p24A, A TYPE I TRANSMEMBRANE PROTEIN, CONTROLS ARF1-DEPENDENT RESENSITIZATION OF PROTEASE-ACTIVATED RECEPTOR-2 BY INFLUENCE ON RECEPTOR TRAFFICKING

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Protease-activated receptor-2 (PAR-2), the second member of the G protein-coupled PAR family, is irreversibly activated by trypsin or tryptase, and then targeted to lysosomes for degradation. Intracellular presynthesized receptors stored at the Golgi apparatus repopulate the cell surface after trypsin stimulation, thereby leading to rapid resensitization to trypsin signaling. However, the molecular mechanisms of the exocytic trafficking of PAR-2 from the Golgi apparatus to the plasma membrane remain largely unclear. Here, we show that p24A, a type I transmembrane protein, which is a crucial constituent of the Golgi apparatus, associates with PAR-2 at the Golgi apparatus. The protein interaction occurs between the N-terminal region of p24A (residues 1-105; p24A-GL (GOLD domain with a small linker)) and the second extracellular loop of PAR-2. After receptor activation, PAR-2 dissociates from p24A. Importantly, we found that ADP-ribosylation factor 1 regulated the dissociation process and initiated PAR-2 trafficking to the plasma membrane. Conversely, overexpression of the fragment p24A-GL, but not other mutants containing the functional coiled-coil domain of p24A, arrested PAR-2 at the Golgi apparatus and inhibited receptor trafficking to the plasma membrane, which consequently prevented resensitization of PAR-2. These findings identify a new function of p24A as a regulator of signal-dependent trafficking that regulates the life cycle of PAR-2. Thus, we reveal a new molecular mechanism underlying resensitization of PAR-2.

G protein-coupled receptors (GPCRs)¹ constitute a large family of seven-transmembrane domain spanning receptors that regulate diverse biological responses within cells via heterotrimeric G proteins and downstream effectors (1). Protease-activated receptor-2 (PAR-2), the second member of the G protein-coupled PAR family, is involved in inflammation, pain, cell proliferation and anti-apoptosis (2-4). A unique activation mechanism is responsible for PAR-2 activation by irreversible proteolytic cleavage by serine proteases, such as trypsin and tryptase (2). Thus, a new N-terminus is unmasked acting as a tethered ligand, which can interact with the second extracellular loop of the receptor and thereby initiate multiple signal transductions. A synthetic receptor-activating peptide (AP) with the sequence identical to that of the tethered ligand domain can also fully activate the receptor, bypassing receptor proteolysis (2). Upon activation, PAR-2 is rapidly sorted to early endosomes, and then translocated to lysosomes where it is degraded (5). Therefore, PAR-2 resensitization requires the repopulation of cell-surface receptors from the presynthesized and newly synthesized PAR-2 that are stored at the Golgi apparatus (6). It has been shown that the GTPase Rab11a is partially involved in resensitization of PAR-2 (7). However, the precise molecular mechanisms underlying exocytic PAR-2 transport from the Golgi apparatus to the plasma membrane are still poorly characterized.

The mammalian p24 family, which comprises type I transmembrane proteins of the early secretory pathway, is divided into p25 (α), p24A (β), p23 (γ) and p26 (δ) subfamilies (8). p24A, a major member of the p24 family,
has been shown to localize in membranes of the intermediate compartment, cis-Golgi network and endoplasmic reticulum (ER) (8-11). The protein p24A has a large N-terminus at the lumen and a highly conserved, short cytoplasmic C-tail at the cytosol. The cytoplasmic tail of p24A, containing conserved hydrophobic (FF) and basic (RR) motifs, is able to bind to coat protein (COP) I and COPII subunits (8,12,13). This suggests that p24A is an important constituent of coatomers (14). The importance of p24A on transport vesicles is also supported by the evidence that p24A, via its cytoplasmic tail, is able to interact with ADP-ribosylation factor 1 (ARF1) (12,15,16), resulting in initiation of transport vesicle assembly and cargo packaging (17). The coiled-coil domain located at the N-terminus of p24A has been shown to mediate the formation of a hetero-oligomeric complex between p24 proteins, which is a pre-requisite for localization and cycling of p24 proteins in the early secretory pathway (9,18,19). These previous studies suggest that p24A, acting as cargo receptor as well as coat protein receptor, is involved in biogenesis of transport vesicles and subsequent protein trafficking between the ER and the Golgi apparatus and within the Golgi apparatus (13,20-22). However, no specific cargo proteins for p24A have been identified so far (22). Conversely, data from Caenorhabditis elegans and Saccharomyces cerevisiae implicate that p24A functions as quality control in the early secretory pathway, but might not directly mediate cargo protein sorting (23,24). Therefore, the precise functions of p24A are still a puzzle.

Here, we show for the first time that a GPCR, PAR-2 can be identified as a specific cargo protein of p24A. Moreover, we determined the interaction domains on both partner proteins. Importantly, studies on the physiological function of the interaction revealed that p24A regulates exocytic trafficking of PAR-2 from the Golgi apparatus to the plasma membrane. p24A serves in the biosynthetic pathway as a signal-dependent retention and release component to control subsequent receptor resensitization, which is initiated by activation of the small GTPase ARF1. Our findings provide novel functional insights into the physiological role of p24A, and also elucidate a molecular mechanism underlying PAR-2 resensitization.

**EXPERIMENTAL PROCEDURES**

**Plasmid constructs.** The FLAG tag (DYKDDDDK) followed by trypsin cleavage site of human PAR-2 amplified by polymerase chain reaction (PCR) was cloned into pEAK-HA (hemagglutinin epitope) vector, and then the proopiromelanocortin signal sequence was introduced to the N-terminus of FLAG-PAR-2 fusion construct. pVL1392-EL2-GST, pcDNA-p24Amyc, pEGFP-p24A and pEGFP-p24A deletion mutants all were constructed by standard PCR cloning. All of the cDNAs were mutated at the stop codon and conjugated with the tag at the C-terminus. Primer sequences are available on request. Other constructs have been described previously (25). pGEX-VHS-GAT and pGEX-VHS were kindly provided by J. S. Bonifacino (National Institutes of Health). The DNA sequences of plasmid constructs were confirmed to be in-frame by ABI 310 sequencer.

