Low Molecular Weight Protein Tyrosine Phosphatase and Caveolin-1: Interaction and Isoenzyme-Dependent Regulation†

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ABSTRACT: Low molecular weight protein tyrosine phosphatases (LMW-PTPs) are small enzymes that are ubiquitous in many organisms. They are important in biological processes such as cell proliferation, adhesion, migration, and invasiveness. LMW-PTP is expressed in mammalian cells as two isoforms (IF1 and IF2) originating through alternative splicing. We have previously shown that IF2 targets lipid rafts called caveolae and interacts with caveolin-1, their major structural protein. Caveolae are cholesterol- and sphingolipid-rich membrane microdomains that have been implicated in a variety of cellular functions, including signal transduction events. Caveolin-1 contains a scaffolding region that contributes to the binding of the protein to the plasma membrane and mediates protein omo- and etero-oligomerization. Interaction of many signaling molecules with the scaffolding domain sequesters them into caveolae and inhibits or suppresses their activities. Caveolin-interacting proteins usually have a typical sequence motif, also present in all the LMW-PTPs, which is characterized by aromatic or large hydrophobic residues in specific positions. We have examined here the interaction of the LMW-PTP isoforms with caveolin-1 and its molecular mechanism, together with the consequences for their tyrosine phosphatase activities. We found that IF1 and IF2 are both capable of interacting with defined regions of caveolin-1 and that their putative caveolin binding sequence motif is not responsible for the association. The formation of LMW-PTP/caveolin-1 complexes is accompanied by modulation of the enzyme activities, and the inhibitory effect elicited against IF1 is stronger than that against IF2. The caveolin scaffolding domain is directly involved in the observed phenomena.

Caveolae are a subset of specialized liquid-ordered domains, referred to as lipid rafts, which are uniquely enriched in various membrane components including cholesterol, sphingolipids, and GPI-anchored proteins (*1*−*3*). The major structural protein of caveolae is caveolin, a 21−24 kDa protein that occurs in three isoforms called caveolin-1, -2, and -3 (*1*, *4*).

Many proteins that reside in caveolae are involved in signal transduction, including some tyrosine kinase receptors (*5*, *6*), G-protein α-subunits (*7*), H-Ras, Src family members (*8*), endothelial nitric oxide synthase (e-NOS)[1] (*9*), and some protein tyrosine phosphatases (*10*). In recent years it has been established that caveolae are multifunctional organelles, playing important roles in a variety of cellular processes mainly through interactions with one of the caveolin proteins and many signaling molecules. Full-length caveolin-1 contains three principal domains: a 101-residue N-terminal domain, a 33-residue membrane spanning region, and a 44-residue C-terminal domain. Both the N- and the C-terminal domains face the cytoplasm, allowing them to interact freely with cytosolic molecules. Usually about 14−16 caveolin monomers assemble into discrete multivalent oligomers; within caveolae these individual caveolin homo-oligomers (4−6 nm particles) can interact with each other or with other molecules to form functional complexes (*11*). Interaction of caveolin with many signaling proteins suppresses their enzymatic activity (*12*). Thus caveolin may organize the formation of caveolae microdomains and regulate signaling events. The interaction of caveolin with itself and other proteins is mediated principally by a region within the N-terminal domain called the oligomerization domain (residues 60−101). Within this region there is a scaffolding domain (residue 82−101) that is responsible for the inhibitory effect of caveolin. Most caveolin-associated proteins possess at least one typical amino acid sequence called the caveolin binding motif, characterized by aromatic or large hydrophobic residues (defined as Φ) in specific positions: ΦXΦXXXXΦ, ΦXXXXΦXXΦ, and ΦXΦXXXXΦXXΦ (*13*).

LMW-PTP is a ubiquitous 18 kDa enzyme that is composed of two active isoforms (IF1 and IF2); these are transcribed from a single gene by alternative splicing (*14*,

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[1] Abbreviations: PTP, protein tyrosine phosphatase; LMW, low molecular weight; IF1, LMW-PTP isoform-1; IF2, LMW-PTP isoform-2; e-NOS, endothelial nitric oxide synthase; GST, glutathione *S*-transferase; pNPP, *p*-nitrophenyl phosphate.

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15). The amino acid sequence from residue 40 to residue 73 is isoform specific. IF1 and IF2 differ in substrate specificity and in their sensitivity, in vitro, to some modulators (*16*), and it is reasonable to suppose that they perform distinct physiological functions. The best characterized isoform is IF2; this enzyme plays a key role in cell proliferation control, and many proteins involved in signaling pathways are among its substrates: PDGF, insulin, and ephrin receptors (*17*, *18*), STAT5 (*19*), p190 RhoGap (*20*), caveolin-1 (*21*), and β-catenin (*22*). LMW-PTP cannot be considered a mere cytosolic enzyme, since it is constitutively present in the cytoskeleton and in caveolin-rich membrane domains, in which it interacts with caveolin-1 (*21*). Upon stimulation by growth factor, c-Src is able to bind and to phosphorylate the cytoskeleton-associated enzyme. Once phosphorylated, LMW-PTP increases its activity about 20-fold, strongly influencing both cellular adhesion and migration (*23*−*25*). The LMW-PTP isoenzymes are also regulated by reversible oxidation of a catalytic cysteine residue (*26*−*28*).

In this paper we show that IF1 and IF2 both interact directly with caveolin-1. The interaction is not mediated by their caveolin binding sequence motif, since recombinant forms mutated in this region are still able to interact with caveolin; it must involve more than one caveolin subdomain. We investigated whether there is a functional consequence of LMW-PTP/caveolin interactions by performing inactivation tests in the presence of the GST/caveolin-1 fusion protein or the synthetic peptide corresponding to the scaffolding domain of caveolin (residues 82−101). We found that caveolin-1 can specifically and differentially modulate the activity of LMW-PTP isoenzymes, causing strong inhibition of the sole IF1; the effect is partly attributable to the scaffolding domain.

EXPERIMENTAL PROCEDURES

Materials. The synthetic peptide corresponding to the scaffolding domain of caveolin (cav[82−101]) was obtained from Neosystem. The synthetic peptide corresponding to the 61−82 domain of caveolin (cav[60−81]), recombinant LAR-PTP, and Tcell-PTP were obtained from Sigma. Monoclonal anti-caveolin-1 antibody (610057) and rabbit polyclonal anti-caveolin (610059) were purchased from BD Transduction Laboratories (Lexington, KY). Anti-GST (sc-138) was purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-LMW-PTP antibody was produced in our laboratory. Dipyridylruthenium(II) was purchased from Aldrich. Lysozyme from hen egg white was from Fluka. All other reagents were the purest commercially available.

Cloning of the cDNA-Encoding Caveolin Subdomain cav-[95−135]. The cDNA construct encoding GST-caveolin subdomain 95−135 (cav[95−135]) was generated by PCR amplification of the full-length bovine sequence cloned into the pGEX-KT cloning vector. Primers for PCR were designed to incorporate *Bam*HI/*Eco*RI restriction sites for subcloning. The cDNAs encoding the fusion proteins were sequenced by the Sanger method to confirm the creation of in-frame fusions devoid of PCR-associated nucleotide incorporation errors.

