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Caveolin-1 interacts with protein phosphatase 5 and modulates its activity in prostate cancer cells

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ABSTRACT

Caveolin-1 is highly expressed in prostate cancer cells, and is implicated in disease progression. Here, we identified protein phosphatase 5 (PP5) as a novel cellular binding partner of caveolin-1 using a pull-down approach in combination with mass spectrometry-based proteomic analyses. *In situ* proximity ligation assays demonstrated co-localization and physical interaction of caveolin-1 and PP5 in the cytoplasm of PC-3 human prostate cancer cells. Using yeast two-hybrid analysis, we found that caveolin-1 interacted with the catalytic domain of PP5. We also found that PP5 activity was elevated about 1.7-fold in the presence of 2 μM caveolin-1, and that the scaffolding domain of caveolin-1 is required for this activation. Our results suggest that caveolin-1 is a novel physiological activator of PP5.

1. Introduction

Caveolin-1 is a major structural component of caveolae, which are specialized plasma membrane invaginations that are involved in multiple cellular processes such as molecular transport, cell adhesion, and signal transduction [1]. Although under some conditions caveolin-1 may suppress tumorigenesis [2], caveolin-1 contributes to malignant progression through various mechanisms [3–5]. High expression of intracellular caveolin-1 is associated with metastasis of human prostate cancer [6,7] and other malignancies, including lung [8], renal [9], and esophageal squamous cell cancers [10]. Thompson et al. showed that caveolin-1 is consistently and strongly overexpressed in metastatic prostate cancer and is secreted in a biologically active form by virulent prostate cancer cells [11]. The cancer-promoting effects of secreted caveolin-1 include anti-apoptotic activities similar to those observed following enforced expression of caveolin-1 [4,11,12]. In addition to showing caveolin-1-mediated autocrine activities, a recent study showed that recombinant caveolin-1 protein is taken up by prostate cancer and endothelial cells *in vitro*, and that recombinant caveolin-1 increases angiogenic activity both *in vitro* and *in vivo* by activating Akt and/or nitric oxide synthase signaling [4]. However, the pro-angiogenic effects of caveolin-1 in prostate cancer development and progression are still largely uncharacterized.

In this study, using immobilized recombinant caveolin-1 as an affinity ligand, we identified protein phosphatase 5 (PP5) as a novel caveolin-1 binding protein in PC-3 human prostate cancer cell lysates.

The 58-kDa phosphoseryl/phosphothreonyl PP5 [13,14] is ubiquitously expressed in all types of mammalian tissue, particularly in the brain [15]. This enzyme contains a C-terminal catalytic domain, which is structurally related to the PP1/PP2A/PP2B family, and an N-terminal regulatory domain consisting of three tetratricopeptide repeats (TPRs) that mediate protein–protein interactions. It has been demonstrated that PP5 interacts with multiple target proteins through the TPR domain [14]. Wild-type native PP5 has very low activity, but is activated after cleavage of the TPR domain. *In vitro* studies indicate that PP5 is activated by ligands of the TPR domain, such as Hsp90 and arachidonyl CoA [16,17]. Despite these data, the definitive natural activator of PP5 remains to be identified.

Here, we showed caveolin-1 directly bind to PP5 in PC-3 human prostate cancer cells and stimulated its activity, suggesting that caveolin-1 may function as an endogenous activator of PP5.

2. Materials and methods

2.1. Materials

The cDNA clones encoding human caveolin-1 and PP5 were purchased from Kazusa DNA Research Institute. Anti-caveolin-1 and anti-PP5 antibodies used for immunoblotting experiments were purchased from Cell Signaling Technology, and the horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody was obtained from Santa Cruz Biotechnology. The synthetic peptide corresponding to residues 82–101 of human caveolin-1 (DGIWKASFTTFTVTKYWFYR-amide) is referred to as CAV(82–101) [18]. Other chemicals of reagent grade were obtained from Sigma, or Nacalai Tesque.

