Rab5a and rab11a mediate agonist-induced trafficking of protease-activated receptor 2

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Rab5a and rab11a mediate agonist-induced trafficking of protease-activated receptor 2. Am J Physiol Cell Physiol 284: C1319–C1329, 2003. First published January 22, 2003; 10.1152/ajpcell.00540.2002.—We evaluated the contribution of rab5a and rab11a to trafficking and signaling of protease-activated receptor 2 (PAR2), a receptor for trypsin and tryptase. Agonists stimulated internalization of PAR2 into early endosomes containing rab5a. Dominant negative rab5aS34N disrupted early endosomes and inhibited agonist-stimulated endocytosis of PAR2. Internalized PAR2 was sorted to lysosomes, and rab5a remained in early endosomes. Rab5a promoted and rab5aS34N impeded resensitization of trypsin-induced calcium mobilization. Rab11a was detected in the Golgi apparatus with PAR2, and PAR2 agonists stimulated redistribution of rab11a into vesicles containing PAR2 that migrated to the cell surface. Dominant negative rab11aS25N was mostly confined to the Golgi apparatus. Although expression of rab11aS25N caused retention of PAR2 in the Golgi apparatus, it did not abolish trafficking of PAR2 to the cell surface. However, expression of wild-type rab11a accelerated both recovery of PAR2 at the cell surface and resensitization of PAR2 signaling. Thus rab5a is required for PAR2 endocytosis and resensitization, whereas rab11a contributes to trafficking of PAR2 from the Golgi apparatus to the plasma membrane.

AGONIST BINDING INDUCES marked alterations in the subcellular distribution of G protein-coupled receptors (GPCRs) (reviewed in Ref. 1). The mechanism and function of receptor trafficking that immediately follows agonist binding have been thoroughly investigated (reviewed in Ref. 6). Agonists of many GPCRs stimulate the translocation of G protein receptor kinases from the cytosol to the plasma membrane, where they phosphorylate receptors. β-Arrestins also translocate from the cytosol and interact with phosphorylated receptors at the plasma membrane. β-Arrestins uncouple receptors from heterotrimeric G proteins to mediate desensitization and are also adaptors for clathrin-dependent endocytosis of receptors, which contributes to desensitization by removing surface receptors. β-Arrestins are also molecular scaffolds that recruit and organize components of the MAP kinase pathway to endosomes containing receptors. Thus the early stages of trafficking of GPCRs and associated proteins are important for signal transduction.

Little is known about the mechanism and function of trafficking of GPCRs once they are internalized. Some receptors for hormones and neurotransmitters, exemplified by the substance P (SP) or neurokinin 1 receptor (NK1R), internalize and then recycle to the plasma membrane (11). In the case of the NK1R, the small GTPase rab5a mediates trafficking from a superficial to a perinuclear sorting region (20). Endosomal acidification dissociates SP, which is degraded in lysosomes, and endosomal phosphatases allow dissociation of β-arrestins (11, 15). These events are required for recycling and resensitization (9). At the other extreme, protease-activated receptors (PARs) are irreversibly activated by proteolysis within the NH2 terminus to expose a tethered ligand domain and are then targeted to lysosomes (2, 8). Prominent stores of PARs within the Golgi apparatus are mobilized to the plasma membrane to allow resensitization. Nothing is known about the mechanisms that control and mediate trafficking of PARs from the Golgi apparatus to the plasma membrane.

We investigated agonist-induced trafficking of PAR2, a receptor for trypsin and mast cell tryptase that is of emerging importance in inflammation and hyperalgesia (24, 28, 29). We evaluated the contribution of the GTPases rab5a and rab11a to PAR2 trafficking and signaling. Rab5a is detected in early endosomes and is required for the proximal steps of endosome formation and the translocation of endosomes to a perinuclear location (4, 19, 26). Rab11a is present in the Golgi apparatus and in recycling endosomes and participates in the recycling of internalized receptors (18, 26, 27) and in the trafficking of proteins from the Golgi apparatus to the plasma membrane (5, 13, 31). Although agonists of PAR2 induce rapid endocytosis of the receptor and mobilization of PAR2 from prominent stores in the Golgi apparatus (2, 8), nothing is known about the role of rab GTPases in this trafficking. Our aims were to 1) examine the role of rab5a in PAR2 endocytosis, 2) determine the contribution of rab11a to translocation of PAR2 from the Golgi apparatus to the plasma membrane, and 3) determine whether this traf-
ficking is required for resensitization of protease signaling.

