Protease-activated receptor-1 (PAR1) contains five N-linked glycosylation consensus sites as follows: three residing in the N terminus and two localized on the surface of the second extracellular loop (ECL2). To study the effect of N-linked glycosylation in the regulation of PAR1 signaling and trafficking, we generated mutants in which the critical asparagines of the consensus sites were mutated. Here, we report that both the PAR1 N terminus and ECL2 serve as sites for N-linked glycosylation but have different functions in the regulation of receptor signaling and trafficking. N-Linked glycosylation of the PAR1 N terminus is important for transport to the cell surface, whereas the PAR1 mutant lacking glycosylation at ECL2 (NA ECL2) trafficked to the cell surface like the wild-type receptor. However, activated PAR1 NA ECL2 mutant internalization was impaired compared with wild-type receptor, whereas constitutive internalization of unactivated receptor remained intact. Remarkably, thrombin-activated PAR1 NA ECL2 mutant displayed an enhanced maximal signaling response compared with wild-type receptor. The increased PAR1 NA ECL2 mutant signaling was not due to defects in the ability of thrombin to cleave the receptor or signal termination mechanisms. Rather, the PAR1 NA ECL2 mutant displayed a greater efficacy in thrombin-stimulated G protein signaling. Thus, N-linked glycosylation of the PAR1 extracellular surface likely influences ligand docking interactions and the stability of the active receptor conformation. Together, these studies strongly suggest that N-linked glycosylation of PAR1 at the N terminus versus the surface of ECL2 serves distinct functions critical for proper regulation of receptor trafficking and the fidelity of thrombin signaling.

Thrombin is a coagulant serine protease generated at sites of vascular injury and in thrombotic disease. Thrombin mediates hemostasis and thrombosis and contributes to inflammation and cancer progression (1, 2). Thus, a better understanding of how thrombin elicits cellular responses could lead to novel therapeutic strategies. Protease-activated receptors (PARs) are G protein-coupled receptors (GPCRs) that play a major role in thrombin-mediated cellular responses (1, 3). Protease-activated receptor-1 (PAR1) is the prototype PAR and the principal effector of thrombin signaling in most cell types. PAR1 is activated when the N terminus is cleaved by thrombin, which creates a new N terminus that acts as a tethered ligand that binds intramolecularly to the receptor to initiate transmembrane signaling (4). Activated PAR1 signals through multiple heterotrimERIC G protein subtypes, including G13, G16, and G12/13, to promote diverse cellular responses in various cell types (5–8). The mechanism by which PAR1 selectively couples to specific G protein subtypes is not known. The current model for activation of GPCR signaling posits that agonist binding within the receptor helical core triggers rearrangements of transmembrane helices that expose cytoplasmic domains to initiate G protein activation (9, 10). However, for the class B GPCRs whose natural ligands are peptide hormones, the extracellular domains have a critical role in ligand binding (11). Although PAR1 is a class A rhodopsin-like GPCR, activation of PAR1 occurs via peptide agonist interaction with the receptor extracellular surface domains. Specifically, the PAR1 second extracellular loop (ECL2) is critical for species-specific ligand interactions and peptide agonist recognition (12, 13). An interesting feature of the ECL2 of PAR1 is the inclusion of two N-linked glycosylation consensus sites in the human receptor, which are absent in the Xenopus receptor. Although many mammalian GPCRs are post-translationally modified with N-linked glycosylation at the N terminus, it is less common for the extracellular loops to contain glycosylation modifications (14). Human PAR2 contains a single N-linked glycosylation consensus site within its ECL2 domain that may serve as a site for glycosylation, but its function is unknown (15, 16). Moreover, whether PAR1 is glycosylated at ECL2 and whether this affects ligand-induced receptor activation are not known.

Human PAR1 contains five potential N-linked glycosylation consensus sites as follows: three reside within the N terminus...
and two are localized in ECL2. Interestingly, several studies have suggested that PAR1 is highly glycosylated in different cell types based on a shift in its apparent molecular weight following glycosidase treatment (17–19). In a yeast strain unable to glycosylate the receptor, PAR1 migrates as a single band close to its predicted molecular weight rather than a broad high molecular weight species typically observed in mammalian cells (18). Moreover, global disruption of glycoprotein synthesis with the drug tunicamycin greatly diminished surface expression of PAR1 in Jurkat T lymphocyte cells (20), suggesting that N-linked glycosylation of PAR1 is important for proper trafficking to the cell surface. However, the actual sites of PAR1 N-linked glycosylation and the importance of such site(s) in the regulation of receptor signaling and trafficking have not been determined.

In this study, we examine for the first time the function of N-linked glycosylation of the PAR1 N terminus and ECL2 in the regulation of receptor signaling and trafficking. Our studies reveal that both the N terminus and ECL2 of PAR1 are targeted for N-linked glycosylation. N-Linked glycosylation of the N terminus of PAR1 contributes less to the overall glycosylation status of the receptor and appears to function in export of the receptor to the cell surface. However, a PAR1 mutant lacking ECL2 N-linked glycosylation displays impaired agonist-induced internalization, whereas constitutive internalization remained intact. Strikingly, we also show that ligand activation of PAR1 NA ECL2 mutant exhibits a marked increase in signaling that is not due to the altered ability of thrombin to cleave the receptor or to defects in signal termination. Rather, the PAR1 NA ECL2 mutant exhibits a greater efficacy in activation of G protein-mediated signaling responses. These findings suggest that glycosylation of PAR1 at the surface of ECL2 influences ligand docking interactions that enhance the stabilization of the active receptor conformation. Taken together, this study reveals for the first time distinct functions for PAR1 N-linked glycosylation at the N terminus versus ECL2 that are critical for the regulation of PAR1 signaling and trafficking.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). The PAR1-activating peptide, SFLLRN, was synthesized as the carboxyl amide and purified by reverse phase-high pressure liquid chromatography at Tufts University Core Facility (Boston). PNGase F, sialidase, and endoglycosidase H were purchased from New England Biolabs, Inc. (Ipswich, MA), Tunicamycin, cycloheximide, carbachol, epidermal growth factor (EGF), and hirudin were obtained from Sigma. Rabbit polyclonal anti-FLAG antibody and mouse monoclonal M1 and M2 anti-FLAG antibodies were purchased from Sigma. The anti-PAR1 WDE mouse antibody was purchased from Beckman Coulter (Fullerton, CA). Anti-PAR1 rabbit polyclonal antibody was described previously (21). The mouse monoclonal anti-EGF receptor LA22 antibody was from Upstate Cell Signaling Solutions/Milipore (Temecula, CA). Mouse anti-early endosome antigen-1 (EEA-1) and trans-Golgi network (TGN) 230 antibodies were purchase from BD Transduction Laboratories. Goat anti-mouse IgG antibody was purchased from Thermo Scientific (Rockford, IL). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Bio-Rad.

