Structure-Function Studies of p38 Mitogen-activated Protein Kinase

LOOP 12 INFLUENCES SUBSTRATE SPECIFICITY AND AUTOPHOSPHORYLATION, BUT NOT UPSTREAM KINASE SELECTION* ^

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Several mitogen-activated protein kinase (MAPK) cascades have been identified in eukaryotic cells. The activation of MAPKs is carried out by distinct MAPK kinases (MEKs or MKKs), and individual MAPKs have different substrate preferences. Here we have examined how amino acid sequences encompassing the dual phosphorylation motif located in the loop 12 linker (L12) between kinase subdomains VII and VIII and the length and amino acid sequence of L12 influence autophasorylation, substrate specificity, and upstream kinase selectivity for the MAPK p38. Conversion of L12 of p38 to an “ERK-like” structure was accomplished in several ways: (i) by replacing glycine with glutamate in the dual phosphorylation site, (ii) by placing a six-amino acid sequence present in L12 of ERK (but absent in p38) into p38, and (iii) by mutations of amino acid residues in loop 12. Two predominant effects were noted: (i) the Xaa residue in the dual phosphorylation motif Thr-Xaa-Tyr as well as the length of L12 influence p38 substrate specificity, and (ii) the length of L12 plays a major role in controlling autophasorylation. In contrast, these modifications do not result in any change in the selection of p38 by individual MAPK kinases.

Protein kinase cascades control a variety of functions in eukaryotic cells (1). Key components of these protein kinase cascades are members of the superfamily of mitogen-activated protein kinases (MAPKs). These serine/threonine kinases act by modulating the activity of cellular proteins such as transcription factors, enzymes including other kinases, cell-surface receptors, and structural proteins (2). In yeast, distinct signal-

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† The abbreviations used are: MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated protein kinase; L12, linker loop 12 between kinase subdomains VII and VIII; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; MKK or MEK, MAPK/ERK kinase; GST, glutathione S-transferase; ATF2, activating transcription factor 2; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein.

Materials and Methods

Mutagenesis of Murine p38—A construct designed to express murine p38 with His at its amino terminus was prepared by ligating the p38 coding region, amplified from p38 cDNA in BlueScript using the polymerase chain reaction (primers gcagccatATGTCGCAGGAGGCC and ccgatccTCAGGACTCCATTTCTTC), with pET14b vector (Novagen, Madison, WI), incorporating NdeI and BamHI sites. A construct designed to express p38 with a flag tag at the amino terminus in a mammalian expression vector (pcDNA3) was prepared as described (10, 26). The p38 mutants shown in Fig. 1 were generated using a polymerase chain
p38 Mutants with Modifications in Loop 12—Sequence comparisons of the known MAPKs revealed that each group has a distinct phosphorylation lip (L12) structure (9, 14). To investigate how the L12 structure is involved in the function of p38, we have created a series of p38 mutants with modifications in this region (shown schematically in Fig. 1). Specifically, we introduced mutations to make p38 more “ERK-like” in this region (shown schematically in Fig. 1). Specifically, we introduced mutations to make p38 more “ERK-like” in the L12 region.

Thus, p38(E) contains an ERK-like Thr-Glu-Tyr dual phosphorylation site; p38(VAP) has 174HTDD177 replaced with DL; and p38(6+VAP) has an additional six-amino acid sequence of p38(6+VAP).

Activation of p38 and p38 Mutants Catalyzed by MKKs—Previous studies have demonstrated that there are distinct MAPK kinases (MEKs or MKKs) responsible for activating ERK, p38, or JNK (or stress-activated protein kinase) (15). It has been suggested by others that the nature of the second amino acid in the Thr-Xaa-Tyr dual phosphorylation motif may play a role in determining the specificity of interactions between MKK and its MAPK substrate (34, 35). To test this hypothesis, we examined the activation of wild-type p38 and various p38 mutants with modifications in Loop 12.
the panel of p38 mutants; MKK3b (36), MKK6b (16), or MEK1 (37) was used for this experiment. Using an in vitro coupled kinase assay, we observed that the enzymatic activity of p38 or p38 mutants was increased when MKK3b or MKK6b was included in the kinase reaction, but not when we used MEK1 (Fig. 2). The extent of activation was nearly the same when each of the mutants was compared with wild-type p38. As expected, MEK1 dramatically increased the activity of ERK2 (Fig. 2, lower panel). In a second approach, we cotransfected MKKs and individual epitope-tagged p38 forms in COS-7 cells to further examine the effects of modifying L12 on the ability of MKKs to activate p38 (Fig. 3A). Consistent with the in vitro experiments, the activity of p38 or p38 mutants was enhanced similarly when cotransfected with MKK3b or MKK6b, but not with MEK1. ERK2 activity was only enhanced when cotransfected with MEK1. Equal amounts of wild-type or modified p38 protein were detected in the immunoprecipitates used for the kinase assay (Fig. 3B).

