Modular structure of a docking surface on MAPK-phosphatases

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Running title: Modular structure of a docking surface of MKPs
Abstract
MAP kinases (MAPKs) must be precisely inactivated to achieve proper functions in the cells. Ten members of dual-specificity phosphatases specifically acting on MAPKs, termed MKP, have been reported. Each member has its own substrate specificity that should be tightly regulated. However, the molecular mechanism underlying the regulation of the specificity is largely unknown. In the MAPK signaling pathways, docking interactions, which are different from transient enzyme-substrate interaction, are known to regulate the enzymatic specificity. Here we have identified and characterized a docking surface of MKPs. Our results show that a docking surface is composed of a tandem alignment of three sub-regions (modules); a cluster of positively charged amino acids, a cluster of hydrophobic amino acids, and a cluster of positively charged amino acids (positive-hydrophobic-positive). This modular structure well fits the docking groove on MAPKs, we have previously identified, and may contribute to regulating the docking specificity of the MKP family. The position, number, and species of charged amino acids in each module including the central hydrophobic sub-region are important factors in regulation of docking to specific MAPKs. This modular structure in the docking interaction may define a novel model of protein-protein interaction that would also regulate other systems.
Introduction

MAP kinase (MAPK) cascades convey a signal in the form of phosphorylation events. MAPKs are phosphorylated by MAP kinase kinases (MAPKKs), phosphorylate various targets, and are dephosphorylated and inactivated by several MAPK-phosphatases (MKPs). There are three major subgroups in the MAPK family, ERK, p38 and JNK/SAPK. ERK is activated mainly by mitogenic stimuli, whereas p38 and JNK/SAPK are activated mainly by stress stimuli or inflammatory cytokines (1-10). The signal must be transduced with high efficiency and specificity. The molecular basis for this accurate signal transduction has been addressed in recent years. MAPKs form a complex with their cognate MAPKKs, substrates, and phosphatases (11-27). The complex formation is distinct from a transient enzyme-substrate interaction through the active center. For example, a complex formation between MEK1 (a MAPKK specific for ERK) and ERK is achieved through an N-terminal portion of MEK1 outside its catalytic domain (12). A C-terminal portion outside the catalytic domain of RSK (a MAPKAPK specific for ERK) is required for a complex formation with ERK2 (16, 19, 28). The ability to form a complex well correlates with the enzymatic specificity (13, 16, 19, 24, 29-33). Such a complex formation is called a docking interaction, which is thought to regulate the enzymatic efficiency and specificity in the MAPK pathways. Recent studies provided cues to understand the molecular nature of the docking interaction. We and others identified a conserved MAPK-docking motif in the primary sequences of MAPK-interacting molecules (11, 14, 16-25). Furthermore, we have identified a docking site on MAPKs, which is located on the opposite side from the active center of the molecules in the steric structure and in the C-terminal portion of MAPKs in the primary sequence (22). As this site is commonly used in the docking interactions with activators, substrates and inactivators, we named it the CD (common docking) domain. These docking interactions through the CD domain might regulate the serial signal transduction of the MAPK cascade.
reactions. The CD domain solely, however, does not determine the docking specificity (22, 23). In search of another site on MAPKs that might regulate the docking specificity, we identified then a site near the CD domain in the steric structure on MAPKs, and called it the ED site (23). When both the CD domain and the ED site of ERK2 are engineered to mimic those of p38, the docking specificity is converted to the p38-type in the case of docking to some MAPKAPKs. We proposed thus a concept of a docking groove, which is composed of the CD domain, the ED site and the surrounding amino acids. While the CD domain is commonly important for every docking interaction, the ED site is differently utilized (23). The next open question is then which region on the MAPK-interacting molecules corresponds to the docking groove of MAPKs.

MKPs belong to a family of dual specificity phosphatases, and specifically dephosphorylate both threonine and tyrosine residues in the P-loop of MAPKs. They share sequence homology and are highly specific for MAPKs, but differ in the substrate specificity, tissue distribution, subcellular localization, and inducibility by extracellular stimuli (34-36). MKPs have been shown to play important roles in regulating the function of the MAPK family (37, 38). In mammals, ten members of MKPs have been reported, and they must be precisely regulated in their substrate specificity to avoid unexpected inactivation of MAPKs. MKPs are mainly composed of two domains, an N-terminal rhodanese-fold and a C-terminal catalytic domain. The former is responsible for their selective docking to members of the MAPK family. As the catalytic domain alone does not show strict selectivity towards the members of MAPK family (13, 31), the N-terminal domain of MKPs plays a major role in regulating their enzymatic specificity in vivo through docking interaction with MAPKs. MKP is unique in this point among MAPK-interacting molecules. For example, even high concentrations of MEK1 (a MAPKK specific for ERK) cannot activate p38 or JNK/SAPK. Therefore, there must be a fine regulatory mechanism for docking of
MKPs to MAPKs. In the N-terminal domain of all the known MKPs, exists a cluster of positively charged amino acids, which has been proposed to be a site corresponding to the CD domain (22, 24). However, whether the docking interaction of MKPs is regulated solely by this cluster alone has not been investigated yet. Here we have identified a novel region in the N-terminal domain of MKPs, that encompasses the cluster, and regulates the docking efficiency and specificity. The region (docking surface) can be subdivided into three sub-domains. Each sub-domain fits each corresponding sub-region of the docking groove on MAPKs. Notably, charged residues in each sub-domain play an essential role in recognition of each specific MAPKs. This modular structure of a docking surface of MKPs proposed here may be a molecular basis explaining the specificity of the action of MKPs towards the MAPK family.
Experimental Procedures

Plasmids

The expression vector used for human CL100/MKP-1 (39), human MKP-7 (24) and rat MKP-3 (30) is pDL-SRα-3XMyC. For ERK2 (Xenopus), JNK2 (rat) and p38α (human), pDL-SRα-HA was used.

