Separate Signals for Agonist-independent and Agonist-triggered Trafficking of Protease-activated Receptor 1*

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Protease-activated receptor 1 (PAR1), a G protein-coupled, protease-activated receptor for the serine protease thrombin, is activated when thrombin cleaves its aminoterminal exodomain. This irreversible mechanism of activation may have necessitated an unusual pattern of receptor trafficking. Unactivated PAR1 cycles tonically between the cell surface and an intracellular pool, providing an intracellular store of uncleaved receptors and allowing repopulation of the surface with uncleaved receptors after thrombin exposure without new receptor synthesis. Activated PAR1 internalizes rapidly and is degraded in lysosomes. We report characterization of a PAR1 mutant that trafficked like the wild-type receptor when activated but did not internalize and recycle in the absence of agonist. This complements a previous study in which a mutant with normal tonic internalization but defective agonist-triggered internalization was described. These observations suggest that the trafficking behaviors of unactivated and activated PAR1 are specified by distinct signals within the receptor and imply that PAR1 internalization in the presence or absence of agonist may be mediated by distinct molecular machinery. PAR1 mutants that did not internalize in the absence of agonist were also shown to localize exclusively to the cell surface and to be defective in their ability to repopulate the cell surface with uncleaved receptors after thrombin exposure. These observations suggest that tonic internalization is necessary for maintenance of the intracellular PAR1 pool.

-Thrombin, a serine protease generated at sites of vascular injury, triggers a variety of cellular responses (1, 2). Such responses are mediated by a family of protease-activated receptors (PARs)3 of which the thrombin receptor PAR1 is the prototype (3–8). PAR1 is a seven-transmembrane G protein-coupled receptor that is activated when thrombin cleaves its aminoterminal exodomain. This cleavage event unmasks a new amino terminus that acts as a “tethered ligand,” binding intramolecularly to a site within the body of the receptor to effect transmembrane signaling (9–13). The synthetic peptide SFLLRN representing the PAR1 tethered ligand sequence can activate PAR1 independent of thrombin and receptor cleavage (9, 14, 15).

The irreversibility of the PAR1 activation mechanism begged the question of its fate after cleavage by thrombin. In unstimulated fibroblasts and endothelial cells, PAR1 resides both on the plasma membrane and in a substantial intracellular pool (16–18). After activation by thrombin or SFLLRN, cell surface PAR1 is rapidly internalized and then sorted predominantly to lysosomes (17, 19), resulting in receptor degradation.2 PAR1 is thus used once and discarded. To maintain or regain the ability to respond to thrombin, a cell must repopulate its surface with uncleaved receptors. In fibroblasts and endothelial cells, this occurs in the absence of new receptor synthesis as receptors in the intracellular pool move to the plasma membrane (16, 17).

The use of antibodies to follow the fate of cell surface PAR1 has been informative (20). In the absence of agonist, antibody-labeled receptors were internalized and then recycled back to the cell surface. A steady-state level of antibody-labeled receptor on the surface of approximately half the initial level was eventually attained. This observation suggested that tonic cycling of receptors might be responsible for both the existence of the intracellular pool and the repopulation of the cell surface with uncleaved receptors observed after thrombin exposure. In the presence of agonist, antibody-labeled receptors internalized to a greater extent and at an apparently greater rate than in the absence of agonist.

The studies described above suggested two distinct modes of PAR1 trafficking: agonist-independent trafficking, which includes agonist-independent internalization and recycling of receptors to the cell surface, and agonist-triggered trafficking, which includes the more rapid internalization of activated receptors and their sorting to lysosomes. This formulation begs the question, at what point does trafficking of activated PAR1 become distinct from that of the naive receptor? Is agonist-triggered internalization simply an acceleration of agonist-independent internalization, or are there two mechanistically different forms of internalization, which use distinct signals? A previous study revealed that human PAR1 truncated at residue 396 was markedly defective in both agonist-independent and agonist-triggered internalization. By contrast, a mutant PAR1 in which serines and threonines (potential phosphorylation sites) in the carboxyl tail were converted to alanines displayed normal agonist-independent internalization but was markedly defective in agonist-triggered internalization (20). We now report characterization of a PAR1 mutant with normally normal agonist-triggered trafficking but markedly defective agonist-independent trafficking. This characterization shows that agonist-independent and agonist-triggered internalization of PAR1 are separable by mutation, suggesting that these processes are probably mediated by distinct recognition...

