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Intra- and intermolecular interactions of the catalytic domains of human CD45 protein tyrosine phosphatase

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Abstract We have investigated protein-protein interaction between distinct domains of the human CD45 cytoplasmic region using a yeast two-hybrid system. Consequently, we have found that the spacer region between two tandem PTP domains specifically interacts with the membrane-distal PTP domain (D2). This interaction is mediated by a stretch of amino acid residues in the carboxyl-terminal half of the spacer region. Although the membrane proximal region does not directly interact with either of the two PTP domains, it appears to function in stabilizing the interaction between the spacer region and D2. We also demonstrate that the interaction between the spacer region and D2 might take place intramolecularly.

Key words: CD45; Protein tyrosine phosphatase; Protein-protein interaction; Two-hybrid system

1. Introduction

CD45 is a transmembrane protein tyrosine phosphatase (PTP) expressed exclusively in nucleated hematopoietic cells, and composed of three distinct portions, a variable extracellular domain, a single transmembrane domain and a large cytoplasmic region containing two tandem PTP domains [1–3]. So far $p56^{Lck}$, $p56^{Lyn}$ and $p59^{Fyn}$ have been identified as physiological substrates of CD45, and these Src-family kinases are activated by CD45 through dephosphorylation at their negative regulatory tyrosine residue [4–10]. Thus, CD45 is assumed to serve as a positive regulator of these key kinases, thereby playing an essential role in the immediate early steps of antigen stimulated signal transduction in T- and B-cells. However, recent investigation has also suggested that CD45 negatively regulated Src-family kinases through dephosphorylation at the positive regulatory tyrosine residue [11,12]. These apparent opposite roles of CD45 may in part be explained by dynamic redistribution of CD45 in the plasma membranes, which would cause inclusion or exclusion of CD45 from a site of receptor engagement [13,14].

In contrast to the well-established physiological roles of CD45, little is known about the mechanisms by which the PTP activity in the cytoplasmic region is regulated. In this context it should be noted that most transmembrane PTPs, including CD45, have two tandem PTP domains in their cytoplasmic region. Interestingly, only the first PTP domain (D1) appeared to contribute to the intrinsic PTP activity of CD45 and little or no PTP activity has been observed with the second PTP domain (D2) [15,16]. However, expression of D1 alone in the absence or truncation of D2 resulted in very low or almost no PTP activity [16]. Thus, both D1 and D2 are necessary for expressing full PTP activity although D1 seems to be principally responsible for PTP activity while the exact role of D2 remains unclear. Contradictory to these observations, recently Felberg and Johnson reported that D1 alone expressed in *Escherichia coli* had substantial PTP activity although the stability and activity of D1 alone were 2–3 fold less than those of D1-D2 [17].

The sequence homology of D2 is less conserved than that of D1 between different transmembrane PTPs, and in some cases the key residues for PTP activity are missing in D2 [2]. These observations, together with the fact that no PTP activity has been detected with D2, suggest that D2 is not an active PTP but might play a regulatory role for D1. In this context, the most plausible possibility may be that D2 is involved in determination of substrate specificity [15]. Recently Kashio et al. reported that the D2 domain has an essential role in interaction with the ζ-chain of T-cell antigen receptor complex (TCR), by which efficient signals are transmitted to the downstream pathway via ZAP-70 [18].

As an attractive possibility for regulatory mechanisms of PTP activity, recent experiments suggest that CD45 might form a homodimer, which modulates its PTP activity. This hypothesis is based on the observation that signal transmission elicited by a chimera protein consisted of extracellular and transmembrane domains of epidermal growth factor receptor (EGF-R), and the cytoplasmic domain of CD45 was modulated by addition of EGF through possible dimerization of the chimera protein [19]. In addition, the hypothesis is strengthened by the crystal structure of a related PTP, PTPα, in which two molecules form a stable dimer by mutually protruding the membrane proximal wedge structure to the catalytic cleft of the opposite molecule [20]. Supporting this hypothesis, recently Felberg and Johnson reported that D1 of murine CD45 can form a homodimer in vitro [17]. In contrast to these observations, D1 of PTPμ and D1-D2 of LAR have been crystallized as a monomeric form [21,22].