Mutagenesis

The mutants used were constructed by PCR based mutagenesis. PCR was performed using pfu polymerase (Stratagene). A DpnI restriction enzyme (Stratagene)-treated PCR product was transformed into E.coli. Positive clones were picked up and mutagenesis was verified by sequencing.

Cell Cultures and Transfection

C2C12 cells or COS7 cells were cultured in Dulbecco's modified Eagle's medium containing 15% or 10% fetal calf serum, respectively. The cells were maintained in 5% CO₂ at 37°C. Cells were split on 35 mm dish or 60 mm dish at 2 x 10⁵ or 5 x 10⁵ cell number per dish, respectively. After 19 h, cells were transfected using Lipofectamine Plus reagent (Gibco BRL) according to the manufacturer's protocol.

Co-immunoprecipitation

Cells were lysed in 50 mM Hepes (pH 7.4), 10% glycerol, 2 mM EGTA, 2 mM MgCl₂, 1% NP-40, 1 mM PMSF and 20 µg/ml aprotinin. Tagged proteins were immunoprecipitated from cell lysates (about 3 x 10⁶ cells in each sample) by incubation with 5 µg of anti-c-Myc antibody (9E10) (Santa Cruz) or 5 µg of anti-HA antibody (12CA5) and protein A-Sepharose beads (25 µl) (Pharmacia) for 2 to 12 h at 4°C. The
precipitates were then washed twice with lysis buffer. The proteins were separated by SDS-PAGE and analyzed by immunoblotting.

**Kinase Assay**

Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 12 mM 2-glycerophosphate, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 1% TritonX-100, 1 mM PMSF, 1 mM sodium vanadate, and 20 µg/ml aprotinin). Tagged proteins were immunoprecipitated from cell lysates (about 1 x 10⁶ cells in each sample) by incubation with 2 µg of appropriate antibody and protein A-Sepharose beads (15 µl) (Pharmacia) for 2 h at 4°C. Each precipitate was washed twice with TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM PMSF, 2 mM DTT, 1 mM sodium vanadate, and 20 µg/ml aprotinin), and then washed with Tris-buffer (20 mM Tris-HCl, pH 7.5). The washed beads were mixed with substrates in a kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 100 µM ATP (2 µCi [γ-³²P]-ATP), and incubated for 10 min at 30°C. The reaction was stopped by addition of Laemmli’s sample buffer. Substrate phosphorylation was detected by autoradiography and BAS 2500 (Fuji Film) after SDS-PAGE.
Results

Both the CD domain and the ED site important for docking interaction of MAPKs with CL100/MKP-1

We previously demonstrated that both the CD domain and the ED site of p38 and ERK MAPKs are important for their interactions with MKP-3 (an MKP specific for ERK) and MKP-5 (an MKP specific for p38 and JNK/SAPK) (23). Here, we extended our analysis to other MKPs, CL100/MKP-1 (also known as 3CH134) (39, 40) and MKP-7 which we have recently identified (24). CL100 is thought to act preferentially on JNK/SAPK and p38, and weakly on ERK. In contrast, like MKP-5, MKP-7 acts specifically on JNK/SAPK and p38, and not on ERK. We used three mutant forms of ERK2: ERK2 SD in which a serine residue in the CD domain (Ser 323) was replaced by aspartic acid to mimic the CD domain of p38; ERK2 TETD in which the ED site (Thr 162 and Thr 163) was converted to that of p38; p38-like ERK2 in which both the CD domain and the ED site were converted to those of p38 (Fig. 1A; see also Ref. 23). As shown in Fig. 1B, while wild-type ERK2 or ERK2 SD did not bind to CL100, ERK2 TETD slightly bound to CL100, and p38-like ERK2 bound to it more strongly. In contrast, none of the ERK2 constructs were able to bind to MKP-7. As expected, wild-type p38 bound to CL100 strongly, and p38 CDm, in which three aspartic acids of the CD domain (Asp 313, Asp 315 and Asp 316) were replaced by asparagine, did not. As reported previously (24), wild-type p38, but not p38 CDm bound to MKP-7 (data not shown). Next, we examined the enzymatic activity of CL100. When co-expressed in the cells, p38-like ERK2 was inactivated by CL100 much more efficiently than was wild-type ERK2 (Fig. 1C). These results clearly show that both the CD domain and the ED site regulate the docking specificity of MAPKs toward CL100.

