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Multiple Independent Functions of Arrestins in the Regulation of Protease-Activated Receptor-2 Signaling and Trafficking

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ABSTRACT

The irreversible proteolytic nature of protease-activated receptor-2 (PAR2) activation suggests that mechanism(s) responsible for termination of receptor signaling are critical determinants of the magnitude and duration of PAR2-elicited cellular responses. Rapid desensitization of activated G-protein-coupled receptors (GPCRs) involves both phosphorylation and binding of arrestins. Arrestins also function as scaffolds and transducers of mitogen-activated protein (MAP) kinase signaling cascades. The PAR2 cytoplasmic tail (C-tail) contains multiple sites of phosphorylation and may be an important determinant for arrestin interaction. Desensitization and internalization of activated PAR2 were markedly impaired in arrestin-deficient cells compared with wild-type control cells. PAR2 C-tail truncation mutants displayed normal agonist-induced internalization, caused rapid distribution of βarr2-GFP to the plasma membrane, and desensitized in an arrestin-dependent manner similar to that of wild-type PAR2. It is interesting that PAR2 C-tail mutants lost the capacity to stably associate with arrestins and consequently, redistributed to endocytic vesicles without βarr2-GFP, whereas internalized wild-type PAR2 remained stably associated with βarr2-GFP in endosomes. Moreover, activated PAR2 caused rapid and prolonged activation of endogenous extracellular signal-regulated kinase (ERK1/2). It was striking that in arrestin-deficient cells, activated PAR2 induced an initial peak in ERK1/2 activity that rapidly declined. The inability of internalized PAR2 C-tail mutants to stably associate with arrestins also resulted in loss of prolonged ERK2 activation. Thus, the PAR2 C-tail regulates the stability of arrestin interaction and kinetics of ERK1/2 activation but is not essential for desensitization or internalization. These findings further suggest that the diverse functions of arrestins in regulating PAR2 signaling and trafficking are controlled by multiple independent interactions involving both the intracellular loops and the C-tail.

Protease-activated receptor-2 (PAR2) is activated by multiple trypsin-like serine proteases, including trypsin, tryptase, and coagulation proteases factors VIIa and Xa, but not by thrombin (Coughlin and Camerer, 2003). Because of the irreversible proteolytic nature of PAR2 activation and the generation of a tethered ligand that cannot diffuse away, mechanisms that contribute to the termination of signaling are critical determinants of the magnitude and kinetics of protease-elicited cellular responses. The regulation of PAR1 signaling has been extensively studied (Trejo, 2003), whereas considerably less is known about PAR2. G-protein-coupled receptors (GPCRs) are initially desensitized by rapid phosphorylation of agonist-occupied receptors by GPCR serine/threonine kinases (GRKs) and other kinases (Krupnick and Benovic, 1998). In many cases, phosphorylation enhances receptor affinity for arrestin, and arrestin binding prevents receptor-G-protein interaction, thereby uncoupling the receptor from signaling. The cytoplasmic carboxyl tail (C-tail) of PAR2 contains multiple potential sites of phosphorylation, including 18 serine and threonine residues (Trejo, 2003), indicating that the C-tail may be a major site of phosphorylation. The function of GRKs in the termination of PAR2 signaling has not been determined; however, the presence of multiple basic amino acids surrounding serine and threonine residues, phospho-acceptor sites, and studies using pharmacological inhibitors of the second-messenger-regulated pro-

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ABBREVIATIONS: PAR, protease-activated receptor; GPCR, G-protein-coupled receptor(s); C-tail, cytoplasmic carboxyl tail; βarr, β-arrestin; KNRK, Kirsten sarcoma virus-transformed rat kidney epithelial cells; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; GRK, G-protein-coupled receptor kinase(s); MEFs, mouse embryonic fibroblasts; IP, inositol phosphate(s); PI, phosphoinositide; βarr, β-arrestin; ELISA, enzyme-linked immunosorbent assay.

tein kinase C suggest a function for this protein kinase in PAR2 desensitization (Bohm et al., 1996a). Activation of PAR2 causes rapid and transient redistribution of β-arrestin1 (βarr1) to the plasma membrane when overexpressed in Kirsten sarcoma virus-transformed rat kidney epithelial (KNRK) cells (Dery et al., 1999). It is interesting, however, that overexpression of βarr1 affected neither the magnitude nor the duration of activated PAR2 mobilization of intracellular Ca^{2+}. βArr1 expression also failed to enhance PAR2 internalization in KNRK cells, whereas $\beta arr1^{319-418}$ C-terminal fragments partially inhibited activated PAR2 internalization. Because this arrestin mutant binds constitutively to clathrin, the major structural component of the endocytic machinery, but not to the receptor, potential nonspecific actions exist. Thus, the molecular mechanism(s) responsible for the regulation of PAR2 signaling and trafficking are not clearly understood.

The nonvisual arrestins arrestin2 and 3 (also termed β-arrestin1 and 2) are ubiquitously expressed and associate with most activated GPCRs at the plasma membrane to facilitate receptor uncoupling from G-proteins and internalization through clathrin-coated pits (Kohout and Lefkowitz, 2003). Several recent studies indicate that arrestins also function as scaffolds that interact with components of the mitogen-activated protein (MAP) kinase cascade, including the extracellular-signal regulated kinases 1 and 2 (ERK1/2) (McDonald et al., 2000; Luttrell et al., 2001). The various functions of arrestins in regulating GPCR signaling and trafficking are controlled in part by the stability of arrestin interaction with activated receptors. In some cases, such as the β_2-adrenergic receptor and others (Barak et al., 1997), arrestins bind activated GPCRs to facilitate desensitization and internalization, then rapidly dissociate from the receptor at the plasma membrane. By contrast, arrestins stably associate with the activated vasopressin V2 and angiotensin II type 1A receptors and consequently internalize together with receptors into early endosomes (Oakley et al., 1999; Luttrell et al., 2001; Tohgo et al., 2003). Stable GPCR-arrestin association is important for regulating the kinetics of receptor recycling and resensitization and to initiate MAP kinase signaling pathways. Activated PAR2 also stably associates with arrestins; together, receptor and arrestins redistribute into endocytic vesicles (Dery et al., 1999), a process required for ERK1/2 activation because inhibition of PAR2 internalization virtually abolishes kinase activation (DeFea et al., 2000). The ability of arrestins to form stable complexes with activated GPCRs is dictated in part by the presence of specific clusters of serine and threonine residues precisely localized in the C-tail region (Oakley et al., 2001). The C-tail of PAR2 contains three distinct clusters of serine and threonine residues; whether the C-tail is important for receptor-arrestin interaction remains to be determined.

In the present study, we investigate the function of the PAR2 C-tail and arrestins in the regulation of receptor signaling and trafficking using C-tail truncation mutants, RNA interference, and mouse embryonic fibroblasts (MEFs) derived from β-arrestin knockout mice (Kohout et al., 2001). Our findings strongly suggest that arrestins function in the desensitization and internalization of activated PAR2 independent of the C-tail region. It is interesting, however, that the PAR2 C-tail is critical for stable arrestin association upon internalization and consequent prolonged ERK1/2 activation, but neither the C-tail nor arrestins are essential for rapid and transient activation of this protein kinase. These studies reveal that the C-tail of PAR2 control cells the stability of arrestin interaction and kinetics of ERK1/2 activation but is not essential for desensitization or internalization of activated PAR2.

Materials and Methods

Reagents and Antibodies. The PAR2-agonist peptide SLIGKV was synthesized as the carboxyl amide and purified by reversed phase high-pressure liquid chromatography (UNC Peptide Facility, Chapel Hill, NC). α-Trypsin treated with tosylamide-2-phenylethyl chloromethyl ketone was from Sigma-Aldrich (St. Louis, MO).

