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Opposing effects of β-arrestin1 and β-arrestin2 on activation and degradation of Src induced by protease-activated receptor 1

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Abstract

Protease-activated receptor 1 (PAR1), a G protein-coupled receptor for thrombin, is irreversibly proteolytically activated. β-Arrestin1 and β-arrestin2 have been reported to have different effects on signal desensitization and transduction of PAR1. In this study, we investigated whether β-arrestin1 and β-arrestin2 regulate Src-dependent activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) induced by PAR1 in HEK 293 cells. Our results show that PAR1-mediated activation of Src and ERK1/2 in HEK 293 cells was increased with overexpression of β-arrestin1 or depletion of β-arrestin2. PAR1-mediated activation of Src and ERK1/2 in HEK 293 cells was decreased or eliminated with depletion of β-arrestin1 or overexpression of β-arrestin2. Furthermore, depletion of β-arrestin2 blocked PAR1-induced degradation of Src. Thus, β-arrestin1 and β-arrestin2 have opposing roles in regulating the activation of Src induced by PAR1. β-Arrestin2 also appears to promote PAR1-induced degradation of Src. This degradation of Src provides a possible mechanism for terminating PAR1 signaling.

Keywords: G protein-coupled receptor (GPCR); Protease-activated receptor 1 (PAR1); β-Arrestin; Src; Extracellular signal-regulated kinase (ERK); Human embryonic kidney 293 cells (HEK 293 cells)

1. Introduction

Thrombin, a serine protease, plays a crucial role in blood coagulation as well as cellular response to injury, inflammation, and wound healing. Many of these cellular actions of thrombin appear to be mediated by protease-activated receptor 1 (PAR1), a G protein-coupled receptor (GPCRs) [1–4]. PAR1 has been shown to be coupled to different G proteins, including the members of Gi/Go, Gq, and G12/13 families, that trigger different signaling pathways [5–9]. Activation of PAR1 is through an unusual irreversibly proteolytic mechanism. When thrombin binds to PAR1 and cleaves it at its N-terminal extracellular domain, the new N-terminus serves as an intramolecular ligand for activation of PAR1 [1–3]. The synthetic agonist peptide, SFLLRN, corresponding to the freshly cleaved N-terminus can also activate PAR1 [1,2].

The signaling of most GPCRs is intimately controlled by a family of arrestins that include visual arrestin and nonvisual arrestins such as β-arrestin1 and β-arrestin2 [10,11]. Upon activation, GPCRs are phosphorylated by G protein-coupled receptor kinases (GRKs) on serine and threonine residues located in the third intracellular loop or the C-terminal cytoplasmic tail [12,13]. The association of a single arrestin with a GRK-phosphorylated receptor uncouples the receptor from its cognate G protein, resulting in receptor desensitization [11,14,15].

β-Arrestin1 and β-arrestin2 serve as adaptor proteins to direct desensitized receptors to clathrin-coated pits for dynamin-

Abbreviations: PAR1, protease-activated receptor 1; ERK1/2, extracellular signal-regulated kinase 1 and 2; HEK 293 cells, human embryonic kidney 293 cells; GPCRs, G protein-coupled receptors; GRKs, G protein-coupled receptor kinases; β2AR, β2-adrenergic receptor; AT1R, angiotensin II type 1 receptor; MAPKs, mitogen-activating protein kinases; JNK3, Jun N-terminal kinase 3; PTX, pertussis toxin; EGFR, epidermal growth factor receptor; PAR2, protease-activated receptor 2; CXCR1, chemokine receptor 1; PI3K, phosphatidylinositol 3-kinase.

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dependent endocytosis [16–19]. β-Arrestins have also been reported to associate with signaling molecules [20]. β-Arrestin1 binds β2-adrenergic receptor (β2AR) and Src to form signaling complexes at clathrin-coated pits [21]. β-Arrestin2 binds angiotensin II type 1 receptor (AT1R) and Src to assist the internalization of AT1R [22]. Both β-arrestins serve as scaffolds in GPCR-induced mitogen-activated protein kinase (MAPK) family signaling pathways, such as extracellular signal-regulated kinase 1/2 (ERK1/2) [23–25] and Jun N-terminal kinase 3 (JNK3) [26]. Hence, β-arrestins are believed to function as desensitizing signals of GPCRs, adaptors for receptor internalization, and scaffold proteins for additional signaling events [27–29].

Similar to most GPCRs, activated PAR1 is rapidly uncoupled from G proteins and internalized by a clathrin-, dynamin-, and phosphorylation-dependent mechanism [30–32]. Activated PAR1 is trafficked to endosome and then to lysosome for degradation with remarkable efficiency [4,33–35]. β-Arrestin1 and β-arrestin2 have been reported to play distinct roles in signal desensitization and transduction of PAR1 [36–39]. Although both β-arrestins are not required for the endocytosis of PAR1, β-arrestin1, but not β-arrestin2, is mainly responsible for desensitizing the Gq signaling of PAR1 [36]. β-Arrestin1 is also required for a rapid activation of Akt induced by PAR1 [37–39]. However, the role of β-arrestin2 in PAR1 signaling is still unclear. In this study, we examined whether β-arrestin1 and β-arrestin2 could associate with PAR1 to form signaling complexes and investigated the respective roles of β-arrestin1 and β-arrestin2 in PAR1-mediated Src and ERK1/2 signaling pathways.

2. Materials

2.1. Materials

Synthetic peptide SFLLRN was synthesized by SynPep (Dublin, CA). Chloroquine, poly-L-lysine (molecular weight: 70,000–150,000), and anti-FLAG M2 affinity gel were purchased from Sigma (St. Louis, MO). The primary antibodies used in this study are anti-FLAG M2 monoclonal antibody from Sigma; ERK2 (D-2) and p-ERK (E-4) antibodies from Santa Cruz (Santa Cruz, CA); anti-Src antibody (clone GD11) from UpState (Charlottesville, VA); phospho-Src (Tyr416) antibody, which recognizes phosphorylated tyrosine 419 on human Src, and phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody from Cell Signaling (Beverly, MA); anti-arrestin (Pan) antibody from Affinity BioReagents (Golden, CO); and tubulin-α Ab-2 antibody (clone DM1A) from NeoMarker (Fremont, CA). The secondary antibodies are peroxidase-conjugated affinipure goat anti-mouse IgG (H+L), peroxidase-conjugated affinipure goat anti-rabbit IgG (H+L), fluorescein (FITC)-conjugated affinipure goat anti-mouse IgG (H+L), and rhodamine (TRITC)-conjugated affinipure goat anti-rabbit IgG (H+L) all from Jackson ImmunoResearch (West Grove, PA). Inhibitors such as PP1, PD98059, U0126, GF109203, pertussis toxin, wortmannin, and AG1478 were purchased from CalBiochem (San Diego, CA).