In order to understand the molecular bases for the regula-

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Abbreviations: PTP, protein tyrosine phosphatase; PCR, polymerase chain reaction; 3-AT, 3-amino-1,2,4-triazole; ONPG, *o*-nitrophenyl β-D-galactopyranoside; CPRG, chlorophenolred-β-D-galactopyranoside; TCR, T-cell antigen receptor complex; EGF-R, epidermal growth factor receptor

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tory mechanisms of PTP activity and roles of D2 we have examined molecular interactions in the cytoplasmic region of CD45 using yeast the two-hybrid system. Consequently, we have found a specific and predominant interaction between the spacer (Sp) region and D2, which might occur intramolecularly. In addition, it appears that the membrane proximal region itself does not directly interact with any of cytoplasmic domains but rather participates in stabilizing the Sp-D2 interaction.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

The cytoplasmic region of human CD45 was dissected into five distinct domains, membrane proximal (Mp, amino acid residues 575[1]–647), PTP-D1 (D1, 647–897), Sp, 897–937), PTP-D2 (D2, 937–1211) and carboxyl-terminal (Ct, 1211–1281) domains (Fig. 1). This conventional delineation has been evolved from delineation of core phosphatase domains (D1 and D2) based on the sequence conservation analysis, and thereby denotes flanking regions as Mp, Sp and Ct [15,16]. The cDNA segments corresponding to each domain were amplified from a human thymus cDNA library (Clontech) by polymerase chain reaction (PCR) and cloned into pCR-Blunt (Invitrogen). The full-length cytoplasmic region was constructed by combining D1 and D2 regions at the *Bsm*BI site in the Sp region. After sequence verification, each DNA segment was recloned into pACT2 and pAS2-1 (Clontech). In all cases both combination of construction with pACT2 and pAS2-1 were examined for two-hybrid interaction. Expression of each fusion protein in yeast cells was confirmed by Western blotting using antibodies against either the Gal4 transactivation domain or DNA-binding domain (Clontech). Random mutagenesis of the Sp region was conducted by a PCR-mediated method using *Taq* DNA polymerase (Takara) [23].

2.2. Two-hybrid assay for protein-protein interaction

A yeast strain YRG-2 (Stratagene Cloning Systems) was made competent for DNA transfection using YEASTMAKER yeast transformation system (Clontech), and transformed with 1 μg of each plasmid DNA. Transformants were selected at 30°C using appropriate auxotrophic markers on SD-medium agar plates (Bio 101). The growth of transformants on His^- plates was investigated as a conventional indication of interactions in the two-hybrid system. 3-Amino-1,2,4-triazole (3-AT), a competitive inhibitor of the yeast His3 protein, was added to the His^- plates with various concentration (0.1–10 mM) to compare the relative strength of interactions. β-Galactosidase activity in each of the transformants was measured according to the published method using either *o*-nitrophenyl β-D-galactopyranoside (ONPG) or chlorophenolred-β-D-galactopyranoside (CPRG) as a substrate [24]. Specific activity and relative activity are the average of several independent assays using at least four independent transformants.

2.3. Other materials and methods

Standard PCR reactions were performed under the error-prone condition using Pfu DNA polymerase (Stratagene Cloning Systems). DNA sequences were determined on an ABI 310 DNA sequencer using PCR-mediated dye terminator methods. Restriction enzymes and ligase were purchased from Toyobo. Other reagents including 3-AT, ONPG and CPRG were purchased from Sigma and of the highest grade available.

3. Results

3.1. Interaction between D1 and D2

In order to examine interaction between the two major domains, D1 and D2, we made four constructs: D1 alone, D1 connected with Mp and Sp, D2 alone and D2 connected with Sp and Ct (Fig. 1). As shown in Fig. 2 (left), Mp-D1-Sp interacted with both Sp-D2-Ct and D2 alone. These interactions were confirmed and semi-quantified by the β-galactosidase activity (Table 1). In both assays Mp-D1-Sp interacted more strongly with D2 alone than Sp-D2-Ct. In contrast, no significant interaction was observed between D1 alone and Sp-D2-Ct or D2 (Fig. 2 and Table 1). These results suggest that Mp and/or Sp are essential for interaction of Mp-D1-Sp with D2.

