The MBP fusion protein restores the activity of the first phosphatase domain of CD45

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Abstract CD45 is a receptor-like protein tyrosine phosphatase critically involved in the regulation of initial effector functions in B- and T-cells. The protein comprises two phosphatase (PTP) domains in its cytoplasmic region. However, whether each PTP domain has enzyme activity by itself or whether both domains are required to build up a functional enzyme is unclear. We have studied different constructions of human CD45 comprising the two PTP domains, both separately and as a single protein, fused to maltose-binding protein (MBP). In apparent contrast with previous studies, we show that the first PTP domain of CD45 (when fused to MBP) may be a viable phosphatase in the absence of the second domain. Phosphatase activity resides in the monomeric form of the protein and is lost after proteolytic cleavage of the fusion partner, indicating that MBP specifically activates the first PTP domain. Furthermore, changes in the optimal pH for activity with respect to wild-type CD45 suggest that protein–protein interactions involving residues in the neighbourhood of the catalytic site mediate enzyme activation.

Key words: Receptor-like protein tyrosine phosphatase; Signal transduction; Maltose-binding protein; Fusion protein

1. Introduction

The activation and regulation of reaction cascades in signalling pathways leading to proliferation and differentiation in eukaryotic cells is related to the balance of tyrosine phosphorylation/dephosphorylation controlled by the action of tyrosine kinases and phosphatases (reviewed in [1,2]). Receptor-like protein tyrosine phosphatases (RPTP) exhibit a modular structure that includes one or two intracellular PTP domains, each homologous to soluble forms of monomeric phosphatases, a single membrane-spanning segment and a variable extracellular domain [3]. The PTP domain is organized as a central eight-stranded β-sheet flanked on both sides by α-helices, as revealed by the crystal structures of the soluble phosphatases PTP1B and Yop51 [4,5]. At the center of the active site, the highly conserved sequence motif (I/V)HCXAGXXR(S/T) contributes the essential nucleophilic cysteine residue and other functional groups required for phosphotyrosine binding and catalysis [6,7].

CD45, a prototypic receptor-like PTPase, is a 180–220 kDa protein highly expressed in hematopoietic cells. CD45 plays a critical role in the response of leukocytes to antigen, where it is involved in the early stages of the signal transduction pathways [8] (for review [9–11]). The two intracellular PTP domains of CD45 share about 40% of sequence identities with each other [3], and both contain a cysteine residue (Cys^{828} in PTP-I; Cys^{1144} in PTP-II) within the conserved sequence motif. However, several mutational studies have indicated that Cys^{828} (but not Cys^{1144}) is critical for phosphatase activity [12,13]. The substitution $Cys^{828} > Ser$ completely abrogated the enzymatic activity in recombinant forms [12–14] and in cells [15], but the replacement $Cys^{1144} > Ser$ in the second PTP domain resulted in a phosphatase with in vitro and in vivo properties similar to those of the wild-type enzyme [13,15]. These results suggest that only the first PTP domain, but not the second, behaves as an active phosphatase. In agreement with these observations, recombinant CD45–domain II alone yielded an inactive protein [13], and several RPTPs lack the conserved cysteine within the second PTP domain [16,17]. Furthermore, in RPTPs such as LAR and HPTPα, the first PTP domain alone displayed a phosphatase activity similar to that of the whole protein [18,19].

However, the first PTP of CD45 domain was found to be inactive when expressed independently [12,13], suggesting that in CD45, unlike other RPTPs, domain II is required for the activity of domain I. This hypothesis is further substantiated by the finding that a single point mutation in the second domain or the deletion of the region linking the two PTP domains totally abrogated the phosphatase activity of CD45 [20]. Contrasting these data, Tan et al. have shown in eukaryotic cells that the second domain of CD45 together with the C-terminal part of the first domain (without the catalytic cysteine) may also be a viable phosphatase [21]. Therefore, it is not clear at present whether the separate PTP domains of CD45 have phosphatase activity by themselves, or whether the enzymatic activity in one or both domains requires specific interdomain interactions.