**MATERIALS AND METHODS**

**Materials.** Unless otherwise specified, all reagents were from Sigma (St Louis, MO) or have been described elsewhere (8, 20). Antibody GM10 recognizing lysosomal acidic membrane protein-1 (LAMP-1) was a gift from Dr. J. Hutton (Cambridge, UK). Rabbit antibody raised to a fusion protein of green fluorescent protein (GFP) and glutathione S-transferase (#9708) was a gift from Dr. J. H. Walsh (CURE/UCLA Antibody Core). Antibody to early endosome antigen 1 (EEA1) was from Transduction Laboratories (Santa Cruz, CA).

**Generation of PAR2 and rab constructs.** Human PAR2 with an NH2-terminal Flag and COOH-terminal HA11 epitope has been described (2, 8). Constructs for wild-type rab5a-GFP, dynamin-pIRE2-GFP, and the dominant negative mutants rab5aS34N-GFP (GTPase-defective binding) and dynamin-K44E-pIRES-GFP (lacks GTPase activity) have been described (20). cDNAs of wild-type rab11a and the GTPase-defective binding mutant rab11aS25N were a gift from Dr. M. Zerial (EMBL, Heidelberg). Canine rab11a and the GTPase-defective mutant rab11aS25N were fused to GFP at the NH2 terminal end using PCR with rab11a and rab11aS25N cDNAs as templates. The forward primer was 5'-GGCCGGAAATTCCATGGGACCCCGAGCAAGCCAGGACG-3' (EcoRI site underlined followed by translation initiation site), and the reverse primer was 5'-GGCCGGGATCTTATATGTTCTGACAGACTGAC-3' (BamHI restriction site underlined followed by stop codon). The PCR products were separated on an agarose gel and isolated using QiaEx extraction kit. PCR fragments and the vector pEGFP-C1 were ligated with T4 DNA ligase overnight at 16°C. Transformed JM109 Escherichia coli cells were selected by Kanamycin. Correct sequences were verified.

**Cell lines.** Kirsten murine sarcoma virus-transformed rat kidney epithelia cells (KNRK) were from American Type Culture Collection (Rockville, MD). KNRK cells were chosen because we have previously investigated PAR2 trafficking and signaling in these cells (2, 7, 8). Generation of KNRK cells stably expressing human PAR2 with NH2-terminal Flag epitope and COOH-terminal HA11 epitope (Flag-PAR2-HA11) has been described (2, 7, 8). KNRK-Flag-PAR2-HA11 cells were transiently transfected by incubation with cDNA encoding wild-type and mutant dynamin, rab5a, rab11a, or empty vector as described (20). Transiently transfected cells were used 3 days after transfection and were prepared for experiments.

**Western blotting.** Expression of rab5a and rab11a was assessed by Western blotting (20). Cells were lysed in Laemmli buffer, lysates were fractionated by SDS-PAGE (12.5%), and proteins were transferred to nitrocellulose membranes. Membranes were incubated with 5% nonfat milk in Tris (20 mM)-buffered saline, pH 7.4, and incubated with antibody to GFP (1:10,000, overnight, 4°C). Membranes were washed and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:50,000, 2 h, room temperature). Controls included preabsorption of the diluted primary antibody with the GFP fusion protein (1–2 μg/ml) for 1 h at 37°C and use of nontransfected KNRK cells.

**Immunofluorescence and confocal microscopy.** The subcellular localization of PAR2 and rabs was determined by immunofluorescence and confocal microscopy (2, 7, 8, 20). Cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) 0.1% BSA with 10 nM trypsin at 37°C, washed, and incubated in trypsin-free medium. Cells were washed in 100 mM PBS, pH 7.4, at 4°C and fixed with 4% paraformaldehyde in PBS for 20 min at 4°C. PAR2 was localized using mouse M1 Flag antibody (10 μg/ml, 18 h, 4°C) or the rabbit HA11 antibody (1:100, 18 h, 4°C). To localize organelles, cells were similarly incubated with mouse LAMP1 antibody (1:10,000), mouse EEA1 antibody (1:1,000), or rabbit mannosidase II antibody (1:5,000). Cells were washed and incubated with goat anti-mouse or goat anti-rabbit antibodies conjugated with Texas red or FITC. Specimens were observed using a BioRad MRC 1,000 confocal microscope. Images were collected at 0.68-μm intervals using a Zeiss ×100 Plan Apo 1.4 NA objective and a zoom from 1.5–2.

