Protease-activated receptor-1 (PAR1) is a G-protein-coupled receptor (GPCR) for the coagulant protease thrombin. Similar to other GPCRs, PAR1 is promiscuous and couples to multiple heterotrimeric G-protein subtypes in the same cell and promotes diverse cellular responses. The molecular mechanism by which activation of a given GPCR with the same ligand permits coupling to multiple G-protein subtypes is unclear. Here, we report that N-linked glycosylation of PAR1 at extracellular loop 2 (ECL2) controls G12/13 versus Gq coupling specificity in response to thrombin stimulation. A PAR1 mutant deficient in glycosylation at ECL2 was more effective at stimulating Gq-mediated phosphoinositide signaling compared with glycosylated wildtype PAR1. In contrast, wildtype PAR1 displayed a greater efficacy at G12/13-dependent RhoA activation compared with mutant receptor lacking glycosylation at ECL2. Endogenous PAR1 rendered deficient in glycosylation using tunicamycin, a glycoprotein synthesis inhibitor, also exhibited increased PI signaling and diminished RhoA activation opposite to native receptor. Remarkably, PAR1 wildtype and glycosylation-deficient mutants were equally effective at coupling to Gq, and β-arrestin-1. Consistent with preferential G12/13 coupling, thrombin-stimulated PAR1 wildtype strongly induced RhoA-mediated stress fiber formation compared with mutant receptor. In striking contrast, glycosylation-deficient PAR1 was more effective at increasing cellular proliferation, associated with Gq signaling, than wildtype receptor. These studies suggest that N-linked glycosylation at ECL2 contributes to the stabilization of an active PAR1 state that preferentially couples to G12/13 versus Gq, and defines a previously unidentified function for N-linked glycosylation of GPCRs in regulating G-protein signaling bias.

Significance

G-protein-coupled receptors (GPCRs) are the largest class of mammalian signaling receptors and mediate vast physiological responses. The capacity to modulate GPCR signaling therapeutically is important for treatment of various diseases, and discovering new aspects of receptor signaling is critical for drug development. Protease-activated receptor-1 (PAR1) is GPCR for thrombin. Similar to other GPCRs, PAR1 is promiscuous and couples to multiple heterotrimeric G-protein subtypes in the same cell. How a single GPCR can couple to multiple G-protein subtypes concurrently has remained an enigma. We demonstrate that N-linked glycosylation of PAR1 regulates G-protein coupling specificity and differentially controls cellular responses. Thus, the status of GPCR glycosylation is a critical determinant for specifying coupling to distinct G-protein subtypes.
with signaling effectors (16, 17). Besides binding of ligands to allosteric sites on the receptor, GPCR interaction with other proteins and segregation into plasma membrane microdomains have been reported to affect receptor bias toward particular effectors. However, whether posttranslational modification of a given GPCR affects ligand-induced coupling to distinct signaling effectors is not known. Here, we report that asparagine (N)-linked glycosylation of PAR1 at ECL2 regulates G_{12/13} versus G_{q} protein coupling specificity, which modulates the robustness of thrombin-induced G_{12/13}-dependent RhoA mediated stress fiber formation and cellular proliferation associated with G_{q} signaling in fibroblasts. These findings are the first, to our knowledge, to define a function for N-linked glycosylation of a GPCR in regulating G-protein signaling bias.

Results

PAR1 Deficient in Glycosylation at ECL2 Exhibits Enhanced G_{q}-Mediated PI Signaling. PAR1 containing amino acid Asn250Ala and Asn259Ala mutations that lacks N-linked glycosylation at ECL2, designated NA ECL2, displayed a greater capacity to stimulate PI hydrolysis compared with wildtype (WT) receptor following exposure to thrombin in HeLa cells (18). Similar differences in thrombin-induced PI hydrolysis were observed in COS-7 cells transiently expressing comparable levels of cell surface PAR1 WT and NA ECL2 mutant (Fig. S1 A and B). To determine whether activated PAR1 coupling to G_{q} protein specifically mediates enhanced PI signaling, siRNA was used to deplete cells of G_{q} expression (Fig. L4). HeLa cells stably expressing PAR1 WT and NA ECL2 mutant were transfected with nonspecific or G_{q}-specific siRNAs and labeled with myo-[^3H]inositol. Thrombin stimulated a greater increase in PI hydrolysis in PAR1 NA ECL2 cells compared with WT cells transfected with nonspecific siRNA that was not due to differences in receptor expression (Fig. 1 A and B). However, both PAR1 WT and NA ECL2 cells deficient in G_{q} expression failed to elicit a thrombin response (Fig. 1 A), indicating that activated PAR1-induced PI hydrolysis is mediated by G_{q} in HeLa cells. To determine if glycosylation of PAR1 at ECL2 affects G_{q}-stimulated PI signaling by modulating receptor association with G_{q} protein, we performed coimmunoprecipitation (co-IP) assays. HeLa cells stably expressing PAR1 WT or NA ECL2 mutant were transiently transfected with different amounts of