2.2. Cell culture, DNA transfection, and siRNA transfection

A PAR1 cDNA containing prolactin signal sequence followed by a FLAG epitope sequence (DYKDDDD) and a plasmid encoding a hygromycin resistance gene were co-transfected into human embryonic kidney 293 (HEK 293) cells [40]. Stable transfectants were selected in 250 μg/mL hygromycin and screened by surface expression of N-terminal FLAG-tagged PAR1 [31]. The cells with stable expression of FLAG-tagged PAR1 were cultured in minimum essential medium (MEM)/Earle's salts supplemented with 10% fetal bovine serum, 100 unit/mL penicillin and 100 μg/mL streptomycin under humidified atmosphere with 5% CO_2 at 37 °C.

DNA transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 2 μg/mL of cDNAs. Transfection of siRNAs was carried out using Lipofectamine 2000, and cells were transfected with 100 nM of siRNA twice in approximately 24-h intervals. The transfection procedures were both according to the manufacturer's instructions (Invitrogen). cDNAs encoding bovine arrestin2 and arrestin3, also known as β-arrestin1 and β-arrestin2, in the mammalian expression vector, pcDNA3.1(+), were generously provided by Dr. J.L. Benovic at Thomas Jefferson University, Philadelphia, PA. Double-stranded siRNA with 19-nucleotide duplex RNA and 2-nucleotide 3′ dTdT overhangs were purchased from Darmacon (Lafayette, CO). The siRNA sequences targeting to β-arrestin1 and β-arrestin2 are 5′-AAAGCCUUCUGCGCGGA-GAAU-3′ and 5′-AAGGACCGCAAAGUGUUUGUG-3′ [41]. A non-silencing RNA duplex, 5′-AAUUCUCCGAACGUGUCACGU-3′, was used as a control [41].

2.3. Treatment of inhibitors

To determine the dependence of Src on PAR1-induced phosphorylation of ERK1/2, HEK 293 cells stably expressing FLAG-tagged PAR1 were pretreated with the following inhibitors before the SFLLRN stimulation. PP1 is a specific inhibitor of Src. PD98095 and U0126, inhibitors of MEK family and MEK1/2, were used to block kinase activity of MEK1/2 to inhibit phosphorylation of ERK1/2. GF109203 is the inhibitor of protein kinase C, a downstream kinase activated by Gq. Pertussis toxin (PTX) catalyzes ADP-ribosylation of guanine nucleotide-binding regulatory proteins Gi/Go to block the activation of Gi/Go. Wortmannin is an irreversible inhibitor of phosphatidylinositol 3-kinase (PI3K) which is activated by $G_{\beta\gamma}$. AG1478 is an inhibitor of epidermal growth factor receptor (EGFR). The inhibitors used for pretreating cells for 90 min were 10 μM PP1, 15 μM PD98059, 8 μM U0126, and 2 μM AG1478. The inhibitors used for pretreating cells for 15 min were 1 μM GF109203 and 1 μM wortmannin. PTX, with the concentration of 1 μg/mL, was used for pretreating cells for 20 h.

2.4. Immunoprecipitation and Western blotting

HEK 293 cells stably expressing FLAG-tagged PAR1 were serum-starved overnight and then stimulated with 100 μM SFLLRN for 5 min. Cells were then lysed in lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.2 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF. Cell lysates were incubated with anti-FLAG M2 affinity gel at 4 °C for 4 h. The gel was washed with lysis buffer to eliminate non-specific binding proteins and incubated with 150 ng/μL 3× FLAG peptide (Sigma) in TBS at 4 °C for 30 min to elute the bound proteins. The proteins of interest in the eluted samples or cell lysates were detected by Western blotting.

Whole cell lysates or immunoprecipitated samples were denatured in 2× SDS sample buffer by heating at 65 °C for 10 min or at 95 °C for 5 min depending on the samples, separated on 9% SDS-PAGE, and then transferred onto PVDF membrane. Chemiluminescent detections were performed using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). For analyzing proteins in whole cell lysates, the membranes were first probed for Src or phospho-ERK1/2 and then stripped with strip buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% SDS, and 0.7% mercaptoethanol at 55 °C for 20 min. The stripped membranes were re-probed for tubulin or ERK2 as controls for equal loading.

2.5. Confocal microscopy

HEK 293 cells stably expressing FLAG-tagged PAR1 were grown on poly-L-lysine-coated coverslips for 24 h and serum-starved for 1 h. For visualization of the cellular trafficking of FLAG-tagged PAR1, cells were incubated with 0.8 μg/mL M2 anti-FLAG antibody at 4 °C for 1 h and then

exposed to 100 μM SFLLRN at 37 °C for indicated periods of time. Cells were kept on ice in the following procedures to stop internalization of PAR1 and to reduce degradation and de-phosphorylation of cellular proteins. Cells were fixed with 4% PFA in PBS for 15 min and were permeabilized and quenched with PBS containing 0.5% Triton X-100, 150 mM sodium acetate, and 1% bovine serum albumin for 20 min. For analyzing the co-localization of PAR1 and the proteins of interest, cells were then incubated with anti-arrestin antibody, anti-phospho-Src (Tyr416) antibody, or anti-phospho-ERK1/2 antibody in 1:200 dilutions at 4 °C for 2 h. After incubation with secondary antibodies, cells were washed with ice-cold PBS and mounted with prolong antifade mounting kit (Molecular Probe, Eugene, OR). Images of cells were collected using a Zeiss Axioskop2 LSM510 confocal microscopy fitted with a Plan-Neofluar 100×/N.A. 1.30 objective.

2.6. Statistical analysis

Intensities of the protein bands detected by Western blotting were quantified with the use of Science Lab 2003 Multi Gauge Ver. 2.2. (Fujifilm) and those of unstimulated samples were defined as basal. The quantitative results are expressed in fold increase over basal and represented as the mean ± S.D. of three repetitions. Statistical significance compared with control was determined by paired *t*-test.

3. Results

3.1. Co-localization of internalized PAR1 and β-arrestins

To determine whether β-arrestins are associated with PAR1 and necessary for PAR1 endocytosis, distributions of PAR1 and β-arrestins in HEK 293 cells were detected by confocal microscopy. In these experiments, HEK 293 cells stably expressing FLAG-tagged PAR1 were transfected with both siRNAs of β-arrestin1 and β-arrestin2 to knock down the expression of β-arrestins. Non-silencing siRNA was used as a

