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Some protein tyrosine phosphatases target in part to lipid rafts and interact with caveolin-1

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Abstract

A profile-based search of the SWISS-PROT database reveals that most protein tyrosine phosphatases (PTPs) contain at least one caveolin-1-binding motif. To ascertain if the presence of caveolin-binding motif(s) in PTPs corresponds to their actual localization in caveolin-1-enriched membrane fractions, we performed subcellular fractionating experiments. We found that all tested PTPs (PTP1B, PTP1C, SHPTP2, PTEN, and LAR) are actually localized in caveolin-enriched membrane fractions, despite their distribution in other subcellular sites, too. More than 1/2 of LAR and about 1/4 of SHPTP2 and PTP-1C are localized in caveolin-enriched membrane fractions whereas, in these fractions, PTP-1B and PTEN are poorly concentrated. Co-immunoprecipitation experiments with antibodies specific for each tested PTP demonstrated that all five phosphatases form molecular complexes with caveolin-1 in vivo. Collectively, our findings propose that particular PTPs could perform some of their cellular actions or are regulated by recruitment into caveolin-enriched membrane fractions. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: PTP; PTPase; Caveolin; Caveolae; Caveolin-binding protein

Protein tyrosine phosphorylation is a key mechanism for eukaryotic cell regulation, because it is involved in several important processes such as cellular metabolism, proliferation, differentiation, and oncogenic transformation [1]. In the cell, protein tyrosine phosphorylation levels are balanced by the counteraction of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The PTK family consists of receptor and non-receptor enzymes: the former localized in the plasma membrane, and the latter in other cell compartments. The PTP super-family consists of four main families: the tyrosine-specific phosphatases, the VH1-like dual specificity phosphatases, the cdc25 phosphatases, and the low molecular weight phosphatases (LMW-PTPs). Despite their extremely limited sequence similarity, all share an active site motif consisting of a cysteine and an arginine separated by five residues (CXXXXXR, where C and R are essential residues and X is any amino acid). All PTPs have an identical catalytic mechanism, which involves the formation of a cysteinyl-phosphate covalent intermediate [2].

Although receptor PTKs were considered to be the principal enzymes regulating mitogenic protein phosphorylation cascades, the presence of Src homology-2 (SH2) domains in specific PTPs and the receptor-like structure of some membrane PTPs clearly indicate that PTPs too are regulated in the cell. Furthermore, a number of recent reports indicate additional regulation mechanisms for PTPs, such as the action of NO and other reactive oxygen species (ROS) on the active site cysteine, which determine the transient inactivation of these enzymes [3–8].

Caveolae are flask-shaped plasma membrane invaginations with characteristic diameter in the 50–100 mm range [9,10]. Though caveolae are present in most cells, they are particularly abundant in terminally differentiated one such as the adipocytes, endothelial, and skeletal muscle cells. Recent studies have revealed the presence of at least three mammalian caveolin sub-types, caveolin-1, caveolin-2, and caveolin-3, with different tissue distribution ([11], and citations therein). The interaction between caveolin-1 and other proteins is mediated by a short stretch of the membrane-proximal region (or caveolin-1 scaffolding domain), formed by residues 82–101, which recognizes and binds proteins containing the

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sequence motif ψXψXXXXψ or ψXXXXψXXψ, where ψ is an aromatic residue and X is any amino acid [12].

We carried out a search in the SWISSPROT database, and found that at least one caveolin-1-binding motif is contained in most PTPs. Furthermore, we demonstrated that all five tested PTPs are in reality associated to the caveolin-enriched membrane fractions, despite being localized in other cellular sites as well. Since caveolae seem to regulate several signaling molecules, our findings suggest that PTPs are also regulated by association to caveolae during signaling.

Materials and methods

Materials. Polyclonal anti-SHPTP2 (sc-280) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies directed against caveolin-1 (C13630, polyclonal), PTP1B (P-18020, monoclonal), PTP1C (P17320, monoclonal), PTEN (P96520, monoclonal), LAR (L33420, monoclonal), and gelsolin were purchased from Transduction Laboratories.