We next asked whether this same pattern is observed in a setting where activation is initiated by an extracellular stimuli. To investigate this, CHO-K1 cells were transiently transfected with flag-tagged p38, p38 mutants, or ERK2, and cells were treated with 0.4 M sorbitol, UV light, epidermal growth factor + insulin, or 20% fetal calf serum. The transiently expressed epitope-tagged MAPKs were immunoprecipitated with the M2 antibody, and the resultant immunoprecipitates were analyzed for kinase activity. High osmolarity or UV light enhanced the kinase activity of p38 and all p38 mutants (Fig. 4A). Addition of an ERK-like loop structure did not result in conversion of p38 to respond to growth factor signals that activate ERK. Thus, the totality of these data suggest that neither the Thr-Xaa-Tyr motif nor the L12 structure is a critical structural feature directing the selectivity of the upstream kinase.

Autophosphorylation of p38 and p38 Mutants—Histidine-tagged recombinant p38 expressed in E. coli appears to undergo an autophosphorylation reaction since it is phosphorylated on tyrosine (Fig. 5). Although at present this event cannot be linked to any physiologically relevant changes, the parameter of autophosphorylation provides us with an additional way to evaluate structure-function relationships of p38. We used anti-phosphotyrosine antibody in Western blots to analyze the extent of tyrosine phosphorylation of recombinant wild-type p38 or p38 mutants with the modification in the L12 region. We observed that the proteins with added amino acids in L12 have substantially more phosphotyrosine when compared with equal amounts of wild-type p38 (Fig. 5, A and B). The same results were obtained using two different anti-phosphotyrosine monoclonal antibodies, 4G10 and FB2. The level of phosphotyrosine in p38(6+), p38(6+E), or p38(VAPD6+LE) is similar to that found in recombinant ERK2 expressed and isolated identically to the means used to obtain p38 forms.

Autophosphorylation of p38 or its modified forms can also be detected by incubation of recombinant protein with [γ-32P]ATP

\[^3\]MKK3b is a 347-amino acid spliced form of MKK3; MKK6b is a 334-amino acid spliced form of MKK6.
under conditions of an in vitro kinase assay; p38(6+), p38(6+E), and p38(VAPD6+LE) display enhanced autophosphorylation when compared with wild-type p38 or the other p38 mutants (Fig. 5C). Similar results were obtained when recombinant proteins expressed in mammalian cells were used (data not shown). These data suggest that the length of L12 controls the structure of p38 in ways that regulate the autophosphorylation reaction, while the nature of the Xaa residue in the Thr-Xaa-Tyr dual phosphorylation site has little or no influence on this activity of p38.

Effect of Sequence Modifications of L12 on p38-Substrate Interaction—We next asked whether changes in L12 influence the substrate specificity of p38. To investigate this, we performed in vitro kinase assays using the following proteins as substrates: ATF2 (38), ELK1 (30), c-Myc (31), PHAS-1 (39), and myelin basic protein (MBP). Each of these proteins can be phosphorylated by p38 (Fig. 6), with the exception of GST used as a control for the GST fusion proteins (data not shown). Equal quantities of kinase (see Fig. 5A) and substrate protein (Fig. 6B) were used in each kinase assay. The level of substrate phosphorylation was observed to differ substantially when wild-type p38 and its modified forms were compared (Fig. 6A). MBP, Myc, and ELK1 contain the consensus Pro-Xaa-Ser/Thr-Pro sequence, which has been defined as the consensus recognition site for ERK1/2 (40–42). The kinase activity of p38 toward these three proteins was increased by the insertion of six residues or by a Gly to Glu mutation. However, p38(6+E) and p38(VAPD6+LE) do not show this enhanced activity, but have reduced substrate phosphorylation. ATF2 and PHAS-1, which have different primary structure at the phosphorylation site when compared with MBP, Myc, or ELK1, behaved differently insofar as p38-catalyzed phosphorylation is concerned. p38(E) and p38(6+) each demonstrated markedly reduced phosphorylation of ATF2 when compared with that produced by wild-type p38. The p38(6+E) and p38(VAPD6+LE) mutants also showed an even greater reduction in ATF2 phosphorylation, suggesting that the two modifications may synergize in terms of their effect on substrate recognition and/or catalysis; p38(6+) and p38(6+E) had enhanced activity toward PHAS-1. The mutation of HTDD (p38(VAP)) or EM (p38(DL)) does not lead to any change in the substrate specificity. The differential phosphorylation of these substrates by p38 and some p38 mutants cannot be due to a general change in enzymatic activity because differing effects on phosphorylation by p38 and its mutants were substrate-dependent.

