The Cytoplasmic Tails of Protease-activated Receptor-1 and Substance P Receptor Specify Sorting to Lysosomes versus Recycling*

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The G protein-coupled receptor (GPCR) for thrombin, protease-activated receptor-1 (PAR1), is activated when thrombin cleaves its amino-terminal exodomain. The irreversibility of this proteolytic mechanism raises the question of how desensitization and resensitization are accomplished for thrombin signaling. PAR1 is phosphorylated, uncoupled from signaling, and internalized after activation like classic GPCRs. However, unlike classic GPCRs, which internalize and recycle, activated PAR1 is sorted to lysosomes. To identify the signals that specify the distinct sorting of PAR1, we constructed chimeras between PAR1 and the substance P receptor. Wild-type substance P receptor internalized and recycled after activation; PAR1 bearing the cytoplasmic tail of the substance P receptor (P/S) behaved similarly. By contrast, wild-type PAR1 and a substance P receptor bearing the cytoplasmic tail of PAR1 (S/P) sorted to lysosomes after activation. Consistent with these observations, PAR1 and the S/P chimera were effectively down-regulated by their respective agonists as assessed by both receptor protein levels and signaling. Substance P receptor and the P/S chimera showed little down-regulation. These data suggest that the cytoplasmic tails of PAR1 and substance P receptor specify their distinct intracellular sorting patterns after activation and internalization. Moreover, by altering the trafficking fates of PAR1 and substance P receptor, one can dictate the efficiency with which a cell maintains responsiveness to PAR1 or substance P receptor agonists over time.

Thrombin, a multifunctional serine protease generated at sites of vascular injury, regulates a variety of cellular processes important in cardiovascular biology and disease (1). The actions of thrombin on human platelets, endothelial cells and fibroblasts are mediated at least in part by a family of G protein-coupled protease-activated receptors (2–5). Protease-activated receptor-1 (PAR1) is prototypical of this family (6–8). PAR1 is activated by an irreversible proteolytic mechanism in which thrombin binds to and cleaves the amino-terminal exodomain of the receptor. Receptor cleavage results in the generation of a new amino terminus that functions as a tethered ligand by binding to the body of the receptor to cause transmembrane signaling (2, 9, 10). A soluble peptide with the sequence SFLLRN representing the first six amino acids carboxyl-terminal to the cleavage site (P′–P′) mimics the tethered ligand of PAR1 and acts as a PAR1 agonist. The irreversible proteolytic mechanism of PAR1 activation raises the questions of how this receptor is shut off and how cells maintain the ability to respond to thrombin over time.

The β2-adrenergic receptor has served as a prototype for the molecular events responsible for GPCR desensitization and resensitization (Refs. 11–13; reviewed in Ref. 14). Most activated GPCRs are desensitized initially by rapid phosphorylation of the agonist-occupied form of the receptor. Phosphorylated receptor then binds arrestin, which prevents receptor interaction with G proteins, thereby uncoupling it from effectors. Arrestin may also mediate the interaction of β2-adrenergic receptor with clathrin-coated pits, thereby promoting internalization of activated receptors (15, 16). Within endosomes the β2-adrenergic receptor dissociates from its ligand, is dephosphorylated, and recycles back to the cell surface competent to signal again. In this case trafficking serves to remove activated receptor from the cell surface and to return the receptor to the surface in a off state, ready to respond again to ligand.