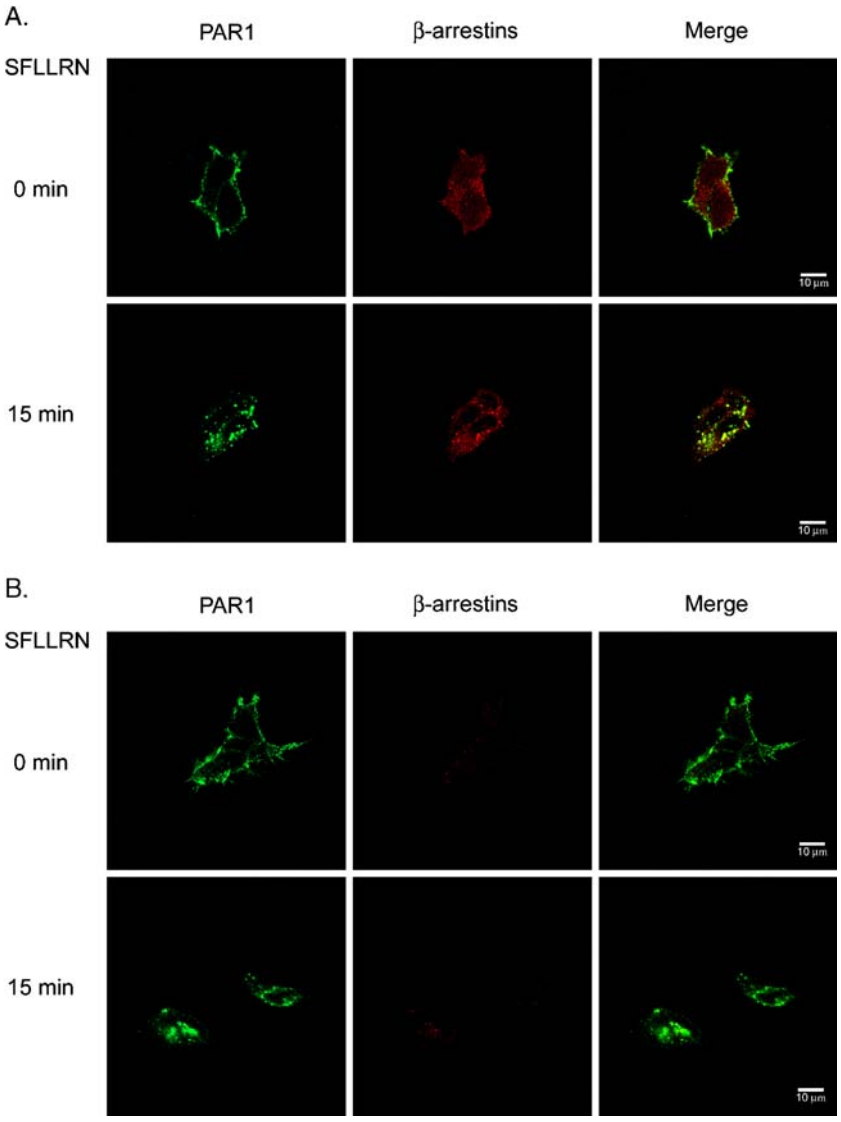

Fig. 1. Cellular trafficking of PAR1 and β-arrestins. HEK 293 cells stably expressing FLAG-tagged PAR1 were either transfected with non-silencing siRNA as a control (A) or with both siRNAs of β-arrestin1 and β-arrestin2 (B). Seventy-two hours after transfection, FLAG-tagged PAR1 (green) on the cell surface was labeled by M2 anti-FLAG antibody. Cells were then incubated in the absence or presence of 100 μM SFLLRN for 15 min at 37 °C and then fixed, permeabilized, and immunostained with anti-arrestin antibody to detect endogenous β-arrestins (red). Co-localization of FLAG-tagged PAR1 and β-arrestins was represented as yellow in the merge images. The images were collected by confocal microscopy. Similar results were observed in two separate experiments.

control. FLAG-tagged PAR1 on the cell surface was labeled with M2 anti-FLAG antibody to track the internalization of PAR1. PAR1 was located at the cell surface in both knockdown and control cells before SFLLRN agonist stimulation (Fig. 1A and B, upper panels). After 15 min of stimulation, PAR1 was internalized into cells as the green puncta (Fig. 1A and B, lower panels). In control cells, β-arrestins were co-localized with PAR1 after stimulation (Fig. 1A, lower panel). These results indicate that β-arrestins are associated and trafficked with PAR1 into endocytic vesicles after SFLLRN stimulation. Our result in this knockdown experiment shows that β-arrestins are not required for the endocytosis of PAR1 in HEK 293 cells, which is consistent with the previous observation [36].

3.2. PAR1-induced activation and degradation of Src

Our finding that β-arrestins are associated with activated PAR1 suggests the possible involvement of β-arrestins in PAR1-induced signaling pathways. Before investigating the signaling events of β-arrestins under the stimulation of PAR1, we examined the ability of PAR1 to activate Src and ERK1/2 in HEK 293 cells. After 5 to 15 min of SFLLRN stimulation, the

