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The subcellular localization of 3-phosphoinositide-dependent protein kinase is controlled by caveolin-1 binding☆

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Abstract

3-Phosphoinositide-dependent protein kinase 1 (PDK1), a member of the serine/threonine kinase family, has been demonstrated to be crucial for cellular survival, differentiation, and metabolism. Here, we present evidence that PDK1 is associated with caveolin-1, a 22-kDa integral membrane protein, which is the principal structural and regulatory component of the caveolae membranes in COS-1. First, we noted the presence of two potential caveolin-1 binding motifs (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306}) in the PDK1 catalytic domain. Using a pull-down approach, we observed that PDK1 interacts physically with caveolin-1 both in vivo and in vitro. Second, we detected the co-localization of PDK1 and caveolin-1 via confocal microscopy. The localization of PDK1 to the plasma membrane was disrupted by caveolin binding. Third, in transient transfection assays, interaction with caveolin-1 induced a substantial reduction in the in vivo serine/threonine phosphorylation of PDK1, whereas the caveolin-1 binding site mutant (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306} change to ^{141}AFVKLAFTA^{149} and ^{299}ADAPEFLA^{306}) did not. Furthermore, a caveolin-1 scaffolding peptide (amino acids 82–101) functionally suppressed the self-phosphorylation and kinase activities of purified recombinant PDK1 protein. Thus, our observations indicated that PDK1 binds to caveolin-1 through its caveolin-binding motifs, and also that the protein–protein interaction between PDK1 and caveolin-1 regulates PDK1 self-phosphorylation, kinase activity, and subcellular localization.

Keywords: 3-Phosphatidyl-dependent kinase 1; Caveolin; Protein–protein interaction; Protein phosphorylation; Subcellular localization

3-Phosphoinositide-dependent kinase 1 (PDK1) is a member of the serine/threonine kinase family. PDK1 plays a central role in activating AGC subfamily members [1,2]. PDK1 was originally identified as a kinase which could phosphorylate Akt kinase on its activation loop at ^{308}Thr [3–5]. Recent studies have also demonstrated that PDK1 also phosphorylates the equivalent residues on PKC isoforms [6], p70 S6K [7], SGK1 [8], and also the p90 ribosomal S6 kinase [9]. Therefore, PDK1 functions as a hub kinase which activates the AGC superfamily of serine/threonine kinases [10]. In the case of Akt kinase, PDK1 promotes Akt kinase ^{308}Thr phosphorylation and activation via membrane co-localization, mediated through the pleckstrin homology (PH) domain which recruits both PDK1 and Akt kinase to the plasma membrane [3–5,10]. The precise

☆ *Abbreviations:* PDK1, 3-phosphatidyl-dependent kinase 1; PH, pleckstrin homology; *Cav*, Caveolin; PI3K, phosphoinositide 3-OH kinase.

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mechanisms underlying the PDK1-dependent phosphorylation and activation of the other AGC family protein kinases remain to be determined [11–17].

PDK1 contains a PH domain in its C-terminal, which binds to the inositol rings of PIP2 and 3. Thus, the subcellular localization of PDK1 to the plasma membrane seems to be dependent on the activity of PI3K [18,19]. Furthermore, PDK1 itself is a member of the AGC subfamily of protein kinases and is phosphorylated on its own activation loop at ^{241}Ser (equivalent to that of Akt ^{308}Thr) [20]. Park et al. [21] reported that Src/Fyn tyrosine kinase phosphorylates at the ^{9}, ^{373}, and ^{376}Tyr residues of PDK1, and activates it through its PH domain, inducing plasma membrane localization. Taniyama et al. reported that the calcium-activated tyrosine kinase Pyk2 functions as a scaffold for the Src-dependent phosphorylation of PDK1 at ^{9}Tyr, which permits the phosphorylation of ^{373} and ^{376}Tyr by Src. This critical function of Pyk2 is further supported by the observation that Pyk2 and tyrosine-phosphorylated PDK1 are found to co-localize in focal adhesions as a result of stimulation with angiotensin II [22]. However, contractively, other data reported that the activity of PDK1 was stimulated by treatment with growth factors or chemokines in a PI3K-independent manner [23]. In addition, the subcellular localization of PDK1 appeared to be independent of the growth factors and chemokines. According to results of these previous studies, IGF-1 stimulation caused no further activation or plasma membrane localization of PDK1, and PDK1 was concluded to be constitutively active [24]. Thus, until now it has remained unclear as to exactly how PDK1 activity and its subcellular localization are regulated through its PH domain [18–24].

Caveolin-1, a 22–24 kDa integral membrane protein composed of cytoplasmic N- and C-termini and a central intra-membrane domain, is thought to be a major structural component of caveolae (small caves, as they are sometimes called, are invaginations of the plasma membrane), but is also found in the cytoplasm [25,26]. A 20-amino acid juxtamembrane region—also called the scaffolding domain—in the N-terminal region of the protein (residues 82–101) has been shown to mediate caveolin's association with the bulk of the relevant signaling molecules [27,28]. Included in these molecules are the G-proteins, H-ras, Fyn, Erk-2, epidermal growth factor, platelet-derived growth factor, TNF receptor associated factor (TRAF) 2, eNOS, PKC isoforms, insulin and Neu (c-ErbB2) receptors, and Src family serine kinases [29–34]. Two other caveolins (2 and 3) have also recently been characterized [33]. Binding to caveolin-1 through the scaffolding domain is sufficient for inhibition of the in vitro kinase activity of c-Src, Bruton's tyrosine kinase/Bmx, or for maintenance of the inactive conformation of G-proteins [29,35,36]. The functional significance, therefore, of a bona fide caveolin binding motif in these proteins warrants functional characterization, particularly with respect to its role in signal transduction. It is surmised that caveolin may function as a negative regulator for many signaling proteins [29–34]. For insulin receptor signaling, however, caveolin exerts an activation function instead [37]. Thus, caveolin regulates the binding partner protein function case by case, depending on the properties of the binding partner protein [25–37].

Upon visual inspection of PDK1 amino acid sequence with the caveolin-1 binding motifs, we noticed the presence of two potential caveolin-binding motifs (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306}) in its catalytic domain [3]. Consequently, we set out to determine whether or not PDK1 is capable of interaction with caveolin-1. Here, our results demonstrated that caveolin-1 interacts with wild type PDK1 through the potential caveolin-1 binding motifs in the catalytic domain. In addition, we presented evidence suggesting that interaction with caveolin1 mediates the subcellular localization of PDK1, and also leads to the down-regulation of PDK1 self-phosphorylation and its kinase activity. Taken together, our observations might shed some light on the molecular mechanism(s) underlying PDK1 regulation, activation, and signaling.