Like the β2-adrenergic receptor, PAR1 is rapidly phosphorylated and uncoupled from signaling after activation (17, 18). PAR1 is also internalized after activation (19–21). However, unlike classical GPCRs, activated PAR1 is sorted predominantly to lysosomes after internalization (19, 22). To begin to understand the function of intracellular trafficking of PAR1 in regulation of signaling, we first sought to identify the domain(s) that specifies sorting of activated PAR1 to lysosomes. We constructed chimeras between PAR1 and the classic GPCR for substance P (SPR) by exchanging their cytoplasmic carboxyl tails. SPR, also known as the neurokinin-1 receptor, behaves like the β2-adrenergic receptor and other classic GPCRs; it is activated reversibly by the peptide substance P, internalized, and then recycled back to the plasma membrane (23, 24). Remarkably, exchanging the cytoplasmic carboxyl tails (C-tails) of PAR1 and SPR switched their trafficking behaviors after activation. SPR bearing the C-tail of PAR1 (S/P chimera) internalized upon activation and sorted to lysosomes like wild-type PAR1. Conversely, PAR1 bearing the C-tail of SPR (P/S chimera) internalized upon activation but recycled back to the plasma membrane like wild-type SPR. Recycling versus lysosomal sorting of activated receptors correlated with the extent to which signaling was down-regulated over time. Cells express-
ing the wild-type substance P receptor and P/S chimera were capable of responding to their cognate agonists even after prolonged agonist exposure. By contrast, signaling by wild-type PAR1 and the S/P chimera was significantly down-regulated. Taken together, these data strongly suggest that the cytoplasmic tails of PAR1 and SPR specify their distinct intracellular sorting fates after activation. Moreover, the P/S and S/P chimeras provide reagents for identifying the molecular mechanisms by which PAR1 is sorted to lysosomes and for determining the importance of this process for terminating PAR1 signaling.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**Monoclonal anti-FLAG antibodies, M1 and M2, were purchased from Eastman Kodak. Rabbit polyclonal anti-FLAG antibody was obtained from Santa Cruz Biotechnology. Polyclonal 1809 antibody was raised to a peptide representing the his-rhodamino sequence in the amino-terminal exodomain of PAR1 (6). The GM10 monoclonal antibody against the lysosomal membrane glycoprotein (lgp120) was generously provided by John Hutton (University of Colorado, Boulder, CO) and Samuel A. Green (University of Virginia, Charlottesville, VA) (25). Texas Red-conjugated goat anti-mouse antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody were purchased from Molecular Probes (Eugene, OR). GM10 and M2, were purchased from Eastman Kodak. Rabbit polyclonal antibody 1809 (1:200) was used for immunostaining PAR1 and P/S chimera, and the rabbit polyclonal anti-FLAG antibody (1:100) was used for staining SPR and S/P chimera. Following agonist treatment at 37 °C, cells were fixed for 1 h at 4 °C with 1% paraformaldehyde in PBS and then permeabilized in 100% methanol at −20 °C for 30 s. Cells were then washed three times with PBS containing 1% nonfat dry milk, 150 mM sodium acetate, pH 7.0, followed by another three washes with PBS containing only 1% nonfat dry milk (blocking buffer). Following these washes, cells were incubated with the lgp120 antibody GM10 for 1 h at 25 °C, washed, and then incubated with Texas Red-X goat anti-mouse and fluorescein goat anti-rabbit secondary antibodies for 1 h at 25 °C. Finally, cells were washed four times with PBS then once with Molecular Probes SlowFade equilibration buffer. One drop of SlowFade anti-fade reagent was added to each slide before mounting the coverslip. Confocal images were collected using a Nikon PCM 2000 laser scanning confocal system configured with an Eclipse E900 microscope fitted with a CFI Plan Achromat 60× oil objective (Nikon, Corp.). Fluorescein and Texas Red-X images were captured sequentially at 1024 × 1024 resolution with 2× optical zoom in 2 s and processed using C-IMAGING 1280 system (Compix, Inc.). The final image composite was created using Adobe Photoshop 4.0.

**Immunofluorescence microscopy** was used to follow internalization and recovery of cell surface receptors. Transfected Rat1 fibroblasts were plated on glass coverslips (22 × 22 mm) and treated as described below for cell surface ELISA. Following fixation with paraformaldehyde, cells were incubated with fluorescein-conjugated goat anti-mouse secondary antibody for 1 h at 25 °C. Cells were then washed four times with PBS and once with SlowFade equilibration buffer, and then one drop of SlowFade reagent was added to each coverslip before mounting. Images were acquired using a Nikon Microphot-FXA fluorescence microscope fitted with a PlanApo 100× oil objective (Nikon Corp.).

**Cell Surface ELISA—**To follow the cohort of cell surface receptors, transfected Rat1 fibroblasts plated in 24-well dishes (Falcon) were incubated with 3 μg/ml M1 anti-FLAG antibody diluted in DMEM/bovine serum albumin/HEPES for 1 h at 4 °C. Cells were washed, warmed to 37 °C, and then exposed to agonists in media containing 50 μM NECA. This concentration of NECA was previously shown to inhibit new receptor synthesis in transfected Rat1 cells (19). Following various treatments, cells were fixed in 4% paraformaldehyde for 5 min at 4 °C and then washed twice with PBS. Cells were then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (1:1000) for 1 h at 25 °C, washed three times in PBS, and incubated with the horseradish peroxidase substrate 1-Step ABTS (2, 2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid) (Pierce). After 15 min, an aliquot was removed, and the optical density was read at 405 nm using a Molecular Devices microplate spectrophotometer.