Protein determination. Protein concentration was assayed by the bicinchoninic acid method (BCA-kit) purchased from Sigma.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was performed according to Laemmli [13].

Cell culture. Human A431 cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum and antibiotics (penicillin, streptomycin).

Detergent-free purification of caveolin enriched membrane fractions. Low-density caveolin-enriched membrane fractions were isolated as described by Song et al. [14]. Briefly, one confluent 100-mm dish, washed twice with ice-cold phosphate-buffered saline (PBS: 10 mM sodium phosphate and 0.15 M NaCl, pH 7.2), was scraped into 0.5 ml of sodium carbonate buffer (500 mM sodium carbonate, pH 11, 25 mM 4-morpholineethanesulfonic acid, 150 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cells were homogenized extensively using a Dounce homogenizer (50 strokes). The homogenate was then adjusted to 45% sucrose by the addition of 0.65 ml of 80% sucrose in 25 mM (morpholinoethane sulfonic acid (MES), 150 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and then placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed above the 45% layer, by adding 2.5 ml of 35% sucrose and 1.3 ml of 5% sucrose, both in 250 mM sodium carbonate, pH 11, 25 mM MES, 150 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin. The gradient was centrifuged at 170,000*g* for 20 h using a Beckman SW50.1 rotor. For the analysis of the resulting gradient, 0.35 ml fractions were collected from the top to the bottom of the gradient. The insoluble pellet (fraction 15) was dissolved into 50 μl of Laemmli sample buffer. The proteins contained in the fractions were separated by SDS–PAGE, and transferred onto a polyvinylidene difluoride membrane. Blots were probed first with specific antibodies, and then with secondary antibodies conjugated with horseradish peroxidase, washed, and developed with the enhanced chemiluminescence kit.

Immunoprecipitation experiments. Cells were cultured in 100 mm dishes were washed with PBS, lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 60 mM *n*-octylglucoside, 2 mM EDTA, 1 mM orthovanadate, 100 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and insoluble material was removed by centrifugation at 10,000*g* for 10 min. For immunoprecipitation, protein concentration of the lysate was adjusted to 1 mg/ml by diluting with the lysis buffer, and 1 mg total protein was used in each experiment. Then specific anti-PTP antibodies (1 μg) were added, and the mixtures were incubated overnight at 4 °C. After 1-h incubation with protein A–Sepharose beads at 4 °C, the immunocomplexes were collected and washed extensively (three times) with lysis buffer. The beads were suspended in 20 μl of two-fold concentrated Laemmli electrophoresis buffer (without 2-mercaptoethanol), separated by SDS–PAGE and electroblotted onto polyvinylidene difluoride membranes for detection.

Results

Most eukaryotic PTPs contain one or more potential caveolin-1-binding motifs

A profile-based search of the SWISS-PROT database, Eukaryota section, using the program PATTINPROT at PBIL (Pôle Bio-Informatique Lyonnais), revealed that most of known eukaryotic PTPs and dual specificity protein phosphatases contain one or both the caveolin-1-binding motifs (ψXψXXXXψ or ψXXXXψXXψ) described by Couet et al. [12]. This finding implies that some PTPs are potentially recruited into caveolae, and suggests that some of them elicit a part of their cellular functions or are regulated within these lipid raft membrane domains.

To investigate the possible localization into caveolin-enriched membrane fractions of the PTPs containing putative caveolin-1-binding domains, we have selected five human PTPs: PTP1B, PTP1C, SHPTP2, PTEN, and LAR, taking into account that A431 cells were known to express these PTPs. Table 1 shows the potential caveolin-1-binding motifs contained in the above PTPs.