We have studied different constructions containing the two phosphatase domains of CD45, both separately and as a single protein, fused to the maltose-binding protein (MBP). We found that the first PTP domain of CD45 when expressed as a MBP fusion protein is an active phosphatase in the absence of the second domain, and that the enzymatic activity is lost after proteolytic cleavage of the fusion partner, suggesting that specific interactions with an external factor are required to stabilize the active conformation.

2. Materials and methods

2.1. Materials

p-Nitrophenylphosphate (*p*-NPP), 2-β-mercaptoethanol (2-ME), dithiotreitol (DTT) and D-maltose were purchased from Sigma. Pefabloc® from Boehringer Mannheim. Factor Xa, antiserum anti-MBP and amylose resin were from New England BioLabs. Bradford reagent from Bio-Rads, and material for SDS-polyacrylamide gel elec-

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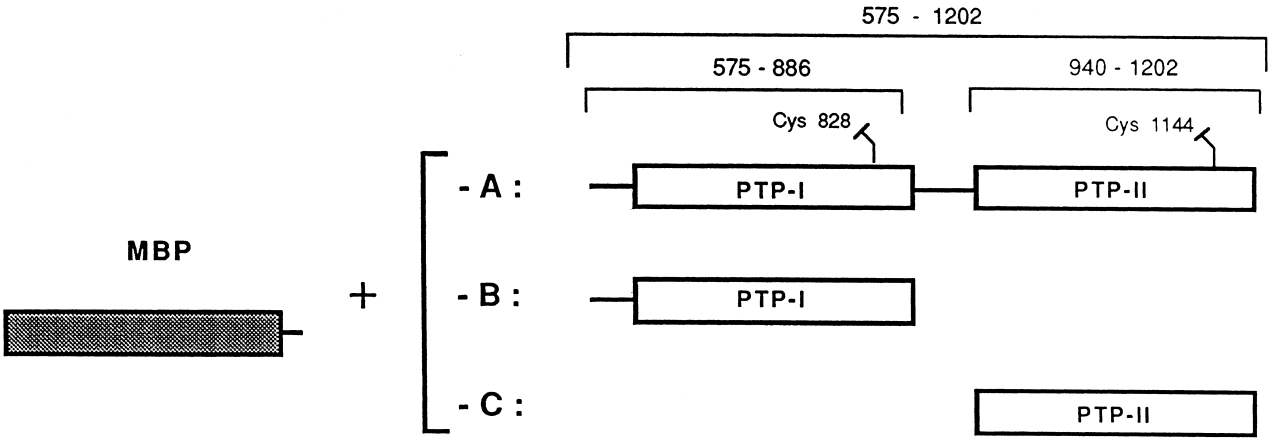

Fig. 1. Different constructions of human CD45. MBP: maltose-binding protein. Numbering of residues as in Streuli et al. (1987)[22].

trophoresis, gel filtration and ion exchange chromatography from Pharmacia.

2.2. MBP–CD45 fusion constructs

Three fusion constructs of human CD45 (PTP domain-I, PTP domain-II and PTP domain-I+II) were generated according to the boundaries proposed by Streuli et al. [22] (Fig. 1). Thus, MBP–PTP-I includes amino acid residues 575–886 (K_{575}IYDL.....QALVE$_{886}$) of human CD45, MBP–PTP-II includes residues 940–1202 (Q_{940}EENKS... ...DVIAS$_{1202}$), and MBP–PTP-I+II includes residues 575–1202 (K_{575}IYDL... ...DVIAS$_{1202}$). The sequences were PCR amplified from an expression plasmid containing the entire human CD45 T200 coding sequence using the primers: 5′-GGAATTCAGATCTAAAATCTATGATCTACAT-3′ and 5′-GGAATTCAGATCTTTACTGATTGTATTCCACCAA-3′ (PTP-I); 5′-CGGGATCCCAAGAAGAAAATAAAAGT-3′, and 5′-CGGGATCCTTAGCTGGCAATGACGTCATA-3′ (PTP-II) and 5′-GGAATTCAGATCTAAAATCTATGATCTACAT-3′ and 5′-GGAATTCAGATCTTTAGCTGGCAATGACGTCATA-3′ (PTP-I+II).