**Phosphoinositide Hydrolysis—**Cells plated in 12-well dishes (Falcon) were labeled overnight with 2 μCi/ml [3H]myo-inositol in DMEM containing 1 mg/ml bovine serum albumin. Cells were washed and treated as described in the legend to Fig. 6, and the formation of inositol phosphates was assayed as reported previously (28).
PAR1 C-tail is designated S/P chimera. Upon activation PAR1 is phosphorylated, rapidly internalized, and sorted predominantly to lysosomes (open oval) where it is degraded. By contrast, SPR resides largely at the plasma membrane in the absence of agonist. Upon activation, SPR is phosphorylated, internalized into an early endosomal compartment (speckled oval), and then recycled back to the cell surface. PAR1 bearing the PAR1 C-tail is designated “P/S chimera” and SPR bearing the SPR C-tail is designated “S/P chimera” (Fig. 1).

RESULTS AND DISCUSSION

Degradation of Wild-type and Chimeric Receptors—To identify the domain(s) that specify the distinct intracellular sorting fates of activated PAR1 and SPR (Fig. 1A), we made chimeras in which the C-tails of these receptors were exchanged carboxyl to their putative palmitoylation sites. PAR1 bearing the SPR C-tail is designated “P/S chimera” and SPR bearing the PAR1 C-tail is designated “S/P chimera.”

To determine whether wild-type or chimeric receptors were sorted to a degradative pathway upon activation, we incubated receptor-expressing cell lines in the presence or absence of agonist for 90 min and then assessed the amount of receptor protein in cell lysates by immunoblot using anti-FLAG antibody; each lane represents an equivalent amount of cell lysate. Similar findings were observed in three separate experiments. Phosphorylation of PAR1 (E), SPR (F), P/S chimera (G), or the S/P chimera (H) in stably transfected Rat1 fibroblasts is shown. Cells were incubated in the presence or absence (Ctrl) of PAR1 agonist SFLLRN (100 μM) or substance P (100 nM) as indicated for 90 min at 37 °C. Where indicated, 10 μM cycloheximide was included during the incubation period (+CHX). Cell lysates were then analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using M1 anti-FLAG antibody; each lane represents an equivalent amount of cell lysate. Similar findings were observed in three separate experiments. Phosphorylation of PAR1, SFLLRN, 100 nM substance P, or media alone (Ctrl) for 3 or 90 min at 37 °C. Cycloheximide (10 μM) was included during the incubation periods. Cell lysates from an equivalent number of cells were prepared, and receptors were immunoprecipitated as described under “Experimental Procedures.” Receptor immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography. No phosphorylated proteins were detected in immunoprecipitates from untreated Rat1 fibroblasts (Rat1 lane in G and H). Similar results were observed in two independent experiments.

bearing the SPR C-tail (P/S chimera) behaved very much like wild-type SPR in this assay. The PAR1 agonist peptide SFLLRN caused a decrease in the mobility of the P/S chimera but little or no loss of P/S receptor protein (Fig. 2C). Immunoblot of lysates prepared from cells expressing a SPR bearing the C-tail of PAR1 (S/P chimera) revealed three transfection-dependent bands (Fig. 2D). Exposure of S/P expressing cells to trypsin at 4 °C caused a decrease in the intensity of the intermediate ~50-kDa band but not the other bands (data not shown), suggesting that only the 50-kDa form of the S/P chimera was expressed on the cell surface and cleaved by extracellular amino termini and were stably expressed in Rat1 fibroblasts.
Stably transfected Rat1 cells expressing epitope-tagged PAR1, SPR, P/S chimera, and S/P chimera were used to determine receptor half-lives and to examine the effect of lysomotropic agents on receptor degradation. Cells were left untreated or treated with 100 μM SFLLRN or 100 μM substance P for 0.5, 1, 3, 6, or 12 h, lysed, and analyzed by immunoblot to determine receptor half-lives. Cells were incubated in media alone or in media containing 0.1 mM chloroquine or 50 mM NH4Cl and treated for 90 min with agonists to determine the effect of lysomotropic agents on receptor degradation. M1 FLAG antibody and 125I sheep anti-mouse antibody were used for immunoblotting. Immunoblots were quantitated using the Molecular Dynamics Storm imager system. The half-life data are the mean (n = 2) from a representative experiment. The experiment with the lysosomal inhibitors was replicated twice. ND, not determined; +, with agonist; −, without agonist.