**Cell culture and transfection.** Human embryonic kidney (HEK) cells 293 and primary rat astrocytes were cultured as described (25,26), and transfected using magnet assisted transfection (IBA GmbH, Germany) (4,25).

To activate PAR-2, cells were rinsed with Hank’s solution, and stimulated with the specific peptide agonist, human PAR-2 AP SLIGKV-NHB2 (100 μM, Bachem, for human PAR-2), or with the most potent peptide agonist 2-furoyl-LIGRLO-NHB2 (50 μM, Bachem, for rat PAR-2) in serum-free medium. To prevent receptor resensitization, cells were pretreated with 10 μg/ml brefeldin A (Calbiochem) for 30 min prior to agonist stimulation.

**Protein-protein interaction in vitro and in vivo.** To detect protein interaction in vitro, we performed the glutathione S-transferase (GST) pull-down assay (25). Cell extracts containing myc-tagged p24A or green fluorescent protein (GFP)-tagged p24A derivatives were incubated overnight with GST fusion protein of PAR-2 derivatives immobilized on glutathione-Sepharose beads (GE Healthcare), followed by western blot analysis with anti-myc antibody (Invitrogen) or anti-GFP antibody (Cell signaling technology). Immunoprecipitation was also carried out as described (25), to test protein interaction in vivo. Cell extracts were incubated overnight with antibodies indicated in the presence of protein A-Sepharose (GE Healthcare) or protein G-agarose beads (Sigma), followed by western blot analysis.
with anti-myc antibody (Invitrogen), anti-HA antibody (Cell signaling technology) or anti-PAR-2 (C-17) antibody (Santa Cruz).

**Immunofluorescence.** Immunofluorescence analysis was carried out by using a LSM510 confocal laser scanning microscope (Carl Zeiss, Germany) (25). Detailed protocols are described in Supplementary data.

**siRNA.** siRNA-based p24A knockdown, performed using magnet assisted transfection (IBA GmbH, Germany) in HEK293-PAR-2-HA cells (25), was evaluated by reverse transcription (RT)-PCR and western blot at 48 h after transfection. p24A siRNA labeled with Alexa Fluor 488 (5’CCGGAUGUCCACCAUGACUdTdT3’) was designed based on the mRNA sequence (GenBank accession No.: X92098) and synthesized by Qiagen. ARF1 ON-TARGETplus SMARTpool siRNA containing four individual siRNAs that target different regions of ARF1 open reading frame was obtained from Dharmacon (5’UGACAGAGACGUGUAAAC3’; 5’CGGCGAGAUCAAGACAA3’; 5’ACGAUCCUCUACAAGCUUA3’; 5’GAACCAAGUAGUACCCGA3’). Control siRNA labeled with Alexa Fluor 488 (5’UUCUCGAACGUGUCAGCUUdTdT3’), which could not target any genes, was obtained from Qiagen.

**Cellular ARF1-GTPase assay.** Recombinant fusion proteins GST-VHS-GAT and GST-VHS as well as GST were expressed in *Escherichia coli* BL21 cells, extracted in lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) supplemented with 1 mg/ml lysozyme (Sigma) and protease inhibitor Cocktail (Roche Diagnostics, Germany, one tablet per 50 ml), and purified with glutathione-Sepharose beads (GE Healthcare). Primary rat astrocytes were treated with 50 μM 2-furoyl-LIGRLO-NH₂ for the indicated times and lysed in modified RIPA buffer (26). The resulting cell lysates were incubated overnight with equal amounts of GST fusion proteins immobilized on glutathione-Sepharose beads, followed by western blot with anti-ARF1 antibody (Epitomics, USA). Total levels of each ARF1 in 2% of whole cell lysates used for precipitation were also determined by western blot.

**Calcium imaging.** PAR-2-induced intracellular calcium mobilization in single cells was determined using the calcium sensitive fluorescent dye Fura-2 AM (2 μM) (27). Cells expressing GFP or GFP-tagged truncated p24As were chosen by the fluorescence microscope at 460 nm-excitation wavelength.

**Statistical analysis.** Data were expressed as mean ± S.E. Differences within multiple groups were examined by one-way analysis of variance. *p*<0.05 was considered significant.

**RESULTS**

**Analysis of the interaction of PAR-2 with p24A.** In a profile search for PAR-2-interacting proteins in the yeast two-hybrid system we used human PAR-2 as bait (25). There we identified p24A (residues 43-181, GenBank™ accession No.: X92098), a type I transmembrane protein of the early secretory pathway, to interact with PAR-2. To confirm the finding from the yeast system, we then performed the co-immunoprecipitation assay in transfected HEK293 cells co-expressing PAR-2-HA and p24Amyc. PAR-2-HA was precipitated by anti-HA antibody from transfected HEK293 cell lysates, and the interaction of PAR-2-HA with p24A was analyzed by western blot using the anti-myc antibody. As shown in Fig. 1A, p24A was specifically immunoprecipitated from HEK293-PAR-2-HA+p24Amyc cells (lane 6), but not in the negative control wild-type HEK293 cells (lane 4) and HEK293-p24Amyc cells (lane 5). The nitrocellulose membrane was reprobed with the anti-HA antibody to confirm the specificity of the immunoprecipitation of PAR-2-HA. These results suggest that p24A interacts with PAR-2 within cells.

Next, we asked whether the interaction of PAR-2 with p24A could occur in a relevant physiological system and whether this interaction was species-specific. We performed immunoprecipitation experiments with anti-p24A antibody in primary rat astrocytes. The interaction between PAR-2-HA and p24A was analyzed by western blot using the anti-myc antibody. As shown in Fig. 1B, the anti-p24A antibody strongly precipitated PAR-2 from rat astrocytes (lane 2), whereas the control antibody (IgG) did not (lane 1). Immunoprecipitation by anti-PAR-2 antibody from rat astrocyte lysates served as positive control (Fig. 1B, lane 3). Similar results were also observed in analogous experiments carried out with primary rat cortical neurons (Fig. 1C). These results confirm the existence of an endogenous interaction between PAR-2 and
p24A in a native system. Moreover, this interaction is not restricted to the human proteins.

