DEF Pocket in p38α Facilitates Substrate Selectivity and Mediates Autophosphorylation*

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Background: p38α MAP kinase has two recognition sites, the CD domain and DEF pocket.

Results: DEF pocket mutations decreased phosphorylation levels of several substrates and autophosphorylation capabilities.

Conclusion: The DEF pocket directs selective substrate activation and also mediates autophosphorylation.

Significance: Understanding how substrates interact selectively with the two p38α docking sites could become the basis for the design of inhibitors.

Signaling processes are primarily promoted by molecular recognition and corresponding protein-protein interactions. One of the key eukaryotic signaling pathways is the MAP kinase cascade involved in vital cellular processes such as cell proliferation, differentiation, apoptosis, and stress response. The principle recognition site of MAP kinases, the common docking (CD) region, forms selective interactions with substrates, upstream activators, and phosphatases. A second docking site, defined as the DEF site interaction pocket (DEF pocket), is formed subsequent to ERK2 and p38α activation. Both crystal structures of p38α in its dually phosphorylated form and of intrinsically active mutants showed the DEF pocket, giving motivation for studying its role in substrate activation and selectivity. Mutating selected DEF pocket residues significantly decreased the phosphorylation levels of three p38α substrates (ATFII, Elk-1, and MBP) with no apparent effect on the phosphorylation of MK2 kinase. Conversely, mutating the CD region gave the opposite effect, suggesting p38α substrates can be classified into DEF-dependent and DEF-independent substrates. In addition, mutating DEF pocket residues decreased the autophosphorylation capability of intrinsically active p38α mutants, suggesting DEF-mediated trans-autophosphorylation in p38α. These results could contribute to understanding substrate selectivity of p38α and serve as a platform for designing p38α-selective DEF site blockers, which partially inhibit p38α binding DEF-dependent substrates, whereas maintaining its other functions intact. In this context, preliminary results using synthetic peptides reveal significant inhibition of substrate phosphorylation by activated p38α.

Controlling vital cellular processes such as proliferation, differentiation, apoptosis, and stress response are primarily mediated by protein kinases. Abnormal activities of kinases are often associated with human diseases (1). Thus, understanding kinase mechanisms of activation and regulation is a pivotal goal in biological research. There are several manners by which cells regulate kinase activity such as timing of activation, specificity, and down-regulation. All these processes involve protein-protein interactions with partner proteins such as scaffold proteins, upstream activators, substrates and phosphatases, which are generally mediated through recognition sites on both interacting molecules.

One of the central signaling cascades in eukaryotic cells is the mitogen-activated protein kinase (MAPK) pathway, which is involved in most essential cellular processes. The MAPKs are activated via dual phosphorylation by their upstream activators, the MKKs. Consequently, MAPKs phosphorylate many substrates in the cytoplasm and nucleus including transcription factors, tumor suppressors, and other kinases (2). More than 200 substrates have been identified for MAPKs that are involved in numerous cellular processes. Phosphorylation of different substrates activates different signaling pathways and consequent phenotypes (3, 4). As with many enzymes, the interactions between MAPKs and substrates, upstream activators and down-regulators are mainly dictated by recognition (docking) regions in both interacting molecules. Two principle docking regions have been identified for substrates on MAPKs affecting substrate specificity and selectivity. The principle docking site identified in all MAP kinases is defined as a common docking (CD) region that selectively interacts with corresponding substrates, upstream activators, and phosphatases. This domain comprises a central cluster of 2–3 acidic residues with several hydrophobic residues in its vicinity (5–7). The CD site is located on the surface of the MAPK in the region connecting the C’ and N’ kinase lobes, distal from the catalytic site. Molecules that bind the CD region contain a complementary interacting site, termed the D-site (also defined as the DEJL domain and δ-domain), and are characterized by a consensus cluster of 2–3 positively charged residues with several proximate hydrophobic residues (7–11).

