Regulation of Protease-activated Receptor 1 Signaling by the Adaptor Protein Complex 2 and R4 Subfamily of Regulator of G Protein Signaling Proteins

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Background: The function of the clathrin adaptor AP-2 in the regulation of GPCR coupling to G protein signaling is not known.

Results: AP-2 controls GPCR signaling by modulating receptor surface expression and, unexpectedly, through RGS protein recruitment to G proteins.

Conclusion: AP-2 has diverse functions in the regulation of GPCR signaling.

Significance: AP-2 provides a new mode of GPCR signal regulation.

The G protein-coupled protease-activated receptor 1 (PAR1) is irreversibly proteolytically activated by thrombin. Hence, the precise regulation of PAR1 signaling is important for proper cellular responses. In addition to desensitization, internalization and lysosomal sorting of activated PAR1 are critical for the termination of signaling. Unlike most G protein-coupled receptors, PAR1 internalization is mediated by the clathrin adaptor protein complex 2 (AP-2) and epsin-1, rather than β-arrestins. However, the function of AP-2 and epsin-1 in the regulation of PAR1 signaling is not known. Here, we report that AP-2, and not epsin-1, regulates activated PAR1-stimulated phosphoinositide hydrolysis via two different mechanisms that involve, in part, a subset of R4 subfamily of “regulator of G protein signaling” (RGS) proteins. A significantly greater increase in activated PAR1 signaling was observed in cells depleted of AP-2 using siRNA or in cells expressing a PAR1420AKKAA424 mutant with defective AP-2 binding. This effect was attributed to AP-2 modulation of PAR1 surface expression and efficiency of G protein coupling. We further found that ectopic expression of R4 subfamily members RGS2, RGS3, RGS4, and RGS5 reduced activated PAR1 wild-type signaling, whereas signaling by the PAR1 AKKAA mutant was minimally affected. Intriguingly, siRNA-mediated depletion analysis revealed a function for RGS5 in the regulation of signaling by the PAR1 wild type but not the AKKAA mutant. Moreover, activation of the PAR1 wild type, and not the AKKAA mutant, induced GoQ association with RGS3 via an AP-2-dependent mechanism. Thus, AP-2 regulates activated PAR1 signaling by altering receptor surface expression and through recruitment of RGS proteins.

The coagulant protease thrombin is generated in response to vascular injury and in thrombotic disease where it promotes hemostasis, thrombosis, and inflammatory responses. Thrombin drives fibrin deposition and mediates cellular responses through a family of protease-activated G protein-coupled receptors (GPCRs)2 (1). Protease-activated receptor 1 (PAR1) is the family prototype and the predominant mediator of thrombin signaling in most cell types. Thrombin cleaves the N terminus of PAR1, unmasking a new N-terminal domain that functions as a peptide ligand by binding to the receptor, inducing a conformational change that facilitates coupling to heterotrimeric G proteins (2). Because of the proteolytic mechanism of activation and generation of a tethered ligand that cannot diffuse away, signaling by PAR1 is tightly regulated. Similar to other GPCRs, activated PAR1 signaling is rapidly desensitized by phosphorylation and β-arrestin binding, which uncouples the receptor from heterotrimeric G protein signaling (3, 4). Activated PAR1 is then internalized from the cell surface, sorted directly to lysosomes, and degraded, which prevents continued signaling by previously activated receptors that return to the cell surface with their tethered ligands intact (5). In metastatic breast carcinoma, activated PAR1 is internalized and recycled back to the cell surface rather than sorted to lysosomes (6). Consequently, activated PAR1 signals persistently and promotes breast carcinoma invasion and tumor growth in vivo (7). These findings indicate that internalization and lysosomal sorting of PAR1 are important for regulating the magnitude and duration of G protein signaling.

In contrast to many classic GPCRs, PAR1 internalization occurs through clathrin-coated pits independent of β-arrestins (4). Several other GPCRs have also been shown to internalize independently of β-arrestins (8). We showed previously that the clathrin adaptor protein complex 2 (AP-2) and epsin-1 are
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Reagents and Antibodies—Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). The PAR1 peptide agonist Ser-Phe-Leu-Arg-Asn (SFLLRN) was synthesized as the carboxyl amide and purified by reverse phase high-pressure liquid chromatography at the Tufts University Core Facility (Boston, MA). Carbachol and UTP were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-FLAG antibody was purchased from Rockland Immunochemicals (Gilbertsville, PA). Mouse monoclonal M2 anti-FLAG antibody and anti-β-actin were purchased from Sigma-Aldrich. The anti-PAR1 C5433 rabbit polyclonal antibody has been described previously (13). Rabbit polyclonal anti-Gαq (C-19) antibody and anti-epsin-1 (H-130) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated secondary goat anti-mouse and anti-rabbit antibodies were obtained from Bio-Rad. Monoclonal anti-HA antibody conjugated to HRP was from Roche. The anti-AP50 (μ2) monoclonal antibody was obtained from BD Biosciences.