**Flow cytometry.** PAR2 at the cell surface was quantified by flow cytometry (7, 8). Cells were incubated in DMEM/BSA with 10 nM trypsin or 500 μM SLIGRL-NH2 for 0–30 min at 37°C. Cells were washed and incubated in agonist-free medium. The cells were washed in PBS and fixed with 3% paraformaldehyde. Suspended cells were incubated in 20 mM Tris-buffered saline containing 1 mM Ca2+ and 5% normal goat serum with mouse Flag M1 antibody (10 μg/ml, overnight, 4°C). Cells were washed and incubated with 2 μg/ml phycoerythrin-conjugated goat anti-mouse IgG antibody. Cells were analyzed using a Facsscan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Fluorophores were excited at 488 nm, and emission was collected at 530/30 for GFP (to gate for rab-expressing cells) and 575/25 nm for phycoerythrin.

**Measurement of intracellular calcium concentration.** PAR2 signaling was evaluated by measuring cytosolic [Ca2+] with fura 2-AM (2, 7, 8). To assess desensitization and resensitization, cells were exposed to 10 nM trypsin or vehicle (control) in Hank’s 0.1% BSA for 15 min at 37°C, washed 3 times, and then challenged a second time with 10 nM trypsin 0–30 min after washing. The response to the second challenge was measured.

**Statistical analysis.** Results are expressed as means ± SE of n > 3 experiments. Differences were analyzed by one-way analysis of variance and Student-Newman-Kuels test, with P < 0.05 considered to be significant.

**RESULTS**

**Expression and localization of PAR2.** We have previously reported that PAR2 with extracellular Flag and intracellular HA11 is fully functional (2, 8). Trypsin stimulates internalization of PAR2 into early endosomes containing transferrin, and PAR2 is then transported for degradation to lysosomes containing LAMP1 (2, 8). Stored PAR2 colocalizes with mannosidase II, a marker for the Golgi apparatus (2, 8).

By using antibodies to the Flag and the HA11 epitopes, we were able to discriminate between intact and cleaved PAR2. The antibody to the extracellular Flag would detect only uncleaved receptor, because trypsin cleavage removes the proximal Flag epitope. The antibody to the intracellular HA11 epitope would detect both intact and cleaved PAR2. KNRK-PAR2 cells were incubated with 10 nM trypsin for 0 and 15 min at 37°C. In unstimulated cells (0 min), Flag and HA11 colocalized at the plasma membrane and in intracellular pools in the Golgi apparatus (Fig. 1A). After 15 min with trypsin, Flag was completely removed from the plasma membrane (Fig. 1B). HA11 was detected in prominent intracellular vesicles, which did not contain Flag, and at low levels at the cell surface.
Thus trypsin cleaves PAR2 to remove the Flag and induces endocytosis of the cleaved receptor. Flag can thus be used to detect intact receptor, whereas HA11 detects both intact and cleaved PAR2.

Expression and localization of rab5a and rab11a. KNRK cells stably expressing PAR2 were transiently transfected with wild-type rab5a and rab11a or the dominant negative mutants rab5aS34N and rab11aS25N, all tagged with GFP. We analyzed rab expression by Western blotting using a GFP antibody. Single proteins were detected with apparent molecular masses of 55 kDa, consistent with the expected size of the rab proteins (27 kDa) and GFP (28 kDa) (Fig. 2).

To localize wild-type and dominant negative mutants of rab5a and rab11a, we stained cells using antibodies with markers of early endosomes, the Golgi apparatus, and lysosomes. EEA1 is an endosomal protein that interacts with rab5a. In unstimulated cells, rab5a was detected in early endosomes containing EEA1 and was also diffusely localized in the cytosol (Fig. 3A). In contrast, rab5aS34N was only found in the cytosol (Fig. 3B). Expression of rab5aS34N disrupted the endosomal location of EEA1, whereas EEA1 was present in distinct endosomes in nontransfected cells (Fig. 3B, asterisk). Thus rab5a is required to maintain the structure of early endosomes. Mannosidase II is a marker of the Golgi apparatus that colocalizes with PAR2 (2, 8). In unstimulated cells, rab11a was detected in a perinuclear region in the cytosol and vesicles and was partially colocalized with mannosidase II in the Golgi apparatus (Fig. 4A). In contrast, rab11aS25N was diffusely located in the cytosol and was strongly colocalized with mannosidase II (Fig. 4B). Thus both rab11a and PAR2 are colocalized in the Golgi apparatus. LAMP1 is a marker for lysosomes. To evaluate lysosomal targeting, we incubated cells with 10 nM trypsin for 30 min at 37°C and localized PAR2-HA11, rab5a, rab11a, and LAMP1. Rab5a and rab11a were not colocalized with LAMP1 and thus do not traffic to lysosomes (not shown). As previously found (2, 8), PAR2-HA11 was colocalized with LAMP1 in lysosomes.