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¶ The abbreviations used are: PAR, protease-activated receptor; PBS, phosphate-buffered saline; WT, wild-type PAR1.

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5 J. Trejo and S. R. Coughlin, manuscript in preparation.
The amino acid sequences (single-letter code) of the cytoplasmic tails of wild type and mutant receptors. The amino acid sequences of PAR1 mutants are shown to the right of the designations used for each mutant. The sequences begin with the first residue after the putative seventh transmembrane segment of the receptor and extend to its carboxyl terminus (9). Residues 397 and 406 are indicated. The sequence of the cytoplasmic tail of WT is shown in the top row. In the Y397Z mutant, residue 397 is replaced with a stop, thus truncating the receptor at residue 396. In the other mutants, residues that are different from those in WT are underlined.

Experimental Procedures

Materials—M1 anti-FLAG monoclonal antibody, which binds the epitope DYKDDDDK in a calcium-dependent manner (21), was from Eastman Kodak and was used at a concentration of 2.5 µg/ml unless noted. Horseradish peroxidase-coupled goat anti-mouse antibody was from Bio-Rad (Hercules, CA) and was used at a 1:1,000 dilution. The PAR1 agonist peptide SFLRN was synthesized with a carboxyl amide and purified by high-pressure liquid chromatography. Thrombin was from Enzyme Research Laboratories (South Bend, IN), and hirudin was from Sigma.

Plasmid Construction—The “WT” receptor referred to below is an otherwise wild-type human PAR1 containing a FLAG epitope at its amino terminus (22). All mutant receptors (Fig. 1) were generated by oligonucleotide-directed mutagenesis (23) of WT to create amino acid changes or, for “Y397Z,” the substitution of a stop codon for residue 397 (20). All receptors were subcloned into the mammalian expression vector pBj1 (provided by Mark Davis, Stanford University, Stanford, CA).

Cell Culture—Cell culture media were obtained from the University of California San Francisco cell culture facility. Cells were routinely grown in Dulbecco’s modified Eagle’s medium supplemented with 3 mg/ml glucose, 10% bovine calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone. Assay medium was Dulbecco’s modified Eagle’s medium supplemented with 3 mg/ml glucose, 1 mg/ml bovine serum albumin, and 10 mM HEPES buffer, pH 7.4. Phosphate-buffered saline (PBS) contained 0.1 mg/ml CaCl2 and MgCl2, 0.2 mg/ml KH2PO4 and KCl, 8 mg/ml NaCl, and 2.2 mg/ml Na2HPO4. PBS-EDTA contained 0.4 mg/ml EDTA and lacked CaCl2 and MgCl2.

Unless noted, −16 h before each assay, cells were split into 24-well Primaria dishes (Falcon, Lincoln Park, NJ) that were previously coated with 5 µg/well fibronectin (Sigma) to improve cell adherence. Three wells treated identically were then used to derive individual data points.

Generation of Rat1 Cell Lines—Rat1 cells were stably transfected, and individual clones were isolated as described previously (20, 22). Clones expressing the various receptors on the cell surface at similar levels, as determined by anti-FLAG antibody binding, were chosen. Typically, Y397Z was expressed at 53% of the surface level of WT, AG397–406 at 70% of the surface level of WT, and all other mutants (Fig. 1) at 75–90% of the surface level of WT. Antibody binding to untransfected Rat1 cells was typically 1.5% of binding to WT-expressing cells (values are the average of data from all experiments shown in which surface binding was measured).

