The Intracellular Trafficking of the G Protein-coupled Receptor TPβ Depends on a Direct Interaction with Rab11*

Emilie Hamelin1, Caroline Thériault, Geneviève Laroche, and Jean-Luc Parent2

From the Service de Rhumatologie, Faculté de Médecine and Centre de Recherche Clinique-CHUS, Université de Sherbrooke, Sherbrooke, Québec J1H SN4, Canada

Intracellular trafficking pathways of cell surface receptors following their internalization are the subject of intense research efforts. However, the mechanisms by which they recycle back to the cell surface are still poorly defined. We have recently demonstrated that the small Rab11 GTPase protein is a determinant factor in controlling the recycling to the cell surface of the β-isof orm of the thromboxane A2 receptor (TPβ) following its internalization. Here, we demonstrate with co-immunoprecipitation studies in HEK293 cells that there is a Rab11-TPβ association occurring in the absence of agonist, which is not modulated by stimulation of TPβ. We show with purified TPβ intracellular domains fused to GST and HIS-Rab11 proteins that Rab11 interacts directly with the first intracellular loop and the C-tail of TPβ. Amino acids 335–344 of the TPβ C-tail were determined to be essential for the interaction of Rab11 with this receptor domain. This identified sequence appears to be important in directing the intracellular trafficking of the receptor from the Rab5-positive intracellular compartment to the perinuclear recycling endosome. Interestingly, our data indicate that TPβ interacts with the GDP-bound form, and not the GTP-bound form, of Rab11 which is necessary for recycling of the receptor back to the cell surface. To our knowledge, this is the first demonstration of a direct interaction between Rab11 and a transmembrane receptor.

The family of G protein-coupled receptors (GPCRs)1 constitute the largest, most ubiquitous and versatile superfamily of cell surface receptors (1). These proteins, which contain seven transmembrane domains, are responsible for communicating stimuli from the environment to the cell in order to produce a broad variety of physiological responses. They respond to a wide range of stimulants including light, hormones, neurotransmitters, and odorants, and generate responses as diverse as cell contraction, secretion, chemotaxis, and proliferation. It is well known that different molecular mechanisms regulate GPCRs signaling cascades. Following agonist binding, many GPCRs undergo agonist-induced phosphorylation, internalization, and resensitization or down-regulation, resulting in a control of the expression of cell surface receptors as well as agonist sensitivity (2). Our laboratory is interested in the mechanisms that regulate the intracellular trafficking of the thromboxane A2 receptor (TP), a rhodopsin-like GPCR.

The prostanoid thromboxane A2 (TXA2) mediates morphological changes and aggregation of platelets. It is also known as a potent broncho- and vasoconstrictor as well as an inducer of vascular smooth muscle cells proliferation and hypertrophy (3). A variety of cardiovascular, pulmonary, and kidney diseases are the result of numerous pathological states associated with defects in TXA2 synthesis or in its receptor function (4–8). TP is encoded by a single gene on chromosome 19p13.3 that is alternatively spliced in the C terminus resulting in two isoforms, TPα (343 amino acids) and TPβ (407 amino acids), which share the first 328 amino acids (9–11). Stimulation of TP results in the production of the second messenger inositol phosphate through phospholipase Cβ (PLCβ) as a consequence of the activation of the Gsα(11) family of G proteins (4). Coupling of TP to other G proteins such as Gαs, Gαq, Gα12, and Gα13 was also reported (4, 12, 13).

GPCRs are subjected to a tight regulation by a diversity of cellular mechanisms (14–23). Among these, the regulation of intracellular trafficking of these receptors by Rab GTPases is the subject of intense research efforts. Rab GTPases are associated with specific intracellular compartments and direct the trafficking pathways of intracellular vesicles. For example, Rab11 was shown to be associated with post-Golgi membranes, including the trans-Golgi network, and the perinuclear recycling endosome, which is proposed to regulate the slow return of recycling receptors to the plasma membrane (24, 25). Perinuclear recycling endosomes participate in the recycling of several cell membrane proteins, such as TPβ, the angiotensin II type 1A receptor, the V2 vasopressin receptor, the m4 muscarinic acetylcholine receptor, the somatostatin receptors, the chemokine receptor CXCRI2, the TGF-β receptor, the neurokinin 1 receptor, and the protease-activated receptor 2, which all traffic through the Rab11-dependent slow recycling pathway (23, 26–33).

