related transcription factor Elk-1 and that double mutations that affect this region in Elk-1 do not affect targeting by ERK2 (6, 7).

To extend this model, further tests of specificity determinants are required. This is particularly important in substrates like SAP-1, which are phosphorylated by two different kinases, where, in comparison with MEF2A, compensatory mutations in different residues are likely to occur to retain phosphorylation by p38 kinases and to introduce specificity for ERK2. For example, the model predicts that the acquisition of an LXL motif is likely to be one such determinant. Several features of the model can also be tested to validate it. First, it would be predicted that residues could be introduced between the basic and hydrophobic regions of MEF2 without affecting its efficacy as a p38 substrate. Furthermore, it is predicted that, by strengthening the hydrophobic triplet in poor p38 substrates (e.g. Elk-1 and MEF2D), they will become better substrates. It has been previously shown that two acidic regions on the surface of p38 MAPks likely represent substrate-binding regions (13, 14). Thus, by enhancing the basic region in the substrates to promote binding to the acidic regions on the kinases, it is predicted that enhanced binding and substrate phosphorylation should ensue.

Multiple Regions of the SAP-1 D-domain Specify Docking of p38—Ala scanning mutagenesis indicated that residues throughout the SAP-1 D-domain act to specify phosphorylation by p38 (Fig. 3B). However, this domain is partially conserved in the related transcription factor Elk-1 (Fig. 5A), which does not appear to be targeted by p38 MAPks (6). Thus, changes within this docking site likely determine this differential substrate specificity for p38. To identify the locations of such determinants, a series of chimeric proteins were made in which either the wild-type D-domains of SAP-1 or Elk-1 or composite D-domains were fused to the transcriptional activation domain of MEF2A (Fig. 5C, left). The MEF2D domain used (amino acids 283–413) contains the phosphorylation sites of p38, but lacks its natural docking domain. The composite domains contained residues from either the N-terminal (containing the basic and LXL motifs) or the C-terminal (containing the hydrophobic triplet) end of the docking site of each protein. Each of the mutant proteins was subsequently tested as a p38 substrate.

Initially, we tested phosphorylation of chimeric proteins containing the wild-type D-domains of SAP-1 or Elk-1 by p38 over a time course (Fig. 5B). Phosphorylation of SAPD-MEF2A was readily detectable after 15 min and subsequently increased over a 60-min period (lanes 1–4). In contrast, levels of ElkD-MEF2A phosphorylation were lower at all time points analyzed (lanes 5–8). Thus, differences in the D-domains of SAP-1 and Elk-1 are responsible for dictating differential phosphorylation by p38.

Next, we compared the phosphorylation of the SAPD-MEF2A and ElkD-MEF2A chimeric proteins with that observed with chimeras containing composite SAP-1 and Elk-1 D-domains (Fig. 5C). The level of SAPD-MEF2A phosphorylation was ~50% of that observed with wild-type MEF2A, indicating that, although representing an efficient p38 docking motif, the SAP-1 D-domain is weaker than the D-domain observed in MEF2A. In contrast, ElkD-MEF2A phosphorylation was reduced to a level similar to that of MEF2AΔD, which lacks a docking site, thereby confirming the absence of p38 targeting by the Elk-1 D-domain. The composite D-domains gave rise to intermediate levels of substrate phosphorylation, with ESD-MEF2A showing more phosphorylation by p38 than SED-MEF2A. As ESD-MEF2A contains the hydrophobic triplet from
SAP-1, this further underscores the importance of this motif in targeting of p38β2. However, the increased phosphorylation of the SED-MEF2A chimera by p38β2 clearly supports a role for the N-terminal half of the SAP-1 docking site in p38β2 targeting. Thus, residues from both the N- and C-terminal ends of the docking domains of SAP-1 and Elk-1 are important in specifying their differential phosphorylation by p38β2.

To further probe the roles of the different submotifs in the docking domains of SAP-1 and Elk-1, we analyzed the effect of point mutations in the Elk-1 D-domain in its natural context. In this case, mutations were introduced to alter the Elk-1 D-domain to resemble the analogous domain in SAP-1 in either the LXL motif (Elk-1(D318G/E320G)) or the hydrophobic triplet (Elk-1(G329I)) (Fig. 6A). Mutation of the hydrophobic triplet had little effect on Elk-1 phosphorylation by p38β2 (Fig. 6B) or ERK2 (Fig. 6C), indicating that the increases observed in ESD-MEF2A (Fig. 5C) were not solely due to altering this motif. However, in contrast, alterations in the LXL motif led to increases in Elk-1 phosphorylation by p38β2 (Fig. 6B) and reciprocal decreases in the efficiency of phosphorylation by ERK2 (Fig. 6C). Thus, in Elk-1, the local context of the LXL motif plays a major role in blocking p38β2 targeting while promoting ERK2 targeting.

Collectively, our data demonstrate that multiple motifs in the docking domains contribute to the specificity of MAPK targeting to Elk-1 and SAP-1. However, the local context LXL motif has been shown to have an important role in dictating their differential responses to p38β2 and ERK2.