**cDNAs and Cell Lines**—A cDNA encoding human PAR1 with an N-terminal FLAG epitope sequence cloned into pB) vector was described previously (22) and used for site-directed mutagenesis. Mutations were introduced into FLAG-tagged PAR1 by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) and confirmed by dideoxy sequencing (Moores Cancer Center Core Facility, La Jolla, CA). HeLa cells stably expressing FLAG-tagged PAR1 wild type and mutants were generated and maintained as described previously (22). The immortalized human endothelial cell line EA.hy926 were grown and cultured as we described previously (23). HeLa cells were transiently transfected with a total plasmid amount of 0.4 μg per 24 wells or 2 μg per 6 wells using Lipofectamine reagent (Invitrogen), according to the manufacturer's instructions, and were assayed 48 h after transfection.

**PAR1 Sequence Alignment**—A sequence alignment was performed using the EMBL-EBI ClustalW2 program. The following were used: *Rattus norvegicus* (NCBI reference sequence NM_012950.2); *Mus musculus* (NCBI reference sequence NM_010169.3); *Cricetulus longicaudatus* (GenBank™ accession number X61958.1); *Homo sapiens* (NCBI reference sequence NM_001992.3); *Papio hamadryas* (GenBank™ accession number AF028727.1); *Equus caballus* (NCBI reference sequence XM_001503957.2); *Canis lupis familiaris* (NCBI reference sequence XM_546059.2), and *Xenopus laevis* (NCBI reference sequence NM_001085783.1).

**PAR1 Immunoprecipitation and Immunoblotting**—HeLa cells stably expressing FLAG-tagged PAR1 wild type or mutants were plated at 5 × 10⁵ cells per well in 6-well culture dishes and grown overnight at 37 °C. Cells were washed and incubated with or without agonists diluted in DMEM containing 1 mg/ml BSA and 10 mM HEPES, pH 7.4, for various times at 37 °C. Cells were then washed with cold phosphate-buffered saline and lysed with Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM NaPP, 1% Triton X-100) containing freshly added protease inhibitors. Cell lysates were solubilized for 1.5 h at 4 °C and centrifuged at 14,000 rpm for 20 min at 4 °C. The total amount of protein in cell lysates was quantified using a bicinchoninic acid protein assay reagent (Thermo Scientific), and equivalent amounts of lysates were used for immunoprecipitation with the M2 anti-FLAG antibody or goat anti-mouse IgG control. Immunoprecipitates were analyzed by SDS-PAGE and transferred to membranes, and PAR1 was detected by immunoblotting with anti-PAR1 antibody. Immunoblots were developed with enhanced chemiluminescence (GE Healthcare), imaged by autoradiography, and quantitated with ImageJ software.

To assess PAR1 degradation, cells were pretreated with 10 μM cycloheximide for 30 min at 37 °C and then incubated with or without agonists in serum-free media containing 10 μM cycloheximide for various times at 37 °C. Cells were washed, solubilized with Triton X-100 lysis buffer, and processed, and the total amount of PAR1 remaining was determined by immunoblotting as described above.
Cell Surface ELISA—HeLa cells stably expressing FLAG-tagged PAR1 wild type or mutants were plated at $1 \times 10^5$ cells per well in a fibronectin coated 24-well culture dish and grown overnight at 37 °C. Cells were washed in serum-free DMEM and then treated with agonists, 100 μM S1L-RLRN or 10 μM thrombin, diluted in DMEM/BSA/HEPES at 37 °C for various times. After treatments, cells were placed on ice, washed with phosphate-buffered saline, and then fixed with 4% paraformaldehyde for 5 min at 4 °C. Cells were washed and then incubated with polyclonal anti-FLAG antibody diluted 1:1000 or rabbit polyclonal anti-PAR1 C5433 diluted 1:250 in DMEM/BSA/HEPES for 1 h at room temperature. Cells were washed and then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h at room temperature and then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h at room temperature and washed extensively. The amount of secondary antibody bound was determined by incubation with one-step 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Thermo Scientific) substrate for 10–20 min at 25 °C. An aliquot was removed, and the absorbance (A) was determined at 405 nm using a Molecular Devices SpectraMax Plus microplate reader (Sunnyvale, CA).

Deglycosylation of PAR1—HeLa cells stably expressing FLAG-tagged PAR1 or PAR2 wild type were plated at $5 \times 10^5$ cells per well for 6-well dishes or $1.0 \times 10^5$ cells per well for a 24-well culture dish coated with fibronectin and grown overnight at 37 °C. Cells were washed and then treated with 0.5 μg/ml tunicamycin (diluted in DMSO) or vehicle control in serum-free DMEM for 16 h at 37 °C. After incubation, cells were either lysed with Triton lysis buffer and immunoblotted for PAR1 expression or fixed with 4% paraformaldehyde and processed for cell surface ELISA as described above.

Glycosidase Incubations—For PNGase F treatments, cells stably expressing FLAG-tagged PAR1 or endogenous PAR1 were plated at $5 \times 10^5$ cells per well in a 6-well culture dish and grown overnight at 37 °C. Cells were lysed with Triton lysis buffer, processed, and then immunoprecipitated with M2 anti-FLAG antibody, anti-PAR1 WEDEE antibody, or goat antimouse IgG as described above. PAR1 immunoprecipitates were then resuspended in 100 μl of 1× glycoprotein denaturing buffer (0.5% SDS, 40 mM dithiothreitol), heated at 100 °C for 10 min, vortexed, and then centrifuged for 30 s at 25 °C. Beads were resuspended, and 20-μl aliquots were added to each reaction tube. PNGase F reaction mixture was prepared using 2 μl of enzyme per 20 μl of sample volume and added to reaction tubes. Samples were incubated at 37 °C for various times, then an equal volume of 2× sample buffer (62.5 mM Tris-HCl, 10% glycerol, 5% SDS, 0.01% bromphenol blue) was added, and the analysis of PAR1 was determined by immunoblot.