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2.2. Expression and purification of caveolin-1 and PP5

Full-length human caveolin-1 (residues 1–178) was amplified by PCR and sub-cloned into the *NdeI* and *XhoI* sites of the pET21b vector incorporating a His_6-tag into the C-terminus of the protein. Expression and purification of the His_6-tagged caveolin-1 was performed as described previously [19]. Similarly, human full-length PP5 (residues 1–499) was amplified by PCR and sub-cloned into the pET21b vector. The constructs were transformed into *Escherichia coli* BL21(DE3) cells, which were grown at 37 °C in LB medium containing 50 μg/mL ampicillin, and were then induced for 5 h with 1 mM IPTG at 28 °C. His_6-tagged PP5 was purified using Ni Sepharose 6 Fast Flow chromatography (GE healthcare) followed by anion exchange chromatography. Purity of samples was confirmed to be >95% using SDS–PAGE and Coomassie brilliant blue (CBB) staining.

2.3. Cell culture

PC-3 human prostate cancer cells (American Type Culture Collection) were seeded at 5×10^4 cells/well onto 4-well LabTek chambers (Nunc) and were maintained for 24 h in high glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin, and 4 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Isolation of caveolin-1-binding proteins

Purified His_6-tagged caveolin-1 was conjugated to SulfoLink Coupling beads (Pierce) according to the manufacturer's protocol. Cultured PC-3 cells (approximately 80% confluence) were rinsed twice with phosphate-buffered saline (PBS), were harvested and lysed with 50 mM Tris–HCl buffer (pH 8.0) containing 1% Triton X-100, 5 mM EDTA, and a protease inhibitor cocktail (Roche Diagnostics), and were sonicated. The cell lysate was centrifuged at 13,000×*g* at 4 °C for 30 min to remove insoluble debris. The cell lysate was mixed with His_6-caveolin-1-conjugated beads (25 μl of a 50% suspension), incubated at 4 °C for 1 h, and the beads were then washed five times with lysis buffer. Proteins bound to His_6-caveolin-1-conjugated beads were recovered by treatment with 40 μl of 1.5× Laemmli's sample buffer (75 mM Tris–HCl, pH 6.8, 1.5% SDS, 15% glycerol, and 0.015% bromophenol blue) for 10 min at 95 °C, separated by electrophoresis using SDS–PAGE (4–12%), and stained with CBB. Control experiments were performed using the same procedures with a L-Cys-immobilized column.

2.5. Peptide mass fingerprinting analysis

Pieces of CBB-stained gel were washed three times with 25 mM NH_4HCO_3 in 50% (v/v) acetonitrile for 10 min each, dehydrated by the addition of acetonitrile, and dried in a vacuum. Dried gel pieces were treated with a solution containing 25 mM NH_4HCO_3 and 10 mM dithiothreitol for 1 h at 56 °C, washed with 25 mM NH_4HCO_3, and incubated in a solution containing 25 mM NH_4HCO_3 and 10 mg/mL iodoacetamide for 45 min at room temperature in the dark. After the gel was washed once with 25 mM NH_4HCO_3, twice with 25 mM NH_4HCO_3 in 50% (v/v) acetonitrile for 10 min, and then dried again; the reduced/alkylated proteins were digested by treating the gel with 10 μg/mL Tosyl-Phe Chloromethyl Ketone (TPCK)-treated trypsin (Promega) in 50 mM NH_4HCO_3 at 37 °C for 16 h. Digested peptides were extracted with 5% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile, and subjected to peptide mass fingerprinting using a Bruker Autoflex instrument (Bruker Daltnics) with an α-cyano-4-hydroxyl-*trans*-cinnamic acid matrix (Sigma). All MALDI-TOF mass spectra were calibrated externally using a standard peptide mixture (Bruker Daltnics). Peptide mass fingerprints were searched against the National Center for Biotechnology Information non-redundant mammalian database using ProFound [20], and were confirmed using a Mascot search engine [21]. One missed cleavage per peptide was allowed.

