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Negative regulation of protease-activated receptor 1-induced Src kinase activity by the association of phosphocaveolin-1 with Csk

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Abstract

Protease-activated receptor 1 (PAR1), a G protein-coupled receptor (GPCR) for thrombin, has been correlated with cell proliferation. PAR1 is activated by the irreversibly proteolytic cleavage, internalized via clathrin-coated pits, and then sorted to lysosomes for degradation. Caveolae play important roles in both signaling transduction and internalization of several GPCRs. However, the role of caveolae in cellular signaling and trafficking of PAR1 is still unclear. In this study, we show that PAR1 was partially localized in caveolae. Disruption of caveolae by cholesterol depletion did not inhibit PAR1 internalization, indicating that internalization of PAR1 was not via caveolae. Of interest, activation of PAR1 resulted in the phosphorylation of caveolin-1, a principal component of caveolae, on tyrosine 14 by a Gi-linked Src kinase pathway and p38 mitogen-activated protein kinase. Analysis of immunoprecipitates from cells stimulated by PAR1 showed that phosphocaveolin-1 but not caveolin-1 with mutation at tyrosine 14 could bind to Csk. In addition, phosphocaveolin-1 could not bind to CskS109C mutant with the defective SH2 domain. These results indicated that phosphocaveolin-1 was associated with the SH2 domain of Csk in response to PAR1 activation. The association further resulted in a rapid decrease in Src kinase activity. Thus, PAR1-induced Src activation is negatively regulated by recruiting Csk through phosphocaveolin-1. Our results also reveal that phosphocaveolin-1 represents a novel effector of PAR1 to downregulate Src kinase activity. The downregulation of PAR1-induced Src activation mediated by phosphocaveolin-1 provides an additional mechanism for the termination of PAR1 signaling at its downstream molecules.

Keywords: Protease-activated receptor 1 (PAR1); G protein-coupled receptor (GPCR); Caveolae; Caveolin-1; Phosphocaveolin-1; Src; C-terminal Src kinase (Csk)

1. Introduction

Protease-activated receptor 1 (PAR1), a G protein-coupled receptor (GPCR), mediates many actions of thrombin, including haemostasis, thrombosis, and cell proliferation. Thrombin-mediated cleavage at a specific site of the N-terminal domain of PAR1 unmasks an internal ligand to activate the receptor [1–3]. Due to the irreversibly proteolytic activation, activated PAR1 is rapidly internalized via clathrin-coated pits and sorted to lysosomes to terminate its signaling [4,5]. A synthetic peptide, SFLLRN, can mimic the action of thrombin and fully activate PAR1 [6]. After activation, PAR1 couples to several hetero-trimeric G proteins and initiates downstream signaling pathways to mediate the actions of thrombin on cells [7]. In breast carcinoma cells, the level of PAR1 expression has been correlated with the degree of invasiveness [8]. In colon cancer cell lines, Src kinase has been shown to play a permissive role for PAR1-mediated cell proliferation [9]. At the molecular level, PAR1-induced Src kinase activation lies between Gi protein and the downstream Ras/mitogen-activated protein kinase cascade,

Abbreviations: PAR1, protease-activated receptor 1; Csk, C-terminal Src kinase; GPCR, G protein-coupled receptor; MAP, mitogen-activated protein; EGFR, epidermal growth factor receptor; BSA, bovine albumin; MES, 4-morpholineethanesulfonic acid; PTX, pertussis toxin; IPTG, isopropyl β-D-thiogalactopyranoside; ABTS, 2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonic acid; FITC, fluorescein isothiocyanate; HEK 293 cells, human embryonic kidney 293 cells; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; PFA, paraformaldehyde; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Cbp, Csk-binding protein.

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which in turn results in PAR1 gene transcription and subsequent cell proliferation [10,11]. Therefore, the regulation of PAR1-induced Src activation might play important roles in proliferation and invasion of tumor cells.

Caveolin-1 was first identified in v-Src transformed chicken embryo fibroblasts as a major tyrosine-phosphorylated protein [12]. This 21–24 kDa integral protein is the chief component of caveolae [13]. Caveolae appear as flask-shaped invaginations of 50–100 nm in the plasma membrane and constitute a subset of lipid rafts in cells with high concentrations of cholesterol [14,15]. Caveolar function is critically dependent on the level of cholesterol in the plasma membrane. Increasing evidence indicates that certain GPCRs, such as somatostatin receptor 2, endothelin receptor type A, and A1 adenosine receptor are endocytosed via caveolae [16–19]. In addition to serving as vesicular transporters, the function of caveolae has also been implicated in organizing and regulating the cellular signaling events of GPCRs. Angiotensin II type 1 receptor has been reported to move to caveolae to transactivate epidermal growth factor receptor (EGFR) [20,21]. Oxytocin receptors localized inside or outside caveolin-enriched microdomains couple to different G proteins and lead to different temporal patterns of mitogen-activated protein (MAP) kinase activation and EGFR transactivation [22]. Direct interaction of caveolin-1 with the heterotrimeric G proteins and its downstream molecules such as Src kinase has been shown to be able to negatively regulate their functions [23]. Thus, caveolin-1 may be critical in regulating the signaling cascade of GPCRs in caveolae.

Caveolin-1 has been reported to be phosphorylated by Src kinase on tyrosine 14 [24]. Insulin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), angiotensin II, and cellular stress can also induce this phosphorylation [25–30]. Phosphocaveolin-1 is localized in close to proximity to focal adhesions [31]. However, the functional significance of phosphocaveolin-1 is not clear. Under oxidative stress, phosphocaveolin-1 serves as a docking protein for recruiting C-terminal Src kinase (Csk) to Src kinase [29,32]. Csk, a tyrosine kinase, phosphorylates the negative regulatory site of tyrosine 527 of the Src family kinase and keeps it in an inactive conformation [33,34]. Such recruitment of Csk by phosphocaveolin-1 is important in the feedback regulation of Src kinase activity. In addition to oxidative stress, whether other stimuli mediate the regulation of Src kinase activity by the association of phosphocaveolin-1 with Csk remains to be determined.

Several GPCRs are localized in caveolae, in which their downstream signaling can be regulated. Also, some GPCRs are internalized via caveolae. However, it is not known whether caveolae are also involved in the cellular signaling and internalization of PAR1. In the present study, we first examined whether PAR1 was localized in caveolae and internalized via caveolae. We then investigated whether activation of PAR1 can stimulate the phosphorylation of caveolin-1 on tyrosine 14 and determine its signal transduction pathways. Finally, we addressed the functional significance of phosphocaveolin-1 in response to PAR1 activation by examining the ability of phosphocaveolin-1 to recruit Csk through the SH2 domain of Csk and then to provide the feedback regulation of Src kinase activity. We herein report the molecular mechanism by which PAR1 regulates Src kinase activity through the phosphorylation of caveolin-1.

2. Materials and methods

2.1. Materials

Synthetic peptide SFLLRN was synthesized by SynPep Crop. (Dublin, CA). Bovine albumin (BSA), 4-morpholineethanesulfonic acid (Mes), sucrose, nystatin, methyl-beta-cyclodextrin, pertussis toxin (PTX), and isopropyl β-D-thiogalactopyranoside (IPTG) were from Sigma (St. Louis, MO). PP1 and SB203580 were from Calbiochem (Darmstadt, Germany). One-step ABTS (2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) was purchased from Pierce Biotechnology Inc. (Carlsbad, CA). Alexa 488-conjugated albumin was from Invitrogen Corp. (Carlsbad, CA). Anti-PAR1 1809 rabbit antiserum was generously provided by Dr. Shaun R. Coughlin at University of California, San Francisco, CA [35]. Polyclonal anti-caveolin antibody and monoclonal anti-phosphocaveolin-1 (clone 56), anti-clathrin (clone 23), and anti-Csk (clone 52) antibodies were from BD Bioscience (San Jose, CA). Polyclonal anti-caveolin-1 antibody (sc-894) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal anti-phospho-p38, anti-phospho-Src tyrosine 527 and anti-nonphospho-Src tyrosine 527 antibodies were from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal anti-FLAG M1 antibody was from Sigma. Tubulin-α Ab-1 (clone DM1A) antibody was from NeoMarker (Fremont, CA). Horseradish peroxidase-conjugated affinipure goat anti-mouse or anti-rabbit antibodies, fluorescein (FITC)-conjugated affinipure goat anti-mouse IgG and rhodamine (TRITC)-conjugated affinipure goat anti-rabbit IgG were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