Amplified PCR fragments were ligated to pPDXa [23] which had been previously linearized with *Bam*HI, treated with calf intestinal phosphatase, and gel purified. pPDXa contains the coding sequence for maltose binding protein (MBP) under the control of the maltose promoter and is a derivative of pMALcRI (New England BioLabs, Beverly, MA). All the plasmids were transformed into competent *mal*E$^-$ PD28 cells according to established protocols [24].

2.3. Expression and purification of recombinant forms of human CD45

Cultures of PD28 cells, induction and isolation of fusion proteins were performed at 30°C using protocols previously described [25]. Protein purification was achieved by affinity chromatography in amylose resin as described by the furnisher, followed by ion exchange chromatography using a MonoQ column (Pharmacia) equilibrated in a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 2 mM DTT. The presence of different MBP–CD45 forms was evidenced by anti-MBP Western blot. Cleavage of purified fusion proteins was carried out by overnight incubation at 4°C with Factor Xa (1:100, w:w). The PTP domains were separated from MBP by anion exchange chromatography as described above. Protein purification was tested by SDS-PAGE and the respective concentrations measured by Bradford colorimetric assay.

2.4. PTPase assays

Kinetics of Michaelis-Menten were studied using *p*-NPP (10 mM final) as substrate in 50 mM imidazol (pH 7.0), 150 mM NaCl, 1 mM EDTA, and 0.1% 2-ME (total volume of 50 μl) at 37°C. The reaction was allowed to proceed for 5–20 min and quenched with 500 μl of 1 M NaOH. The amount of dephosphorylated substrate was calculated from the absorbance at 405 nm assuming a molar extinction coefficient $E_{405} = 18\,000\ M^{-1}\ cm^{-1}$ [6]. Michaelis-Menten constants were calculated using the non-linear regression program ENZFITTER [26].

The effect of pH on the PTPase activity of the recombinant forms of CD45 was studied using 10 mM *p*-NPP as substrate under similar conditions as above. Buffers used for these tests were as follows: pH 4–5.25, 100 mM acetate; pH 5.5–6.25, 100 mM citrate; pH 6.5–7.25, 100 mM imidazol; pH 7.5–8.5, 100 mM Tris-HCl. All buffers contained 1 mM EDTA, 0.1% 2-ME and 50 mM NaCl. pH dependence data were fitted by non-linear least-squares regression using the program KaleidaGraph 3.0.

3. Results and discussion

The cytoplasmic region of CD45 (PTP-I+II) as well as each separate PTP domain (PTP-I and PTP-II) (Fig. 1) were expressed as MBP fusion proteins in bacteria under control of the maltose promoter, and purified to near homogeneity (Fig. 2). Each construction was tested for PTPase activity using *p*-NPP as a substrate. Recombinant proteins containing the entire cytoplasmic region of CD45 (MBP–PTP-I+II and PTP-I+II) displayed a phosphatase activity (Table 1) comparable

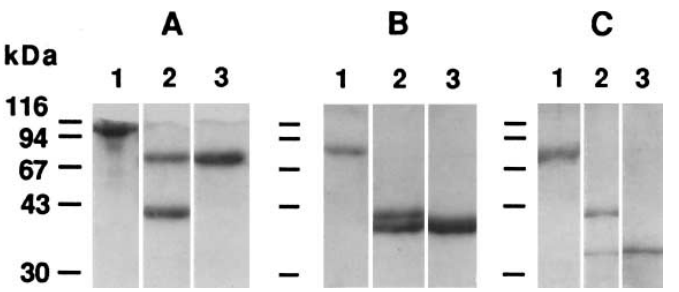

Fig. 2. SDS-PAGE analysis of MBP–CD45 constructions. A: MBP–PTP-I+II; B: MBP–PTP-I; C: MBP–PTP-II. Lane 1: after amylose affinity chromatography. Lane 2: after factor Xa treatment. Lane 3: after anion exchange chromatography.