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2 The abbreviations used are: CD, common docking; MBP, myelin basic protein.
Biochemical Characterization of p38α DEF Pocket

site is formed by local conformational changes following activation (dual phosphorylation) and consists of hydrophobic residues (Fig. 1A). The DEF pocket is shaped as a hydrophobic cavity designed to accommodate a distinctive hydrophobic segment in substrates defined as the “docking site for ERK FXF” (F-site or DEF site). The F-site in substrates is generally characterized by two Phe residues separated by one amino acid (FXF motif) located 6–20 amino acids downstream to the substrate phosphoacceptor (12–15).

Mutational analysis of ERK2 docking regions emphasized their importance in substrate selectivity. Mutating selected residues in the DEF pocket in ERK2 resulted in decreased phosphorylation levels of Elk-1 and c-Fos transcription factors, whereas phosphorylation of RSK (kinase) was not affected (6, 16). In contrast, the D319N (“sevenmaker” (17, 18)) mutation in the CD region resulted in decreased RSK phosphorylation but did not affect Elk-1 and c-Fos phosphorylation levels, indicating different substrate preferences (6, 16).

Little is known about the availability and function of the DEF pocket in p38α, yet a F-site was identified in the p38α protein kinase. Phosphorylation levels of Elk-1 and c-Fos transcription factors, indicated by a peptide derived from the Elk-1 F-site, displays inhibitory properties on ATFII phosphorylation (12–15).

Expression, purification, and crystallization protocols of the p38α/2 (Y239A + ML194–5AA + CI228–9AA) penta-mutant were conducted as previously described for the p38α (22, 24). Crystallographic data were collected at the European Synchrotron Radiation Facility (ESRF) (see Table 1), integrated and scaled using the HKL suite (25). The structure of the penta-mutant was solved via molecular replacement using p38α as the search model and further refined at the resolution range of 50–1.6 Å using Phenix (26) (Table 1).

Protein Expression and Purification for in Vitro Kinase Assay—Protein expression and purification of p38α proteins and GST-ATF2 were conducted as previously described (27). The GST-Elk1 (amino acid 310–428), GST-Elk1ΔD (amino acid 329–428), and GST-MK2 (pGEX-MK2-K76R) were expressed in Escherichia coli as with the p38α proteins. The cell cultures of GST-Elk1 and GST-MK2 were grown at 30 °C for 20 and 5 h, respectively. Cells were collected by centrifugation and washed in phosphate-buffered saline (PBS) and the pellet was then stored at −20 °C. The frozen pellet was gently thawed on ice, and suspended in PBS and protease inhibitors mixture (Sigma P8849). After mechanical disruption of the cells using a microfluidizer (model M-110 EHIS, Microrofluidics Corp., Newton, MA) the lysate was centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant, containing the soluble proteins was loaded on a glutathione-Sepharose column (Amersham Biosciences), washed in PBS, and eluted using 50 mM Tris buffer, pH 8, and 20 mM glutathione. The protein solution was then dialyzed overnight against 12.5 mM Hepes buffer, pH 7.5, 100 mM KCl, 6.25% glycerol, and 1 mM diithiothreitol (DTT). After dialysis, the protein concentration was determined using the Bradford method and the purified protein was then divided into aliquots, flash-frozen in liquid nitrogen, and stored at −80 °C. MBP (Sigma M-1891) was dissolved with 50 mM Tris buffer, pH 8.

In Vitro Kinase Assay—the paper-spotted kinase reactions were performed as previously described (27). In parallel, a quality assay was done in which samples from the paper-spotted kinase reactions were mixed with Laemmli sample buffer and boiling at 100 °C for 5 min. The assay samples were run on SDS-PAGE stained with Coomassie staining and then expose to x-ray film. The kinetic kinase assays were carried out for 10 min with substrate concentrations ranging between 0 and 140 μg (0–75 μM for GST-ATFII or 0–85 μM for GST-Elk-1). The peptide competitive kinase assay was performed using increasing concentrations (0.1 μM to 1 mM) of peptides derived from the Elk-1 F-site (APRSFLAKSLQFPPSS) or a mutated F-site (APRSFLAKSLAQASSPSS) as a negative control.