Docking Domain Specificity Determinants in the MEF2 Transcription Factors—In contrast to SAP-1, MEF2A is phosphorylated by p38α/β2, but not ERK2. Alanine scanning indicated that Asp772, located downstream of the basic region, plays an important role in inhibiting phosphorylation by p38β2. Furthermore, the presence of negatively charged residues downstream of the basic region in Elk-1 caused inhibition of its phosphorylation by p38β2 (Fig. 6). Thus, one potential role for amino acids in this position might be to limit the potency of p38β2 targeting. Recently, acidic patches on p38 MAPKs have been shown to be important for targeting of substrates, thereby implying the importance of the basic regions in docking domains (13, 14). To study the importance of basic residues in the docking domain, we introduced two basic residues into the MEF2A docking site (Fig. 7A). The introduction of basic residues led to a large increase in MEF2A phosphorylation by p38β2 (Fig. 7A). Binding of p38 MAPKs to MEF2A is difficult to detect. However, the MEF2A mutant MEF2A(N267R/D272K) exhibited a large increase in recruitment of p38β2 in kinase binding assays (Fig. 7B). Thus, the strengthening of the basic region clearly enhances targeting by p38β2.

Another key difference between SAP-1 and MEF2A is the absence of an LXL motif in MEF2A. This has important implications for the spacing requirements of the basic and hydrophobic regions, and it is predicted that the introduction of an LXL motif into MEF2A should not affect phosphorylation by p38β2 (Fig. 4). Indeed, such a mutation in MEF2A, MEF2A(+GL), had little effect on MEF2A phosphorylation by p38β2 (Fig. 7A), demonstrating that the spacing between these two motifs is indeed flexible. The weak phosphorylation of MEF2A by ERK2 was not enhanced by the introduction of an LXL motif, indicating that additional determinants are required to confer this property on the MEF2A D-domain (data not shown).

Unlike MEF2A, MEF2D is not phosphorylated efficiently by p38 MAPKs (21). Inspection of the region corresponding to the p38 docking domain suggests one reason for this might be that the hydrophobic triplet is poorly conserved in MEF2D (Fig. 7C). We therefore tested this by introducing reciprocal mutations of residues in this region between MEF2A and MEF2D (Fig. 7C). The MEF2A mutant MEF2A(T277P/P278S) exhibited greatly reduced phosphorylation by p38β2 to levels observed with MEF2D. Conversely, the MEF2D mutant MEF2D(T274I/S275P) exhibited greatly enhanced phosphorylation by p38β2 to levels above those observed with MEF2A. The introduction of the single mutation T275I within the hydrophobic triplet led to an intermediate level of phosphorylation, equivalent to that obtained with MEF2A (data not shown). These results therefore confirm the critical role of the hydrophobic region in the MEF2A docking site in specifying phosphorylation by p38 MAPKs. However, as predicted from the alanine scanning experiments, residues C-terminal to this motif also contribute to MAPK targeting.

DISCUSSION

Docking domains play a major role in dictating the specificity of substrate phosphorylation by MAPKs (reviewed in Refs. 4 and 5). These docking domains are typically short peptides (<20 amino acids) that specify phosphorylation by one MAPK or a subset of MAPKs. Here, we have studied the structure of these domains and how targeting specificity is generated in the transcription factors Elk-1, SAP-1, and MEF2A.

Structure of MAPK Docking Domains

Alignments of MAPK docking domains found in substrates such as transcription factors and downstream kinases reveal the existence of clusters of conserved residues. These clusters are characterized regarding residue type rather than sequence identity. For example, in SAP-1, three distinct submotifs can be identified, including a basic region, an LXL motif, and a hydrophobic region. The importance of these motifs in dictating phosphorylation by p38 MAPKs is supported by the fact that mutations in these motifs result in either increased or decreased phosphorylation of the corresponding docking domain.
phobic triplet (see Fig. 4A). However, different MAPK substrates such as Rsk1 are rich in basic residues, but lack the other two features (5). Other transcription factors like MEF2A retain the basic and hydrophobic regions, but lack the intervening LXL motif (Fig. 4A). Based on our mutational analysis of SAP-1 and MEF2A, several conclusions can be drawn about the roles of the individual submotifs.

The Basic Region—The basic region is clearly an important targeting determinant. Mutations of basic residues reduced the efficiency of substrate phosphorylation by both the ERK and p38 MAPKs (Figs. 1–3). Furthermore, an increase of net positive charge in this region in Elk-1 (Figs. 5 and 6) and MEF2A (Figs. 1 and 7) led to enhanced phosphorylation by p38\(\beta_2\). Reductions in positive charge led to decreases in SAP-1 phosphorylation by ERK and p38 MAPKs, although among basic residues, Arg318 is more important for SAP-1 phosphorylation by p38\(\beta_2\). However, MEF2A lacks an extensive basic region (Fig. 4A), and introduction of additional basic residues resulted in enhanced binding and phosphorylation by p38 MAPKs (Fig. 7). This result suggests that MEF2A has evolved a targeting domain that is suboptimal for targeting of p38 MAPKs, thereby promoting the temporary interactions that are required for transient responses. On the other hand, SAP-1 has a very strong basic region (Fig. 4A), which presumably compensates for the required reductions elsewhere in the D-domain to permit ERK2 binding.