2.2. cDNAs and cell lines

The cDNA encoding FLAG-tagged PAR1 in the pBJ vector was a generous gift of Dr. Shaun R. Coughlin [36]. A kinase inactive mutant of p38 MAP kinase (p38AF) was generously provided by Dr. Amira Klip at University of Toronto, Canada [37]. The cDNAs encoding Csk and CskS109C mutant in pEF-HA vectors were the generous gifts of Dr. Walter Eckhart at Salk Institute, La Jolla, CA [38]. A cDNA encoding wild-type human caveolin-1 in the pCEP4 vector was a gift from Dr. Harold A. Chapman at University of California, San Francisco, CA. The cDNAs encoding human caveolin-1 was subjected to site-directed mutagenesis by polymerase chain reaction (PCR) to convert codon 14 from Tyr (TAC) to Phe (TTC) and the PCR fragments were then subcloned into the pCEP4 vector, resulting in the human caveolin-1/Y14F mutant. Mutagenesis was done by Te-Jung Lu at National Cheng Kung University, and the mutation was confirmed by DNA sequencing analysis. Human embryonic kidney 293 (HEK 293) cells stably expressing FLAG-tagged PAR1 and COS7 cells were cultured in minimum essential medium with Earle's salts and Dulbecoo's modified Eagle's medium (Invitrogen), respectively, and supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) in a humidified atmosphere containing 5% CO_2.

2.3. Transfection methods

For transient transfection, COS7 or HEK 293 cells were transfected with 0.8 μg of DNA and 1.6 μl of Lipofectamine 2000 (Invitrogen) in 24-well dishes according to the manufacturer's instructions (Invitrogen). To transfect cells in different formats, the amounts of Lipofectamine 2000 and DNA were scaled up in proportion to the relative surface area of the cultured dishes.

2.4. Detergent-free purification of caveolin-enriched membrane fraction

COS7 cells with 95% of confluence in 150-mm dishes transiently transfected with 60 μg plasmids of FLAG-tagged PAR1 were used to prepare caveolin-enriched membrane fractions by a detergent-free sodium carbonate method, essentially as described previously [39]. Briefly, cells were scraped into 2 ml of

500 mM sodium carbonate buffer, pH 11.0, containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM Na_3VO_4. The cell suspension was sonicated thirty times with 60% amplitude and 0.5 cycle on ice using an UP 50H ultrasonicator (Dr. Hielscher GmbH, Teltow, Germany), and the insoluble material was removed by centrifugation. The supernatant was adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in Mes-buffered saline (MBS) containing 25 mM Mes, pH 6.5, and 0.15 M NaCl, overlaid with 4 ml of 35% sucrose and 4 ml of 5% sucrose both in MBS containing 250 mM sodium carbonate, and centrifuged at 4 °C for 18 h at 39,000 rpm in a Beckman SW41 rotor. After centrifugation, twelve 1-ml fractions were collected, and aliquots of each fraction were subjected to SDS-PAGE and western blot analysis with either anti-FLAG M1 or anti-caveolin antibodies.

2.5. Internalization assays

HEK 293 cells stably expressing FLAG-tagged PAR1 were plated in 24-well cell culture plates (BD Primaria™) at the density of 3×10^5 cells per well and grown overnight. Cells were then incubated in serum-free medium containing 20 mM HEPES, pH 7.4, and 1 mg/ml BSA at 37 °C for 1 h. After serum starvation, cells were pretreated with 400 mM sucrose, 7.5 mM methyl-beta-cyclodextrin, or 50 μg/ml nystatin for 90 min and then were treated in the absence or presence of 100 μM SFLLRN at 37 °C for the indicated times. Cells were fixed with 4% paraformaldehyde (PFA) at 4 °C for 10 min and washed once with phosphate-buffered saline (PBS). Then, cells were incubated with anti-FLAG M1 antibody for 1 h, washed and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody for 1 h. Finally, cells were washed and incubated with one-step ABTS (the substrate of horseradish peroxidase) for 30 min. The aliquots were transferred to a 96-well dish and the optical density was then determined at 405 nm using a model 550 microplate reader (BioRad Laboratories, Inc., Hercules, CA).

2.6. Confocal microscopy

COS7 cells transiently transfected with FLAG-tagged PAR1 were split onto coverslips in a 6-well dish. Cells were pre-incubated in serum-free medium containing 20 mM HEPES, pH 7.4, and 1 mg/ml BSA at 37 °C for 1 h and incubated in the absence or presence of 100 μM SFLLRN for 30 min or 50 μg/ml Alexa 488-conjugated albumin for 5 or 30 min. Cells were washed three times with PBS, fixed with 4% PFA at 4 °C for 10 min, washed twice with the quench buffer (PBS, pH 7.4, containing 1 mg/ml BSA and 150 mM sodium acetate), and permeabilized with the quench buffer containing 0.1% saponine at room temperature for 20 min. Cells were incubated with anti-FLAG M1 antibody or 1809 rabbit antiserum to label PAR1, anti-caveolin antibody to label caveolin, or anti-clathrin antibody to label clathrin for 1 h. Cells were then washed and incubated with species-specific fluorophore-conjugated secondary antibodies for 1 h at room temperature. Cells were washed four times with PBS and mounted with the *SlowFade* anti-fade reagent (Invitrogen). Confocal images were collected using a Zeiss Axioplan2 LSM510 confocal microscope fitted with a Plan-Neofluar 40X/NA 0.75 objective or a Plan-Neofluar 63X/NA 1.2 water immersion objective. The final composite image was created using Adobe Photoshop 7.0.

2.7. Immunoprecipitation

FLAG-tagged PAR1 was immunoprecipitated by using the FLAG immunoprecipitation kit (Sigma). Briefly, confluent COS7 cells in 100-mm dishes transiently cotransfected with 12 μg plasmids of human caveolin-1 together with 12 μg plasmids of FLAG-tagged PAR1 or pBJ vector were serum starved for 24 h. Cells were lysed in ice-cold lysis buffer containing 50 mM Tris–HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, 1% triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM Na_3VO_4. The insoluble material was removed by centrifugation at 13,000 ×*g* for 15 min prior to the incubation with anti-FLAG M2 affinity gel at 4 °C for 2 h. The FLAG-tagged proteins were eluted by the incubation in 100 μl of Tris-buffered saline (TBS) containing 150 ng/μl 3X FLAG peptide (Sigma) at 4 °C for 1 h. The eluted proteins were recovered by centrifugation at 12000 ×*g* for 2 min, and the supernatant was subjected to SDS-PAGE and western blot analysis.

HA-tagged Csk was immunoprecipitated by using the anti-HA immunoprecipitation kit (Sigma). Briefly, confluent HEK 293 cells in 100-mm dishes transiently transfected with 12 μg plasmids of HA-tagged Csk or HA-tagged CskS109C together with 12 μg plasmids of human caveolin-1, human caveolin-1/Y14F or pCEP4 vector were incubated in the absence or presence of 100 μM SFLLRN at 37 °C for 10 min, washed with PBS, and lysed in ice-cold CelLytic M buffer (Sigma) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM Na_3VO_4. The insoluble material was removed by centrifugation, and the supernatant was incubated with 10 μl of anti-HA-agarose for 2 h with gentle mixing at 4 °C. HA-tagged Csk and its associated proteins were eluted by incubating with 100 μl of sample buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% SDS, and 10% glycerol at 95 °C for 5 min. The eluted proteins were recovered by centrifugation at 12000 ×*g* for 2 min, and the supernatant was subjected to SDS-PAGE and western blot analysis.