The autophosphorylation kinase assay was performed in a similar buffer as the paper-spotted kinase assay with no substrate, where each reaction contained 1.25 μg of purified protein in a final volume of 25 μL. Reactions were carried out for durations of 0, 15, 30, and 60 min at 30 °C and terminated by cooling to 4 °C and adding 6 μL of 5X Laemmli sample buffer and then heating to 100 °C for 5 min. The assay samples were run on SDS-PAGE with Coomassie staining and then exposed to x-ray film. For quantifying the autophosphorylation levels the rele-

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of the p38α Mutants and Structural Analysis—Site-directed mutagenesis was performed by polymerase chain reaction according to the recommendations of the manufacturer. Mutagenesis was performed on the human p38α cDNA subcloned into a pET-28b (Novagen) vector downstream and in-frame with the hexahistidine coding sequence. All mutated cDNAs were verified by sequencing the entire p38α cDNA.

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vant bands for the dried SDS-PAGE were counted using a scintillation counter running a $^{32}$P Cherenkov program.

Western Blot Analysis—For the Western blot analysis, 0.2 µg of purified recombinant protein were heated at 100 °C for 5 min, separated by SDS-PAGE, and then transferred to a nitrocellulose membrane. After incubating the membrane with the appropriate antibodies, specific proteins were visualized using an enhanced chemiluminescence detection reagent and then monitored by exposing the membranes to x-ray film. The antibodies used in the assays were as follows: goat anti-p38 from Santa Cruz Biotechnology, rabbit anti-phospho-p38, rabbit anti-MK2 (3042S), rabbit anti-phospho-MK2 (3007S), and rabbit anti-Elk-1 from Cell Signaling (9182), rabbit anti-phospho-Elk-1 from Santa Cruz Biotechnology (SC8406), anti-HA tag from 12CA5 hybridomas and mouse anti-phosphothreonine from Cell Signaling (9386S); and anti-phosphotyrosine from 4G10 hybridomas.

Cell Culture and Luciferase Assay—The transfected recombinant p38α cDNAs containing an HA tag were cloned into pCFL vectors (Invitrogen). The active MKK6 double mutant (MKK6-EE) with an HA tag was cloned into the pBabe plasmid. The transfected recombinant MK2 and Elk-1 cDNAs were cloned into the pΔCR and pEXV3 plasmids (28). HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Biological Industries, BeitHa’emek, Israel) and incubated at 37 °C in 5% CO₂. Mouse embryonic fibroblasts cells, lacking p38α (MEFα−/−), were grown in a similar medium solution as described above, supplemented with nonessential amino acids, sodium pyruvate, and β-mercaptoethanol (Invitrogen). The MEF cells were transfected using the TurboFect™ transfection reagent (Fermentas) according to the manufacturer’s instructions and HEK293 cells were transfected using the calcium-phosphate method. Cells were washed with phosphate-buffered saline 48 h post-transfection, harvested by Laemmli’s buffer, and then scraped using rubber policeman.

For the luciferase assay, MEF cells were plated on 12-well plates (0.1 × 10⁶ cells/well). The cells were transfected with 2 µg of pCFL containing wild type p38α or the different mutants, 0.1 µg of 6× AP-1-luc, 0.2 µg of pBabe-MKK6-EE, and 30 ng of Renilla luciferase (pRL-TK) as a control for transfection efficiency. The cells were harvested 48 h post-transfection and the luciferase activity was measured using the dual luciferase reporter assay system (Promega).

RESULTS

Selection of DEF Pocket Mutants—The conformational changes shown for the Tyr323 intrinsically active mutants resulted in the formation of the DEF site interaction pocket in the C-lobe (22, 24). This pocket acquires a similar contour to the DEF pocket in p38α using homologous amino acids (21, 23) (Fig. 1).