Materials and methods

Reagents. The protease inhibitor cocktail was obtained from Roche Molecular Biochemicals in tablet form. All phosphatase inhibitors were purchased from Sigma. Anti-PDK1 rabbit polyclonal and mouse monoclonal antibodies were purchased from Cell Signaling. Monoclonal and polyclonal antibodies against caveolin-1, PDK1, and phospho ^{241}Ser PDK1 were purchased from Transduction Laboratories (Lexington, KY) or Cell Signaling (Beverly, MA). The non-ionic detergent *n*-octyl glucoside was purchased from Sigma–Aldrich.

Cell culture and transfections. COS-1 cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1000 U penicillin–streptomycin (Gibco-BRL). COS-1 cells were maintained in RPMI 1640 supplemented with 10% (v/v) FBS and antibiotics. Transfections were carried out using the FuGENE 6 Reagent (Roche Molecular Biochemicals), according to the manufacturer's instructions.

Plasmid constructs and recombinant proteins. Plasmid pcDNA3.1/GS encoding human PDK1 cDNA was obtained from Dr. Alessi (Dundee University). Deletion mutant PDK1 1-249, PDK1 1-556, PDK1 250-556, and PDK1 100-446 were cloned into pTrcHis-TOPO by PCR with primers (up 5′-ATGGCCAGGACCACCAGCCAG-3′ and down 5′-AACGTACTGCGCTGTTCCCAC-3′; up 5′-ATGGCCAGGACCACCAGCCAG-3′ and down 5′-TCACTGCACAGCGGCGTCCGG-3′; up 5′-tctccagagctgctcacggag-3′ and down 5′-TCACTGCACAGCGGCGTCCGG-3′; up 5′-ttctttgttaagctttacttc-3′ and down 5′-GTTTCCGCCAGCCTGCTTGAC-3′, respectively). In order to generate the caveolin-binding motif mutant, PDK1 *cav* construct, mutagenic primers (up 5′-ACCCTCCTCGGCCCGAAATGGTGCTCTGAACAAA-3′ and down 5′-TTTGTTCAGCTCCATTTCGGGGCCGAGGAGGGT-3′; up 5′-GGAAGCTGACGCTCCAGAAAAATTCGCCCCTAAG-3′ and down 5′-CTTAGGGGCGAATTTTTCTGGAGCAGCTTCC-3′), and a QuikChange Multi Mutagenesis

Kit (Stratagene, West Cedar, TX) were utilized according to the manufacturer's instructions. Plasmid pcDNA3.1/GS, encoding for human caveolin-1 cDNA, was obtained from Dr. Ryu (Pohang University, Korea). Caveolin-1 was cloned into a prokaryotic expression vector pET100/D-TOPO (InVitrogen, Carlsbad, CA) by PCR, using two primers (up 5′-CACCATGGAGAAGACTGAGC-3′ and down 5′-TTAGTTTTCAGCCCCTTCTGC-3′) and was re-cloned into the *Eco*RI/*Xba*I sites of the eukaryotic expression vector pcDNA3 flag (C-term; InVitrogen, Carlsbad, CA). His-tagged recombinant proteins for PDK1, truncated PDK1 lacking the catalytic domain, and PDK1 *cav* mutant were purified from *Escherichia coli* BL21 (DE3), after performing PCR with two primers (up 5′-AAAAGAATTCAAGCTTTA TGGA-3′ and down 5′-CCTACTTGTCCTGCATCTTC-3′). All constructions were confirmed by DNA sequencing.

Caveolin-derived synthetic peptides. As in the previous report of Vargas et al. [36], a synthetic peptide corresponding to the caveolin-1 scaffolding domain (amino acids 82–101) termed peptide 1 (DGIWK ASFTTFTVTKYWFYR) and the scrambled version, peptide 2 (WGIDKAFFTTSTVTYKWFRY), were purchased from Peptron (Daejeon, Korea).

Immunoprecipitation. Cells were routinely analyzed 48 h post-transfection. Cells were rinsed with ice-cold phosphate-buffered saline and resuspended in 1 ml of extraction buffer [10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 5 mM DTT, 100 mM NaCl, 1.0% Triton X-100, 60 mM *n*-octyl glucoside, 1 mM vanadate, 100 μM molybdate, 20 mM sodium fluoride, and protease inhibitor cocktail (1 tablet per 10 ml extraction buffer)]. The pre-cleaned lysate was incubated for 1 h at 4 °C with the appropriate antibody, and the resulting immune complexes were collected on protein A–Sepharose beads (Pharmacia). Immune complexes were then captured by centrifugation, washed extensively in lysis buffer, and solubilized with 2× sample buffer, prior to loading onto 10% SDS–PAGE gel.

PDK1 pull-down assay. Whole cell lysates of COS-1 cells transiently expressing caveolin-1 were pre-cleaned with Ni^{2+}–NTA agarose beads, and incubated with 1 μg of each His tagged recombinant PDK1 at 4 °C for 2 h on an end-over-end rotating shaker, in order to allow for the association of PDK1 protein and caveolin-1. The associated protein complexes were collected using the slurry of the Ni^{2+}–NTA agarose beads and washed extensively. After resuspension in 2× Laemmli sample buffer, samples were analyzed on 10% SDS–PAGE.

Immunoblotting. The pull-down or immunoprecipitated PDK1 was resolved on 10% SDS–PAGE gels and transferred to nitrocellulose membranes. The membranes were then incubated in blocking buffer (5% dried skim milk in PBS and 0.05% Tween 20), and probed with specific antibodies, followed by horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected with the chemiluminescence Western blotting detection system (Pierce, Rockford, IL).

In vitro PDK1 self kinas activity and kinase assay. One hundred nanograms of purified recombinant PDK1 protein from *E. coli* was incubated with the caveolin-1 scaffolding peptides, at concentrations of 2.5, 5, and 10 μM, in a standard kinase reaction buffer. In brief, reactions were performed in a 20 μl kinase buffer (20 μM Hepes, pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 200 μM cold ATP) for 10 min at room temperature. The reactions were stopped by the addition of 8 μl of 4× sample buffers. Ten microliters of reaction sample was resolved on 10% SDS–PAGE gels and transferred to nitrocellulose membranes. The serine self-phosphorylated PDK1 protein was detected using an anti-phospho ^{241}Ser PDK1 antibody (Cell Signaling, MA) and the chemiluminescence Western blotting detection system (Pierce, Rockford, IL). The non-radioactive kinase assay for PDK1 was performed according to the protocol established by the Promega (Wisconsin, USA) PepTag Non-radioactive Protein Kinase C (PKC) Assay System, with the exception of the substrate peptides. The FITC conjugated Akt peptide (FITC-AATFTGTLQYMAPEFAAF), used as a substrate for the PDK1 kinase assay, was purchased from Peptron (Daejeon, Korea). FITC-labeled oligopeptide (0.5 μg) was incubated with 10 μl of differentially treated cell lysates, the cell lysates, or PDK1, in 25 μl of protein kinase reaction buffer (20 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol, 0.2 mM EGTA, 20 μM ATP, and 1 μg phosphatidylserine) at 30 °C for 30 min. The reactions were stopped by heating at 95 °C for 10 min. The phosphorylated peptide was separated on 0.8% agarose gel at 100 V for 15 min. The phosphorylated products gained one more negative charge and then migrated to the anode. After electrophoresis, the gel was photographed on a transilluminator. The optical density of the phosphorylated product was measured by densitometry. Alternatively, the radioactive PDK1 kinase assays were performed for 30 min at 30 °C in a 25-μl reaction volume containing [20 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol, 0.2 mM EGTA, and 1 μg phosphatidylserine, 50 μM [γ-^{32}P]ATP (1000–2000 cpm/pmol; Amersham)], and 30 μM Akt peptide (AATFTGTLQYMAPEFAAF) as a substrate for PDK1 substrate [14].