Like all GTPases of the Ras family, Rab11 functions as a molecular switch. In the active GTP-bound state, it interacts with downstream effector proteins. Rab11-binding protein (Rab11BP) or rabphilin 1 was the first effector identified to interact with the GTP-bound form of Rab11 (34), followed by Myosin Vb (35). Recent studies isolated a family of Rab11-interacting proteins (FIPs) that may mediate Rab11 effects in recycling endosomes. These include Rip11, Rab11-FIP1, Rab11-FIP2, Rab11-FIP3/Efrin, Rab11-FIP4, and RCP (36–41). All of these proteins share similar Rab11 binding sites within a C-terminal coiled-coil motif (36, 42), and they all possess the ability to influence sorting of vesicles through endosomes. Finally, actin (43) and Sec15 (44) were also recently demonstrated as effectors of Rab11.

Specific sequences in the C terminus of many GPCRs have been recognized as potential sites required for the efficient recycling of internalized receptors back to the plasma membrane. For example, sequences in the C terminus of the dopamine D1, β2-adrenergic, µ-opioid, human

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2 Recipient of a CIHR New Investigator Award. To whom correspondence should be addressed: Service de Rhumatologie, Faculté de Médecine, Université de Sherbrooke, 3001 12e Ave. Nord, Fleurimont, QC J1H 5N4, Canada. Tel.: 819-564-5264; Fax: 819-564-5265; E-mail: jean-luc.parent@USherbrooke.ca.

3 The abbreviations used are: GPCR, G protein-coupled receptor; TP, thromboxane A2 receptor; TXA2, thromboxane A2; GST, glutathione S-transferase; GFP, green fluorescent protein; GDP/βS, guanosine 5’-[β,γ-methylene]diphosphate; GTP-5S, guanosine 5’-y- thio)diphosphate; ICL, intracellular loop of TPβ; HA, hemagglutinin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney.

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luteinizing hormone, and V2 vasopressin receptors were previously shown to act like recycling signals (31, 45–49). We previously demonstrated that TPβ undergoes constitutive and agonist-induced internalization (16, 19), and we showed that constitutive internalization of TPβ helps to maintain an intracellular pool of receptors in the Rab11-positive recycling endosome from which they recycle to the cell surface to preserve agonist sensitivity (23). In the present study, we demonstrate a direct interaction between TPβ and Rab11. To our knowledge, this is the first report of an interaction between Rab11 and a membrane receptor involved in internalization and subsequent recycling.

**EXPERIMENTAL PROCEDURES**

*Reagents.—* The hemagglutinin (HA)-specific polyclonal antibody and protein A-agarose were from Santa Cruz Biotechnology. GST-specific polyclonal antibody was purchased from Zymed Laboratories Inc. The FLAG-M2-specific monoclonal antibody, GDP-binding specific polyclonal antibody was from Bethyl Laboratories, whereas HA-specific antibody was from Invitrogen and HindIII and ligated into the pRSET A vector (Invitrogen) digested with the same enzymes. Here, we propose that the identified Rab11-TPβ interaction directs the intracellular trafficking of the receptor. Our findings have potential implications in the wide array of membrane proteins undergoing internalization and subsequent recycling.