Monoclonal M1 anti-FLAG antibody was purchased from Sigma-Aldrich. Monoclonal anti-phospho-p44/42 MAP (ERK1/2) kinase and polyclonal anti-p44/42 MAP (ERK1/2) kinase antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). The polyclonal anti-β-arrestin antibody A1CT was generously provided by R. J. Lefkowitz (Duke University, Durham, NC). Anti-actin antibody was obtained from Sigma-Aldrich. Horseradish peroxidase conjugated goat anti-mouse and anti-rabbit secondary antibodies were purchased from Bio-Rad (Hercules, CA). Alexa-488 and Alexa-594 conjugated goat anti-mouse antibodies were from Molecular Probes (Eugene, OR).

cDNAs and Cell Lines. A cDNA encoding wild-type human PAR2 containing an amino terminal FLAG epitope was generously provided by S. Coughlin (University of California, San Francisco, CA). PAR2 C-tail truncation mutants were generated by introducing stop codons at the indicated residues by site-directed mutagenesis using QuikChange (Stratagene, La Jolla, CA), and specific mutations were confirmed by dideoxy sequencing. The plasmids encoding green fluorescence protein (GFP) fused to either βarr1 or βarr2 were kindly provided by M. Caron (Duke University). The GFP-tagged ERK2 cDNA plasmid was provided by A. Howe (University of Vermont, Burlington, VT).

HeLa and COS-7 cells were maintained as described previously (Trejo et al., 2000; Chen et al., 2004). MEFs derived from wild-type and β-arrestin knockout mice were kindly provided by R.J. Lefkowitz (Duke University). MEFs stably expressing FLAG-tagged PAR2 were generated as described previously (Paing et al., 2002).

Transient Transfection. COS-7 cells were plated at 4×10^4 cells per well in 24-well dishes (Falcon; BD Biosciences Discovery Labware, Bedford, MA), and HeLa cells were plated at 2×10^5 cells per well in 12-well dishes (Falcon), and grown overnight. Cells were transiently transfected with a total of either 0.4 μg or 0.8 μg of plasmids per well of 24-well or 12-well dishes, respectively, using LipofectAMINE Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Small Interfering RNA Transfections. HeLa cells were plated in 24-well dishes at 1×10^5 cells per well. Cells were transfected with 60 nM siRNAs using LipofectAMINE 2000 according to the manufacturer's instructions, and experiments were performed 48 h after transfections. The following siRNAs were from Dharmacon (Lafayette, CO) and used to target specific mRNA sequences: β-arrestin1 siRNA (5′- AAAGCCUUCUGCGCGGAGAAU-3′) (position 439–459 from the start ATG), β-arrestin2 siRNA (5′-AAGGACCGCAAAGUGUUUGUG-3′) (position 148–168 from the start ATG), and nonspecific siRNA (5′-GGCUACGUCCAGGAGCGCACC-3′).

Cell Surface ELISA. Transiently transfected COS-7 or HeLa cells were treated with or without agonist, fixed, and the amounts of cell surface PAR2 wild type or mutants were determined by ELISA (Paing et al., 2004). PAR2 internalization in wild-type and β-arrestin-deficient MEFs was determined as described previously (Paing et al., 2002).

Phosphoinositide Hydrolysis. COS-7 cells transiently cotransfected with FLAG-PAR2 wild type or C-tail truncation mutants and

either βarr1, βarr2, or pcDNA were labeled with 1 μCi/ml [*myo*-^{3}H]inositol (American Radiolabeled Chemicals, St. Louis, MO) and incubated in the absence or presence of agonist for various times, and [^{3}H]inositol phosphates (IPs) were measured as reported previously (Paing et al., 2002). MEFs stably expressing FLAG-tagged PAR2 wild type were plated in 24-well dishes (Falcon), treated with or without agonists, and amounts of [^{3}H]IPs formed were determined as we described previously (Paing et al., 2002).

Immunofluorescence Confocal Microscopy. MEFs and transiently transfected HeLa cells were grown on fibronectin-coated glass coverslips (22 × 22 mm), exposed to agonist, fixed, and processed for immunofluorescence microscopy as described previously (Gullapalli et al., 2004). Images were collected using a Fluoview 300 laser-scanning confocal microscope imaging system (Olympus, Tokyo, Japan), configured with an IX70 fluorescent microscope fitted with a PlanApo 60× oil objective (Olympus). The final composite images were created in Adobe Photoshop CS (Adobe Systems, Mountain View, CA).

Immunoblotting. Cell lysates were prepared as described previously (Trejo and Coughlin, 1999), and protein concentrations were determined using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Equivalent amounts of cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred, and the membranes were immunoblotted with A1CT rabbit polyclonal anti-β-arrestin antibody or monoclonal anti-phospho-p44/42 MAP (ERK1/2) kinase antibody. Membranes were washed, incubated with species-specific secondary antibodies-conjugated to horseradish peroxidase, washed again, developed with enhanced chemiluminescence (Amersham Biosciences Inc., Piscataway, NJ), and imaged by autoradiography. To detect total p44/42 MAP (ERK1/2) kinase or actin, membranes were reprobed with rabbit polyclonal anti-p44/42 MAP (ERK1/2) kinase antibody or anti-actin antibody.

Results

Toward understanding the regulation of PAR2 signaling and trafficking, we first determined whether receptor internalization is dependent on arrestins by examining agonist-induced loss of surface receptor in MEFs derived from β-arrestin knockout mice (Kohout et al., 2001). A comparable amount of surface FLAG-tagged PAR2 was detected in stably transfected wild-type and arrestin-null MEFs (Fig. 1A). In wild-type MEFs expressing both isoforms of β-arrestins, ~40% of PAR2 was internalized from the cell surface after 60 min of exposure to agonist-peptide SLIGKV (Fig. 1A), consistent with the extent of PAR2 internalization observed in other cell types (Dery et al., 1999). By contrast, in cells that lacked expression of both β-arrestin isoforms, agonist-induced PAR2 internalization was completely abolished (Fig. 1A). Internalization of activated PAR2 assessed by immunofluorescence confocal microscopy is consistent with an arrestin-dependent pathway for receptor internalization. In wild-type MEFs, a significant redistribution of PAR2 from the plasma membrane to endocytic vesicles was detected after 30 min of SLIGKV exposure (Fig. 1B), whereas activated PAR2 failed to internalize in MEFs lacking arrestins (Fig. 1B). Similar findings were observed in other independent clones of wild-type and arrestin-deficient MEFs (data not shown). Together, these findings strongly suggest that arrestins are essential for agonist-stimulated PAR2 internalization.

We next evaluated the function of arrestins in the regulation of PAR2 signaling by comparing agonist-induced phosphoinositide (PI) hydrolysis in MEFs lacking arrestin expression to wild-type control cells. Activated PAR2 stimulates PI hydrolysis in multiple cell types (Bohm et al., 1996b; Seatter et al., 2004), an effect attributed to $G\alpha_q$ coupling to phospholipase C-β. Cells expressing similar amounts of PAR2 labeled with [*myo*-^{3}H]inositol were incubated in the absence or presence of trypsin for various times, and the accumulation of [^{3}H]IPs was measured. The initial rate of trypsin-induced PI hydrolysis was similar in both wild-type and βarr1,2-deficient MEFs (Fig. 2), suggesting that PAR2 initial coupling to G-protein signaling is comparable in both cell types. After 30 min of agonist exposure, a ~7-fold increase in PI hydrolysis was detected in wild-type MEFs (Fig. 2). However, cells that lacked both isoforms of β-arrestin had a marked ~19-fold increase in [^{3}H]IPs after 30 min of agonist exposure (Fig. 2), suggesting that arrestins are essential for efficient receptor uncoupling from G-protein signaling. Activation of PAR2 with SLIGKV also induced greater signaling in βarr1,2-null cells compared with wild-type control cells (data not shown). Thus, in the absence of arrestins, rapid termination of PAR2 signaling is markedly impaired.