No significant interactions were observed in homomeric combinations between the longer and shorter versions of D1

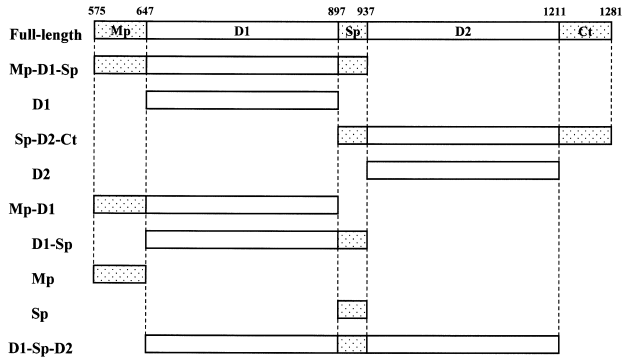

Fig. 1. Map of DNA segments carried by plasmids for the yeast two-hybrid system. The cytoplasmic region of human CD45 was dissected into five distinct domains, membrane proximal (Mp), PTP-D1 (D1), spacer (Sp), PTP-D2 (D2) and carboxyl-terminal (Ct) domains. Horizontal boxes represent domains carried by each plasmid. Numbers indicate the amino acid residue at the boundary of each domain.

Table 1
β-Galactosidase reporter quantification of protein-protein interactions between distinct domains of CD45

pAS2-1	pACT2	β-Galactosidase[a]
Vector	vector	0.03
p53	SV40-LT	11.53
Mp-D1-Sp	Sp-D2-Ct	0.52
Mp-D1-Sp	D2	4.73
D1	Sp-D2-Ct	0.00
D1	D2	0.03
Mp-D1-Sp	Mp-D1-Sp	0.09
D1	D1	0.06
D1	Mp-D1-Sp	0.05
Sp-D2-Ct	Sp-D2-Ct	0.01
D2	D2	0.03
D2	Sp-D2-Ct	0.04
Mp-D1	Sp-D2-Ct	0.07
Mp-D1	D2	0.03
D1-Sp	Sp-D2-Ct	0.04
D1-Sp	D2	0.40
Mp	Sp-D2-Ct	0.03
Mp	D2	0.03
Sp	Sp-D2-Ct	0.01
Sp	D2	0.85
Mp	Mp-D1-Sp	0.03
Mp	D1	0.04
Sp	Mp-D1-Sp	0.06
Sp	D1	0.06
Full-length	Full-length	0.02
D1-Sp-D2	D1-Sp-D2	0.03

[a]Values for β-galactosidase represent the specific activity calculated as described [24].

[1] The number of amino acid residues of human CD45 was counted from the amino-terminus of the mature protein devoid of a signal sequence.

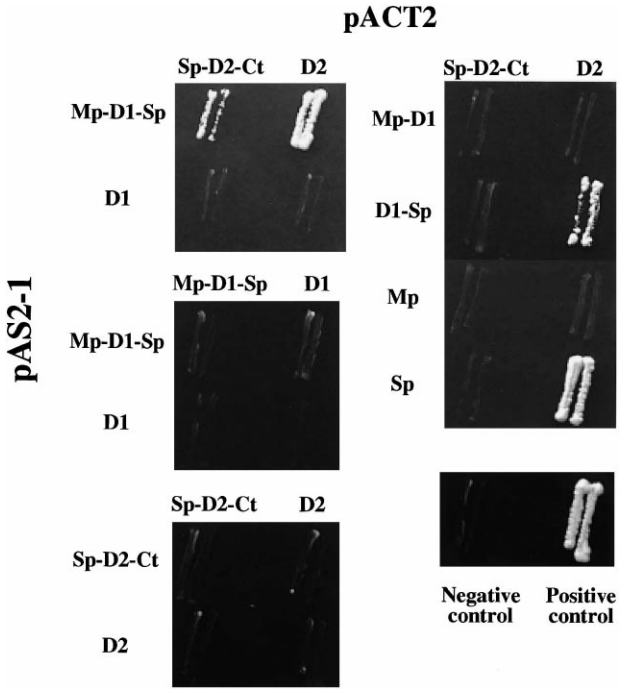

Fig. 2. Growth reporter assay for yeast two-hybrid interactions between distinct domains of CD45. Each pACT2 (horizontal lanes) and pAS2-1 (vertical lanes) construct was cotransfected into a yeast strain YRG-2. Transformants were streaked on SD plates devoid of histidine and incubated at 30°C for 4 days. The plates contained 10 mM 3-AT. The negative and positive controls for the two-hybrid interaction correspond to cotransfections of pACT2 and pAS2-1, and SV40-LT and p53, respectively.

and D2 (Fig. 2 (left) and Table 1). However, weak interactions between Mp-D1-Sp and Mp-D1-Sp, D1 and D1, and Mp-D1-Sp and D1 were detected in growth reporter assay under the less stringent condition of only 0.1 mM 3-AT (data not shown).