Rab5a-mediated endocytosis of PAR2. To examine the involvement of rab5a in the trypsin-induced trafficking of PAR2, KNRK-PAR2 were transiently transfected with wild-type or dominant negative rab5a. Cells were incubated with 10 nM trypsin for 15 min at 37°C, washed, and incubated in trypsin-free medium.
for 0 or 10 min. PAR2 was detected using the HA11 antibody with Texas red secondary antibody, and rab5a was detected with GFP.

In unstimulated cells, rab5a was diffusely localized in the cytosol and prominently detected in vesicles, consistent with localization in early endosomes (Fig. 5A). After 15 min with trypsin (Fig. 5B) or 10 min recovery (Fig. 5C), rab5a was still prominently localized in early endosomes. At these times, PAR2-HA11 was also detected in endosomes containing rab5a (Fig. 5, B and C). Thus trypsin induces internalization of PAR2 into rab5a containing early endosomes.

Rab5aS34N was diffusely localized in the cytosol and not detected in endosomes of unstimulated cells (Fig. 6A). Treatment with trypsin did not alter this distribution (Fig. 6, B and C). In unstimulated cells expressing rab5aS34N, PAR2-HA11 was detected at the plasma membrane and in intracellular stores (Fig. 6A). In these cells, PAR2-HA11 remained at the plasma membrane after 15 min with trypsin (Fig. 6B) and after...
10 min of recovery (Fig. 6C). In contrast, trypsin induced internalization of PAR2-HA11 in cells not expressing rab5aS34N (Fig. 6, B and C, asterisk). Thus rab5a mediates endocytosis of PAR2.

Quantification of rab5a-mediated endocytosis of PAR2. We quantitatively assessed the contribution of rab5a to agonist-induced endocytosis of PAR2 by flow cytometry with the Flag antibody. Cells were incubated with 500 μM SLIGRL-NH₂ (PAR2 agonist that does not remove the Flag epitope) for 0, 5, or 30 min at 37°C, fixed, and processed for flow cytometry. In cells expressing PAR2 alone, incubation with SLIGRL-NH₂ induced a time-dependent loss of surface Flag, indicative of receptor endocytosis (Fig. 7). This endocytosis was unaffected by expression of rab5a. However, expression of rab5aS34N caused retention of PAR2 at the cell surface (%surface Flag compared with 0 min: 5 min, control 78.2 ± 1.7%, rab5aS34N 95.9 ± 1.8%, P < 0.05 compared with control; 30 min, control 52.5 ± 2.5%, rab5aS34N 77.9 ± 6.2%, P < 0.05 compared with control). We compared the contribution of rab5a with that of dynamin, a GTPase that mediates endocytosis of many GPCRs. Expression of wild-type dynamin did not alter PAR2 endocytosis, whereas dominant negative dynaminK44E caused significant retention of PAR2 at the cell surface (30 min, control 52.5 ± 2.5%, dynaminK44E 62.0.9 ± 3.8%; P < 0.05 compared with control; Fig. 7). DynaminK44E also retained PAR2 at the cell surface as determined by microscopy (not shown). Thus rab5a and dynamin mediate agonist-induced endocytosis of PAR2.

Rab11a-mediated trafficking of PAR2. There are prominent stores of PAR2 in the Golgi apparatus. Because PAR2 is degraded in lysosomes after endocytosis, mobilization of stored PAR2 is required to replenish the plasma membrane. To examine the involvement of rab11a in this trafficking, KNRK-PAR2 were transiently transfected with wild-type or dominant negative rab11a. Cells were incubated with 10 nM trypsin for 15 min at 37°C, washed, and incubated in trypsin-
free medium for 0 or 60 min. PAR2 was detected using the Flag antibody, which only interacts with intact receptor and with Texas red secondary antibody, and rab11a was detected with GFP.