| Receptor       | Half-life | Receptor protein remaining |
|----------------|-----------|---------------------------|
|                | h         |                          |
|                |           | No inhibitor | Chloroquine | NH4Cl |
| PAR1           | SFLLRN    | 9           | 0.5         | SFLLRN |
| SPR            | Substance P | 9           | 6           | ND     |
| P/S chimera    | SFLLRN    | 7           | 3           | ND     |
| S/P chimera    | Substance P | 4           | 0.6         | Substance P |

Effect of agonist and lysosomal hydrolase inhibitors on receptor degradation

These data are consistent with the hypothesis that the 50-kDa band represents a properly processed and activatable form of this receptor chimera. Interestingly, the 50-kDa band was the only form markedly reduced in cells incubated with substance P (Fig. 2D), consistent with the notion that it undergoes agonist-dependent sorting to a degradation pathway (see below).

The structural basis for misfolding or improper processing of the other species of S/P protein is unknown. Of note, when analogous chimeras were made between PAR1 and β2-adrenergic receptor, no functional chimeric receptors were obtained (data not shown). It is possible that in such chimeras, interactions between the C-tail and the body of the receptor led to misfolding or misprocessing or that conflicting trafficking signals interfered with their biogenesis. By contrast, generation of chimeras between PAR1 and SPR did yield receptors capable of mediating signaling, and we have chosen to focus on the agonist-dependent trafficking behaviors of these chimeras in the current study to avoid events involving improperly processed receptors.

**Phosphorylation of Wild-type and Chimeric Receptors**—PAR1 was phosphorylated within 3 min of exposure to agonist peptide SFLLRN, but no phosphorylated receptor was detected in PAR1-expressing cells after prolonged exposure to agonist (Fig. 2E). Only the middle band representing the S/P chimera showed phosphorylation upon addition of ligand, again consistent with this band representing functional chimeric receptor expressed at the cell surface (Fig. 2H). As with wild-type PAR1, little or no detectable phosphorylated S/P chimera was detected after 90 min of exposure to agonist. These findings are consistent with the nearly complete degradation of PAR1 after 90 min of agonist treatment (Fig. 2A). Wild-type SPR was also rapidly phosphorylated upon exposure to agonist, but in contrast to PAR1, phosphorylated SPR was still detected after prolonged exposure of cells to substance P (Fig. 2F). SPR phosphorylation was reversible within 30 min of agonist withdrawal (data not shown), and new receptor synthesis was blocked in these studies, thus detection of phosphorylated SPR after 90 min of constant exposure to agonist is consistent with ongoing activation and phosphorylation of recycled receptors. The P/S chimera behaved like SPR in this regard, showing continued phosphorylation in the presence of agonist peptide SFLLRN and reversible phosphorylation upon removal of peptide agonist (Fig. 2G and data not shown).

**Sorting of Wild-type and Chimeric Receptors to Lysosomes**—We next examined the effect of lysosomal inhibitors on agonist-induced changes in the half-lives of wild-type and chimeric receptors in these cell lines as described under “Experimental Procedures.” Incubation with agonist decreased the half-life of wild-type PAR1 by a remarkable 18-fold (Table I). Similarly, the S/P chimera 50-kDa band showed a marked ~7-fold decrease in half-life in the presence of agonist. By contrast, wild-type SPR and P/S chimera showed only modest decreases in half-life of ~0.5- and ~2-fold, respectively, upon exposure to agonist. To test whether agonist-promoted degradation of PAR1 and the S/P chimera required active lysosomal hydrolases, we incubated cells with chloroquine or NH4Cl and then assayed the amount of receptor protein remaining after incubation in the presence or absence of agonist. Chloroquine or NH4Cl did not block internalization of PAR1 or SPR (data not shown). However, the striking agonist-induced decreases in cellular PAR1 and S/P chimera protein levels were substantially attenuated by these lysosomal hydrolase inhibitors (Table I). These data are consistent with the hypothesis that PAR1 and the 50-kDa form of the S/P chimera are degraded in lysosomes after activation and internalization.