![Fig. 1. PAR1 NA ECL2 mutant exhibits enhanced G_{q}-mediated PI hydrolysis.](image-url)
Goαq plasmid. PAR1 was immunoprecipitated, and coassociated Goαq was detected by immunoblotting. PAR1 NA ECL2 exhibited a greater capacity to co-IP with Goαq basally compared with WT receptor or IgG control (Fig. 1C, lanes 1, 4, and 7). Remarkably, thrombin induced a twofold increase in PAR1 WT–Goαq association compared with control cells (Fig. 1D, lanes 1–3). Although PAR1 NA ECL2 mutant exhibited a statistically greater capacity to interact with Goαq basally (Fig. 1C and D), receptor–Goαq coassociation was not further increased by thrombin stimulation (Fig. 1D, lanes 4 and 5). The PAR1 NA ECL2 mutant expressed in COS-7 cells also showed enhanced association with Goαq protein compared with WT receptor (Fig. S1C), indicating that the observed findings are not cell type specific.

In many cell types, PAR1 and Goαq localize to caveolae (19, 20), plasma membrane lipid rafts enriched in cholesterol and caveolin-1. To exclude the possibility that altered PAR1 WT versus NA ECL2 mutant activation of Goαq protein might be due to differential localization in caveolae, sucrose gradient fractionation was used. A similar distribution of PAR1 WT, NA ECL2, and Goαq proteins in caveolin-1 enriched and nonenriched fractions was observed (Fig. S1D). These data suggest that thrombin-activated PAR1 lacking glycosylation at ECL2 displays an enhanced capacity to couple to Goαq-dependent PI signaling in different cell types that is not due to alterations in receptor expression or distribution to caveolae.

PAR1 NA ECL2 Displays Diminished G12/13-Mediated RhoA Signaling.

To examine whether glycosylation at ECL2 regulates PAR1 coupling to other G-protein subtypes, we examined RhoA activation, an effector of G12/13 proteins. HeLa cells stably expressing comparable PAR1 WT and NA ECL2 at the cell surface were stimulated with thrombin, and activation of RhoA was measured using GST-rototkin Rho-binding domain (RBD) pull-down assays (21). Thrombin induced a robust increase in RhoA activation at 2.5 min in PAR1 WT cells that subsided after 15–30 min (Fig. 2A, lanes 3–6), whereas untransfected cells not expressing PAR1 were not responsive (Fig. 2A, lanes 1 and 2). In contrast, RhoA activation was significantly reduced in thrombin-treated PAR1 NA ECL2 cells examined over the same time frame (Fig. 2A, lanes 3–10). Consistent with these findings, activated PAR1 WT was more potent at stimulating RhoA activation than the NA ECL2 mutant assessed at earlier time points (Fig. S2A). The peptide agonist SFLLRN also significantly increased RhoA activation in PAR1 WT cells compared with NA ECL2 expressing cells (Fig. 2B, lanes 4–9), indicating that neither thrombin binding nor proteolytic cleavage contribute to differential RhoA activation. Compared with PAR1 NA ECL2 mutant, RhoA activation induced by thrombin-activated PAR1 WT was also markedly increased in COS-7 cells (Fig. S2B). These data suggest that activated PAR1 WT has a greater capacity to induce RhoA signaling compared to mutant receptor deficient in glycosylation at ECL2.