**Analysis of the domains of PAR-2 and p24A responsible for the interaction.** To map the domain of PAR-2 responsible for the interaction with p24A, we next performed *in vitro* GST pull-down assays. Wild-type and truncated PAR-2-GST fusion proteins, which are depicted in Figure 2A, were generated in Sf9 cells and purified by glutathione-Sepharose beads. Either wild-type or truncated PAR-2-GST fusion proteins on glutathione-Sepharose beads were further incubated overnight with the lysate of HEK293 cells expressing p24Amyc, and then the precipitates were analyzed for p24Amyc by western blot using the anti-myc antibody. As shown in Fig. 2B, p24A interacted with both PAR-2Δ(246-397)-GST (Δ(246-397)) and PAR-2Δ(1-213)-GST (Δ(1-213)) with binding capacities comparable to that of the wild-type PAR-2-GST (WT). The lack of interaction of p24A with GST confirmed the specificity of the precipitation (Fig. 2B). Since p24A is an intracellular protein, it is primarily thought that the intracellular loops and the carboxyl tail (C-tail) of the receptor would be required for the interaction. Therefore, we tested the interaction of p24A with the intracellular loops and the C-tail of PAR-2 in the GST pull-down assay. Interestingly, all the intracellular loops and the C-tail of PAR-2 failed to interact with p24A (Fig. 2C). Instead, we made the astonishing discovery that the second extracellular loop of PAR-2 (EL2) strongly interacted with p24A (Fig. 2D).

Next, we determined the domain of p24A responsible for the interaction with PAR-2 in the GST pull-down assay. The wild-type and a series of truncated p24A-GFP fusion proteins were constructed and expressed in HEK293 cells, as depicted in Figure 2E. Cell extracts containing the various p24A derivatives were incubated overnight with the wild-type PAR-2-GST immobilized on glutathione-Sepharose beads. Subsequent western blot analysis, using the anti-GFP antibody, demonstrated that deletion of the N-terminal luminal part of p24A (ΔN) completely abolished the interaction with PAR-2-GST in the GST pull-down assay (Fig. 2F). However, proteins with C-terminal deletion (ΔC) and deletions of both the transmembrane domain and the C-terminal domain (ΔCT), in either case efficiently interacted with PAR-2-GST (Fig. 2F). The lack of interaction of PAR-2-GST with GFP confirmed the specificity of precipitation (Fig. 2F). These data strongly suggest that the N-terminus of p24A is required for the interaction with PAR-2.

The GOLD (Golgi dynamics) domain located at the N-terminus of p24A is supposed to bind to cargo proteins (28). Thus, we further tested whether the GOLD domain is involved in the interaction with PAR-2 in the GST pull-down assay. As expected, p24AΔGOLD-GFP (AG) failed to interact with PAR-2 (pull-down in Fig. 2G), suggesting that the GOLD domain is required for the interaction with PAR-2. However, the single GOLD domain (G) only weakly bound to PAR-2 (Fig. 2G). Therefore, the GOLD domain is necessary, but not sufficient to interact with PAR-2. Further experiments demonstrated that p24A-GL-GFP (GL) which contains the GOLD domain and a neighboring linker, indeed strongly interacted with PAR-2 (pull-down in Fig. 2G). Identical results were also observed when we used PAR-2EL2-GST in the GST pull-down assay, showing that p24A-GL-GFP directly bound to the second extracellular loop of PAR-2 (Supplementary Fig. 1). Therefore, these findings clearly demonstrate that p24A-GL (residues 1-105) is responsible for the interaction with PAR-2.

Taken together, our data show that the N-terminal region of p24A (residues 1-105) specifically interacts with the second extracellular loop (EL2) of PAR-2.

**p24A interacts with PAR-2 at the Golgi apparatus.** To localize the interaction of PAR-2 with p24A in the cells, we next performed *in vivo* immunofluorescence staining in co-transfected HEK293 cells. Using the antibody against HA, PAR-2-HA was predominantly detected at the plasma membrane, and located in intracellular stores as well (Fig. 3A). By staining with the anti-myc antibody, p24Amyc was shown to be distributed in the cytosol, but strongly located at the Golgi apparatus (Fig. 3B), as revealed by colocalization with the Golgi apparatus marker GM130 (Fig. 3C) in the merged picture (Fig. 3D). The merged image clearly shows that p24A closely colocalized with the intracellular PAR-2, but not with the cell-surface receptor (Fig. 3D, indicated by arrowheads). Moreover, GM130 staining demonstrates that GM130 additionally colocalized with the intracellular PAR-2 and p24A (Fig. 3D, indicated by arrowheads).
Therefore, these data clearly indicate that the interaction of PAR-2 with p24A occurs at the Golgi apparatus.

In further experiments we used p24A small interfering RNA (siRNA). p24A siRNA was shown to specifically knock down p24A mRNA and protein expression. The levels were reduced by ~60% in HEK293-PAR-2-HA cells (Fig. 4A). Immunostaining demonstrated that p24A knockdown completely eliminated the intracellular PAR-2, but had no effect on cell-surface receptors (Fig. 4B). Interestingly, we found that GM130 staining became diffuse in the cytosol in p24A-deficient HEK293-PAR-2-HA cells (Fig. 4B, given by arrowheads), indicating that the Golgi apparatus was disrupted by p24A knockdown. However, p24A knockdown had no effect on the organization of the ER, as shown by staining with the ER marker BiP (Fig. 4B). These results reveal that p24A is a crucial structural component of the Golgi apparatus. The redistribution of the intracellular PAR-2 might be due to the disruption of the structure of the Golgi apparatus by p24A siRNA. ARF1 regulates the interaction of PAR-2 with p24A. To examine the functional significance of the interaction, we evaluated whether activation of PAR-2 affects the interaction with p24A. As shown by the co-immunoprecipitation experiment in Fig. 5A, the precipitation of p24A was significantly reduced at 30 min after stimulation with the specific peptide agonist of PAR-2, the human PAR-2 AP, compared to that in unstimulated cells (0 min). The reduction in interaction was similarly observed at 60 min after receptor activation (Fig. 5A). The data summarized in the plot demonstrate that activation of PAR-2 significantly reduced the interaction with p24A, by 64% at 30 min, and by 57% at 60 min, respectively (Fig. 5A). Similar results were observed in HEK293-PAR-2-HA+p24Amyc cells treated with the PAR-2 agonist protease trypsin (data not shown). Therefore, these data indicate that p24A is dissociated from PAR-2 after receptor activation.