Assay of Internalized Antibody—Cells in each well to be assayed were washed with 0.5 ml assay medium and incubated at 4 °C for 5 min. Cells were then incubated with 0.25 ml assay medium containing anti-FLAG antibody at 4 °C for 1 h. They were then washed twice with assay medium and incubated at 37 °C in assay medium with or without 100 µM SFLRN. Next, surface antibody was removed by a brief incubation in PBS-EDTA (binding of the M1 anti-FLAG antibody requires calcium (21)). The cells were then lysed in 1% Triton X-100, and the amount of anti-FLAG antibody in each lystate was determined by a “sandwich” enzyme-linked immunosorbant assay as described previously (20). Briefly, anti-FLAG antibody in lysates was captured by immobilized goat anti-mouse antibody in 96-well dishes and then detected by free goat anti-mouse antibody coupled to horseradish peroxidase. The start times for the initial incubations with anti-FLAG antibody were staggered so that all procedures subsequent to the incubation at 37 °C were performed simultaneously.

Assay of Surface Receptor Levels—After various treatments, the cells in each well were washed in 0.5 ml PBS, fixed in 0.5 ml PBS containing 2% paraformaldehyde for 5 min at room temperature, and then washed twice with PBS. Cells were next incubated for 1 h at room temperature in 0.25 ml assay medium containing anti-FLAG antibody and washed twice in PBS. Cells were then incubated with 0.25 ml assay medium containing horseradish peroxidase-coupled goat anti-mouse antibody, washed twice with PBS, and incubated in One Step 2,2’-azine-dil(3-ethylbenzthiazolinesulfonate) solution (Pierce). The absorbance of this solution at 405 nm was measured using a Molecular Devices (Menlo Park, CA) microplate spectrophotometer. For the experiment shown in Fig. 5, live cells were incubated with anti-FLAG antibody and, after various treatments, were fixed, and the amount of antibody bound to the surface was determined as described above starting with the incubation with secondary antibody.

Assays of Receptor Degradation—Cells plated in 12-well dishes were lysed in sample buffer containing 2% SDS. Each lysate was homogenized by passage through a 27-gauge needle and then applied to a 9% polyacrylamide gel. After electrophoresis, protein was transferred to a nitrocellulose filter for immunoblotting. Blotting buffer was 50 mM Tris, pH 7.4, 100 mM NaCl, 0.05% Tween 20, 1 mM CaCl2, and 5% nonfat dry milk (milk was omitted for washes). Filters were incubated overnight at 4 °C in blotting buffer and then incubated for 2 h at room temperature in blotting buffer with 2.5 µg/ml anti-FLAG antibody. Filters were then washed four times in blotting buffer and then incubated for 1 h at room temperature in blotting buffer with 2 µg/ml 125I-coupled sheep anti-mouse antibody (DuPont New England Nuclear). After four additional washes in blotting buffer, filters were exposed to x-ray film.

Immunostaining and Microscopy—All manipulations were performed at room temperature. Wash buffer was PBS containing 1% nonfat dry milk. Cells plated on glass coverslips were fixed in PBS containing 2% paraformaldehyde for 5 min and washed once in PBS. Some cells were then permeabilized by a 30-s incubation in methanol (at −20 °C) followed by two washes in PBS. Next, cells were washed three times for 5 min in wash buffer supplemented with 150 mM NaOAc, pH 7.0, and then washed three times for 5 min in wash buffer. Cells were next incubated for 1 h in wash buffer containing 5 µg/ml fluorescent isothiocyanate-coupled goat anti-mouse antibody (Life Technologies, Inc.) and then washed five times for 5 min in PBS. Coverslips were then mounted on microscope slides after the application of Slowfade reagent (Molecular Probes, Eugene, OR). Cells were photographed on a Nikon Microphot-FXA fluorescence microscope.

Results

Truncation of the cytoplasmic tail of human PAR1 by introduction of a stop codon at residue 397 (Y397Z) yielded a receptor that signaled robustly but was markedly defective in both agonist-independent and agonist-triggered internalization. By contrast, truncation at residue 407 (K407Z) yielded a receptor with near wild-type trafficking behavior (20). Toward further defining the roles of amino acids 397–406, a mutant receptor (AG397–406) in which the 10 residues in this region were replaced with a mixture of alanines and glycines was constructed.