The binding of arrestins to activated GPCRs involves multiple interactions with the second and/or third intracellular loops and C-tail (Gurevich and Gurevich, 2004). The molecular determinants responsible for arrestin interaction with activated PAR2 are not known. To determine whether sequences in the PAR2 C-tail region are important for arrestin association, we examined a series of C-tail truncation mu-

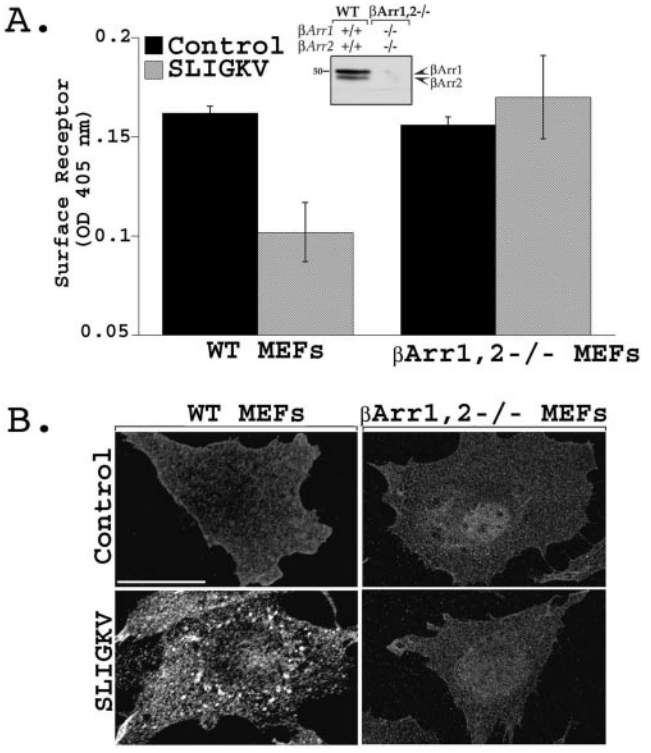

Fig. 1. β-arrestins are essential for agonist-induced PAR2 internalization. A, wild-type (WT) and β-arrestin-deficient MEFs stably expressing FLAG-tagged PAR2 were incubated with or without 100 μM SLIGKV for 60 min at 37°C. Cells were fixed, and the amount of PAR2 remaining on the cell surface was measured by ELISA and used as an index for receptor internalization. The data (mean ± S.D.; $n = 3$) are representative of three different experiments. The inset confirms β-arrestin expression in wild-type cells and loss of expression in βarr1,2-null cells. B, MEFs stably expressing FLAG-tagged PAR2 were incubated in the absence of presence of 100 μM SLIGKV for 30 min at 37°C. Cells were then fixed, immunostained for PAR2, processed, and imaged by confocal microscopy. The imaged cells are representative of many cells examined in two independent experiments. Scale bar, 10 μm.

tants (Fig. 3A). We first determined whether PAR2 C-tail mutants were expressed at the cell surface to the same extent as wild-type receptor. COS-7 cells were transiently transfected with either FLAG-tagged PAR2 wild type or mutants, and receptor surface expression was assessed by ELISA. PAR2 wild type showed significant expression at the cell surface compared with vector control (Fig. 3B), whereas the expression of the severely truncated S348Z mutant was virtually undetectable. Surface expression of PAR2 C-tail truncation mutants K368Z, H379Z, and Y386Z, lacking various portions of the C-tail region, was comparable with that of the wild-type receptor, although expression of C361Z mutant was slightly diminished (Fig. 3B). A similar expression pattern of PAR2 wild type and C-tail truncation mutants was observed in transiently transfected HeLa cells (data not shown).

We next evaluated signaling responses of PAR2 wild type and mutants by assessing agonist-induced PI hydrolysis in transiently transfected COS-7 cells. Cells labeled with [*myo*-^{3}H]inositol were incubated in the absence or presence of saturating concentrations of trypsin for 30 min at 37°C, and [^{3}H]IPs were then measured. In cells expressing PAR2 wild type, the addition of agonist induced a ~7-fold increase in IP accumulation compared with untreated control cells (Fig. 3C). Activated PAR2 K368Z, H379Z, and Y386Z mutants also stimulated a robust ~10-fold increase in IP production, a response greater than that of the wild-type receptor (Fig. 3C). In contrast, a ~5-fold increase in PI hydrolysis was observed in PAR2 C361Z mutant expressing cells after 30 min of agonist exposure, consistent with the level of surface receptor expression observed in these cells (Fig. 3, B and C). Activation of wild-type and mutant PAR2 with SLIGKV elicited signaling responses comparable with those observed with trypsin (data not shown). Similar responses were observed in HeLa cells transiently expressing PAR2 wild type and C-tail mutants (data not shown). By contrast, neither trypsin nor the peptide agonist SLIGKV caused significant IP accumulation in vector-transfected control COS-7 or HeLa cells (data not shown). Together, these findings suggest that PAR2 C-tail truncation mutants retain the capacity to couple to G-protein signaling in both COS-7 and HeLa cells.

To determine whether the C-tail is essential for receptor endocytosis, we examined agonist-induced loss of surface PAR2 wild type and C-tail truncation mutants in transiently transfected cells. COS-7 cells were incubated in the absence or presence of saturating concentrations of SLIGKV for 60 min at 37°C and fixed, and the amount of PAR2 remaining on the cell surface was quantitated by ELISA and used as a measure of receptor internalization. In cells expressing wild-type PAR2, agonist induced a ~40% loss of surface receptor (Fig. 4A), similar to that reported in other cell types (Dery et al., 1999). To our surprise, agonist also triggered a ~30 to 40% decrease in surface levels of PAR2 C-tail mutants after 60 min of exposure, a response comparable with that observed with the wild-type receptor (Fig. 4A). Moreover, both PAR2 wild-type and C-tail mutant internalization was increased ~30 to 40% by agonist in transiently transfected HeLa cells (Fig. 4B), suggesting that internalization of PAR2 C-tail truncation mutants is not cell type-specific. Together, these findings suggest that the C-tail of PAR2 is not essential for agonist-induced receptor internalization.

We next used GFP-tagged βarr2 to examine the association of arrestins with PAR2 C-tail truncation mutants in transiently transfected HeLa cells. In the absence of agonist,

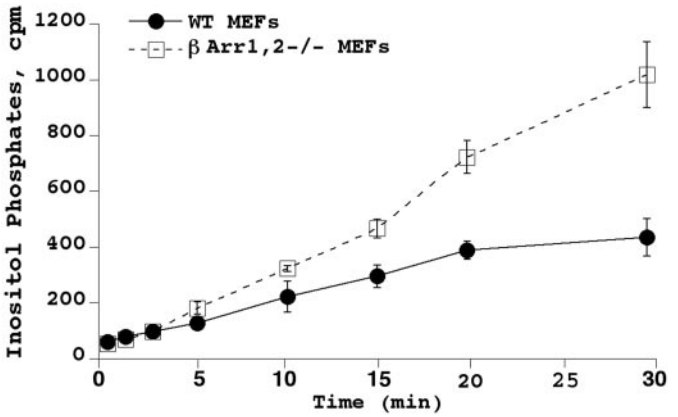

Fig. 2. PAR2 signaling to phosphoinositide hydrolysis induced by trypsin is enhanced in β-arrestin-deficient MEFs. Wild-type (WT) and β-arrestin-deficient cells were labeled with [*myo*-^{3}H]inositol and incubated in the absence or presence of 10 nM trypsin for various times at 37°C, and the amounts of [^{3}H]IPs formed were then measured. The values (mean ± S.D.; n = 3) for PAR2 surface expression in wild-type and βarr1,2−/− MEFs were 0.207 ± 0.0416 and 0.268 ± 0.066, respectively. The amount of antibody binding to untransfected MEFs was 0.05 ± 0.008 optical density units.