The DEF pocket in p38α, as in ERK2, consists of 13 residues from several segments, including the activation loop (residues 180–182), the loop connecting P+1 and F-helix (residues 190–203), the G-helix (residues 227–237), and the MAP kinase insert region (residues 240–262) (Fig. 1). Site-directed mutagenesis was done on selected residues to examine the role of the DEF pocket residues for p38α substrate recognition and selectivity. Of the 13 residues forming the DEF pocket, three are the Thr-Gly-Tyr phosphorylation motif (180–182) and two (Arg189 and Trp197) stabilize the active form. Mutating these residues drastically decrease p38α activity altogether (29). Two additional residues (Leu195 and Leu232) are buried in the internal part of the pocket and are probably considered to play a minor role in substrate binding. Based on the structural and biochemical analyses we have elected to mutate 5 amino acid residues obtaining three distinct variants (p38αY258A, p38αY194A, p38αY258A+ML194–5AA, and p38αHI228–9AA).

The Role of the DEF Pocket in p38α Substrate Selectivity—The mutants in the DEF pocket were first examined for their capability to be phosphorylated and activated in vitro by MKK6, ERK2, and 4G10 hybridomas. The results indicate that all mutants were phosphorylated similarly to p38α (not shown). Additionally, the crystal structure of the DEF pocket pentamer (inactive p38αY258A+ML194–5AA+HI228–9AA), determined at a resolution of 1.66 Å (Table 1), shows a highly similar overall fold compared with the nonactivated p38α (not shown).
Biochemical Characterization of p38α DEF Pocket

The activities of the DEF pocket mutants were assayed for their ability to phosphorylate in vitro four p38α substrates, GST-ATF-II, GST-Elk-1, GST-MAPKAPK2 (GST-MK2), and MBP. The results show that some of the DEF pocket mutations display differences in their ability to phosphorylate different substrates. In this context, p38αY258A displayed a reduced capability in phosphorylating ATF-II, Elk-1, and MBP (~25% of p38αwt activity), but it phosphorylates MK2 at levels almost identical to those of p38αwt (Fig. 2). The p38αML194–5AA double mutant has shown very low activity toward all four substrates although still capable of phosphorylating MK2 to some extent (13% of p38αwt activity). The p38αML194–5AA double mutant showed somewhat decreased activity toward Elk-1 and MBP (to about ~72% of the activated wild type) but had no activity-decreasing effect on ATFII and MK-2 (Fig. 2). In summary, ATFII, Elk-1, and MBP were more affected by the DEF pocket mutations than MK2. Also, the ML194–5AA double mutation resulted in a notable decrease in activity toward all substrates. Met194 and Leu195 are located in the αEF helix near the αEF/αF loop, a conserved functional region in all kinases that contribute to stabilization of the active form of the kinase, and has also been shown to be critical for p38α activity (30). One could thus assume that the double mutant affects the overall catalytic properties of p38α rather than substrate selectivity.

We further determined the catalytic parameters of p38αY258A and p38αHI228–9AA against ATFII and Elk1 (Table 2). The mutants display differences in the catalytic parameters in comparison to p38αwt with a high correlation to the kinase assay results (Table 2). In this context, the p38αY258A mutant displays a decrease of the specificity constant (K_m/k_act) to ~23 and 40% compared with the wild type (for the ATFII and Elk1 substrates, respectively). The K_m/k_act values of the p38αHI228–9AA mutant are 107 and 83% compared with the wild type (for the ATFII and Elk1 substrates, respectively).

**TABLE 1**

| Data collection and refinement statistics | p38αY258A+ML194–5AA+HI228–9AA |
|------------------------------------------|---------------------------------|
| PDB entry | 4GEO |
| Space group | P2_1,2,2. |
| Unit cell parameters (Å) | a = 66.9, b = 74.6, c = 74.8 |
| Resolution range (Å) (last resolution shell) | 52.0–1.66 (1.69–1.66) |
| Unique reflections | 44,163 |
| Redundancy | 4.3 |
| R_{int}(l)^a | 5.1% (44.9%) |
| Completeness | 98.2 (99.1) |
| I/σ | 40 |
| Number of protein atoms | 2622 |
| Number of ligand atoms | 40 |
| Number of solvent atoms | 326 |
| R-factor | 0.217 |
| R-free^b | 0.255 |
| Average B-factor (Å^2) | Protein: 27.1, Solvent: 35.5 |

Root mean square deviation from ideality

| Bond length | 0.015 Å |
| Bond angle | 1.49^c |

Ramachandran plot (PROCHECK)

| Favorable | 92.8% |
| Allowed | 6.8% |
| Generously allowed | 0.3% |
| Disallowed | 0.0% |

^a R_{int}(l) = Σ(l−|l|)/Σl.