To directly test whether the activated receptors and receptor chimeras sorted to lysosomes, we used confocal microscopy to determine whether these receptors co-localized with a lysosomal marker in an agonist-dependent manner. Cells were incubated in the presence or absence of agonist for 30 min, fixed, immunostained for receptor protein (Fig. 3, green) and the lysosomal integral membrane protein lgp120 (Fig. 3, red), and then imaged by confocal microscopy. In the absence of agonist, little co-localization (Fig. 3, yellow) of receptor and lgp120 was seen in cells expressing either wild-type or chimeric receptors. After incubation with agonist, co-localization (Fig. 3, yellow) of both PAR1 and the S/P chimera with lgp120 was easily detected (Fig. 3, A and D). These data are consistent with at least a fraction of the S/P chimera (presumably the functional 50-kDa form) undergoing agonist-triggered internalization and sorting to lysosomes. At face value, these data suggest that information specifying the sorting of activated PAR1 to lysosomes resides in its C-tail. In contrast to activated PAR1 and S/P chimera, wild-type SPR and P/S chimera failed to show any co-localization with the lysosomal marker lgp120 following 30 min of exposure to agonist. Taken together with the half-life and lysosomal hydrolase inhibitor data cited above, this observation suggests that replacing the C-tail of PAR1 with that of the SPR prevents its sorting to lysosomes and its agonist-dependent degradation.

**Internalization and Recycling of Wild-type and Chimeric Receptors**—To determine whether replacing the C-tail of PAR1 with that of the SPR conferred recycling of the activated chimeric receptor, we followed internalization and recycling of receptor-bound antibody (Fig. 4). Rat1 fibroblasts stably transfected with wild-type or chimeric receptors bearing a FLAG
epitope at their amino termini were incubated with the calcium-dependent M1 FLAG antibody for 60 min at 4 °C (Fig. 4, t0); under these conditions, antibody was bound to cell surface receptor but did not internalize. Cells were then washed to remove unbound antibody and incubated at 37 °C for 90 min in the presence or absence of agonist (Fig. 4, t1). After this incubation, surface-bound antibody was removed by washing cells briefly with PBS/EDTA, agonist was removed, and the reappearance of previously internalized receptor-bound antibody was followed over the next 60 min. The amount of antibody on
the cell surface at various times was quantitated by cell surface ELISA. Antibody binding in these studies was dependent on transfection with cDNAs encoding the FLAG-tagged receptors. In cells expressing PAR1 (in which the FLAG epitope is cleaved by the receptor from thrombin), antibody binding was ablated by thrombin treatment (17). M1 antibody bound to the same FLAG epitope displayed at the extracellular amino-terminal of PAR1, SPR, or the chimeras showed distinct recycling properties depending upon the receptor to which it was bound. Studies examining internalization of receptor-bound antibody have yielded results concordant with studies of loss of receptor from the cell surface (21). Lastly, when receptor-bound antibody internalized and then returned to the cell surface, it could be reinternalized by again exposing the cells to agonist (data not shown). These data strongly suggest that antibody detected on the cell surface in these studies represents receptor-bound antibody and that such antibody does not interfere with either agonist-induced receptor internalization or recycling.

In PAR1-transfected cells not exposed to agonist during the 90-min incubation, the amount of receptor-bound antibody on the cell surface declined to a new steady state approximately two-thirds of the initial level (Fig. 4A, t1). It is unlikely that this decrease in surface-bound antibody was due to antibody dissociation or antibody-induced internalization of receptor, because parallel experiments with cells expressing wild-type SPR bearing the same amino-terminal epitope showed little loss of surface-bound antibody (Fig. 4B, t1). Rather, these findings are consistent with tonic internalization of cell surface PAR1 and equilibration with an intracellular pool as previously reported (19, 21).

Exposure to the PAR1 agonist peptide SFLLRN during the 90-min incubation caused a 90% decrease in the level of PAR1-bound antibody on the cell surface (Fig. 4A, t1) consistent with agonist-triggered internalization and a lack of receptor recycling. By contrast, the addition of substance P caused a more modest decrease in SPR cell surface levels consistent with internalization and recycling of this receptor (Fig. 4B, t1).