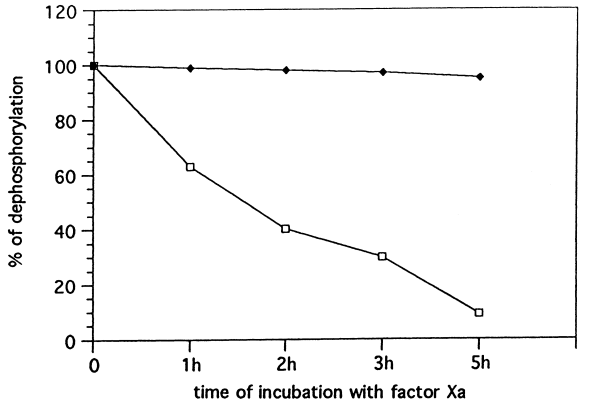

Fig. 3. Progressive loss of PTPase activity of MBP–PTP-I after treatment with factor Xa. MBP–PTP-I (□) and MBP–PTP-I+II (♦) were incubated with factor Xa at different times and their respective PTPase activities determined afterwards. pNPP (10 mM final) was used as substrate. Each point represents the mean value of quadruplicated experiences and was calculated as the percentage of dephosphorylation of pNPP exhibited. 100% of activity was considered as the activity displayed in identical conditions of incubation but in the absence of factor Xa.

to similar constructions studied by other authors [6,27,28]. Also, as expected, no enzymatic activity was detected in the recombinant proteins lacking the first phosphatase domain (MBP–PTP-II and PTP-II). However, MBP–PTP-I was found to be an active phosphatase, in contrast with previous work that reported the lack of catalytic activity of PTP-I in the absence of the second domain [12,13]. Both MBP–PTP-I and MBP–PTP-I+II displayed K_m values in the mM range, which are similar to the values reported by other authors for CD45 and other PTPases [6,27,28]. As shown in Table 1, V_{max} and k_{cat}/K_m values of MBP–PTP-I and MBP–PTP-II differ in about one order of magnitude, indicating that the absence of the second domain influences, but does not abrogate, the phosphatase activity of the first PTP domain. Similar results were obtained when two pY-peptides from hirudin and gastrin (Tyrosine Phosphatase Assay Kit, Boehringer) were used as substrates (data not shown).

Functional studies of different CD45 constructions suggested that specific interactions with the second domain are necessary to have a first active domain [13,20,21]. In a similar way, the phosphatase activity of MBP–PTP-I protein (in the absence of the second domain) might be accounted for either by specific interactions of PTP-I with MBP or by protein dimerization as observed, for example, in receptor-associated protein tyrosine kinases [29,30]. In order to test whether the covalently linked MBP was involved in PTP-I activation, MBP–PTP-I was treated with Factor Xa to separate the fusion partners. This experiment revealed a progressive loss of PTPase activity following the addition of Factor Xa (Fig. 3), indicating that the first PTP domain alone was not an active phosphatase. On the other hand, cleavage of the MBP moiety from the fusion protein containing the entire cytoplasmic region did not significantly affect the PTPase activity.