To determine if the differences in RhoA activation exhibited by PAR1 WT versus NA ECL2 mutant are mediated by G12/13 proteins, siRNAs were used to deplete cells of G12 and/or G13 expression. PAR1 WT and NA ECL2 mutant-expressing HeLa cells were transfected with nonspecific, G12, G13, or G12 and G13 siRNAs and then stimulated with thrombin. Immunoblotting analysis indicates that siRNAs specifically depleted G12 or G13 protein in PAR1 WT and NA ECL2 cells (Fig. 3A and B). Thrombin-activated PAR1 WT caused a significant increase in RhoA activation in nonspecific siRNA control cells that was virtually abolished in cells depleted of either G12 or G13 proteins (Fig. 3A, lanes 5–8). The modest increase in RhoA activation observed in thrombin-treated PAR1 NA ECL2 siRNA transfected control cells was also significantly inhibited in G12 and G13 knockdown cells (Fig. 3B, lanes 5–8). These findings indicate that PAR1-induced RhoA activation is dependent on G12/13 proteins.

We next examined if PAR1 WT and NA ECL2 mutant showed differences in G12/13 association using co-IP. HeLa cells stably expressing PAR1 WT and NA ECL2 mutant were transiently transfected with increasing amounts of G12/13 plasmid. Cells were then stimulated with thrombin and immunoprecipitated, and the presence of coassociated G12 protein was detected. PAR1 WT showed greater association with G12 compared with NA ECL2 mutant basally when expressed at low levels (Fig. 4A, lanes 1–5 and 7–9) and after thrombin stimulation (Fig. 4B, lanes 6 and 10). PAR1 WT also exhibited a preference for G13 association compared with the NA ECL2 mutant both in the presence and absence of thrombin stimulation (Fig. S3). Bioluminescence resonance energy transfer (BRET) measurement

Fig. 2. PAR1 WT and NA ECL2 differentially activate RhoA. FLAG-PAR1 WT and NA ECL2 mutant HeLa cells displaying similar cell surface expression (WT, 0.363 ± 0.079; NA ECL2, 0.263 ± 0.049, OD units) were stimulated with 10 nM α-Th (A) or 100 μM SFLLRN (B), lysed, and processed for GST-RBD pull-down assays, and activated RhoA was detected by immunoblotting. UT cells were processed similarly. The data (mean ± SD; n = 3) were normalized to total RhoA, are representative of three independent experiments, and were significant (*P < 0.05; **P < 0.001). NS, not significant.
was also used to assess PAR1–Gαi2 association in living cells. COS-7 cells were transiently transfected with either full-length PAR1 WT or NA ECL2 mutant fused to YFP at the C terminus and Gαi2–Rluc that yielded optimal expression (Fig. S4). Cells were then either left untreated or treated with thrombin, and the net BRET signal was quantified. Thrombin induced a statistically significant increase in the net BRET response elicited by PAR1 WT–YFP and Gαi2–Rluc compared with untreated control cells (Fig. 4B), suggesting that the activated PAR1 WT–Gαi2 complex undergoes a conformational change. In contrast, thrombin failed to induce a change in BRET signal in cells coexpressing PAR1 NA ECL2 and Gαi2–Rluc (Fig. 4B). These studies suggest that activation of PAR1 glycosylated at ECL2 results in a conformational state that preferentially couples to Gαi13.

**Glycosylation of PAR1 at ECL2 Does Not Affect G_i Coupling or β-arrestin-1 Recruitment.** In addition to Gq and G12/13, PAR1 is known to signal through the Gi protein in various cell types (1, 9). To investigate whether glycosylation of PAR1 at ECL2 regulates coupling to Gi, we examined PAR1–Gi association by BRET. COS-7 cells were transiently transfected with a constant amount of Gαq–Rluc and increasing amounts of either PAR1 WT–YFP or NA ECL2–YFP, and the net BRET was determined. A hyperbolic increase in net BRET was observed as the ratio of PAR1 WT–YFP to Gαq–Rluc expression was increased (Fig. 5A), suggesting a specific interaction. PAR1 NA ECL2–YFP and Gαq–Rluc saturation curves also yielded a hyperbolic increase in the net BRET signal (Fig. 5A), suggesting that glycosylation of PAR1 at ECL2 does not affect basal association with Gi protein. We next examined whether thrombin induced a change in PAR1–Gi association. In COS-7 cells coexpressing equivalent amounts of either PAR1 WT–YFP or NA ECL2–YFP together with Gαq–Rluc (Fig. S5 A and B), the addition of thrombin resulted in a rapid and transient increase in net BRET that peaked at 1 min and returned to baseline (Fig. 5B). These findings indicate that activated PAR1 coupling to Gi is not affected by glycosylation at ECL2. These results were confirmed by examining PAR1–Gi association using co-IP. Activation of either PAR1 WT–YFP or NA ECL2–YFP with thrombin resulted in a marked fourfold increase in Gαq–Rluc association compared with untreated control cells (Fig. 5C), consistent with the equal capacity of both PAR1 WT and NA ECL2 to associate with Gi protein.