A possible site on CL100/MKP-1 that interacts with the ED site

Examining the primary sequence of CL100, we noticed another site, near the previously
identified site (residues 50 to 58) presumably interacting with the CD domain (22), that is composed of two positively charged amino acids and surrounding hydrophobic amino acids (residues 71 to 75 of CL100 (human); see Fig. 2A). We hypothesized that this site is a site corresponding to the ED site, because the ED site of p38 is composed of two negatively charged amino acids. To test this hypothesis, we created a mutant form of CL100 (CL100LMGML), in which two arginine residues (Arg 72 and Arg 74) were replaced by methionines (Fig. 2A). While wild-type CL100/MKP-1 bound to p38-like ERK2, CL100LMGML scarcely bound (Fig. 2B and C). These results suggest the possible interaction of the ED site with Arg 72 and Arg 73 of CL100.

Arginines 53, 54, 55, 72 and 74 of CL100/MKP-1 are utilized in the docking interaction with p38 and JNK/SAPK

Next, we tested whether Arg 72 and Arg 74 of CL100/MKP-1 are important for the interaction with the MAPK family members (ERK2, p38 and JNK/SAPK). As shown in Fig. 3B, CL100LMGML showed a significantly decreased ability to bind to p38α, JNK2 or p38-like ERK2 than did wild-type CL100. These results show that Arg 72 and Arg 74 are utilized in the docking interaction between CL100 and MAPKs. Neither the wild-type nor the LMGML mutant CL100 bound to ERK2. To confirm that the Arg 53, Arg 54 and Arg 55 of CL100, which are suggested to constitute the site corresponding to the CD domain of MAPKs, are essential for docking, we created mutants, in which two or three of these arginines were replaced by methionines (Fig. 3A). While wild-type CL100 strongly bound to p38α, CL100MMR or CL100MMM did not (Fig. 3C). The docking ability of the both mutants to JNK2 was also reduced compared with wild-type CL100, but the mutants still bound to JNK2 significantly (Fig. 3C). These results show that Arg 53, Arg 54 and Arg 55 of CL100/MKP-1 are important for docking to both p38 and JNK, but their contribution is less for JNK than p38. CL100MMR or CL100MMM did not bind to p38-like ERK2, implying that the
docking mode of p38-like ERK2 is similar to that of p38.

**A docking region of CL100/MKP-1**

Comparing the primary sequences of the known MKPs, we noticed that the region corresponding to residues 51 to 81 of CL100 is relatively well conserved (Fig. 4A) and that the region can be divided into three sub-regions (modules): in the first N-terminal region, a cluster of positively charged residues exists to which the negatively charged amino acids of the CD domain of MAPKs might bind; in the second central region, hydrophobic residues such as leucine, isoleucine and valine are abundant and conserved; in the third C-terminal region, positively charged residues and often surrounding hydrophobic residues exist. The third sub-region is most diverse in the amino acid composition. We hypothesized that this modular structure of an assumed docking surface of MKPs fits the docking groove of MAPKs, and regulates the docking specificity towards MAPKs (Fig. 4B). To examine whether the residues in each sub-region (module) are utilized in the docking interaction, we created several mutant forms of CL100, in which some of conserved or non-conserved residues in each sub-region were replaced by alanines or methionines (Fig. 5A). As shown in Fig. 5B, both the hydrophobic amino acids (Ile 51 and Val 52) and positively charged amino acids in the first sub-region of CL100 are essential for its docking interaction with p38. Although three amino acids seem to be involved in the docking interaction with JNK2 to same extent, their contribution was small as compared with the case of p38 (compare the lane “wt” with those of “MMR”, “MMM” and “AARRR”). The hydrophobic amino acids of the second sub-region are crucially important for the docking interaction with p38 and JNK2 (see the lanes of “MLAA” and “IVAA”). The positively charged amino acids (Arg 72 and Arg 74) and the surrounding hydrophobic amino acids (Leu 71 and Leu 75) in the third sub-region are important for docking to both p38 and JNK2, these amino acids, however, are differently utilized in each docking interaction (see the lanes
of “LRGML”, “LMGRL”, “LMGML”, “ARGRL”, “LRGRA” and “ARGRA”).

Generally, the mutations in the third sub-region had severer effects on the interaction with JNK2 than that with p38. For example, while CL100LMGRL bound to p38 as efficiently as wild-type CL100 did, it bound to JNK2 much more weakly than wild-type CL100 did. Next, we mutated arginines in the first and third sub-regions to lysines. The R to K mutation is supposed to scarcely induce gross conformational changes in the protein, as is the case with the K to R mutation that is routinely used to produce a kinase-dead form of protein kinases in general. As shown in Fig. 5C, while the first sub-region mutants, CL100KKR and CL100KKK, showed a reduced ability to bind to p38, they bound to JNK2 as efficiently as wild-type CL100 did. In contrast, while the third sub-region mutant CL100LKGKL bound to p38 as efficiently as wild-type CL100 did, it showed a reduced ability to bind to JNK2. These results suggest that our results obtained here reflect local changes in the direct docking surface of the protein. Our results indicate that each sub-regions in the docking region of CL100 are differently utilized in docking interaction with each members of the MAPK family: the first sub-region is essential for the docking interaction with p38, but not with JNK; the second sub-region is essential for the docking to both p38 and JNK; the third sub-region is essential for the docking to JNK, but less important for p38. Thus, this region comprising the three sub-regions (residues 51 to 81 of CL100/MKP-1) is utilized in the docking interaction. We call this region a docking surface.