The possibility that the enzymatic activity of MBP–PTP-I activation could arise from protein dimerization was also investigated, since previous experimental evidences indicated that CD45 could be regulated by dimerization. For example, using cross-linking reagents in YAC-1 cell lysates, Takeda et al. detected CD45 homodimers apparently induced by a CD45-associated protein [31]. Furthermore, epidermal growth factor (EGF)-induced dimerization of an artificial EGF/CD45 chimera expressed in CD45-deficient T-cell line caused the loss of antigen-dependent activation [32]. Using gel filtration chromatography at different MBP–PTP-I concentrations, we observed monomers, dimers and higher oligomers of MBP–PTP-I. However, we have only found PTPase activity in fractions

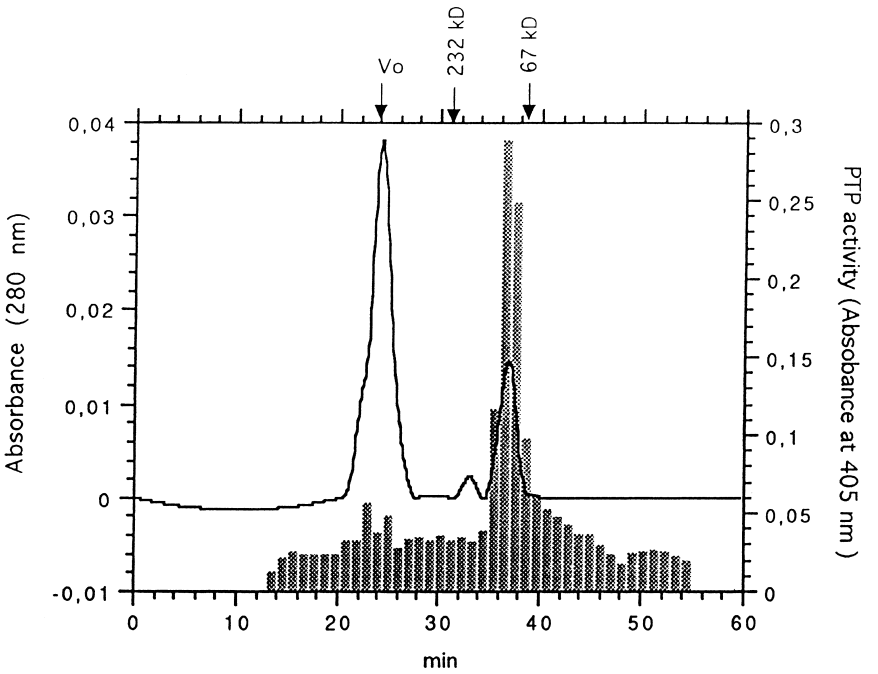

Fig. 4. The phosphatase activity is associated with the monomeric form of MBP–PTP-I after denaturation/renaturation. Aggregated (inactive) MBP–PTP-I was treated with 2.5% SDS and 5 M Urea and renatured by gel filtration (Superdex 200 SMART®). The fractions corresponding to the monomer, dimer and higher oligomeric forms were incubated with *p*-NPP (10 mM final) at 37°C for 12 h. The apparent PTP activity is represented by bars.