2.6. In situ proximity ligation assay (PLA)

PC-3 cells (5×10^4 cells) were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature followed by permeabilization with 0.1% Triton X-100 in PBS. Fixed and permeabilized cells were incubated overnight with primary antibodies, anti-PP5 rabbit polyclonal antibody (Cell Signaling Technology) and anti-caveolin-1 mouse monoclonal antibody (Santa Cruz). The combination of rabbit isotype control IgG (Millipore) and anti-caveolin-1 mouse monoclonal antibody, was used as a control. The Duolink *in situ* PLA was performed according to the manufacturer's instructions (Olink Bioscience). Images were observed with an ECLIPSE Ti microscope (Nikon) and were analyzed using NIS-Elements 3.1 imaging software (Nikon).

2.7. Yeast two-hybrid method

We used the Matchmaker Gold System (Clontech) with the GAL4-based yeast two-hybrid system to identify interactions of caveolin-1 and PP5. For the bait protein, cDNA encoding human caveolin-1 (residues 1–101) was amplified by PCR, and the PCR product was sub-cloned into *Nde*I and *Eco*RI sites of the pGBKT7 vector downstream of the GAL4 DNA-binding domain. The construct was transformed into the *Saccharomyces cerevisiae* Y2H Gold strain using the lithium acetate method. The cDNA encoding human PP5 (residues 1–499), the TPR domain of PP5 (residues 1–130), and the catalytic domain of PP5 (residues 170–499) were amplified by PCR and subcloned into *Nde*I and *Eco*RI sites of the pGADT7 vector to obtain PP5(1–499)/pGADT7, PP5(1–130)/pGADT7, and PP5(170–499)/pGADT7 that expressed proteins fused to the GAL4 activation domain. These constructs were then transformed into Y187 strains and mated with the Y2H Gold strain that was transformed with caveolin-1/pGBKT7. Subsequently, these strains were cultured on L-Trp and L-Leu free minimal media supplemented with 125 ng/mL aureobasidin A and 40 μg/mL 5-bromo-4-chloro-3-indolyl-α-D-galactosidase (X-α-Gal). In this assay, a positive control was generated by transforming the p53/pGBKT7 clone into the Y2H Gold strain and mating with the Y187 strain transformed with SV40 Large T antigen in pGADT7-vectors. As a negative control, the p53/pGBKT7 transformed Y2H Gold strain was mated with the lamin/pGADT7 transfected Y187 strain, and the progeny were used to confirm specificity of the interaction.

2.8. Phosphatase assays

The activity of PP5 was assayed with p-nitrophenyl phosphate (pNPP) as previously described [16,22] with minor modifications. In brief, the assay mixture (0.1 mL) containing 50 mM pNPP, 10 mM $MnCl_2$, and the indicated compounds in 100 mM Tris, pH 8.0, was allowed to equilibrate at 25 °C. Subsequently, PP5 (0.6 μg) was pre-incubated in the presence or absence of varied concentrations of caveolin-1 (1–178) or CAV(82–101) at 4 °C for 30 min, and was then added to the assay mixture. The production of p-nitrophenol (pNP) was determined by monitoring absorbance at 410 nm for 10 min. Activity was expressed as moles of pNP produced per μg of PP5 per min. The concentration of pNP was determined as described above using a molecular absorption coefficient of 15.1×10^3 mol of pNP L^{-1} cm^{-1}.