cDNAs and Cell Lines—A cDNA encoding human PAR1 WT and C-tail AKKA mutant containing an N-terminal FLAG epitope cloned into the pBJ vector have been described previously (13, 20). An HA epitope-tagged Gαq (14) placed within an internal loop was provided by Dr. Philip Wedegaertner (Thomas Jefferson University, Philadelphia, PA). HA-tagged RGS2, RGS4-C2S, and RGS5-C2S have been described previously (21). HA-tagged RGS3 was purchased from the Missouri S&T CDNA Resource Center (Rolla, MO).

HeLa cells expressing the FLAG-tagged PAR1 WT or AKKA mutant were generated and maintained as described previously (20). Human umbilical vein endothelial-derived EA.hy926 cells were maintained as described (22). COS7 cells were grown in DMEM containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

siRNA—HeLa cells stably expressing FLAG-tagged PAR1 WT or AKKA mutant were plated at 0.5–1 × 10⁵ cells/well in fibronectin-coated 24-well plates and grown overnight at 37 °C. HeLa cells were transiently transfected with 50 nM of nonspecific, epsin-1, μ2, or RGS4 siRNAs or 100 nM RGS2-, 100 nM RGS5-, or 200 nM RGS3-specific siRNA using Lipofectamine 2000 or Oligofectamine 2000 or Oligofectamine according to the instructions of the manufacturer (Invitrogen). Endothelial cells were plated at 3.5 × 10⁵ cells/well in fibronectin-coated 12-well plates and grown overnight at 37 °C. Endothelial cells were transiently transfected with 50 nM nonspecific or μ2-specific siRNA using Oligofectamine. The nonspecific siRNA 5′-CTAGTCCAGGA-GCCGACC-3′, μ2 siRNA 5′-CTAGATGCCCTTGGGCTCA-3′, and epsin-1 siRNA 5′-GGAAGCGCCGGAGTCATT-3′ have been described previously (13). The RGS2-specific siRNA 5′-AACAGTGGGTCTCTCACTGAA-3′, RGS3-specific siRNA 5′-CAGACGATAGACATCGGA-3′, RGS4-specific siRNA 5′-AACATGCTAGAGCCTACAATA-3′, and RGS5-spe...
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cific siRNA 5′-AACGAGAGCAATGACTATTTA-3′ were purchased from Qiagen (Valencia, CA).

Immunoblotting—Protein concentrations were determined from total cell lysates using BCA (Thermo Fisher Scientific, Rockford, IL). Equivalent amounts of cell lysates were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the appropriate antibodies.

Phosphoinositide (PI) Hydrolysis—HeLa cells stably expressing FLAG-tagged the PAR1 WT or AKKAA mutant were plated at 1 × 10⁶ cells/well in fibronectin-coated 24-well plates and grown overnight at 37 °C. After transfection, cells were labeled with 1 μCi/ml myo-[3H]inositol (American Radiolabeled Chemicals, St. Louis, MO) overnight, treated with or without agonist in the presence of 20 mM lithium chloride (LiCl) for various times at 37 °C, and then accumulated [3H]inositol phosphates (IPs) were measured from 3 wells/time point of an independent experiment as described previously (4). To determine the desensitization rates, cells were labeled as described above. Cells were then stimulated with 10 nM thrombin for 10 min at 37 °C, LiCl was added as indicated, and the accumulation of [3H]IPs was measured as described previously (23).

Immunoprecipitation—COS7 cells were plated at 1.5 × 10⁶ cells/60-mm dish and grown overnight at 37 °C. Cells were transfected with HA-tagged Gaq and FLAG-tagged PAR1 WT or AKKAA mutant for 48 h, serum-starved for 1 h, and then incubated with or without agonist at 37 °C. Cells were washed with ice-cold PBS and lysed in Triton X-100 lysis buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, and 1% Triton X-100) supplemented with 100 μg/ml PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin, and 10 μg/ml benzamidine. Cell lysates were cleared by centrifugation, protein concentrations were determined, and equivalent amounts of cell lysates were immunoprecipitated with anti-FLAG antibody overnight at 4 °C. Immunoprecipitates were washed with lysis buffer, and proteins were eluted in 2× Laemmli buffer (62.5 mM Tris-HCl, (pH 6.8), 10% glycerol, 5% SDS, 0.01% bromophenol blue). Immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-Gaqq1, antibody or anti-FLAG antibody to detect PAR1 expression. Cell lysates were immunoblotted with anti-Gaqq1 and anti-β-actin antibodies as controls. Immunoblot analyses were developed using ECL, exposed to film, and quantified by densitometry using ImageJ.