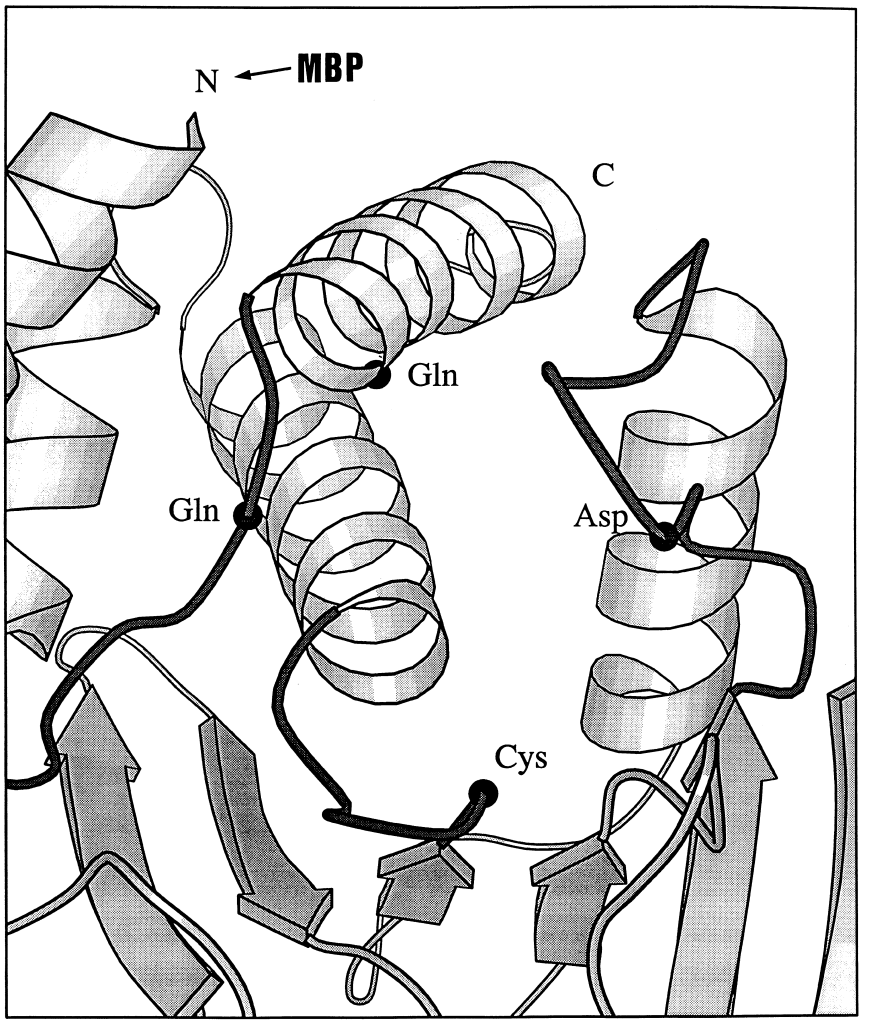

Fig. 5. Ribbon model of the active site of monomeric phosphatases. The nucleophilic cysteine residue is labelled. In our construction, the MBP moiety is fused to the NH_2 terminus of the PTP domain (indicated with an arrow) and could therefore interact with neighbouring PTP loops (shown in darker colour) containing functionally critical residues (Asp, Gln). The figure was drawn with MOLSCRIPT [36].

corresponding to the monomeric form, even after denaturation/renaturation of MBP–PTP-I (Fig. 4). These observations are consistent with the crystallographic study of RPTPα, which demonstrated that homodimerization of the first PTP domain inactivated the enzyme by blocking the access of substrate to the catalytic site [33].

At this point, our results demonstrated that: (i) the N-terminal PTP domain of CD45 can be an active phosphatase in the absence of the second domain, (ii) the PTP activity is only evidenced when PTP-I covalently bound to MBP (or to PTP-II in the wild-type enzyme) and (iii) the monomeric form of MBP–PTP-I is responsible for the phosphatase activity. In the light of these results, we may speculate that MBP activates the first PTP domain of CD45 through specific protein–protein interactions. This interaction probably involves contact residues close to the active site cleft and could compensate, at least partially, the putative contacts induced in wild-type CD45 by the presence of the second PTP domain. A structural model of the MBP–PTP-I protein provides additional support to this hypothesis. In the fusion protein, the C-terminal region of MBP is connected through a 22 amino acid-long linker to the N-terminal segment of PTP-I, a region which folds into an α-helix close to the substrate-binding cleft in monomeric phosphatases (Fig. 5). In particular, two loops of the PTP domains close to the N-terminal α-helix contain important functional residues. One of these loops has two glutamine residues (corresponding to Gln^{872} and Gln^{876} in CD45–PTP-I) which are highly conserved in the family of protein tyrosine