Some PTPs associate with caveolin-enriched membrane domains in vivo, and coimmunoprecipitate with caveolin-1

We lysed near-confluent human A431 cells and then separated the caveolae-enriched membrane domains from other cellular components, using the density gradient centrifugation method described in Materials and methods. Fifteen fractions were collected from the top of the gradient. A constant volume from each fraction was analyzed by SDS–PAGE and Western blotting with anti-caveolin-1 antibody. All the results shown in Fig. 1 demonstrate that the caveolin-1 band is essentially restricted to fractions 5–7 indicating the position of the caveolin-enriched membrane domains in the gradient. To ascertain the fractions containing the various PTPs (PTP1B, PTP1C, SHPTP2, PTEN, and LAR), we performed Western blot analysis with specific antibodies directed against each PTP. In all cases both the caveolin-enriched membrane domains (fractions 5–7) and the bulk cell fractions (fractions 9–15) contained the above PTPs, although their relative abundance is different. Fig. 3 summarizes the data obtained by densitometry: greater than 60% of total LAR is localized in caveolin-enriched membrane fractions that contain, however, only about

Table 1
Caveolin-1-binding motifs

Accession No.	PTP	Position	Motif
O00633	PTEN *Homo sapiens*	271–278	FHFWVNTF
P29350	PTP-1C *H. sapiens*	206–213	FVYLRQPY
Q06124	SH-PTP2 *H. sapiens*	416–423	WQYHFRTW
P10586	LAR (precursor) *H. sapiens*	531–538	IMYELVYW
		1355–1362	FTWENSNL
P18031	PTP-1B *H. sapiens*	174–182	FHYTTWPDF
P10586	LAR (precursor) *H. sapiens*	1785–1793	IRQFQFTDW