3. Results

3.1. Identification of novel caveolin-1 binding partners in PC-3 cells

To identify novel caveolin-1-associated proteins, the lysates of PC-3 human prostate cancer cells, in which caveolin-1 is abundantly expressed [6,7], were pulled down with His_6-caveolin-1-conjugated beads. Pull-down complexes were suspended in Laemmli's sample buffer and analyzed by SDS–PAGE. Staining with CBB revealed at least three prominent bands at approximately 90-, 60-, and 45-kDa (Fig. 1A, right lane). Peptide mass fingerprinting analysis of tryptic fragments of the ~60-kDa protein using MALDI-TOF MS and MASCOT search engines returned PP5 as the best hit; exceeding the significance threshold for the database search. Major mass peaks detected by MS analysis, and the corresponding amino acid sequences of human PP5, were as follows: *m/z* 744.608 for ^{25}RAEELK^{30}, *m/z* 1050.86 for ^{39}AKDYENAIK^{47}, *m/z* 1164.947 for ^{101}AASNMALGKFR^{111}, *m/z* 1382.958 for ^{179}LEDGKVTISFMK^{190}, *m/z* 1802.968 for ^{184}AFALWSAVTPLTFTR^{197}, and *m/z* 2508.585 for ^{372}QPPDSGPMCDLLWSDPQPQNGR^{393} (GenBank ID: AAH00750.4; amino acid sequence for human PP5). Total peptide coverage corresponded to 26% of the predicted peptide sequence with a ProFound expectation score of 1.3×10^{-3}. The identity of the ~60-kDa protein was confirmed to be PP5 by immunoblotting. Subsequently, lysates were mixed with His_6-caveolin-1-conjugated beads or with L-Cys-conjugated beads as a control. The polyclonal anti-PP5 antibody revealed a major 58-kDa band from His_6-caveolin-1-bound materials (Fig. 1B, right lane). This band was not present in elutes from control beads (Fig. 1B, left lane). Again, these data identified PP5 as a ~60-kDa protein bound to His_6-caveolin-1-conjugated beads. Mass spectrometry analysis indicated that the other bands, ~90-kDa and ~45-kDa proteins are transcription factor IIIC and casein kinase 1δ, respectively. Both proteins might also be of great interest as novel caveolin-1-binding partners. However, direct interactions between caveolin-1 and these proteins have not yet been confirmed and require further investigation.

3.2. Caveolin-1 is associated with PP5 in PC-3 cells

To determine whether caveolin-1 interacts with PP5 in intact cells we used a Duolink *in situ* proximity ligation assay. PC-3 cells were stained with anti-caveolin-1 mouse monoclonal and anti-PP5 rabbit polyclonal antibodies or control anti-rabbit isotype IgG antibody. The secondary antibodies were modified by addition of complementary oligonucleotides that interact when they are in close proximity. This interaction was detected using PCR with a fluorochrome-based detection method. Endogenous caveolin-1 was co-localized with PP5, and the majority of binding interactions were found in the cytoplasm (Fig. 2A). In contrast, the negative control revealed negligible nonspecific binding of PLA probes (Fig. 2B). These data indicate that caveolin-1 directly interacts with PP5 in PC-3 cells.

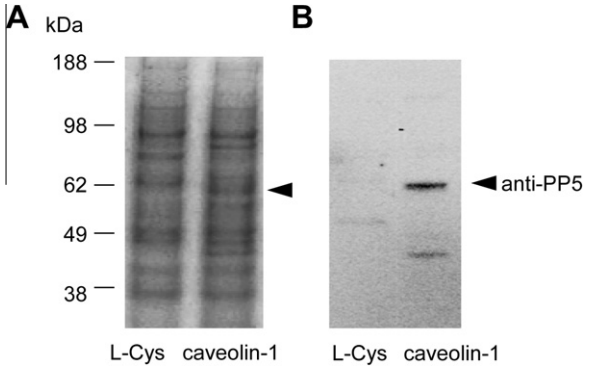

Fig. 1. Isolation of caveolin-1-binding proteins from human prostate cancer cells. Cell lysates prepared from PC-3 human prostate cancer cells were incubated with His_6-caveolin-1 conjugated Sulfo Link beads or control L-Cys conjugated beads. Elutes from His_6-caveolin-1 and L-Cys control beads were resolved by SDS–PAGE and were visualized by Coomassie blue staining (A), or Western blotting using a polyclonal human anti-PP5 antibody (B). The arrow indicates a specific 58-kDa band, which was identified as PP5 by mass spectroscopy. Molecular weight markers are shown on the right.