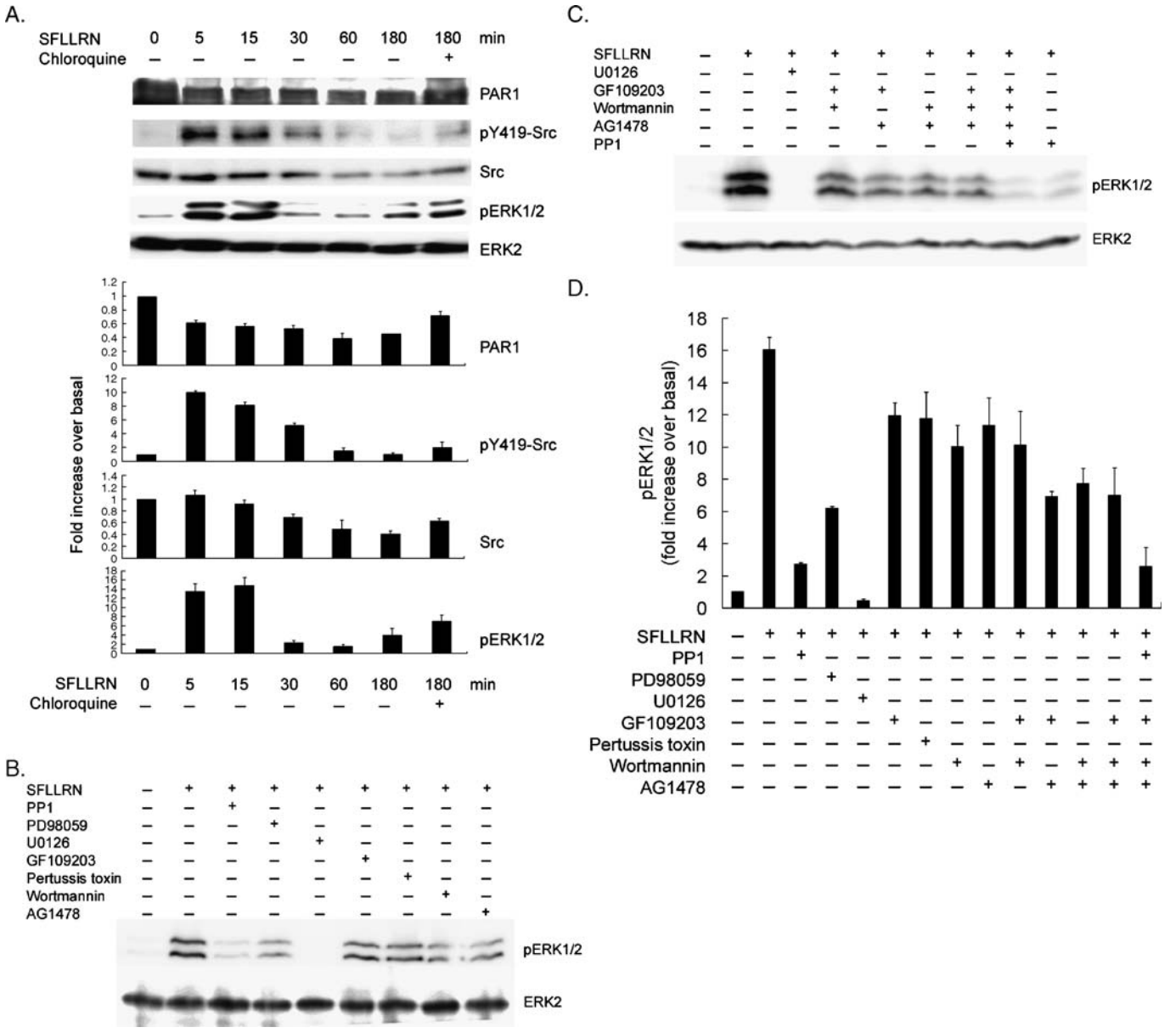

Fig. 2. Signal transduction of PAR1 in HEK 293 cells. HEK 293 cells stably expressing FLAG-tagged PAR1 were serum-starved overnight and then were pretreated with indicated drugs and stimulated with 100 μM SFLLRN at 37 °C. (A) Cells were not stimulated or stimulated with SFLLRN for 5, 15, 30, 60, or 180 min (5, 15, 30, 60, or 180) or were pretreated with 100 μM chloroquine for 1 h and then stimulated with SFLLRN for 180 min. Western blots were probed for FLAG-tagged PAR1, phospho-Tyr419-Src (pY419-Src), Src, phospho-ERK1/2 (pERK1/2), and ERK2. The quantitative results are expressed in fold increase over basal and represented as the mean ± S.D. of three repetitions. (B and C) Cells were not stimulated or stimulated with SFLLRN for 5 min or were pretreated with indicated inhibitors described under Materials and then stimulated with SFLLRN for 5 min. Western blots were probed for phospho-ERK1/2 and ERK2. (D) The amount of phospho-ERK1/2 (with regard to B and C) was quantified and the results are expressed in fold increase over basal and represented as the mean ± S.D. of three repetitions.

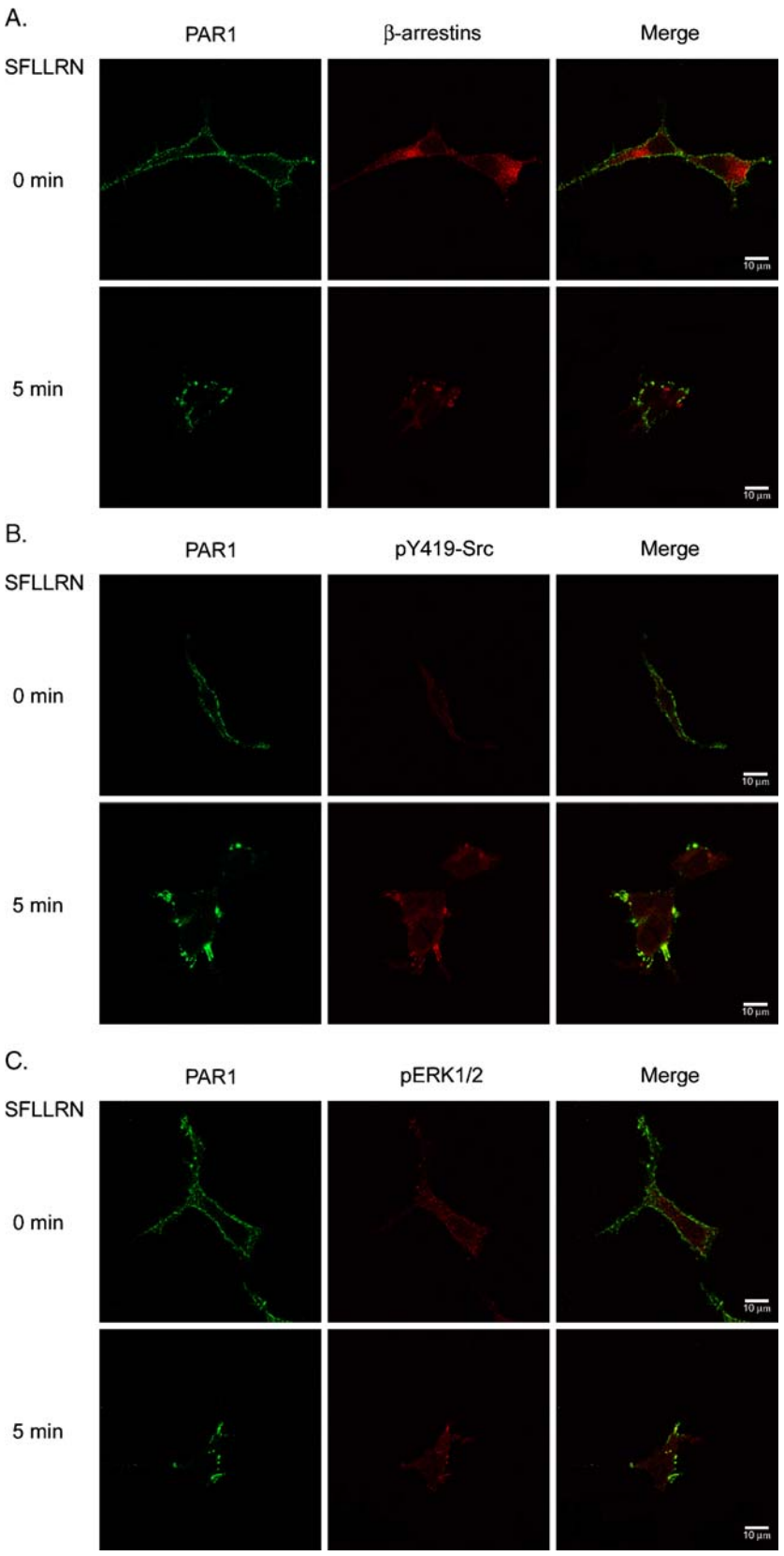

Fig. 3. Co-localization of PAR1 and β-arrestins, phospho-Tyr419-Src, or phospho-ERK1/2. HEK 293 cells stably expressing FLAG-tagged PAR1 were pre-incubated with M2 anti-FLAG antibody to label FLAG-tagged PAR1 (green) on the cell surface. Cells were then incubated in the absence or presence of 100 μM SFLLRN at 37 °C for 5 min and then fixed, permeabilized, and immunostained with anti-arrestin antibody (A), anti-phospho-Tyr419-Src antibody (B), or anti-phospho-ERK1/2 antibody (C). β-Arrestins, phospho-Tyr419-Src (pY419-Src), and phospho-ERK1/2 (pERK1/2) were represented as red, and protein co-localization was represented as yellow in the merge images. The images were collected by confocal microscopy. Similar results were observed in two separate experiments.

phosphorylation of Src and ERK1/2 was increased (Fig. 2A). Following the stimulation, PAR1 underwent lysosomal degradation, which was blocked by a lysosome inhibitor, chloroquine (Fig. 2A). More than 40% of the amount of Src was degraded after 60 min of SFLLRN stimulation while chloroquine also partially blocked this degradation of Src (Fig. 2A). These results indicate that PAR1 induces activation of Src and ERK1/2. PAR1 also appears to down regulate the activation of Src by promoting degradation of Src in HEK 293 cells.