In unstimulated cells, rab11a was diffusely distributed throughout the cytosol and enriched at the plasma membrane and in areas of the Golgi network (Fig. 8A). Exposure to trypsin for 15 min induced marked redistribution of rab11a from the cytosol to prominent perinuclear vesicles (Fig. 8B). After 60 min in trypsin-free medium, rab11a was also detected in vesicles immediately beneath the plasma membrane (Fig. 8C). In unstimulated cells, PAR2-Flag was present at the plasma membrane (Fig. 8A). Exposure to trypsin for 15 min removed surface Flag due to PAR2 cleavage, and PAR2-Flag was detected in perinuclear vesicles containing rab11a (Fig. 8B, arrows). These vesicles were larger than those found in nontransfected cells (Fig. 8B, asterisk). After 60 min of recovery, PAR2-Flag was present in vesicles just beneath the plasma membrane that contained rab11a (Fig. 8C). Thus trypsin cleaves PAR2 at the cell surface and triggers mobilization of intact receptors to vesicles containing rab11a that migrate toward the cell surface. The results suggest that rab11a is cotransported with PAR2 from the Golgi network to the cell surface.

In unstimulated cells, rab11aS25N was diffusely localized throughout the cytosol and prominently detected in the Golgi apparatus (Fig. 9A). In contrast to wild-type rab11a, incubation with trypsin did not alter the distribution of rab11aS25N (Fig. 9, B and C). Although PAR2-Flag was present at the plasma membrane of unstimulated cells, there was pronounced localization in the Golgi apparatus (Fig. 9A, arrows). After incubation with trypsin for 15 min, PAR2-Flag was mostly present in the Golgi apparatus where it colocalized with rab11aS25N (Fig. 9B). In contrast to cells expressing wild-type rab11a (Fig. 8B), PAR2-Flag...
was not found in distinct vesicles in cells expressing rab11aS25N. However, after 60 min of recovery, PAR2-Flag was still detected in the Golgi apparatus and was also present at the plasma membrane (Fig. 9C). These data show that disruption of rab11a induces prominent localization of PAR2 within the Golgi apparatus but does not prevent the mobilization of intact PAR2 to the plasma membrane after stimulation with trypsin. After 60 min, rab11aS25N was detected in vesicles immediately beneath the plasma membrane, but these vesicles did not contain detectable PAR2 (Fig. 9C).

**Quantification of rab5a- and rab11a-mediated trafficking of PAR2.** Mobilization of PAR2 from the Golgi apparatus is necessary for the replenishment of surface receptors (2, 8). To quantitatively assess the contribution of rab5a and rab11a to this recruitment, we used flow cytometry with the Flag antibody. Cells were incubated with 10 nM trypsin for 15 min at 37°C, washed, and incubated in trypsin-free medium for 0, 10, or 30 min. Surface Flag immunoreactivity was measured by flow cytometry. In control cells expressing PAR2 alone, exposure to trypsin strongly desensitized the response to a second challenge with trypsin immediately after washing, and this desensitization was unaffected by expression of wild-type or dominant negative rab5a or rab11a (Fig. 11). In control cells, the trypsin response resensitized with time (%response to vehicle-treated cells: 10 min, 32.2 ± 0.6%; 30 min, 40.1 ± 0.7%). Expression of rab5a accelerated resensitization at 30 min (control 40.1 ± 0.7%, rab5a 58.6 ± 4.2%, P < 0.05 compared with control), and rab5aS34N markedly inhibited resensitization at 10 min compared with control cells (control 32.2 ± 0.6%, rab5aS34N 10.8 ± 1.7, P < 0.05 compared with control). These results suggest that rab5a-dependent trafficking accelerates resensitization. Rab11a also accelerated resensitization at 30 min (control 40.1 ± 0.7%, rab11a 67.3 ± 12.5%, P < 0.05 compared with control). Rab11aS25N diminished resensitization 10 min after stimulation (control 32.2 ± 0.6%, rab11aS25N 22.6 ± 3.7%), but the differences were not significant. This observation is consistent with the finding that rab11aS25N did not prevent recovery of PAR2 at the cell surface.