In addition to G proteins, many GPCRs display bias toward the multifunctional β-arrestin adaptor proteins (22). Since the β-arrestin-1 isoform is the principal regulator of thrombin-activated PAR1 signaling (23, 24), recruitment of β-arrestin-1 to PAR1 was examined by BRET. Intriguingly, PAR1 WT and NA ECL2 expressed at similar levels were equally effective at recruiting β-arrestin-1 following thrombin stimulation (Fig. 5 D and E and Fig. S5C), indicating that glycosylation does not affect receptor–β-arrestin-1 association. Together, these data suggest that unlike G12/13 and Gq, PAR1 deficient in glycosylation at ECL2 displays no bias toward Gi or β-arrestin-1.

**Quantifying PAR1 signaling bias.** The operational model of agonism was next used to quantify the G-protein coupling bias of PAR1 WT versus NA ECL2 mutant (25, 26). The concentration–response curves of Gq-stimulated PI hydrolysis, G12/13-induced RhoA activation, and Gi-activated PAR1 association (BRET) (Fig. S6) were normalized to receptor expression and fitted to the Black–Leff model of agonism to obtain the dissociation constant of the agonist–receptor complex (Kd) and an estimation of the τ transducer constant. The parameters τ and Kd provide an approximation of the signal transduction efficiency and intrinsic agonist efficacy (Tables S1 and S2). The transduction coefficient for each pathway was then calculated as log(τ/Kd). To determine the relative bias of PAR1 NA ECL2 mutant to WT receptor, Gi BRET response was used as the reference pathway, because Gi displayed the least difference between WT and NA ECL2 mutant receptor. Each of the PAR1 WT and NA ECL2 mutant G-protein signaling assays were performed in the same cell type with comparable cell surface expression (Fig. S6). To compare thrombin-induced Gq and G12/13 signaling pathway bias between the PAR1 WT and NA ECL2 mutant, the Δ(log(τ/Kd)) was calculated (Table 1 and Fig. S6). The calculated biases (Δ(log(τ/Kd)) values) indicate that PAR1 NA ECL2 mutant is 0.58-fold less effective at coupling to the G12/13–RhoA pathway compared with WT receptor, whereas PAR1 NA ECL2 is 5.27-fold more effective at stimulating Gq-induced PI hydrolysis than WT receptor. These findings strongly suggest that N-linked glycosylation of PAR1 at ECL2 regulates G12/13-versus Gq-protein bias signaling.
Glycosylation-deficient endogenous PAR1 displays G-protein signaling bias. PAR1 is expressed in endothelial cells and signals through G_{q} and G_{12/13} to promote inflammatory responses (27). To determine if glycosylation of endogenous PAR1 regulates G-protein signaling bias in human cultured endothelial cells, we used the pharmacological inhibitor tunicamycin, which blocks the first step in glycoprotein synthesis. Glycosylated PAR1 migrates as a broad ~75-kDa protein that was reduced to its predicted molecular weight of ~40 kDa following treatment with tunicamycin (Fig. 6A, lanes 3 and 5), consistent with previous studies (18). Tunicamycin also caused partial loss of PAR1 surface expression (Fig. S7A); however, the majority of the receptor trafficked to the cell surface. This was confirmed by examining PAR1’s susceptibility to cleavage by thrombin at 4 °C (18), which caused a shift in the size of the major PAR1 species in both control and tunicamycin-treated cells (Fig. 6A, lanes 3–6). In tunicamycin-treated endothelial cells expressing deglycosylated endogenous PAR1, thrombin induced a significantly greater increase in PI hydrolysis compared with control cells expressing the glycosylated native receptor (Fig. 6B). In striking contrast, thrombin caused a marked response in RhoA activation under control conditions that was virtually ablated in cells treated with tunicamycin (Fig. 6C). To ensure that tunicamycin does not globally affect cell signaling, epidermal growth factor (EGF)-induced ERK1/2 activation was examined and shown to be equivocal in control and tunicamycin-treated cells (Fig. S7B). These findings provide evidence that N-linked glycosylation of PAR1 at ECL2 promotes enhanced G-protein-mediated PI signaling and diminished RhoA activation in a natural context.