Immunofluorescence and confocal microscopy. COS-1 cells were seeded overnight at 60% confluence onto culture slides coated with human fibronectin (Becton–Dickinson, MA). The following day, cells were transfected with the PDK1/GFP construct and allowed to grow for an additional 48 h. The cells were washed several times with ice-cold PBS and fixed in 2% paraformaldehyde for 10 min. The fixed cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked for 2 h in PBS containing 0.1% BSA-C (Aurion, The Netherlands) and 0.1% Tween. Following incubation with a polyclonal antibody against caveolin-1, the cells were washed and stained further with a conjugated donkey anti-rabbit IgG prior to processing the slides for immunofluorescence. After an additional 20 min of incubation at 37 °C, the cells were fixed, permeabilized, and decorated with either an anti-caveolin-1 or PDK1 antibody. As a secondary antibody, Alexa Fluor 568 or 488-conjugated donkey anti-rabbit or anti-mouse (Molecular Probes, Eugene, OR) was used. Confocal microscopy analysis was performed as previously described [40], except with the optics (Bio-Rad, MRC-1024, and UK) at the Center for Experimental Research Facilities of Chungbuk National University.

Results

PDK1 interacts with caveolin-1 in vitro

Caveolin-binding motifs are present in most G α subunits and the kinase domains of many families of serine and serine/threonine kinases (Src family kinases; PKC; MAP kinase; EGF-R; insulin receptor; and PDGF receptor) [29–34]. Two related caveolin-1 binding motifs (ΦXΦXXXXΦ and ΦXXXXΦXXΦ, where Φ is an aromatic amino acid) are found in most caveolae-associated proteins [36]. With this information, we found that PDK1 contains two potential caveolin-binding motifs (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306}) within its catalytic domain (Fig. 1A). Thus, the presence of a highly conserved caveolin-1 binding motif in PDK1 clearly suggests that PDK1 is one of the caveolin-1 binding proteins [3].

In order to determine which PDK1 interacts with caveolin-1, we constructed PDK1 deletion mutants (Fig. 1A) which included/excluded two related caveolin-1 binding motifs. Each His-PDK1 fusion protein was purified with Ni^{2+}–NTA agarose beads, and incubated with COS-1 cell lysate, in order to yield a binding

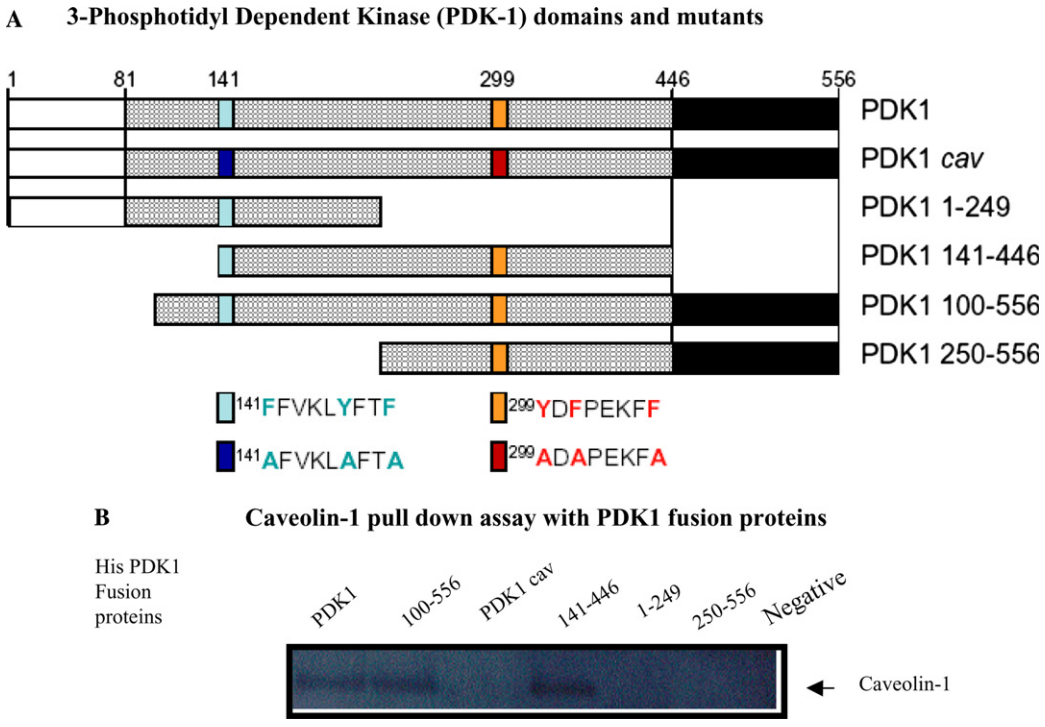

Fig. 1. PDK1 domain and Caveolin 1-binding motif. (A) PH domain (solid) located in the C-terminus. PDK1 contains two well-conserved caveolin-1 binding motifs, ^{141}FFVKLYFTF^{149} (cyan) and ^{299}YDFPEKFF^{306} (orange), within its protein kinase domain (hatched). Four deletion mutants were prepared in order to define the binding region. PDK1 *cav*, a mutant in the caveolin-1 binding motifs, was also prepared, and the mutated sequences are indicated as ^{141}AFVKLAFTA^{149} (dark blue) and ^{299}ADAPEKFA^{306} (dark red). (B) The pull-down assay was performed with the recombinant PDK1 proteins. Whole cell extracts of COS-1 cells were incubated with equal quantities of each His-PDK1 fusion protein expressed in *E. coli*. Caveolin-1 bound to PDK1 fusion protein was detected by using an anti-caveolin-1 antibody. *E. coli* containing His fusion expression vector only was used as the negative control.

with caveolin-1. As expected with the two potential candidate caveolin-binding motifs in Fig. 1A, wild-type PDK1, PDK1 100–556, and PDK1 141–446 brought down caveolin-1 from COS-1 cell lysates in high quantities, while the N-terminal PDK1 1–249 and C-terminal PDK1 250–556 fusion proteins did not (Fig. 1B). Furthermore, the PDK1 *cav* mutant (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306} were changed to ^{141}AFVKLAFTA^{149} and ^{299}ADAPEKFA^{306}) did not result in an appreciable pull down of caveolin-1 (Fig. 1B). *E. coli* containing the His fusion expression vector (pET100/D-TOPO) only was used as a negative control.