Site-Directed Mutagenesis. Oligonucleotide-mediated mutagenesis was performed with the Unique Restriction Elimination Site (USE) mutagenesis kit as described by the manufacturer (Amersham Pharmacia Biotech). The mutagenic primers converting the putative caveolin binding motif of the LMW-PTP isoenzymes from ITKEDFATF to ITKEDFATQ (F85Q) and from ITKEDFATF to ITKEDMATQ (F82M/F85Q) were 5′CTTCGTGACCCAGGACTACATCCTG3′ and 5′GTGACAAAATATTGGTTTTACCGC3′. The IF1 and IF2 mutants were verified by DNA sequencing by the Sanger method.

Purification of GST-Fusion Proteins. All GST-fusion proteins were expressed in *Escherichia coli* (BL21 strain; Novagen, Inc.). The GST-caveolin fusion proteins (GST-cav, GST-cav[1−60], GST-cav[95−135], and GST-cav-[135−178]) and the GST protein were purified by affinity chromatography on glutathione−agarose as described by Frangioni and Neel (*29*). Human recombinant LMW-PTP isoenzymes and PTP-1B were prepared as GST-fusion proteins or not, as described previously (*30*).

Cell Culture. Murine endothelial H-end cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, penicillin, streptomycin, and L-glutamine.

Cell Lysis and Immunoprecipitation. We produced cell lysates in native and in denaturing conditions. Native conditions: A culture dish (100 mm) of confluent cells was rinsed with phosphate-buffered saline (PBS) and lysed with 10 mM Tris, pH 7.4, 60 mM octyl glucoside, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 1 μg/mL leupeptin, and 1 μg/mL aprotinin (buffer A). The sample was maintained in constant agitation for 30 min at 4 °C. After sonicate briefly, the sample was centrifuged at 16000*g* for 10 min.

Denaturing conditions: A culture dish (100 mm) of confluent cells was rinsed with phosphate-buffered saline (PBS) and lysed with 1 mL of boiling 1% SDS in buffer A. The lysate was transferred to a 1.5 mL microcentrifuge tube and boiled for an additional 5 min. After sonication briefly, the sample was centrifuged at 16000*g* for 10 min. Lysates (2 mg of protein) were incubated overnight with 2 μg of anti-caveolin antibody. After 1 h incubation with protein A−Sepharose beads at 4 °C, the immunocomplexes were collected and washed three times with buffer A. The samples were resuspended in 50 mM β,β-dimethylglutarate, pH 7.0, 150 mM NaCl, 1 mM EDTA, and 5 mM dithiothreitol.

In Vitro Interaction. IF1 or IF2 (2 μg) was added to caveolin-1 immunoprecipitated from H-end cell lysates, obtained in native conditions and in denaturing ones. After 1 h incubation at room temperature, samples were extensively washed and then analyzed by SDS−PAGE, and proteins were revealed by silver staining.

Pull-Down Assay. H-end cell lysates (1 mL) containing 1.2 mg of protein were added to 10 μg of GST or GST-fusion proteins (GST-IF1, GST-IF2, GST-IF2/F85Q, GST-IF2/F82MF85Q) bound to glutathione−agarose. After 16 h incubation at 4 °C, samples were extensively washed and then analyzed by western blotting using anti-caveolin-1 and anti-GST antibodies.

Determination of the Effects of GST/Caveolin-1 and of Synthetic Peptides on Phosphatase Activity. To examine the effect of caveolin-1 on LMW-PTP activity, variable amounts (0.1−1 μM) of GST/caveolin-1 were incubated at 37 °C with purified IF1 or IF2 isoforms (0.1 μM) in 50 mM β,β-dimethylglutarate, pH 7.0, for 5 min. Enzymatic activities

were monitored at 37 °C by the addition of 5 mM pNPP. The sole GST was used as control. Analogous tests were also performed on some typical PTPs: PTP1B, LAR, and Tcell PTP. The LMW-PTP isoenzyme activities were assayed in the same experimental conditions with the synthetic peptides (cave[61−82] and cav[82−101]) or vehicle (dimethyl sulfoxide), using the concentration range of 0.1−35 μM.

Experiments testing the effects of synthetic peptides as a function of ionic strength were carried out by adding 0.1 or 0.5 M NaCl to the experimental mixtures, using the same protocol and only a selected peptide concentration; the molar ratio ([peptide]/[LMW-PTP]) in the tests was 100.

LMW-PTP Inhibition Kinetics. LMW-PTP activity was assayed as described above on *p*-nitrophenyl phosphate (1−25 mM) in the absence or in the presence of various cav-[82−101] peptide concentrations: 5, 10, and 25 μM. Kinetic constants were calculated using the FigSys software (Biosoft, U.K.) by nonlinear regression analysis of data fitted to the Michaelis−Menten kinetic function. For the determination of K_i, data were analyzed by fitting to models describing different inhibitor types.

Interaction of Caveolin Subdomains with LMW-PTP Isoenzymes. All of the experiments were performed in 50 mM β,β-dimethylglutarate, pH 7.0, 1 mM EDTA, and 5 mM dithiothreitol with or without 150 mM NaCl. GST-caveolin subdomains and GST-IF1 or GST-IF2 were respectively incubated with the pure LMW-PTP isoenzymes and caveolin synthetic peptides in equimolar concentration (10 μM). GST was used as control in all the experiments. After 30 min incubation at room temperature, glutathione−agarose beads were added. After 30 min the samples were extensively washed and then analyzed by SDS−PAGE. We used respectively 12% and 18% acrylamide gels. Proteins were revealed by silver staining.

MALDI Spectra. Mass spectra were obtained on a MALDI-TOF OmniFLEX (Bruker DALTONICS) instrument. IF1 and IF2 (1 μM) were incubated in the presence or in the absence of the cav[82−101] peptide (100 μM) in 10 mM β,β-dimethylglutarate, pH 7.0, for 5 min. The samples were mixed with an equal volume of sinapinic acid in 0.1% trifluoroacetic acid/CH_3CN, 2/1(v/v), and deposed on the probe. Mass spectra were produced by using the following parameters: delay time, 450 ns; ion focus, 9.6 kV; ion source 1, 19 kV; ion source 2, 16.7 kV.

The mass spectrum of the cav[82.101] peptide was obtained by using α-cyano-4-hydroxycinnamic acid (α-CHCA) as the matrix. Volumes of 0.5 μL of peptide solution [5 pmol/μL in 0.1% trifluoroacetic acid/CH_3CN, 2/1 (v/v)] were deposed on the target after dilution 1/1 with a saturated solution of α-CHCA. Parameters: delay time, 500 ns; ion focus, 9.6 kV; ion source 1, 19 kV; ion source 2, 13.2 kV; reflector, 20 kV.