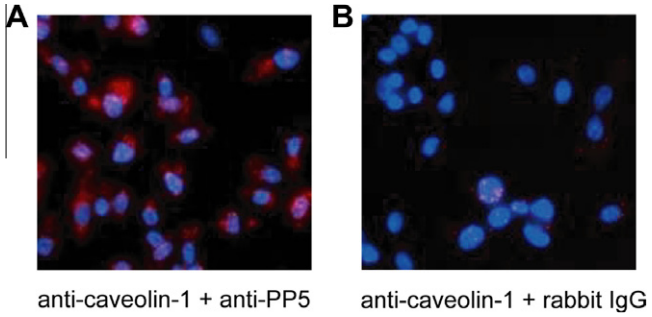

Fig. 2. Interactions between caveolin-1 and PP5 in PC-3 cells shown by the Duolink *in situ* proximity ligation assay. The *in situ* PLA method was used to observe interactions of caveolin-1 and PP5 in PC-3 cells as described in Section 2. Primary antibody pairs, anti-PP5 (A) or control anti-IgG (B) with anti-caveolin-1 were used for the experiment. Dual binding by pairs of corresponding proximity probes and secondary antibodies with attached oligonucleotides generates a red blob if the two antibodies are in close proximity. The cells were counterstained with 4′,6-diamidino-3-phenylindole (blue) to visualize the nucleus.

3.3. Caveolin-1 interact with the catalytic domain of PP5

The PP5 protein contains a C-terminal catalytic domain that is structurally related to the PP1/PP2A/PP2B family, and an N-terminal TPR domain that usually mediates protein–protein interactions with other proteins [14]. Interestingly, the catalytic domain of PP5 possesses previously described consensus caveolin binding sites [23], which are conserved among several species, including rats and humans, and are found in other members of the PP1/PP2A family (Fig. 3A). In yeast two-hybrid experiments, we defined the domains responsible for interactions between caveolin-1 and PP5. The N-terminal cytoplasmic domain of caveolin-1 (residues 1–101) was constructed as a bait protein using pGBKT7 vectors, while full-length PP5, the N-terminal TPR domain of PP5 (residues 1–130), and the catalytic domain of PP5 (residues 170–499) were constructed as prey proteins using pGADT7 vectors. Bait plasmid caveolin-1(1–101)-pGBKT7 transformed Y2H Gold cells were mated with the Y187 strains transformed with each prey plasmid, and were cultured on L-Trp and L-Leu free minimal media that was supplemented with aureobasidin A and X-α-Gal. As shown in Fig. 3B, numerous colonies formed from the progeny of caveolin-1(1–101)/pGBKT7-Y2H Gold and PP5/pGADT7-Y187 yeast strains (Fig. 3B-a). Yeast colonies also grew from the progeny of the caveolin-1(1–101)/pGBKT7-Y2H Gold and PP5(170–499)/pGADT7-Y187 strains (Fig. 3B-b). In contrast, no yeast colonies were seen from the progeny of caveolin-1(1–101)/pGBKT7-Y2H Gold and PP5(1–130)/pGADT7-Y187 strains (Fig. 3B-c). These results indicate that caveolin-1 interacts directly with the catalytic domain of PP5, but not via the TPR domain.

3.4. Effect of Caveolin-1 on PP5 activity

The effect of caveolin-1 on PP5 activity was examined to clarify whether caveolin-1 regulates its activity. As shown in Fig. 4, PP5 activity was enhanced by caveolin-1 in a dose-dependent manner, with a 1.7-fold increase in PP5 activity in the presence of 2 μM caveolin-1. The caveolin-1 residues 82–101, which comprise the caveolin scaffolding domain [23], reportedly play an essential role