Next, we asked by which mechanisms the cell surface PAR-2 signals are transmitted to p24A, to induce protein dissociation from PAR-2 after receptor activation. p24A is able to interact with inactive ARF1-GDP (12,16), and activation of ARF1 would initiate transport vesicle assembly and cargo packaging, thereby resulting in protein trafficking along the secretory pathway (17). Therefore, we hypothesized that activated ARF1 might regulate protein dissociation between PAR-2 and p24A. To test this assumption, rat astrocytes were stimulated with rat PAR-2 AP (50 μM) for the indicated time periods (Fig. 5B), and then cell lysates were used to measure the activated ARF1. The latter was precipitated specifically by GST-VHS-GAT in a GST pull-down assay. The GAT domain of GGA3 (Golgi-localized, γ ear-containing ARF-binding protein 3) has been shown to specifically interact with the activated ARF1 (29), and can thus serve as a tool to demonstrate ARF1 activation.

Activation of PAR-2 by PAR-2 AP time-dependently induced ARF1 activation in rat astrocytes (Fig. 5B). The maximal activation of ARF1 was observed at 20 min after stimulation with PAR-2 AP (summary in Fig. 5B), which was an early event prior to protein dissociation between PAR-2 and p24A. GST-VHS, the domain of GGA3 with no ARF1-binding, and GST were not able to pull down ARF1-GTP (Fig. 5B), confirming the specificity of precipitation by GAT domain. Therefore, these data clearly demonstrate that ARF1 can be activated after stimulation with PAR-2 agonists. To further study whether activated ARF1 initiates protein dissociation between PAR-2 and p24A after receptor activation, we tested the effect of brefeldin A on the interaction of PAR-2 with p24A. Brefeldin A is an inhibitor of the guanine nucleotide exchange factors (GEFs), and can prevent ARF1 activation within cells (30). To this aim, HEK293-PAR-2-HA+p24Amyc cells were pretreated with brefeldin A, followed by human PAR-2 AP stimulation. Here, we found that brefeldin A completely prevented protein dissociation of p24A from PAR-2 after PAR-2 activation, both at 30 and 60 min (Fig. 5C), indicating that activation of ARF1 results in protein dissociation of PAR-2 from p24A. When the cell surface PAR-2 has been activated and internalized, the intracellular PAR-2 should be triggered to be sorted to the plasma membrane for receptor resensitization. Brefeldin A has been previously shown to inhibit PAR-2 resensitization (6). Therefore, our data suggest that protein dissociation between PAR-2 and p24A initiated by activated ARF1 might be required for PAR-2 resensitization.


p24A regulates post-Golgi transport of PAR-2 and is involved in receptor resensitization. To clarify whether the dissociation of PAR-2 from p24A is involved in receptor resensitization, we next interfered with the dissociation process. Since p24A siRNA had side effects (disruption of the Golgi structure, Fig. 4B), we applied dominant-negative p24A, which is an alternative strategy for loss-of-function studies, to achieve retention of PAR-2 at the Golgi apparatus. Our binding data above clearly revealed that p24A-GL-GFP had strong binding activity to PAR-2. However, this mutant lacks the coiled-coil domain and the cytoplasmic tail, two key functional domains of p24A, which are required for protein transport in the early secretory pathway (13,18,19). Therefore, we reasoned that overexpression of p24A-GL-GFP would constitutively arrest PAR-2 at the Golgi apparatus, and prevent receptor trafficking to the plasma membrane.

To test this hypothesis, we studied the effect of p24A-GL-GFP on PAR-2 trafficking along the post-Golgi pathway by confocal microscopy. A new PAR-2 construct, containing the FLAG tag in front of the N-terminal trypsin cleavage site, was generated. Then we could monitor trafficking of intact PAR-2 during receptor recovery with FLAG staining. HEK293-FLAG-PAR-2 cells transiently transfected with p24A-GL-GFP were incubated with 100 nM trypsin for 15 min, washed and further incubated for 0-60 min in trypsin-free medium. In control cells, FLAG-PAR-2 is located at the plasma membrane, as well as at the Golgi apparatus (Fig. 6A, CON). We also found that p24A-GL-GFP predominantly resided at the Golgi apparatus, similar to that of wild-type p24A (Fig. 6A). Moreover, p24A-GL-GFP closely colocalized with the intracellular FLAG-PAR-2 at the Golgi apparatus (Fig. 6A), suggesting that p24A-GL-GFP had the proper localization within cells, as the wild-type p24A did.

After stimulation with trypsin, the FLAG tag of the cell-surface receptor was cleaved. With FLAG staining, we detected only the intracellular PAR-2 at the Golgi apparatus, but no PAR-2 at the plasma membrane (Fig. 6A, 0 min). Likewise, the intact PAR-2 was not able to be detected at the cell surface in p24A-GL-GFP overexpressing HEK293-FLAG-PAR-2 cells upon trypsin stimulation (Fig. 6A). After 30 min recovery, FLAG-PAR-2 was repopulating the plasma membrane in HEK293-FLAG-PAR-2 cells (Fig. 6A, indicated by white arrowheads). However, overexpression of p24A-GL-GFP sequestered FLAG-PAR-2 at the Golgi apparatus and prevented FLAG-PAR-2 trafficking to the cell surface (Fig. 6A, indicated by green arrows). This inhibitory effect was much clearer at 60 min (Fig. 6A, indicated by green arrows).

The intracellular intact PAR-2 at the Golgi apparatus that is sorted to the plasma membrane should contribute to receptor resensitization (6). Thus, we studied whether retention of PAR-2 at the Golgi apparatus by p24A-GL-GFP affected receptor resensitization, by measuring the intracellular calcium mobilization, which represents the functional response upon receptor activation. To this end, HEK293-PAR-2-HA cells were challenged with human PAR-2 AP (100 μM) for 15 min to desensitize the cell-surface receptors, washed and exposed to repeated 60 s-stimuli of PAR-2 AP, at 5-60 min after the pre-pulse challenge. After 5 min, the calcium response to the second PAR-2 AP exposure was 65% of the control level (Fig. 6B). The response almost completely resensitized to control levels at 20-60 min after the first exposure (Fig. 6B). However, overexpression of p24A-GL-GFP significantly inhibited the PAR-2 AP-induced calcium mobilization at 20-60 min of recovery time (dark green curve in Fig. 6B). This suggests that p24A-GL-GFP prevented resensitization of PAR-2. These results indicate that protein dissociation of PAR-2 from p24A is a major pre-requisite for receptor resensitization.