2.8. Western blot analysis

To detect phosphocaveolin-1, cells were lysed in 500 mM sodium carbonate buffer, pH 11.0, containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM Na_3VO_4 [40]. The cell suspension was sonicated thirty times with 60% amplitude and 0.5 cycle on ice using an UP 50H ultrasonicator. To detect other proteins of interest, cells were lysed with lysis buffer containing 50 mM NaH_2PO_4, pH 7.2, 150 mM NaCl, 6 mM sodium deoxycholate and 1% NP-40. The insoluble material was removed by centrifugation at 12000 ×*g* for 15 min. Protein concentration was determined by the BCA assay kit (Pierce). Samples containing equal amounts of proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Western blotting was performed using primary antibodies against the proteins of interest, and horseradish peroxidase-conjugated secondary antibodies (1:3000 dilution) were used to visualize bound primary antibodies by an enhanced chemiluminescence assay (ECL) (Amersham, Piscataway, NJ).

3. Results

3.1. Association of PAR1 and caveolin-1

To investigate whether PAR1 is localized in caveolae, three approaches were used. In the first approach, caveolin-enriched fractions were separated from the bulk of cellular membranes and cytosolic proteins by using an established method based on the buoyant-density properties of caveolae [39]. COS7 cells transiently transfected with FLAG-tagged PAR1 were lysed in a detergent-free sodium carbonate buffer, and the lysates were fractionated on a discontinuous sucrose gradient. Equal volumes of each of the twelve collected fractions were subjected to western blot analysis for PAR1 and endogenous caveolin, an integral component of caveolar membrane. Because of the property of light buoyancy, caveolar membrane was floated between 5–35% of sucrose gradient. As shown in Fig. 1A, caveolin was predominantly recovered in the fractions 4 and 5 (5–35% of sucrose gradient). Before SFLLRN stimulation, a portion of PAR1 also appeared in the caveolin-enriched fractions 4 and 5, and the rest of PAR1 was recovered in the fractions 7 to 12. However, after SFLLRN stimulation for 30 min, the distribution of PAR1 was similar to that before the stimulation (Fig. 1A). The results suggest that PAR1 is partially localized in caveolae both before and after agonist stimulation. Second, we attempted to determine whether PAR1 and caveolin-1 were physically associated with each other in COS7 cells by immunoprecipitation. In cells cotransfected with the plasmids of human caveolin-1 and FLAG-tagged PAR1, significant amounts

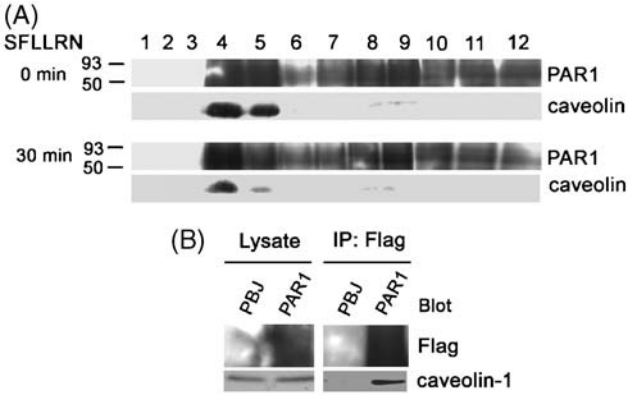

Fig. 1. Association of PAR1 and caveolin-1 in COS7 cells. A) Co-fractionation of PAR1 and caveolin. COS7 cells transiently transfected with FLAG-tagged PAR1 were serum starved, incubated in the absence or presence of 100 μM SFLLRN at 37 °C for 30 min, homogenized, and fractionated on a discontinuous sucrose gradient. Sucrose-gradient fractions were collected from top to bottom, and equal volumes of each fraction were immunoblotted with anti-FLAG M1 or anti-caveolin antibodies. B) Coimmunoprecipitation of FLAG-tagged PAR1 and caveolin-1. COS7 cells were cotransfected with the plasmids of human caveolin-1 and FLAG-tagged PAR1 or the plasmids of human caveolin-1 and pBJ vector. After 48-h transfection, PAR1 was immunoprecipitated (IP) with anti-FLAG M2 affinity gel. Whole cell lysates and immunoprecipitates (IP) were immunoblotted with anti-FLAG M1 or anti-caveolin antibody. Similar results were obtained in two independent experiments.

of caveolin-1 and PAR1 were detected when FLAG-tagged PAR1 was immunoprecipitated with anti-FLAG M2 affinity gel. However, almost no caveolin-1 could be detected in the control cells cotransfected with human caveolin-1 and pBJ vector (Fig. 1B). Therefore, PAR1 might directly interact with caveolin-1 in COS7 cells. In the final approach, the distribution of PAR1 and the endogenous caveolin was examined by confocal microscopy in COS7 cells transiently transfected with FLAG-tagged PAR1. We found that PAR1 was partially colocalized with caveolin along the cell periphery without agonist stimulation (Fig. 2A, a–c). Taken together, these results indicate that a portion of PAR1 is localized in caveolae at the cell membrane and directly interacts with caveolin-1 even in the absence of PAR1 agonist.

3.2. Caveolae-independent and clathrin-dependent internalization of PAR1

Partial localization of PAR1 in caveolae raises the possibility that PAR1 might be internalized via caveolae after agonist stimulation. Confocal microscopy was employed to examine whether PAR1 is colocalized with caveolin-1 in COS7 cells after agonist stimulation. In cells transfected with FLAG-tagged PAR1, exposure to SFLLRN for 30 min caused internalization of PAR1 into endocytic vesicles (green), whereas little or no internalized receptor was colocalized with caveolin (red) (Fig. 2A, d–f). In contrast, the internalized PAR1 (green) was colocalized with clathrin (red) in the presence of agonist stimulation (Fig. 2B, d–f). Since the uptake of albumin has been reported to be via caveolae [41], we then further determined whether the machinery of caveolin-mediated internalization is normal by monitoring the albumin uptake in COS7 cells transiently transfected with FLAG-tagged PAR1. As the time of incubation with albumin increased from 5 to 30 min, additional Alexa 488-conjugated albumin (green) was colocalized with caveolin (red) (Fig. 2C). Thus, the machinery of caveolin-mediated internalization in these cells is normal. These results indicate that PAR1 might not be internalized via caveolae but through a clathrin-dependent mechanism in COS7 cells.

To further investigate whether caveolae are involved in PAR1 internalization, nystatin and methyl-beta-cyclodextrin, sterol-binding agents, were used to block caveolae formation in HEK 293 cells stably expressing PAR1 [14,15]. The levels of PAR1 on the cell surface were determined by modified ELISA. When cells were stimulated with SFLLRN for 5 min, approximately 75% of PAR1 was rapidly internalized into the cells, and the amount of internalized PAR1 almost reached a maximum. In cells pretreated with nystatin or methyl-beta-cyclodextrin, PAR1 was also rapidly internalized into the cells after SFLLRN stimulation, and the rate of PAR1 internalization was similar to that in the control cells. On the contrary, only about 20% of PAR1 was internalized into the cells pretreated with sucrose, which is known to specifically inhibit clathrin-dependent endocytosis [42,43]. The rate of internalization of PAR1 in cells pretreated with sucrose was much slower than that in the control cells (Fig. 2D). This result indicates that internalization of PAR1 is not inhibited by the disruption of caveolae but is clathrin-dependent in HEK 293 cells. Thus, internalization of PAR1 is not via caveolae in both COS7 and HEK 293 cells.

3.3. Characterization of PAR1-induced phosphorylation of caveolin-1 on tyrosine 14 in COS7 cells and HEK 293 cells

Since PAR1 is partially localized in caveolae, we then determined whether activated PAR1 could induce phosphorylation of caveolin-1 on tyrosine 14. The time course and dose response of PAR1-induced phosphorylation of caveolin-1 were studied in COS7 cells transiently transfected with FLAG-tagged PAR1 and HEK 293 cells stably expressing FLAG-tagged PAR1. After stimulation with 100 μM SFLLRN, phosphorylation of caveolin-1 on tyrosine 14 was transiently induced in both COS7 and HEK 293 cells (Fig. 3A). Such induction started at about 5 min and reached a maximum at around 15 min (Fig. 3A). The phosphorylation of caveolin-1 decreased gradually after 20 min (Fig. 3A). When both cells were stimulated with increased concentration of SFLLRN from 10 to 500 μM for 15 min, caveolin-1 was phosphorylated in a concentration-dependent manner with pronounced effect at the SFLLRN concentration of 100 and 500 μM (Fig. 3B). The phosphorylation of caveolin-1 induced by PAR1 was not due to the protein expression of caveolin-1, since the expression of caveolin-1 did not change under the SFLLRN stimulation. The equivalent levels of tubulin also verified the uniform loading (Fig. 3A and B, the bottom panels). These results indicate that activated PAR1 could induce phosphorylation of caveolin-1 on tyrosine 14.