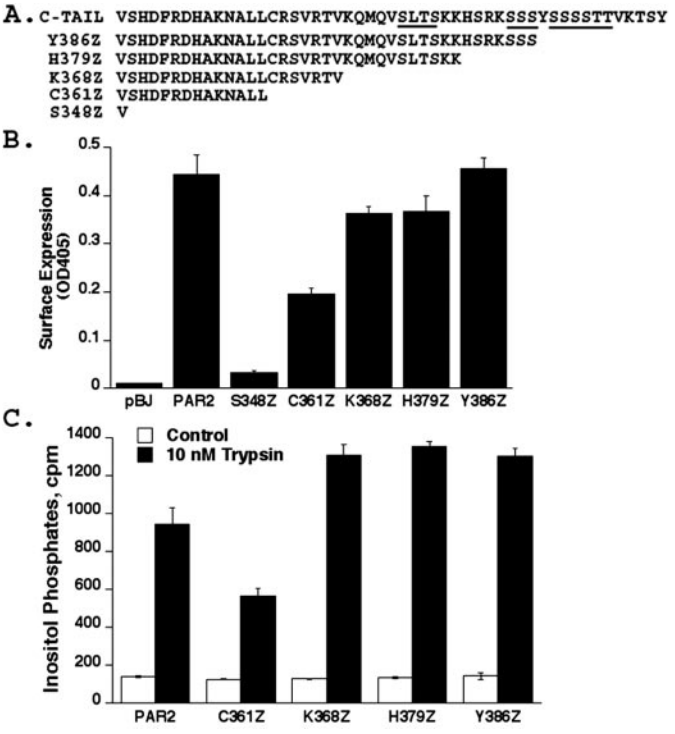

Fig. 3. Cell surface expression and signaling by PAR2 wild type and C-tail mutants. A, amino acid sequence of human PAR2 C-tail region, clusters of serines and threonine residues are shown (underlined). PAR2 C-tail truncation mutants are designated by the amino acid corresponding to the codon, which was replaced by a stop codon (Z). B, COS-7 cells transiently transfected with FLAG-tagged PAR2 wild type, C-tail truncation mutants, or pBJ vector were fixed, and the steady-state amounts of cell surface receptors were determined by ELISA. C, COS-7 cells transiently expressing PAR2 wild type and C-tail mutants were labeled with [*myo*-^{3}H]inositol and incubated in the absence or presence of 10 nM trypsin for 30 min at 37°C, and the amounts of [^{3}H]IPs accumulated were then measured. The data shown are the mean ± S.D. for triplicates in one experiment and are representative of three independent experiments.

PAR2 wild type localized primarily to the cell surface, whereas βarr2-GFP was uniformly distributed in the cytoplasm of cells (Fig. 5A). The addition of SLIGKV for 5 min caused a rapid and marked redistribution of βarr2-GFP to the plasma membrane, which showed significant colocalization with activated PAR2 (Fig. 5A), consistent with published studies using other cell types (Dery et al., 1999). It is interesting, however, that in cells expressing PAR2 C-tail truncation mutants, a 5-min agonist exposure also induced a marked redistribution of βarr2-GFP to the plasma membrane, which robustly colocalized with activated receptor (Fig. 5, B–D). A similar pattern of βarr1-GFP redistribution was induced by agonist in wild-type and mutant PAR2-expressing cells, whereas neither βarr2-GFP nor βarr1-GFP was redistributed by SLIGKV in untransfected control cells (data not shown). These results indicate that the PAR2 C-tail is not essential for initial recruitment of arrestins to activated receptors and are consistent with a role for arrestins in agonist-triggered internalization of PAR2 C-tail truncation mutants.

To determine whether the PAR2 C-tail is essential for arrestin-mediated uncoupling of receptor from G-protein signaling, we examined agonist-induced PI hydrolysis in transiently transfected COS-7 cells coexpressing PAR2 and either βarr1 or βarr2. Transfected COS-7 cells were incubated in the absence or presence of trypsin for 30 min at 37°C, and [^{3}H]IPs were measured. After 30 min of agonist exposure, a marked ~10-fold increase in PI hydrolysis was detected in cells expressing PAR2 wild type (Fig. 6). In contrast, agonist-stimulated signaling was markedly impaired in cells expressing PAR2 and either βarr1 or βarr2; a ~5- and ~4-fold increase in IP accumulation was detected after 30 min of agonist exposure (Fig. 6). It is remarkable that coexpression of either βarr1 or βarr2 with PAR2 C-tail truncation mutants also significantly attenuated agonist-induced signaling responses similar to that observed with wild-type PAR2 (Fig. 6). Neither βarr1 nor βarr2 enhance agonist-induced internalization of PAR2 wild type or C-tail truncation mutants in these cells (data not shown), suggesting that the effects of arrestins on PAR2 signaling are independent of receptor trafficking. Together, these data support the idea that the

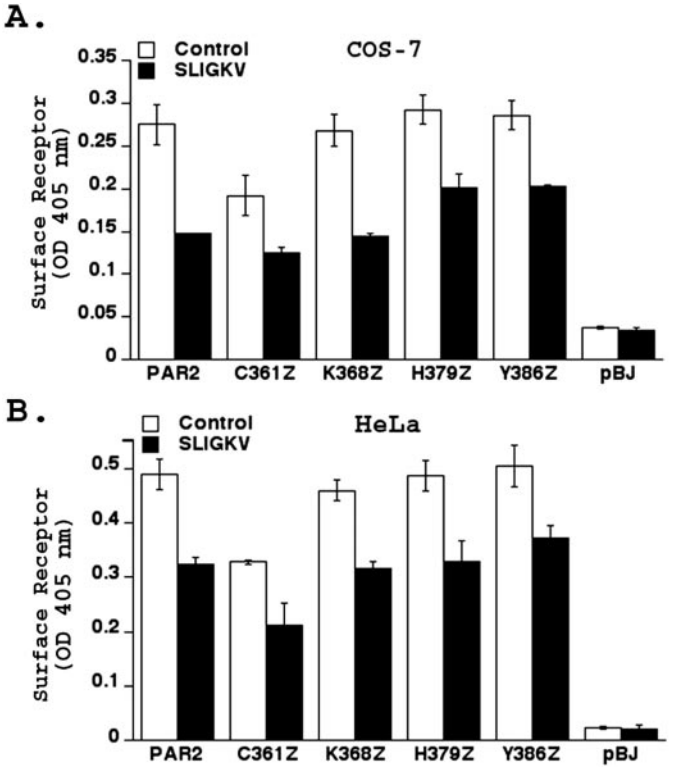

Fig. 4. Agonist-induced internalization of PAR2 wild type and C-tail truncation mutants. COS-7 (A) and HeLa cells (B) transiently expressing PAR2 wild type, C-tail mutants, or pBJ vector were incubated in the absence (Control) or presence of 100 μM SLIGKV for 60 min at 37°C. Cells were then fixed, and the amounts of receptor remaining on the cell surface were quantitated by ELISA. The results shown are the mean ± S.D. of an individual experiment performed in triplicate and are representative of three separate experiments.