To ascertain whether p24A-GL-GFP specifically inhibited resensitization of PAR-2, we also tested the effect of p24AΔC-GFP and p24AΔCT-GFP on PAR-2 resensitization in HEK293-PAR-2-HA cells. p24AΔC-GFP and p24AΔCT-GFP both contain the coiled-coil domain that is important for p24A expression, localization and function (9,18,19). Localization studies demonstrated that both truncated proteins had the same intracellular distribution as wild-type p24A, and they also strongly colocalized with PAR-2-HA at the Golgi apparatus (Supplementary Fig. 2, indicated by arrowheads). Expectedly, p24AΔC-GFP and p24AΔCT-GFP both were not able to significantly reduce resensitization of PAR-2 (Fig. 6B).

Next, we investigated whether p24A-GL-GFP had a similar effect on PAR-2
resensitization in the native system. For that purpose, primary rat astrocytes were stimulated
with rat PAR-2 AP (50 \( \mu \)M) for 2 min, washed, and challenged with a second pulse of rat
PAR-2 AP at 1-60 min after the first exposure. The calcium response to PAR-2 AP
stimulation completely resensitized at the 20-60 min recovery period in astrocytes, which
was largely prevented by overexpression of p24A-GL-GFP (Fig. 6C). Overexpression of
GFP had no effect on PAR-2 resensitization in astrocytes (Fig. 6C). These data confirm that
the dissociation between PAR-2 and p24A is mandatory for resensitization of PAR-2 in the
native system.

Taken together, p24A regulates intact PAR-2 trafficking from the plasma membrane, and thereby controls
resensitization of PAR-2.

**ARF1 is essential for PAR-2 resensitization.** To further test the role of ARF1 in PAR-2 resensitization, we studied PAR-2 trafficking in ARF1-deficient cells by using ARF1 siRNA. By RT-PCR and western blot, we found that ARF1 siRNA, but not control siRNA, largely reduced ARF1 expression at both mRNA and protein levels by ~80% in HEK293-FLAG-PAR-2 cells (Fig. 7A). Under these conditions, HEK293-FLAG-PAR-2 cells were incubated with 100 nM trypsin for 15 min, to cleave the FLAG tag off the cell surface PAR-2. Cells were then washed and further incubated in trypsin-free medium for 60 min- and 120 min-recovery. With FLAG staining, we found that the intact PAR-2 still resided at the intracellular stores and was not detectable at the cell surface in ARF1-deficient HEK293-FLAG-PAR-2 cells after 60 min and 120 min of recovery (indicated by green arrows in the lower panels, Fig. 7B). In contrast, PAR-2 appeared at the cell surface in HEK293-FLAG-PAR-2 cells transfected with control siRNA after 60 min-recovery (indicated by white arrowheads). The plasma membrane localization of PAR-2 was more dominant at 120 min after recovery (indicated by white arrowheads in the upper panels, Fig. 7B). These data clearly reveal that ARF1 activation is required for PAR-2 trafficking from the Golgi apparatus to the plasma membrane, and subsequent receptor resensitization.

**DISCUSSION**

GPCRs constitute the largest family of cell-surface receptors that respond to a diverse
variety of extracellular stimuli, including proteases, neurotransmitters, hormones, light,
odor and taste (1). The endocytic and exocytic trafficking of GPCRs regulates spatial and
temporal receptor signaling. PAR-2, after activation, is internalized and then targeted to
lysosomes for degradation (31). Intracellular presynthesized receptors stored at the Golgi
apparatus repopulate the cell surface, thereby leading to rapid resensitization to trypsin
signaling (6). Therefore, the exocytic receptor trafficking from the Golgi apparatus to
the plasma membrane plays a pivotal role in PAR-2 resensitization. Here, we for the first time
report that p24A, a member of the p24 family, interacts with PAR-2 at the Golgi apparatus,
and regulates the exocytic trafficking of PAR-2 to the cell surface and subsequent receptor
resensitization.

p24A functions as both cargo receptor and coat protein receptor and is involved in
protein transport and quality control along the biosynthetic pathway (22). In the present
study, domain mapping analysis demonstrates that the N-terminal lumenal region of p24A
(residues 1-105) containing the GOLD domain and a neighboring linker, recognized PAR-2.
However, we found that the GOLD domain alone, which was previously postulated by
computational sequence analysis to bind to cargo proteins, was not sufficient to target the
cargo. It may be that the small linker is necessary to complete the proper folding of the
GOLD domain. Nevertheless, our findings clearly determined the direct cargo-binding
region of p24A. Further immunostaining data demonstrate that the interaction of PAR-2 with
p24A selectively occurred at the Golgi apparatus. Considering their binding properties
at the Golgi lumen, here we suggest that PAR-2 is the first identified specific vesicular cargo
protein of p24A. In addition, we found that PAR-2 also strongly interacted with p23,
another member of the p24 family, suggesting that PAR-2 might be also selected by other p24
proteins in the biosynthetic pathway.

The intracellular C-tail of GPCRs contains several sorting signals and determines
protein trafficking of GPCRs within cells. A highly conserved motif, FxxxXxxxF, was
identified as an ER-export signal for the dopamine D1 receptor (32). The FxxxxxxLL
motif also functioned in mediating GPCR export from the ER (33). The tyrosine-based
sorting signal YxxL has been shown to mediate PAR-1 internalization (34). The C-tail
of PAR-1 is also involved in receptor sorting to lysosomes for degradation (35). Recent studies demonstrate that the extracellular N-terminal YS motif was involved in α2-adrenergic receptor export from the Golgi apparatus (36). Therefore, the conserved motifs at the extracellular N-terminus and the intracellular C-terminus are crucial for intracellular trafficking of GPCRs. Interestingly, the N-terminus, C-tail, intracellular loops and transmembrane domains of PAR-2 all failed to interact with p24A. Strikingly, here we show that the second extracellular loop of PAR-2 bound to the N-terminal lumen of p24A. Therefore, our findings provide the new insight that the second extracellular loop of PAR-2 could harbor a novel sorting signal which is recognized by p24 proteins and is involved in exocytic receptor trafficking.