3.2. Interaction between Sp and D2

Since Mp and/or Sp appear to be involved in interaction

Table 2
Effect of mutations on Sp-D2 interaction

Mutation in Sp	Relative activity[a]
Wild	1.00
S898C	0.80
E899Q	0.06
Y903F	0.63
L904P	0.08
M907V	0.82
K908R	0.59
E915G	0.58
S917P	0.12
E920K	0.15
A921T	0.15
F923L	0.05
R925G	0.12
L926P	0.03
P927S	0.09
R933W	1.02
T934A	0.70
H936N	0.78

[a]Relative activity was calculated from the specific activity of β-galactosidase.

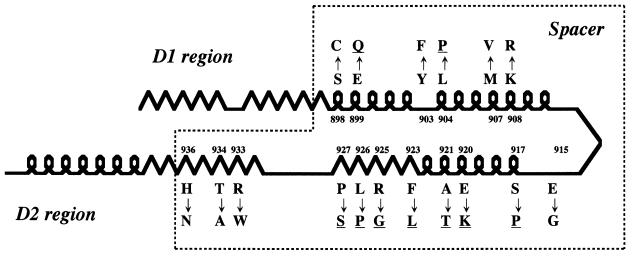

Fig. 3. Positive and negative mutations in the Sp region for interaction with D2. Possible secondary structure of the Sp region was depicted based on Chou and Fasman prediction [27]. Mutations which abrogated the interaction between Sp and D2 are underlined.

with D2 we made truncated constructs that carried either Mp or Sp with D1 (Fig. 1). As shown in Fig. 2 (right) and Table 1, D1-Sp interacted with D2 but Mp-D1 did not. Thus, Sp is assumed to predominantly participate in interaction with D2. It should be noted that D1-Sp did not interact with Sp-D2-Ct as predicted from the previous results indicating that D1-Sp was less competent to interact with D2 than Mp-D1-Sp. We have further truncated the carboxyl-half of Sp from D1-Sp, which resulted in loss of interaction with D2 (data not shown).

Finally, we expressed either Mp or Sp alone to examine if Sp directly interacted with D2. As demonstrated in Fig. 2 (right), Sp alone could directly interact with D2 although it did not interact with Sp-D2-Ct, while Mp alone interacted with neither D2 nor Sp-D2-Ct. Activity in the β-galactosidase assay was consistent with these observations (Table 1). Interestingly, neither Mp nor Sp interacted with D1 or Mp-D1-Sp at all (Table 1).

3.3. Effect of point mutation on interaction between Sp and D2

To further validate direct interaction between Sp and D2 and to identify the Sp amino acid residues involved in the interaction, we have randomly and site-directedly introduced single amino acid replacement in Sp (Fig. 3). Of seventeen single mutations created, which correspond to about half of the residues in Sp, nine mutations resulted in dramatic decrease in Sp interaction with D2 (Table 2). The same results were obtained when these mutations were re-introduced in Mp-D1-Sp (data not shown). Interestingly, seven negative mutations were clustered in a single stretch of the primary structure (Fig. 3). These results indicate that the interaction between Sp and D2 is specific.

3.4. Dimerization of CD45 cytoplasmic region

Given the strong interaction between Sp and D2, we examined dimerization of the full-length cytoplasmic region of CD45. The full-length cytoplasmic region and its truncated variant D1-Sp-D2 were cloned into pACT2 and pAS2-1 (Fig. 1). Neither full-length nor D1-Sp-D2 showed homomeric interaction in yeast two-hybrid assays (Table 1). However, the truncated variant D1-Sp-D2 showed a weak tendency to form a homodimer under less stringent conditions, as observed with the homomeric combinations of D1 and Mp-D1-Sp (data not shown).