PAR1 NA ECL2 Exhibits Reduced RhoA-Mediated Stress Fiber Formation and Enhanced Cellular Proliferation. To test whether the effects of N-linked glycosylation on PAR1 differential coupling to G_{q} versus G_{12/13} impacts cellular responses, we examined actin stress fiber formation. Serum-deprived HeLa cells expressing PAR1 WT and NA ECL2 mutant were stimulated with thrombin, stained with phalloidin-TRITC to visualize F-actin filaments and imaged by confocal microscopy. Thrombin caused a marked increase in actin stress fiber formation in PAR1 WT cells (Fig. 7A), whereas the response was significantly diminished in NA ECL2 cells (Fig. 7A). Inhibition of RhoA activation with C3 toxin virtually abolished thrombin-induced actin stress fiber formation in PAR1 WT cells (Fig. 7B), consistent with G_{12/13}-induced RhoA-mediated stress fiber formation as previously reported (27). These results suggest that N-linked glycosylation of PAR1 at ECL2 regulates preferential coupling to G_{12/13} and induction of RhoA-mediated stress fiber formation.

Thrombin activation of PAR1 promotes G_{q}-dependent mitogenic responses in fibroblasts (28, 29). To determine if N-linked glycosylation of PAR1 at ECL2 affects thrombin-induced cellular proliferation, [3H]thymidine incorporation was measured to assess DNA synthesis in fibroblasts. In these studies, mouse lung fibroblasts derived from Par1^{−/−} gene knockouts stably expressing PAR1 WT or NA ECL2 mutant were deprived of serum and incubated with thrombin, and the amount of [3H]thymidine incorporation was quantified. Remarkably, fibroblasts expressing PAR1 NA ECL2 displayed a higher basal level of [3H]thymidine incorporation compared with WT fibroblasts (Fig. 7C), despite lower cell surface expression of PAR1 NA ECL2 compared with WT receptor (Fig. S7C). Moreover, a substantially greater increase in [3H]thymidine incorporation was observed in thrombin-stimulated PAR1 NA ECL2 fibroblasts relative to untreated control or WT fibroblasts (Fig. 7D), whereas the cells responded equivocally to serum stimulation (Fig. 7D). Together, these findings strongly suggest that N-linked glycosylation of PAR1 at ECL2 regulates preferential coupling to G_{12/13} versus G_{q} proteins, which modulate the robustness of thrombin-induced cellular responses in various cell types.

Discussion
In the present study, we define a novel function for N-linked glycosylation of a GPCR in regulation of G-protein signaling bias. A PAR1 mutant deficient in glycosylation at ECL2 favors coupling to G_{q}-mediated PI signaling over G_{12/13}-induced
RhoA activation, opposite of the glycosylated WT receptor. Moreover, endogenous PAR1 lacking glycosylation exhibited an enhanced G-protein-mediated PI response and reduced RhoA activation, in contrast to native receptor. Intriguingly, both PAR1 WT and mutant were equally effective at coupling to Gi and β-arrestin-1. N-linked glycosylation of PAR1 at ECL2 also enhanced thrombin-induced RhoA-mediated stress fiber formation and attenuated cellular proliferation in fibroblasts, consistent with preferential coupling to G12/13 versus Gq proteins. These studies are the first, to our knowledge, to show that N-linked glycosylation of a GPCR is critical for G-protein coupling specificity.

The best described function for N-linked glycosylation of mammalian GPCRs is in proper folding of the nascent protein during translation and export to the cell surface. The majority ∼90% of Class A GPCRs contain N-linked glycosylation N–X–S/T consensus sequences within their N terminus, whereas only ∼30% of the receptors contain consensus sites within the extracellular loops (30). The extent of glycosylation and full utilization of consensus sites likely varies with a given GPCR. PAR1 contains five consensus sites for N-linked glycosylation, three in the N terminus and two in ECL2, and all appear to be modified by glycosylation (18). We previously showed that glycosylation of PAR1 at the N terminus and not the ECL2 is important for efficient transport to the cell surface (18). In addition to cell surface export, other studies suggest a function for N-linked glycosylation in GPCR dimerization. N-linked glycosylation was shown to contribute to the stabilization of bradykinin-B2 receptor homodimerization (31), whereas β1- and α2-adrenergic receptor (AR) heterodimerization was inhibited by glycosylation (32). Although PAR1 NA ECL2 appears largely as a monomer based on immunoblotting analysis (Figs. 1, 4, and 5), a minor higher molecular weight species was sometimes evident. However, we failed to confirm a difference in PAR1 WT versus NA ECL2 mutant dimerization. Another study reported that N-linked glycosylation of the sphingosine-1-phosphate receptor effects caveolae localization (33). Surprisingly, PAR1 distribution into caveolae was not affected by loss of glycosylation at ECL2 (Fig. S1D). Thus, these findings suggest that glycosylation of PAR1 at ECL2 likely serves a distinct function not related to surface export, dimerization, or partitioning into caveolae.