3.3. Dependence of Src on PAR1-induced phosphorylation of ERK1/2

PAR1-mediated phosphorylation of ERK1/2 has been reported to be regulated by Src-dependent pathway in fibroblasts [42]. Our results show that, in HEK 293 cells, PAR1 induces transient activation of Src and ERK1/2. HEK 293 cells were pretreated with various inhibitors to examine whether PAR1-induced phosphorylation of ERK1/2 is regulated through Src or through PAR1-coupled G protein-mediated signaling pathways. After pretreatment with PP1, a specific inhibitor of Src, most of SFLLRN-induced phosphorylation of ERK1/2 was abolished (Fig. 2B and D). Such inhibition effect of PP1 was comparable to those of PD98059 and U0126, inhibitors of MEK1/2 which phosphorylates ERK1/2 (Fig. 2B and D). These results confirm that PAR1-induced phosphorylation of ERK1/2 is mediated by Src-dependent signaling pathway in HEK 293 cells. Four inhibitors, pertussis toxin (PTX), GF109203, wortmannin, and AG1478, were used to determine the involvement of G protein- and transactivated epidermal growth factor receptor (EGFR)-mediated signaling pathways in PAR1-induced phosphorylation of ERK1/2. Pretreatment of HEK 293 cells with these inhibitors individually partially decreased the phosphorylation of ERK1/2 (Fig. 2B and D). To rule out the involvement of PAR1-activated G proteins and EGFR pathways upstream of Src on ERK1/2 phosphorylation, multi-inhibitor cocktails were added to HEK 293 cells in the next experiment. Different combinations of GF109203, wortmannin, and AG1478 did not abolish all SFLLRN-stimulated phosphorylation of ERK1/2 (Fig. 2C and D). However, in cells pretreated with PP1 with the three inhibitors combined or pretreated with PP1 alone, SFLLRN-stimulated phosphorylation of ERK1/2 was almost completely blocked (Fig. 2C and D). Thus, blocking the activity of Src by PP1 alone successfully inhibited PAR1-induced phosphorylation of ERK1/2. These results indicate that signaling initiators other than G proteins and transactivated EGFR may be upstream of Src and involved in PAR1-induced, Src-dependent phosphorylation of ERK1/2.

3.4. Interaction of activated PAR1 with β-arrestins, Src, and ERK1/2

We hypothesized that β-arrestins may act as the signaling initiators to regulate PAR1-induced, Src-dependent phosphorylation of ERK1/2 by forming signaling complexes with PAR1. To test this hypothesis, co-localization and co-immunoprecipitation were used to examine the existence of signaling complexes of PAR1. The co-localization of PAR1 with β-arrestins, activated Src, and activated ERK1/2 was examined by confocal microscopy. Before SFLLRN stimulation, PAR1 was located at the cell surface, and β-arrestins were distributed in the cytosol (Fig. 3A; upper panels). Some phosphorylated Src and ERK1/2, not silenced by serum starvation, were distributed in the cells (Fig. 3B and C; upper panels). After 5 min of SFLLRN stimulation, phosphorylation of Src and ERK1/2 was evoked. PAR1 was observed as clusters of green puncta and was co-localized with β-arrestins, phosphorylated Src, and phosphorylated ERK1/2 at the cell membrane (Fig. 3A, B, and C; lower panels). This result indicates that β-arrestins, phosphorylated Src, and phosphorylated ERK1/2 are recruited to the activated PAR1 to form signaling complexes at the cell membrane.

To immunoprecipitate FLAG-tagged PAR1 and its associated proteins in HEK 293 cells, anti-FLAG M2 affinity gel was used. Before SFLLRN stimulation, some β-arrestins and Src were co-immunoprecipitated with PAR1 (Fig. 4). After 5 min of SFLLRN stimulation, increased amounts of β-arrestins and Src were co-immunoprecipitated with PAR1. Phosphorylated Src and phosphorylated ERK1/2 were detected in the immunoprecipitates (Fig. 4). These results show that activation of PAR1 promotes the translocation of β-arrestins, Src, and ERK1/2 from the cytosol to the cell membrane to form signaling complexes with PAR1.

3.5. β-Arrestin1- and β-arrestin2-mediated activation and degradation of Src

The finding that activated PAR1 interacts with β-arrestins, Src, and ERK1/2 indicates that β-arrestins may mediate PAR1-induced activation of Src and ERK1/2. To test this hypothesis, overexpression of β-arrestin1 and β-arrestin2 in HEK 293 cells

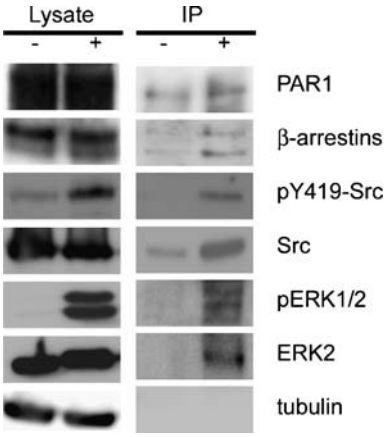

Fig. 4. Co-immunoprecipitation of proteins in PAR1-containing signaling complexes. HEK 293 cells stably expressing FLAG-tagged PAR1 were serum-starved overnight and then were not stimulated or stimulated with 100 μM SFLLRN for 5 min. FLAG-tagged PAR1 was immunoprecipitated from the whole cell lysates with M2 anti-FLAG affinity gel, and proteins in the cell lysates (lysate) and immunoprecipitates (IP) were detected by Western blotting using M2 anti-FLAG antibody for PAR1 (PAR1) and anti-β-arrestins, phospho-Tyr419-Src (pY419-Src), anti-Src, phospho-ERK1/2 (pERK1/2), anti-ERK2, and anti-tubulin antibodies. The experiments were repeated twice with similar results.