HeLa cells stably expressing FLAG-tagged PAR1 wild type or mutant were prepared as described for the PNGase F assay. Sialidase, endoglycosidase H, and PNGase F reaction mixtures were prepared using 4, 2, or 2 μl of the enzymes, respectively, per 20-μl sample volume and added to reaction tubes. Samples were incubated at 37 °C for 16 h, and then an equal volume of 2× sample buffer was added. PAR1 analysis was determined by immunoblot.

Thrombin Cold Cleavage Assay—HeLa cells expressing FLAG-tagged PAR1 wild type or mutants were plated at $5 \times 10^5$ cells per well in 6-well culture dishes and grown overnight, washed with serum-free DMEM, and placed on ice for 30 min. Cells were then incubated with cold DMEM with or without 30 nm thrombin, and incubated for 15 min on ice. Cells were lysed, and PAR1 was immunoprecipitated with M2 anti-FLAG antibody and immunoblotted as described above.

PAR1 Internalization Assay—HeLa cells stably expressing FLAG-tagged PAR1 wild type or mutants were plated at $1 \times 10^5$ cells per well in a fibronectin-coated 24-well culture dish and grown overnight at 37 °C. After incubation, cells were washed and then incubated for 1 h on ice with the Ca$^{2+}$-dependent M1 anti-FLAG antibody diluted in DMEM containing 1 mg/ml BSA, 1 mM CaCl$_2$, and 10 mM HEPES, pH 7.4. Under these conditions, only cell surface PAR1 is labeled with antibody. The remaining unbound antibody was washed away, and then prewarmed DMEM was added to the cells for various times at 37 °C. The remaining bound antibody was then stripped from the cell surface by washing four times with Ca$^{2+}$/Mg$^{2+}$-free phosphate-buffered saline containing 1 mM EDTA. Cells were lysed with Triton lysis buffer (3% BSA, 1% Triton X-100, 5 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl, pH 7.4), and the accumulated intracellular antibody was measured by ELISA as described previously (24).

Immunofluorescence Microscopy—HeLa cells stably expressing FLAG-PAR1 wild type and mutants were plated at a density of $1.5 \times 10^5$ cells per well on fibronectin-coated glass coverslips in 12-well dishes. Cells were fixed and either left alone or permeabilized and immunostained for PAR1 using an anti-FLAG polyclonal antibody (Rockland, MD) and processed for microscopy as described previously (22). Images were collected using an Olympus DSU spinning disk confocal microscope configured with a PlanApo 60× oil objective and a Hamamatsu ORCA-ER digital camera. Fluorescent images of X-Y sections at 0.28 μm were collected using Intelligent Imaging Innovations Slidebook 4.2 software, and the composite was configured using Adobe Photoshop CS3.

Thrombin Cleavage Assays—We determined the rate at which thrombin cleaves FLAG-tagged PAR1 wild type and mutants essentially as described previously (25). Cells were plated at $1 \times 10^5$ cells per well in a fibronectin-coated 24-well culture dish and grown overnight. Cells were washed and then incubated in the presence or absence of 1 or 10 nM thrombin for various times at 37 °C. Cells were fixed with 4% paraformaldehyde, and the amount of uncleaved PAR1 was detected with the polyclonal anti-FLAG antibody and cell surface ELISA.

Phosphoinositide Hydrolysis—HeLa cells stably expressing FLAG-tagged PAR1 wild type or mutants were plated at $1 \times 10^5$ cells per well in a fibronectin-coated 24-well culture dish and grown overnight at 37 °C. Cells were then labeled with 1 μCi/ml myo-[3H]inositol (American Radiolabeled Chemicals, St. Louis) diluted in serum- and inositol-free DMEM containing 1 mg/ml BSA overnight. Cells were washed and treated with or without agonists in DMEM containing 20 mM lithium chloride (LiCl) for various times at 37 °C, and accumulated [3H]inositol phosphates (IPs) were measured as described previously (24).

To determine whether recycling of activated PAR1 continued to signal, cells labeled with myo-[3H]inositol were treated with DMEM with or without 10 nM thrombin for 1 h at 37 °C.
PAR1 ECL2 N-Linked Glycosylation

Results

conservation of N-linked glycosylation sites with only one present in the N terminus compared with the H. sapiens (human) receptor.

Whether the putative N-linked glycosylation sites of PAR1 were conserved between species using Clustal W2 alignment. We found that among eight different species, the second N-linked glycosylation sites of PAR1 have the least conservation of N-linked glycosylation sites with only one present in the N terminus compared with the H. sapiens (human) receptor. Next, we examined the glycosylation status of human PAR1 ectopically expressed in HeLa cells by examining its susceptibility to cleavage by PNGase F, an enzyme that cleaves all N-linked glycosylation except for those with an α-(1,3)-linked core fucose modification. HeLa cells expressing FLAG-tagged PAR1 were lysed and immunoprecipitated with M2 anti-FLAG antibody or IgG control and then incubated with PNGase F for various times at 37 °C. In untreated control cells, PAR1 migrated as a broad band between ~64 and 98 kDa (Fig. 2A, lane 3), consistent with that observed previously (22), but significantly greater than the predicted molecular mass of ~40 kDa. In contrast, after incubation with PNGase F, PAR1 migrated comparable with its predicted molecular mass of ~40 kDa (Fig. 2A, lanes 4–6), suggesting that PAR1 is modified extensively by N-linked glycosylation. Endogenous PAR1 immunoprecipitated from human EA.hy926 endothelial cells also migrated as a high molecular weight band that was reduced to its predicted molecular weight after PNGase F treatment (Fig. 2B, lanes 2–4). To further confirm that PAR1 is modified by N-linked glycosylation, we examined the mobility of PAR1 following treatment with tunicamycin, a drug that blocks the first step of glycoprotein synthesis and functions as a global inhibitor of N-linked glycosylation. In cells treated with tunicamycin, PAR1 migrated at ~40 kDa (Fig. 2C, lane 4) similar to that observed with PNGase F treatment (Fig. 2A) and distinct from the broad high molecular weight band observed with untreated PAR1. These findings strongly suggest that PAR1 is extensively glycosylated and is consistent with that reported previously (17–19).

