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POINT MUTATION IN THE SECOND PHOSPHATASE DOMAIN OF CD45 ABROGATES TYROSINE PHOSPHATASE ACTIVITY

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CD45 is a transmembrane protein tyrosine phosphatase that possesses two phosphatase domains in its cytoplasmic region. Whether both domains function independently as phosphatase enzymes or whether both domains interact to form an active enzyme is unclear. A point mutation of a critical cysteine residue in domain I is known to abolish CD45 activity, implying that the catalytic activity resides in domain I. In this report, mutational analysis of purified, recombinant CD45 cytoplasmic domain protein was performed. It was found that a single amino acid change in domain II (glutamine 1180 to a glycine) resulted in an inactive phosphatase enzyme, whereas two other point mutations in the membrane proximal region of the molecule had no effect on activity. Deletion of the region linking the two phosphatase domains also abolished enzymatic activity. Amino acids crucial for phosphatase activity thus reside in both phosphatase domains of CD45, illustrating that the phosphatase domains of CD45 do not act independently, but are both required for the phosphatase activity of CD45.

Unlike all soluble cytoplasmic protein tyrosine phosphatases (PTPs) sequenced to date, the majority of transmembrane phosphatases have two phosphatase domains that are located in the cytoplasmic region of the protein (reviewed in 1). The functional significance of these two repeated phosphatase domains is unknown. Initial mutagenesis studies on the transmembrane PTP's, LAR and CD45, indicated that mutation of a highly conserved cysteine in domain I, but not

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Abbreviations: PTP, protein tyrosine phosphatase; LAR, leukocyte antigen-related protein; LRP, leukocyte common antigen-related protein; HPTP, human protein tyrosine phosphatase; EGFR, epidermal growth factor receptor; Nickel-NTA agarose, Nickel-nitrilotriacetic acid; FPLC, fast protein liquid chromatography; MBP, myelin basic protein; RCML, reduced, carboxymethylated, maleylated lysozyme; BCA,bicinchoninic acid.

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in domain II, resulted in the loss of PTP activity (2,3), implying the first PTP domain of CD45 and LAR, but not the second, was catalytically active. This highly conserved cysteine is present in virtually all protein tyrosine phosphatase domains and has been shown to be the active site residue in PTP1 and in domain I of LAR (4,5). Crystallographic studies have recently shown that in PTP1B and Yop51 protein tyrosine phosphatases, this cysteine, which is part of a highly conserved region (V/IHCXXGXXRS/TG), is located at the center of the phosphate binding loop (6,7).

Further studies in T cell lines expressing chimeric EGFR/CD45 molecules containing mutations of these conserved cysteines have indicated that the catalytic activity and ability to restore T cell signaling events is dependent upon the presence of the presumed catalytic cysteine in domain I, but not the cysteine in domain II (8). This study also implies that the catalytic activity of CD45 resides exclusively in domain I . Indeed, recombinant bacterial expression of the first phosphatase domain alone of three transmembrane phosphatases, LAR, the human homologue of LRP (HPTPα), and HPTPζ, generated an active phosphatase enzyme (3,9,11-13), indicating that for the phosphatases, domain I can function independently of domain II. In the case of LAR, the recombinant protein containing domain I alone had almost indistinguishable activity from the two domain LAR protein (10,11), whereas individual domains of HPTPα both had considerably less activity than the two domain recombinant protein (12). However, expression of individual phosphatase domains of CD45 in either in bacteria or in an *in vitro* transcription/translation system indicated that neither domain had catalytic activity when expressed alone (3,14). In these mutational studies, a deletion of 78 amino acids from the carboxy terminus of CD45 had no effect on activity but an additional deletion of 13 amino acids from carboxy region of domain II abolished enzyme activity, suggesting that an intact domain II, in addition to domain I was required for activity (14). Thus unlike LAR and HPTPα, the first phosphatase domain of CD45 was not active in the absence of the second domain and neither PTP domain was active in the absence of the other, implying that both domains are integral components of the phosphatase enzyme.

However, other studies in eukaryotic cells have implied that domain II of CD45 may have independent catalytic activity (15). In these studies, a full length form of human CD45 lacking residues 825-933, which include the presumed catalytic cysteine in domain I, was expressed in eukaryotic cells and phosphatase activity was detected. In addition, a proteolytic fragment that was thought to contain only the C-terminal portion of domain I and domain II of CD45 also had enzymatic activity and showed substrate specificities that were distinct from the intact CD45 molecule (15).