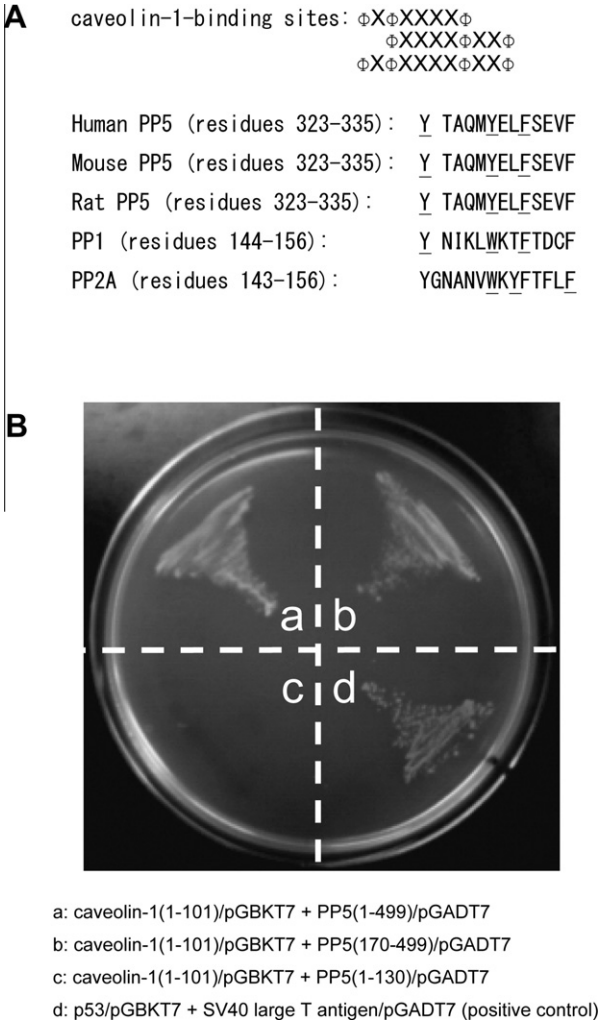

Fig. 3. Identification of a direct PP5-interacting domain on caveolin-1. (A) Caveolin-1 consensus binding sites and putative caveolin-1 binding sites in catalytic domains of human, mouse, rat PP5, human PP1, and human PP2A. Aromatic residues (Φ) and any amino acid (X) in the caveolin-1-binding sites are indicated, and the aromatic residues in PP5, PP1, and PP2A are underlined. (B) In yeast two-hybrid assays, the pray plasmid, the (a) PP5(1–499)/pGADT7, (b) PP5(170–499)/pGADT7 and (c) PP5(1–130)/pGADT7 constructs were transformed into the Y187 strain and mated with caveolin-1(1–101)/pGBKT7 transformed Y2H Gold yeast, and were cultured on L-Trp and L-Leu free minimal media supplemented with 125 ng/mL aureobasidin A and 40 μg/mL X-α-Gal. The p53/pGBKT7-Y2H Gold strain was mated with SV40 Large T antigen/pGADT7-Y187 (d) as the positive control.

in caveolin-related protein–protein interactions [24]. To investigate the regulation of PP5 by the caveolin scaffolding domain, we prepared a synthetic peptide of this segment; designated as CAV(82–101). PP5 activity was also stimulated by CAV(82–101) in a dose-dependent manner (Fig. 4).

4. Discussion

In the present study, we identified PP5 as a novel cellular binding partner of caveolin-1 using a pull-down approach in combination with mass spectrometry-based proteomic analyses (Fig. 1). Using the Duolink *in situ* proximity ligation assay, which is based on close juxtaposition of antibodies for each of the interacting proteins, we verified that caveolin-1 and PP5 interact in PC-3 cells. Data from these experiments clearly show co-localization of the two proteins throughout the cell and indicate that these protein complexes reside predominantly in the cytoplasm of PC-3 cells (Fig. 2).

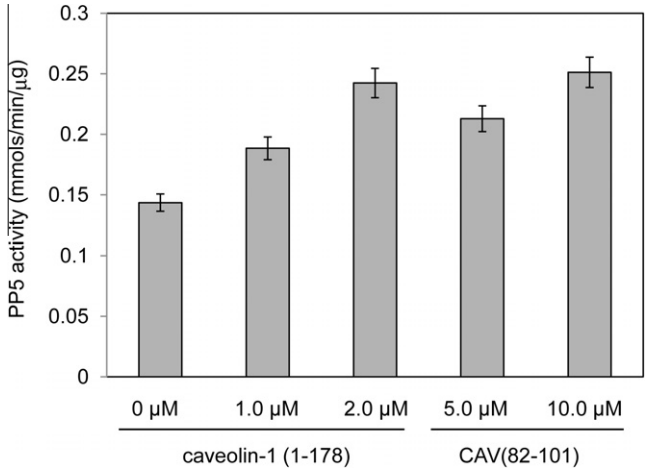

Fig. 4. Stimulation of recombinant PP5 activity by caveolin-1. PP5 activity was measured as described in Section 2. Recombinant PP5 activity was measured after pre-incubated with full-length caveolin-1 (residues 1–178) at 0, 1.0, and 2.0 μM, and with CAV(82–101) at 5.0 and 10.0 μM at 4 °C for 30 min.