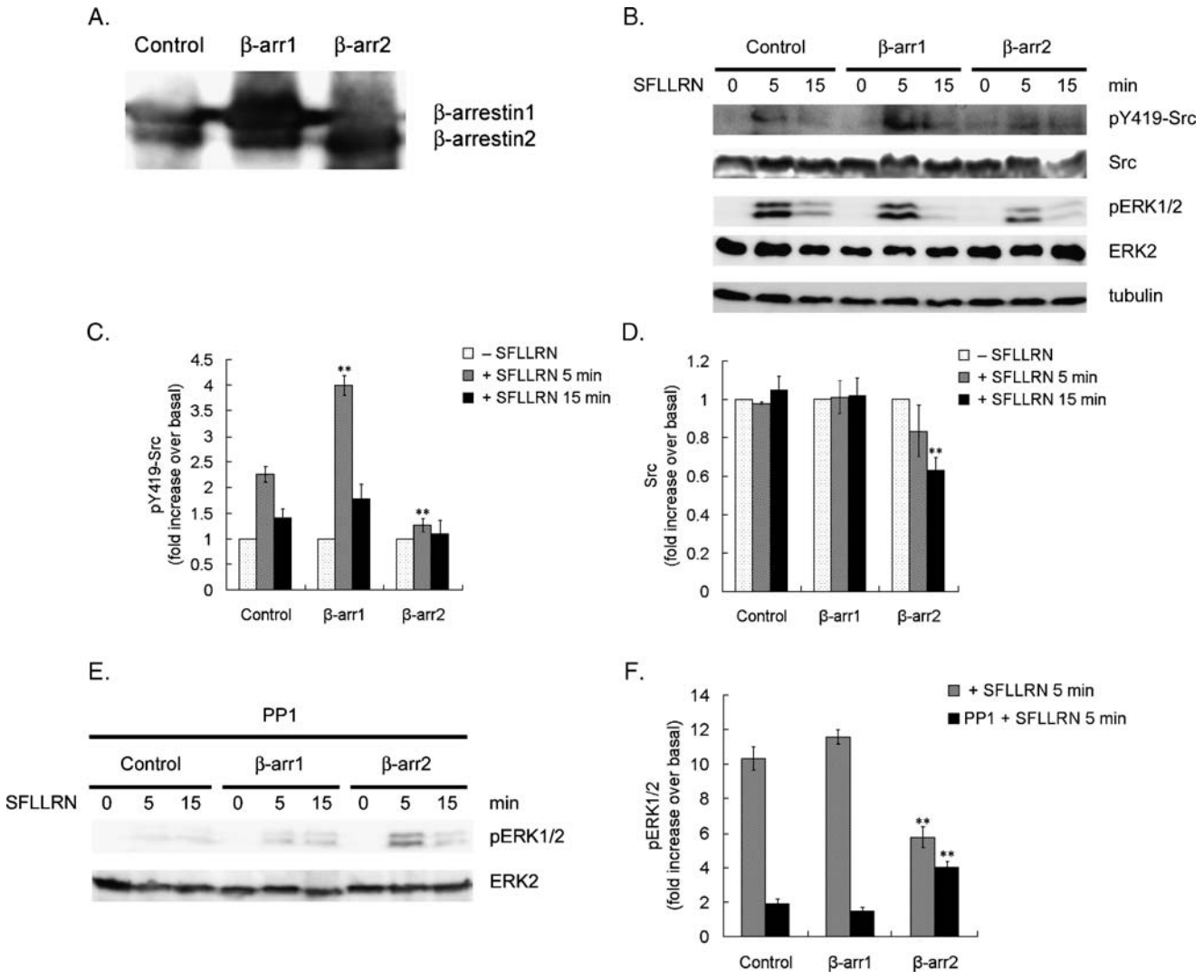

Fig. 5. Effects of overexpression of β-arrestins in PAR1-mediated phosphorylation of Src and ERK1/2. HEK 293 cells stably expressing FLAG-tagged PAR1 were transfected with β-arrestin1 (β-arr1), β-arrestin2 (β-arr2), or pcDNA3.1(+) (control), and cellular extracts were examined for the overexpression of β-arrestins by Western blotting (A). Sixty hours after transfection, cells were serum-starved overnight and then stimulated with 100 μM SFLLRN at 37 °C for 5 or 15 min (B). After stimulation, cellular extracts were analyzed by Western blotting and the blots were probed for phospho-Tyr419-Src (pY419-Src), Src, phospho-ERK1/2 (pERK1/2), ERK2, and tubulin. The amounts of phospho-Tyr419-Src (C) and Src (D) were quantified and the results are expressed in fold increase over basal and represented as the mean ± S.D. of three repetitions (**$P<0.005$ vs. control). Sixty hours after transfection, cells were serum-starved overnight and pretreated with 10 μM PP1 for 90 min and then stimulated with 100 μM SFLLRN at 37 °C for 5 or 15 min (E). After stimulation, cellular extracts were analyzed by Western blotting and the blots were probed for phospho-ERK1/2 (pERK1/2) and ERK2. The amount of phospho-ERK1/2 (F, with regard to B and E) was quantified and the results are expressed in fold increase over basal and represented as the mean ± S.D. of three repetitions (**$P<0.005$ vs. control).

stably expressing PAR1 were used to investigate the function of β-arrestins in PAR1-mediated Src activation. As shown in Fig. 5A, the expression of β-arrestin1 or β-arrestin2 was increased in cells transfected with their respective cDNAs. In control cells, SFLLRN-induced phosphorylation of Src was triggered after 5 min of stimulation, and the level of phosphorylation was reduced after 15 min of stimulation (Fig. 5B and C; control). When β-arrestin1 was overexpressed in cells, SFLLRN-induced phosphorylation of Src was hyperactively triggered after 5 min of stimulation (Fig. 5B and C; β-arr1). However, when β-arrestin2 was overexpressed in cells, about 40% of the amount of Src was degraded after 15 min of SFLLRN stimulation, and SFLLRN-induced phosphorylation of ERK1/2 was inhibited to about half of that in control cells (Fig. 5B and D; β-arr2). Thus, the rate of degradation of Src is faster in cells overexpressing β-arrestin2 than in control cells or in cells overexpressing β-arrestin1.

To further determine whether β-arrestins mediate Src-dependent, PAR1-induced phosphorylation of ERK1/2, Src inhibitor, PP1, was used to block Src-mediated phosphorylation of ERK1/2 in HEK 293 cells. When β-arrestin1 was overexpressed, PP1 efficiently blocked SFLLRN-induced phosphorylation of ERK1/2 (Fig. 5E and F; β-arr1). However, when β-arrestin2 was overexpressed, PP1 did not block SFLLRN-induced phosphorylation of ERK1/2 (Fig. 5E and F; β-arr2). This result indicates that after the stimulation of PAR1, β-arrestin1 may mediate Src-dependent phosphorylation of ERK1/2. In contrast, β-arrestin2 may provide additional Src-

independent phosphorylation of ERK1/2 in HEK 293 cells. These results suggest that β-arrestin1 promotes PAR1-induced activation of Src, and β-arrestin2 accelerates PAR1-induced degradation of Src.