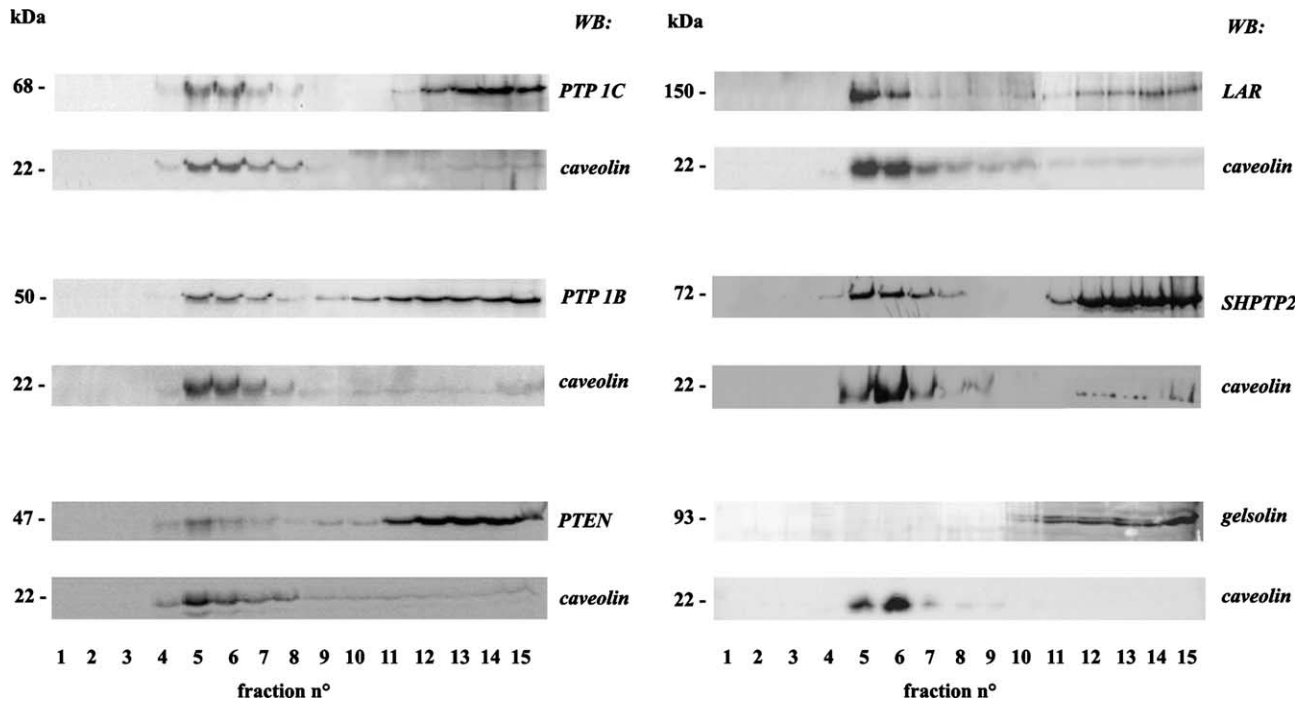

Fig. 1. In human A431 cells, PTP1B, PTP1C, SHPTP2, PTEN, and LAR in part co-fractionate with caveolin-enriched membrane domains and in part with other cellular fractions. Cells were subjected to sub-cellular fractionation on a discontinuous sucrose density gradient after homogenization in alkaline buffer at the conditions described in Materials and methods. Sucrose gradients were fractionated by collecting 0.35 ml fractions from the top, and 30 μl of each fraction were subjected to immunoblot analysis with specific antibodies directed against caveolin-1, PTP1B, PTP1C, SHPTP2, PTEN, and LAR. Note that caveolin-enriched membrane domains (fractions 5–7) are separated from gelsolin (fractions 11–15). One representative experiment for each PTP is shown ($n = 3$).

10% of PTP1B and PTEN. Furthermore, about 30% of total PTP-1C and SHPTP2 are localized in caveolin-enriched membrane domains of A431 cells. The result of a control experiment performed with gelsolin (a cytosolic actin-binding protein) confirms its extra-caveolar localization (Figs. 1 and 3). Additionally, we found that more than 99% of total cellular proteins were excluded from the caveolin-rich membrane fractions (data not shown).

To determine whether each PTP (PTP1B, PTP1C, SHPTP2, PTEN, and LAR) interacts directly with caveolin-1 in vivo, we performed co-immunoprecipitation experiments human A431 cell lysate. Immune-complexes were collected on protein A–Sepharose, separated by SDS–PAGE, and immunoblotted using antibodies specific for caveolin-1 as well as those specific for each PTP. The results shown in Fig. 2 demonstrate that all tested PTPs co-immunoprecipitate with caveolin-1, thus indicating that they are complexed in vivo with caveolin-1 in human A431 cells. In contrast, the immunoprecipitate obtained with antibodies directed against gelsolin (a cytosolic actin-binding protein) does not contain caveolin-1 (Fig. 2).

Discussion

Protein phoshorylation on specific tyrosine residues is the main mechanism that controls important cellular processes such as cell growth, proliferation, locomotion,