**Plasmid Constructions**—The Rab11 cDNA from pcDNA3-HA-Rab11 (16) was digested with EcoRI and XhoI and ligated into the pcDNA3-FLAG vector (16) digested with the same enzymes. (His)6-tagged Rab11 was used to produce a His-tagged HA-Rab11 protein was purified using Ni-NTA-agarose resin (Qiagen) according to the manufacturer's instructions. The other TPβ constructs were all generated by PCR using pcDNA3-HA-TPβ DNA (16) as template. The mutant constructs pGEX-4-T1-TPβCT (329–407), pGEX-4-T1-TPβCT (334–407), and pGEX-4-T1-TPβCT (345–407) were prepared with the primer SP6 (5′-CATAC-GATTATGTTACACTATAG-3′) and the following primers, respectively: R329For (5′-GGAGAATTCGAGCTACCTGGGTATCACCCG-3′), W334For (5′-GGAGAATTCGAGCTACCTGGGTATCACCCG-3′), A345For (5′-GGAGAATTCGAGCTACCTGGGTATCACCCG-3′), and A345Rev (5′-GGAGAATTCGAGCTACCTGGGTATCACCCG-3′). The TPβ (Δ335–344) mutant was generated from pcDNA3-HA-TPβ construct (16) by PCR using the following primers to generate the fragment 1–334, T7 (5′-TTAATAAGCTCAGCTATGAGG-3′) and TPβ-334Rev (5′-CTCTGTGAGCTCAGCTATGAGG-3′), and the following primers to create the fragment 345–407, TPβ-345For (5′-CTGTCAGCTGAGCTCAGCTATGAGG-3′) and TPβ-345Rev (5′-CTGTCAGCTGAGCTCAGCTATGAGG-3′) and SP6. Both fragments were joined by the PCR extension method. The mutant receptor was subcloned in pcDNA3-HA and pcDNA3-FLAG digested with EcoRI and Xhol. Integrity of the coding sequences of all the constructs was confirmed by dideoxy sequencing.

**Immunoprecipitations**—6-Well plates of HEK293 cells were transfected with pcDNA3, pcDNA3-HA-TPβ, pcDNA3-HA-TPβR328stop, pcDNA3-HA-TPβ (Δ335–344), and pcDNA3-FLAG-Rab11 in the different combinations indicated under "Results." 48-h post-transfection, the cells were incubated for 60 min at 37 °C in the presence or the absence of 1 μM U46619 prior to harvesting. The cells were then rinsed with ice-cold phosphate-buffered saline (PBS) and harvested in 500 μl of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM Na3PO4, 5 mM EDTA) supplemented with protease inhibitors (9 mM pepstatin, 9 mM antipain, 10 mM leupeptin, and 10 mM chymostatin) (Sigma). After the cells were incubated in lysis buffer for 60 min at 4 °C, the lysates were clarified by centrifugation for 20 min at 14,000 rpm at 4 °C. 4 μg of specific polyclonal antibodies were added to the supernatant. After 60 min of incubation at 4 °C, 75 μl of 50% protein A-agarose pre-equilibrated in lysis buffer was added, followed by an overnight incubation at 4 °C. Samples were then centrifuged for 1 min in a microcentrifuge and washed three times with 1 ml of lysis buffer. Immunoprecipitated proteins were eluted by addition of 50 μl of SDS sample buffer followed by a 60-min incubation at room temperature. Initial lysates and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting using specific antibodies.

**Recombinant Protein Production and Binding Assays**—The pRSET A-HA-Rab11 construct was used to produce a His-tagged HA-Rab11 fusion protein in OverExpressTM C41(DE3) Escherichia coli strain (Avi-Tag) by following the manufacturer's instructions. The recombinant HA-Rab11 protein was purified using Ni-NTA-agarose resin (Qiagen) as indicated by the manufacturer. All the constructs in the pGEX-4-T1 vector (Amersham Biosciences) listed above were used to produce A Rab11-TPβ Interaction Directs TPβ Recycling

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GST-tagged mutants of TPβCT or ICL fusion proteins also in the Over-Express™ C41(DE3) E. coli strain. All the recombinant mutants of TPβCT and ICL were purified using glutathione-Sepharose™ 4B (Amersham Biosciences) as indicated by the manufacturer. Purified recombinant proteins were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. 50 μg of glutathione-Sepharose-bound GST mutants of TPβCT or ICL were incubated with 50 μg of purified (His)₆-HA-Rab11 in binding buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Igepal) supplemented with proteases inhibitors (9 mM pepstatin, 9 mM antipain, 10 mM leupeptin, and 10 mM chymostatin) (Sigma) and 5 mM dithiothreitol. The binding reactions were then washed four times with binding buffer. SDS sample buffer was added to the binding reactions, and the tubes were boiled for 5 min. The binding reactions were analyzed by SDS-PAGE, and immunoblotting was performed with the indicated specific antibodies or by Ponceau Red S staining.