**DISCUSSION**

The rab GTPases comprise a large family of proteins that are required for trafficking, fusion, and targeting of vesicles (reviewed in Refs. 17 and 23). Most information about the role of rabs in receptor trafficking derives from observations of recycling receptors, such as the transferrin receptor. Little is known about their contribution to trafficking of GPCRs, especially the PARs, which undergo agonist-induced endocytosis and lysosomal degradation rather than recycling (2, 8).

We evaluated the role of rab5a and rab11a in endocytosis and recycling of PAR2 by expression of dominant negative mutants that lack GTPase activity. We found that trypsin stimulated endocytosis of PAR2 to early endosomes containing rab5a and that rab5aS34N inhibited endocytosis of PAR2. Thus rab5a is required for agonist-induced endocytosis of PAR2. We observed

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**Fig. 7. Agonist-induced loss of surface PAR2.** Cells expressing PAR2 alone (control) or PAR2 with wild-type or dominant negative rab5a or rab11a were incubated with 500 μM SLIGRL-NH₂ for 0, 5, or 30 min at 37°C. Surface Flag immunoreactivity was measured by flow cytometry. Results are expressed as a percentage of the surface Flag immunoreactivity of untreated cells. *P < 0.05 compared with control cells expressing PAR2 alone (n = 3 experiments). Rab5aS34N inhibited removal of surface Flag.
prominent stores of PAR2 in the Golgi apparatus, where it was partially colocalized with rab11a. After exposure to trypsin, there was a prominent redistribution of rab11a into vesicles containing PAR2, which migrated to the cell surface. Rab11aS25N induced retention of PAR2 in the Golgi apparatus but did not prevent the recruitment of receptors to the cell surface. However, expression of wild-type rab11a accelerated both recovery of PAR2 at the cell surface and resensitization of PAR2 signaling. Thus rab11a participates in trafficking of PAR2 to the cell surface from the Golgi apparatus. To our knowledge, this is the first report of the role of rab proteins in the trafficking of a PAR.

Rab5a-mediated trafficking of PAR2. Our results show that rab5a mediates agonist-induced endocytosis of PAR2. Using confocal microscopy and immunocytochemistry, we found that rab5a colocalized with EEA1 and is thus located in early endosomes. In unstimulated cells, PAR2 was detected at the plasma membrane and in the Golgi apparatus. Exposure to trypsin stripped detectable Flag immunoreactivity from the cell surface, confirming PAR2 cleavage and activation. Trypsin stimulated the redistribution of the cleaved PAR2, detected with the HA11 antibody, from the plasma membrane to early endosomes containing rab5a. Importantly, expression of rab5aS34N disrupted the integrity of early endosomes and caused the retention of cleaved PAR2 at the cell surface. These results were confirmed by using flow cytometry to quantify the loss of Flag from the cell surface after exposure to the PAR2-activating peptide SLIGRL-NH₂. In cells only expressing PAR2, SLIGRL-NH₂ induced a marked loss of surface Flag, indicative of receptor endocytosis. Overexpression of wild-type rab5a did not affect endocytosis. In contrast, rab5aS34N markedly inhibited endocytosis in response to activating peptide. Expression of dominant negative dynaminK44E also inhibited endocytosis of PAR2 to a
comparable extent as rab5aS34N. Thus both dynamin and rab5a mediate agonist-induced redistribution of PAR2 from the cell surface to early endosomes. We have previously shown that H9252-arrestin, which serves as an adaptor protein for clathrin, also mediates endocytosis of PAR2 (8). Agonists of PAR2 stimulate the rapid redistribution of H9252-arrestin1 from the cytosol to the plasma membrane, where H9252-arrestin interacts with phosphorylated PAR2. H9252-arrestins are adaptor proteins for clathrin-mediated endocytosis of PAR2, and dynamin is required for formation of vesicles from clathrin-coated pits. Rab5a most likely mediates the more distal steps of endosome formation and the trafficking of endosomes from the cell surface to a perinuclear region. Rab5a is known to be an important mediator in formation of clathrin-coated vesicles, and functional rab5a is required for the subsequent fusion of these vesicles with early endosomes and for the maintenance of the organelle structure of early endosomes (10, 16, 30). In support of our observations, β-arrestins, dynamin, and rab5a mediate endocytosis of the certain other GPCRs, notably the NKIR (8, 20), dopamine D2 receptor (12), β2 adrenergic receptor (21), and the m4 muscarinic acetylcholine receptor (30). Rab5a is also required for the downregulation of the κ opioid receptor (14). In the case of the neurokinin 1 receptor, rab5a also mediates trafficking of the receptor from the plasma membrane to perinuclear endosomes (20).