These results demonstrated unequivocally that PDK1 interacts with caveolin-1 through two caveolin-1 binding motifs (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306}), and that both caveolin-1 binding motifs of PDK1 are required for interaction with caveolin-1.

PDK1 interacts with caveolin-1 in the COS-1 cell

Since the PDK1 kinase domain fusion protein containing two well-conserved caveolin-1 binding motifs pulls down caveolin-1 in vitro (see Fig. 1B), we set out to determine whether the endogenous PDK1 formed a protein complex with caveolin-1 in COS-1 cells. As shown in Fig. 2A, we ascertained that the PDK1 immunoprecipitate contained caveolin-1. Antibodies directed against caveolin-1 were also able to successfully capture PDK1 from the same lysates, corroborating the hypothesis that the two proteins are indeed physically associated (Fig. 2B). Furthermore, we attempted to determine whether PDK1 exists together with caveolin-1 in the cell with a confocal microscopy experiment. The results indicated that the endogenous PDK1 (green) and caveolin-1 (red) are, indeed, co-localized in the cytosol (yellow) (Fig. 2C). These findings strongly suggest that endogenous PDK1 interacts with caveolin-1 in the COS-1 cell.

In the previous results shown in Fig. 1, we observed that PDK1 interacts with caveolin-1 through two caveolin-1 binding motifs (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306}) in vitro. To further confirm that PDK1 interacts with caveolin-1 through these caveolin-1 binding motifs in vivo, we transfected GFP-PDK1 or GFP-PDK1 *cav* into COS-1 cells, and observed that only GFP-PDK1 was able to specifically bind to caveolin-1 (Fig. 2D, left lane). In stark contrast to the results obtained with GFP-PDK1, GFP-PDK1 *cav* utterly failed to interact with caveolin-1 (Fig. 2D, right lane), indicating that the kinase domain is the crucial factor with regard to the interaction between PDK1 and caveolin-1.