Photoinduced Cross-Linking. IF1, IF2, and lysozyme (1 μM) in 50 mM β,β-dimethylglutarate, pH 7.0, were incubated in the presence or in the absence of the cav[82−101] peptide (100 μM). After 5 min of incubation at room temperature, 60 μM dipyridylruthenium(II) and 100 μM ammonium persulfate were added. Irradiation was performed for 30 s in 1.7 mL Eppendorf tubes positioned parallel to the beam of light at a distance of 10 cm from a 150 W incandescent lamp. After irradiation, samples were quenched with 50 μL of 4× Laemmli sample buffer, heated to 100 °C for 5 min, and analyzed by SDS−PAGE. Proteins were revealed by silver staining.

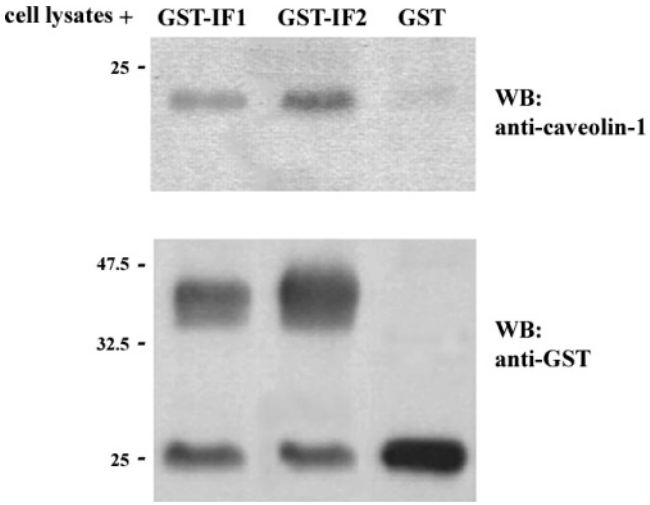

FIGURE 1: In vitro binding of LMW-PTP isoenzymes to cellular caveolin-1. 1 mL of H-end cell lysates containing 1.2 mg of protein was added to 10 μg of GST or GST-IF1 and GST-IF2 fusion proteins bound to glutathione−agarose. After 16 h incubation at 4 °C, samples were extensively washed and then subjected to SDS−PAGE and immunoblotting with anti-caveolin-1 and anti-GST antibodies. Cellular caveolin-1 associated strongly with the GST-IF1 and GST-IF2 fusion proteins but not with GST alone. Data are representative of two separate experiments.

Data Analysis. Results shown are referred to at least three separate experiments performed in duplicate. The data are expressed as the mean ± standard error of mean (SEM). Statistical analysis of the data was performed by Student's *t*-test. p values <0.05 were considered significant.

RESULTS

Both LMW-PTP Isoenzymes Directly Interact with Caveolin-1. To investigate the interaction between the LMW-PTP isoenzymes and caveolin-1, we used two distinct approaches: (i) pull-down experiments, using immobilized GST-LMW-PTP fusion proteins and cell lysates (the experimental details are described in Experimental Procedures), and (ii) in vitro binding assays using caveolin-1 immunoprecipitated in "native" rather than in "denaturing" conditions and pure IF1 and IF2 isoenzymes. Figure 1 shows that incubation of H-end cell extracts with GST alone or with GST-LMW-PTP fusion proteins resulted in the specific binding of IF1 and IF2 to cellular caveolin-1. To show that this is a direct interaction, we immunoprecipitated caveolin-1 from cell lysates produced (as described above) in two different ways. Denatured lysates were obtained by treating cells with boiling lysis buffer containing high concentrations of detergents (see Experimental Procedures for details); this procedure allows us to disrupt all protein complexes. Caveolin-1 immunoprecipitates were linked to Sepharose−protein A and were then incubated with IF1 or IF2. After washing, SDS−PAGE analysis was performed, revealing proteins by silver staining. Figure 2, which shows the positions of the bound proteins in relation with molecular weight standards and with pure IF1 and IF2, reveals that caveolin-1 always formed a stable complex with LMW-PTP isoenzymes. The association did not depend on other docking

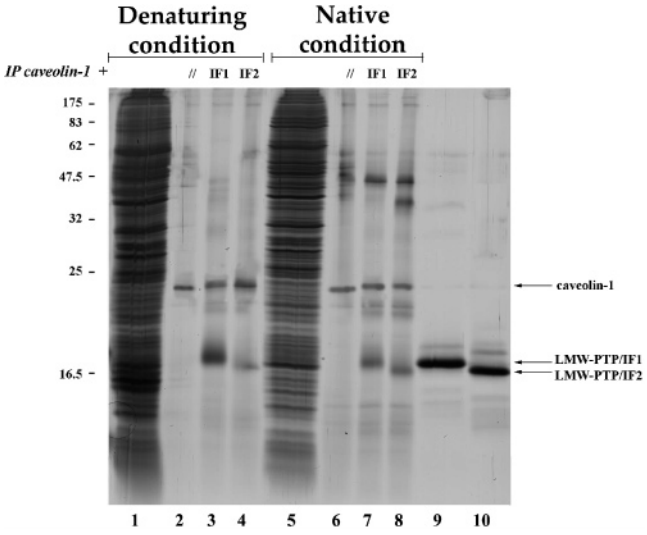

FIGURE 2: The LMW-PTP isoenzymes directly interact with caveolin-1. 2 μg of IF1 or IF2 was added to caveolin-1 immunoprecipitated from 2 mg of H-end cell lysates, obtained both in native conditions (buffer A: 10 mM Tris, pH 7.4, 60 mM octyl glucoside, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin) and in denaturing ones (boiling buffer A, containing 1% SDS). After 1 h incubation at room temperature, samples were extensively washed and then analyzed by SDS−PAGE. Proteins were revealed by silver staining. Lanes: 1, 20 μg of total lysate (denaturing conditions); 2, immunoprecipitated caveolin-1; 3, washed immunoprecipitated caveolin-1 + IF1; 4, washed immunoprecipitated caveolin-1 + IF2; 5, 20 μg of total lysate (native conditions); 6, immunoprecipitated caveolin-1; 7, washed immunoprecipitated caveolin-1 + IF1; 8, washed immunoprecipitated caveolin-1 + IF2; 9, IF1 positive control; 10, IF2 positive control.

molecules but occurred directly, and it was a specific binding. The enzymes never interacted with cell lysates (native and denaturing) immunoprecipitated by control IgG or with the sole anti-caveolin antibody (data not shown).