To examine RGS and Ga protein association, HeLa cells expressing FLAG-tagged PAR1 WT or AKKAA mutant were plated at 1.5 × 10⁶ cells/60-mm dish and grown overnight at 37 °C. Cells were transfected with HA-tagged RGS3 for 48 h or with 50 nM siRNA targeting the μ2-adaptin subunit in combination with HA-tagged RGS3 for 72 h. Cells were serum-starved for 1 h and incubated with or without agonist at 37 °C. Cells were lysed in Triton lysis buffer, and equivalent amounts of cell lysates were immunoprecipitated with anti-Gaqq1 antibody. Immunoprecipitates were processed, and membranes were probed with anti-HA antibody to detect RGS3 associated with Gaqq1, developed with ECL, and quantitated by densitometry using ImageJ.

Reverse Transcription Polymerase Chain Reaction—The first-strand cDNA was generated from total mRNA extracted from either HeLa or endothelial cells using SuperScript® II reverse transcriptase (RT), an engineered version of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) and oligo(dT)₁₂₋₁₈ following the instructions of the manufacturer (Invitrogen). The RT enzyme was omitted from the cDNA synthesis reaction in the negative (-) control samples. The first-strand cDNA was amplified by PCR using primers specific for all conventional RGS mRNA transcripts. The RGS primer sequences and predicted PCR amplicon sizes have been described previously (24). The PCR amplification products were resolved by 1.8% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining.

Cell Surface ELISA—HeLa cells stably expressing FLAG-tagged PAR1 WT or AKKAA mutant were plated at 1 × 10⁵ cells/well in fibronectin-coated 24-well plates and grown overnight at 37 °C. After transfection, cells were placed on ice for 10 min and washed once with ice-cold PBS. Cells were fixed in 4% paraformaldehyde for 5 min on ice and washed twice with PBS. Cells were incubated with anti-FLAG antibody or anti-PAR1 antibody for 60 min at room temperature. Cells were washed twice with medium and incubated with HRP-conjugated secondary antibody for 60 min at room temperature. Cells were washed three times with PBS before incubating with 1-step 2’,2’-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (Thermo Fisher Scientific, Rockford, IL). The absorbance of an aliquot was read at 405 nm using a Molecular Devices SpectraMax Plus microplate reader (Sunnyvale, CA).

Data Analysis—Data were analyzed using GraphPad Prism 4.0 and JMP statistical software. Statistical analysis was determined by performing Student’s t test, one-way ANOVA, Dunnett’s multiple test, Tukey’s post hoc honest significant difference (HSD) test, or two-way ANOVA and Bonferroni post-test.

RESULTS

AP-2 Regulates Activated PAR1 Signaling—In recent work, we showed that activated PAR1 internalization is mediated by AP-2 and epsin-1, not β-arrestins (4, 10), suggesting that they may regulate receptor signaling. However, precisely how AP-2 or epsin-1 function in the regulation of PAR1 signaling is not known. Previous studies indicate that activated PAR1 stimulates Gaq, mediated phospholipase C-induced PI hydrolysis in numerous cell types (25, 26). Thus, to examine the function of AP-2 and epsin-1 in PAR1 signaling, HeLa cells stably expressing PAR1 WT were transfected with nonspecific, μ2-, and/or epsin-1-specific siRNAs, labeled with myo-[3H]inositol, incubated with or without thrombin for various times, and then the accumulation of [3H]IPs was measured as described (4). Activated PAR1 signaling rapidly declined and reached near steady state at 2.5 min in cells transfected with nonspecific or epsin-1-specific siRNA (Fig. 1A), indicating that epsin-1 is not required for the regulation of PAR1 signaling. In contrast, activation of PAR1 in cells lacking AP-2 resulted in a marked increase in PI hydrolysis (Fig. 1A), which was comparable with cells depleted of both AP-2 and epsin-1. The increase in PAR1 signaling observed in AP-2 deficient cells also correlated with an increase...
in PAR1 expression at the cell surface (Fig. 1A). Unlike PAR1, activation of the G_{q/11}-linked muscarinic receptors with carbachol in AP-2-depleted HeLa cells failed to affect the signaling response compared with nonspecific siRNA-transfected control cells (Fig. 1B). To confirm these findings, we examined the effects of AP-2 depletion on endogenous PAR1 signaling in a cultured human endothelial EA.h926 cell line. A 60-min incubation with thrombin caused a significantly greater accumulation of [^{3}H]IPs in endothelial cells deficient in AP-2 expression compared with control cells (Fig. 1C), whereas signaling by UTP (an endogenous agonist for the purinergic P2Y_{1} or P2Y_{4} GPCRs) resulted in comparable signaling responses regardless of AP-2 expression (D). Elevated expression of endogenous PAR1 at the cell surface was also observed in AP-2-depleted cells (Fig. 1C). Thus, increased expression of PAR1 at the cell surface may contribute, in part, to greater signaling observed in AP-2-deficient cells.