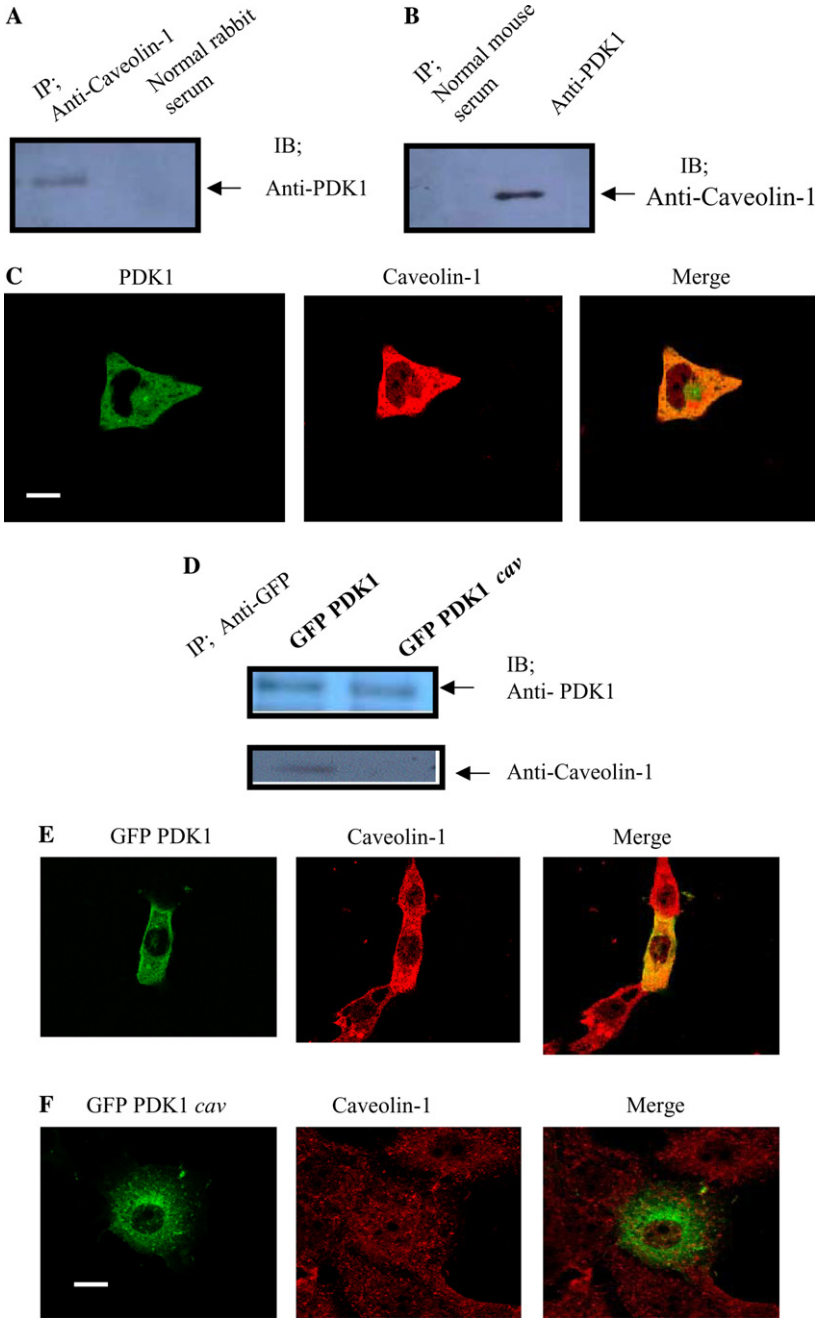

Fig. 2. Interaction between PDK1 and caveolin-1 in COS-1 cells. (A) Following immunoprecipitation (IP) using an anti-caveolin antibody, an immunoblot (IB) was performed using an antibody against PDK1. (B) The immunoprecipitated PDK1 complexes were applied to the immunoblot, using an anti-caveolin-1 antibody. For the negative control, normal mouse serum was used for immunoprecipitation. (C) Confocal fluorescence micrographs showing the endogenous PDK1 in COS-1 cells. Caveolin-1 was visualized by immunofluorescence in fixed and permeabilized cells using polyclonal antibodies to human caveolin-1/PDK1 and Alexa Fluor 568 conjugated donkey anti-rabbit IgG or Alexa Fluor 488 conjugated mouse anti-rabbit IgG. The yellow pattern resulting from the merging of red and green colors indicates the co-localization of both proteins at a specific region of the plasma membrane. (D) COS-1 cells were transiently transfected with expression vectors, GFP-PDK1 or GFP-PDK1 *cav*. Following immunoprecipitation (IP) using an anti-GFP antibody, either caveolin-1 or PDK1 was detected with the immunoblot (IB) using an antibody against caveolin-1 or PDK1. (E) Confocal fluorescence micrographs of GFP-PDK1 in COS-1 cells. Caveolin-1 was visualized by immunofluorescence in fixed and permeabilized cells using a polyclonal antibody against human caveolin-1 and Alexa Fluor 568 conjugated donkey anti-rabbit IgG. The yellow pattern resulting from the merging of red and green colors indicates co-localization of both proteins at a specific region of the plasma membrane or cytoplasm, similar to the results obtained for endogenous PDK1 shown in Fig. 1C. (F) Confocal fluorescence micrographs of GFP-PDK1 *cav* in COS-1 cells. Caveolin-1 was visualized by immunofluorescence in fixed and permeabilized cells using a polyclonal antibody against human caveolin-1 and Alexa Fluor 568 conjugated donkey anti-rabbit IgG. In this test, no yellow color appeared. GFP-PDK1 *cav* was localized mainly in the peri-nuclear region, rather than in the plasma membrane.

In order to gain a better understanding of the effects of interactions between PDK1 and caveolin-1, confocal microscopy was performed (Figs. 2E and F). In a finding consistent with the endogenous PDK1 results shown in Fig. 2C, we observed that exogenous GFP-PDK1 (green) and caveolin-1 (red) were localized together, both in the cytosol and in the plasma membrane (yellow) (Fig. 2E). Surprisingly, however, the color of the exogenous GFP-PDK1 *cav* (green) did not appear to be merging with that of the caveolin-1 (red) (Fig. 2F), suggesting that these two proteins were not co-localized in the cell, probably due to the mutations in the caveolin-1 binding sites. We also observed that the GFP-PDK1 *cav* (green) was localized proximally to the perinuclear region (Fig. 2F), rather than to the plasma membrane, where GFP-PDK1 was mainly found to be localized (Fig. 2E). This suggests that PDK1 *cav* does not form a protein complex with caveolin-1 in the cell, and is consistent with the results of the coimmunoprecipitation experiment shown in Fig. 2D. It also indicates that the defection of PDK1 *cav* binding with caveolin-1 disrupts its subcellular localization into the plasma membrane.

Together, these data strongly suggested that caveolin-1 interacts with PDK1 through its binding consensus motifs (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306}) in the catalytic domain of PDK1, and the protein–protein interaction between PDK1 and caveolin-1 is a requirement for its localization to the plasma membrane.

The binding of caveolin-1 down-regulates both the self-phosphorylation and kinase activity of PDK1

As shown in Figs. 1 and 2, it was demonstrated that the kinase domain of PDK1 also contributes to the physical interaction site with caveolin-1. In order to characterize the functional consequences of this protein–protein interaction, we transiently expressed GFP-PDK1 or GFP-PDK1 *cav* in the COS-1 cell and compared their phosphorylation status. For the monitoring of PDK1 expression, we used an anti-PDK1 antibody (Fig. 3A). In the same samples, we used an anti-caveolin-1 antibody to determine whether caveolin-1 is coimmunoprecipitated with exogenous PDK1. Consistent with the results shown in Fig. 2D, we observed caveolin in the GFP-PDK1 immunocomplex, but not in GFP-PDK1 *cav* (Fig. 3B). This indicates that PDK1 forms a protein complex with caveolin-1 through its two caveolin-1 binding motifs (^{141}FFVKLYFTF^{149} and ^{299}YDF PEKFF^{306}) in the catalytic domain. It was reported that PDK1 self-phosphorylates on the ^{241}Ser residue in its activation loop, in a fashion similar to the activation mechanisms of other AGC family protein kinases [8–10]. Thus, we used an anti-phospho ^{241}Ser PDK1 antibody to determine the phosphorylation of GFP-PDK1 and GFP-PDK1 *cav* [20,21]. As shown in Fig. 3C, the results of Western blotting with

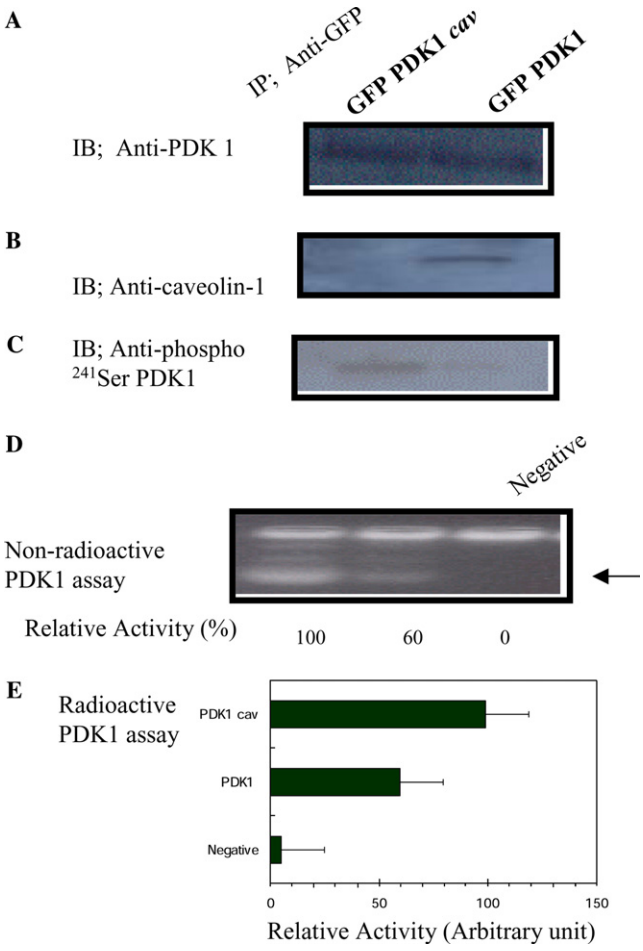

Fig. 3. Caveolin-1 suppresses PDK1 self-kinase activity in COS-1 cells. cells were transfected with GFP-PDK1 and GFP-PDK1 *cav* (caveolin-1 binding site mutant). Following immunoprecipitation (IP) with GFP antibody, the immuno-complexes were resolved on 10% SDS–PAGE gels, and the immunoblots (IB) were performed with the specific antibodies. (A) The amount of both PDK1 and PDK1 *cav* in these experiments was monitored with an anti-PDK1 antibody. (B) The self-phosphorylation of PDK1 was measured with an anti-phospho ^{241}Ser PDK1 antibody. (C) The co-immunoprecipitated caveolin1 by PDK1 was detected with a caveolin-1 antibody. (D) Non-radioactive PDK1 kinase assay was performed using the synthetic FITC-conjugated peptide (FITC-AATFTGTLQYMAPEFAAF). The average of the three repeat PDK1 kinase activities is indicated below. The arrow indicates the phosphorylated peptide. The negative control lacking PDK1 is shown in the right lane. (E) Radioactive PDK1 kinase assay was performed using synthetic peptide (AATFTGTLQYMAPEFAAF). The average of five repeat PDK1 kinase activities is indicated.