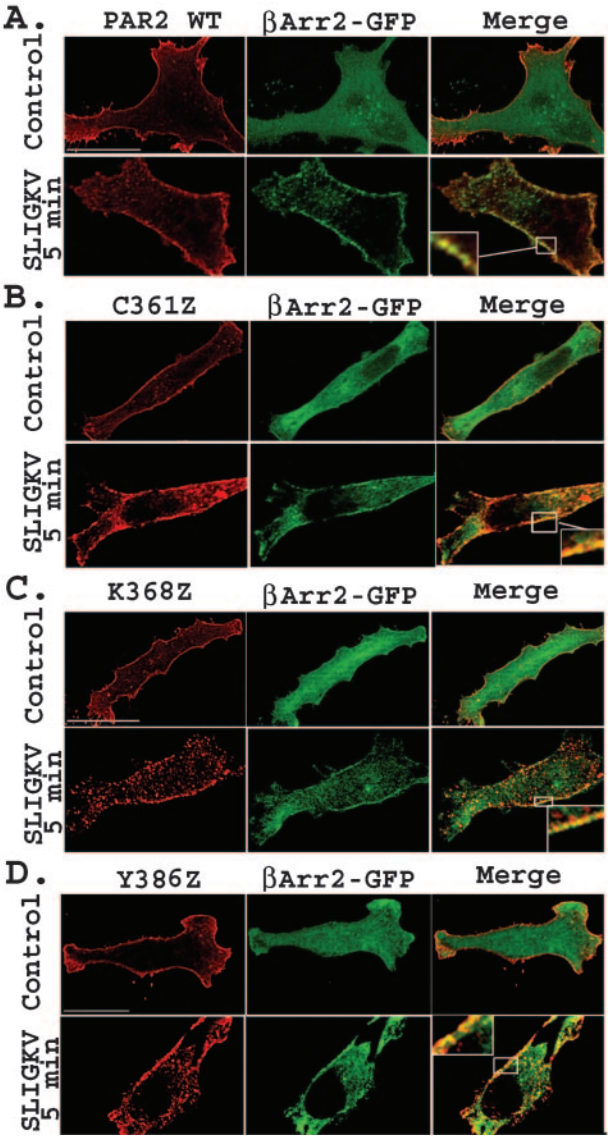

Fig. 5. βarr2-GFP is rapidly recruited to activated PAR2 wild type and C-tail truncation mutants. HeLa cells transiently expressing PAR2 wild type (WT) (A), C361Z mutant (B), K368Z mutant (C), or Y386Z mutant (D), together with βarr2-GFP, were incubated in the absence (Control) or presence of 100 μM SLIGKV for 5 min at 37°C. Cells were fixed and processed for immunofluorescence microscopy. These images are representative of many cells examined in at least three independent experiments. Note the prominent colocalization (yellow) of activated PAR2 wild type and C-tail truncation mutants with βarr2-GFP in the merged images. The insets are magnifications of boxed areas. Scale bar, 10 μm.

PAR2 C-tail is not critical for the ability of arrestins to desensitize activated receptor signaling.

The PAR2 C-tail contains three distinct clusters of serine and threonine residues (Fig. 3A), which are predicted to mediate stable interaction with arrestins (Oakley et al., 2001). We therefore examined whether the receptor C-tail is important for stable PAR2-arrestin interaction by examining the association of βarr2-GFP with internalized receptor. In the absence of agonist, PAR2 was localized predominantly at the plasma membrane, whereas βarr2-GFP was found diffusely distributed in the cytoplasm and failed to significantly colocalize with receptor (Figs. 5A and 7). However, the addition of SLIGKV for 30 min caused a marked redistribution of PAR2 wild type and βarr2-GFP into endocytic vesicles that showed robust colocalization (Fig. 7). PAR2 C-tail truncation mutants were also markedly redistributed to endocytic vesicles after 30 min of agonist exposure, consistent with agonist-induced loss of surface receptor shown in Fig. 4. In striking contrast to the wild-type receptor, however, βarr2-GFP failed to internalize into endocytic vesicles together with activated PAR2 C-tail truncation mutants lacking either all three serine/threonine clusters or only the distal carboxyl terminal cluster (Figs. 3A and 7). Similar findings were observed with βarr1-GFP (data not shown). Thus, the C-tail seems to be a critical determinant for activated PAR2-arrestin stable association after internalization.

Inhibition of activated PAR2 internalization by the dominant-negative $\beta arr1^{319-418}$ fragment virtually abolishes ERK1/2 activation (DeFea et al., 2000). Toward understanding the function of arrestins in PAR2-mediated ERK1/2 activation, we first determined whether arrestins are essential for endogenous ERK1/2 activation using MEFs lacking arrestin expression. In wild-type MEFs expressing PAR2 and arrestins, the addition of trypsin stimulated a rapid and robust ~3.3-fold increase in ERK1/2 activity at 5 min that remained substantially elevated for more than 60 min (Fig. 8A). SLIGKV also caused prolonged ERK1/2 activation in wild-type MEFs (data not shown). In arrestin-null cells expressing surface PAR2 levels comparable with wild-type cells, trypsin also induced a rapid and robust ~2.8-fold increase in ERK1/2 activity at 5 min. In striking contrast to wild-type cells, MEFs deficient in arrestin expression failed to sustain prolonged ERK1/2 activation after incubation with either trypsin or SLIGKV (Fig. 8B and data not shown). Similar results were obtained in other independently derived wild-type and βarr1,2-null clonal cell lines stably expressing PAR2. The ability of PAR2 to cause prolonged activation of ERK1/2 in wild-type MEFs is not simply the result of a defect in G-protein uncoupling, because receptor desensitization and internalization remains intact in these cells (Figs. 1 and 2). Together, these findings suggest that arrestins have a critical role in PAR2-mediated prolonged activation of ERK1/2, whereas the initial peak of ERK1/2 activation seems to be independent of arrestin expression.

The loss of prolonged ERK1/2 activation by PAR2 in arrestin-deficient cells could be caused by defective internalization. It is also possible that the ability of PAR2 to induce prolonged ERK1/2 activation is not only linked to internalization but also to stable association of receptor with arrestin in endocytic vesicles. To distinguish between these possibilities, we examined ERK activation by PAR2 C-tail truncation mutants that internalize but fail to stably associate with arrestins in endosomes. HeLa cells transiently transfected with PAR2 wild type or C-tail K368Z truncation mutant, together with GFP-ERK2, were incubated with trypsin for various times at 37°C, and activation of ERK2 was assessed by immunoblotting with phospho-ERK1/2 antibodies. In cells expressing PAR2 wild type, agonist induced a rapid and robust ~3-fold increase in ERK2 activation at 5 min followed by a plateau that remained elevated ~2-fold above basal levels for more than 30 min (Fig. 9A). The PAR2 C-tail truncation K368Z mutant expressing cells also showed a rapid ~3-fold increase in ERK2 activity at 5 min (Fig. 9B). However, in contrast to wild-type receptor, PAR2 C-tail mutant signaling to ERK2 declined rapidly to basal levels within 20 min of agonist exposure (Fig. 9B). Together, these results indicate that activated PAR2 stable association with arrestins is important for the ability of receptor to induce prolonged ERK1/2 activation.

To determine whether βarr1 and βarr2 are essential for activated endogenous PAR2 induced-ERK1/2 activation in HeLa cells, we used siRNA to selectively deplete cells of these proteins. We first determined whether siRNA targeted against specific βarr1 and βarr2 mRNA sequences were ef-

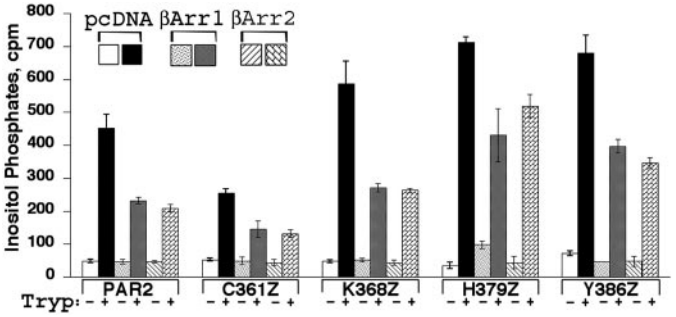

Fig. 6. The PAR2 C-tail is not essential for β-arrestin-mediated termination of receptor signaling. COS-7 cells transiently transfected with PAR2 wild type or C-tail mutants and either βarr1, βarr2, or pcDNA vector were labeled with [*myo*-^{3}H]inositol and incubated in the absence (Ctrl) or presence of 10 nM trypsin (Tryp) for 30 min at 37°C. The amounts of [^{3}H]IPs accumulated were then measured. The data shown (mean ± S.D.; $n = 3$) are from one experiment that is representative of three separate experiments.