^b Test set consists of 5% for all data.

The specificity of substrate to the CD docking region in p38α was examined by mutating Asp316 to Asn as in ERK2D316N (17, 18). Western blot analysis showed that the phosphorylation level of MKK-activated p38αD316N was similar to that of p38αwt indicating that Asp316 is not critical for the interaction of p38α with its upstream activator MKK6 in vitro (Fig. 3a). The ability of the activated mutants to phosphorylate each of the four substrates was determined by the kinase assay against the different substrates that show that the activated p38αD316N phosphorylates MK2 at a lower level (29%) than p38αwt. Conversely, p38αD316N activity toward ATFII, Elk-1, and MBP was increased by 11–18% (Fig. 3b). Catalytic studies of the p38αD316N mutant revealed a similar kinetic profile to that of p38αwt (Table 2). These results indicated that substrates that are less influenced by mutation in the CD domain are more dependent on interactions with the DEF pocket and vice versa. A truncated fragment of the GST-fused Elk-1 substrate (GST-Elk1ΔD) was used to corroborate this result. This substrate lacks the D-domain region of Elk-1 but includes the F-site region. GST-Elk1ΔD was phosphorylated by p38α to a higher level than Elk-1, which had both docking domains (Fig. 3c). In addition, the degree of phosphorylation of GST-Elk1ΔD by p38α DEF pocket mutants was similar to that previously observed for GST-Elk1 (Fig. 3d).

The Role of the DEF Pocket in p38α Autophosphorylation—

The alternative activation modes of p38α and the intrinsically active mutants have been shown to induce autophosphorylation and subsequent activation (22, 24, 31, 32). Autophosphorylation of the intrinsically active mutants previously identified in p38α (Asp176, Phe237, and Tyr232 sites) occurs in trans and may involve interactions with the DEF pocket (22, 24). This assumption was examined by combining and assaying the intrinsically active mutations and the DEF pocket mutations of p38α. The autophosphorylation activity of each intrinsically active mutant was normalized to 100% activity and the wild type molecule was used as a negative control for basal (low) autophosphorylation activity. A notable decrease in the autophosphorylation capability was found for all the combined mutants, probably resulting from mutations at the DEF pocket (Fig. 4a). More specifically, the Y258A and H228A/I229A mutations resulted in decreased autophosphorylation levels (after 60 min) to 16–60% compared with the autophosphorylation level of the intrinsically active mutants. A more dramatic effect was found for the M194A/L195A mutants that exhibited only a 4–5% autophosphorylation level (Fig. 4A). Western blot analyses revealed that the decreased phosphorylation levels of both Thr and Tyr residues resulted from mutations in the DEF pocket (Fig. 4B).

The F-site of MAPK substrates is considered to accommodate aromatic amino acids 6–20 residues downstream to the substrate phosphoacceptor (12–15). The activation loop of p38α contains two aromatic residues (Trp187 and Tyr188) 7 and 8 residues downstream to the Thr-180 phosphoacceptor (Fig. 5a). Because autophosphorylation of p38α was shown to occur...
in trans, it is plausible that the aromatic region may interact with a DEF pocket of another p38\(\alpha\) molecule directing the phosphoacceptors toward the active site thus facilitating auto-phosphorylation. The p38\(\alpha^{wt}\) and DEF pocket mutants (Y258A, ML194–5AA, and HI228–9AA) were initially activated in vitro by MKK6\(^{EE}\). For each substrate the activity of activated p38\(\alpha^{wt}\) was normalized to 100% and nonactivated wild type was used as a negative control. Mutagenesis of Tyr\(^{258}\) and ML194–5 into Ala decreased the phosphorylation level of ATFII to approximately 25% and 3%, respectively. In contrast, the double mutant HI228–9AA had no significant influence on ATFII phosphorylation levels. For MK2 only, mutagenesis of ML194–5AA dramatically decreased the phosphorylation levels. Both Elk-1 and MBP have a similar phosphorylation profile, whereas all three mutants show decreased phosphorylation levels to approximately 25%, 4, and 72% for Y258A, ML194–5AA, and HI228–9AA, respectively. The results show the average of two independent experiments (each in triplicates) and error bars are shown. 