N-Linked glycosylation of many transmembrane proteins, including GPCRs, is important for proper folding during translation and export to the cell surface (27, 28). Thus, we first determined whether global disruption of PAR1 N-linked glycosylation affected receptor export to the cell surface using tunicamycin. PAR1 cell surface expression was substantially reduced in tunicamycin-treated cells compared with untreated control cells (Fig. 3), suggesting that global disruption of PAR1 N-linked glycosylation affects the cell surface using tunicamycin. PAR1 cell surface expression was substantially reduced in tunicamycin-treated cells compared with untreated control cells (Fig. 3), suggesting that global disruption of PAR1 N-linked glycosylation affects transport of the receptor to the cell surface as reported previously (20). To control for pleiotropic effects of tunicamycin on protein transport, we examined cell surface expression of PAR2. In contrast to PAR1, PAR2 surface expression was surprisingly unaffected in tunicamycin-treated HeLa cells (Fig. 3), although immunoblot analysis confirmed efficient deglycosylation of the receptor after tunicamycin treatment (Fig. 3, inset). These findings suggest that glycosylation of PAR1, but not PAR2, is important for export to the cell surface in HeLa cells. However, whether N-linked glycosylation of PAR1 occurs largely at the N terminus, second extracellular loop, or both is not known. Moreover, whether N-linked glycosylation serves specific functions in the regulation of PAR1 signaling and trafficking is not known.

Cells were washed, and medium was replaced with new media containing 20 mM LiCl. Cells were incubated for 1 h at 37 °C, and accumulated [3H]IPs were measured as described (24).

Data Analysis—Data were analyzed using Prism software, and statistical analysis was determined using the Prism data analysis tool as noted. Statistical analysis was determined by performing Student’s t test, one-way ANOVA, and Dunnett multiple comparison test, or two-way ANOVA and Bonferroni post tests.

Results

The post-translational modification of nascent proteins by N-linked glycosylation occurs via covalent attachment of saccharides to asparagine residues of the consensus site N(S/T)(X)N (where X is any amino acid except proline) (26). Human PAR1 contains five potential consensus sites for N-linked glycosylation as follows: three are localized in the N terminus and two reside in the second extracellular loop (Fig. 1). We first determined whether the five putative N-linked glycosylation sites of PAR1 were conserved between species using Clustal W2 alignment. We found that among eight different species, the second N-linked glycosylation sites of PAR1 are the most conserved (Fig. 1).

Interestingly, X. laevis (frog) was the species with the least conservation compared with H. sapiens (human) PAR1 N-linked glycosylation sites, although the species P. hamadryas (baboon) and C. lupus familiaris (dog) sites were completely conserved. Whether the putative N-linked glycosylation sites of PAR1 are each modified by glycosylation or have specific functions important for regulation of PAR1 signaling and trafficking is not known.

Next, we examined the glycosylation status of human PAR1 ectopically expressed in HeLa cells by examining its susceptibility to cleavage by PNGase F, an enzyme that cleaves all N-linked glycosylation except for those with an α-(1,3)-linked core fucose modification. HeLa cells expressing FLAG-tagged PAR1 were lysed and immunoprecipitated with M2 anti-FLAG antibody or IgG control and then incubated with PNGase F for various times at 37 °C. In untreated control cells, PAR1 migrated as a broad band between ~64 and 98 kDa (Fig. 2A, lane 3), consistent with that observed previously (22), but significantly greater than the predicted molecular mass of ~40 kDa. In contrast, after incubation with PNGase F, PAR1 migrated comparable with its predicted molecular mass of ~40 kDa (Fig. 2A, lanes 4–6), suggesting that PAR1 is modified extensively by N-linked glycosylation. Endogenous PAR1 immunoprecipitated from human EA.hy926 endothelial cells also migrated as a high molecular weight band that was reduced to its predicted molecular weight after PNGase F treatment (Fig. 2B, lanes 2–4). To further confirm that PAR1 is modified by N-linked glycosylation, we examined the mobility of PAR1 following treatment with tunicamycin, a drug that blocks the first step of glycoprotein synthesis and functions as a global inhibitor of N-linked glycosylation. In cells treated with tunicamycin, PAR1 migrated at ~40 kDa (Fig. 2C, lane 4) similar to that observed with PNGase F treatment (Fig. 2A) and distinct from the broad high molecular weight band observed with untreated PAR1. These findings strongly suggest that PAR1 is extensively glycosylated and is consistent with that reported previously (17–19).

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To begin to delineate the function of potential N-linked glycosylation of the PAR1 N terminus versus ECL2, we generated PAR1 mutants lacking N-linked glycosylation sites only in the N terminus (NA NTer) or second extracellular loop (NA ECL2)
We first determined whether mutants were expressed at the cell surface comparable with wild-type PAR1 by quantifying the amount of cell surface receptor with ELISA. Interestingly, cells expressing the NA NTer PAR1 mutant showed significantly less cell surface expression compared with PAR1 wild type (WT) or NA ECL2 mutant (Fig. 4A). Thus, PAR1 appears to be modified by glycosylation at the N terminus critical for efficient export to the cell surface. We next examined the mobility of PAR1 wild type and mutants by SDS-PAGE. The migration of PAR1 NA NTer was altered compared with wild type or NA ECL2 mutant (Fig. 3A). Interestingly, however, a substantial amount of PAR1 NA NTer mutant was retained in an intracellular compartment compared with wild type or NA ECL2 mutant (Fig. 3B, lower panels). We next examined the localization of PAR1 NA NTer to specific intracellular compartments. Confocal microscopy studies revealed that intracellular PAR1 NA NTer localized primarily to the trans-Golgi network indicated by marked co-localization with TGN230 and minimally to endocytic vesicles co-stained with EA1 (Fig. 4C). These findings suggest that glycosylation of the PAR1 N terminus is critical for efficient export to the cell surface. We next examined the mobility of PAR1 wild type and mutants by SDS-PAGE. The migration of PAR1 NA NTer was altered compared with wild-type receptor suggesting that the N terminus is indeed modified by glycosylation (Fig. 3A, inset and 3B, lower panels). In contrast, PAR1 NA ECL2 mutant, a receptor lacking both N-linked glycosylation sites in the second extracellular loop, expressed at the cell surface comparable with wild-type receptor (Fig. 4A) but displayed a marked difference in mobility compared with PAR1 wild type and NA NTer mutant (Fig. 3A, lanes 3, 9, and 11). Interestingly, PAR1 mutants defective in only one of the ECL2 N-linked glycosylation sites, N250A or N259A, also showed a shift in mobility similar to the NA NTer PAR1 mutant (Fig. 5A, lanes 5, 7, and 9). Thus, PAR1 appears to be modified by N-linked glycosylation at both the N terminus and ECL2, with the ECL2 serving as the major site for N-linked glycosylation.