3.4. Involvement of p38 MAP kinase in PAR1-induced phosphorylation of caveolin-1

It has been known that the phosphorylation of caveolin-1 can be mediated by p38 MAP kinase [30]. In addition, p38

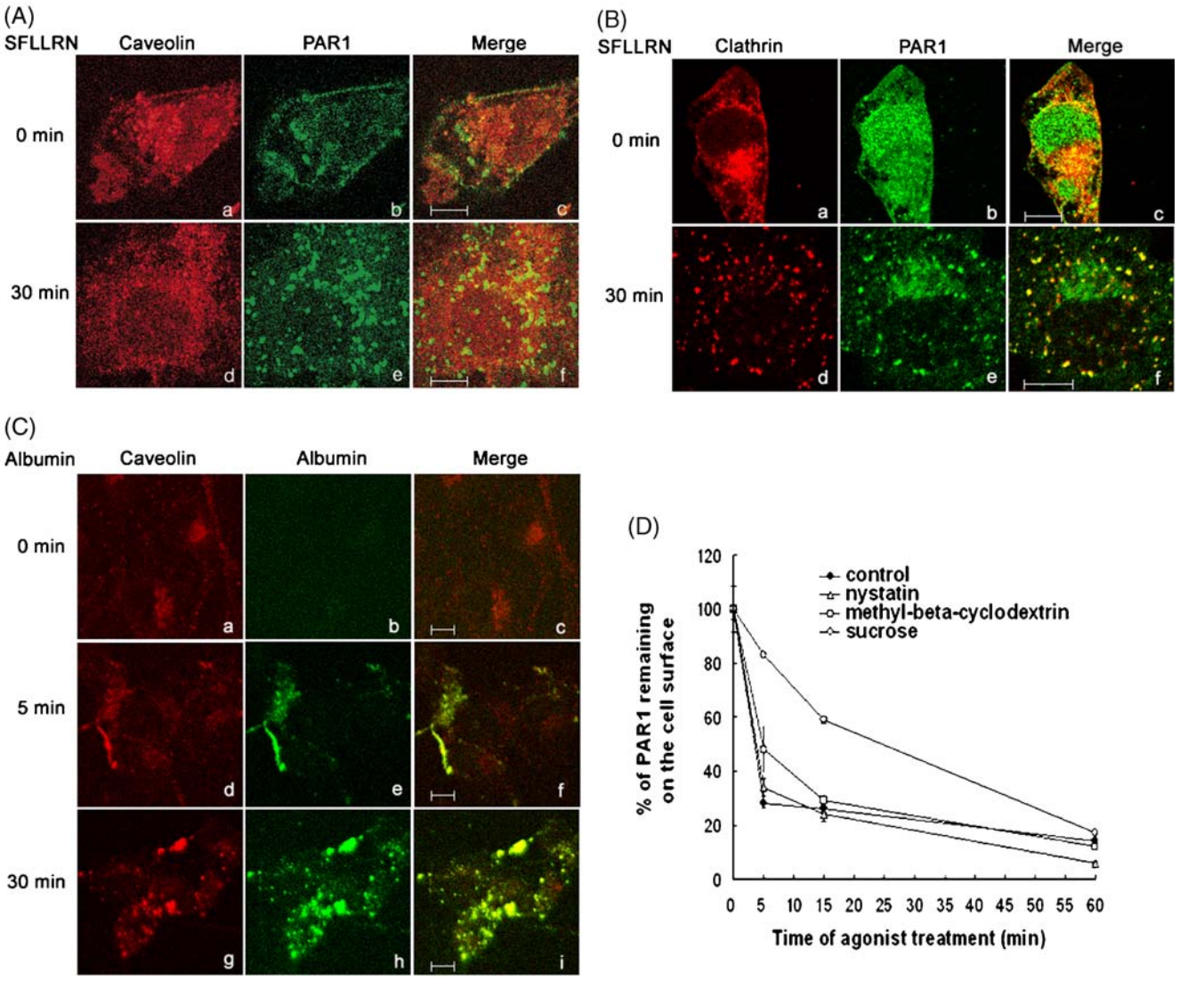

Fig. 2. Caveolae-independent and clathrin-dependent internalization of PAR1. A) Colocalization of PAR1 and caveolin. COS7 cells transiently transfected with FLAG-tagged PAR1 were grown on coverslips and incubated in the absence or presence of 100 μM SFLLRN at 37 °C for 30 min. Cells were then immunostained for PAR1 (*green*) or caveolin (*red*). B) Colocalization of PAR1 and clathrin. Cells were treated as in A and immunostained for PAR1 (*green*) or clathrin (*red*). C) Colocalization of albumin and caveolin. COS7 cells transiently transfected with FLAG-tagged PAR1 were incubated in the absence or presence of 50 μg/ml Alexa 488-conjugated albumin at 37 °C for 5 or 30 min. Cells were then immunostained for caveolin (*red*). Protein colocalization is represented as *yellow* on the merged image. Scale bar, 10 μm. D, Effect of pharmacological inhibitors on the internalization of PAR1. HEK 293 cells stably expressing PAR1 were pre-incubated in the absence (●) or presence of 50 μg/ml nystatin (Δ), 7.5 mM methyl-beta-cyclodextrin (□), or 400 mM sucrose (○) for 90 min. They were then stimulated with 100 μM SFLLRN at 37 °C for 5, 15, or 60 min. Levels of the receptor on the cell surface were determined by performing modified ELISA. The amount of PAR1 on the cell surface at the time zero was designated as the 100% point. Results shown are triplicate determinations from a single experiment that was repeated twice with essentially similar results.

MAP kinase is activated by PAR1 in platelets and several established cell lines [44–46]. To determine whether p38 MAP kinase is involved in PAR1-induced phosphorylation of caveolin-1 on tyrosine 14, a selective p38 MAP kinase inhibitor, SB203580, was used. The p38 MAP kinase activity was inhibited in HEK 293 cells pretreated with SB203580 for 2 h prior to SFLLRN stimulation for 15 min (Fig. 4A, the bottom panel). Moreover, this pretreatment also resulted in partial inhibition of the phosphorylation of caveolin-1 stimulated by SFLLRN (Fig. 4A and B).

To further verify the involvement of p38 MAP kinase in PAR1-induced phosphorylation of caveolin-1 on tyrosine 14, a dominant-negative form of p38 MAP kinase with the replacement of two activating phosphorylation sites by alanine and phenylalanine (p38AF) was used. In these experiments, HEK 293 cells were transiently transfected with a plasmid containing a gene encoding the dominant negative p38 MAP kinase under the control of the *lac* operon. Treatment of the cells with IPTG overrides the transcriptional repression, thereby allowing expression of this dominant-negative p38 MAP kinase. The cells were left untreated or treated for 48 h with IPTG and then stimulated with SFLLRN for 15 min. In cells without IPTG treatment, the levels of phosphorylation of both p38 MAP kinase and caveolin-1 increased under SFLLRN stimulation (Fig. 4C