Table 1
Kinetic constants for dephosphorylation of *p*-NPP by different CD45 constructions

	K_m (mM)	V_{max} (μmol/min)	k_{cat}/K_m (s^{-1} M^{-1})	°C
MBP–PTP-I[a]	0.8 ±0.2	22.98 ± 2.1	1 233	37
MBP–PTP-I+II	1.75 ± 0.24	348.6 ±1.2	12 297	37
PTP-I+II[b]	4.8	36	11 000	25

[a]Values were corrected assuming that only the monomeric form of MBP–PTP-I was an active PTPase.
[b]Values taken from Cho et al. (1993)[27].

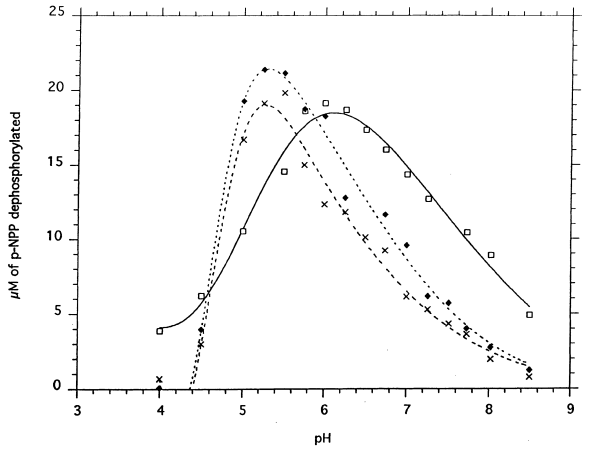

Fig. 6. Dephosphorylation of pNPP for the different active forms of CD45 as a function of pH. The solid line and (□) represents MBP–PTP-I, the dashed line and (X) represents PTP-I+II, and the dot line and (♦) represents MBP–PTP-I+II.

phosphatases and which have been proposed to be involved in substrate binding interactions [4,5]. A second loop close to the N-terminal α-helix of monomeric phosphatases contains the putative proton donor (Asp^{796} in CD45) and undergoes a significant conformational change upon the binding of phosphotyrosine to the active cleft (reviewed in [7]). Given their proximity to the N-terminal α-helix (and therefore to MBP), these loops might be involved in interdomain contacts, that could account for PTP-I activation.

Modifications of the local structural environment close to, or within, the active site cleft might affect the pKa value of functionally critical residues. The study of the pH dependence of the enzymatic activity could therefore provide an indirect evidence about MBP-contact regions in the neighbourhood of the PTP-I active site. As expected, the two constructions containing the entire cytoplasmic region (MBP–PTP-I+II and PTP-I+II) displayed similar bell-shaped profiles with a maximum at pH 5–5.2 (Fig. 6). These values are within the pKa ranges observed for other PTPases [6] and confirm our previous results (Fig. 3) showing that the presence of MBP did not affect the catalytic properties of PTP-I+II. However, the absence of the second PTP domain caused a displacement of the activity curve towards higher pH values, with an optimal activity at 5.8–6.2 for the MBP/PTP-I protein. These differences are apparently due to a change in the ascending slope of the activity curve and suggest that a region near the active site of the PTP-I domain might directly interact with the MBP moiety in the fusion protein. In the case of wild-type CD45, similar interactions between the two PTP domains could serve to regulate the PTPase activity [13,20,21], although other mechanisms of regulation, such as phosphorylation and external ligand binding have also been proposed [34,35]. Ultimate validation of this hypothesis can only be provided by further biochemical and structural studies of CD45 and other members of the RPTP family.

In conclusion, our results demonstrate that the first PTP domain of CD45 may be an active phosphatase in the absence of the second domain. However, a functional PTP-I domain requires specific protein–protein interactions with an additional factor (MBP or PTP-II), a particular feature of CD45 that may differentiate it from other receptor-like transmembrane phosphatases.

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