Activated PAR1 Signaling Efficiency, but Not Desensitization, Is Regulated by AP-2—We next examined the signaling properties of PAR1 in cells lacking AP-2 expression. The concentration effect curves for thrombin at PAR1 expressed in control or AP-2-deficient cells was determined by incubating cells labeled with myo-[^{3}H]inositol with varying concentrations of thrombin. The accumulation of [^{3}H]IPs was then measured. The effective thrombin concentration to stimulate a half-maximal response was markedly decreased in AP-2-deficient cells compared with control cells (Fig. 2A). In addition, activation of PAR1 in cells lacking AP-2 expression resulted in an enhanced maximal signaling response compared with siRNA-transfected control cells (Fig. 2A). We also examined whether the efficacy or potency of carbachol-stimulated muscarinic receptor signaling was affected in AP-2-deficient cells. Using PAR1-expressing cells, the concentration-effect curves for carbachol stimulation of muscarinic receptor signaling were determined in control and AP-2-deficient cells. The EC_{50} values for carbachol-stimulated IP accumulation at 10 min were comparable under both conditions (Fig. 2B). The maximal signaling response induced by saturating carbachol concentrations were also similar (Fig. 2B). These results suggest that AP-2 depletion does not globally affect GPCR signaling activity. We also examined whether the differences in PAR1 signaling were related to desensitization. To assess PAR1 desensitization in control and AP-2-depleted cells, we measured the extent of PAR1 signaling activity remaining after various times of thrombin incubation and found that the apparent rates of desensitization were not significantly different (Fig. 2C). Together, these findings suggest that AP-2 specifically modulates activated PAR1 signaling activity independently of receptor desensitization.
A PAR1 AKKAA Mutant Exhibits Enhanced G Protein Coupling Efficiency—To determine whether AP-2 regulates PAR1 signaling via binding to the C-tail tyrosine motif, we utilized a PAR1 AKKAA tyrosine motif mutant that exhibits defective basal AP-2 binding in vitro (13). We examined whether AP-2 retained the capacity to regulate PAR1 AKKAA mutant signaling. HeLa cells stably expressing the PAR1 AKKAA mutant displayed a marked increase in PI hydrolysis following activation with saturating concentrations of thrombin in control siRNA-transfected cells (Fig. 3A). Activated PAR1 AKKAA mutant signaling was similarly enhanced in AP-2-deficient cells (Fig. 3A), suggesting that an intact tyrosine motif is important for AP-2-negative regulation of PAR1 signaling. To further examine differences in PAR1 WT versus AKKAA mutant sig-

FIGURE 2. Activated PAR1 signaling efficiency, and not desensitization, is regulated by AP-2. HeLa cells expressing PAR1 WT were labeled with myo-[3H]inositol incubated with varying concentrations of thrombin for 5 min (A) or carbachol (B) or 10 min (C) at 37 °C. The concentration effect curve (mean ± S.D., n = 3) shown is a representative experiment. The EC50 values (mean ± S.E.) from multiple independent experiments are shown in the bar graph. The differences in thrombin-stimulated signaling were significant as determined by two-way ANOVA and Bonferroni post-tests (*, p < 0.05; **, p < 0.01; ***, p < 0.001). The differences in EC50 values were determined by Student’s t test (***, p < 0.001), ns, not significant; Ctrl, control; M, molar. C, PAR1 WT-expressing HeLa cells labeled with myo-[3H]inositol were incubated with 10 nm thrombin for 10 min at 37 °C (1), and LiCl was added after various times of thrombin preincubation (2). [3H]IPs formed were then measured. The data (mean ± S.D., n = 3) are representative of three independent experiments. The difference in EC50 was significant (***, p < 0.001) as determined by Student’s t test. M, molar. C, HeLa cells transiently expressing varying amounts of PAR1 WT or PAR1 AKKAA mutant were labeled with myo-[3H]inositol, incubated with 10 nm or 1 nm thrombin for 10 min at 37 °C, and [3H]IPs formed were measured. The data (mean ± S.D., n = 3) are representative of three independent experiments. Surface expression of PAR1 (mean ± S.D., n = 3) was determined by ELISA in the same experiment.