It is well known that the second extracellular loop of PAR-2 is a key domain for receptor activation. It intramolecularly interacts with the extracellular N-terminal tethered ligand domain of the receptor generated by proteolytic cleavage by proteases, thereby auto-activating the receptor (2). Therefore, our data suggest that novel receptor antagonists could be developed, based on the N-terminal amino acid sequence of p24A, as indicated by preliminary studies (3).

p24A is abundant in membranes of the intermediate compartment, cis-Golgi network and ER (9,11). We here show that p24A knockdown specifically led to the redistribution of GM130 that functions as a structural element of the Golgi apparatus, without any effect on the ER. Therefore, our data provide new functional evidence that p24A is a crucial structural constituent for the organization of the Golgi apparatus.

Importantly, we here found that p24A interacted with PAR-2 in a PAR-2 agonist-dependent manner. p24A was significantly dissociated from PAR-2 at 30 min after agonist stimulation. At this time point, PAR-2 was repopulating the plasma membrane and the cells were almost completely resensitized. Conversely, once the protein dissociation between PAR-2 and p24A was prevented by the loss-of-function mutant p24A-GL-GFP, both exocytic trafficking of PAR-2 and receptor resensitization were significantly blocked. These results indicate that p24A plays a pivotal role in regulating exocytic trafficking of PAR-2 from the Golgi apparatus to the plasma membrane. In addition, we found that the localization of p24A was not affected upon PAR-2 activation (data not shown). This excludes the possibility that p24A directly mediates PAR-2 transport.

Thus, our data support a model that delineates a novel role of p24A, distinct from its hitherto known functions (Fig. 8). In unstimulated cells, p24A traps and retains PAR-2 at the Golgi apparatus to exclude the receptor from transport vesicles that are operated in the biosynthetic pathway. Upon cell surface PAR-2 activation, p24A loses its ability to interact with the intracellular PAR-2 at the Golgi apparatus. This facilitates PAR-2 to be incorporated into transport vesicles, which then deliver PAR-2 to the plasma membrane for resensitization. p24A was initially proposed to regulate protein transport in the early secretory pathway, because of its property of divergent luminal domains to recognize cargo and the ability of its C-tail to bind to COPI and COPII (8,12,13,21). Later the opposite hypothesis was raised for p24A functions. Studies on Caenorhabditis elegans and Saccharomyces cerevisiae demonstrate that p24A may serve as quality control to restrict the incorporation of unfolded or mutant proteins into COP vesicles, but p24A seems not to be essential for protein transport in the early secretory pathway (23,24). Here, our data also suggest that p24A does not directly mediate cargo (PAR-2) transport. It retains PAR-2 at the Golgi apparatus, but releases the intracellular receptor for the exocytic trafficking after PAR-2 agonist exposure. This implies that p24A might be a “signal worker” to control post-Golgi transport of cargo. Thus, the next question arose what might be the signal to start the delivery of receptor from the Golgi store.

The small GTPase Rab families have been shown to be extensively involved in endocytic and exocytic trafficking of GPCRs (37). The member Rab11 is well characterized to mediate post-Golgi transport of rhodopsin to the apical membrane surface (38), and to regulate recycling of several GPCRs through recycling endosomes (37). Studies on PAR-2 trafficking revealed that Rab11a was partially involved in post-Golgi transport of PAR-2 and subsequent resensitization (7). In the present investigation, we established the role of ARF1, another key GTPase that controls the intracellular trafficking machinery, on PAR-2 transport. We found that ARF1 regulated the protein dissociation between PAR-2 and p24A.
after PAR-2 activation and initiated PAR-2 trafficking from the Golgi apparatus to the plasma membrane. The importance of ARF1 in transport vesicle formation and protein transport has been emphasized (17). The C-tails of p23 and p24A recruit inactive ARF1-GDP to membranes of the Golgi apparatus, resulting in ARF1 activation by GEF (15). The activated ARF1-GTP is likely to induce the dissociation of p23 from p24A, thereby initiating coated vesicle assembly and cargo packaging (39). Therefore, the loss-of-function mutant p24A−GL-GFP that cannot interact with ARF1 and accept PAR-2 signaling from ARF1 was able to arrest PAR-2 at the Golgi apparatus and thus blocked receptor resensitization after trypsin exposure. In contrast, both p24AΔC-GFP and p24AΔCT-GFP have the coiled-coil domain that enables the formation of oligomeric complexes between the truncated protein and endogenous full-length p24 proteins. Thus, both truncated proteins were still able to talk with ARF1 recruited by endogenous p24 proteins, and did not influence resensitization of PAR-2. Our present data demonstrate that brefeldin A prevents PAR-2 trafficking to the plasma membrane. It has been reported that the large ARF GEFs, including BIG1, BIG2 and GBF1, are sensitive to brefeldin A (40-42). Thus, brefeldin A can inhibit activation of ARF1, ARF3 and ARF5 (17). Although we established the role of ARF1 in PAR-2 trafficking, we still cannot exclude the effect of other brefeldin A-inhibited ARFs on PAR-2 resensitization. In addition, further studies have to define which ARF1 GEF is activated upon PAR-2 activation. Overall, ARF1 is a key factor that mediates cell surface PAR-2 signals to intracellular transport vesicles, thereby regulating receptor trafficking from the Golgi apparatus to the plasma membrane and subsequent resensitization. This pathway is outlined in the model in Fig. 8.

In conclusion, we for the first time identified PAR-2 as a specific cargo protein of p24A. Furthermore, we determined the cargo-binding region of p24A and the sorting motif of PAR-2. Significantly, we found that p24A selectively associated with the intracellular PAR-2 at the Golgi apparatus, and regulated post-Golgi transport of PAR-2 to the plasma membrane. This controlled subsequent physiological resensitization upon receptor activation. Importantly, the small GTPase ARF1 was involved in initiation of PAR-2 trafficking. Therefore, our findings elucidate an important mechanism underlying post-Golgi transport of PAR-2, and have wide implications for PAR-2 physiology as well as for biological functions of p24 proteins.