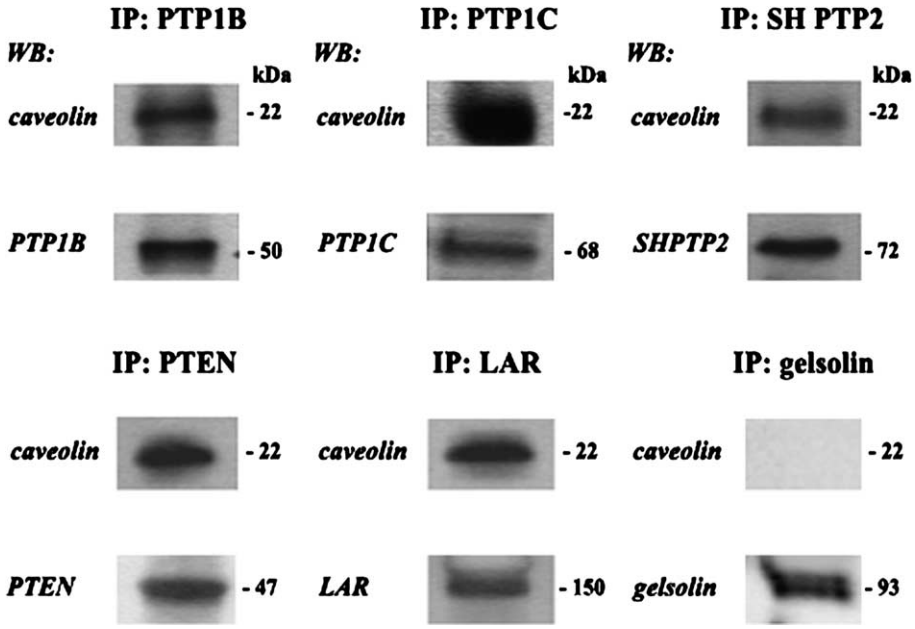

Fig. 2. In human A431 cells, PTP1B, PTP1C, SHPTP2, PTEN, and LAR co-immunoprecipitate with caveolin-1. PTPs were immunoprecipitated from the human A431 cell lysate in separate experiments using specific antibodies directed against each particular PTPs. The immunoprecipitates were collected by the addition of protein A–Sepharose beads and analysed by SDS–PAGE. Caveolin-1 was immunodetected by the anti-caveolin-1 antibody, whereas the various PTPs were immunodetected by a set of antibodies, each directed against a particular PTP. One representative experiment for each PTP is shown ($n = 3$).

differentiation, transformation, and metabolism. Two kinds of enzymes (PTKs and PTPs) are involved in balancing the tyrosine phosphorylation level of cellular proteins. Until recently, most studies were focused on the regulation of the activity of PTKs, because PTPs were initially believed to be few in number and to have little substrate specificity; furthermore, they were thought to exert a mere "housekeeping" role [15]. On the contrary, PTPs form a large family subjected to sophisticated modes of regulation, and playing critical role in controlling a wide range of signaling pathways [15]. In addition, recent findings indicate certain PTPs to be highly specific, not only for a particular protein substrate but also for specific phosphorylation sites ([15], and citations therein). In some cases their specificity is conferred by intracellular targeting, but there are also examples in which PTP catalytic domains display high specificity on the recognition of substrates. Tonks and Nee [15] have suggested that PTP specificity is actually governed by targeting and catalytic domains acting in a combinatorial fashion.

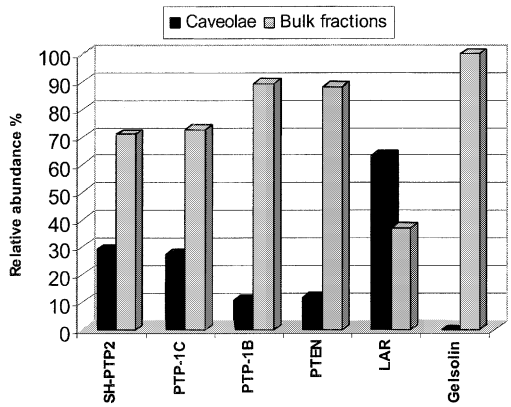

Fig. 3. Densitomentric analysis of PTP1B, PTP1C, SHPTP2, PTEN, LAR, and gelsolin contained in caveolae-enriched membrane domains and in bulk cellular fractions. Densitometric analysis was performed on the images reported in Fig. 1, using the Quantity One software (BioRad).

This study offers evidence that most PTPs contain one or more potential caveolin-1 binding-sequence-motifs, which are generally thought to be involved in targeting proteins to the caveolae rafts. Caveolin-enriched membrane fractions are rich in cholesterol, glycosphingolipids, and sphingomyelin. Caveolin-1, a 22–24 kDa protein, is a major integral membrane component of caveolae. Caveolin-1 is ubiquitous and is involved not only in the recruitment of protein to caveolae, but also in regulating the activities of several signaling molecules such as etherotrimeric G-proteins, Src kinases, nitric oxide synthase, epidermal growth factor, and platelet-derived growth factor receptors, as well as of protein kinase C. All these signaling molecules contain at least one of the sequence signature motifs described above. Binding of these proteins with caveolin-1 may produce negative or positive regulation of their activity ([10], and citations therein).