Thus, at present, it is not clear whether CD45 represents a distinct class of protein tyrosine phosphatase that requires two phosphatase domains for enzymatic activity, whether domain II may regulate the activity and substrate specificity of domain I, or whether each domain has distinct phosphatase activities. In order to distinguish between these possibilities, we set out to further localize the regions and amino acids in the CD45 cytoplasmic domain that were absolutely required for enzymatic activity. The cytoplasmic domain of murine CD45 was expressed in a bacterial expression system, purified using a simple two-step procedure, and was determined to be catalytically active. Three mutant forms of CD45 were produced by site-directed mutagenesis and expressed in bacteria. These recombinant proteins were purified and tested for phosphatase

activity. It was shown that deletion of the region between the two phosphatase domains and a point mutation in domain II both resulted in the total loss of enzymatic activity. Two point mutations in the membrane proximal region had no deleterious effect on activity. This is the first indication that a point mutation in the second phosphatase domain of CD45 can abrogate enzymatic activity and further supports the two domain phosphatase model, in which crucial residues in both domain I and domain II are required for the phosphatase activity of CD45.

Materials and Methods

Materials--Mutant constructs were sequenced using the Sequenase kit from U.S. Biochemicals and [^{35}S]-αdATP (1000Ci/mmol) was from New England Nuclear-DuPont. Nickel-NTA-Agarose was from Qiagen and Sephadex G-25M PD-10 and MonoQ columns were from Pharmacia. The p59^{fyn} phosphopeptide (TATEPQpYQPGENL) was a kind gift from Dr I. Clark-Lewis; the sequence was derived from the carboxyterminal region of p59^{fyn} and it was synthesized and purified essentially as described in (16).

Buffers--Bacterial pellets were lysed in buffer 1 (0.1%Triton X-100, 20mM Tris pH7.5, 150mM NaCl, 20mM Imidazole pH7.2, 0.025% β-mercaptoethanol). Elution buffer (buffer 2) for the Nickel column was 1M Imidazole, pH7.2 and equilibration buffer (buffer 3) for the PD10 column was 0.1%Triton X-100, 20mM Tris pH8.0, 150mM NaCl. Buffers A and B for the FPLC were 0.1% Triton X-100, 20mM Tris pH8.0 and 0.1% Triton X-100, 20mM Tris pH8.0, 2M NaCl. All above buffers involved in the purification process contained 0.2mM PMSF, 1.0μg/ml pepstatin, 1.0μg/ml leupeptin, and 1.0μg/ml aprotinin. The phosphatase buffer used was 50mM Imidazole pH7.2, 1mM EDTA, 0.1% β-mercaptoethanol.

Bacterial Strains and Plasmids--Escherichia coli strains used were CJ236 and DH5αF' (Biorad), XL-1 Blue (Stratagene) and BL21(DE3) obtained from Dr. F. Studier (17). The bacterial expression pET-3D vector, obtained from Dr. F.Studier (17), was modified by removing a Nco I-Bam HI fragment and replacing it with linker oligonucleotides encoding a six histidine tag and the Factor Xa cleavage site, IEGR. The cytoplasmic domain of murine CD45 in pBluescript SK$^+$ vector (Stratagene) was used for mutagenesis (18), subcloned into a shuttle vector containing BglII restriction sites flanking the cytoplasmic domain and was then subcloned into the Bam HI site of the modified pET-3D vector, pET-3D-6HisIEGR.

Oligonucleotide-Directed Mutagenesis--Mutagenesis was performed essentially as described by Kunkel (19). pGGAACTGGTACCCCTCATAGCTGC was used to generate the Q1180G mutant; pGTTCTTCTTCTTATTTTCCACTAAAGC was used to create the spacer deletion removing amino acids 876-931; pGTTCATCTAAATTGGCGCCTCTTTTCTTGC was used to generate SS573,574 to GA mutant; and the C817S mutant was made previously (14). Mutations were screened by restriction enzyme analysis and verified by sequencing. For each mutant, two independent clones were derived and used in expression studies.