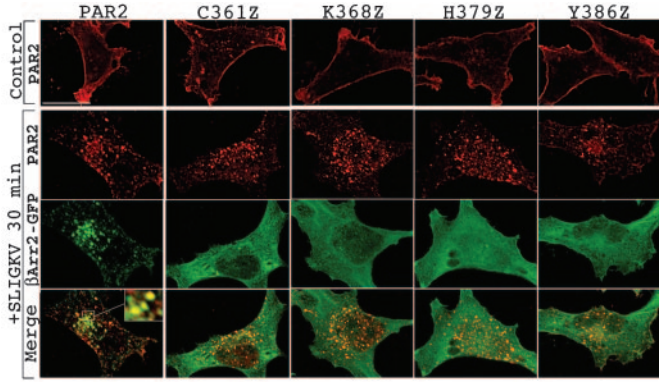

Fig. 7. Activated PAR2 C-tail truncation mutants fail to stably associate with βarr2-GFP in endocytic vesicles. HeLa cells transiently expressing PAR2 wild type or C-tail truncation mutants, together with βarr2-GFP, were incubated in the absence (Control) or presence of 100 μM SLIGKV for 30 min at 37°C. Cells were fixed, immunostained for PAR2, and then imaged by immunofluorescence confocal microscopy. The images shown are representative of many cells examined in three different experiments. Scale bar, 10 μm.

fective at reducing β-arrestin expression. HeLa cells were transiently transfected with βarr1, βarr2, or nonspecific siRNA, and the amounts of β-arrestin protein remaining were then determined. Immunoblots of untransfected HeLa cell lysates revealed a greater amount of βarr1 compared with βarr2 expression (Fig. 10A). The apparent differences in the amounts of the individual β-arrestin isoforms may be caused by the greater affinity of A1CT antibody for the βarr1 protein (Kohout et al., 2001). The expression of βarr1 was virtually abolished in cells transfected with siRNA directed against βarr1 mRNA, compared with cells transfected with nonspecific siRNA or untransfected control cells (Fig. 10A). βarr2 siRNA also caused a significant and specific decrease in βarr2 protein, whereas cells transfected with both βarr1 and βarr2 siRNA were essentially depleted of β-arrestin proteins (Fig. 10A). Neither βarr1- nor βarr2-specific siRNAs affected actin expression in the same cells.

We next examined whether βarr1 and βarr2 proteins are necessary for PAR2-stimulated prolonged increase in ERK1/2 activation in HeLa cells. HeLa cells transiently transfected with βarr1, βarr2, or nonspecific siRNA were incubated with trypsin for various times at 37°C, and activation of ERK1/2 was assessed by immunoblotting. In cells transfected with nonspecific siRNA, agonist induced a rapid ~5-fold increase in ERK1/2 activity that remained elevated ~4-fold above basal for at least 30 min (Fig. 10, B and C). Cells transfected with siRNA specific to either βarr1, βarr2,

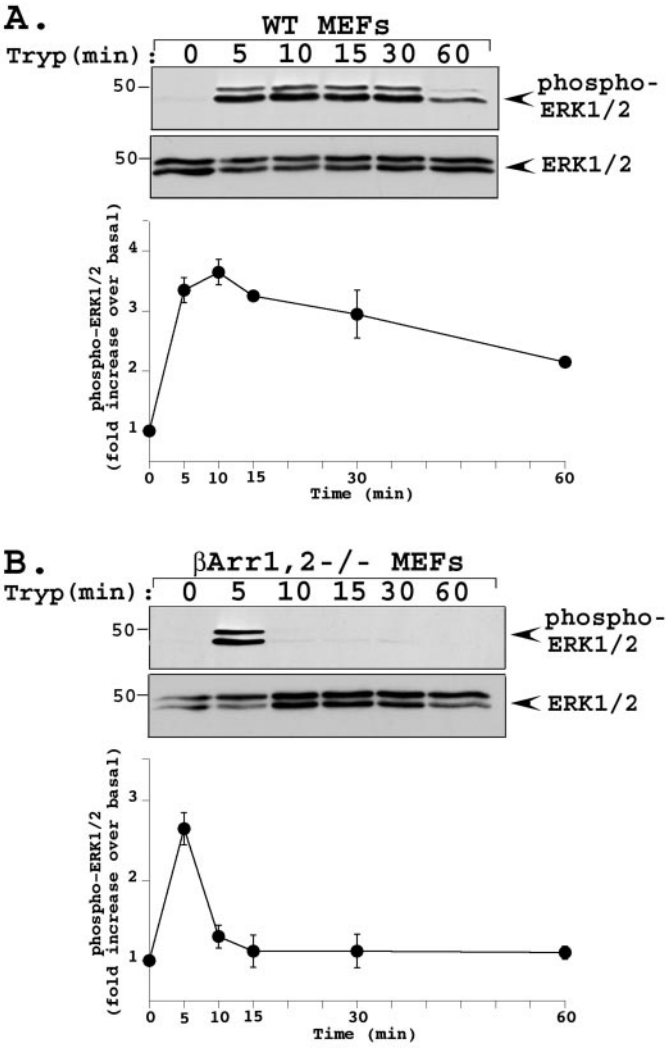

Fig. 8. β-arrestins are essential for PAR2-mediated prolonged ERK1/2 activation. Serum-deprived wild-type (WT) (A) and βarr1,2−/− (B) cells expressing similar amounts of surface PAR2 were exposed to 10 nM trypsin (Tryp) for various times at 37°C. An equivalent amount of cell lysates were then resolved by SDS-polyacrylamide gel electrophoresis, and ERK1/2 activation was determined by immunoblotting with anti-phospho-p44/42 MAP (ERK1/2) antibodies. Membranes were stripped and reprobed with anti-p44/42 MAP (ERK1/2) antibody to control for equal loading. The time course of agonist-stimulated endogenous ERK1/2 activation shown is a representative experiment. The quantitative results are expressed as -fold increase over basal and represent the mean ± S.E. of three separate experiments.