**TABLE 2**

|                | GST-ATFII | GST-Elk-1 |
|----------------|-----------|-----------|
|                | \(K_m\)  | \(K_{cat}\) | \(K_{cat}/K_m\) | \(K_m\)  | \(K_{cat}\) | \(K_{cat}/K_m\) |
| wt             | 119 100 0.84 | 472 6 0.013 |
| Y258A          | 297 57 0.19 | 244 1 0.005 |
| HI228–9AA      | 445 400 0.89 | 201 2 0.011 |
| D361N          | 114 100 0.87 | 483 7 0.014 |

a significant decrease in their autophosphorylation capabilities (Figs. 4c and 5b). An in vitro kinase assay was performed using ATFII as a substrate to examine the influence of the W187A mutation on the intrinsic activity of the active mutants. The ATFII phosphorylation assay showed a dramatic decrease in phosphorylation levels. The activation capability of the p38\(\alpha^{D176A/W187A}\) mutant was decreased by a factor of nine, whereas the p38\(\alpha^{F327S/W187A}\) showed no activity (Fig. 5C).

**Cell Culture Assay of p38\(\alpha\) DEF Pocket Mutants**—To examine whether the DEF pocket analyzed in this study plays a role in living cells, the relevant mutants were transiently expressed in p38\(\alpha^{H9251}\) ME Cells with co-expression of MKK6\(^{EE}\). Western blot analysis showed that the DEF mutants were phosphorylated in situ by MKK to a similar level as the wild type protein (Fig. 6a). The effect of the DEF pocket mutants on phosphorylation of MK2 and Elk-1, which were co-transfected, was examined, showing that the phosphorylation levels of MK2 were not affected by the mutagenesis of Tyr\(^{258}\) or His\(^{228}\)–Ile\(^{229}\) but the
phosphorylation level of Elk-1 was significantly decreased by mutagenesis of Tyr258. For the p38\(\alpha\)/H9251 ML194–5AA mutant, no phosphorylation was observed for either of the substrates. Taken together, these observations are of high correlation with the in vitro results.

p38\(\alpha\) intrinsically active mutants have been shown to be spontaneously phosphorylated in cell culture (33). HEK293 cells were transfected with the D176A intrinsically active mutant or combined with DEF pocket mutants to determine whether autophosphorylation is mediated by the DEF pocket. The p38\(\alpha\)^D176A/ W187A mutant was also examined. Western blot analysis showed a decrease in the spontaneous autophosphorylation of D176A, probably resulting from mutations in the DEF pocket or W187A mutation (Fig. 6B).

We then examined if formation of the DEF pocket in p38\(\alpha\) is required for the natural activation of the p38\(\alpha\) cascade by determining the ability of the DEF pocket mutants to induce transcription of a reporter gene driven by an AP-1-responsive cis element. These elements serve as binding sites for transcription activators of the AP-1 family (34, 35) and p38\(\alpha\) was shown to stimulate AP-1 activity via several of its components (e.g. ATF2 and cAMP-response element-binding protein). HEK293 were co-transfected with the different p38\(\alpha\) mutants, MKK6\^{EE}, and the AP1-luciferase constructs. There was a significant decrease in luciferase activity for the p38\(\alpha\)^Y258A but not the p38\(\alpha\)^HI228–9AA mutant. As expected, the p38\(\alpha\)^ML194–5AA mutant decreased luciferase activity to the basal activity similar to the activity measured for an empty vector (Fig. 6C).