The LXL Motif—The LXL motif in SAP-1 is clearly important for targeting by both the ERK and p38 MAPKs (Fig. 3). However, as this region is lacking in MEF2A, it is not absolutely required for p38 targeting. Consistent with this view is that the reintroduction of the LXL motif into MEF2A did not affect its phosphorylation by p38\(\beta_2\) (Fig. 7), indicating that the spacing between the basic region and hydrophobic triplet is relatively unimportant. However, conversely, in substrates like SAP-1, where a spacer is present, residues in this spacer play essential roles (Fig. 3). The introduction of charged residues next to and within the LXL motif of SAP-1/Elk-1 also affected targeting specificity (Figs. 5 and 6), demonstrating the importance of the local context of this motif. However, in the context of the MEF2A docking domain, the introduction of the LXL motif is not sufficient to enhance the weak ERK-mediated phosphorylation of this protein.

The Hydrophobic Region—Alanine scanning mutagenesis revealed an important role for the hydrophobic triplet in SAP-1 and MEF2A in phosphorylation by both the ERK and p38 MAPKs (Figs. 1 and 4). However, in SAP-1, Leu331 within this motif is clearly not important for ERK-mediated phosphorylation. Similarly, alanine insertions at the equivalent position in Elk-1 do not affect ERK-mediated phosphorylation (6). Thus, residues in the hydrophobic motif play an important role in specificity determination and are particularly important for the phosphorylation of p38 substrates. This is underlined by the observation
that MEF2D can be converted into an efficient p38 substrate by strengthening its hydrophobic region (Fig. 7).

Proline Residues—Several proline residues are present within the docking domains of SAP-1 and MEF2A, suggesting that these domains are unlikely to fold into defined secondary structure elements. However, the majority of these residues play important roles in MAPK targeting (Figs. 1, 3, and 7). One attractive role for these residues would be structural, to direct the docking domain peptides in the correct trajectory to interact with the surface of the kinase. Such a critical role would explain the importance of prolines such as Pro328 in SAP-1, the mutation of which has the most severe consequences. An alternative but not mutually exclusive role would be that these residues make specific contacts with the kinases.

Finally, it is important to emphasize that, although the contributions of individual submotifs can be recognized, the interplay between these motifs is of critical importance. Residues outside these motifs can play important roles, and changes in one submotif can be compensated for by alterations elsewhere in the docking domain. This is particularly important when considering how dual targeting specificity is generated in substrates, as observed in SAP-1.

Implications for Predicting Docking Domain Specificity

It has previously been demonstrated that it is possible to predict the existence of MAPK docking domains (12). However, although this is possible, subsequent predictions of the specificity of targeting dictated by these domains is difficult. The results of this study highlight these inherent difficulties, as residues throughout the motifs differentially contribute to MAPK targeting. However, the model we have generated should aid in the prediction of targeting specificity.

Another implication of our results is that, although docking domains might be identified (such as in MEF2D), these domains might be nonfunctional. In the case of MEF2D, residues have been introduced that block p38 targeting and presumably have regulatory consequences. For example, MEF2D might become phosphorylated by p38 MAPKs only as a heterodimer with MEF2A or MEF2C, which will contribute a docking domain. A similar scenario has been demonstrated for Jun family members (26).

Substrate Recognition motifs in MAPKs

The model proposed for the structure of the docking domains found on transcription factor substrates (Fig. 4B) has important implications for the cognate surfaces found on the kinases. For example, p38 MAPKs must have three different motifs that recognize the basic region, LXL motif, and hydrophobic triplet. In an alternative recognition mode, the p38 MAPKs would use only two such regions for recognizing substrates such as MEF2A that lack the LXL motif. This also suggests that the three motifs are not located on a linear epitope across the surface of the kinase and that the basic domain and hydrophobic recognition motifs must be located quite close together on the kinase. A further prediction is that the three binding motifs on the kinase will be acidic, partially hydrophobic, and hydrophobic to accommodate the three submotifs found in the docking domains. Attractive candidate motifs for binding to the basic regions are the previously identified acidic patches (CD and DE domains) (13, 14). The first of these is proposed to be important in all MAPK-substrate interactions (presumably via the basic domain), whereas the DE motif is specifically involved in recognizing p38 substrates. However, although SAP-1 contains an extensive basic region, the reduced basic region in MEF2A suggests that these acidic regions on the p38 MAPKs might not be important for interaction with its docking domain.

Conclusions

Collectively, our data provide a compelling model for the structure of MAPK docking domains and the specificity determinants embedded within these domains. These results should therefore be of wide significance for studies on other MAPK-substrate interactions.

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