Although dynaminK44E and rab5aS34N inhibited endocytosis of PAR2, they did not affect desensitization of agonist-induced mobilization of intracellular Ca2+. These results, therefore, confirm the general view that receptor endocytosis is not the principal mechanism of homologous desensitization to repeated challenge with agonists of GPCRs. However, expression of rab5aS34N

Fig. 9. Localization of dominant negative rab11aS25N-GFP and PAR2-Flag. Rab11aS25N-GFP was transiently expressed in KNRK-PAR2 cells. Cells were unstimulated (A) or incubated with 10 nM trypsin for 15 min (B), followed by 60 min of recovery in trypsin-free medium (C). PAR2 was detected using the Flag antibody. In unstimulated cells, rab11a-GFP was detected in the cytosol and prominently in the Golgi apparatus. PAR2-Flag was very prominent in the Golgi apparatus of these cells (arrows). The distribution of rab11aS25N was not changed by incubation with trypsin. After 15 min with trypsin, PAR2-Flag was cleared from the plasma membrane and was prominently found in the Golgi apparatus. PAR2-Flag was still detected in the Golgi apparatus after 60 min of recovery, but intact receptors were also found at the plasma membrane (arrowheads). Scale bar = 10 μm.
inhibited the resensitization of trypsin-induced signaling of PAR2. This result suggests that rab5a-dependent endocytosis and intracellular trafficking of cleaved PAR2 promotes resensitization. The mechanism of this effect remains to be determined. We have previously reported that agonists of PAR2 induced endocytosis and lysosomal degradation of the receptor (2, 8). Resensitization of cellular responses to trypsin requires mobilization of the prominent stores of PAR2 that are found in the Golgi apparatus (2, 8). PAR1 desensitizes and resensitizes in a similar manner (22, 25). Thus rab5a-dependent trafficking of PAR2 could stimulate the mobilization of PAR2 from intracellular stores to the plasma membrane. However, rab5aS34N did not affect the recovery of intact PAR2 at the plasma membrane after exposure of cells to trypsin, which would argue against this mechanism. Alternatively, rab5aS34N could disrupt the recruitment of accessory proteins that are required for PAR2-dependent signaling, but this possibility remains to be explored. Dynamin and rab5a-dependent endocytosis and trafficking of the NKIR are also required for resensitization of responses to SP (20). Resensitization of the NKIR requires receptor endocytosis, dissociation of SP and β-arrestins in endosomes, and recycling of the receptor to the plasma membrane (9).

Rab11a-mediated trafficking of PAR2. Several observations suggest that rab11a contributes to agonist-induced trafficking of PAR2 from intracellular stores, such as the Golgi apparatus. In unstimulated cells, PAR2 was prominently detected in the Golgi apparatus, where it was only partially colocalized with rab11a. Activation of PAR2 induced mobilization of PAR2 from the Golgi apparatus to the plasma membrane and stimulated a marked redistribution of rab11a to vesicles containing intact PAR2 at or close to the plasma membrane. The mechanism of PAR2-dependent redistribution of rab11a remains to be determined. A contribution of rab11a to PAR2 trafficking is supported by the finding that overexpression of rab11a accelerated the replenishment of intact PAR2 at the plasma membrane of trypsin-treated cells and promoted resensitization of trypsin-stimulated mobilization of intracellular Ca2+. We have previously shown that mobilization of PAR2 from the Golgi apparatus is required for resensitization of responses to trypsin (7). In support of our results, rab11 also mediates insulin-stimulated transport of GLUT4 and insulin-regulated amino peptidase from storage pools to the cell surface (13, 31). Rab11 also contributes to the transport of vesicular stomatitis virus (VSV) G protein from the Golgi apparatus to the plasma membrane (5). Thus rab11 appears to play a general role in mediating the transport of storage proteins to the plasma membrane.

Our results indicate that rab11a is not solely responsible for trafficking of PAR2 from the Golgi apparatus to the cell surface and for resensitization of responses to trypsin. Although overexpression of rab11aS25N caused marked retention of PAR2 in the Golgi apparatus, it neither abolished the recovery of intact PAR2 at the cell surface nor prevented agonist-induced Ca2+ mobilization in cells pre-exposed to trypsin. Additional studies are required to define other mechanisms of PAR2 trafficking and resensitization.
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