**Radioligand Binding Assays**—Competition binding curves were done on HEK293 cells expressing wild-type and mutant receptor species. Cells were harvested and washed twice in Buffer A (10 mM Hepes, pH 7.6, 129 mM NaCl, 8.9 mM NaHCO₃, 0.8 mM KH₂PO₄, 0.8 mM MgCl₂, 5.6 mM dextrose, 0.38% sodium citrate, pH 7.4, 5 mM EDTA, and 5 mM EGTA). Binding reactions were carried out on 1.5 x 10⁵ cells in a total volume of 0.1 ml in the same buffer with 10 nM [³H]SQ29548 (a TXA2 antagonist) and increasing concentrations of U46619 or nonradioactive antagonist. Cells transfected with pcDNA3 alone were studied concurrently to determine background. Following incubation with the primary antibody, cells were washed three times with TBS and blocked again with TBS/BSA, 1%, for 15 min. The binding reactions were then washed four times with binding buffer. SDS sample buffer was added to the binding reactions, and the tubes were boiled for 5 min. The binding reactions were analyzed by SDS-PAGE, and immunoblotting was performed with the indicated specific antibodies or by Ponceau Red S staining.

**Measurement of Cell Surface Receptor Loss**—For quantification of cell surface receptor loss, ELISA assays were performed as described (16, 19). HEK293 cells were plated at 8 x 10⁵ cells per 60-mm dish, transfected with 4 μg of DNA, and 24-h after the transfections into 6 wells of a 24-well tissue culture dish coated with 0.1 mg/ml poly(l-lysine) (Sigma). On the following day, the cells were washed once with phosphate-buffered saline (PBS) and incubated in the presence or the absence of 100 nM of U46619 for different times at 37°C in prewarmed DMEM supplemented with 0.5% BSA and 20 mM Hepes, pH 7.5. Then, the medium was removed, and the cells were fixed in 3.7% formaldehyde/TBS (20 mM Tris, pH 7.5, 150 mM NaCl) for 5 min at room temperature. Cells were washed three times with PBS and nonspecific binding blocked with PBS/BSA, 1%, for 45 min. HA-specific monoclonal antibody was then added at a dilution of 1:1000 in PBS/BSA, 1%, for 60 min. Following incubation with the primary antibody, cells were washed three times with PBS and blocked again with PBS/BSA, 1%, for 15 min. Incubation with goat anti-mouse-conjugated alkaline phosphatase diluted 1:1000 in PBS/BSA, 1%, was carried out for 60 min. The cells were washed three times with PBS and 250 μl of a colorimetric alkaline phosphatase substrate was added. Plates were then incubated at 37°C until a yellow color appeared. A 100-μl aliquot of the colorimetric reaction was taken, stopped by the addition of 100 μl of 0.1 N NaOH, and read at 405 nm using a Titertek Multiskan MCC/340 spectrophotometer. Cells transfected with pcDNA3 alone were studied concurrently to determine background.

**Immunofluorescence Microscopy**—Immunofluorescence microscopy assays were performed as described (23). 911 cells were seeded at a density of 1.2 x 10⁶ cells per 60-mm plate. The next day, cells were transiently transfected with pcDNA3 alone (control) or cotransfected with pcDNA3-Rab11 or GFP-Rab5 and either pcDNA3-HA-TPβ or pcDNA3-HA-TPβ (A335-344). On the following day, 2 x 10⁵ cells were transferred onto coverslips and further grown overnight. Cells were treated or not with 100 nM U46619 for 120 min at 37°C in DMEM supplemented with 0.5% BSA and 20 mM Hepes, pH 7.5. Cells were washed with PBS and then fixed with 3% paraformaldehyde in PBS for 10 min at room temperature, washed with PBS, and incubated in quenching solution (50 mM Tris, pH 8, 100 mM NaCl) for 5 min at room temperature. Cells were then permeabilized 20 min with 0.1% Triton X-100 in PBS and blocked with 0.1% Triton X-100 in PBS containing 5% nonfat dry milk for 30 min at room temperature. Cells were incubated with HA-specific monoclonal and Rab11-specific polyclonal antibodies where applicable at a dilution of 1:500 in blocking solution for 60 min at room temperature. Cells were washed twice with PBS and blocked again with 0.1% Triton X-100 in PBS containing 5% nonfat dry milk for 30 min at room temperature. Goat anti-mouse Rhodamine Red-conjugated and anti-rabbit Oregon Green 488-conjugated secondary antibodies were added at a dilution of 1:200 in blocking solution for 60 min at room temperature. The cells were washed four times with permeabilization buffer, two times with PBS, and coverslips were mounted using Vectashield mounting medium (Vector Laboratories) and examined on a Nikon Eclipse TE2000-U inverted fluorescence microscope using a plan fluor ×40/0.75 objective. Image acquisition was done using a Hamamatsu Photonics C4742-95-12ER camera and Simple PCI high performance imaging software, and processed with Adobe Photoshop.