Caveolin-1 Specifically Inactivates LMW-PTP Isoenzymes. To determine whether interactions of LMW-PTP isoenzymes with caveolin-1 alter their catalytic activities, we incubated equal quantities of purified IF1 and IF2 with variable amounts of the GST alone or GST-caveolin-1 fusion protein. Phosphatase activities were then determined. As shown in Figure 3, the full-length caveolin-1 strongly inhibited IF1; a molar ratio of 7 sufficed for almost complete inactivation of this isoenzyme. In the same conditions, only about 30% inactivation was observed for IF2. (Caveolin-1 in vivo assembles into discrete multivalent oligomers, and a molar ratio of 7−8 may be representative of physiological conditions.) At low GST/LMW-PTP molar ratio, we observed that GST alone activated both IF1 and IF2 via an unknown mechanism. This phenomenon could explain why we observed an apparent activation in IF2, which shows less sensitivity to the inhibitory action of the caveolin-1. To further assess the importance of these results, we performed analogous inactivation tests on other typical PTPs: PTP1B, LAR, and Tcell PTP. Figure 4 demonstrates that the effect elicited against IF1 is much more significant than for the other enzymes analyzed.

The Scaffolding Domain of Caveolin-1 Forms Stable Complexes with IF1 and IF2 and Modulates Their Enzymatic Activity. Caveolin-1 has been shown to interact directly with

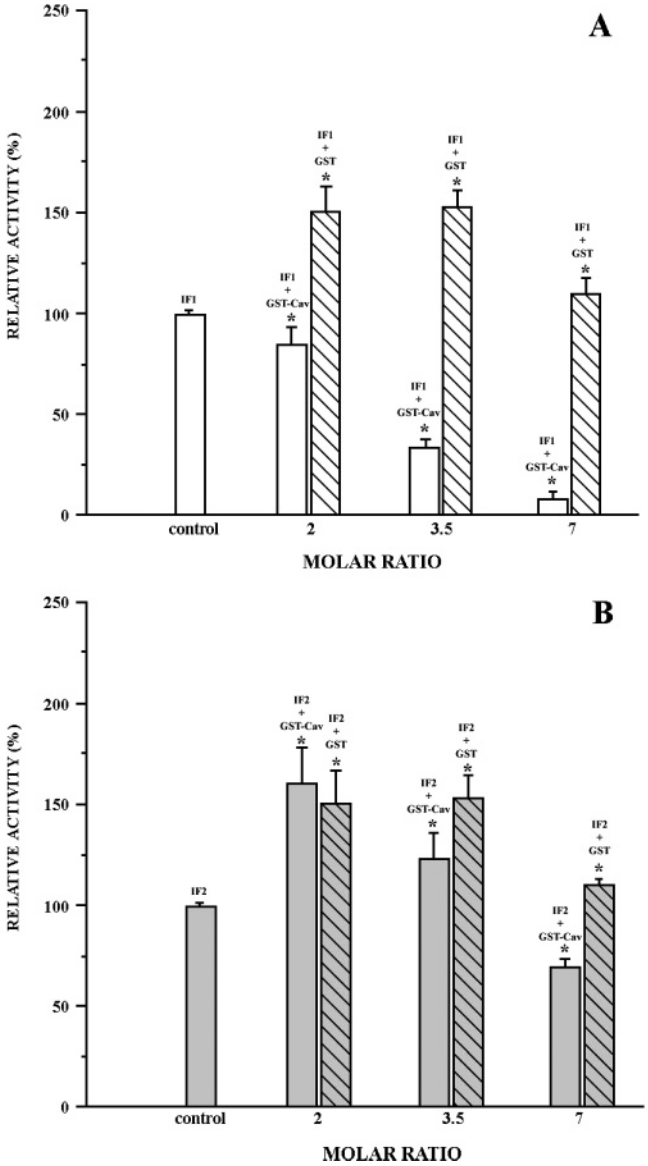

FIGURE 3: Effect of GST-caveolin 1 fusion protein on LMW-PTP activity. 0.1 μM IF1 (panel A) or IF2 (panel B) was incubated with different amounts of GST or GST-caveolin 1 fusion protein for 5 min at 37 °C. LMW-PTP activities were then determined by adding *p*-nitrophenyl phosphate as substrate. The molar ratio indicates GST or GST-caveolin concentration/LMW-PTP concentration. Results shown represent the means of three experiments in duplicate ± SEM. *, $p < 0.05$ compared with the control group, represented by the sole enzyme.

a variety of cytoplasmatic signal transducing molecules through the common proximal region of its NH_2-terminal cytoplasmic domain, called the scaffolding domain (cav[82−101]). Accordingly, we determined whether a synthetic peptide derived from this motif is the region responsible for the observed inactivation. To test the possibility that the scaffolding domain of caveolin-1 was capable of directly interacting with the LMW-PTP isoenzymes, we performed three experiments. We incubated two synthetic peptides corresponding to caveolin-1 sequence regions 61−82 (cav-[60−81]) and 82−101 (cav[82−101], the scaffolding domain) with GST and GST-LMW-PTP fusion proteins (GST-IF1 and GST-IF2). The tests were performed in the presence of 0.15 M NaCl. In all experiments the GST-fusion proteins or GST alone were prebound to agarose beads. After

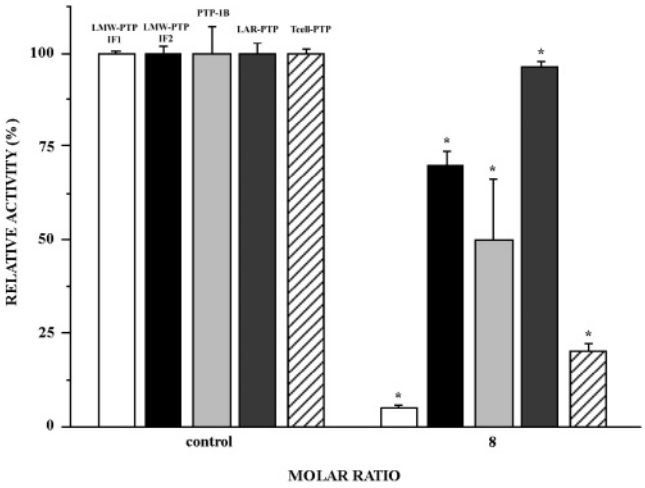

FIGURE 4: Comparative caveolin-1 effect on some PTPs. 0.1 μM indicated PTPs were incubated with 0.8 μM GST or GST-caveolin 1 fusion protein for 5 min at 37 °C. Phosphatase activities were determined on pNPP. The molar ratio indicates GST or GST-caveolin concentration/PTP concentration. Results shown represent the means of three experiments in duplicate ± SEM. *, $p < 0.05$ compared with the control groups, represented by each enzyme alone.