Fig. 1. Amino acid sequences of the cytoplasmic tails of wild type and mutant receptors.
with anti-FLAG antibody for 1 h at 4 °C. After unbound antibody was removed, the cells were incubated at 37 °C for 30 min in the absence (hatched bars) or presence (filled bars) of 100 μM SFLLRN. Next, antibody remaining bound to receptors on the cell surface was removed by incubation with EDTA, and the cells were lysed. The amount of antibody in the lysates was then measured as an index of receptor internalization. The amount of antibody detected in lysates from cells not incubated at 37 °C (open bars) reflects the small amount of antibody that the EDTA treatment failed to remove. Results are expressed as a percent of the amount of antibody initially bound to the surface of cells expressing each receptor. Initial binding was defined as the amount of bound antibody present after the 4 °C incubation described above. The values shown are the averages and S.E.s from three separate experiments in which triplicate determinations were made. In the absence of agonist, the internalization of every mutant except G401A,Q402A,L403A was significantly less (p < 0.02) than that of WT. In the presence of agonist, only Y397Z internalization was significantly less (p < 0.01) than that of WT. Significance was determined by two-way analysis of variance followed by a Bonferroni t test.

(Fig. 1). Stably transfected Rat1 cell lines were used to compare the trafficking pattern of AG397–406 with that of wild-type PAR1 (WT) and the Y397Z mutant. A FLAG epitope preceded the amino-terminal exodomain of each of these receptors; this permitted recognition of receptors at the cell surface by anti-FLAG antibody and elimination of the epitope from surface receptors by thrombin cleavage (17, 22).

To measure both agonist-independent and agonist-triggered internalization in the same assay, cells were incubated with anti-FLAG antibody at 4 °C to exclusively label receptors on the cell surface. After removal of unbound antibody and subsequent incubation at 37 °C, antibody remaining bound to receptors on the cell surface was stripped away by EDTA treatment. The amount of antibody present in cell lysates was then detected by Western blotting with anti-FLAG antibody. Molecular masses (in kilodaltons) are indicated to the left. When cycloheximide was omitted, significant degradation of WT and AG397–406 was still apparent, but slightly more receptor was detected in lysates from cells treated with SFLLRN (data not shown). This experiment was replicated three times.

Agonist-triggered and agonist-independent internalization. Rat1 cells expressing the indicated receptors were incubated with anti-FLAG antibody for 1 h at 4 °C. After unbound antibody was removed, the cells were incubated at 37 °C for 30 min in the absence (hatched bars) or presence (filled bars) of 100 μM SFLLRN. Next, antibody remaining bound to receptors on the cell surface was removed by incubation with EDTA, and the cells were lysed. The amount of antibody in the lysates was then measured as an index of receptor internalization. The amount of antibody detected in lysates from cells not incubated at 37 °C (open bars) reflects the small amount of antibody that the EDTA treatment failed to remove. Results are expressed as a percent of the amount of antibody initially bound to the surface of cells expressing each receptor. Initial binding was defined as the amount of bound antibody present after the 4 °C incubation described above. The values shown are the averages and S.D.s of triplicate points. These results provide an index of agonist-independent trafficking, because the reappearance of antibody-labeled receptors indicates that an intracellular pool protected from the EDTA treatment was generated and that receptors subsequently recycled back to the cell surface. This experiment was replicated three times.

Effect of agonist on the steady-state level of receptor on the cell surface. Cells expressing wild-type PAR1 (circles), Y397Z (squares), or AG397–406 (triangles), were incubated in medium containing 100 μM SFLLRN at 37 °C for the indicated times. Cells were then fixed, and the amount of cell surface receptor was measured. For each mutant, the amount of receptor detected was divided by the amount detected on cells not exposed to agonist to derive the percent remaining. The values shown are the averages and S.D.S of triplicate points. This experiment was replicated three times.