References


FOOTNOTES

Acknowledgements

We thank Dr. R. Blum (University of Munich) for critical comments on the manuscript and for providing the anti-p24A antibody. We thank Dr. J. S. Bonifacino (National Institutes of Health) for pGEX-VHS and pGEX-VHS-GAT plasmids. We are grateful to E. Busse for the excellent technical assistance and Dr. R. Stricker for helpful discussions.

1The abbreviations used here are: GPCR, G protein-coupled receptor; AP, activating peptide; ARF1, ADP-ribosylation factor 1; COP, coat protein; C-tail, carboxyl tail; ER, endoplasmic reticulum; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin epitope; HEK, human embryonic kidney cells; PAR-2, protease-activated receptor-2; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA

2W. Luo, Y. Wang, and G. Reiser, unpublished data.

3W. Luo, Y. Wang, and G. Reiser, unpublished data.
**Figure Legends**

**Fig. 1. Interaction of p24A with PAR-2 in vivo.** (A). HEK293 cells were stably transfected with p24A/myc or co-transfected with PAR-2-HA and p24A/myc. The whole cell lysates from HEK293-PAR-2-HA+p24A/myc cells, as well as wild-type HEK293 cells (negative control) and HEK293-p24A/myc cells (negative control) were immunoprecipitated (IP) by anti-HA antibody in the presence of protein A-Sepharose beads and the immunocomplex was detected by western blot using the anti-myc antibody and anti-HA antibody. Lysate represents 2% of cell lysates used in the immunoprecipitation assay. (B-C). Endogenous interaction in primary rat astrocytes (B) and primary rat cortical neurons (C). The whole cell lysates were immunoprecipitated (IP) by anti-p24A antibody, anti-PAR-2 (C-17) antibody or control antibody (IgG) in the presence of protein G-agarose beads and the immunocomplex was detected by western blot with the anti-PAR-2 antibody.

**Fig. 2. Domain analysis of PAR-2 and p24A responsible for the interaction.** (A). Schematic representation of full-length and truncated PAR-2-GST tested for the binding to p24A/myc, with the relative binding strength indicated as ++ (strong) or – (no). The positions of amino acids of PAR-2 are noted below the constructs. (B-D). Truncated PAR-2-GST fusion proteins were tested for binding to p24A/myc in the GST pull-down assay. The whole cell lysates from HEK293-p24A/myc cells were incubated overnight with GST protein or truncated PAR-2-GST fusion proteins immobilized on glutathione beads followed by western blot with the anti-myc antibody. Lysate shows 1% of whole cell lysates used for the pull-down experiment. (E). Schematic representation of full-length and truncated p24A-GFP tested for the binding to PAR-2-GST, with the relative binding strength indicated as ++ (strong), + (weak) or – (no). The positions of amino acids of p24A are noted below the constructs. (F-G). Truncated p24A-GFP fusion proteins were tested for binding to PAR-2-GST in the GST pull-down assay. The whole cell lysates from HEK293 cells stably transfected with full-length or truncated p24A-GFP constructs were incubated overnight with PAR-2-GST fusion protein immobilized on glutathione beads followed by western blot with the anti-GFP antibody. Lysate shows 0.5% of whole cell lysates used for the pull-down experiment.

**Fig. 3. Colocalization of PAR-2 with p24A at the Golgi apparatus.** HEK293 cells stably co-transfected with PAR-2-HA and p24A/myc were fixed, permeabilized, stained, and imaged by confocal microscope. PAR-2-HA (red) was visualized by anti-HA antibody and Alexa Fluor 568 goat anti-rabbit IgG. p24A (cyan) was visualized by anti-myc antibody and Alexa Fluor 633 goat anti-chicken IgG. GM130 (green) was visualized by anti-GM130 antibody and Alexa Fluor 488 goat anti-mouse IgG. The merged image reveals the overlap of PAR-2, p24A and GM130 (indicated by arrowheads). Scale bar, 10 μm.

**Fig. 4. Effect of p24A on the constitution of the Golgi apparatus and the ER.** (A). Evaluation of p24A siRNA. HEK293-PAR-2-HA cells were transfected with control siRNA and p24A siRNA at the concentrations indicated, respectively. At 48 h after transfection, p24A knockdown was determined by RT-PCR and western blot analysis. GAPDH served as an internal control for PCR, and β-tubulin I served as loading control for western blot. The density was quantitated and normalized to control. Mean ± S.E. ***p < 0.001, compared with control. (B). p24A knockdown disrupted the structure of the Golgi apparatus (shown by GM130 staining), but had no effect on the organization of the ER (shown by BiP staining). HEK293-PAR-2-HA cells transfected with indicated siRNAs labeled with Alexa Fluor 488 (100 nM) were fixed, permeabilized, stained, and observed by a confocal microscope. PAR-2-HA (red) was visualized by anti-HA antibody and Alexa Fluor 568 goat anti-rabbit IgG. GM130 (cyan, Golgi marker) or BiP (cyan, ER marker) was visualized by anti-GM130 antibody or anti-BiP antibody, respectively, and Alexa Fluor 633 goat anti-mouse IgG. Transfected cells were observed by Alexa Fluor 488 fluorescence (green). Scale bar, 20 μm.

**Fig. 5. Effect of ARF1 on the interaction of PAR-2 with p24A.** (A) HEK293-PAR-2-HA+p24A/myc cells were treated with 100 μM human PAR-2 AP for indicated time periods in serum-free medium. The whole cell lysates were immunoprecipitated (IP) by anti-HA antibody in the presence of protein A-Sepharose beads, and the immunocomplex was detected by western blot using the anti-myc
antibody and anti-HA antibody. Lysate shows 3% of whole cell lysates used for the immunoprecipitation experiment. The density was quantitated and normalized to control (0 min). Mean ± S.E. **p < 0.01, compared with control. (B). Primary rat astrocytes were treated with rat PAR-2 AP (50 μM) for the indicated times and lysed. The endogenous ARF1-GTP in the resulting cell lysates were precipitated by GST-VHS-GAT in the GST pull-down assay, and quantitated by western blot with the anti-ARF1 antibody. GST and GST-VHS served as negative control. The density was quantitated and normalized to control (0 min). Mean ± S.E. **p < 0.01, compared with control. (C). HEK293-PAR-2-HA+p24Amyc cells were pretreated with 10 μg/ml brefeldin A for 30 min, followed by stimulation with 100 μM human PAR-2 AP for indicated time periods in serum-free medium. The whole cell lysates were immunoprecipitated (IP) by anti-HA antibody in the presence of protein A-Sepharose beads, and the immunocomplex was detected by western blot using the anti-myc antibody and anti-HA antibody. Lysate shows 3% of whole cell lysates used for the immunoprecipitation experiment. The density was quantitated and normalized to control (0 min). Mean ± S.E.