After this initial incubation in the presence or absence of agonist, antibody was stripped from the cell surface with PBS/EDTA, and recovery of internalized antibody-receptor complexes was followed. In PAR1-expressing cells, little recovery of antibody was seen in cells that had not been preincubated with agonist. Similarly, in cells exposed to agonist, there was little recovery of internalized antibody despite the large cohort of receptor-bound antibody that had been previously internalized (Fig. 4A). These data are consistent with the hypothesis that activated PAR1 is internalized and sorted to lysosomes with the bound antibody. In SPR-expressing cells, there was relatively little recovery of antibody in cells that had not seen agonist. However, in striking contrast to PAR1-expressing cells, SPR-expressing cells that had been pretreated with agonist showed substantial recovery of antibody on the cell surface with time (Fig. 4B). Re-addition of substance P caused rapid internalization of this surface-bound antibody (data not shown); thus recovered antibody likely reflects the reappearance of SPR on the cell surface. These data are consistent with agonist-dependent sequestration of SPR and its recycling to the cell surface after removal of agonist, known properties of this
FIG. 5. Internalization and recovery of wild-type and chimeric receptors examined by immunofluorescence microscopy. Stably transfected Rat1 fibroblasts expressing PAR1 (A), SPR (B), P/S chimera (C), or the S/P chimera (D) were treated as described in the legend to Fig. 4 and examined by fluorescence microscopy. Images of cells preincubated with M1-FLAG antibody that were either left untreated (Control) or treated with agonists (100 μM SFLLRN or 100 nM substance P) for 90 min are indicated by $t_1$. Following agonist treatment, remaining surface-bound antibody was removed with PBS/EDTA, images are indicated by $t_R = 0$ min. Fluorescent image (left) shows substantial decrease in antibody binding, the adjacent phase-contrast image (right) is of the identical field. At 60 min, recovery of receptor-bound antibody to the cell surface was then examined, images are indicated by $t_R = 60$ min. Constant exposure times of 20 s for PAR1 (A), 30 s for SPR (B), 30 s for P/S chimera (C), and 30 s for S/P chimera (D) were used to collect fluorescent images. The amount of fluorescent staining in $t_R = 0$ min was similar
classic GPCR (23, 24).

Remarkably, the P/S chimera behaved very much like the wild-type SPR in this assay; cells expressing the P/S chimera clearly exhibited the phenomenon of an agonist-dependent recoverable pool of receptor-bound antibody (Fig. 4C). Taken together with the results shown in Figs. 2 and 3, these data suggest that the P/S chimera undergoes agonist-dependent internalization but then recycles back to the cell surface instead of sorting to lysosomes like wild-type PAR1. By contrast, the S/P chimera behaved like PAR1 in this assay, with a profound agonist-dependent loss of surface receptor-bound antibody and no agonist-dependent recoverable pool (Fig. 4D). These observations suggest that the S/P chimera fails to recycle after agonist-triggered internalization and are consistent with its sorting to lysosomes (see Fig. 3D).

Studies using fluorescence microscopy supported the ELISA results in Fig. 4. Treatment of PAR1-expressing cells with SFLLRN caused almost complete internalization of PAR1-bound antibody from the cell surface (Fig. 5A, t1). After this initial internalization little return of antibody to the cell surface was detected in either untreated or agonist-treated cells (Fig. 5A). The S/P chimera behaved like wild-type PAR1 (Fig. 5D), consistent with lysosomal sorting of internalized antibody-receptor complexes. Untreated SPR-expressing cells also showed little recovery of receptor-bound antibody over time (Fig. 5B). Strikingly, however, a substantial recovery of SPR-bound antibody was observed in SPR-expressing cells that had been treated with agonist (Fig. 5B). The P/S chimera also displayed a robust agonist pretreatment-dependent recovery of receptor-bound antibody on the cell surface (Fig. 5C). These observations are consistent with agonist-induced internalization and recycling of SPR and the P/S chimeric receptor to the cell surface.