an anti-phospho ^{241}Ser PDK1 antibody from the immunoprecipitant with an anti-GFP antibody revealed that the ^{241}Ser phosphorylation of GFP-PDK1 in vivo was reduced to about 60% that of GFP-PDK1 *cav*, even though the same amount of GFP-PDK1 of GFP-PDK1 *cav* was used (Fig. 3A). This suggests that the association of PDK1 with caveolin-1 (Fig. 3B) is related to its ^{241}Ser residue self-phosphorylation (Fig. 3C). However, these results could not rule out kinase activity change of PDK1 *cav* by site-directed mutagenesis.

Therefore, we compared the kinase activity of PDK1 *cav* with that of PDK1 (Figs. 3D and E).

In order to determine whether caveolin-1 is responsible for the down-regulation of PDK1's kinase activity, we utilized FITC conjugated synthetic substrate peptide (FITC-AATFTGTLQYMAPEFAAF) derived from the combination of both the Akt kinase activation (T) loop (AATFTGTLQYMAPE) and the PDK1-interacting fragment (FAAF) of PKA [14,19]. Interestingly, as shown in Fig. 3D, the kinase activity of PDK1 was about 60% of the kinase activity of PDK1 *cav*. In order to verify these non-radioactive PDK1 assay results (Fig. 3D), we also performed a PDK1 assay with Akt1 peptide (AATFTGTLQYMAPEFAAF) and [γ-^{32}P]ATP, as shown in Fig. 3E. With the radioactive PDK1 assay method, we observed that the kinase activity of wild type PDK1 was reduced to about 60% of the kinase activity of PDK1 *cav* (Fig. 3E). Similar to the results of the self-phosphorylation activity, this result suggested that the binding of PDK1 to caveolin-1 results in a reduction of the kinase activity of PDK1. Interestingly, although the localization of PDK1 *cav* to the plasma membrane was disrupted (Fig. 2F), both the self-phosphorylation activity (Fig. 3C) and the kinase activity (Figs. 3D and E) of PDK1 *cav* underwent increases. Therefore, it is abundantly clear that the kinase activity of PDK1 bound to caveolin-1 is less active than that of the caveolin-1 free PDK1, and also that the protein–protein interaction with caveolin-1 results in the negative control of the kinase activity of PDK1.

Together, these data suggest that PDK1 (in a complex with caveolin-1) is less self-phosphorylated on the ^{241}Ser residue than is PDK1 *cav* (not in a complex with caveolin-1) in vivo, and that the kinase activity of PDK1 is less active than that of PDK1 *cav*, largely due to differences in caveolin-1 binding.

The caveolin-1 peptide also down-regulates both self-phosphorylation and kinase activity of PDK1 in vitro

In order to further determine whether the scaffolding domain of caveolin-1 is also responsible for down-regu-

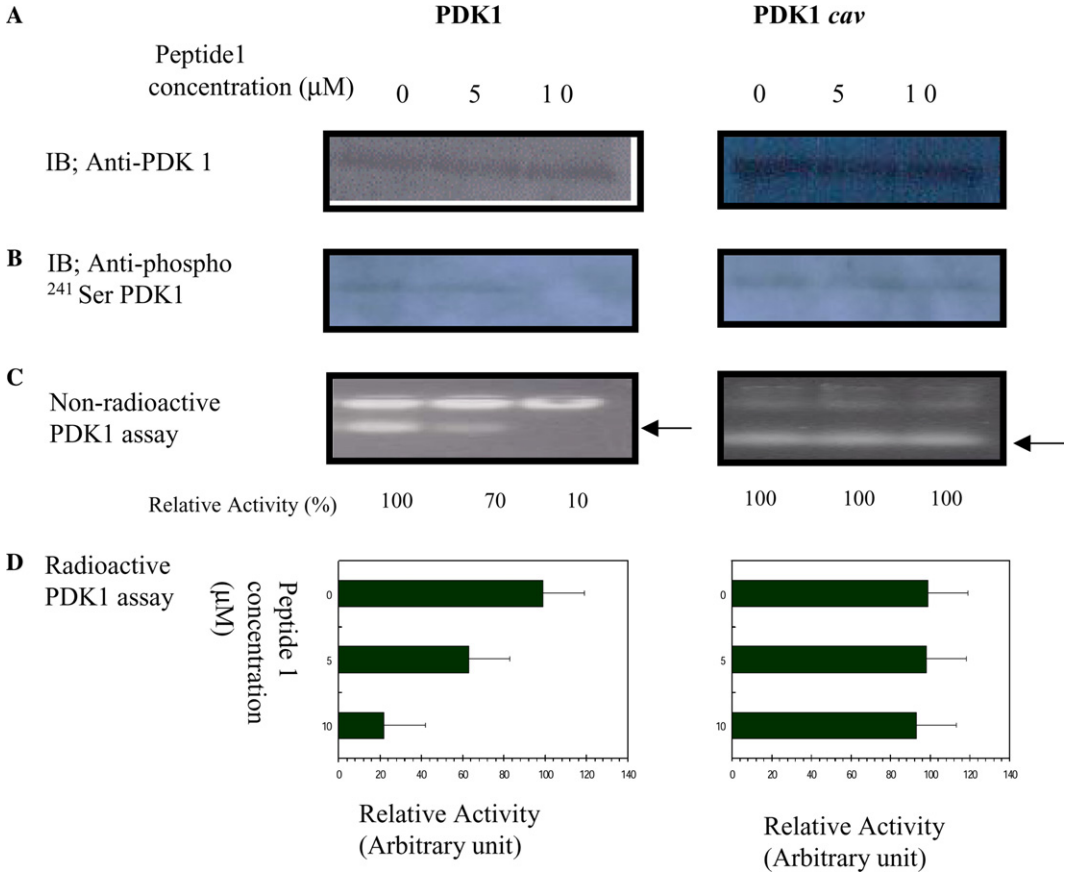

Fig. 4. Caveolin-1 peptide suppresses PDK1 kinase activity to Akt kinase ^{308}Thr residue and its self-phosphorylation at ^{241}Ser residue in vitro. Inhibition of the self-kinase activity of the Ni+ bead purified recombinant His PDK1 (left panel) and His PDK1 *cav* (right panel) from *E. coli* by caveolin-1 peptide. His PDK1 and His PDK1 *cav* were purified, as described under Materials and methods. (A) The amount of both PDK1 and PDK1 *cav* in these experiments was monitored by immunoblot (IB) using PDK1 antibody. (B) After incubation with peptide1 (0, 5, and 10 μM) for 30 min (see Materials and methods), PDK1 kinase self-phosphorylation was measured with IB using an anti-phospho ^{241}Ser PDK1 antibody. Non-radioactive (C) and radioactive (D) PDK1 kinase assays were performed according to the methods described in Figs. 3D and E.