4. Discussion

Although the tyrosine phosphatase activity of CD45 is be-

lieved to be a key factor in T-cell signal transduction, the mechanisms of phosphatase regulation are not yet fully understood. In the present paper, we demonstrated intra- and intermolecular interactions between distinct cytoplasmic domains of human CD45. Our results clearly indicate that the Sp region between two tandem phosphatase domains (D1 and D2) interact with D2. The interaction appears to be specific since no interaction of Sp with D1 was observed in spite of significant sequence homology between D1 and D2. The essential role of Sp identified in this study is consistent with the observation that deletion of Sp from the murine CD45 cytoplasmic region abolishes PTP activity in vitro [25]. In addition, recent publication of the crystal structure of LAR D1-D2 described the close proximity of Sp to D2 in a single molecule [22]. Recently Felberg and Johnson reported binding of D1 to D2 in in vitro binding assays [17], however, their D1 protein contained both the membrane proximal (Mp) region and Sp. Therefore, the binding of D1 to D2 observed in previous studies may likely be mediated by Sp embedded in the D1 protein.

We have further identified a series of point mutations in the carboxyl-terminal half of Sp, which abolish the interaction between Sp and D2. The Sp region is predicted to form a hairpin structure having a β-turn in the middle of the structure (Fig. 3 and [22]). Interestingly, most of negative mutations are clustered in one side of the hairpin structure. Although the negative interaction between mutant Sp and D2 might be due to a gross conformational change of Sp, the carboxyl-terminal half of Sp is assumed to exhibit direct contact with D2 as a distinct domain since several different mutations in a restricted region abrogated the interaction. Supporting this notion, limited but significant sequence homology was observed only in the carboxyl-terminal region of Sp while overall conservation among Sp regions of various two-domain PTPs was low [2]. Presently we do not know about the particular domains and residues in D2 responsible for binding to Sp. In this context, it may be worth noting that truncation of carboxyl-terminal 23 residues of D2 or a point mutation in the relevant region abolished PTP activity of murine CD45 [16,25]. Thus, the carboxyl-terminal region of D2 may be a possible target for interaction with Sp. Indeed, the closest proximity is observed between the Sp and two α-helix structures in the carboxyl-terminus of D2 in the crystal structure of LAR [22].

Previously, many people anticipated that the CD45 cytoplasmic region might have a tendency to homodimerize through a direct interaction between Mp and D1. This speculation has evolved from a small number of indirect indications: (i) PTPα, which is structurally similar to CD45, crystallizes to form a dimer in which the two molecules interact via mutual protrusion of the membrane proximal wedge structure (Mp) to the catalytic cleft of the opposite molecule [20], (ii) an Mp mutation modulates ligand induced signal transmission elicited by a chimera protein consisting of EGF-R and CD45 [26]. However, in the present study we observed neither interaction between Mp alone nor Mp-D1 and D1. Although we can not exclude the possibility that Mp and D1 are unstable or incorrectly folded when expressed solely, these results may suggest that either different mechanisms operate in regulation of the catalytic activities of CD45 and PTPα, or Mp simply affect the catalytic activity of D1 through intra- and/or intermolecular interactions between Sp and D2. Nonetheless, Mp appear to support and/or stabilize the interaction between Sp and D2 since the interaction between Sp or D1-Sp and D2 was much weaker than that between Mp-D1-Sp and D2. Alternatively, Mp may stabilize the conformation of D1 (and D1-Sp) to interact with D2 as suggested in the LAR crystal structure [22]. Consistent with this indication, deletion of Mp from the murine CD45 cytoplasmic region results in the loss of PTP activity in vitro [16].

Finally, we observed that Sp could interact only with D2 alone, not with Sp-D2-Ct. This outcome may indicate that the Sp region in Sp-D2-Ct intramolecularly interacts with D2, thereby preventing intermolecular interaction between Sp and Sp-D2-Ct. Taken together, we would speculate that the cytoplasmic region of CD45 may exist primarily as a monomer. This idea is supported by the observations that active CD45 D1-D2 enzyme exists as a monomer in vitro [17] and LAR D1-D2 crystallized as a monomer [22]. The intramolecular folding via interaction between Sp and D2 may encourage this active state. In certain cases such as ligand binding or phosphorylation, dimerization may be induced concomitantly with release of the intramolecular folding, in which case the phosphatase activity is negatively regulated via intermolecular interaction between Mp and the catalytic center in D1.

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