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FIGURE 3. The activated PAR1 AKKAA mutant with defective AP-2 binding exhibits enhanced G protein coupling efficiency. A, HeLa cells expressing the PAR1 AKKAA mutant transfected with nonspecific (ns) or μ2-adaptnspecific siRNAs labeled with myo-[3H]inositol were incubated with 10 nm thrombin for varying times at 37 °C, and [3H]IPs formed were measured. The data (mean ± S.D., n = 3) are representative of three independent experiments. The difference in EC50 was significant (***, p < 0.001) as determined by Student’s t test. M, molar. C, HeLa cells transiently expressing varying amounts of PAR1 WT or PAR1 AKKAA mutant were labeled with myo-[3H]inositol, incubated with 10 nm or 1 nm thrombin for 10 min at 37 °C, and [3H]IPs formed were measured. The data (mean ± S.D., n = 3) are representative of three independent experiments. Surface expression of PAR1 (mean ± S.D., n = 3) was determined by ELISA in the same experiment.

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naling, thrombin concentration effect curves were performed. HeLa cells expressing the PAR1 WT or AKKAA mutant labeled with \textit{myo-[3H]inositol} were incubated with varying concentrations of thrombin for 10 min at 37 °C, and then the accumulation of \([3H]\) IPs was examined. The effective concentration of thrombin to stimulate a half-maximal response was modestly but significantly greater for the PAR1 WT compared with the AKKAA mutant (Fig. 3B), suggesting that the activated PAR1 AKKAA mutant couples more efficiently to G protein signaling.

To further examine this effect, the relationship between the amount of PAR1 WT and AKKAA mutant expressed at the cell surface to the extent of PI hydrolysis was determined. As the level of PAR1 WT expression at the cell surface was increased (by transfection of cDNA), a greater accumulation of \([3H]\) IPs was detected following stimulation with either saturating or subsaturating concentrations of thrombin (Fig. 3C). Thrombin activation of the PAR1 AKKAA mutant resulted in a similar increase in thrombin-induced PI hydrolysis compared with the wild-type receptor expressed at comparable levels (Fig. 3C). Thus, the amount of PAR1 present on the cell surface contributes to the extent of thrombin-stimulated PI hydrolysis, suggesting that the greater capacity of PAR1 AKKAA mutant to stimulate G protein signaling (Fig. 3A) is attributed, at least in part, to increased surface expression (Fig. 1).

The \textit{PAR1} Wild Type and AKKAA Mutant Associate with \(G_q\) Similarly—To determine whether differences in PI signaling exhibited by the PAR1 WT and AKKAA mutant were due to \(G_q\) association, we examined receptor interaction with \(G_q\) by coimmunoprecipitation using COS7 cells. COS7 cells were utilized because they exhibit high transfection efficiency and because transfected PAR1 stimulates PI hydrolysis following incubation with thrombin (23). Cells were transiently cotransfected with the FLAG-tagged PAR1 WT or AKKAA mutant together with or without \(G_q\) containing an internal HA epitope tag. Cell lysates were immunoprecipitated with anti-FLAG antibody. HA-G\(q\) associated with PAR1 WT or AKKAA mutant was quantitated and expressed as a fraction of PAR1 WT (A) or untreated control cells (B). The PAR1 WT and AKKAA mutant were because of \(G_q\) association, we examined receptor interaction with \(G_q\) by coimmunoprecipitation using COS7 cells. COS7 cells were utilized because they exhibit high transfection efficiency and because transfected PAR1 stimulates PI hydrolysis following incubation with thrombin (23).
interaction with Gaq occurred basally, and the amount of Gaq coimmunoprecipitated was similar with both the wild-type and mutant receptor (Fig. 4A). Moreover, stimulation of either the PAR1 WT or AKKAA mutant with peptide agonist failed to affect the extent of receptor association with Gaq (Fig. 4B). These findings suggest that PAR1 association of Gaq is not likely to contribute to differences observed between wild-type and mutant receptor signaling.

**Signaling by the PAR1 Wild Type and AKKAA Mutant Is Differentially Regulated by RGS Proteins**—The regulation of GPCR signaling occurs through multiple mechanisms and at both the receptor and G protein effector levels. The “regulator of G protein signaling” (RGS) proteins accelerate the hydrolysis of GTP by the Gα subunit and, thereby, function as negative regulators of G protein signaling (14). To determine whether PAR1 WT and AKKAA mutant signaling is differentially regulated by RGS proteins, the expression of RGS proteins in the HeLa and human EA.hy926 endothelial cell lines were first profiled using RT-PCR and primers that detect all conventional RGS mRNA transcripts, as described previously (18). Only a subset of transcripts for conventional RGS proteins were detected in HeLa and endothelial cells (Fig. 5). These included RGS2, RGS3, RGS4, and RGS5 of the R4 family of RGS proteins, which are known to effectively regulate Gq subtype Gα subunits (27). The RZ family members RGS19 and RGS20 and the R12 family member RGS10 were also detected in HeLa and endothelial cells (Fig. 5) but are not critical regulators of Gq signaling (28).