A docking surface of MKP-7 is utilized in docking interaction with MAPKs.

We then examined whether a corresponding region in MKP-7 is also utilized in its docking interaction. MKP-7 is specific for JNK and p38. Wild-type MKP-7 efficiently bound to both p38 and JNK2 (Fig. 6B). The first sub-region mutants of MKP-7, MKP-7RRR and MKP-7LRR, in which Lys 55 of the first sub-region was replaced by arginine and leucine, respectively (Fig. 6A), showed a decreased ability to
bind to p38. The latter showed a more decreased ability than the former. Remarkably, these two mutants bound to JNK2 as efficiently as did wild-type MKP-7 (Fig. 6B). Thus, the mutations in the first sub-region of MKP-7 induce severer effects on its interaction with p38 than that with JNK. This situation is similar to that in CL100. The third sub-region mutants of MKP-7, MKP-7MHMV and MKP-7KHKA, in which lysines or Val 77 in the third sub-region was mutated, respectively (Fig. 6A), bound to p38 as efficiently as did wild-type MKP-7, whereas they showed a decreased ability to bind to JNK2 (Fig. 6B). Thus, the mutations in the third sub-region of MKP-7 resulted in severer defects in the docking interaction with JNK than with p38. The third sub-region of MKPs is therefore in common important for docking to JNK and is less important for docking to p38. However, for docking to p38, the third sub-region has some more important role in the case of CL100 than in the case of MKP-7.

**Efficiency of the enzymatic action of various mutants of CL100/MKP-1 and MKP-7**

We measured the enzymatic ability of various mutants of MKPs described above. When expressed in cells, wild-type CL100 strongly inactivated p38, but CL100MMM, which fails to dock to p38 (see Fig. 5B) did not inactivate p38 efficiently (Fig. 7A, left). Both wild-type CL100 and CL100MMM inactivated JNK2 efficiently; CL100MMM was slightly less effective (Fig. 7A, right). Wild-type MKP-7 and MKP-7MHML also efficiently inactivated p38, and MKP-7LRR was less effective (Fig. 7B, left). While wild-type MKP-7 and MKP-7LRR inactivated JNK2 efficiently, MKP-7 MHML had a reduced ability to inactivate JNK2 (Fig. 7B, right). Collectively, the efficiency of enzymatic ability of various mutants of CL100 and MKP-7 towards p38 and JNK2 (see Fig. 7) correlated well with their docking ability towards each member of MAPKs (see Fig. 5 and Fig. 6).
A charged amino acid residue in the second sub-region participates in docking interactions.

Examining the primary sequence of the second sub-region (module) of MKPs, we noticed that a charged residue exists in the center of the module (Fig. 4A and 7A). MKPs acting on JNK/SAPK and p38 have a negatively charged amino acid (Asp or Glu), and MKPs acting on ERK have a positively charged one (Arg). We discuss later about PAC-1 (see Discussion). JNK/SAPK, but not p38, has a positively charged amino acid near the CD domain in the docking groove (Fig. 4B; see also the crystal structure of JNK3 (PDB Id: 1JNK)) (40). We speculated that this positively charged amino acid interacts with the negatively charged amino acid in the second module of MKPs. To examine this hypothesis, we created CL100ER and MKP-7EA, in which the negatively charged amino acid was replaced by arginine and alanine, respectively (Fig. 8A). MKP-7TREA was created to mimic the sequence of the second module of MKP-3 (see discussion). CL100ER, MKP-7EA and MKP-7TREA bound to p38 with the same or higher efficiency comparing with wild-type ones, but their ability to bind to JNK2 was significantly reduced (Fig. 8B). Thus, the negatively charged amino acid in the second module of MKPs is important for the proper docking to JNK, but not for p38, as we hypothesized. ERK has a negatively charged amino acid at the same position (Fig. 4B; see also the crystal structure of ERK2 (PDB Id: 1ERK)) (42). When the positively charged amino acid of the second module of MKP-3 was replaced by a negatively charged one (MKP-3RE), the ability to bind to ERK2 was reduced (Fig. 8B). Collectively, the charged amino acid residue located in the center of the second module of MKPs is important for the proper docking to JNK/SAPK or ERK.
Discussion