Knockdown of β-arrestin1 and β-arrestin2 was also used to confirm these different effects of the two β-arrestin isoforms in HEK 293 cells. As shown in Fig. 6A, the expression of β-arrestin1 or β-arrestin2 was abolished in cells transfected with their respective siRNAs. In control cells, which were transfected with non-silencing siRNA, SFLLRN-induced phosphorylation of Src was triggered after 5 min of stimulation and reduced after 15 min of stimulation (Fig. 6B, and C; control). SFLLRN-induced phosphorylation of ERK1/2 was initiated after 5 min of stimulation and extended to 15 min (Fig. 6B and D; control). When β-arrestin1 was knocked down and β-arrestin2 remained in cells, the basal level of phosphorylated Src and ERK1/2 was raised, and SFLLRN-induced phosphorylation of Src was inhibited (Fig. 6B and C; β-arr1$^-$). SFLLRN-induced phosphorylation of ERK1/2 in β-arrestin1-knockdown cells was less than that in control cells (Fig. 6B and D; β-arr1$^-$). When β-

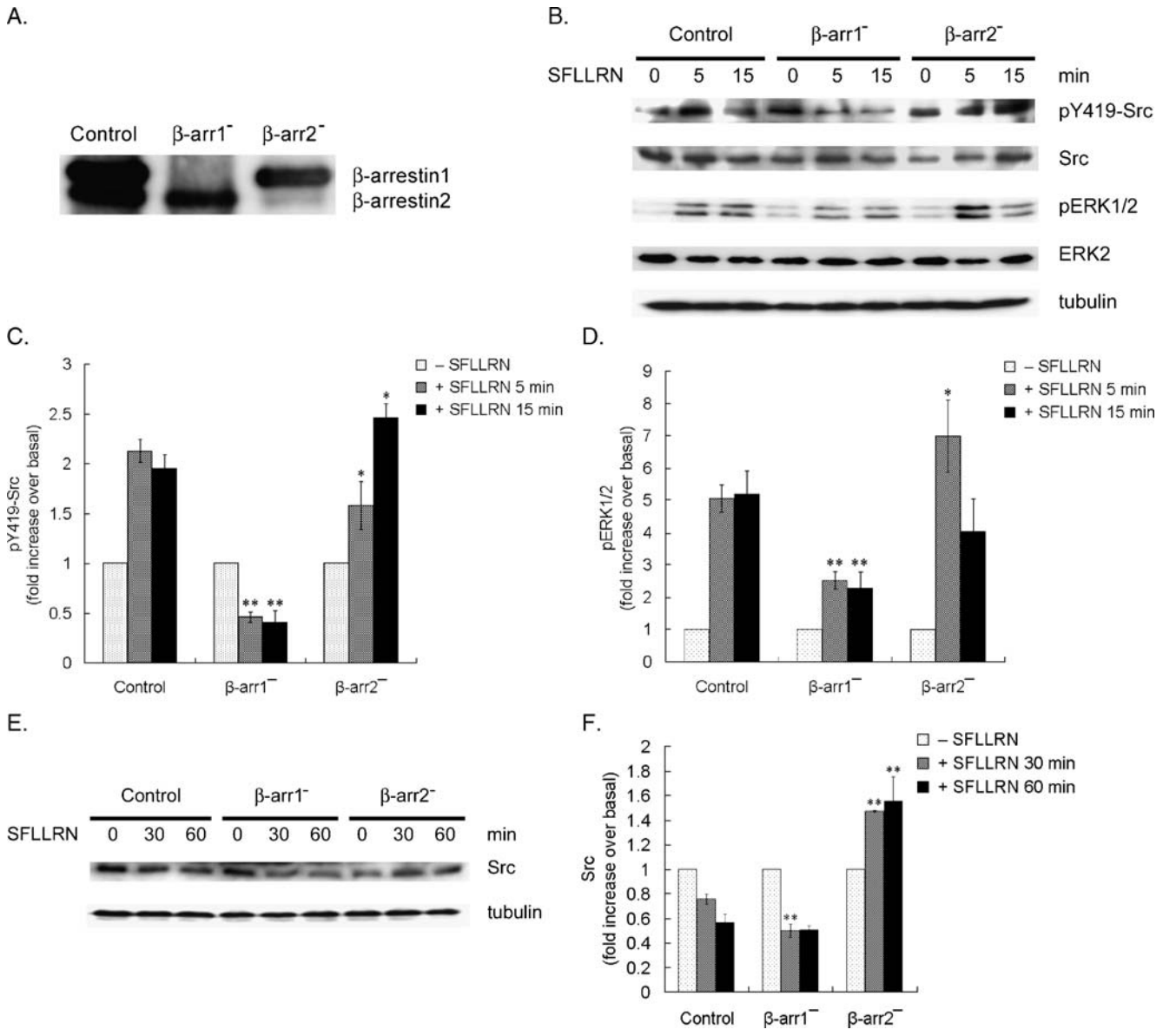

Fig. 6. Effects of knockdown of β-arrestins in PAR1-mediated phosphorylation of Src and ERK1/2. HEK 293 cells stably expressing FLAG-tagged PAR1 were transfected with non-silencing siRNA (control) or siRNAs of β-arrestin1 (β-arr1$^-$) or β-arrestin2 (β-arr2$^-$), and cellular extracts were examined for the expression of β-arrestins by Western blotting (A). Sixty hours after transfection, cells were serum-starved overnight and then stimulated with 100 μM SFLLRN at 37 °C for 5 or 15 min (B). After stimulation, cellular extracts were analyzed by Western blotting and the blots were probed for phospho-Tyr419-Src (pY419-Src), Src, phospho-ERK1/2 (pERK1/2), ERK2, and tubulin. The amounts of phospho-Tyr419-Src (C) and phospho-ERK1/2 (D) were quantified and the results are expressed in fold increase over basal and represented as the mean ± S.D. of three repetitions (*P < 0.05 or **P < 0.005 vs. control). Sixty hours after transfection, cells were serum-starved overnight and then stimulated with 100 μM SFLLRN at 37 °C for 30 or 60 min (E). After stimulation, cellular extracts were analyzed by Western blotting and the blots were probed for Src and tubulin. The amount of Src (F) was quantified and the results are expressed in fold increase over basal and represented as the mean ± S.D. of three repetitions (**P < 0.005 vs. control).

arrestin2 was knocked down and β-arrestin1 remained in cells, the basal level of phosphorylated Src and ERK1/2 was also increased (Fig. 6B; β-arr2^{-}). SFLLRN-induced phosphorylation of Src was triggered after 5 min of stimulation and further increased after 15 min of stimulation (Fig. 6B and C; β-arr2^{-}). SFLLRN-induced phosphorylation of ERK1/2 in β-arrestin2-knockdown cells was evoked at 5 min of stimulation, which was more than that in control cells (Fig. 6B and D; β-arr2^{-}). However, the phosphorylation of ERK1/2 was reduced after 15 min of stimulation (Fig. 6B and D; β-arr2^{-}). These results indicate that when only β-arrestin1 remained in cells, SFLLRN-induced phosphorylation of Src was increased and its kinase activity was enhanced to activate ERK1/2. When only β-arrestin2 remained in cells, SFLLRN-induced phosphorylation of Src was inhibited, and SFLLRN-induced phosphorylation of ERK1/2 was also blocked.