DISCUSSION

The functionality of living cells requires tight regulation of the proteins mediating the different signals. One of the key signaling pathways in eukaryotic cells is the MAPKs cascade. Two main regulation modes of signaling are mediated by protein-protein interactions in the MAPK signaling cascade. One involves direct interactions between proteins at specific recognition sites, whereas the other is directed by scaffold proteins.

For MAPKs, it has been shown that the specificity for substrates is exclusively derived from docking site interactions rather than interactions in the active site (36, 37). Of the two

FIGURE 3. a, Western blot analysis of the MKK6\^{EE}-activated p38\(\alpha\)^D316N mutant using the anti-p-p38 antibody (upper image). The analysis reveals similar phosphorylation levels to those of the p38\(\alpha\) wild type indicating that Asp316 is not critical for the interaction of p38\(\alpha\) with its upstream activator MKK6. Anti-p38 antibody verified the amount of proteins assayed (lower image). b, In vitro paper-spotted kinase assay of the activated D316N mutant against the four substrates in comparison to activated p38\(\alpha\)^wt, which was set to 100% for each substrate. The results show a significant decrease in the phosphorylation level of only MK2 to ~30%, whereas the levels for the remaining substrates are increased. The results show the average of two independent experiments (each in triplicates) and error bars are shown. c, qualitative results of the kinase assay comparing the phosphorylation levels of Elk-1 (left) and Elk-1ΔD (right) lacking the D-domain region (amino acid 310–328) by activated p38\(\alpha\)^wt. Elk-1ΔD is phosphorylated to a higher degree than Elk-1 (upper image). Coomassie staining verifies equal amounts of each substrate (lower image). d, quantitative results of the kinase assay of the DEF pocket mutants and the D316N mutant against Elk1ΔD reveals similar phosphorylation levels as observed for the Elk1, which might imply that the CD docking region is less essential for the Elk1 interaction with p38\(\alpha\). The results show the average of two independent experiments (each in triplicates) and error bars are shown.

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characterized MAP kinase docking sites, the CD region is the principal region accountable for MAP kinase specificity toward upstream activators, substrates, and phosphatases. The DEF pocket, the second docking site, is distinctive for substrates formed mainly in the active dually phosphorylated state of the ERK and p38, and located in the C-lobe proximal to the catalytic site.

Here, the role of the DEF pocket in substrate selectivity in p38α was examined. Activation of the four substrates by p38α is influenced differently by DEF pocket mutations and could be classified into two main groups. The first includes the MK2 whose phosphorylation was not affected by DEF pocket mutations (p38αY258A, p38αML194–5AA, and p38αH1228–9AA) and did not contain putative F-site residues near its phosphorylation site. The second group including ATFII, Elk-1, and MBP, can be defined as substrates whose phosphorylation was mediated by interactions with the DEF pocket of p38α. This classification into two groups was also supported by experiments where Asp316, one of the essential acidic residues of the CD region, was mutated into Asn. D316N significantly decreased the phosphorylation of MK2 but not the other three substrates. The notion that these two spatially segregated docking regions interact differently with substrates has already been suggested for ERK2 (16, 38).

In this context, it was already shown in vivo and in vitro that phosphorylation of transcription factors c-Fos and Elk-1 is principally mediated by DEF pocket interactions, whereas phosphorylation of RSK (90-kDa ribosomal S6 kinase, a member of the MAPKAPK family) is mediated by the CD domain (16). In addition, it was also shown that in ERK2, the induction of epithelial to mesenchymal transformation is mediated by DEF pocket-dependent signaling events (38). The results for ERK2 showed that the phosphorylation of transcription factors is DEF-dependent, whereas the activation of kinase substrates is DEF-independent, as we now also show for p38α. The results of the luciferase assay indicate that the DEF pocket is also essential for activating the p38α signaling cascade. Thus interactions through any of the two docking regions in p38α can mediate different signaling events. In addition, we have shown that a short peptide derived from Elk-1 containing the FQF motif shows an inhibitory effect in ATFII phosphorylation, whereas the mutated peptide lacking the FQF (AQA) motif did not (Fig. 7). These results suggest that the DEF site binders could be optimized to become selective p38α inhibitors.