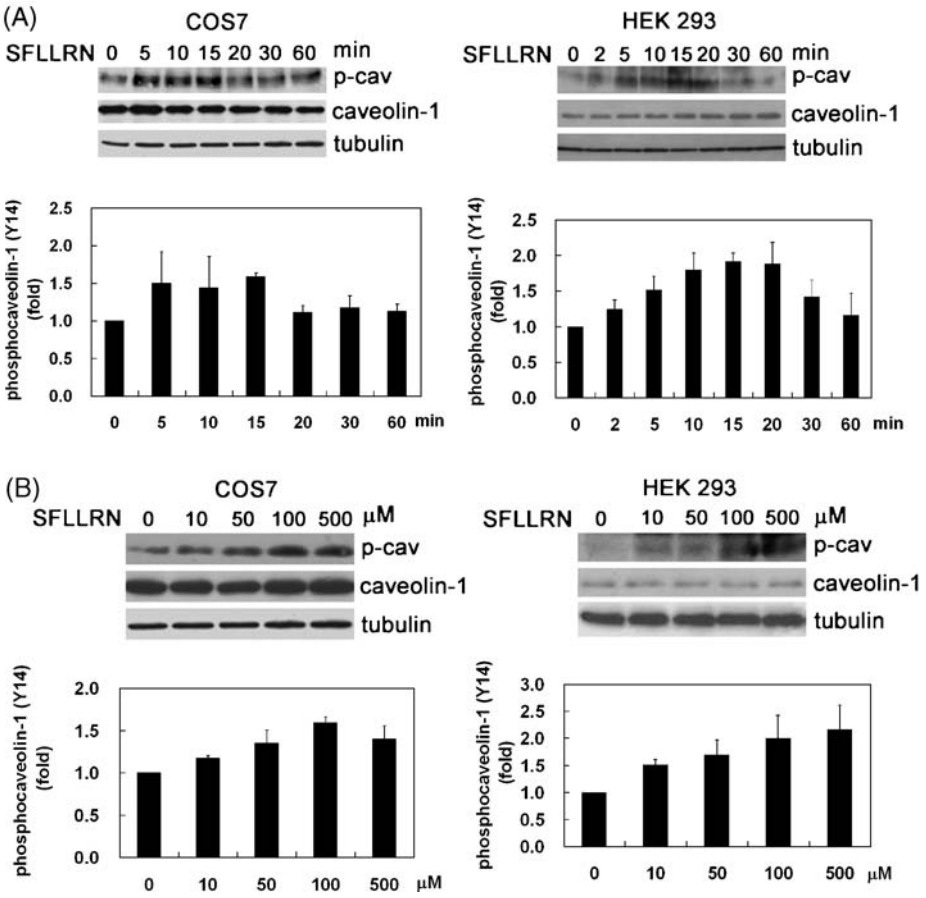

Fig. 3. Characterization of PAR1-induced phosphorylation of caveolin-1 on tyrosine 14 in COS7 and HEK 293 cells. A) Time course of PAR1-induced phosphorylation of caveolin-1 on tyrosine 14. COS7 cells transiently transfected with FLAG-tagged PAR1 and HEK 293 cells stably expressing FLAG-tagged PAR1 were serum starved for 24 h. Both cells were incubated with 100 μM SFLLRN at 37 °C from 2 to 30 min. Cell extracts were treated as described under Materials and methods and assayed by western blotting. Phosphorylation of caveolin-1 (p-cav) was detected by using phosphospecific caveolin-1 monoclonal antibody that recognized only tyrosine 14-phosphorylated form of caveolin-1. Caveolin-1 was detected with polyclonal anti-caveolin-1 antibody, and α-tubulin was detected with monoclonal antibody to ensure equal loading. The amounts of phosphocaveolin-1 were determined from densitometric analysis. Relative amounts of phosphocaveolin-1 were calculated by defining phosphocaveolin-1 in cells without any treatment as 1 and represented as the mean ± S.E. of three independent experiments. B) Dose response of PAR1-induced phosphorylation of caveolin-1 on tyrosine 14. After serum starvation for 24 h, both COS7 and HEK 293 cells were incubated with 10 to 500 μM SFLLRN at 37 °C for 15 min. Cell extracts were assayed by western blotting and the relative amounts of phosphocaveolin-1 were analyzed and represented as described in A.

and D). Expression of the dominant negative p38 MAP kinase by IPTG treatment attenuated the phosphorylation of both p38 MAP kinase and caveolin-1 (Fig. 4C and D). These results indicate that p38 MAP kinase could mediate PAR1-induced phosphorylation of caveolin-1 on tyrosine 14.

3.5. Involvement of the Src family kinase in PAR1-induced phosphorylation of caveolin-1

Tyrosine 14 of caveolin-1 was identified as the principal site of phosphorylation by c-Src [24]. Since PAR1 could stimulate Src kinase activity [10], it is possible that the Src family kinase is involved in PAR1-induced phosphorylation of caveolin-1 on tyrosine 14. Pretreatment of HEK 293 cells stably expressing PAR1 with PP1, a Src family kinase inhibitor, for 90 min completely blocked the phosphorylation of caveolin-1 after SFLLRN stimulation for 15 min (Fig. 5), indicating that activation of the Src family kinase was involved in PAR1-induced phosphorylation of caveolin-1. Thus, PAR1-induced phosphorylation of caveolin-1 on tyrosine 14 is mediated by the Src family kinase. To determine whether Src kinase is also involved in PAR1-induced activation of p38 MAP kinase, the phosphorylation of p38 MAP kinase was examined. In cells pretreated with PP1 prior to SFLLRN stimulation, phosphorylation of p38 MAP kinase was partially inhibited (Fig. 5, the lowest panel). Thus, PAR1-induced Src kinase activity could mediate the activation of p38 MAP kinase. These results suggest that PAR1-induced phosphorylation of caveolin-1 on tyrosine 14 is mediated by Src kinase and its downstream p38 MAP kinase.

3.6. Involvement of PTX sensitive G protein in PAR1-induced phosphorylation of caveolin-1

Pertussis toxin (PTX) has been reported to prevent Gi proteins from interacting with the receptors [47]. Since PTX has been reported to partially inhibit PAR1-induced Src kinase activity [10], it is possible that Gi protein acts upstream of Src kinase to mediate the phosphorylation of caveolin-1 in response to PAR1

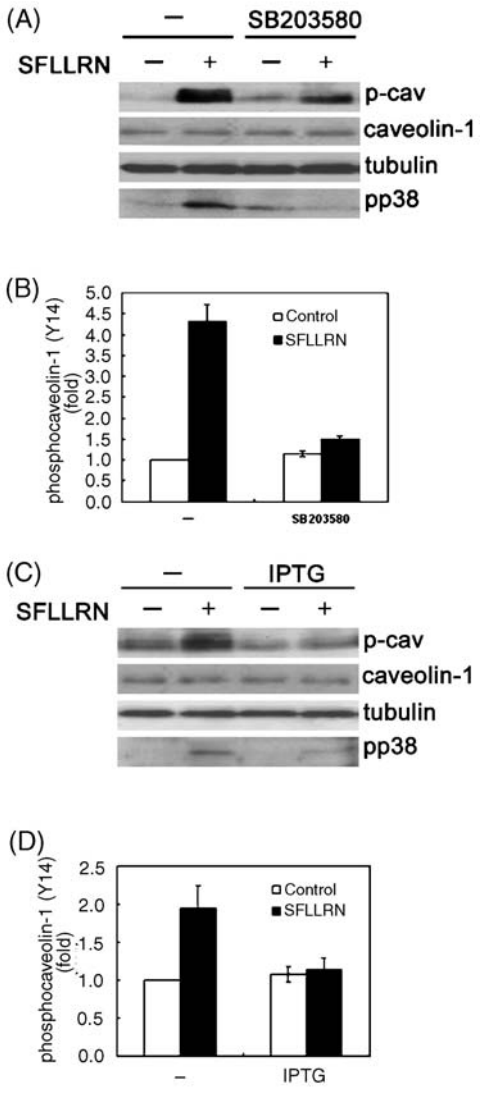

Fig. 4. Involvement of p38 MAP kinase in PAR1-induced phosphorylation of caveolin-1. A) Effect of SB203580 on PAR1-induced phosphorylation of caveolin-1. HEK 293 cells stably expressing PAR1 were serum starved for 24 h and then preincubated in the absence or presence of SB203580 5 μM for 2 h. Cell extracts were assayed by western blotting as described in Fig. 3. Phospho p38 MAPK (pp38) was detected with a phosphospecific p38 polyclonal antibody that only recognized the active phosphorylated form of p38 MAP kinase. B) Quantification of the amounts of phosphocaveolin-1 obtained in A. Relative amounts of phosphocaveolin-1 were calculated by defining phosphocaveolin-1 in cells without the treatment of both SFLLRN and SB203580 as 1. The quantitative results were represented as the mean ± S.E. of three independent experiments. C) Effect of dominant negative p38 MAP kinase on PAR1-induced phosphorylation of caveolin-1. HEK 293 cells transiently transfected with a IPTG-inducible p38 dominant negative mutant were treated with 5 mM IPTG for 48 h to induce the expression of dominant negative p38 MAP kinase (p38AF). After IPTG treatment for 24 h, cells were serum starvation for 24 h. Both sets of cells were stimulated in the absence and presence of 100 μM SFLLRN at 37 °C for 15 min. Cell extracts were assayed by western blotting as described in A. D) Quantification of the amounts of phosphocaveolin-1 obtained in C. Relative amounts of phosphocaveolin-1 were calculated by defining phosphocaveolin-1 in cells without the treatment of both of SFLLRN and IPTG as 1. The quantitative results were represented as the mean ± S.E. of two independent experiments.