Glycosylation can also influence GPCR–ligand interactions. PAR2, a GPCR related to PAR1, is cleaved and activated by trypsin-like serine proteases but not by thrombin (1). PAR1 WT and NA ECL2 are equally effective at coupling to Gi and β-arrestin-1. (A) COS-7 cells coexpressing increasing PAR1 WT–YFP or NA ECL2–YFP with a constant amount of Gαi–Rluc were analyzed by BRET. Data are representative of three independent experiments. (B) COS-7 cells coexpressing PAR1 WT–YFP or NA ECL2–YFP and Gαi–Rluc were treated with 10 nM α-Th, and BRET was determined. Data (mean ± SD; n = 3) are representative of three independent experiments. (C) COS-7 cells coexpressing PAR1 WT–YFP or NA ECL2–YFP and Gαi–Rluc were stimulated with 10 nM α-Th, immunoprecipitated, and immunoblotted. Data (mean ± SD; n = 3) are from three independent experiments and were significant (***P < 0.001). (D) COS-7 cells coexpressing PAR1 WT–YFP or NA ECL2–YFP and β-arrestin-1–Rluc were stimulated with 10 nM α-Th, and BRET was determined. The data (mean ± SD; n = 3) are representative of three independent experiments. (E) PAR1 surface expression (mean ± SD; n = 3) was measured by ELISA.
Intriguingly, differential glycosylation of the gonadotrophin follicle-stimulating hormone (FSH) ligand modulates the capacity of the cognate FSH receptor to couple to G<sub>i</sub> versus G<sub>q</sub> signaling (35). These findings indicate that naturally occurring heterogeneity of glycosylation of certain peptide hormones can affect GPCR-biased signaling. However, whether naturally occurring glycosylation of GPCRs affects G-protein signaling has not been previously reported.

Significant efforts have been made toward understanding the mechanisms by which certain GPCRs couple to multiple distinct G-protein subtypes in the same cell, but it remains poorly understood. Our results suggest that N-linked glycosylation of PAR1 at ECL2 regulates G-protein coupling specificity. The ECL2 of other class A GPCRs has also been shown to control ligand-directed effects (36, 37). Thus, we hypothesize that ECL2 of other class A GPCRs likely to exist as populations of receptors containing distinct glycan structures even when expressed in the same cell (39). The contribution of these diverse structures to GPCR

glycosylation of PAR2 at the N terminus was shown to affect trypsin but not trypsin cleavage (34), indicating that glycosylation directly affects protease recognition and receptor activation. We previously showed that N-linked glycosylation of PAR1 at either the N terminus or ECL2 has no effect on the rate of receptor cleavage by thrombin (18). Intriguingly, differential glycosylation of the gonadotrophin follicle-stimulating hormone (FSH) ligand modulates the capacity of the cognate FSH receptor to couple to G<sub>i</sub> versus G<sub>q</sub> signaling (35). These findings indicate that naturally occurring heterogeneity of glycosylation of certain peptide hormones can affect GPCR-biased signaling. However, whether naturally occurring glycosylation of GPCRs affects G-protein signaling has not been previously reported.

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function is not known. PAR1 is extensively glycosylated but the nature and diversity of N-glycan modification have not been determined. Importantly, naturally occurring mutations in N-linked glycosylation consensus sequences of Rhodopsin have been linked to retinitis pigmentosa (40, 41), indicating that modulation of GPCR glycosylation status can contribute to disease progression. However, mutations in PAR1 N-linked glycosylation sites have not been identified. In summary, our findings demonstrate for the first time, to our knowledge, that N-linked glycosylation of a GPCR specifies coupling to distinct G-protein subtypes in the same cell.