Within the DEF-dependent subgroup of the three substrates, there are differences in their respective phosphorylation levels, which could result from variations in their F-sites. Only Elk-1 contains the characterized FXF canonical motif (defined as the F-site) downstream to its phosphorylation sites (12, 19). Hydrophobic/aromatic residues (Phe-Leu and Phe-Ser-Trp), which may serve as F-sites, were found in ATFII and MBP sequences,
respectively, downstream to their phosphorylation site (Fig. 5a). Although they do not contain the canonical FXF motif, these hydrophobic segments may bind the DEF pocket although they would be accommodated differently in the hydrophobic DEF pocket of p38α/H9251. Presumably each substrate interacts somewhat differently with the DEF pocket, thus contributing to the specificity of the each substrate. The kinetic experiments for substrates Elk-1 and ATFII also support this assumption because the kinetic parameters of the DEF pocket mutants differ. The variation in the binding regions in the substrates that participate in binding the DEF pocket could also indicate a certain plasticity of the latter, which could also provide indications of how autophosphorylation in p38α/H9251 occurs.

Activation by autophosphorylation is one of the main self-regulating mechanisms of kinases occurring either in cis (intramolecular) or trans (intermolecular). For many kinases autophosphorylation occurs upon stimulation or inhibition (ligand binding, phosphorylation, etc.) (39). Although it was previously thought that throughout evolution MAPKs lost their autophosphorylation capabilities, recent results show that p38α and ERK2 can also be autophosphorylated. The alternative activation pathways in p38α/H9251 are probably the best indication for the involvement of autophosphorylation in their activation (31, 32, 40). In addition, intrinsically active mutants of p38αs and ERKs are also shown to be activated by autophosphorylation (24, 27, 41–43). For p38α/H9251 it was shown that autophosphorylation of intrinsically active mutants and TCR-induced activation occur in trans (24, 44). Two aromatic residues, Trp187 and Tyr188, downstream to the Thr180 phosphorylation site of p38α/H9251 form a putative p38α F-site (Fig. 5a). The autophosphorylation kinase assays reveal that mutating either the DEF pocket residues or Trp187 to Ala significantly decreases the autophosphorylation capability of the intrinsically active mutants, as was also shown in cell culture assays. In addition, the W187A mutation combined with the intrinsically active mutants resulted in a dramatic decrease in the intrinsic activity probably due to low autophosphorylation levels. The autophosphorylation results of the DEF pocket mutants and W187A experimentally validate.
results show high coloration to the 5AA mutant, no phosphorylation was observed for both substrates. These phosphorylation levels of Elk-1 but no effect on the MK2. Conversely, for the ML194 –9AA mutant shows a slight decrease in the phosphorylation capabilities of p38α.

The HI228 –9AA mutant shows a slight decrease in the phosphorylation levels of Elk-1 but no effect on the MK2. The HI228 –9AA mutant was normalized to 100%. These results clearly show a significant decrease in ATFII phosphorylation to ~50% by the F-site peptide with no apparent effect by the negative control peptide.

### FIGURE 7. Inhibition of the ATFII phosphorylation activity of dually phosphorylated p38αwt by Elk-1 F-site derived peptides.

p38αwt was initially activated in vitro by MKK6EE and the phosphorylation levels of ATFII in the presence of increasing concentrations of the peptides (between 0.1 μM to 1 μM) were monitored by a paper-spotted kinase assay. The Elk-1 F-site-mutated peptide, where the FQF motif was mutated into AQA, was used as a negative control (the full sequence is shown in the upper part of the figure). The phosphorylation levels of ATFII in the presence of 0.1 μM Elk-1 F-site mutant was normalized to 100%. These results clearly show a significant decrease in ATFII phosphorylation to ~50% by the F-site peptide with no apparent effect by the negative control peptide.

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