Agonist-triggered degradation. Cells expressing wild-type PAR1 (left two lanes), AG397–406 (middle two lanes), or Y397Z (right two lanes) were incubated in assay medium containing 10 μM cycloheximide in the absence (−) or presence (+) of 100 μM SFLLRN for 90 min at 37 °C. Cell lysates were prepared and subject to Western blotting with anti-FLAG antibody. Molecular masses (in kilodaltons) are indicated to the left. When cycloheximide was omitted, significant degradation of WT and AG397–406 was still apparent, but slightly more receptor was detected in lysates from cells treated with SFLLRN (data not shown). This experiment was replicated three times.
observed in the absence of agonist peptide, and greater internalization was observed in the presence of agonist (Fig. 2). By contrast, very little internalization of Y397Z was observed in either the presence or absence of agonist (Fig. 2). Like Y397Z, AG397–406 displayed little internalization in the absence of agonist, but, strikingly, AG397–406 displayed nearly wild-type levels of internalization in the presence of agonist (Fig. 2). AG397–406 mediated thrombin and agonist peptide-triggered phosphoinositide hydrolysis in stably transfected Rat1 cells at least as effectively as WT (data not shown). Thus AG397–406 has a selective defect in agonist-independent internalization.

To further investigate the contribution of amino acids 397–406 to PAR1 internalization, additional mutants containing substitutions within this region were analyzed in stably transfected Rat1 cells (Figs. 1 and 2). Substitutions were not made at the serines between 397 and 406, because such mutants were predicted to be uninformative based on the previous observation of normal agonist-independent internalization of a receptor in which all the serines in the cytoplasmic tail were mutated (20). Three mutants in which substitutions were made at clusters of two or three positions (Fig. 1) all displayed roughly wild-type levels of internalization in the presence of agonist, consistent with results obtained with AG397–406 (Fig. 2). All three also displayed defects in agonist-independent internalization, although of varying extents. A mutant in which conservative substitutions were made at all 10 residues between 397 and 406 (CONS397–406; Fig. 1) displayed agonist-independent and agonist-triggered internalization nearly identical to AG397–406 (data not shown). These results provide further evidence that amino acids 397–406 of PAR1, although dispensable for agonist-triggered internalization, are critical for agonist-independent internalization.

Two additional assays of agonist-triggered trafficking were performed. First, the effect of receptor activation on the steady-state level of cell surface receptor was determined (Fig. 3). As expected, incubation with the PAR1 agonist peptide SFLLRN resulted in a decrease in the level of wild-type PAR1 detected on the cell surface but little decrease in the level of Y397Z. AG397–406 disappeared from the cell surface at least as rapidly as WT, confirming that it is capable of normal agonist-triggered internalization. PAR1 activation accelerates its degradation, presumably because of the sorting of activated receptors to

**Fig. 6. Subcellular localization of receptors by immunofluorescence.** Cells expressing wild-type PAR1 are shown in the left panels, and cells expressing AG397–406 are shown in the right panels. The cells shown in the top row were fixed but not permeabilized before staining to reveal surface receptors only. The cells in the middle row were incubated with 20 nM thrombin in assay medium for 30 min at 4 °C, thereby cleaving the FLAG epitope from the cell surface but not intracellular receptors; this reduced surface staining to an undetectable level (data not shown). Cells were then fixed and permeabilized before staining to reveal intracellular receptors. The cells in the top and middle rows were photographed with identical exposure times under fluorescence illumination using a 100× objective. The phase-contrast photomicrographs shown in the bottom row represent the same fields photographed under fluorescence for the middle row. The results shown are representative of observations made in three separate studies.
lysosomes (17, 19). To measure receptor degradation, cells were incubated for 90 min with cycloheximide to inhibit protein synthesis in the presence or absence of agonist peptide, and then the total amount of receptor present in cell lysates was determined by Western blotting. WT was nearly undetectable in a lysate from agonist-treated cells, whereas only a slight decrease in the level of Y397Z was observed (Fig. 4). AG397–406 underwent degradation like WT, again displaying normal agonist-triggered trafficking.