lation of both the self-phosphorylation on its ^{241}Ser and the kinase activity of PDK1 in vitro, we used recombinant His-PDK1and His-PDK1 *cav*, which were purified from recombinant *E. coli* using Ni^{2+}–NTA agarose beads. As shown in Fig. 4B, the caveolin-1 scaffolding peptide (peptide 1) completely inhibited the self-kinase activity (Fig. 4B, left) and kinase activity (Figs. 4C and D, left) of PDK1 at a 10 μM concentration. This contrasts with the results seen with PDK1 (Figs. 4B–D, left), in which peptide 1 exhibited no inhibitory effects on self-phosphorylation (Fig. 4B, right) or kinase activity (Figs. 4C and D, right) of PDK1 *cav* at three different concentrations. With both the non-radioactive (Fig. 4C) and the radioactive (Fig. 4D) PDK1 assay methods, we observed that the kinase activity of PDK1 (but not PDK1 *cav*) underwent a reduction, depending on the peptide 1 concentration. Thus, similar to the results shown in Fig. 3, these results (Figs. 4B–D) showed unequivocally that caveolin-1 binding inhibited the self-phosphorylation and kinase activity of PDK1 in vitro. Furthermore, these results suggested that phosphorylation on the ^{241}Ser residue of PDK1 (Fig. 4B) is strongly correlated with its kinase activity (Figs. 4C and D). We monitored the amounts of both PDK1 and PDK1 *cav* in these experiments via Western blot (Fig. 4A). However, a scrambled version of the peptide (peptide 2) had no effect on PDK1's self-kinase or kinase activity, even at concentrations as high as 10 μM (data not shown).

In summary, our data indicated that PDK1 binds to caveolin-1 (as its new partner protein) through its catalytic domain (131–320 aa), which contains two conserved caveolin binding motifs (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306}), and that interaction with caveolin-1 regulates both PDK1 kinase activity and its subcellular localization.

Discussion

The role of 3-phosphoinositide-dependent protein kinase 1 (PDK1) in signal transduction has been most clearly characterized in the context of PI3K-Akt kinase signaling, in which it functions as a hub protein kinase [10]. In the present report, we described the in vitro and in vivo interactions of PDK1 (a member of the AGC kinase family) with caveolin-1 (a membrane-organizing coat protein). Using the pull-down method, co-immunoprecipitation, and confocal microscopy analysis, we demonstrated the strong physical association occurring between PDK1 and caveolin-1 (Figs. 1–3). In addition, we presented clear evidence that this interaction is mediated through both caveolin-1 binding motifs (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306}). We also demonstrated here that caveolin-1 down-regulates the self-phosphorylation of PDK1 and its kinase activity on the Akt1 peptide (AATFTGTLQYMAPEFAAF) (Fig. 3). Furthermore, a caveolin-1 scaffolding peptide (but not a control peptide) was sufficient to completely inhibit both the self-kinase activity of PDK1 and its kinase activity (Fig. 4).

Although our data suggest that the interaction of PDK1 and caveolin-1 reduces the kinase activity of PDK1 and controls its subcellular localization, our findings also raise several questions regarding the interaction of PDK1 and caveolin-1. It remains unknown as to whether the self-phosphorylation or the kinase activity of PDK1 to its substrate protein kinases was actually necessary for its functional interaction with caveolin-1 in vivo. Further experiments are clearly warranted in order to gain a greater understanding of the biological implications regarding the high degree of conservation of a well-defined caveolin-1 binding motif in PDK1. Moreover, it remains for researchers to characterize the fashion and mechanisms by which the interactions between PDK1 and caveolin-1 are controlled, depending on physiological conditions. In addition, it remains to be determined whether PDK1 mutation (^{141}FFVK LYFTF^{149} and ^{299}YDFPEKFF^{306} are changed to ^{141}AFVKLAFTA^{149} and ^{299}ADAPEKFA^{306}) itself affects kinase and self-phosphorylation activity, regardless of protein–protein interactions with caveolin-1. It is also necessary to ascertain whether the phosphorylation of caveolin-1 is necessary for the activation and/or regulation of PDK1, or for the interaction between PDK1 and caveolin-1.

It has been reported that PDK1 possesses a hydrophobic pocket in the small lobe of its catalytic domain similar to that of PKA, which interacts with S6K1, SGK1, PKCζ, and PKC related kinase-2 [6–12]. Furthermore, mutations of a central residue in the predicted pocket, ^{155}Leu near the PDK1 caveolin-1 binding motif, prevented PDK1 from phosphorylating and activating S6K1 and SGK1 without affecting its ability to phosphorylate either PKB or a short peptide substrate encompassing the activation loop of PKB [4]. They termed the hydrophobic pocket on the kinase domain of PDK1 the PDK1-interacting fragment (PIF)-pocket, after the name of the first discovered PDK-binding AGC-kinase hydrophobic motif-containing peptide [5,6]. They also suggested that the PIF-pocket in PDK1 functions as a docking site, enabling PDK1 to interact with some of its physiological substrates [7,8]. For this reason, we designed a synthetic fluorescein-conjugated peptide (FITC-AATFTGTLQ YMAPEFAAF) for PDK1 kinase assay as a PDK1 substrate (Figs. 3 and 4). The peptide consists of an Akt kinase T loop (AATFTGTLQYMAPE) and PKA PIF (FAAF). Without the PIF motif (FAAF) of PKA, the Akt kinase T loop (AATFTGTLQYMA PE) peptide alone was not efficiently phosphorylated by PDK1 (data not shown).