In this study, we have identified a region on MKPs, that serves as a surface for docking to MAPks. We tentatively call the region the docking surface. Recently, the solution structure of the N-terminal domain (rhodanese-fold) of MKP-3 was reported (43). In agreement with our results, their results with 2D $^{13}$N-edited transverse relaxation-optimized spectroscopy (TROSY) showed that the docking surface region of MKP-3 constitutes a direct interaction surface for binding to ERK2. We propose here that the docking surface is composed of three modules. The first module consists of positively charged amino acids surrounded by hydrophobic amino acids. This module has been proposed to be a direct binding site for the CD domain. This is because, firstly, this module is conserved in all MKPs, as is the case with the CD domain in MAPks. Secondly, the number of the consecutive positively charged amino acids in the first module well corresponds to the number of the negatively charged amino acids in the CD domain of MAPks. Supporting this idea, the ability of MKP-5 to dock to p38 was reduced in proportion to reducing the number of positively charged amino acids by neutral ones (22). The CL100KKK mutant, in which all the three arginine residues of the first module were replaced by lysines, had a more decreased ability to bind to p38 than the CL100KKR mutant, in which the first two arginine residues were replaced by lysines (Fig. 5). On the other hand, the first module does not necessarily play an essential role in the docking interaction with JNK/SAPK. Even when all the positively charged amino acids of the first module of CL100 were replaced by methionines, the docking ability of CL100 to JNK2 was not so much reduced comparing with that to p38. Recently, Slack et al. (25) reported essentially the same results for CL100. The same is true in the case of MKP-2, MKP-5, or MKP-7 (22, 44, and this study). Collectively, the first module is essential for the docking interaction with p38 or ERK, but not with JNK/SAPK. The second module is mainly composed of hydrophobic residues. These hydrophobic residues are important for the docking
interaction of CL100 with both p38 and JNK/SAPK. They are well conserved in all MKPs, and are suitable for binding to the cluster of aromatic amino acids surrounding the CD domain in the docking groove of MAPKs. There is one charged amino acid residue in the center of the second module. CL100, MKP-2, MKP-5, MKP-7 and hVH5 (MKPs capable of acting on JNK/SAPK) have a negatively charged amino acid at this position (see Fig. 4A). The other MKPs (MKPs acting mainly on ERK) have a positively charged amino acid. The negatively charged amino acid in the second module of MKPs acting on JNK/SAPK is suitable for interacting with a positively charged amino acid in the docking groove of JNK/SAPK near the CD domain, which has been shown to relatively protrude from the neighboring amino acids (41, and see Fig. 4B). Supporting this idea, when the negatively charged amino acid in the second module was replaced by a neutral or positively charged one, the ability of these mutant forms of MKPs to bind to JNK2 was significantly reduced (Fig. 8B). Their ability to dock to p38 was not weakened, but was rather strengthened. This may be because p38 has negatively charged amino acids at the corresponding site in the docking groove (see Fig. 4B). ERK also has a negatively charged amino acid at the position. MKPs acting on ERK have a positively charged amino acid in the second module. When the positively charged amino acid of MKP-3 was converted to a negatively charged one, the docking to ERK2 was significantly reduced (Fig. 8B). PAC-1 (45, 46) is reported to act mainly on p38 and less efficiently on JNK/SAPK (47). This might be consistent with the fact that PAC-1 has a positively charged amino acid instead of a negatively charged amino acid in the second module (see Fig. 4A). The third module is composed of positively charged amino acids and hydrophobic amino acids. The amino acid composition of this module is less conserved in MKPs than that of the other two modules. The third module is differently utilized in each docking interaction. While the third module is important for CL100 to dock to p38, it is less important for MKP-7. However, for the both MKPs, this module is essential for docking to JNK/SAPK. The
ED site on MAPKs is utilized in some docking interactions, but not in others (26). Then, we speculate that the third module is a site corresponding to the ED site (Fig. 9). Collectively, the docking surface of MKPs is a strong candidate region corresponding to the docking groove of MAPKs. Our model presented here can explain the molecular basis for the docking specificity towards three major members of MAPKs (ERK, p38 and JNK/SAPK) and further support the model of the docking groove (Fig. 9).

We created several chimeric proteins of MKPs, in which their docking surface was exchanged with that of other MKPs (data not shown). Chimeric MKP-7 containing the docking surface of CL100 or MKP-3, chimeric CL100 containing the docking surface of MKP-3 or MKP-7, and chimeric MKP-3 containing the docking surface of CL100 or MKP-7 were examined for their ability to dock to MAPKs. However, all the mutant forms of MKPs failed to bind to any of MAPKs (ERK2, p38 or JNK2) (data not shown). This may imply that the swapping of the docking surface may induce gross conformational changes. In addition, we produced several other mutant forms of MKPs and examined their ability to bind to MAPKs. MKP-3RRR (L63R) and MKP-3RRR_RE (L63R, R74E), which are expected to mimic CL100 in its docking surface, did not bind to p38 or JNK2 (data not shown; see also Fig. 8 for the mutation introduced in each protein). MKP-7LRR, MKP-7EA, MKP-7TREA or CL100ER did not bind to ERK2 (data not shown; see Fig. 6 and 8 for the mutation introduced in each protein). We further created additional mutant forms of CL100 and MKP-7 to mimic MKP-3, namely, CL100LRR (R53L), CL100RLYER (R53L, E63R), MKP-7LRRYEA (K55L, E67A), MKP-7LRRYTREA (K55L, T66R, E67A), MKP-7LRRYQK (K55L, Q60K), MKP-7LRRYQKYTEA (K55L, Q60K, E67A) and MKP-7RLYQKYTEA (K55L, Q60K, T66R, E67A). However, none of them showed an enhanced ability to dock to ERK2 (data not shown). For most proteins, it was difficult to change their binding specificity to another molecule. It was often observed that mutations introduced in the binding surface result in either neutral phenotypes or a general loss in the binding. This may
be interpreted as follows. The high-affinity binding may generally require efficient exploitation of the multiple potential interactions available on the binding partner, in which each amino acids involved in each interaction are appropriately enmeshed in the docking surface of the binding partners. Then, mutations designed to introduce new interactions may often result in disruption of the binding because they may induce distortion in the appropriate configuration of each amino acids involved.