Expression and Purification of Recombinant Proteins--500ml of Luria broth supplemented with 100μg/ml ampicillin was inoculated with a fresh culture of BL21(DE3) containing the appropriate pET vector and grown at 37°C, 180rpm. When the O.D. at 600nm of the culture was ~0.6 to 0.8, the culture was induced with IPTG to a final concentration of 0.1mM and left to grow for ~12 hours at 30°C, 180rpm. The culture was then centrifuged at 4000g for 10min at 4°C, and the pellet resuspended in 10ml of buffer 1. The solution was frozen in a dry ice/ethanol bath and thawed slowly at room temperature; this step was repeated twice more. 100μg DNase I was added and the solution kept at 37°C under constant agitation until no longer viscous. The solution was then spun at 10000g for 20min at 4°C and the supernatant retained, filtered using a low protein binding 0.2μm filter and was loaded onto a column containing Nickel chelate beads (Ni^{2+}-NTA agarose, 1.0ml bed volume). The column was washed with buffer 1 and bound proteins were eluted with buffer 2 and then passed through a PD10 column previously equilibrated with buffer 3. The PD10 eluent was filtered using a low protein binding 0.2μm filter and loaded onto a Mono Q column. FPLC was performed using buffers A and B under a linear gradient ranging from 7.5% to 30% buffer B. Protein fractions were run on an SDS PAGE gel and were tested for PTP activity. Active fractions were stored in 50% glycerol at -80°C.

Phosphatase Assays--Phosphatase assays were performed at 30°C as previously described (20). Briefly, an aliquot of purified CD45 protein was diluted in PTP buffer and 10μl was added directly to 100μl microtitre wells (Half-Area, Tissue Culture Treated, flat bottomed 96 well

microtitre plates, Costar Corporation) for each timepoint tested. Addition of 10µl of phosphatase buffer containing the fyn peptide at a concentration of 3mM, initiated the reaction and addition of 80µl of malachite green reagent stopped the reaction. Release of inorganic phosphate was measured by determining absorbance at 650nm using a multiwell plate reader and compared to a standard curve of amounts of inorganic phosphate.

ProteinDetermination--Protein amounts were measured using the BCA protein assay (Pierce). Amounts of protein present on SDS PAGE gels were compared by densitometric scanning and analysis of coomassie stained bands (PDI systems).

Results and Discussion

Expression and purification of soluble recombinant CD45 cytoplasmic domain-proteins

The cytoplasmic domain of murine CD45 was expressed in bacteria. Six histidines were incorporated at the amino terminus of the molecule, followed by the Factor Xa recognition sequence, IEGR, and then residues 564-1268 of murine CD45. Expression of the six histidine tag allowed the protein to be recovered from the soluble fraction by attachment to a Nickel affinity column. Elution from this column in 1M imidazole resulted in substantially purified recombinant protein, and a 30-40 fold purification (see Table 1). A second purification step involving ion-exchange chromatography separated the remaining proteins, yielded approximately a 100 fold purification and generated a single protein band of apparent molecular mass of 95kDa (see Figure 1). This protein reacted with antisera raised against the cytoplasmic domain of CD45 (18) and had protein tyrosine phosphatase activity. The specific activity of this recombinant enzyme was 60µmol/min/mg and a K_m value of 200µM was obtained using a 13 amino acid phosphopeptide derived from the carboxy terminal region of $p59^{fyn}$ as a substrate. Approximately 2mg/litre of purified recombinant protein was obtained. This purification procedure is a simple two step procedure which generates a single protein band and as such offers a significant improvement over existing methods to purify recombinant CD45 from *E. Coli*, which have used multiple steps and have not generated a single protein species (10,11). More recently, an enzymatically active recombinant CD45 cytoplasmic domain protein was purified from yeast using a multi-step procedure (21).

Recombinant murine CD45 cytoplasmic domain and four mutant proteins were expressed and purified from bacteria. A CD45 cytoplasmic domain protein with point mutation in domain II (glutamine 1180 to glycine) and a protein with two point mutations in the membrane proximal region (serine 573 and serine 574 to a glycine and alanine respectively) were expressed and

Table 1. **Purification of murine recombinant cytoplasmic CD45 from 500mls of *E. coli* culture.** The purification procedure has been repeated several times and the values given in this table are representative values derived from a single experiment.

Sample	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Total activity (µmol/min)	Specific activity[a] (µmol/min/mg)	Total purification	Yield (%)
Soluble Cell Lysate	12	31.8	382	249	0.65	-	100
Imidazole Eluent	3.0	1.5	4.6	105	22.8	35.1	42
Mono Q Eluent	5.0	0.21	1.1	62.9	60.0	92.3	25

[a]All activity values were derived using the fyn peptide at a final concentration of 3.0mM.