Caveolin-1 is phosphorylated on tyrosine-14 by the oncogenic viral Src kinase, and is associated with normal cellular Src and other Src family tyrosine kinases [16,17]. The functional consequences of tyrosine phosphorylation of caveolin-1 are not well understood, although several reports suggest some involvement of caveolin phosphorylation in several cellular processes [18–25].

To assess whether the presence of caveolin-binding motifs in PTPs actually determines their association with caveolin-1 and their recruitment into caveolin-enriched membrane domains, we have selected five PTPs (PTP1B, PTP1C, SHPTP2, PTEN, and LAR), all expressed in human A431 cells, and performed two kinds of experiments: (i) sucrose gradient centrifugation to test their eventual localization into caveolin-enriched membrane fractions, and (ii) immunoprecipitation of caveolin-1–PTP complexes using antibodies specific to each PTP. Four out of the five PTPs (PTP1B, PTP1C, SHPTP2, and PTEN) contain one canonical caveolin-1-binding motifs, whereas human LAR contains three similar motifs (see Table 1). In their study on caveolin-1-binding motifs Couet et al. [12] reported that almost 10% of the selected 15-mer peptides in their library contained only two aromatic residues. They also reported that Gαq contained valine and leucine as substitutes for aromatic residues and that it nevertheless coimmunoprecipitates with caveolin-1. Furthermore, Carman et al. [26] reported that all known G-protein-coupled receptor kinases contained the conserved caveolin-1-binding motif **I/LXXXXFXXF**.

The results reported in Fig. 1 show that all tested PTPs are localized in part in caveolin-enriched membrane domains and in part in other cellular fractions. Furthermore, the co-immunoprecipitation results shown in Fig. 2 demonstrate that in A431 cell caveolin-1 is able to form molecular complexes with all the tested PTPs. Taken together our findings reveal that the potential caveolin-1-binding motifs contained in the five PTPs expressed in A431 cells are indeed involved in binding with caveolin-1, which may determine the recruitment of these PTPs into caveolin-enriched membrane domains.

Previous findings have demonstrated that the two other PTPs were localized in caveolin-enriched membrane domains: CD45 [27] and LMW-PTP [28]. CD45 is a receptorial PTP involved in both T- and B-cell signaling, whereas LMW-PTP is a small enzyme involved in down-regulating PDGF and insulin receptors and in regulating citoskeleton rearrangement. CD45 contains one canonical caveolin binding motif (ψXψXXXXψ), whereas LMW-PTP, which is able to associate with caveolin-1 in vivo [28], contains a caveolin-1-binding motif identical to that found in the G-protein coupled receptor kinases [26].

First Liu and co-workers [29] reported that multiple caveolae proteins acquire phosphotyrosine when PDGF binds to its receptor. Successively, other authors described that other caveolae-recruited proteins (such as endothelial nitric oxide synthase, epidermal growth factor, STAT3, the low-density lipoprotein receptor-related protein, and dynamin [30–35]) are phosphorylated during normal or pathological signaling. Collectively, these reports demonstrate the presence of several tyrosine phosphorylated proteins in the caveolae rafts; their phosphorylation may cause changes in their cellular localization or activity [30,32,35]. In some cases, the protein tyrosine kinases phosphorylating caveolae proteins are known, but only one report indicates a caveolae target substrate for a particular PTP, the LMW-PTP [28]. It is well known that a large number of genes codify for PTPs, but the cellular targets for each PTP are generally unknown. The presence of multiple protein substrates phosphorylated on tyrosine into caveolae suggests that their phosphorylation state is regulated not only by the action of protein tyrosine kinases, but also by certain PTP family members able to be recruited into caveolae. Our results demonstrate that most PTPs contain potential caveolin-binding motifs probably involved in their recruitment into caveolin-enriched membrane fractions. We put forward the idea that some PTPs play a part of their cellular actions on caveolar protein substrates.

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