Wild-type ERK2 could not bind to CL100, although the docking groove of ERK2 may be able to fit the docking surface of CL100. This may be because the affinity is not strong enough for a tight docking. Then, when the electrostatic interaction is strengthened by adding three more negatively charged amino acids in the docking groove of ERK2, ERK2 becomes to bind to CL100 strongly. In other words, CL100 can inactivate ERK2 when an excess amount of the molecule exists. This might account for the published results showing that CL100 could inactivate ERK2 when overexpressed in cultured cells or Xenopus oocytes (40, 48, 49). Note that the expression level of CL100 is low in Fig. 1C in the present study. As the docking surface of MKP-7 might not fit the docking groove of ERK2, the docking ability between MKP-7 and ERK2 could not be changed by merely adding three more negatively charged amino acids in the docking groove of ERK2. The fitness might be determined by the overall conformation of the docking surface and/or even the whole molecule. We should also keep it in mind that enzymatic reactions are regulated by their subcellular localization. CL100/MKP-1 is nuclear, and ERK2 is known to translocate from the cytoplasm to the nucleus upon stimulation (39, 50). Then, these two molecules might accumulate in the nucleus enough to associate with each other, even though their affinity is weak.

Our results presented here show that charged amino acid residues play an important role in determining the binding affinity and specificity of MKPs, and strongly suggest that the electrostatic interactions play a crucial role in the docking interactions between
MKPs and MAPKs. This molecular mechanism might provide a model for studying protein-protein interactions.
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Footnotes

1The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MKP, MAPK-phosphatase; TBS, Tris-buffered saline.
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**Figure Legends**

**Fig. 1.** The CD domain and the ED site regulate the docking interaction between MAPKs and CL100/MKP-1.  

**A.** The primary sequences of the ED site and the CD domain of p38 and ERK2 are shown. The numbers shown are the sequences of *Xenopus* ERK2 and human p38. The mutations introduced in p38-like-ERK2 (p38L-ERK2) are also shown.  

**B.** The binding abilities between MAPKs and CL100/MKP-1 or MKP-7ΔC. MKP-7ΔC is described elsewhere (24). Lysates of C2C12 cells each transfected with HA-wild-type ERK2 (wt), HA-ERK2 SD (S323D), HA-ERK2 TETD (T157E, T158D), HA-p38-like ERK2 (p38L) (T157E, T158D, S323D), HA-wild-type p38 (wt), or HA-p38 CDm (D313N, D315N, D316N) were mixed with the lysates of C2C12 cells transfected with Myc-CL100/MKP-1 or Myc-MKP-7ΔC, and the mixture was subjected to immunoprecipitation with anti-HA antibody. The lysates of C2C12 cells transfected with Myc-CL100/MKP-1 (3 plates of 60-mm dish) or Myc-MKP-7ΔC (2 plates of 60-mm dish) were equally divided into 6 or 4 samples, respectively. Co-immunoprecipitated Myc-CL100/MKP-1 or Myc-MKP-7ΔC was detected (αMyc (IP:αHA)). Comparable amounts of HA-MAPKs were immunoprecipitated in each lanes (αHA (IP:αHA)).  

**C.** An expression plasmid of HA-wild-type ERK2 (wt), or HA-p38-like ERK2 (p38L) (0.5 µg for a 35-mm dish) was transfected into COS7 cells with the increasing amounts of SRα-Myc-CL100/MKP-1. Plasmid concentrations were maintained constant using SRα empty plasmids. After 36 h, the cells were stimulated by 10% FCS for 20 min after incubation in serum-free medium for 16h. Immune complex kinase assays were then performed using myelin basic protein. Phosphorylation of substrates was detected by autoradiography (Activity). The amounts of HA-ERK2 in each immunoprecipitate were determined by Western blotting (αHA (IP)). The expression level of Myc-CL100/MKP-1 in each lane was also determined (αMyc (whole)). Note that the expression levels of CL100/MKP-1 are low.
Fig. 2  Arg 72 and Arg 74 are important for the docking interaction with p38-like ERK2.  
A, The primary sequences of CL100/MKP-1 is shown. The numbers shown are the sequence of human CL100/MKP-1. Shaded amino acids indicate the site presumed to be involved in the docking interaction. The site presumed to bind to the CD domain is underlined. Arg 72 and Arg 74 were replaced by methionines in the CL100LMGML mutant. This region locates in the N-terminal portion of MKPs in the rhodase homologous domain (RHOD; rhodanese-fold) outside the catalytic domain.  