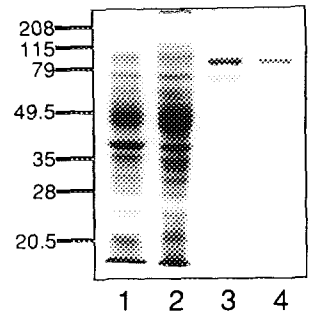

Figure 1. **SDS-PAGE analysis of the purification of recombinant cytoplasmic CD45.** Lane 1: whole cell lysate, lane 2: soluble cell lysate fraction, lane 3: imidazole eluent from the Nickel-NTA column, and lane 4: post Mono Q fractions containing purified cytoplasmic CD45. Protein samples were fractionated on a 10% SDS polyacrylamide gel and stained with Coomassie Blue.

purified, as was a CD45 cytoplasmic mutant lacking the protein sequence separating the two phosphatase domains (the spacer region, amino acids 876 to 931) and a CD45 cytoplasmic domain protein with a point mutation in domain I (cysteine 817 to serine) that had previously been shown

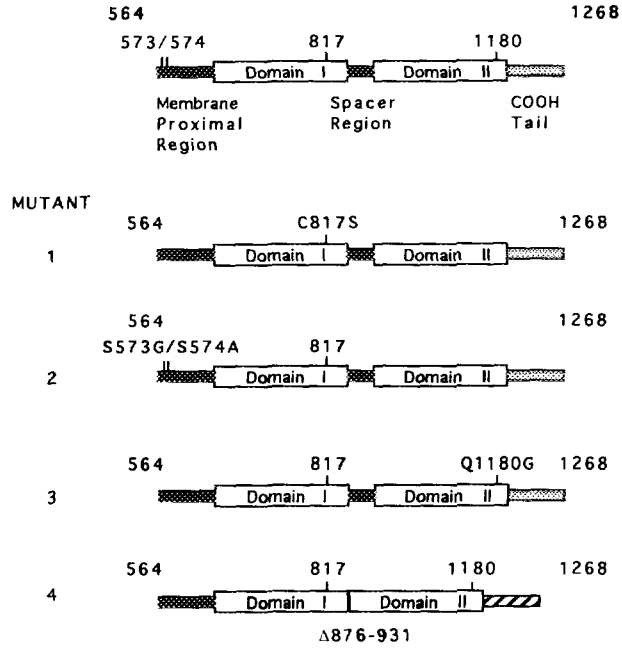

Figure 2. **Schematic representation of the murine CD45 cytoplasmic domain and the mutant CD45 cytoplasmic domain proteins.** The recombinant CD45 protein is shown at the top and is divided into 5 distinct protein domains; the membrane proximal region, phosphatase domain I, the spacer region, phosphatase domain II and the carboxy terminal tail. The amino acids are numbered according to the sequence of the mouse CD45(ABC) isoform (22). Four mutant CD45 proteins (1-4) are shown illustrating the mutation made in each case. Mutant 1 has cysteine 817 mutated to serine (C817S); mutant 2 has serines 573 and 574 mutated to glycine and alanine, respectively (SS573,573GA); mutant 3 has glutamine 1180 changed to a glycine (Q1180G) and mutant 4 has amino acids 876 to 931 deleted (Δ876-931, spacer region deletion).