To assess the activity of the R4 family members on PAR1 signaling, HeLa cells were cotransfected with PAR1 and HA-tagged RGS proteins of the R4 family. RGS2, RGS4, and RGS5 were tagged with an HA epitope at the C terminus, whereas RGS3 contained three tandem HA epitopes at the N terminus. These findings suggest that PAR1 association of RGS3 is observed to cause a modest but significant increase in activated PAR1 wild-type signaling (Fig. 6, A and B). In contrast, only coexpression of RGS5 was observed to cause a modest but significant decrease in PAR1 AKKAA mutant signaling, although its overall effect was reduced substantially with its effect at wild-type PAR1 (Fig. 6, B and C). These findings suggest that RGS2, RGS3, RGS4, and RGS5 function as negative regulators of activated PAR1-induced PI signaling, whereas the PAR1 AKKAA mutant with defective AP-2 interaction is less sensitive to RGS protein regulation of agonist-stimulated G protein signaling.

To confirm RGS protein function on PAR1 signaling, we examined whether depletion of individual endogenous R4 family members by siRNA affects PAR1 signaling. We employed transfection conditions optimized for effective siRNA-mediated depletion of individual epitope-tagged RGS proteins expressed in HeLa cells (Fig. 7A) and then measured the accumulation of [3H]IPs following activation of the PAR1 wild type or AKKAA mutant with thrombin for 60 min. To control for variations in signaling because of changes in PAR1 expression, the data were normalized to the amount of receptor detected on the cell surface in the different transfection conditions. In cells expressing wild-type PAR1 alone, the extent of thrombin-induced PI hydrolysis increased with the amount of wild-type PAR1 expressed at the cell surface (Fig. 6B), whereas coexpression of varying amounts of RGS2, RGS3, RGS4, and RGS5 proteins significantly reduced thrombin-stimulated PI hydrolysis (Fig. 6B). Untransfected cells not expressing PAR1 or RGS proteins were stimulated with thrombin in parallel and showed a minimal increase in PI hydrolysis (Fig. 6, A and C). In contrast, only coexpression of RGS3 was observed to cause a modest but significant decrease in PAR1 AKKAA mutant signaling, although its overall effect was reduced substantially compared with its effect at wild-type PAR1 (Fig. 6, B and C).
likely that other R4 family members have redundant functions or important functions in the regulation of PAR1 signaling in other cell types.

Coimmunoprecipitation experiments were then performed to determine whether RGS proteins form a complex with endogenous Goq following activation of PAR1. The expression

FIGURE 6. Activated PAR1 signaling is negatively regulated by the R4 family of RGS proteins. A, domain structure of the HA-tagged R4 family of RGS proteins expressed in HeLa and endothelial cells. HeLa cells transiently expressing FLAG-tagged PAR1 WT and AKKAA mutant together with increasing amounts of HA-tagged RGS proteins, pcDNA3 vector, or untransfected (UT) control were either lysed or processed as described in B and C. Cell lysates were immunoblotted (IB) with anti-HA antibody to detect RGS protein expression or with anti-actin antibodies as a control. B and C, cells labeled with myo-[3H]inositol were incubated with 10 nM thrombin for 60 min at 37 °C, and [3H]IPs formed were measured. The data (mean ± S.D., n = 3) are representative of three independent experiments. The amount of PAR1 expressed on the cell surface (mean ± S.D., n = 3) for each transfected condition was determined by ELISA. The results are plotted as the fraction of [3H]IPs formed relative to the maximal response versus the amount of PAR1 expressed on the cell surface. The difference between the PI signaling normalized to receptor surface expression (mean ± S.D., n = 3) observed with PAR1 WT expressed alone in cells compared with cells coexpressing the various RGS proteins was significant as determined by single ANOVA and Tukey’s HSD post hoc test (**, p < 0.01). However, only coexpression of RGS3 with the PAR1 AKKAA mutant caused a significant decrease in PI signaling compared with PAR1 AKKAA expressed alone as determined by single ANOVA and Tukey’s HSD post hoc test (**, p < 0.01).
of RGS5 is low in HeLa cells, and because robust expression of RGS proteins is necessary for the coimmunoprecipitation analysis with Gq, we used the HA-tagged RGS3 protein (100 kDa), which was more easily detectable in post-precipitation immunoblotting as compared with the other RGS proteins. HeLa cells stably expressing the PAR1 WT or AKKAA mutant were transiently transfected with HA-tagged RGS3, stimulated with or without thrombin for 5 min, lysed, and immunoprecipitated with an anti-Gq antibody. The presence of RGS3 was detected by immunoblotting. Activation of wild-type PAR1 with thrombin enhanced the association of Gq and RGS3 (Fig. 8A). In contrast, thrombin stimulation of the PAR1 AKKAA mutant failed to increase RGS3 protein association with Gq (Fig. 8A). These findings suggest that AP-2 may function in PAR1-induced interaction of Gq with RGS3. To assess the function of AP-2 in agonist-induced RGS3 protein association with Gq, we used siRNA to deplete cells of AP-2. However, in AP-2-deficient cells, the interaction of PAR1 failed to cause Gq association with RGS3 (Fig. 8B). Taken together, these findings suggest that AP-2 may function as an adaptor to facilitate RGS protein recruitment to Gq protein.