B, The binding between CL100/MKP-1 and ERK2. Lysates of C2C12 cells each transfected with HA-wild-type ERK2 (wt) or HA-p38-like ERK2 (p38L) were mixed with the lysates of C2C12 cells transfected with Myc-wild-type CL100/MKP-1 (wt) or Myc-CL100LMGML mutant (LMGML), and the mixture was subjected to immunoprecipitation with anti-HA antibody. The lysates of C2C12 cells transfected with Myc-wild-type CL100/MKP-1 or Myc-CL100LMGML (2 plates of 60-mm dish each) were equally divided into 3 samples. Co-immunoprecipitated Myc-CL100/MKP-1 was detected (αMyc (IP: αHA)). Comparable amounts of HA-ERK2 were immunoprecipitated in each lanes (αHA (IP: αHA)). The expression levels of Myc-CL100/MKP-1 were examined (left panel).  

C, The experiments were performed as in B, except that the proteins were immunoprecipitated with anti-Myc antibody.

Fig. 3  The importance of R53, R54, R55, R72 and R74 of CL100/MKP-1 in the docking interaction with several MAPKs.  
A, The mutations introduced in CL100/MKP-1 are indicated.  
B, The bindings between Myc-CL100LMGML and HA-MAPKs were examined as in Fig. 1B. The proteins co-immunoprecipitated with HA-MAPKs were examined by immuno-blotting using anti-Myc antibody (αMyc (IP: αHA)). Comparable amounts of HA-MAPKs were immunoprecipitated (lower
left panel). The expression levels of Myc-CL100/MKP-1 were also examined (lower right panel). C, The binding between Myc-CL100MMR (R53M, R54M) or CL100MMM (R53M, R54M, R55M) and HA-MAPKs were examined as in A.

Fig. 4 The docking surface of MKPs A, Primary sequences of the region (the docking surface) on MKPs corresponding to residues 50 to 77 of CL100/MKP-1 are aligned. The red characters indicate positively charged amino acids. The blue characters indicate negatively charged amino acids. The green characters indicate hydrophobic amino acids. Bold-types indicate conserved amino acids. This region locates in the rhodanese homology domain (RHOD; rhodanese-fold) outside the catalytic domain. This region can be divided into three modules (module 1, 2 and 3). B, The docking surface might bind to the docking groove of MAPKs. Red circles indicate the CD domain, and blue circles indicate the ED site. Negatively charged amino acids or positively charged ones in the docking groove is indicated as – or +. Aromatic residues are indicated as ar. This schematic was created on the basis of the crystallographic data of MAPKs (41, 42, 51, 52).

Fig. 5 The positively charged amino acids and hydrophobic amino acids in the docking surface are both important for the docking interaction of CL100/MKP-1. A, Amino acids replaced in each mutant form of CL100/MKP-1 are shown. CL100MMR (R53M, R54M), CL100MMM (R53M, R54M, R55M), CL100AARRR (I51A, I52A), CL100MLAA (M56A, L58A), CL100IVAA (I61A, V62A), CL100LMGRL (R68M), CL100LRGML (R70M), CL100LMGML (R68M, R70M), CL100ARGRL (L67A), CL100LRGRA (L71A), CL100ARGRA (L67A, L71A), CL100KKR (R53K, R54K), CL100KKK (R53K, R54K, R55K) and CL100LKGKL (R68K, R70K) were tested. Three modules are indicated by underlines. B, C, The bindings between several mutant forms of CL100/MKP-1 and p38 (vs. p38) or JNK/SAPK (vs. JNK2) were
examined. Lysates of C2C12 cells each transfected with HA-p38, or HA-JNK2 were mixed with the lysates of C2C12 cells transfected with Myc-CL100/MKP-1, and the mixture was subjected to immunoprecipitation with anti-HA antibody. The lysates of C2C12 cells (9 plates of 60-mm dish) transfected with HA-p38 or HA-JNK2 were divided equally into 18 samples (150 μl each), and then mixed with the lysates (1/2 of 60-mm dish) of C2C12 cells expressing Myc-CL100/MKP-1 (1/2 of 60-mm dish). Co-immunoprecipitated Myc-CL100/MKP-1 was detected (αMyc (IP:αHA)). The expression levels of Myc-CL100/MKP-1 were examined (expression).

**Fig. 6** The docking surface of MKP-7  
*A,* Primary sequence of the docking surface of MKP-7 (human). The amino acids replaced in each mutant form of MKP-7 are indicated. MKP-7RRR (K55R), MKP-7LRR (K55L), MKP-7MMHV (K74M, K76M) and MKP-7KHKA (V77A) were created. MKP-7ΔC was used. Each module is separately indicated by underlines. The first and third modules (underlined with bold-type) were examined.  
*B,* The bindings of the mutant forms of Myc-MKP-7ΔC to HA-p38 or HA-JNK/SAPK were examined as in Fig. 5B.