incubation with potential ligands, beads were extensively washed, and the samples were analyzed by SDS−PAGE. Proteins were revealed by silver staining. Figure 5A shows that there is no interaction between cav[60−81] and the proteins tested, but the scaffolding domain may interact not only with IF1 and IF2 but also with the beads alone. To further assess the formation of noncovalent complexes between the LMW-PTP isoenzymes and the scaffolding domain of caveolin-1, we analyzed IF1 and IF2 by MALDI-TOF mass spectrometry, both alone and in the presence of the cav[82−101] peptide. The spectra shown in Figure 5C,D demonstrate that pure LMW-PTP isoenzymes were able to bind the scaffolding domain by forming detectable complexes with appropriate molecular weight. Panel B of the figure shows the mass spectra of the cav[82−101] peptide. We also performed chemical cross-linking experiments triggered by light in the presence of tris-bipyridylruthenium (*31*). The method is based on a process that involves brief photolysis of the tris-bipyridylruthenium(II) dication with visible light in the presence of the electron acceptor ammonium persulfate and the protein of interest, either preincubated or not with the cav[82−101] peptide. In this way, preexisting noncovalent complexes were fixed. The samples were then analyzed by SDS−PAGE followed by silver staining. Panels E and F of Figure 5 show that both IF1 and IF2 complexed 1:1 with cav[82−101]. We can also notice that the presence of the peptide prevented the formation of noncovalent dimeric form, usually existing in solution in equilibrium with the monomeric form (*32*). To assess the specificity of the observed phenomenon, we performed analogous experiments by using the lysozyme protein; no protein/peptide complexes were noticed (Figure 5G). The functional consequences of the observed bindings were determined by testing IF1 and IF2 activities in the presence or absence of the cav[82−101] peptide or the control peptide cav[60−81]. Figure 6 reveals that cav[82−101] inactivates LMW-PTP isoenzymes dose-dependently in a fashion similar to the inhibition previously demonstrated using the full-length caveolin-1, but with reduced efficiency. For direct determination of K_i, experiments were performed at varied substrate and inhibitor concentrations. Nonlinear regression analysis of the data obtained for the LMW-PTP isoenzymes performed by FigSys software reveals that cav[82−101] peptide is a mixed-type inhibitor with determined K_i values of 13 ± 1.5 μM for IF1 and 155 ± 20 μM for IF2. Graphical analysis clearly supports the mixed-type inhibition pattern, with lines dissecting in the first quadrant in a Lineweaver−Burk plot (Figure 7). We repeated some inhibition tests in the presence of 0.1 and 0.5 M NaCl in order to verify the influence of the ionic strength of the medium on the binding of the scaffolding domain peptide. As shown in Figure 8 the cav[82−101]-IF1 association is prevalently mediated by hydrophobic interactions, since the inhibitory effect on phosphatase activity persists also at high ionic strength. On the other hand, the inhibitory effect on IF2 is suppressed at high ionic strength, suggesting that the binding is prevalently mediated by ionic interactions.

Caveolin-1 Contains More than One Region That May Be Involved in LMW-PTP Binding. To see if other caveolin-1 domains could support a direct interaction with the LMW-PTP isoenzymes, we employed a panel of bacterially expressed GST-fusion proteins corresponding to the caveolin-1 regions not yet analyzed: cav[1−60], cav[95−135], and cav[135−178]. Each expressed protein was subjected to SDS−PAGE, followed by silver staining to reveal the purified protein and any degradation products or additional proteins that were present. We found that the expressed proteins were the major species present in each sample. We incubated both IF1 and IF2 purified from *E. coli* with equal amounts of GST and the GST-fusion proteins GST-cav[1−60], GST-cav[95−135], and GST-cav[135−178]. As seen in Figure 9, pure IF1 and IF2 did not bind to GST or to cav[1−60] but interacted with the C-terminal motif (cav-[135−178]) and with the membrane proximal one (cav[95−135]), suggesting that the caveolin-1/LMW-PTP interaction is more complex than supposed and involves more than the scaffolding domain.

The Putative Caveolin-1 Binding Motif of LMW-PTP Is Not Involved in the Association with Caveolin-1. By using the caveolin-1 scaffolding domain as a receptor to select random peptide ligands, Couet et al. deduced three similar caveolin binding sequence motifs: ΦXΦXXXXΦ, ΦXXXXΦXXΦ, and ΦXΦXXXXΦXXΦ (where Φ is an aromatic or a large hydrophobic residue) (*13*, *33*). One of these motifs is present in all of the LMW-PTPs in the sequence region 77−85. To verify whether this short sequence can serve as a ligand for caveolin-1, we created two GST-IF1 and GST-IF2 fusion proteins mutated within this region: F85Q and F82M/F85Q. We used both the single mutants and the double mutants to perform pull-down experiments, as described above. Figure 10 shows the results; the putative caveolin binding sequence motif of the LMW-PTP is not involved in the binding, because all of the mutants were still able to associate with caveolin-1.

DISCUSSION

Caveolae and their principal constituent proteins, caveolins, have an important role in many cellular processes, including signal transduction pathways such as those triggered by EGF,