**RESULTS**

**The C-terminal Tail of TPβ Interacts Directly with the Small Rab11 GTPase**—Recently, we showed by co-localization experiments performed by immunofluorescence microscopy that both constitutive and agonist-triggered internalization resulted in the targeting of TPβ to the Rab11-positive recycling endosome. We also demonstrated that Rab11 regulates the recycling of TPβ (23). Because of the very strong co-localization of the two proteins and of the dramatic effect of Rab11 on TPβ cell surface expression (23), we suspected a putative interaction between TPβ and Rab11. To verify this hypothesis, we first performed an in vitro binding assay using the purified recombinant C-terminal tail of TPβ in fusion with GST (GST-TPβCT) along with the purified recombinant HA-Rab11 fused to His (His)₆-HA-Rab11). The TPβ C terminus was first chosen to perform this test because only TPβ, but not TPs, displays constitutive and agonist-induced trafficking, which is encoded by specific regions found in this receptor domain (16, 19). The results shown in Fig. 1A illustrate that Rab11 binds specifically to glutathione-Sepharose-bound GST-TPβCT and not to glutathione-Sepharose-bound GST.

To investigate the interaction between Rab11 and TPβ in a cellular context, we performed immunoprecipitation experiments in HEK293 cells transfected with pcDNA3-FLAG-Rab11 and pcDNA3-HA-TPβ in the presence or absence of stimulation with U46619 (a stable TP agonist). Cell lysates were incubated with a HA-specific polyclonal antibody and protein A-agarose, and immunoprecipitation reactions were then analyzed by immunoblotting with a FLAG-specific monoclonal antibody. Our results demonstrate that Rab11 was co-immunoprecipitated with TPβ in the presence or absence of agonist (Fig. 1B). Similar data were obtained when immunoprecipitations were carried out on lysates of cells stably expressing low levels of HA-tagged TPβ (80,000 receptors/cell) and Western blots probed with a polyclonal anti-Rab antibody to detect the endogenous protein (Fig. 1C). This suggests that the interaction between Rab11 and TPβ occurs in both constitutive and agonist-triggered trafficking of TPβ, and that this interaction is not modulated by agonist stimulation. Rab4 failed to co-immunoprecipitate with TPβ in the same experimental conditions, demonstrating specificity of the Rab11-TPβ interaction (not shown). Taken together, our data
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Rab11 Binds to Amino Acids 335–344 of the C-terminal Tail of TPβ—To screen the site of the Rab11 interaction on the TPβ C-tail, we generated several C terminus mutant constructs of the receptor that we introduced in the pGEX-4T-1 vector (Fig. 2A). Determination of the site of interaction was performed by GST pull-down assays using the purified recombinant protein of the TPβ C-tail mutants in fusion with GST (GST-TPβCT) along with the purified recombinant HA-Rab11 protein fused to His ((His)₆-HA-Rab11). The binding reactions were analyzed by immunoblotting with a HA-specific monoclonal antibody to detect the presence of the His₆-HA-Rab11. Our results demonstrate that the constructs containing the amino acids 312–328 and 312–332 of TPβ did not bind Rab11. However, the presence of five additional residues in the GST-TPβCT 312–337 protein restored Rab11 binding properties comparable to the full-length GST-TPβCT protein (Fig. 2B). Our data also show that the amino acid 345–407 construct failed to interact with Rab11. On the other hand, we observed that the TPβCT-(329–407) and TPβCT-(334–407) mutant constructs bound Rab11 (Fig. 2B). This suggests that amino acids 335–344 contain the putative site on the C-tail of TPβ required for the interaction with Rab11.