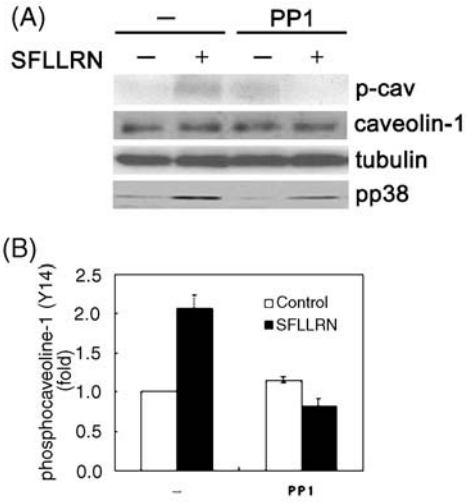

Fig. 5. Involvement of Src family kinase in PAR1-induced phosphorylation of caveolin-1. A) Effect of PP1 on PAR1-induced phosphorylation of caveolin-1. HEK 293 cells stably expressing PAR1 were serum starved for 24 h. Cells were preincubated in the absence or presence of 10 μM PP1 for 90 min, and both sets of cells were stimulated with 100 μM SFLLRN at 37 °C for 15 min. Cell extracts were assayed by western blotting as described in Fig. 4. B) Quantification of the amounts of phosphocaveolin-1 obtained in A. Relative amounts of phosphocaveolin-1 were calculated by defining phosphocaveolin-1 in cells without the treatment of both SFLLRN and PP1 as 1. The quantitative results were represented as the mean ± S.E. of four independent experiments.

activation. To determine whether Gi protein is involved in PAR1-induced phosphorylation of caveolin-1, HEK 293 cells stably expressing PAR1 were pretreated with PTX for 20 h to examine its

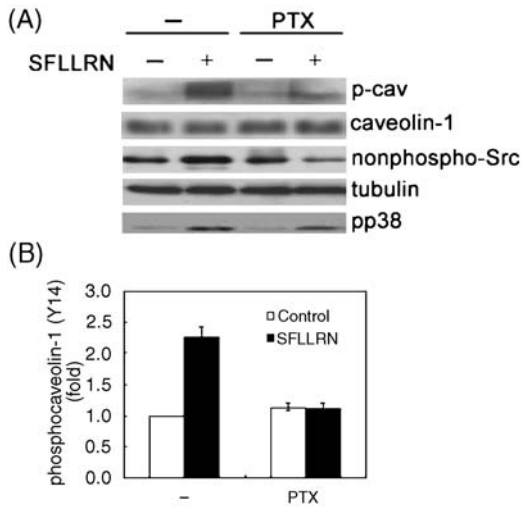

Fig. 6. Involvement of PTX-sensitive G protein in PAR1-induced phosphorylation of caveolin-1. A) Effect of PTX on PAR1-induced phosphorylation of caveolin-1. HEK 293 cells stably expressing PAR1 were serum starved for 24 h. Cells were preincubated in the absence or presence of 100 ng/ml PTX for 20 h and then were stimulated with 100 μM SFLLRN at 37 °C for 15 min. Cell extracts were assayed by western blotting as described in Fig. 4. Nonphosphorylated Src (nonphospho-Src) at tyrosine 530 was detected by a nonphospho-Src polyclonal antibody which recognized the active form of Src. B) Quantification of the amounts of phosphocaveolin-1 obtained in A. Relative amounts of phosphocaveolin-1 were calculated by defining phosphocaveolin-1 in cells without the treatment of both SFLLRN and PTX as 1. The quantitative results were represented as the mean ± S.E. of three independent experiments.

effect on phosphorylation of caveolin-1. As shown in Fig. 6, stimulation of PAR1 by SFLLRN resulted in the increase of the phosphorylation of caveolin-1 on tyrosine 14. The same treatment also resulted in the increased levels of nonphospho-tyrosine 530 of Src (tyrosine 527 in avian Src), an active form of Src. However, pretreatment of the cells with PTX inhibited both effects induced by PAR1 (Fig. 6). The result indicates that a Gi-linked Src kinase pathway is involved in PAR1-induced phosphorylation of caveolin-1 on tyrosine 14. In this experiment, the phosphorylation of p38 MAP kinase was also examined to determine whether Gi protein is involved in PAR1-induced activation of p38 MAP kinase. In cells pretreated with PTX prior to SFLLRN stimulation, phosphorylation of p38 MAP kinase was partially inhibited (Fig. 6, the lowest panel). These results indicate that PAR1-induced activation of p38 MAP kinase is mediated by a Gi-linked Src kinase pathway. Thus, p38 MAP kinase may act downstream of Gi-linked Src kinase to mediate PAR1-induced phosphorylation of caveolin-1 on tyrosine 14.

3.7. Negative regulation of Src kinase activity by the association of phosphocaveolin-1 with Csk after PAR1 activation

Tyrosine 14-phosphorylated caveolin-1 has been reported to recruit Csk and then inactivate Src kinase by phosphorylation of