To confirm that FLAG-tagged PAR1 wild type and mutants are expressed at the cell surface, we incubated cells with a saturating concentration of thrombin at 4°C; under these "cold-cleavage" conditions, thrombin efficiently cleaves all surface-localized receptors resulting in the loss of FLAG epitope, but the receptor is unable to internalize as described previously (29). HeLa cells stably expressing FLAG-tagged PAR1 wild type or mutants were chilled to 4°C and then incubated with or without 30 nM thrombin for 15 min at 4°C. Cells were lysed,
FIGURE 4. PAR1 harboring mutations in the N terminus or ECL2 N-linked glycosylation consensus sites are differentially transported to the cell surface. A, PAR1 N terminus (NTer) and second extracellular loop (ECL2) mutants were generated by mutating the critical asparagines (N) to alanine (A) or aspartate (D). HeLa cells were transiently transfected with FLAG-tagged PAR1 WT, NA NTer, NA ECL2 mutant, or pBJ vector, and the amount of cell surface expression was determined by ELISA. The data shown (mean ± S.D.; n = 3) are expressed as the absorbance (OD) values measured at 405 nm. Similar findings were observed in three independent experiments. A significant difference between WT and NA NTer cell surface expression was detected (*, p < 0.01) by Dunnett multiple comparison test. B, HeLa cells expressing PAR1 WT, NA NTer, or NA ECL2 were fixed and either left alone (unpermeabilized) or treated with thrombin and then permeabilized (Perm), processed, and immunostained for PAR1 using a polyclonal anti-FLAG antibody. The cells were imaged by confocal microscopy. Scale bar, 10 μm. C, PAR1 NA NTer-expressing HeLa cells were fixed, permeabilized, and immunostained for PAR1 NA NTer or endogenous EEA1 or TGN230 and examined by confocal microscopy. The images shown are representative of many cells examined. Co-localization of PAR1 NA NTer with endogenous TGN230 is indicated by the yellow color in the merge image.
immunoprecipitated, and then immunoblotted for FLAG-tagged PAR1 using the polyclonal anti-FLAG antibody. To our surprise, all PAR1 N-linked glycosylation mutants were susceptible to thrombin cold cleavage comparable with wild-type receptor (Fig. 5A, see “+” lanes). These findings suggest that minimal N-linked glycosylation at either the N terminus or ECL2 is sufficient to facilitate trafficking of PAR1 to the cell surface and that neither mutants are globally defective in protein folding or trafficking.

We next evaluated the sensitivity of PAR1 NA Nter and ECL2 mutants to cleavage by various glycosidases to determine whether the mutant receptors indeed retained N-linked glycosylation and the nature of the glycosylation modifications. The mobility of PAR1 wild type and mutants was unchanged after incubation with sialidase, an enzyme that cleaves terminal sialic acid of glycoproteins, suggesting that either the receptors are not modified with sialic acid or that the loss of sialic acid is not sufficient to cause a change in receptor mobility (Fig. 5, B, lanes 2 and 6, and C, lanes 6 and 10). Treatment with endoglycosidase H, which cleaves between the two N-acetylglucosamine subunits directly proximal to the asparagine residue, caused a shift in the mobility of the immature receptor form that appears as a minor species migrating below the fully glycosylated PAR1 wild type and mutants (Fig. 5, B, lanes 3 and 7, and C, lanes 7 and 11). Interestingly, incubation with PNGase F caused a dramatic shift in mobility of PAR1 wild type, NA Nter, and NA ECL2 mutants to the predicted molecular weight of the unmodified receptor (Fig. 5B, lanes 4 and 8, and C, lanes 8 and 12). These findings lend further support to the idea that the PAR1 N terminus and ECL2 domains are both functional targets for modification with N-linked glycosylation.

Previous studies indicate that activation of PAR1 occurs via ligand docking interactions with residues localized in the second extracellular loop (12, 13). Thus, to determine whether N-linked glycosylation of PAR1 within the ECL2 has a function in receptor activation, we examined the capacity of activated wild-type and mutant receptors to stimulate phosphoinositide (PI) hydrolysis. Activated PAR1-stimulated PI hydrolysis occurs predominantly by $G_{a1}$ coupling to phospholipase $\beta$-mediated, as well as $G_{a12/13}$- out and phospholipase $\epsilon$-mediated, hydrolysis of phosphoinositides (3, 30, 31). We initially compared the rates of agonist-induced PI hydrolysis in HeLa cells stably expressing similar amounts of PAR1 wild type and NA ECL2 mutant on the cell surface (Fig. 6A). Cells were incubated with a saturating concentration of thrombin for various times at 37 °C, and total $[^{3}H]$IPs were measured. After 60 min of agonist exposure, an ~4-fold increase in PI hydrolysis was detected in wild-type PAR1-expressing cells, whereas a significantly greater ~6-fold increase in signaling was measured in cells expressing the PAR1 NA ECL2 mutant lacking N-linked glycosylation in the second extracellular loop (Fig. 6A). In cells expressing similar amounts of both PAR1 wild type and NA ECL2 mutant, stimulation of endogenous muscarinic acetylcholine receptors with carbachol resulted in comparable changes in PI hydrolysis (Fig. 6B), indicating that there are no cell clone-specific defects in G protein signaling. Moreover, the activation of PAR1 with the peptide agonist also caused enhanced signaling of PAR1 NA ECL2 compared with wild-type PAR1 (Fig. 6B). In contrast to PAR1 NA ECL2, thrombin activation of PAR1 NA Nter caused a significant but modest increase in signaling compared with the wild-type receptor (Fig. 6C). Together, these data suggest that N-linked glycosylation of PAR1 at ECL2 is important for ligand-receptor interaction and activation of G protein signaling.