**DISCUSSION**

GPCR signaling is precisely regulated through various mechanisms mediated by β-arrestins, which function to uncouple the receptor from G protein signaling and promote receptor internalization (29). However, not all GPCRs utilize β-arrestins for desensitization or internalization. We showed previously that internalization of activated PAR1 occurs independently of β-arrestins and requires the clathrin adaptor proteins AP-2 and epsin-1 (4, 10). However, it remained unclear whether AP-2 or epsin-1 regulates PAR1 signaling. Here, we now report that AP-2, and not epsin-1, regulates PAR1 signaling through two distinct mechanisms. Depletion of AP-2 by siRNA or expression of a PAR1 mutant defective in AP-2 binding resulted in enhanced signaling, which correlated with elevated PAR1 expression at the cell surface. Thus, AP-2 regulates PAR1 signaling by modulating the amount of receptor expressed at the cell surface. Our data also revealed that certain isoforms of the R4 subfamily of RGS proteins control PAR1 signaling through an AP-2-dependent mechanism that involves agonist-stimulated recruitment of RGS proteins to Gq protein. These findings suggest a second mechanism by which AP-2 regulates activated PAR1 signaling, namely through the formation of an RGS-G protein complex that efficiently shuts off signaling.

AP-2 is a stable complex, composed of four distinct subunits, that binds to phosphatidylinositol-4,5-bisphosphate and has established functions in clathrin-coated pit assembly and cargo recruitment (30). However, the function of AP-2 in regulating signaling responses is less clear. Previous studies showed that the μ2-adaptin subunit of AP-2 interacts with the type I 4-phosphate 5-kinase core domain to affect phosphatidylinositol-4,5-bisphosphate synthesis (31). Moreover, the binding of endocytic cargo proteins to the μ2-adaptin subunit results in the potent stimulation of type I 4-phosphate 5-kinase activity.
Clathrin Adaptor AP-2 Regulates GPCR Signaling

In addition to its established function as an endocytic clathrin adaptor protein, AP-2 appears to regulate post-endocytic trafficking. AP-2 has been shown to associate with the N-formyl peptide receptor, a GPCR, on perinuclear endosomes via β-arrestins to facilitate recycling (33). Intriguingly, however, depletion of endogenous AP-2 resulted in the initiation of apoptosis induced by multiple GPCR-specific ligands. These findings suggest that AP-2 has a critical role in GPCR recycling that appears to be linked to cell survival. Recently, AP-2 and phosphatidylinositol clathrin assembly lymphoid-myeloid have been shown to regulate the cellular level of the Alzheimer amyloid precursor protein cleaved C-terminal fragment via the autophagy pathway (34). These findings indicate that AP-2/phosphatidylinositol clathrin assembly lymphoid-myeloid function as autophagic adaptors that recognize and recruit cargo from the endocytic pathway to the LC-3-mediated degradation pathway. Given the emerging evidence that AP-2 has diverse regulatory functions in signaling and trafficking, we hypothesized that AP-2 might regulate activated PAR1-induced G protein signaling.