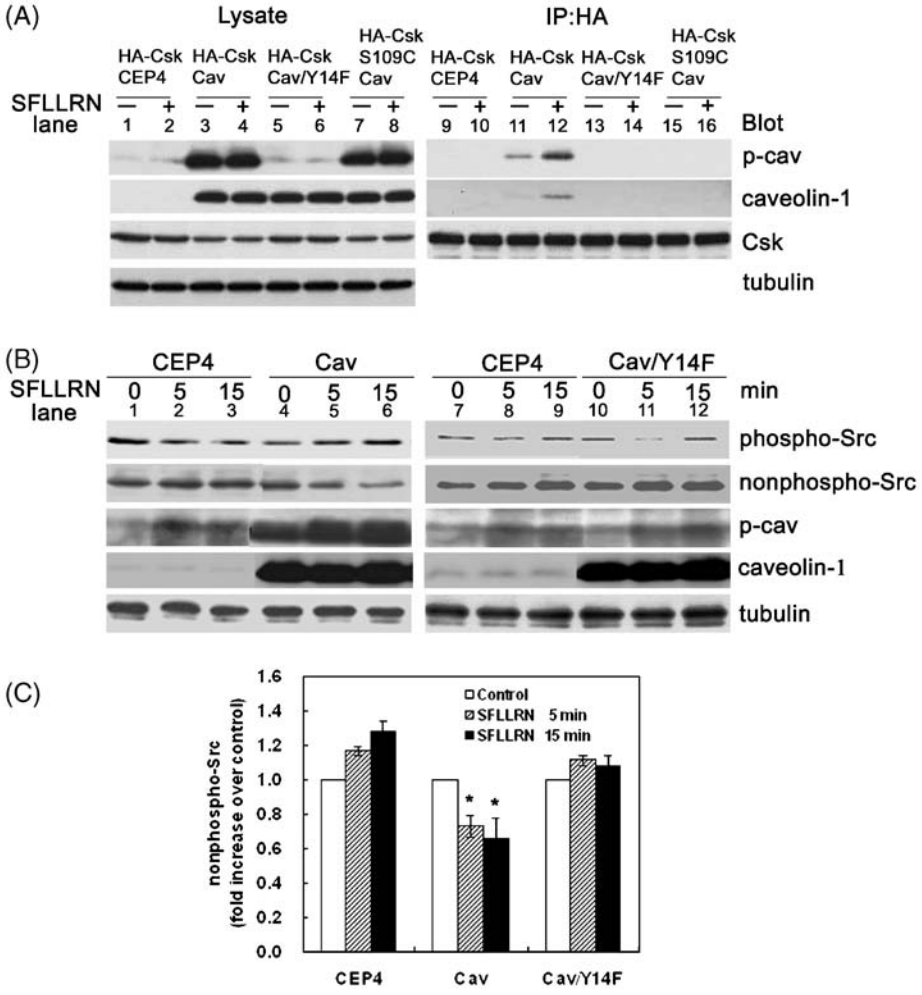

Fig. 7. Negative regulation of Src kinase activity by the association of phosphocaveolin-1 with Csk after PAR1 activation. A) Association of phosphocaveolin-1 and Csk. HEK 293 cells stably expressing PAR1 were transfected with the plasmids of HA-tagged Csk (HA-Csk) or HA-tagged CskS109C (HA-CskS109C) together with the plasmids of caveolin-1 (Cav), caveolin-1/Y14F (Cav/Y14F), or pCEP4 vector. After 48 h transfection, cells were serum starved for 24 h and incubated in the absence or presence of 100 μM SFLLRN at 37 °C for 10 min. Cells were subjected to immunoprecipitation with HA-conjugated agarose beads for the isolation of HA-tagged Csk or HA-tagged CskS109C. Whole cell lysates and immunoprecipitates (IP) were subjected to SDS-PAGE and assayed by western blotting. Csk was detected by monoclonal anti-Csk antibody. Phosphocaveolin-1, caveolin-1 and tubulin were detected as described in Fig. 3. B) Attenuation of Src kinase activity through the phosphorylation of tyrosine 530 of Src. HEK 293 cells stably expressing PAR1 were transiently transfected with caveolin-1 (Cav), caveolin-1/Y14F (Cav/Y14F), or pCEP4 vector. After 48 h transfection, HEK 293 cells were serum starved for 24 h and then incubated in the absence or presence of 100 μM SFLLRN at 37 °C for 5 or 15 min. Cell lysates were subjected to SDS-PAGE and assayed by western blotting. Nonphosphorylated Src (nonphospho-Src) or phosphorylated Src (phospho-Src) at tyrosine 530 were detected by nonphospho-Src or phospho-Src polyclonal antibodies that recognized the active or inactive form of Src, respectively. Phosphocaveolin-1, caveolin-1 and tubulin were detected as described in Fig. 3. C) Quantification of the amounts of nonphospho-Src obtained in B. The amounts of nonphospho-Src were determined from densitometric analysis of unstimulated and stimulated cells transiently transfected with caveolin-1, caveolin-1/Y14F, or pCEP4 vector, and shown as fold increase over control. The quantitative results were represented as the mean ± S.E. of three independent experiments. Statistical significance compared with CEP4 was determined using a paired *t* test (*$p<0.05$).

its tyrosine 527 under oxidative stress [29]. We first examined whether Csk is associated with phosphocaveolin-1 through its functional SH2 domain in response to PAR1 activation. In these experiments, HEK 293 cells were transfected with caveolin-1 or caveolin-1/Y14F mutant together with Csk or CskS109C mutant. Caveolin-1/Y14F mutant that could not be phosphorylated at tyrosine 14 was used to determine the binding function of phosphotyrosine; CskS109C mutant with a defect in the FLVRES motif of the SH2 domain was used to examine the requirement of SH2 domain to bind with phosphotyrosine-containing proteins. Both Csk and CskS109C mutant contained a hemagglutinin epitope tag allowing them to be separated from the endogenous Csk protein during immunoprecipitation. The association of phosphocaveolin-1 and Csk was examined by performing immunoprecipitation of HA-tagged Csk from the cells. In whole cell lysates, phosphocaveolin-1 was dramatically induced in HEK 293 cells overexpressing caveolin-1 with either HA-tagged Csk or HA-tagged CskS109C in the absence and presence of SFLLRN stimulation (Fig. 7A, lanes 3, 4, 7, and 8). In cells overexpressing caveolin-1/Y14F and HA-tagged Csk, only endogenous phosphocaveolin-1 was detected, despite an overexpression of caveolin-1 (Fig. 7A, lanes 5 and 6).

In HA-tagged Csk immunoprecipitates, additional phosphocaveolin-1 and caveolin-1 could be detected only from cells overexpressing caveolin-1 and Csk after SFLLRN stimulation for 10 min (Fig. 7A, lane 12). However, neither phosphocaveolin-1 nor caveolin-1 was detected from cells coexpressing HA-tagged Csk with caveolin-1/Y14F mutant or with its control pCEP4 vector (Fig. 7A, lanes 9, 10, 13, and 14). These results indicate that in response to PAR1 activation, phosphocaveolin-1 could bind to Csk, whereas caveolin-1 could not bind to Csk if its tyrosine 14 was mutated to phenylalanine. To further determine whether the SH2 domain of Csk is essential for the binding of phosphocaveolin-1, HA-tagged Csk immunoprecipitates from cells coexpressing CskS109C, a mutant with defective SH2 domain, and caveolin-1 in HEK 293 cells were examined. No phosphocaveolin-1 was detected in HA-tagged CskS109C immunoprecipitates (Fig. 7A, lanes 15 and 16), despite an overinduction of phosphocaveolin-1 in the cell lysates (Fig. 7A, lanes 7 and 8). These results indicate that the SH2 domain of Csk was necessary to bind tyrosine 14-phosphorylated caveolin-1 after PAR1 activation.

Next, we determined whether the recruitment of Csk by phosphocaveolin-1 could phosphorylate tyrosine 530 of Src to downregulate PAR1-induced Src activation. The levels of phosphorylation of tyrosine 530 of Src in HEK 293 cells transfected with caveolin-1, caveolin-1/Y14F, or control pCEP4 vector were examined after SFLLRN stimulation. In cells transiently transfected with caveolin-1/Y14F or control pCEP4 vector, the levels of phosphorylation of tyrosine 530 of Src decreased after SFLLRN stimulation for 5 min (Fig. 7B, lanes 2, 8, and 11). By contrast, in cells transiently transfected with caveolin-1, the levels of phosphorylation of tyrosine 530 of Src increased, which was associated with an overinduction of phosphocaveolin-1, with SFLLRN stimulation from 5 to 15 min (Fig. 7B, lanes 5 and 6). The increase of phosphorylation of tyrosine 530 of Src in cells overexpressing caveolin-1 suggests that PAR1-induced Src activation might be inhibited in these cells. We then examined the activity of Src kinase by detecting the levels of nonphospho-tyrosine 530 of Src, an active form of Src. In this experiment, caveolin-1/Y14F mutant was used to differentiate whether the inhibitory effect of PAR1-induced Src activation is resulted from the association of phosphocaveolin-1 with Csk or from direct inhibition of caveolin-1 on Src kinase activity. In cells transiently transfected with caveolin-1/Y14F or control pCEP4 vector, the level of activated Src increased with SFLLRN stimulation from 5 to 15 min (Fig. 7B, lanes 2, 3, 8, 9, 11 and 12, and C), even though caveolin-1 was overexpressed in cells transfected with caveolin/Y14F as compared with the control cells, but the induction of phosphocaveolin-1 was similar in both cells (Fig. 7B, lanes 7–12). By contrast, in cells transiently transfected with caveolin-1, the level of activated Src decreased, which was associated with an overinduction of phosphocaveolin-1, with SFLLRN stimulation from 5 to 15 min (Fig. 7B, lanes 5 and 6, and C). Thus, overinduction of phosphocaveolin-1 after SFLLRN stimulation downregulates PAR1-induced Src kinase activity. The results indicate that Src kinase activity is negatively regulated through the recruitment of Csk by phosphocaveolin-1 in response to PAR1 activation.