Autophosphorylation and Enzymatic Activity—We next investigated whether the increased autophosphorylation observed with the p38 mutants p38(6+), p38(6+E), and p38(VAPD6+LE) is reflected in a similar change in enzymatic activity toward specific protein substrates. We found that a higher level autophosphorylation of p38(6+), p38(6+E), and p38(VAPD6+LE) did not correlate with phosphorylation of MBP and PHAS-1 (Fig. 7). Similar results were obtained with the other substrates shown in Fig. 6 (data not shown). These data show that there are different mechanisms in controlling the autophosphorylation and substrate recognition/phosphorylation.
DISCUSSION

Although all MAPKs are regulated by dual phosphorylation on adjacent Thr and Tyr residues, each has its distinct set of upstream activators. Whereas the members of this enzyme family have >40% overall identity, each displays very distinct substrate specificities. A structural basis for these upstream and downstream specificities has not been currently determined. Here we have utilized p38 and ERK2 to investigate structural features that may control recognition by upstream activators or may determine substrate specificity. Using recombinant DNA techniques, we have modified p38 in the phosphorylation lip L12, the locus of major differences between p38 and ERK2, to an ERK-like structure. This permitted us to evaluate the influence of such modifications on the activation and function of p38 as well as on its ability to undergo autophosphorylation.

Since this structural domain contains the dual phosphorylation sites, the target of the upstream MAPK kinase, this region of MAPK must interact with the activating kinase. Differences in the L12 structure of the known MAPKs suggested that the Xaa residue in the dual phosphorylation motif may play a role in determining the specificity of interactions with MKK (34, 35). Our data indicate that neither the Xaa residue in the Thr-Xaa-Tyr motif in p38 nor the other residues present in L12 are critical in controlling p38 activation regardless of whether this is tested in fully in vitro experiments or in cells.

After submission of this paper, two other studies provided data supporting this contention. Brunet and Pouyssegur (31) expressed a p38-ERK chimeric protein that demonstrated that the amino terminus of p38 plays a predominant role in determining interactions with the upstream activator and that L12 has no role in this event. Robinson et al. (43) showed that Xaa in the dual phosphorylation motif of ERK does not have any role in the upstream kinase selectivity and that the length of the lip of ERK2 has only a small effect on the ability of MEK1 to phosphorylate ERK2. Therefore, although amino acid residues in the phosphorylation lip interact with MKK, the MKK specificity is determined by a p38-MKK interaction involving other domain(s). These may include portions of the amino terminus of p38 (31).

Information from the three-dimensional structure of p38 suggests that a surface groove of p38 is likely to be in contact with protein substrates (24, 25). This groove is composed of several helices and the carboxyl-terminal domain. Based on a
MAPK when there is a prior autophosphorylation on tyrosine. It is possible that a serine/threonine kinase can partially activate autophosphorylation may contribute to the activation of MAPK. We suggest that a physiological substrate of p38 should be a preferred substrate for unmodified p38, but not for the L12 mutants. Therefore, such p38 mutants may be useful tools to test the specificity of suspected p38 substrates. For example, the stronger phosphorylation of ATP2 by wild-type p38 than by p38 mutants suggests that there is a specific interaction between this protein and p38.

In vitro autophosphorylation has been studied in the ERK group of MAPKs, where it was shown to be a unimolecular reaction occurring primarily on tyrosine (45-52). From the results of crystallographic studies, it is clear that the Thr residue in the Thr-Glu-Tyr phosphorylation site of ERK is on the surface, while the Tyr residue is buried in a large hydrophobic pocket. The Tyr residue is located near a putative catalytic base, Asp147 (Asp150 in p38), and γ-phosphate of bound ATP. This may account for the ability of ERK to catalyze its efficient autophosphorylation. The shortened length of L12 in p38 results in the Thr-Gly-Tyr dual phosphorylation site being present on the surface of the protein. This may cause a decrease in the flexibility of the loop that prevents the conforma-

In summary, our data demonstrate that although the sequences in loop 12 account for major differences between p38 and ERK2, this phosphorylation site does not appear to direct MKK specificity. A change in the length of L12 affects the structure of p38 in a way that alters the level of autophosphorylation. In addition, modifications of the lip sequence lead to specific changes in substrate selectivity. These data suggest that the phosphorylation lip may be involved in p38-substrate interaction. This finding would not have been predicted from what is known about the cyclic AMP-dependent protein kinase-substrate interaction (44). How the phosphorylation lip is involved in substrate recognition awaits further investigation.

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Loop 12 and p38 MAPK Function

The insufficient activation of p38 by autophosphorylation suggests that the phosphorylated amino acids may not reflect phosphorylations at all critical regulatory sites; this is the case for ERK (46). Although this study does not provide a physiological role for autophosphorylation, we can speculate that the autophosphorylation may contribute to the activation of MAPK in cells. Although it is well known that dual specificity kinases are responsible for the activation of MAPKs (34, 53), it is still possible that a serine/threonine kinase can partially activate MAPK when there is a prior autophosphorylation on tyrosine.
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