Rab11 Does Not Promote Recycling of the TPβ-(Δ335–344) Mutant Receptor—We were then interested in determining the role of this Rab11 interaction site in the intracellular trafficking of TPβ. The results from the binding assay (Fig. 2B) prompted us to create a TPβ deletion mutant where the Rab11 interaction site is deleted, TPβ-(Δ335–344). TPβ and the TPβ-(Δ335–344) mutant display similar binding affinities for the specific TP antagonist SQ29548, with respective \( K_d \) values of 11.3 ± 1.5 and 14.2 ± 1.3 nM, indicating that the C-tail does not affect the binding properties of these receptors, as shown previously (11, 15, 16, 50). Signaling of the TPβ-(Δ335–344) mutant was not altered as determined by inositol phosphate production (not shown), supported by our earlier report that a TPβ mutant truncated after residue 328 had the same EC₅₀ as the wild-type receptor in this assay (16). We previously reported that co-expression of Rab11 promoted recycling of TPβ back to the cell surface following agonist stimulation resulting in an apparent inhibition of receptor internalization (23). Thus, we wanted to compare the internalization of the TPβ-(Δ335–344) mutant to wild-type TPβ, and to assess if internalization of TPβ-(Δ335–344) was affected by co-expression of Rab11 like the wild-type receptor. Fig. 3 shows the results of an assay measuring cell surface receptor loss by ELISA following a time course of stimulation with U46619 in HEK293 cells transfected with HA-TPβ or HA-TPβ-(Δ335–344) and either Rab11 or pcDNA3 constructs. Like we observed before (23), co-expression of Rab11 promoted TPβ recycling resulting in the apparent inhibition of TPβ internalization from the cell surface. Interestingly, the cell surface receptor loss of the TPβ-(Δ335–344) mutant was not affected as significantly as the wild-type receptor by co-expression of Rab11, indicating that Rab11 could not efficiently promote recycling of this receptor mutant to the cell surface. Also worthy of note is the fact that there was a greater loss of TPβ-(Δ335–344) mutant receptors from the cell surface compared with wild-type TPβ when both constructs were co-transfected with pcDNA3. This supports the idea that the TPβ-(Δ335–344) mutant

demonstrate that Rab11 interacts directly with the C-terminal tail of TPβ in an agonist-independent manner.

**FIGURE 1.** The C-terminal tail of TPβ interacts with Rab11. A, the binding assay was performed using purified glutathione-Sepharose-bound GST-TPβCT and purified recombinant (His)₆-HA-Rab11 proteins. The recombinant (His)₆-HA-Rab11 binding was detected by the use of a HA-specific monoclonal antibody (upper panel), and the GST-TPβCT protein present in the binding reaction was detected with a GST-specific polyclonal antibody (middle panel), as detailed under "Experimental Procedures." B, co-immunoprecipitation experiments were performed in HEK293 cells transfected with pcDNA3-FLAG-Rab11 and pcDNA3-HA-TPβ and stimulated or not with 1 μM U46619 for 60 min at 37 °C. Immunoprecipitations of the receptor were carried out using a HA-specific polyclonal antibody and immunoblotting was performed with HA-specific or FLAG-specific monoclonal antibodies as described under "Experimental Procedures." C, co-immunoprecipitation experiments of endogenous proteins were performed in HEK293 cells stably expressing pcDNA3 or pcDNA3-HA-TPβ. The cells were stimulated or not with 1 μM U46619 for 60 min at 37 °C and immunoprecipitations of the receptor were carried out using a HA-specific polyclonal antibody. Immunoblotting was performed with HA-specific monoclonal or Rab11-specific polyclonal antibodies. Data presented are representative of three different experiments. **IP**, immunoprecipitation; **IB**, immunoblotting.
receptors do not recycle back to the cell surface resulting in a greater disappearance of mutant receptors than wild-type TPβ from the cell surface over time. The results presented in Fig. 3 indicate that the Rab11-interaction site on TPβ C-tail is important in targeting the receptor to the Rab11-positive recycling endosome to ensure its return to the cell surface membrane.