Caveolin-1 is highly expressed in prostate cancer cells and is implicated in disease progression [4,11]. The membrane-associated protein caveolin-1, is secreted by prostate cancer cells and taken up by caveolin-1-negative tumor cells and endothelial cells, leading to the stimulation of specific angiogenic and anti-apoptopic pathways [4,11]. Although the mechanisms of these effects are largely unknown, multiple studies indicate that Akt activation plays an important role in caveolin-1-mediated prostate carcinogenesis [4,12]. The first demonstration of a direct association between caveolin-1 and Akt indicated that the overexpression of caveolin-1 inhibited the serine/threonine phosphatases PP1 and PP2A in human prostate cancer cells [12]. These interactions, which were likely mediated through caveolin-1 binding to the scaffolding domain-binding sites on PP1 and PP2A, and inhibition of their activities, led to significantly increased phosphorylation of Akt and sustained activation of downstream oncogenic Akt targets [12]. Interestingly, the present data demonstrate that caveolin-1 interacts with PP5 and enhances its activity (Fig. 4). Since PP5 has been shown to act as a suppressor of ASK1 [25] and p53 [26], the observed increase in cell survival associated with PP5 overexpression is consistent with the suppression of these apoptotic signaling cascades by PP5. Although it is not clear if PP5 plays a role in the development of human cancers, a recent study reported a strong correlation between PP5 expression and tumor growth in a tumor xenograft mouse model [27]. Recently, a protein microarray analysis of mantle cell lymphomas also revealed elevated PP5 expression [28], implicating PP5 in other cancers. In addition, *PP5* mRNA silencing did not reduce Akt phosphorylation, suggesting that PP5 does not directly phosphorylate Akt [29]. Our findings reveal a novel role of caveolin-1 mediated PP5 activation in prostate cancer cells that may be relevant to the progression of various cancers.

The present data indicate that caveolin-1 associates with the catalytic domain of PP5 but not the TPR domain (Fig. 3). In addition, domain mapping shows that associations of various signaling molecules with caveolin-1 are mediated by a conserved caveolin scaffolding domain located in the membrane-proximal region [23]. This domain recognizes the well-defined caveolin-binding motifs, ΦXΦXXXXΦ or ΦXXXXΦXXΦ, in which Φ is a hydrophobic residue and X is any amino acid residue. The catalytic domain of PP5 contains this motif, which is conserved in rat and human PP5, and in other members of the PP1/PP2A family (Fig. 3A). Interestingly, PP5 activity was enhanced by both CAV(82–101) and caveolin-1(1–178) in a dose-dependent manner, further indicating

that PP5 interacts with caveolin-1 via the caveolin-1 scaffolding domain (Fig. 4). Unlike PP1 and PP2A, basal activity of PP5 is extremely low *in vitro* [13,14]. While the mechanisms of PP5 stimulation *in vivo* remain unclear, a number of PP5 activators have been identified from *in vitro* studies [16,22]. For example, arachidonic acid and long-chain fatty acyl-CoA esters stimulate full-length PP5 with half-maximal activation (AC_{50}) values between 1 and 125 μM [16,22]. It is important to note that these values are much greater than physiological concentrations of these potential ligands. However, in other recent studies, the C-terminal of Hsp90 also stimulated PP5 activity by approximately 7-fold, with an AC_{50} of ~6 μM [17]. Interestingly, all of these known activators interact with the TPR domain, whereas caveolin-1 associates with the catalytic domain of PP5 through the scaffolding domain binding site. Although the activation of PP5 by caveolin-1 is less potent than that of other known activators, the molecular mechanisms of PP5 activation by caveolin-1 and the functions of this interaction in carcinogenesis are worthy of further *in vivo* investigation.

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