In this study, we found that AP-2 has a role in controlling PAR1 signaling by modulating receptor expression at the cell surface and through recruitment of RGS proteins. We showed previously that constitutive internalization of PAR1 is mediated by the μ2-adaptin subunit of AP-2 that binds directly to a C-tail distal tyrosine-based motif. In HeLa cells and endothelial cells, siRNA-mediated depletion of AP-2 caused a significant increase in PAR1 expression at the cell surface and enhanced thrombin-stimulated signaling. A PAR1 AKKAA mutant defective in AP-2 binding exhibited similar effects, suggesting that the amount of PAR1 present on the cell surface is related to the extent of signaling. In invasive breast carcinoma cells, increased expression of PAR1 has been correlated with invasion and metastasis (6, 35). We discovered that PAR1 trafficking is severely altered in metastatic breast carcinoma but not in non-metastatic or normal breast epithelial cells. Consequently, PAR1 is not sorted to lysosomes but, rather, internalized and recycled back to the cell surface and results in sustained signaling and enhanced cellular invasion. Thus, maintaining an appropriate amount of PAR1 at the cell surface is critical for proper signaling and appropriate cellular responses.

**FIGURE 8. AP-2 mediates activated PAR1-stimulated Gαq-RGS protein complex formation.** A, HeLa cells stably expressing FLAG-tagged PAR1 WT or AKKAA mutant were transiently transfected with HA-tagged RGS3 (3xHA-RGS3) and incubated with 10 nM thrombin (α-Th) for 5 min at 37 °C. Equivalent amounts of cell lysates were immunoprecipitated (IP) with anti-Gαq antibody, and coassociated RGS3 was detected with anti-HA antibody. Cell lysates were probed with anti-HA, anti-actin, and anti-PAR1 antibody as controls. IB, immunoblot. B, HeLa cells stably expressing FLAG-tagged PAR1 WT were transfected with nonspecific (ns) or μ2-adaptin-specific siRNA together with HA-tagged RGS3. Cells were then incubated with 10 nM thrombin for 5 min at 37 °C, and immunoprecipitated as described above. Cell lysates were immunoblotted with anti-μ2-adaptin antibody to evaluate the efficiency of AP-2 depletion. The data are representative of three independent experiments.
Clathrin Adaptor AP-2 Regulates GPCR Signaling

In addition to modulating PAR1 surface expression, AP-2 also appears to regulate the recruitment of RGS proteins to facilitate termination of PAR1 signaling. The R4 subfamily of RGS proteins functions mainly as GTPase-accelerating proteins for Goq/11 and Goaip proteins (14). We found that a subset of R4 subfamily of RGS proteins, including RGS2, RGS3, RGS4, and RGS5, are expressed in HeLa and endothelial cells. In addition, ectopic expression of RGS2, RGS3, RGS4, and RGS5 markedly attenuated PAR1 signaling, whereas the PAR1 AKKAA mutant defective in AP-2 binding was unaffected. Intriguingly, only siRNA-mediated depletion of RGS5 caused a significant increase in thrombin-activated PAR1 signaling, whereas loss of RGS2, RGS3, and RGS4 expression had no effect. However, signaling by the PAR1 AKKAA mutant was not affected by the loss of RGS5 expression, suggesting that an intact AP-2 binding site is important for regulation by the RGS5 protein. These findings further indicate that RGS5 has a unique function in PAR1 signaling in HeLa cells on the basis of the siRNA-mediated depletion approach. Interestingly, a prior siRNA screen of RGS protein specificity at PAR1 signaling examined in HEK293 cells revealed an important role for RGS2 and RGS8 but not for any other R4 family members (18). RGS8 expression was not detected in HeLa or endothelial cells, but RGS2 expression was confirmed (Fig. 5). Previous studies have clearly established a role for RGS2 in regulation of thrombin signaling in vitro and in vivo (36), suggesting that the failure of RGS2 depletion to affect thrombin signaling in HeLa cells may be due to cell type-specific responses. Indeed, in platelets, thrombin signaling (mediated predominantly by PAR1) occurs through regulation of RGS18 phosphorylation, which modulates its interaction with 14-3-3, spinophilin, and SHP-1 to control G protein signaling (37, 38). However, RGS18 expression was not detected in HeLa and endothelial cells in this study, suggesting that RGS proteins are expressed and regulated uniquely in distinct cell types.

In summary, our work illustrates an important function for the clathrin adaptor AP-2 in the regulation of PAR1 signaling. AP-2 controls the level of PAR1 present at the cell surface, which correlates with the magnitude of signaling, at least for Goq-stimulated PI hydrolysis. In addition, AP-2 modulates RGS protein recruitment to Goq protein in response to PAR1 activation and, thereby, provides an additional mode by which GPCR signaling can be regulated. Future studies will be important to determine whether other GPCRs that associate with AP-2, such as the N-formyl peptide receptor or thromboxane A2 receptor TPβ, are regulated similarly (33, 39). The precise mechanism by which AP-2 affects RGS protein recruitment to G proteins remains unclear and is an important future pursuit.

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