**Impaired Trafficking of the TPβ-(Δ335–344) Mutant toward the Rab11-Positive Recycling Endosome**—We demonstrated before that TPβ accumulated in a Rab11-positive perinuclear intracellular compartment following constitutive and agonist-induced internalization from which it recycled back to the cell surface (19, 23). We then wanted to assess whether the co-localization of TPβ and Rab11 was conserved when the amino acids 335–344 were deleted. Cells transfected with HA-TPβ or HA-TPβ-(Δ335–344) constructs were treated with agonist or vehicle. The cells were then processed for immunofluorescence microscopy, as detailed under “Experimental Procedures.” Fig. 4A shows prominent co-localization of TPβ with Rab11 in the perinuclear region following 120 min of agonist-promoted (U46619) receptor internalization (lower panel), as we showed previously (23). Co-localization of TPβ with Rab11 is also detected at 0 min (upper panel) and 120 min of vehicle-treatment (middle panel) because of the labeling of total receptors and of the constitutive internalization of the receptor (23). In contrast, co-localization of TPβ-(Δ335–344) with Rab11 in the presence or absence of agonist is almost non-existent (Fig. 4B, middle panel). Interestingly, agonist treatment of TPβ-(Δ335–344) receptors that constitutively internalized failed to co-localize with the Rab11-positive compartment (Fig. 4B, middle panel). Interestingly, agonist treatment of TPβ-(Δ335–344) receptors that constitutively internalized failed to co-localize with the Rab11-positive compartment (Fig. 4B, middle panel). We then proceeded to assess targeting of the wild-type and mutant receptors to the Rab5-positive intracellular compartment. Co-localization studies in cells co-expressing GFP-tagged Rab5 and either HA-tagged TPβ or TPβ-(Δ335–344) revealed that the TPβ-(Δ335–344) mutant could be targeted to the Rab5-positive compartment like the wild-type receptor (Fig. 5, A and B). One could spec-
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FIGURE 4. Targeting of TPβ to a Rab11-positive compartment is impaired when amino acids 335–344 of the TPβ C terminus are deleted. 911 cells transiently expressing HA-TPβ (A) or HA-TPβ-(A335–344) (B) were allowed to undergo constitutive (vehicle) (middle panels) or agonist-induced (100 nM U46619) (lower panels) internalization for 120 min at 37 °C. Receptors and Rab11 were visualized by incubating the cells with mouse monoclonal anti-HA and a rabbit anti-Rab11 polyclonal antibodies, followed by Rhodamine-conjugated anti-mouse and Oregon Green 488-conjugated anti-rabbit antibodies, respectively. The cells were then processed for fluorescence microscopy as described under “Experimental Procedures.” Results are representative of three independent experiments.
FIGURE 5. TPβ(-Δ335–344), like wild-type TPβ, is targeted to a Rab5-positive compartment. 911 cells transiently co-expressing GFP-Rab5 and either HA-TPβ (A) or HA-TPβ(-Δ335–344) (B) were allowed to undergo constitutive (vehicle) or agonist-induced (100 nM U46619) internalization for different times at 37 °C and processed for fluorescence microscopy as described under "Experimental Procedures." Results are representative of three independent experiments.
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ulate that the TPβ-(Δ335–344) mutant was not targeted to the Rab11-positive compartment because it was rather diverted to lysosomes and degraded. Total receptor protein levels and receptor degradation experiments demonstrated that the TPβ-(Δ335–344) mutant was not degraded more rapidly than the wild-type receptor (data not shown). These data show that the TPβ-(Δ335–344) mutant can traffic to the Rab5-positive endosome after internalization but then fails to proceed toward the Rab11-associated perinuclear recycling endosome. Thus, our results indicate that the region comprised of amino acids 335–344 of TPβ is involved in the interaction with Rab11 and in proper trafficking of the receptor from the Rab5-positive intracellular compartment to the Rab11-associated recycling endosome.