**Fig. 7** Enzymatic activities of several mutant forms of MKPs towards p38 and JNK/SAPK were examined.  
*A,* An expression plasmid of HA-p38 (0.5 μg for a 35-mm dish) or HA-JNK2 (0.25 μg for a 35-mm dish) was transfected into COS7 cells with SRα-Myc-CL100/MKP-1 (0.25 μg for a 35-mm dish). Plasmid concentrations (1 μg for a 35-mm dish) were maintained constant using SRα empty plasmids. Wild-type CL100/MKP-1 and CL100MMM were examined. After 20 h, the cells were stimulated by 0.4 M sorbitol for 10 min. Immune complex kinase assays were then performed using myelin basic protein. Phosphorylation of substrates was detected by BAS2500 (Fuji Film) (Activity). The amounts of HA-MAPK in each immunoprecipitate were determined by Western blotting (αHA (IP)). The amounts of
Myc-CL100/MKP-1 in each lane were also determined (αMyc (whole)). Note that the expression levels of CL100/MKP-1 were low.  

**B,** The enzymatic ability of MKP-7 was examined as in A. Wild-type MKP-7, MKP-7RL and MKP-7MHM were examined. 0.125 µg of SRα-Myc-MKP-7 was used for a 35-mm dish. MKP-7ΔC was used. Note that the expression levels of MKP-7 were low.

**Fig. 8** A charged residue in the second module of MKPs is required for efficient docking to JNK/SAPK or ERK.  

**A,** The amino acids replaced in each mutant form of MKPs are shown. CL100ER (E63R), MKP-7EA (E67A), MKP-7TREA (T66R, E67A), MKP-3RE (R74E) were created. MKP-7ΔC was used.  

**B,** The bindings of the mutant forms of MKPs to p38, JNK/SAPK or ERK2 were examined as in Fig. 5B.

**Fig. 9** Schematic representation of the docking interaction between MAPKs and MKPs.  

**A,** The docking groove of MAPKs consisting of the CD domain and the ED site binds to the docking surface of MKPs, which can be subdivided into three modules.  

**B,** Each module of the docking surface of MKPs determines the specificity towards MAPKs.
**A**

ED site

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CD domain

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**B**

CL100 MKP-7 CL100

IgG

αMyc (IP:αHA)

IgG

αHA (IP:αHA)

**C**

ERK2 wt  p38L-ERK2

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Activity

αHA (IP)

αMyc (whole)

CL100

Fig. 1, Tanoue et al.
A

CL100/MKP-1

N

RHOD
catalytic domain

C

wt TIVRRRAKGMGLEHIVPNAELRGMLA

LMGML

B

CL100 wt LMGML

expression

CL100 wt LMGML

αMyc (IP:αHA)

αHA (IP:αHA)

ERK2 wt P38L wt P38L

C

expression

wt p38L

ERK2

αHA (IP:αMyc)

αMyc (IP:αMyc)

CL100 wt LMGML wt LMGML

Fig. 2, Tanoue et al.
A

CL100/MKP-1

50

TIVRRRAKGMLEHIVPNAELRGRLLA

wt

MMR

MM

MM

MM

LMGML

M M

B

CL100

wt

LMGML

IgG

αMyc (IP:αHA)

IP

αHA (IP)

CL100

expression

αMyc (whole)

C

CL100

wt

MMR

MMM

IgG

αMyc (IP:αHA)

IP

αHA (IP)

expression

αMyc (whole)

Fig. 3, Tanoue et al.
A

RHOD catalytic domain

CL100  TIVRRRAK-G--AMGLEHV--P--NAELRGLL
MKP-2   TIVRRRAK-G--SVSEQLP--AEEEVARL
PAC-1   ALLRRRAARGPPA--VSR--VPL--DEALRT

MKP-3   GIMLRLQK--NLPR--ALFT-RCEDRFR
Pyst2   GLMLRRRKG--NLPI--STIP-NHADKFR
MKP-4   ALLLRLRRG--LSVRLLP--GPLLQP--

MKP-5   KISRSSRLQG--KITVLDSLIS--CREDKDSFR
MKP-7   KLMKRLQIQ--KVLEETIQ--HSAKHKVD
hVH3    SVVLRRARGG--AVSARY--VPL--DEAARL

MKP-5   KISRSSRLQG--KITVLDSLIS--CREDKDSFR
MKP-7   KLMKRLQIQ--KVLEETIQ--HSAKHKVD
hVH5    KLVKRRRLQG--KVTEELIQ--PAARSQVE

module 1 2 3

B

docking groove

ERK

p38

JNK/SAPK

substrate MAPKs

p38  →  JNK/SAPK (ERK)

→  ERK

→  p38

→  JNK/SAPK

Fig. 4, Tanoue et al.
Fig. 5, Tanoue et al.
A

MKP-7

52 79

wt KLMKRRRLQIQKVLITELIQHASAKHVKDI
RRR R
LRR L
MHML MM
KHKA A

B

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Fig. 6, Tanoue et al.
Fig. 7, Tanoue et al.
A

**CL100/MKP-1**

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**MKP-7**

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**CL100/MKP-3**

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B

**CL100**

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**MKP-7**

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**MKP-3**

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Fig. 8, Tanoue et al.
**A**

![Diagram of the docking surface of MKPs](image)

**B**

*The docking surface of MKPs*

![Diagram of protein interactions](image)

Fig. 9, Tanoue et al.
Modular structure of a docking surface on MAPK-phosphatases
Takuji Tanoue, Takuya Yamamoto and Eisuke Nishida

J. Biol. Chem. published online April 12, 2002

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