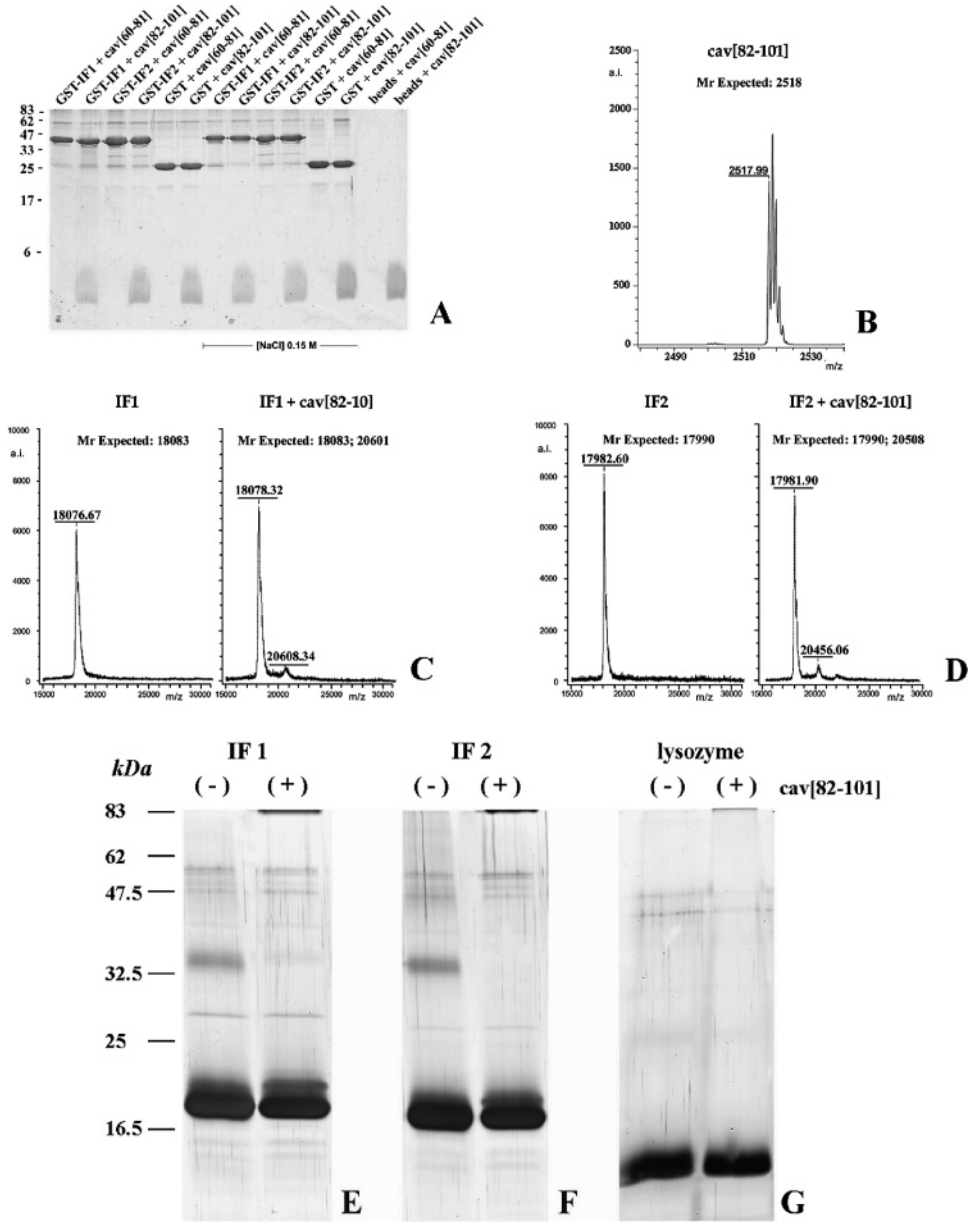

FIGURE 5: Direct interaction of the caveolin-1 scaffolding domain with LMW-PTP isoenzymes. (A) GST-IF1, GST-IF2, and pure GST were incubated with the synthetic peptides cav[60−81] and cav[82−101] in 50 mM β,β-dimethylglutarate, pH 7.0, 1 mM EDTA, and 5 mM dithiothreitol, for 30 min at room temperature. After addition of glutathione−agarose beads, the samples were extensively washed and then analyzed by SDS−PAGE (18% acrylamide). Beads were added to pure peptides, too. In order to verify the salt dependence on the binding, the tests were also performed in the presence of 0.15 M NaCl. Proteins were revealed by silver staining. Data are representative of two independent experiments. (B) MALDI mass spectrum of the cav[82.101] peptide. (C) MALDI mass spectra of IF1 incubated in the presence (right panel) or in the absence (left panel) of the cav[82−101] peptide. (D) MALDI mass spectra of IF2 incubated in the presence (right panel) or in the absence (left panel) of the cav[82−101] peptide. (E−G) The reaction mixtures containing IF1 or IF2 or lysozyme (1 μM) with or without the cav[82−101] peptide (100 μM), 60 μM dipyridylruthenium (II), and 100 μM ammonium persulfate were irradiated with a 150 W incandescent lamp for 30 s. Immediately after irradiation, samples were quenched with 4× Laemmli sample buffer and analyzed by SDS−PAGE followed by silver staining. Data are representative of two separate experiments.

PDGF, and insulin (*2*, *4*, *36*−*38*). Many structurally distinct proteins and signaling pathways are localized within caveolae, suggesting that signal integration, amplification, and desensitization are all regulated in these organelles: they possibly allow efficient interactions between key signaling proteins. In fact, many signals emanating from caveolae, as well as signal modulation and termination, utilize these platforms for integrating information and sorting the elements of signaling cascades. Caveolin-1 acts as a scaffold protein by interacting with numerous molecules and regulates the activation state of many enzymes. Often, this "functional" interaction occurs via a 20 amino acid stretch characteristic of the caveolins, which has been termed the "caveolin scaffolding domain". The LMW-PTP activity is related to several physiological stimuli and to growth factor signaling (*18*, *20*, *23*, *39*−*42*). Two LMW-PTP isoenzymes are usually coexpressed in all mammalian cells, but their precise role is not yet known, in regard to either substrate specificity or functional regulation. By using various complementary approaches, we examined the relation between caveolin-1 and the LMW-PTP isoenzymes. First, our experiments showed that both IF1 and IF2 interact with caveolin-1 through defined regions within each protein and that this binding is not mediated by other proteins complexed with

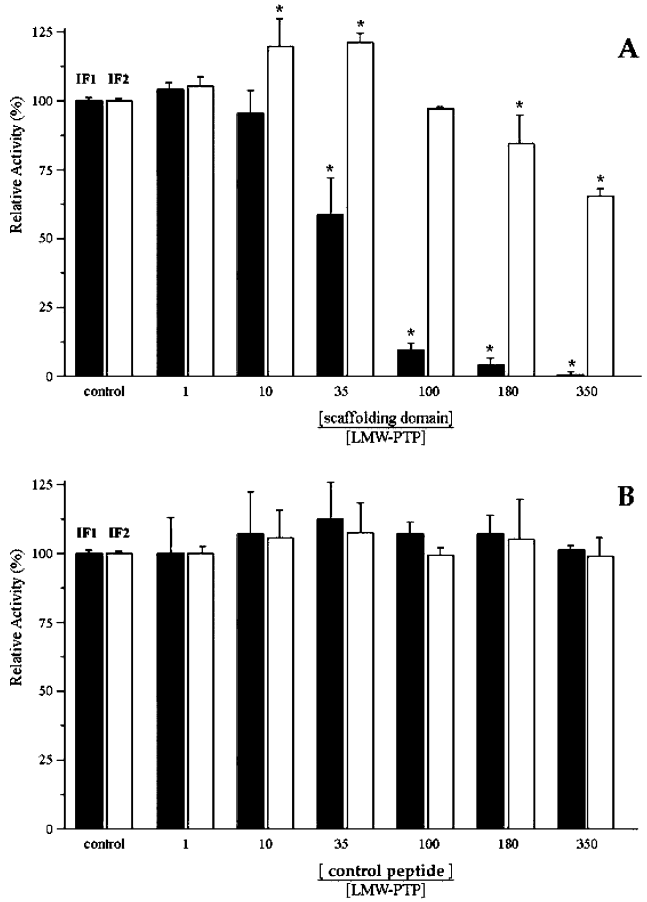

FIGURE 6: Inhibition of LMW-PTP activities by the caveolin scaffolding domain. Variable amounts (0.1−35 μM) of caveolin-1 peptides (cav[82−101, cav[60−81]) were incubated with 0.1 μM LMW-PTP IF1 or LMW-PTP IF2 in 50 mM β,β-dimethylglutarate, pH 7.0, for 15 min at room temperature. Enzymatic activities were monitored at 37 °C by the addition of 5 mM pNPP. In panel A is shown the effects of the scaffolding domain peptide (cav[82−101]) on the phosphatase activity of IF1 and IF2; in panel B is shown the control peptide (cav[60−81]) effects. Results shown represent the means of three experiments in duplicate ± SEM. *, $p < 0.05$ compared with the control groups, represented by the sole enzyme.