As an additional assay of agonist-independent trafficking, cells were incubated with anti-FLAG antibody for 90 min at 37 °C to allow both antibody binding and agonist-independent internalization to occur. Next, antibody bound to cell surface receptors was removed, and the reappearance of antibody-labeled surface receptors was measured after further incubation (Fig. 5). A rapid reappearance of antibody-labeled WT was observed, indicating that antibody-labeled receptors had undergone agonist-independent internalization during the first incubation and that internalized receptors had cycled back to the cell surface during the second. Substantially less reappearance of both Y397Z and AG397–406 was observed, indicating that the two mutants have similar defects in agonist-independent trafficking.

Based on the presence of an intracellular receptor pool in cells expressing a PAR1 mutant defective in agonist-triggered internalization and the absence of this pool in cells expressing a mutant defective in both agonist-triggered and agonist-independent internalization, we previously suggested that the intracellular pool of wild-type PAR1 might be generated by the agonist-independent internalization and recycling of PAR1 between the cell surface and the intracellular compartment (20). Because AG397–406 had a relatively selective defect in agonist-independent internalization, it provided a more precise tool to test this hypothesis. Conventional immunofluorescence microscopy was performed to determine the subcellular distribution of AG397–406 (Fig. 6). WT and AG397–406 were detected at similar levels at the cell surface when unpermeabilized cells were stained with anti-FLAG antibody. Both receptors displayed punctate as well as diffuse surface staining—it is not presently known whether this punctate staining is the result of localization to a particular subcellular structure. As expected, no staining was observed when unpermeabilized cells were treated with thrombin at 4 °C to cleave the FLAG epitope from cell surface receptors (data not shown; see below). When cells expressing WT were treated with thrombin and then permeabilized, staining of the previously reported intracellular pool of receptors was observed (Fig. 6). By contrast, virtually no intracellular staining was detected in cells expressing AG397–406 (Fig. 6), consistent with the hypothesis that agonist-independent internalization is necessary for maintenance of this pool.

The intracellular pool of PAR1 has been proposed to facilitate repopulation of the cell surface with uncleaved receptors after surface receptors are cleaved by thrombin (16, 17). If this is the case, recovery of uncleaved receptors on the cell surface should be impaired in cells expressing PAR1 mutants that do not form an intracellular pool. To test this idea, cells were exposed to thrombin for 30 min at 4 °C, after which uncleaved cell surface receptors bearing FLAG epitopes were nearly undetectable (Fig. 7). The reappearance of uncleaved surface receptors on the cell surface after removal of thrombin and incubation at 37 °C was then measured. A rapid reappearance of uncleaved WT was observed, whereas, as predicted, very little Y397Z or AG397–406 reappeared (Fig. 7). It is unlikely that the reappearance of uncleaved wild-type PAR1 was attributable substantially to new protein synthesis, because cycloheximide was included at a concentration that prevented new receptor syn-

**DISCUSSION**

The results presented above, in combination with those we have reported previously, demonstrate that it is possible to selectively eliminate either or both of the two forms of trafficking displayed by wild-type PAR1 by mutation of its cytoplasmic tail. Y397Z, a mutant that lacks part of the tail, was defective in both agonist-independent and agonist-triggered trafficking (Ref. 20 and this study). A receptor in which the serines and threonines in the tail were mutated was defective in agonist-triggered internalization yet displayed normal agonist-independent internalization (20). The AG397–406 mutant completes this picture with the converse phenotype: defective agonist-independent trafficking but unimpaired agonist-triggered internalization and degradation. In two assays of agonist-independent trafficking, AG397–406 showed defects nearly identical to those of Y397Z (Figs. 2 and 5). By contrast, in three assays of agonist-triggered trafficking, AG397–406 displayed WT-like behavior (Figs. 2–4). Taken together, these observations show that PAR1 agonist-independent and agonist-triggered trafficking patterns are separable by mutation and suggest that separate signals in the PAR1 cytoplasmic tail specify its two distinct modes of internalization and sorting.