Next, we examined whether the initial coupling of activated PAR1 wild type and NA ECL2 mutant coupling to G protein-stimulated PI hydrolysis was affected. The concentration effect curves for thrombin at PAR1 wild type and mutant deficient in N-linked glycosylation at ECL2 were determined by incubating...
cells labeled with myo-[3H]inositol and varying concentrations of thrombin for 5 min at 37 °C, and accumulation of [3H]IPs was measured. The effective concentration of thrombin needed to stimulate half-maximal response after 5 min was significantly different for PAR1 wild type (1.61 ± 0.14 nM) versus NA ECL2 mutant (0.99 ± 0.12) (Fig. 7, A and B). Moreover, activation of PAR1 NA ECL2 mutant caused an enhanced maximal signaling response compared with wild-type receptor (Fig. 7A). Thus, each activated PAR1 mutant lacking N-linked glycosylation on the surface of ECL2 appears to couple longer and more robustly to G protein-mediated PI hydrolysis before receptor signaling is shut off. These findings indicate that each activated PAR1 deficient in ECL2 N-linked glycosylation is more efficacious at coupling to G protein activation than wild-type receptor.

To assess PAR1 desensitization rates, HeLa cells expressing comparable surface amounts of PAR1 wild type and NA ECL2 mutant were exposed to saturating concentrations of thrombin for 10 min at 37 °C. The extent of PAR1 signaling activity remaining after various times of thrombin incubation was then determined by the addition of lithium chloride, and the amounts of [3H]IPs formed were then measured. Thrombin-induced IP formation is not detectable in the absence of lithium chloride (data not shown). In PAR1 NA ECL2 mutant-expressing cells, the rate of desensitization actually occurred more rapidly compared with wild-type receptor cells (Fig. 8A). These findings suggest that despite PAR1 NA ECL2 increased rate of desensitization, activated receptor is still able to induce robust activation of G protein signaling.

It is conceivable that the proteolytically activated PAR1 NA ECL2 mutant can continue to signal if it remains on the cell surface or if it is internalized and recycled back to the cell surface with its tethered ligand intact (32, 33). Therefore, we examined whether proteolytic activation of PAR1 lacking ECL2 N-linked glycosylation with thrombin induced persistent signaling by assaying for the accumulation of [3H]IPs after thrombin withdrawal. HeLa cells expressing comparable amounts of PAR1 wild type and NA ECL2 mutant labeled with myo-[3H]inositol were stimulated with a maximal concentration of thrombin for 1 h in the presence of LiCl, thrombin stimulated significant increases in [3H]IPs in both PAR1 WT and NA ECL2-expressing cells compared with untreated control cells (Fig. 8B, left columns). In cells treated with thrombin and in the absence of LiCl, PI hydrolysis is stimulated, but [3H]IPs are rapidly metabolized and fail to accumulate. To detect ongoing PAR1 signaling, thrombin was removed, and LiCl was then added together with hirudin (a thrombin inhibitor) to allow accumulation of any IPs generated by ongoing phosphoinositide hydrolysis. If receptor signaling were terminated, no IPs would be detected. In cells expressing PAR1 wild type or NA ECL2 mutant, phosphoinositide hydro-
lysis in response to thrombin incubation was virtually abolished after agonist removal indicating that receptor signaling was substantially shut off (Fig. 8B, right columns). Thus, PAR1 NA ECL2 mutant-enhanced signaling is not due to constitutive activation or impaired signal termination.

Next, we examined whether PAR1 NA ECL2 mutant is cleaved by thrombin at the same rate as wild-type receptor to determine whether differences in signaling are caused by distinct PAR1 cleavage rates. HEK cells stably expressing FLAG-tagged PAR1 wild type or NA ECL2 mutant were incubated with 1 or 10 nM thrombin, and the rate of PAR1 cleavage was assessed by monitoring the loss of FLAG epitope over time by ELISA as described previously (25). Both PAR1 wild type, NA ECL2, and NA NTER mutants were efficiently cleaved with similar kinetics at maximal and sub-maximal doses of thrombin (Fig. 8, C and D). These findings strongly suggest that the gain in signaling response observed with PAR1 NA ECL2 mutant is not due to differences in receptor cleavage by thrombin but rather due to stabilization of an active conformation that couples more efficiently to G protein signaling before signaling is shut off.

To determine the role of PAR1 ECL2 N-linked glycosylation in receptor trafficking, we examined both constitutive and agonist-induced receptor internalization. HEK cells expressing PAR1 wild type and NA ECL2 mutant were incubated with the M1 anti-FLAG antibody for 1 h at 4 °C. Under these conditions, only the receptor cohort at the cell surface binds antibody. Cells were washed and then warmed to 37 °C for various times to facilitate PAR1 constitutive internalization. Both PAR1 wild type and NA ECL2 mutant showed a similar slow rate of constitutive internalization with ~12% of the surface receptor cohort being internalized by 20 min (Fig. 9A). These findings are consistent with our previously published studies (21, 34) and indicate that PAR1 constitutive internalization occurs independent of N-linked glycosylation of ECL2. In contrast, activation of PAR1 NA ECL2 mutant with either peptide agonist or thrombin caused a marked delay in activated receptor internalization compared with wild-type receptor (Fig. 9, B and C). To exclude the possibility of a global defect in receptor-mediated endocytosis, we examined internalization of the endogenous EGF receptor in the same HEK cell lines. Activated EGF receptor showed robust internalization in both PAR1 wild type and NA ECL2-expressing cell lines indicating that the endocytic machinery is intact (Fig. 9D). Thus, impaired agonist-induced PAR1 NA ECL2 mutant internalization suggests that N-linked glycosylation of the second extracellular loop is important for stabilizing an active receptor conformation that is efficiently engaged by the endocytic machinery.

We next compared the rates of agonist-induced PAR1 wild-type and NA ECL2 mutant degradation to determine whether N-linked glycosylation mediates endocytic sorting of the receptor. HEK cells expressing FLAG-tagged PAR1 wild type and NA ECL2 mutant were incubated with or without agonist for various times at 37 °C, and the amount of receptor protein remaining was determined by immunoblot analysis. In cells exposed to agonist for 45 min, a significant decrease in the amount of PAR1 wild-type protein was observed, and the detection of receptor protein was virtually abolished at 90 min (Fig. 9, lanes 3–5), consistent with that previously reported. Interestingly, PAR1 NA ECL2 mutant displayed a rate of agonist-induced degradation comparable with the wild-type receptor (Fig. 10). These findings suggest that N-linked glycosylation of PAR1 ECL2 is not important for endocytic sorting and lysosomal degradation of activated PAR1.