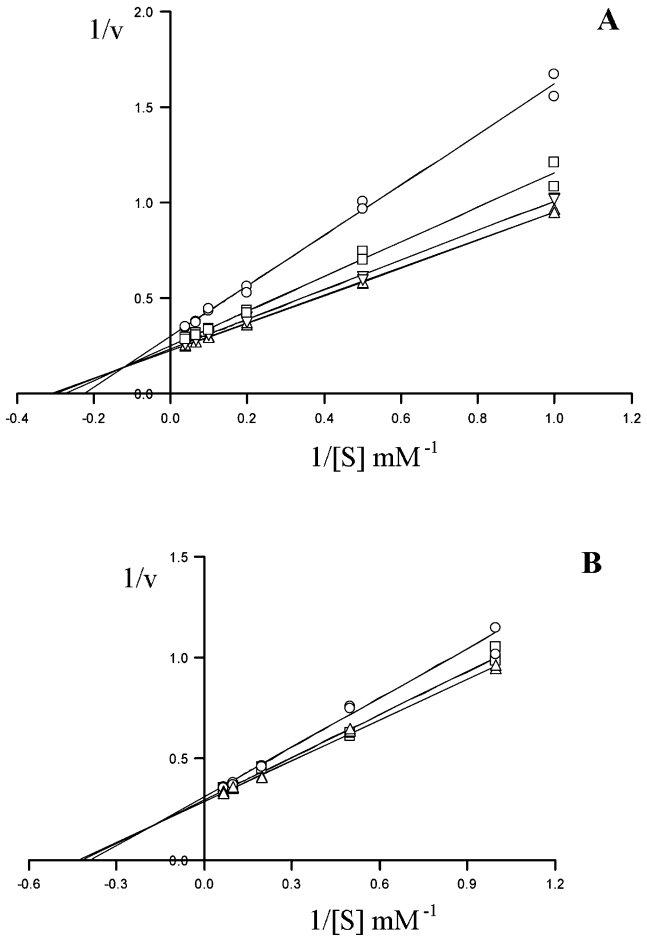

FIGURE 7: Lineweaver−Burk plot for the inhibition of LMW-PTP isoenzymes by the scaffolding domain peptide. The reactions were performed in the absence of inhibitor (Δ) or in the presence of 5 (∇), 10 (□), or 25 μM (○) cav[82−101] peptide. Panel A shows the results obtained for IF1; panel B shows the ones obtained for IF2.

caveolin-1, since caveolin-1 immunoprecipitated from lysates obtained in strongly denaturing conditions is still capable of binding LMW-PTP isoenzymes (Figure 2). The functional consequences of this interaction is the inhibition of only IF1 isoform activity, while IF2 activity is slightly affected (Figure 3). We compare the relative inhibition elicited by caveolin-1 on catalityc activity of some other typical PTPs, and our results (Figure 4) show that IF1 is the enzyme that presents the higher inhibition suggesting a specific in vivo IF1 regulation by caveolin-1. We emphasize that the molar ratio, 1/7, at which we observed a significant inhibitory effect is comparable to the physiological molar ratio between cav-1 and the LMW-PTP isoenzymes inside caveolae. In fact, caveolin-1 forms high molecular mass homo-oligomers of about 350 kDa that contain approximately 14−16 monomers per oligomer. As already described, these oligomers interact with each other and with other molecules via an oligomerization domain (cav[60−101]) and the C-terminal domains, forming a cav-1 reach network on the cytoplasmic side of the plasma membrane (*11*, *43*). Since the scaffolding domain is known to be the site largely responsible for most of the physiological actions of caveolin-1, we tested whether the observed effects were due to this region. By using a peptide encoding the caveolin-1 scaffolding domain cav[82−101] and, as control, another one relative to the contiguous region 60−81, we demonstrated a direct and specific interaction between the scaffolding domain and both LMW-PTP isoenzymes, based on MALDI mass spectrometry and photo-cross-linking experiments (Figure 5). Furthermore, the cav[82−101] peptide specifically and differentially inhibits the activity of the LMW-PTP isoenzymes (Figure 6), confirming the data obtained with full-length caveolin. The effect on IF1 activity is strong, but the dose dependence is not comparable to that observed in the presence of the full-length caveolin-1, suggesting a more complex interaction. Curiously low concentrations of the synthetic peptide cav[82−101], as well as the GST and the GST-caveolin-1 fusion proteins, have an activatory effect on the LMW-PTP isoenzymes. A possible explanation of this phenomenon could come from the analysis of electrophoretic patterns obtained after the cross-linking photoinduced experiments (Figure 5E,F). Probably we observed an apparent activity increase, since the cav[82−101] peptide, which binds to IF1 and IF2 by forming bimolecular complexes, also prevents the formation of inactive oligomeric forms (*32*). This unspecific effect disappears when the specific inhibition of caveolin becomes prevalent. We examined the inhibitory mechanism of the

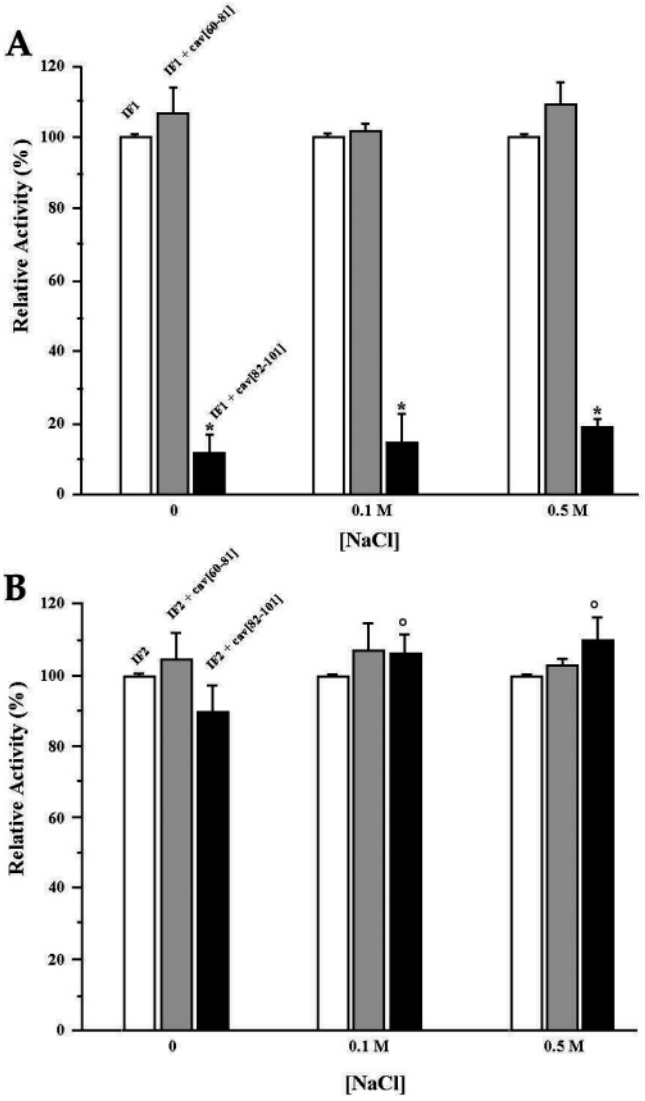

FIGURE 8: Influence of ionic strength on the inhibitory effect of the scaffolding domain of caveolin vs LMW-PTP isoenzymes. LMW-PTP activity was determined after the incubation of 0.15 μM IF1 or IF2 in 50 mM β,β-dimethylglutarate, pH 7.0, with cav-[82−101] or cav[60−81]. The tests were performed in the absence and in the presence of 0.1 and 0.5 M NaCl. In panel A is shown the results obtained for IF1 treated with 15 μM caveolin peptides; in panel B is shown those obtained for IF2 treated with 25 μM caveolin peptides. Results shown are the means of three experiments in duplicate ± SEM. *, $p < 0.05$ compared with the control groups, represented by the sole enzyme. °, $p < 0.05$ compared with the control groups, represented by the enzyme incubated with the peptide in the absence of NaCl.