Since overexpression of β-arrestin2 accelerated PAR1-induced degradation of Src, we then further investigated the effect of β-arrestin2 on degradation of Src after long-term stimulation. In control cells, which were transfected with non-silencing siRNA, the amount of Src gradually decreased at 30 and 60 min after SFLLRN stimulation (Fig. 6E and F; control). When β-arrestin1 was knocked down and β-arrestin2 remained in cells, the amount of Src was also decreased after SFLLRN stimulation (Fig. 6E and F; β-arr1^{-}). In contrast, when β-arrestin2 was knocked down and β-arrestin1 remained in cells, the amount of Src was further increased at 30 and 60 min after SFLLRN stimulation (Fig. 6E and F; β-arr2^{-}). Thus, β-arrestin2 is required for PAR1-induced degradation of Src. Therefore, the two β-arrestin isoforms have opposing effects in regulating PAR1-induced activation and degradation of Src as well as PAR1-induced, Src-dependent phosphorylation of ERK1/2.

4. Discussion

In this study, evidence is presented showing that β-arrestin1 and β-arrestin2 are recruited to the activated PAR1 and that β-arrestin1 and β-arrestin2 also regulate PAR1-induced activation and degradation of Src. After PAR1 activation, β-arrestin1, β-arrestin2, Src, and ERK1/2 are recruited to the receptors to form signaling complexes. Under short-term stimulation of PAR1, β-arrestin1 enhances the activation of Src, leading to the increased phosphorylation of ERK1/2, whereas β-arrestin2 inhibits the activation of Src, resulting in the reduced phosphorylation of ERK1/2. After long-term stimulation of PAR1, β-arrestin2 also appears to promote the degradation of Src. We conclude that β-arrestin1 and β-arrestin2 play opposing roles in regulating PAR1-induced activation and degradation of Src. Such distinct regulation of Src activation mediated by the two β-arrestin isoforms may occur in the signaling complexes of PAR1.

β-Arrestin1 is known to act as an initiator to activate the downstream signaling through the formation of signaling complexes with receptors, which is distinct from GPCR-mediated G protein signal transduction. A previous report showed that stimulation of β2AR can promote the formation of protein complexes containing β2AR, β-arrestin1, and Src [21]. The association of β-arrestin1 and de-phosphorylated Src (Tyr527) enhances the kinase activity of Src as well as β2AR-mediated phosphorylation of ERK1/2 [21]. Similar results have been reported that different GPCRs also recruit β-arrestin1 and signaling molecules. The association of substance P receptor with β-arrestin1 is further involved in the recruitment of Src and ERK1/2 [24]. Also, the formation of signaling complexes containing protease-activated receptor 2 (PAR2), β-arrestin1, and ERK1/2 subsequently activates ERK1/2 [23]. In this study, PAR1-induced phosphorylation of ERK1/2 in HEK 293 cells is primarily Src-dependent and G protein-independent. Stimulation of PAR1 promotes the formation of signaling complexes containing PAR1, β-arrestin1, Src, and ERK1/2. β-Arrestin1 also increases PAR1-induced phosphorylation of Src to enhance the phosphorylation of ERK1/2. Therefore, β-arrestin1, which acts as a signaling initiator, can increase the kinase activity of Src to activate PAR1-induced ERK1/2 cascade.

β-Arrestin2 also directly interacts with Src and recruits it to the activated GPCRs. For instance, activated AT1R was reported to recruit β-arrestin2 and Src. The association of β-arrestin2 and activated Src can further assist the internalization of AT1R [22]. Additionally, β-arrestin2 can directly interact with Hck, a member of the Src family, and lead to Hck activation after interleukin 8-induced activation of chemokine receptor 1 (CXCR1) [43]. Our study shows that activation of PAR1 also induces the formation of signaling complexes containing PAR1, β-arrestin2, Src, and ERK1/2. However, in contrast to β-arrestin1, β-arrestin2 does not lead to Src activation but blocks PAR1-induced activation of Src, and thus reduces the phosphorylation of ERK1/2. This finding reveals a novel function of β-arrestin2 in PAR1 signaling and provides the first example that β-arrestin2 acts as an inhibitory regulator in GPCR-induced activation of Src.

The degradation of active Src is ubiquitin-dependent [44] and is mediated by the Cbl family of ubiquitin E3 ligases [45,46]. After ubiquitination, active Src has been shown to undergo either proteosomal degradation mediated by c-Cbl or lysosomal degradation mediated by Cbl-c [46]. In this study, PAR1-induced degradation of Src can be partially rescued by chloroquine, a lysosome inhibitor. Therefore, Src may be ubiquitinated by the Cbl family and targeted to lysosome for degradation after PAR1 stimulation. We cannot yet exclude the possibility that Src is also degraded through a proteosome-mediated pathway. Our finding that β-arrestin2 promotes PAR1-induced degradation of Src raises the question of how β-arrestin2 is involved in this event. A previous study demonstrated that agonist stimulation of β2AR induces direct interaction between β-arrestin2 and Mdm2, a ubiquitin E3 ligase [47]. This effect causes the ubiquitination of β-arrestin2 and β2AR to assist the internalization and degradation of β2AR [47]. Also, activated PAR2 can interact directly with β-arrestin2 [23]. Ubiquitination and lysosomal degradation of PAR2 are mediated by Src-dependent interaction of PAR2 and c-Cbl [48]. Thus, it is possible that after PAR1 activation, β-arrestin2 might be associated with a ubiquitin E3 ligase to enhance the ubiquitination and degradation of Src. However, the exact

mechanism by which β-arrestin2 assists PAR1-induced degradation of Src remains to be investigated.

Due to the irreversibly proteolytic activation of PAR1, termination of the receptor signaling becomes very critical for temporal fidelity of thrombin signaling. PAR1 signaling can be terminated by β-arrestin1-dependent desensitization and lysosomal degradation of the receptor. Here, we show that β-arrestin2-mediated degradation of Src provides an additional mechanism for terminating PAR1 signaling. After PAR1 activation, Src activity is turned down by its degradation. β-Arrestin2 serves as a required mediator in this event. In addition, β-arrestin2 inhibits PAR1-induced activation of Src. Such effect further results in down regulation of ERK1/2 cascade. β-Arrestin1, however, initiates PAR1-induced activation of Src-dependent ERK1/2 cascade. These findings reveal the new roles of β-arrestin1 and β-arrestin2 in PAR1 signaling in terms of their opposing regulation of Src activation and might also provide new insights in the signaling regulation of other irreversibly activated PARs.

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