DISCUSSION

In this study, we have identified the major sites of PAR1 N-linked glycosylation and defined a function for this post-translational modification in the regulation of receptor activation and trafficking. We show that PAR1 is heavily glycosylated and that elimination of glycosylation by tunicamycin treatment substantially reduced receptor surface expression, consistent with that reported previously (20). These findings suggest that at least partial glycosylation of PAR1 is required for efficient trafficking to the cell surface. Indeed, glycosylation of many transmembrane-spanning proteins is critical for proper folding and transport to the cell surface (27, 35, 36). Although N-linked glycosylation of the PAR2 N terminus has been shown to partially effect export to the cell surface (16), it is also critical for protease-selective signaling (37). In contrast, virtually nothing is known about the function of PAR1 N-linked glycosylation. We now show that PAR1 glycosylation occurs mainly at two sites localized to the second extracellular loop, whereas the sites...
in the N terminus contribute less to the overall glycosylation status of the receptor. Moreover, the lack of glycosylation at the second extracellular loop caused a marked increase in receptor signaling, which is not due to defects in the ability of thrombin to cleave the receptor or impaired receptor desensitization. In fact, the rate of PAR1 ECL2 mutant desensitization is greater than wild-type receptor. In addition, the activated PAR1 mutant lacking second extracellular loop glycosylation displays a slowed initial rate of agonist-induced internalization, whereas the rate of constitutive internalization remained intact. Together these findings suggest that N-linked glycosylation of the PAR1 second extracellular loop is important for proper regulation of ligand-induced receptor activation and endocytosis, whereas glycosylation of the N terminus mediates export to the cell surface.

N-Linked glycosylation is a highly diverse post-translational modification due to the heterogeneity of the carbohydrate chains of glycoproteins (26, 38). Most mammalian GPCRs harbor N-linked glycosylation sites within their N-terminal extracellular domain, whereas glycosylation occurs less commonly on the extracellular loops linking the transmembrane helices. Despite the presence of three N-linked glycosylation consensus sites in the N terminus, we found that modification of the PAR1 N terminus with glycosylation does occur but contributes minimally to the overall glycosylation status of the receptor. However, like many other mammalian GPCRs, our findings suggest that N-linked glycosylation of the PAR1 N terminus is important for receptor trafficking through the biosynthetic pathway and export to the cell surface. Calnexin and calreticulin are chaperone proteins that retain unfolded or unassembled N-linked glycoproteins in the endoplasmic reticulum and may be important for proper trafficking of PAR1 through the endoplasmic reticulum, but this remains to be determined. We also show that the gross pattern of glycosylation remains intact for both the PAR1 N terminus and second extracellular loop, indicating that the receptor mutants are still capable of being processed by the diverse set of enzymes involved in N-linked glycosylation formation in the endoplasmic reticulum and Golgi.

Unlike most classic reversibly activated GPCRs, PAR1 is irreversibly activated through an unusual proteolytic mechanism that results in the formation of a tethered ligand that binds intramolecularly to the receptor to elicit transmembrane signaling (4). The precise mechanism by which the newly formed tethered ligand docks to the extracellular surface of PAR1 to induce a signaling response remains poorly understood. GPCRs that belong to class B bind peptide hormones presumably through critical interactions with the extracellular portions (39, 40). In addition, the extracellular domains of other nonpeptide hormone binding GPCRs belonging to the large class A rhodopsin-like family of GPCRs have been reported to influence ligand interactions (41–43). In the case of both the dopamine and cannabinoid receptor, the second extracellular loop was found to participate in ligand binding, although other extracellular domains participated in ligand recognition of the CCR3 receptor. A more recent study using NMR spectroscopy showed that small molecules that bind within the transmembrane core and display different efficacies toward G protein activation stabilize different conformations of the β2-adrenergic receptor extracellular domain.
The idea that a lack of PAR1 N-linked glycosylation allows the tethered ligand or synthetic peptide agonist to bind the receptor in a manner that induces an active receptor conformation that is more efficient at coupling to G protein signaling.

In addition to thrombin, many other proteases, including serine, cysteine, and metalloproteases can cleave and activate PARs (23, 45). These proteases can function as soluble enzymes similar to thrombin or require membrane-associated cofactors that facilitate membrane localization and/or allosterically modulate protease activity. Interestingly, an N-linked glycosylation site present in the N terminus of PAR2 appears to regulate signaling by the serine protease trypstatin but not trypsin or synthetic peptide agonist (16, 37), suggesting that glycosylation of PARs at the N terminus can dictate protease specificity. Thrombin binds to and cleaves PAR1 with exquisite specificity and recognizes both the N-terminal LDPR ↓ S cleavage site and an acidic region C-terminal to the cleavage site termed the hirudin-like sequence (46). Our results indicate that PAR1 is glycosylated at the N terminus, but the actual site of glycosylation remains to be determined. In addition, the PAR1 NA NTer mutant lacking all potential sites of N-linked glycosylation in the N terminus was cleaved by thrombin as efficiently as the wild-type receptor (Fig. 8D). These findings suggest that N-linked glycosylation is not critical for thrombin cleavage of PAR1, but whether N-linked glycosylation of the PAR1 N terminus affects the capacity of other proteases, such as activated protein C or matrix metalloprotease-1, to cleave and activate PAR1 is not known. In malignant cancer, altered glycosylation of proteins is common and can result from either loss, incomplete, altered, or truncated forms of N-linked glycosylation. The expression of PAR1 is up-regulated in many types of invasive cancers, including breast cancer, due in part to defective receptor trafficking, which leads to persistent signaling, transactivation of ErbB receptors, and breast cancer progression (2, 33, 47). Whether defective glycosylation of PAR1 occurs in malignant tumors and contributes to persistent signaling and breast cancer invasion and metastasis has not been examined.