**Signaling by Wild-type and Chimeric Receptors**—The data presented above reveal substantial down-regulation of PAR1 protein by agonist consistent with lysosomal sorting of activated PAR1. By contrast, the P/S chimeric receptor was long lived in the presence of agonist consistent with its recycling. To determine whether these differences in down-regulation of receptor protein had a functional correlate, we examined the ability of cells expressing PAR1 or the P/S chimera to respond to agonist after a prolonged agonist exposure. In these experiments, transfected Rat1 fibroblasts labeled with [3H]inositol were incubated in the presence or absence of agonist in media without LiCl. Without LiCl, receptor activation does trigger phosphoinositide hydrolysis, but the released inositol phosphates are rapidly metabolized and fail to accumulate. After such pretreatment, agonist was washed out, LiCl was added to the medium, cells were again incubated in the presence or absence of agonist, and inositol phosphate accumulation was measured. Without prior agonist treatment, SFLLRN cause an approximately 8-fold increase in phosphoinositide hydrolysis in cells expressing PAR1. Agonist pretreated cells showed only a small decrease in signaling following agonist pretreatment (B and C). These results are representative of five separate experiments. The initial amount of receptor expressed on the surface of wild-type and chimeric receptor expressing cell lines in this experiment was 0.8 for PAR1, 0.5 for SPR, 1.0 for P/S chimera, 0.5 for S/P chimera, and 0.1 for untransfected Rat1 fibroblasts.

![Graphs A, B, C, D](image_url)
respond to agonist. Taken together these data suggest that, unlike PAR1 itself, the P/S chimera recycles efficiently after activation and is capable of responding to agonist again after such recycling.

The magnitudes of the signaling responses in these cells are also noteworthy. Cells expressing the P/S chimera reliably showed greater absolute levels of phosphoinositide hydrolysis than wild-type PAR1, whereas the S/P chimera showed less than substance P receptor. It is possible that the apparent gain-of-function of the P/S chimera is another manifestation of its ability to recycle and avoid down-regulation during the stimulation period. Conversely, the decreased signaling by the S/P chimera may be a manifestation of its gaining the ability to down-regulate. It is certainly also possible that intrinsic differences in the efficiency of G protein coupling contribute to these phenomena.

These studies demonstrate that the cytoplasmic carboxyl tails of two G protein-coupled receptors, PAR1 and SPR, specify their distinct intracellular sorting patterns following activation. SPR bearing the C-tail of PAR1 (S/P chimera) internalized and sorted to lysosomes upon activation like wild-type PAR1. By contrast, PAR1 bearing the SPR C-tail (P/S chimera) internalized upon activation but recycled back to the plasma membrane like wild-type SPR. These distinct sorting fates correlated with the extent to which agonists down-regulated receptor protein and signaling responses over time. Thus the cytoplasmic tails of these GPCRs specify distinct intracellular sorting fates that dictate the extent to which a cell down-regulates or maintains its ability to respond to their cognate ligands. The ability to confer distinct trafficking and down-regulation patterns by exchanging cytoplasmic tails may provide a useful tool for defining the importance of receptor down-regulation in various responses in transgenic mouse models. Whether exchanging the cytoplasmic tails of other GPCRs will provide such a clean change in agonist-dependent trafficking phenotype is not known.

The P/S chimera also provided an opportunity to evaluate the importance of lysosomal sorting for termination of thrombin signaling. One might predict that the irreversible proteolytic mechanism by which PAR1 is activated necessitates that PAR1 not recycle if temporal fidelity of signaling is to be maintained. We recently showed that SFLLRN-stimulated phosphoinositide hydrolysis in P/S chimera-expressing cells rapidly ceased upon removal of the SFLLRN peptide. However, thrombin-stimulated phosphoinositide hydrolysis in these cells persisted long after removal of thrombin, in marked contrast to the efficient shut off seen for wild-type PAR1 (29). These data suggest that lysosomal sorting of PAR1 is indeed critical for temporal fidelity of signaling to thrombin; such fidelity is presumably important in endothelial cells, fibroblasts, and other cells that may need to respond to thrombin appropriately over time (29).

As noted above, the cytoplasmic tails of PAR1 and SPR appear to dictate their specific intracellular sorting patterns. Recycling is generally thought to be the default pathway for internalized membrane proteins, and specific information is believed to be required for their sorting to lysosomes. If this is the case for GPCRs, the cytoplasmic tail of PAR1 presumably contains such information. Tyrosine- and di-leucine-based motifs have been implicated in the sorting of internalized membrane proteins to lysosomes (30, 31). Sequences highly homologous to such motifs are not obvious in the cytoplasmic tail of PAR1. We have mutated the two tyrosines (Y397A,Y420A) as well as the di-leucine (L423A,L424A) in the cytoplasmic tail of PAR1 to alanines without a dramatic effect on PAR1 down-regulation (data not shown).

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