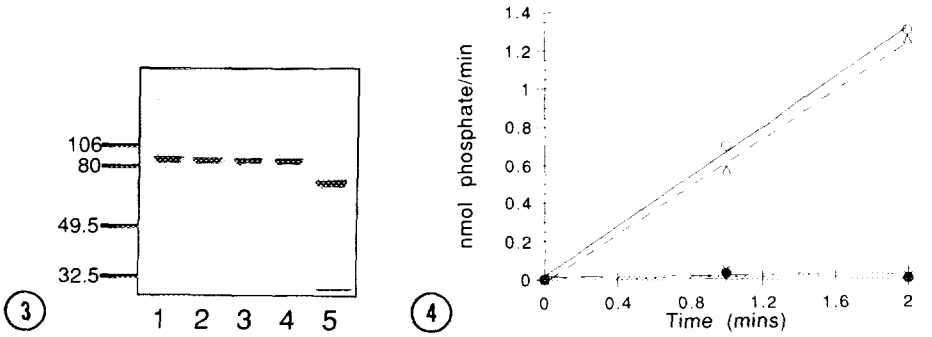

Figure 3. **10% SDS-polyacrylamide gel of equivalent protein amounts for each recombinant CD45 protein.** Bacterially expressed proteins were purified as described and assayed for protein amount using the BCA assay. Equivalent protein amounts were loaded and subjected to SDS-PAGE and then stained with coomassie blue. Lane 1: recombinant cytoplasmic CD45 protein. Lanes 2, 3, 4, and 5 show mutant CD45 cytoplasmic domain proteins, 1-4, respectively (refer to Figure 2): 1(C817S), 2 (SS573,574GA), 3 (Q1180G) and mutant protein 4 (Δ876-931, spacer region deletion).

Figure 4. **Comparison of initial rates of PTP activity for wild type murine cytoplasmic CD45 and the four mutant CD45 cytoplasmic domain proteins.** The symbols represent the following CD45 cytoplasmic proteins: (o) wild type, (x) mutant 1: C817S, (Δ) mutant 2: SS573,574GA (•) mutant 3: Q1180G, and (+) mutant 4: Δ876-931 spacer region deletion. Reaction rates of 10ng of recombinant protein was assayed using fyn peptide at a final concentration of 3.0mM. Time points were taken at 0, 1, and 2 min and nanomoles of inorganic phosphate (PO_4) released were calculated using the malachite green phosphate assay (20).

to abrogate CD45 phosphatase activity (see Figure 2). Approximately 2-4 mgs of purified recombinant protein/litre were obtained for the wild type and point mutation proteins and approximately 0.2mgs of purified protein was obtained for the spacer deletion mutant.

Phosphatase activity of CD45 cytoplasmic domain mutants

The amount of purified recombinant CD45 cytoplasmic domain protein was determined by the BCA protein assay. Equal amounts of protein were taken for each mutant and the initial rates of phosphatase activity determined using fyn phosphopeptide substrate at a final concentration of 3.0mM. Confirmation that equivalent amounts of protein were used to compare phosphatase activities was demonstrated by running equivalent protein amounts on an SDS gel (Figure 3) and by densiometric scanning of the coomassie blue stained bands (data not shown). Comparison of initial phosphatase activities using the fyn phosphopeptide as substrate is shown in Figure 4. These results indicate that two point mutations in the membrane proximal region did not affect phosphatase activity illustrating that not all point mutations have a detrimental affect on CD45 phosphatase activity. Secondly, the mutation of a highly conserved glutamine (1180) to a glycine in domain II generated a CD45 protein with undetectable phosphatase activity. The spacer deletion mutant also generated undetectable levels of activity. These mutants produced similar results to the cysteine to serine mutation in domain I which, as previously shown, also had undetectable levels of activity (see Figure 4). These results indicate that the region separating the two phosphatase domains is required to generate an active enzyme. More surprisingly, it was found that a single amino acid change in domain II abolished the phosphatase activity of CD45, despite the fact that

the catalytic cysteine residue is thought to be located in domain I (2,3,8,14). This data argues against domain I of CD45 having independent catalytic activity and supports the two domain phosphatase model for CD45 where both domains are required and interact to form an active phosphatase enzyme. Interestingly, mutation of the residue adjacent to the glutamine, tyrosine 1181 to phenylalanine, did not adversely affect activity (14), suggesting that glutamine 1180 has a crucial role in the formation of an active enzyme complex. By comparison to PTP1B, for which the crystal structure is known (6), an analogous glutamine (Q266) was found to be located in an α-helix close to the carboxy end of the PTP domain. Glutamine 262 in PTP1B is thought to be involved in an interaction with substrate (6). Whether glutamine 1180 in CD45 interacts with substrate remains to be determined. As a glutamine residue appears in this position in the majority of tyrosine phosphatases, it is likely that this residue has an important function in either the structure or the function of protein tyrosine phosphatases. Of particular interest here is the fact that mutation of this single residue in domain II abrogated an enzymatic activity thought to reside in domain I. This leads us to the conclusion that both domain I and domain II of CD45 contain amino acids essential for enzymatic activity and thus CD45 may represent a distinct class of transmembrane phosphatases which require both phosphatase domains for enzymatic activity.

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