Materials and Methods

Reagents and Antibodies. Human α-thrombin (α-Th) was from Enzyme Research Laboratories. SFLLRN was synthesized at Tufts University Core Facility. Insulin, transferrin, selenous acid (ITS) premix, caveolin-1, and anti-early endosomal antigen-1 (EEA1) antibodies were from BD Biosciences. ERK1/2 antibodies were from Cell Signaling Technologies. C3 transferase toxin was from Cytoskeleton, Inc. Polyclonal and M2 monoclonal anti-FLAG antibody, TRITC-conjugated phalloidin, tunicamycin, EGF, and anti-β-actin antibody were from Sigma. The anti-PAR1 WEDE antibody was from Beckman Coulter. Anti-PAR1 polyclonal antibody was generated against the YEPFWEDEEKNESGLTEYC peptide. RhoA, G\textsubscript{α\textsubscript{q/11}}, G\textsubscript{α\textsubscript{12}}, and G\textsubscript{α\textsubscript{13}} antibodies were from Santa Cruz Biotechnology. Renilla Luciferase antibody was from Millipore. Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies were from Bio-Rad Laboratories.

cDNAs and Cell Lines. HeLa cells stably expressing N-terminal FLAG-tagged PAR1 WT and NA ECL2 were generated as described in ref. 18. HA-G\textsubscript{α\textsubscript{q}} plasmid was from Philip Wedegaertner, Thomas Jefferson University, Philadelphia, PA. G\textsubscript{α\textsubscript{12}}-EE and G\textsubscript{α\textsubscript{13}}-EE constructs were from Dr. John Hepler, Emory University, Atlanta, GA. G\textsubscript{α\textsubscript{12}}-Rluc, G\textsubscript{α\textsubscript{13}}-Rluc, and G\textsubscript{α\textsubscript{13}}-Rluc, and PAR1-YFP plasmids were from Dr. Jean-Philippe Pin, Montpellier University, Montpellier, France. PAR1–YFP NA ECL2 mutant was generated by site-directed mutagenesis using the QuikChange Mutagenesis kit (Stratagene) and confirmed by dideoxy sequencing. COS-7, HeLa, and endothelial cells were cultured as described in refs. 18, 21, and 42.

Fig. 7. PAR1 NA ECL2 exhibits diminished stress fiber formation and enhanced cellular proliferation. (A) FLAG–PAR1 WT or FLAG–NA ECL2 mutant HeLa cells were treated with 10 nM α-Th for 5 min, stained with phalloidin-TRITC, and imaged. Data (mean ± SD; n = 3) for f-actin fluorescence were quantified from four different images of three independent experiments and were significant (*P < 0.05). (Scale bar, 10 μm.) (B) FLAG–PAR1 WT HeLa cells pre-treated with 1.5 μg/mL C3 toxin for 4 h at 37 °C or DMSO were incubated with 10 nM α-Th for 5 min and stained with phalloidin-TRITC, and f-actin fluorescence was quantified. Data (mean ± SD; n = 3) were significant (**P < 0.001). (Scale bar, 10 μm.) (C) Mouse lung fibroblasts expressing FLAG–PAR1 WT or NA ECL2 mutant were incubated without (basal) or (D) with 10 nM α-Th or 2% FBS, and [\textsuperscript{3}H]thymidine incorporation was measured. Basal [\textsuperscript{3}H]thymidine incorporation (mean ± SD; n = 3) is from three independent experiments and was significant (**P < 0.001). Data (mean ± SD; n = 3) are from α-Th-stimulated [\textsuperscript{3}H]thymidine incorporation from three independent experiments and were significant (*P < 0.05). NS, not significant.
Cell Transfections. See SI Materials and Methods.

**PAR1 Immunoprecipitation and Immunoblotting.** Equivalent amounts of lysates from cells grown in six-well plates were processed for PAR1 immunoprecipitation as described in ref. 18.

**PAR1 Cell Surface ELISA.** HeLa and COS-7 cells expressing FLAG–PAR1 WT or NA ECL2 mutant were grown in 24-well plates and processed for cell surface ELISA as described in ref. 18.

**PI Hydrolysis.** Cells were labeled overnight with 1 μCi/mL of myo-[3H]inositol (American Radiolabeled Chemicals) and treated with agonists, and accumulated [3H]IPs were measured as described in ref. 18.

**BRET Assays.** COS-7 cells transiently expressing PAR1 WT–YFP or NA ECL2–YFP and either G protein–Riuc or β-arrestin–1–Riuc were treated with agonists and analyzed by BRET as described in ref. 42.

**RhoA Activity Assay.** Equivalent amounts of lysates were used for GST-RBD pull-down assays as described in ref. 19.