The previous observation that PAR1 truncated at residue 407 had near wild-type levels of agonist-independent and agonist-triggered internalization, whereas truncation at residue 397 caused defects in both functions (20), might at face value suggest that the single region between residues 397 and 406 is necessary for both functions, but it must be kept in mind that both of these mutants lack residues carboxyl to 406. The pres-
ent study suggests that the region between 397 and 406 is indeed necessary for agonist-independent internalization regardless of whether the remainder of the carboxyl tail is present. This region may bind a protein(s) that mediates agonist-independent internalization, but as in all such mutation studies, the possibility that the AG397–406 substitution effects structural changes that influence other recognition sites cannot be excluded. In contrast to the case for agonist-independent internalization, the trafficking behavior of AG397–406 shows that the region between 397 and 406 is not strictly necessary for agonist-triggered internalization. Phosphorylation sites (24) or other structural features located carboxyl to position 406 and preserved in the AG397–406 mutant but not in Y397T presumably contribute to agonist-triggered internalization and are sufficient to mediate this function. Regardless, the ability to generate PAR1 mutants with selective defects in agonist-independent and agonist-triggered internalization suggests that separate recognition events specify these two trafficking behaviors. Future studies will focus on clarifying the exact nature of the receptor structures involved and on identifying the proteins that recognize these signals and specify the fate of PAR1.

The trafficking pattern of AG397–406 provides an additional lesson regarding agonist-triggered internalization of PAR1. Wild-type PAR1 undergoes internalization in the absence of agonist and then recycles back to the cell surface, whereas activated receptors internalize and then are targeted to lysosomes (17, 19, 20). The more rapid loss of PAR1 from the cell surface that occurs in the presence of PAR1 agonists could be accomplished by the more rapid internalization of activated receptors and/or by suppression of the recycling of internalized receptors back to the surface. Because AG397–406 showed virtually no detectable agonist-independent internalization or intracellular pool (Figs. 2, 5, and 6) yet displayed nearly wild-type agonist-triggered internalization (Figs. 2 and 3), it is likely that agonist-triggered internalization of PAR1 reflects an increased rate of movement of activated receptors from the plasma membrane to an intracellular compartment.

The intracellular pool of PAR1 seen in endothelial cells and fibroblasts has been proposed to serve as an intracellular store of PAR1 that is protected from thrombin cleavage and allows repopulation of the cell surface with unactivated receptors without requiring new receptor synthesis (16, 17). The observation that in the absence of agonist PAR1 tonically internalizes and then recycles back to the cell surface suggests that agonist-independent trafficking may be responsible for both the genesis of the intracellular pool and the repopulation of the cell surface with uncleaved receptors (16, 17, 20). Evidence presented here supports this model. First, an intracellular pool was largely absent in cells expressing a PAR1 mutant selectively defective in agonist-independent internalization (Fig. 6) but was previously found to be present in cells expressing a PAR1 mutant selectively defective in agonist-triggered internalization (20). Second, in cells expressing mutants defective in agonist-independent internalization, repopulation of the cell surface with uncleaved receptors after thrombin treatment occurred at a markedly reduced rate compared with cells expressing WT (Fig. 7). Thus loss of agonist-independent internalization correlates with both loss of the intracellular PAR1 pool and loss of efficient repopulation of the cell surface with uncleaved receptors after exposure to thrombin.

The intracellular compartment(s) that functions as a store of protected PAR1 has not been characterized in detail. In human endothelial cells analyzed by immunoelectron microscopy, PAR1 was found to localize predominantly to a continuous tubulovesicular network extending from the perinuclear region to the periphery and relatively weakly to the plasma membrane (18). The present functional characterization of a PAR1 mutant selectively defective in both localization to an intracellular compartment and recycling provides a useful new reagent for determining whether the pools identified in such anatomical studies are associated with the ability of PAR1 to repopulate the cell surface with new receptors.

Classically, G protein-coupled receptors internalize after ligand binding and then recycle back to the cell surface after ligand dissociation (25–30). This process has been shown to allow the resensitization of cells to a particular agonist after its removal (31–33). Because PAR1 is activated in an irreversible manner by thrombin, recycling of activated receptors cannot provide a mechanism of resensitization. However, the intracellular pool of PAR1 and the tonic cycling of unactivated receptors does provide cells with a means to rapidly regain a surface contingent of unactivated receptors after thrombin exposure. Hence, agonist-independent trafficking of PAR1 may be an adaptation necessitated by its unique mechanism of activation.

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