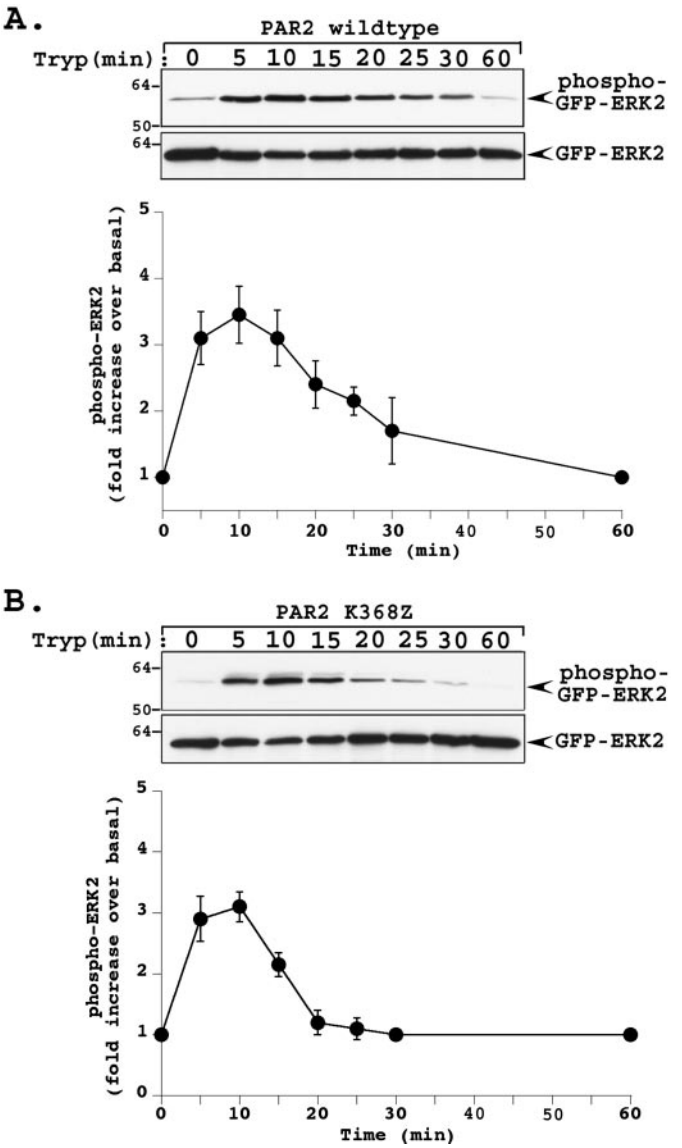

Fig. 9. The PAR2 C-tail is important for prolonged activation of ERK2. Serum-deprived HeLa cells transiently expressing PAR2 wild type (A) or K368Z mutant (B), together with GFP-ERK2, were incubated in the absence or presence of 10 nM trypsin (Tryp) for various times at 37°C. Equivalent amounts of cell lysates were resolved by SDS-polyacrylamide gel electrophoresis, and activation of GFP-ERK2 was determined by immunoblotting with anti-phospho-p44/42 MAP (ERK1/2) antibodies. Immunoblots were reprobed with anti-p44/42 MAP (ERK1/2) antibodies to control for equal loading. The time course of agonist-stimulated GFP-ERK2 activation shown is from a representative experiment. The quantitative results are expressed as -fold increase over basal and represent the mean ± S.E. of three separate experiments.

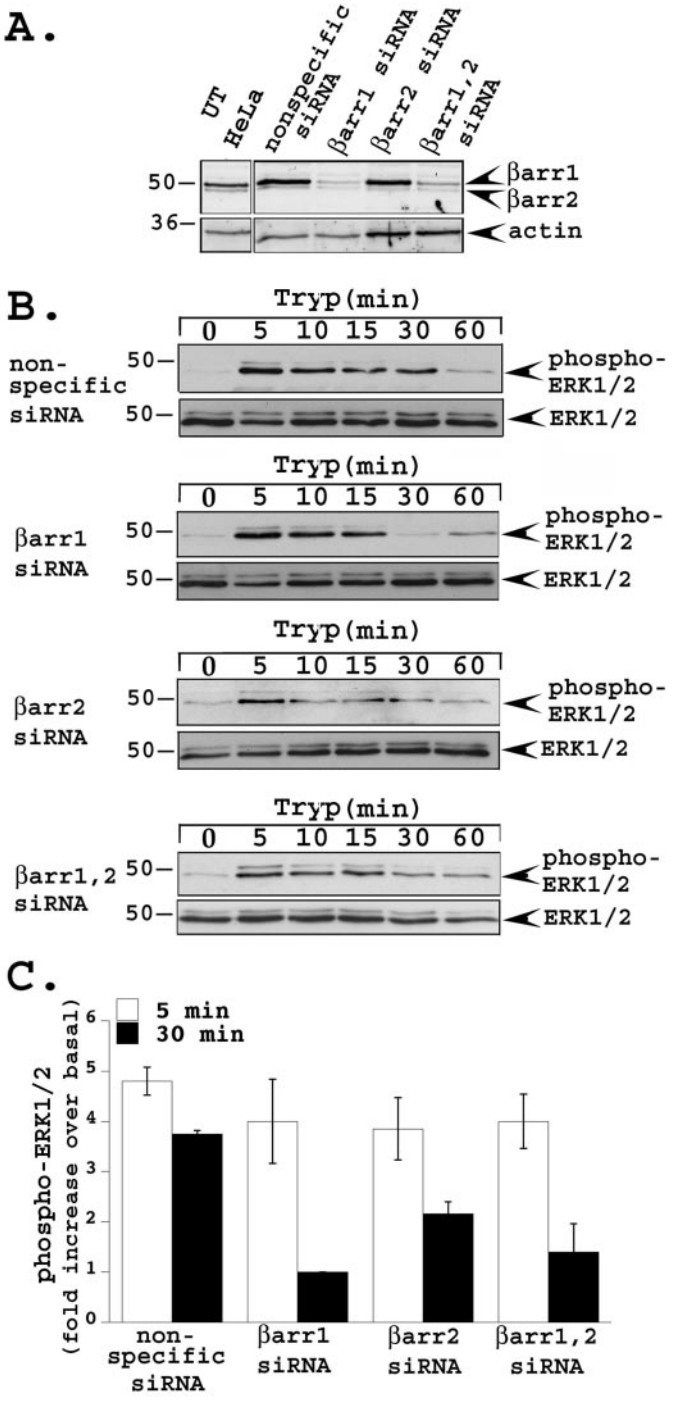

Fig. 10. Depletion of βarr1 and βarr2 protein expression inhibits endogenous PAR2-stimulated prolonged increase in ERK1/2 activation. A, HeLa cells were transiently transfected with 60 nM siRNA targeted to either βarr1, βarr2, or nonspecific mRNA sequences or left untransfected (UT). Cell lysates were prepared and immunoblotted with anti-β-arrestin A1CT antibody. Actin expression was also detected as a loading control. B, HeLa cells transfected with siRNAs were incubated with 10 nM trypsin (Tryp) for various times at 37°C. Equivalent amounts of cell lysates were resolved by SDS-polyacrylamide gel electrophoresis, and activation of endogenous ERK1/2 was assessed by immunoblotting. Immunoblots were reprobed with anti-p44/42 MAP (ERK1/2) antibodies to control for equal loading. The time course of agonist-stimulated ERK1/2 activation shown is from a representative experiment. C, the quantitative results are expressed as -fold increase over basal and represent the mean ± S.E. of three different experiments.

or both showed a ~4-fold initial peak in ERK1/2 activity at 5 min similar to that observed in cells transfected with nonspecific siRNA control (Fig. 10, B and C). However, trypsin-stimulated ERK1/2 activity measured at 30 min showed only a modest ~2-fold increase in βarr2 siRNA transfected cells, whereas the response was virtually abolished in cells transfected with either βarr1 siRNA or both βarr1 and βarr2 siRNA (Fig. 10, B and C). Together, these findings suggest that both βarr1 and βarr2 are critical for activated PAR2-induced prolonged increase in ERK1/2 activity in HeLa cells, but neither protein is essential for the initial peak in ERK1/2 activity.

Discussion

The molecular mechanisms responsible for the regulation of PAR2 signaling and trafficking remain poorly understood. Most activated GPCRs are initially desensitized by rapid phosphorylation and binding of arrestins. Arrestin binding uncouples the receptor from G-proteins, facilitates receptor internalization, and initiates other signaling pathways such as ERK1/2 activation (Shenoy and Lefkowitz, 2003). The binding of arrestins to activated GPCRs involves multiple interactions with the second and/or third intracellular loops and C-tail (Gurevich and Gurevich, 2004). The PAR2 C-tail contains three clusters of serine and threonine residues predicted to form a stable interaction with arrestins (Oakley et al., 2001). Toward understanding the regulation of PAR2 signaling and trafficking, we examined the function of the C-tail in these processes. Our findings strongly suggest that activated PAR2 desensitization and internalization is dependent on arrestins but occurs independently of the C-tail. Activation of PAR2 mutants lacking either the entire or distal portions of the C-tail region caused rapid and transient redistribution of βarr2-GFP to the plasma membrane. Activated PAR2 C-tail truncation mutants also desensitized in an arrestin-dependent manner to the same extent as wild-type receptor in COS-7 cells, and internalization of mutant receptors remained intact. In marked contrast to wild-type receptor, however, PAR2 C-tail truncation mutants lost the capacity to form stable interactions with arrestin and consequently redistributed to endocytic vesicles without βarr2-GFP. Activated PAR2 induced a rapid and transient increase in endogenous ERK1/2 activity in the absence of arrestins, whereas arrestins were essential for PAR2-stimulated prolonged ERK1/2 activation. Consistent with a requirement for PAR2-arrestin stable interaction in promoting prolonged ERK1/2 activation, the C-tail-truncation K368Z mutant internalized but failed to sustain association with arrestin and to elicit prolonged activation of ERK2. Together, these findings demonstrate for the first time an essential role for arrestins in PAR2 desensitization, internalization, and prolonged signaling to ERK1/2 activation. Moreover, our study suggests that the different functions of arrestins in regulating PAR2 signaling and trafficking are controlled by multiple independent interactions involving both the intracellular loops and C-tail.