It is interesting to note that one of the PDK1 caveolin-1 binding motifs (^{141}FFVKLYFTF^{149}) overlaps with the PIF-pocket (112–197 aa) [14,15]. Moreover, the other PDK1 caveolin-1 binding motif (^{299}YDFPEKFF^{306}) is found proximal to the PDK1 self-phosphorylation site (^{241}Ser residue). Thus, we surmised that the binding with caveolin-1 blocks not only the PDK1 substrate protein access to the PIF-pocket, but also PDK1 self-phosphorylation, resulting in the reduction of its kinase activity (Figs. 3 and 4). Other researches have reported that the PDK1 kinase domain also interacts with several other scaffolding proteins, including 14-3-3 and HSP 90 [38,39]. Although the PDK1 binding motif for caveolin-1 (through its 131–320 aa) overlaps with the substrate protein (through its 112–197 aa), 14-3-3 (through its 238–251 aa), or HSP 90 (through its 156–223 aa) [12,34,39], it remains unclear as to whether or not the association of caveolin-1 with PDK1 affects these protein bindings. Recently, Li et al. [41] demonstrated that caveolin-1 over-expression significantly increases the activity of PDK1 and Akt, but not PI3K, in caveolin-1 stimulated cells compared to control cells. They subsequently suggested that caveolin-1 interacts with and inhibits serine/threonine protein phosphatases PP1 and PP2A via interactions with scaffolding domain binding sites, resulting in PDK1 activity increases, and concluded that caveolin-1 is an anti-apoptotic protein. However, it remains to be clarified as to the origin of this discrepancy. In this article, our data clearly indicate that the direct interactions between PDK1 and caveolin-1 reduce both the kinase activity of PDK1 on the synthetic Akt ^{308}Thr peptide and the self-phosphorylation activity at ^{241}Ser of PDK1 (Fig. 4). Therefore, it seems that this difference reflects the affinity between the PDK1 and serine/threonine protein phosphatases to caveolin-1, and the subcellular localization of PDK1 and serine/threonine protein phosphatases to caveolin-1. We also found the caveolin-binding motif (ΦXΦXXXXΦ, where Φ is an aromatic amino acid) in the integrin-linked kinase 1 (ILK1) [42], and observed that the interaction between ILK1 and caveolin-1 results in the inhibition of ILK1 kinase activity (Chun et al., unpublished data). Thus, it seems that caveolin-1 preferentially associates with the inactive conformations of the signaling molecules. Nevertheless, the high affinity of caveolin-1 for the inactive conformation of caveolin-1-interacting proteins is not reflected in PDK1, since the constitutively active PDK1 mutant, PDK1 kinase death, binds readily to caveolin-1 (data not shown). Importantly, the phosphorylation and kinase activity of PDK1 were neither necessary nor required for its functional interaction with caveolin-1. While caveolin-1 has been demonstrated to interact specifically with wild-type c-Src and Ha-Ras, it fails to form a stable complex with their constitutively active counterparts [32,35]. Thus, further experiments are indicated in order to understand the biological implications of the high degree of conservation of a well-defined caveolin-1 binding domain in PDK1. In addition, it will be valuable to ascertain whether or not the serine phosphorylation of caveolin-1 is required for the activation and/or regulation of PDK1. Most importantly, understanding is required of the biological as well as functional implication(s) of PDK1 and caveolin-1 interaction. Thus, we are attempting to determine whether the mutations within the caveolin-1 binding motif might compromise the stability, function, and/or signaling of PDK1.

Caveolin-1 is the principal structural and regulatory component of the caveolar membranes. As lipid rafts are the major functional compartments with regard to signaling, the localization to microdomains, whether caveolae or rafts, could itself be a prerequisite for proper PDK1 signaling. However, caveolin-1 is an integral membrane protein, constituting the major structural component of the caveolae; it is also found in the cytoplasm [36,43]. Our confocal data also showed that caveolin-1 is predominantly localized in the cytoplasm (Fig. 2). We do not currently know why this should be. Since two other caveolin (2 and 3) proteins have been characterized, it cannot be ruled out that each caveolin subtype may play its own functional role with the binding proteins in specific cell lines [33].

Though the caveolin-1 association with PDK1 contributes to PDK1 function via the mediation of its subcellular localization, it remains to be seen whether the differences in PDK1 subcellular localization are due to the differences in cell lines or to differences in the function of PDK1. Because PDK1 also contains a PH domain in its C-terminus which binds to the inositol ring of PIP2 and 3, PDK1 appears to be localized predominantly in the plasma membrane, depending on the activity of PI3K activity [18,19]. However, contractively, other data reported that the PDK1 activity was stimulated in a PI3K-independent manner upon treatment with growth factors and chemokines [23]. In addition, the subcellular localization of PDK1 was also unaffected by treatment with growth factors and chemokines. According to their results, stimulation with IGF-1 caused no further activation or plasma membrane localization of PDK1, and PDK1 was thought to be constitutively active [24]. Further, our confocal data demonstrated that PDK1 *cav*, containing the intact PH domain, is predominantly localized proximally to the nuclear membrane, rather than the plasma membrane (Fig. 2F). Thus, though it remains unclear as to the mechanism by which the PH domain of PDK1 contributes to the mediation of its kinase activity and plasma membrane localization, the interaction between PDK1 and caveolin-1 may also contribute to the localization of PDK1 to the plasma membrane (Figs. 2E and F). It has been reported that the overexpression, and/or constitutive activation of PDK1, results in oncogenic

transformation, culminating in the progression to invasive and metastatic phenotypes [44]. In addition, it has been documented that PDK1 expression and activity are up-regulated in several types of cancers [18,42,43]. However, it remains to be determined whether or not dysfunctions with regard to the interactions occurring between PDK1 and caveolin-1 are related to these diseases.

It appears that the identification of adaptor/substrate proteins and signaling properties of PDK1 provide some indications of possible therapeutic strategies for the inhibition of PDK1 activity. Therefore, further characterization of the biological ramifications of the interaction between PDK1 and caveolin-1 in terms of cell survival, differentiation, and Akt kinase signaling is also required. The activated PDK1 mediates the phosphorylation of a variety of intracellular substrates, most notable of which are: Akt kinase, SGK1, and glycogen synthase kinase-3 [1. 4]. However, many other authentic substrate proteins of PDK1 remain to be identified. Thus, even though the role of PDK1 kinase activity is largely that of a hub kinase in PI3K-Akt kinase signaling, the identification of other PDK1 authentic substrate proteins may facilitate the characterization of the biological functions of PDK1 in the cell.

In conclusion, in this study we identified caveolin-1 as a new binding partner for PDK1 through the motifs (^{141}FFVKLYFTF^{149} and ^{299}YDFPEKFF^{306}) in its catalytic domain. Although the functional significance of this interaction remains poorly understood, the negative regulation of PDK1 activity by caveolin-1 may well represent a relevant consequence of the different signaling pathways in which PDK1 is involved. The assay results of both PDK1 self-phosphorylation activity and the kinase activity of PDK1 reveal that these activities are inhibited upon engagement of caveolin-1 (Figs. 3 and 4). Thus, it appears that the role of caveolin-1 is antagonistic to PDK1 signal transduction. However, though the interaction of PDK1 with caveolin-1 and the role of caveolin-1 as a negative regulator in PDK1 signaling are suggested in this article, the precise control mechanisms underlying the subcellular localization of PDK1 in the caveolae require further characterization in order to gain deeper insight into the overall function of caveolae/caveolin in the PDK1 signal transduction pathway.

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