Fig. 6. Effect of p24A on exocytic trafficking of PAR-2 and receptor resensitization. (A). The loss-of-function mutant p24A-GL-GFP prevented PAR-2 trafficking from the Golgi apparatus to the plasma membrane. HEK293-FLAG-PAR-2 cells were transiently transfected with p24A-GL-GFP for 24 h. After incubation with 100 nM trypsin for 15 min, cells were washed, and further incubated for the indicated times (0, 30, 60 min) in trypsin-free medium. Cells were then fixed, permeabilized, stained, and observed by a confocal microscope. FLAG-PAR-2 (red) was visualized by anti-FLAG antibody and Alexa Fluor 568 goat anti-rabbit IgG. p24A-GL-GFP (green) was visualized by GFP fluorescence. GM130 (cyan) was visualized by anti-GM130 antibody and Alexa Fluor 633 goat anti-mouse IgG. At 30 min- and 60 min-recovery, FLAG-PAR-2 was undetectable at the plasma membrane in transfected cells (green arrows), whereas FLAG-PAR-2 was repopulating the plasma membrane in non-transfected cells (white arrowheads). Scale bar, 10 μm. (B). The loss-of-function mutant p24A-GL-GFP inhibited resensitization of PAR-2 in transfected HEK293 cells. HEK293-PAR-2-HA cells were transiently transfected with p24A-GL-GFP, p24AΔCT-GFP or p24AΔC-GFP for 24 h. After incubation with 100 μM human PAR-2 AP for 15 min, cells were washed, and challenged with repeated PAR-2 AP pulse of 60 s at indicated recovery times. Calcium responses are normalized to control (0 min), and expressed as mean ± S.E. *p < 0.05, ***p < 0.001 compared with control curve. (C). The loss-of-function mutant p24A-GL-GFP inhibited resensitization of PAR-2 in primary rat astrocytes. Primary rat astrocytes were transiently transfected with p24A-GL-GFP or GFP for 24 h. After incubation with 50 μM rat PAR-2 AP for 2 min, cells were washed, and challenged with repeated PAR-2 AP pulse of 60 s at indicated recovery times. Calcium responses are normalized to control (0 min), and expressed as mean ± S.E. *p < 0.05 compared with control curve.

Fig. 7. Effect of ARF1 on PAR-2 resensitization. (A). Evaluation of ARF1 siRNA. HEK293-FLAG-PAR-2 cells were transfected with either control siRNA or p24A siRNA at concentrations indicated. At 48 h after transfection, ARF1 knockdown was determined by RT-PCR and western blot analysis. GAPDH served as an internal control for PCR, and β-tubulin I served as loading control for western blot. The density was quantitated and normalized to control. Mean ± S.E. ***p < 0.001, compared with control. (B). ARF1 knockdown impaired PAR-2 trafficking to the cell surface. HEK293-FLAG-PAR-2 cells transfected with indicated siRNA were incubated with 100 nM trypsin for 15 min, washed, and further incubated for the indicated times (60 and 120 min) in trypsin-free medium. Cells were then fixed, permeabilized, stained, and observed by a confocal microscope. FLAG-PAR-2 was visualized by anti-FLAG antibody and Alexa Fluo 568 goat anti-rabbit IgG. At 60 min- and 120 min-recovery, FLAG-PAR-2 was detected at the intracellular stores, but not at the plasma membrane in ARF1-deficient cells (green arrows), whereas FLAG-PAR-2 was repopulating the plasma membrane in cells transfected with control siRNA (white arrowheads). Scale bar, 10 μm.

Fig. 8. Model for resensitization of PAR-2 regulation by p24A. In unstimulated cells the N-terminus of p24A interacts with the second extracellular loop of PAR-2, and traps the intracellular receptor at the Golgi apparatus, to retain the receptor and exclude it from transport vesicles that are operating in the biosynthetic pathway. Upon PAR-2 activation, the inactive ARF1-GDP is recruited to
the Golgi membrane. ARF1-GDP recruited to the Golgi membrane is activated by the brefeldin A-sensitive GEF. This results in dissociation of PAR-2 from p24A. Subsequently, the intracellular PAR-2 is then incorporated into transport vesicles and consequently sorted to the plasma membrane for receptor resensitization.
Fig. 1
Fig. 2

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B

Lysate  | Pull-down

p24A

C

Lysate  | Pull-down

p24A

D

Lysate  | Pull-down

p24A

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F

Lysate  | Pull-down

GFP WT ΔN ΔC ΔCT

G

Lysate  | Pull-down

GFP WT G GL ΔG

WB: myc 25 kDa

WB: GFP 50 kDa

WB: GFP 25 kDa
Fig. 3

PAR-2-HA  p24Amyc  GM130  Merge
Fig. 4

A

Control siRNA - 100 - (nM)  
p24A siRNA - - 100 (nM)

p24A

GAPDH

Control siRNA - 100 - - (nM)  
p24A siRNA - - 10 100 (nM)

p24A

β-tubulin

B

Control  Control siRNA  p24A siRNA

PAR-2-HA

GM130

Merge

PAR-2-HA

BiP

Merge
Fig. 5

(A) Western blot analysis of p24Amyc and PAR-2-HA under different time points (0, 30, 60 min).

(B) Measurement of ARF1 activation levels at various time points (0, 5, 10, 20, 30, 60 min).

(C) Evaluation of interaction under Brefeldin A treatment compared to vehicle control at time points (0, 30, 60 min).
Fig. 6
Fig. 7

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ARF1

GAPDH

β-tubulin

Control siRNA

ARF1 siRNA

Resensitization

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p24A, a type I transmembrane protein, controls ARF1-dependent resensitization of protease-activated receptor-2 by influence on receptor trafficking
Weibo Luo, Yingfei Wang and Georg Reiser

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