We assessed arrestin function in PAR2 signaling and trafficking using MEFs derived from arrestin knockout mice (Kohout et al., 2001). It was previously reported that arrestins function in the regulation of PAR2 signaling and trafficking based on the observations that overexpression of a $\beta arr1^{319-418}$ fragment, which interacts constitutively with

clathrin but not the receptor, inhibited agonist-induced PAR2 internalization (Dery et al., 1999). In addition, activated PAR2 caused rapid redistribution of βarr1-GFP to the plasma membrane, and both the receptor and βarr1-GFP redistributed to endocytic vesicles in KNRK cells (Dery et al., 1999). However, overexpression of βarr1 failed to affect agonist-triggered internalization and desensitization of activated PAR2 (Dery et al., 1999). In transfected arrestin-deficient MEFs, the rate of PAR2 desensitization was significantly slowed, resulting in a greater accumulation of IPs in βarr1,2-null cells compared with wild-type cells (Fig. 2). Thus, in the absence of arrestins, desensitization of PAR2 signaling is significantly impaired. Consistent with a role for arrestins in PAR2 desensitization, we demonstrate in COS-7 cells, which express low level of arrestins (Menard et al., 1997), that both βarr1 and βarr2 are equally effective at desensitizing PAR2 signaling (Fig. 6). The ability of arrestins to modulate PAR2 signaling is not caused by receptor trafficking or nonspecific effects because neither βarr1 nor βarr2 enhances PAR2 internalization or globally disrupt signaling by $G\alpha_q$ in these cells (data not shown) (Chen et al., 2004). These findings are the first to demonstrate a critical role for arrestins in the termination of PAR2 signaling.

In addition to regulating PAR2 desensitization, arrestins are essential for PAR2 internalization. We found that PAR2 internalization was completely abolished in cells lacking arrestins (Fig. 1), whereas in wild-type MEFs, agonist-triggered PAR2 endocytosis occurred normally. Arrestins interact with phosphorylated GPCRs and bind to clathrin and adaptor protein complex-2 (AP-2) to facilitate receptor internalization through clathrin-coated pits (Ferguson et al., 1996; Goodman et al., 1996). The C-tail of PAR2 contains multiple potential sites for phosphorylation, including three clusters of serine and threonine residues (Fig. 3A), suggesting that the C-tail may be critical for internalization of PAR2. We were surprised to find, however, that our results indicate that the C-tail is not essential for activated PAR2 internalization (Fig. 4). Consistent with these results, a PAR2 C-tail truncation mutant lacking residues beyond serine-363 also internalized in transfected human keratinocytes (Seatter et al., 2004). One report showed that a PAR2 C-tail double point mutant, in which serine-363 and threonine-366 were converted to alanines (δST363/6A), was markedly defective in desensitization and internalization in KNRK cells (DeFea et al., 2000). However, we found that the same PAR2 δST363/6A mutant showed internalization and desensitization comparable with that of wild-type PAR2 in both COS-7 and HeLa cells (data not shown), suggesting that these residues are not important for desensitization and internalization in these cell types.

Our results indicate that arrestins bind to activated PAR2 through multiple interactions involving both the intracellular loops and the C-tail. The binding of arrestin to activated phosphorylated GPCRs involves both activation- and phosphorylation-recognition sites. The activation-recognition domain localized to the amino-terminal portion of arrestin binds to the second and/or third intracellular loops of GPCRs (Wu et al., 1997), whereas the phosphorylation-recognition domain is a positively charged central region of the molecule that binds to receptor associated phosphates (Kieselbach et al., 1994). The sequential binding of arrestin to both the activation- and phosphorylation-recognition sites imparts high affinity binding, whereas engagement of only one site mediates a weaker interaction (Gurevich and Gurevich, 2004). Most GPCRs have multiple potential sites for phosphorylation located in both the intracellular loops and C-tail. The phosphorylation of activated GPCRs by GRKs occurs randomly at these sites, producing a variety of phosphorylated forms of the same receptor in vivo (Kennedy et al., 2001). At least two, and perhaps three, phosphorylated residues are necessary for arrestin to bind to activated GPCR, whereas one phosphorylated residue is insufficient. We hypothesize that high-affinity binding of arrestin to activated PAR2 involves both the intracellular loops and C-tail. The C-tail of PAR2 contains three clusters of serine and threonine residues, and phosphorylation of these residues would generate a region enriched in negative charges sufficient to bind arrestin. However, in the absence of the C-tail, activated PAR2 retains the capacity to bind arrestins based on the observations that activated receptor C-tail truncation mutants caused βarr2-GFP redistribution to the plasma membrane and desensitized in an arrestin-dependent manner like the wild-type receptor. In contrast to wild-type PAR2, however, arrestins failed to remain associated with internalized receptor mutants lacking the C-tail, consistent with a weak binding of arrestin to these receptors. Thus, it is likely that the PAR2 C-tail functions as the phosphorylation-recognition site and the intracellular loops as the activation-recognition domain, although the actual phosphorylation sites in PAR2 have not yet been defined.

The diverse functions of arrestins in regulating PAR2 desensitization, internalization, and prolonged signaling to ERK1/2 activation are controlled in part by the differential binding of arrestin to the receptor. The transient interaction of activated PAR2 with arrestins is sufficient to facilitate uncoupling from G-protein signaling and internalization from the plasma membrane. In contrast, the stable association of PAR2 with arrestins seems to be important for prolonged signaling to ERK1/2 activation. It is interesting, however, that in the absence of arrestins, activated PAR2 retained the capacity to induce a rapid and transient increase in endogenous ERK1/2 activity (Figs. 8 and 10). It is likely that the early transient increase in ERK1/2 activity induced by activated PAR2 involves a G-protein dependent pathway. Consistent with this idea, the angiotensin II type 1A receptor activates ERK1/2 by a rapid and transient G-protein-dependent pathway and a slower and more persistent βarr2-dependent pathway (Wei et al., 2003; Ahn et al., 2004). The molecular mechanisms regulating the distinct temporal modes of ERK1/2 activation by PAR2 are not known.

The findings in this study reveal that the binding of arrestin to activated PAR2 involves multiple independent interactions with the intracellular loops and C-tail. We found that arrestins transiently interact with activated PAR2 independent of the C-tail and is sufficient to promote desensitization and internalization. However, the C-tail is essential for stable PAR2-arrestin interaction and prolonged ERK1/2 activation. These findings suggest that the diverse functions of arrestins in regulating PAR2 signaling and trafficking can be dissociated based on the stability of the receptor-arrestin interaction. Likewise, the C-tail of the D1 dopamine receptor is also not required for rapid arrestin association and desensitization (Kim et al., 2003). The regulation of PAR1 desensitization by arrestins is independent of receptor phosphory-

lation (Chen et al., 2004), and our data with PAR2 C-tail mutants indicate that arrestin binding may also occur without receptor phosphorylation. These findings raise the possibility that perhaps phosphorylation of PARs may not be required for rapid and transient arrestin interaction. In contrast to PAR2, however, arrestins are not essential for activated PAR1 internalization through clathrin-coated pits (Paing et al., 2002) or for thrombin-stimulated ERK1/2 activation in MEFs and in Chinese hamster embryonic fibroblasts (IIC9 cells) (M. M. Paing and J. Trejo, unpublished observations) (Goel et al., 2002). Thus, despite similar proteolytic mechanisms of activation, arrestins are capable of differentially regulating certain aspects of PAR1 and PAR2 signaling and trafficking.

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