4. Discussion

PAR1-induced Src activation plays a key role in cell-proliferative responses. However, the mechanisms by which PAR1 regulates the activity of Src kinase are not completely understood. In this study, we show a novel mechanism for the feedback regulation of Src kinase in PAR1 signaling. After PAR1 activation, Csk negatively regulates Src kinase activity by the association with phosphocaveolin-1. This association occurs through the SH2 domain of Csk by binding with the tyrosine 14-phosphorylated caveolin-1. The finding that PAR1-induced phosphorylation of caveolin-1 requires a Gi-linked Src kinase pathway suggests that G proteins also mediate the phosphorylation of caveolin-1 induced by GPCRs. Since PAR1 is partially localized in caveolae and associated with caveolin-1, the feedback regulation of PAR1-induced Src kinase activity by phosphocaveolin-1 might occur in caveolae. Our findings provide the first evidence for the involvement of caveolin-1 in PAR1 signaling.

Two important phosphorylation sites, tyrosine 416 and tyrosine 527, regulate Src kinase [48]. Src undergoes autophosphorylation at tyrosine 416, and its phosphorylation increases kinase activity. Under basal conditions, most Src is phosphorylated at tyrosine 527, and phosphotyrosine 527 binds intramolecularly with the SH2 domain of Src to keep it in an inactive conformation. The Tyr527Phe Src mutant has been reported to be more active than other forms and can induce anchorage-independent growth in cell culture and in tumors in vivo [49], indicating a key role of tyrosine 527 in the regulation of Src kinase activity. Therefore, downregulation of Src kinase activity by the phosphorylation of tyrosine 527 after PAR1 activation could be important in PAR1 signaling. The scaffolding domain, residues 82–101, of caveolin-1 can directly interact with Src and inhibit its autophosphorylation at tyrosine

416 [50]. This observation raised the possibility that overexpression of caveolin-1 could result in the inhibition of PAR1-induced Src activation. However, our results showed that after PAR1 activation, a transient increase in phosphorylation of caveolin-1 on tyrosine 14 was associated with a rapid decrease in kinase activity of Src only in cells overexpressing caveolin-1 but not in cells overexpressing caveolin-1/Y14F (Fig. 7B and C). Therefore, PAR1-induced phosphorylation of caveolin-1 on tyrosine 14 serves a regulatory role in the Src-kinase feedback loop of transient activation and attenuation.

Csk is not a membrane-associated polypeptide and lacks a membrane-targeting motif. To date, two tyrosine-phosphorylated proteins, caveolin-1 and Csk-binding protein (Cbp), have been reported to recruit Csk from the cytosol to a specific site at the cell membrane to target the Src family kinase [29,32,51]. Of interest, both caveolin-1 and Cbp are located at a specific microdomain called lipid rafts. In our study, activation of PAR1 resulted in a transient increase of phosphorylation of caveolin-1 on tyrosine 14. Phosphocaveolin-1 further recruited Csk to return Src activity back to the basal state. The findings are similar to the previous observations for cells under oxidative stress [29,32]. Thus, transient phosphorylation of caveolin-1 after PAR1 activation might occur in caveolae, a subset of lipid rafts, to fine tune Src kinase activity for the appropriate regulation of cellular responses.

Activation of the p38 MAP kinase pathway, but not the p42/44 MAP kinase pathway, has been reported to be required to induce tyrosine phosphorylation of caveolin-1 in response to hyperosmotic stress [30]. Consistent with their observations, using a specific p38 MAP kinase inhibitor, SB203580, and the dominant negative form of p38 MAP kinase, we found that most of the phosphorylation of caveolin-1 occurred through the activation of the p38 MAP kinase pathway. When we used the specific inhibitors, PD98059 and U0126, of the p42/44 MAP kinase pathway, we did not observe any reduction in tyrosine phosphorylation of caveolin-1 (T.-L. Lu and H.-W. Fu, unpublished observation). Therefore, the signaling pathways of tyrosine phosphorylation of caveolin-1 induced by PAR1 and by hyperosmotic stress might be quite similar. However, the detailed mechanisms remain to be clarified.

Caveolae are important in the regulation of endocytosis and signaling of GPCRs. A number of GPCRs shuttle inside and outside of caveolae upon agonist binding. Some of them are localized in the caveolae independent of agonist binding. For example, endothelin receptor subtype A is mainly in caveolae and enters cells via caveolae [17]. β2-adrenergic receptor is also localized in caveolae but leaves after agonist binding and then is internalized via clathrin-coated pits [52,53]. In our study, PAR1 was partially localized in caveolae and associated with caveolin-1 in the absence of an agonist, indicating that localization of PAR1 in caveolae was ligand-independent. In addition, caveolae were not involved in the internalization of PAR1. Thus, the localization of PAR1 in caveolae should be important in the regulation of PAR1 signaling. Our study showed that Gi protein and Src kinase were all upstream signaling molecules that mediated PAR1-induced phosphorylation of caveolin-1. Both of them were reported to be associated with caveolin-1 and localized in caveolae [23,54]. Therefore, caveolae might serve as compartments for recruiting components involved in PAR1-mediated signaling pathways to support the efficient and rapid coupling of receptors to the system with more than one effector.

Since proteolytic activation of PAR1 is irreversible, the mechanisms that contribute to the termination of receptor signaling are critical in determining the magnitude and duration of thrombin-stimulated signaling in cells. Lysosomal degradation and desensitization of the receptor are the two well-established mechanisms for terminating the signaling of PAR1 at the level of receptors. In our study, new evidence is presented to support that phosphocaveolin-1 could facilitate the termination of PAR1 signaling at the level of its downstream molecules.

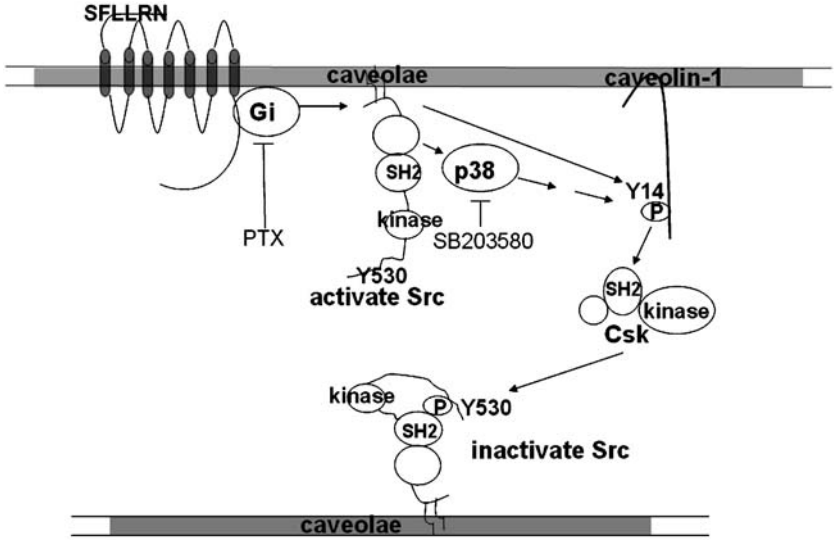

Fig. 8. Proposed signal transduction mechanism underlying PAR1-induced phosphorylation of caveolin-1 and feedback inhibition of Src kinase by the association of phosphocaveolin-1 and Csk. Activation of PAR1 by SFLLRN leads to phosphorylation of caveolin-1 on tyrosine 14 (pY14). The phosphorylation is mediated by PTX-sensitive Gi protein-linked Src kinase. p38 MAP kinase, which acts downstream of Src, is also involved. Phosphorylated caveolin-1 in turn leads to the recruitment of Csk through its SH2 domain to phosphorylate tyrosine 530 (pY530) of Src and attenuate PAR1-induced Src activation. These signaling events might occur in caveolae.

In response to PAR1 activation, phosphocaveolin-1 recruits Csk to downregulate Src kinase activity. The pathways established are summarized in the scheme shown in Fig. 8. After PAR1 activation, caveolin-1 is phosphorylated on tyrosine 14 through a Gi-linked Src kinase pathway and p38 MAP kinase. The association of phosphocaveolin-1 with Csk further attenuates PAR1-induced Src kinase activity. The regulation of PAR1-induced Src kinase activity described here might serve as a paradigm for other GPCR-mediated signaling pathway.

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