scaffolding domain peptide on the LMW-PTP isoenzymes by kinetic analysis, and we determined a K_i value of 13 μM for IF1 and a K_i value of 155 μM for IF2. The pattern of inhibition (Figure 7) was of the mixed type, in which the peptide can bind competitively with the substrate not only at the active site but also at other sites, by forming enzyme−substrate−inhibitor and enzyme−inhibitor complexes during the reaction to reduce the efficiency of catalysis. The inhibitory effect on IF2 ceased at higher ionic strength (Figure 8), consistent with binding not being primarily mediated by hydrophobic interactions. This behavior was not observed for IF1, indicating that the two isoenzymes could interact with caveolin-1 through a different site, which is probably present in the isoform-specific region of sequence

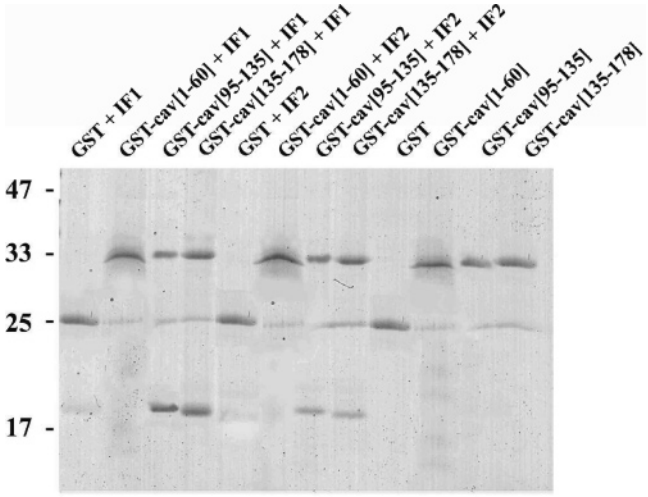

FIGURE 9: Identification of the regions of caveolin-1 which recognize LMW-PTP isoenzymes. GST-caveolin subdomains (cav-[1−60], cav[95−135], cav[135−178]) were incubated in 50 mM β,β-dimethylglutarate, pH 7.0, 1 mM EDTA, and 5 mM dithiothreitol, for 30 min at room temperature, with the LMW-PTP isoenzymes. GST was used as control. After addition of glutathione−agarose beads, the samples were extensively washed and then analyzed by SDS−PAGE (12% acrylamide). Proteins were revealed by silver staining. Data are representative of two independent experiments.

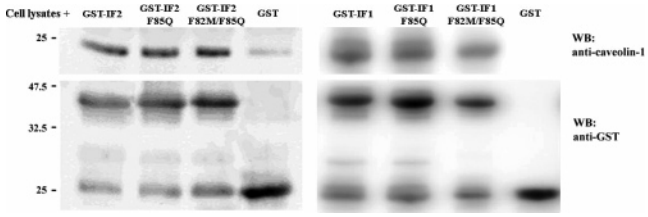

FIGURE 10: In vitro binding of LMW-PTP mutants to cellular caveolin-1. 1 mL of H-end cell lysates containing 1.2 mg of protein was added to 10 μg of GST or GST-fusion proteins (IF1/F82M-F85Q, IF1/F85Q, IF2/F82M-F85Q, IF2/F85Q) bound to glutathione−agarose. After 16 h incubation at 4 °C, samples were extensively washed and then analyzed by immunoblot using anti-caveolin-1 and anti-GST antibodies. Data are representative of two separate experiments.

40−73. Topologically, caveolin-1 is subdivided into three conserved portions: (i) an N-terminal domain (residues 1−101), (ii) a membrane-associated domain (residues 102−135), and (iii) a C-terminal domain (residues 135−178). Caveolin-1 forms a hairpin loop with both the N-terminus and the C-terminus facing toward the cytosol. Most interactions between caveolin-1 and other proteins are mediated by its scaffolding domain (residues 82−101) (*7*), even if other sequence portions can mediate specific bindings. The C-terminal domain is a secondary binding site for some proteins, i.e., a catalytic subunit of protein kinase A and c-Src (*44*−*46*), and the N-terminal sequence 1−61 is responsible of caveolin-1 association with NOSTRIN (*47*). It is not clear if the 102−135 region is really a membrane spanning portion; some data suggest that it may associate with the inner surface of membrane, where it is still able to bind to other proteins including caveolin-2 (*48*) and dynamin-2 (*49*). Interestingly, we found that the LMW-PTP isoenzymes can interact with caveolin-1 in the sequence region 82−178 (Figures 5 and 9), which includes the membrane-associated domain, the scaffolding domain, and the C-terminal domain; analogous behavior has been found for dynamin-2 (*49*). We also found

some evidence that the LMW-PTP interaction site for caveolin-1 is not the typical consensus motif, defined as "caveolin binding sequence motif", present in all of these enzymes in the sequence region 77−85. We performed pull-down experiments by using two kinds of LMW-PTP mutants, in which essential aromatic amino acids (Phe 85 and/or Phe 82) were mutated. We demonstrated that these enzymatic forms, which show no significant differences in K_m and V_{max} values from wild-type ones, were still able to bind caveolin-1 present in cell lysates (Figure 10). This behavior is not anomalous; it is well documented that the caveolin binding sequence motif is not essential for binding caveolin-1 to some other proteins, such as Nostrin (*47*), dynamin-2 (*49*), n-NOS (*50*), e-NOS (*51*), and c-Src (*46*). Another protein that localizes to the caveolae membrane and interacts with caveolin-1 is Spred-1, a tyrosine kinase substrate that inhibits growth-factor-mediated activation of ERK. The association with caveolin-1 occurs through the palmitoylated C-terminus of Spred-1 in which no "caveolin binding motif" is present (*52*). All of our data indicate that caveolin binding requirements are broader than initially hypothesized. The overall cellular role of these interactions remains to be determined; one possibility is that caveolin-1 serves to differentially modulate the LMW-PTP isoenzymes. Perhaps the inhibition of LMW-PTP IF1 by direct interaction with caveolin is a molecular switch to terminate specific signaling processes. Further investigation is needed to settle these issues.

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