In addition to rapid desensitization, trafficking of PAR1 is critical for the fidelity of receptor signaling. PAR1 displays constitutive internalization, a process important for cellular resensitization (21), and agonist-induced internalization, which is

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**FIGURE 9. Agonist-induced PAR1 NA ECL2 mutant internalization but not constitutive internalization is impaired.** A, HeLa cells stably expressing FLAG-tagged PAR1 WT or NA ECL2 mutant were labeled with M1 anti-FLAG antibody for 1 h at 4 °C, washed, and incubated in media without agonist for various times at 37 °C. After incubations, cells were stripped of remaining surface-bound antibody, and internalized antibody was quantitated by ELISA. The data shown (mean ± S.D.; n = 3) are expressed as the fraction of initial cell surface receptor-bound antibody corrected for background = ((value at time x − average 0 min value)/(average untreated value − average 0 min value)) and are the averages from three independent experiments performed in triplicate. HeLa cells stably expressing similar amounts of FLAG-tagged PAR1 WT or NA ECL2 mutant were serum-starved for 30 min and then treated with media containing 100 ng/ml EGF for 1 h at 37 °C, and remaining surface EGF receptor (EGFR) was assayed by ELISA. The data shown (mean ± S.D.; n = 3) are from three independent experiments performed in triplicate.

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**PAR1 ECL2 N-Linked Glycosylation**

The extracellular surface of GPCRs is dynamic and can alter receptor conformation and G protein activation.

Previous studies have shown that the second extracellular loop of PAR1, a class A GPCR, is critical for ligand recognition and signal propagation (12, 13). In these studies, the chimera of the PAR were generated by substituting various extracellular portions of different species and used to examine species-specific receptor-ligand interaction requirements. The results from these studies indicate that the second extracellular loop is a critical receptor domain dictating ligand docking and receptor activation. Our findings reported here now suggest that glycosylation of the second extracellular loop of PAR1 is also a critical determinant for ligand-induced receptor activation and the fidelity of thrombin signaling. Indeed, in the absence of N-linked glycosylation, activation of PAR1 either proteolytically with thrombin or by the addition of synthetic peptide agonist caused a marked increase in signaling compared with wild-type receptor. Remarkably, enhanced signaling by the PAR1 NA ECL2 mutant was not due to a defect in thrombin cleavage of the receptor or desensitization. Thus, our findings support the idea that a lack of PAR1 N-linked glycosylation allows the tethered ligand or synthetic peptide agonist to bind the receptor in a manner that induces an active receptor conformation that is more efficient at coupling to G protein signaling.

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**N-linked glycosylation**

When the idea that a lack of PAR1 N-linked glycosylation allows the tethered ligand or synthetic peptide agonist to bind the receptor in a manner that induces an active receptor conformation that is more efficient at coupling to G protein signaling.

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**Constitutive Internalization**

The idea that a lack of PAR1 N-linked glycosylation allows the tethered ligand or synthetic peptide agonist to bind the receptor in a manner that induces an active receptor conformation that is more efficient at coupling to G protein signaling.

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**Thrombin Binding**

The idea that a lack of PAR1 N-linked glycosylation allows the tethered ligand or synthetic peptide agonist to bind the receptor in a manner that induces an active receptor conformation that is more efficient at coupling to G protein signaling.

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**Thrombin Cleavage**

The idea that a lack of PAR1 N-linked glycosylation allows the tethered ligand or synthetic peptide agonist to bind the receptor in a manner that induces an active receptor conformation that is more efficient at coupling to G protein signaling.

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**Thrombin Cleavage**

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**Thrombin Cleavage**

The idea that a lack of PAR1 N-linked glycosylation allows the tethered ligand or synthetic peptide agonist to bind the receptor in a manner that induces an active receptor conformation that is more efficient at coupling to G protein signaling.
critical for receptor signal termination (35, 48). Interestingly, we found that the initial rate of agonist-induced PAR1 NA ECL2 mutant internalization is impaired compared with wild-type receptor, whereas constitutive internalization remained intact. Moreover, the defect in PAR1 NA ECL2 mutant internalization was observed with thrombin, the physiological relevant agonist, as well as with the synthetic peptide agonist. These findings raise the possibility that glycosylation is important for maintaining distinct active conformations of the receptor that are able to engage the endocytic machinery differently. Both constitutive and agonist-induced PAR1 internalization occur through clathrin-coated pits independent of β-arrestins (22, 24). Interestingly, constitutive internalization of PAR1 requires the clathrin adaptor protein complex-2, whereas agonist internalization is specified by ubiquitination (34). Whether the PAR1 NA ECL2 mutant defective in N-linked glycosylation internalizes through the same pathway as wild-type receptor will be important to determine. In contrast to the defect in PAR1 NA ECL2 internalization, we failed to observe any difference between agonist-induced PAR1 wild type and NA ECL2 mutant lysosomal degradation, suggesting that receptor trafficking through the endocytic pathway occurs independent of receptor glycosylation.

In summary, our studies reveal for the first time a function for N-linked glycosylation of the PAR1 second extracellular loop in the regulation of receptor signaling and trafficking. We have shown that the lack of N-linked glycosylation at the second extracellular loop leads to a gain in PAR1 signaling and impaired trafficking, suggesting an important role for glycosylation in the fidelity of thrombin signaling. In contrast, the glycosylation of the PAR1 N terminus appears to function in proper processing and transport of the receptor through the biosynthetic pathway. Whether defects in PAR1 N-linked glycosylation occur naturally, perhaps in invasive cancer, will be important to examine. In addition, other studies have shown that glycosylation of certain transmembrane proteins mediates localization to lipid rafts (49, 50). We previously showed in endothelial cells that PAR1 localizes to caveolae and is important for protease selective signaling (23). Thus, it will be important to assess whether N-linked glycosylation of PAR1 is important for targeting to caveolae and/or protease-selective signaling in endothelial cells and other cell types.

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FIGURE 10. PAR1 wild type and NA ECL2 mutant display similar rates of agonist-induced degradation. A, HeLa cells stably expressing PAR1 WT or NA ECL2 mutant were incubated with 100 μM SFLFRN for various times at 37 °C. Cells were lysed and PAR1 immunoprecipitated (IP) with M2 anti-FLAG antibody or IgG control. Immunoprecipitates were resolved by SDS-PAGE and transferred to membranes. PAR1 was detected by immunoblotting (IB) with rabbit polyclonal anti-FLAG antibody. Similar findings were observed in three independent experiments. B, amount of PAR1 remaining after agonist treatment detected in immunoblot analysis was quantified using ImageJ software, and the data shown (mean ± S.D.; n = 3) are expressed as the fraction of untreated control and are the averages of three separate experiments.
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