Rab11 Also Interacts with the First Intracellular Loop of TPβ—Having shown that Rab11 associated directly with the TPβ C-tail and that this interaction regulated the trafficking of the receptor, we wanted to ascertain if Rab11 interacted with other intracellular domains of TPβ. Because we observed in Fig. 2B that the membrane-proximal C-tail amino acids 312–328 were not involved in binding Rab11, we decided to perform a co-immunoprecipitation of the TPβ328Stop mutant with Rab11 in HEK293 cells to evaluate the role of the three intracellular loops of the receptor in this interaction. Surprisingly, it can be seen in Fig. 6A that Rab11 co-immunoprecipitated with the TPβ328Stop receptor mutant. This indicated that at least one of the three intracellular loops of the receptor was also interacting with Rab11. The amino acid sequences of the three intracellular loops of TPβ were thus individually expressed in fusion with GST and used in a pull-down assay with purified His-Rab11 protein. The results presented in Fig. 6B show that the first, but not the second and the third, intracellular loop of TPβ also interacts directly with Rab11. The Rab11 interaction seems weaker with the first intracellular loop than with the C-tail of the receptor. This suggests that there are two Rab11 binding sites on TPβ: a strong binding site on the C-tail and a weaker one on the first intracellular loop of the receptor. However, the site on the C-tail appears determinant for proper receptor trafficking.

TPβ Preferentially Interacts with GDP-bound Rab11—It has been shown that many effectors interact with the active GTP-bound state of Rab11. Conversely, some proteins interact preferentially with the GDP-bound form of small GTPases, like β-arrestin 1 and ARF6, or the angiotensin II type 1A receptor (AT1AR) and Rab5 (51, 52). We were thus interested to study if the interaction between TPβ and Rab11 was regulated by the nature of the nucleotide bound to the small G protein. We performed GST pull-down assays using the purified recombinant C-terminal tail of TPβ in fusion with GST (GST-TPβCT) on extracts of HEK293 cells overexpressing either HA-Rab11, HA-Rab11S25N, or HA-Rab11Q70L. Rab11S25N is a Rab11 mutant that displays lower affinity for GTP than for GDP leading to a dominant inhibitory effect, whereas Rab11Q70L is a mutant with reduced GTPase activity, which stabilize the GTP-bound and active conformation of Rab11 (53). The results obtained in Fig. 7A show that Rab11 and Rab11S25N, but not Rab11Q70L, could bind specifically to glutathione-Sepharose-bound GST-TPβCT. Similarly, co-immunoprecipitation experiments in HEK293 cells revealed that HA-TPβ interacted with Rab11 and Rab11S25N but not with Rab11Q70L (Fig. 7B). These results obtained with the Rab11 mutants suggest that TPβ interact with GDP-bound Rab11. To further support these results, we performed GST pull-down assays with the purified recombinant GST-TPβCT protein on cell extracts of HEK293 cells overexpressing HA-Rab11 in the presence or absence of non-hydrolyzable GDPβS and GTPγS analogs. The addition of GDPβS did not alter the TPβ-Rab11 interaction, whereas addition of GTPγS completely abolished the binding of Rab11 to TPβCT (Fig. 7C). Taken together, our data indicate a preferential interaction of the receptor with the GDP-bound form of Rab11.

DISCUSSION

Endocytosis of cell surface membrane receptors is a fine tuned regulatory mechanism essential for the proper function of the cell. Endocytosis participates in the uptake of various ligands, such as ions, nutrients, hormones, neurotransmitters, and immune complexes, and is implicated in the regulation of the different receptors involved in these processes. Internalized receptors can be subjected to degradation, transcytosis, or recycling back to the cell surface. Receptor recycling to the cell surface is crucial in maintaining the cell sensitivity to its environment. Whereas the mechanisms involved in endocytosis are characterized in increasing details, those responsible for receptor recycling are less well defined.
Individual Rab GTPases localized on discrete intracellular compartments regulate unique trafficking pathways of intracellular vesicles. For instance, Rab5 is described as being localized to the plasma membrane, clathrin-coated vesicles, and early endosomes, and controlling formation of clathrin-coated vesicles, endocytosis of clathrin-coated vesicles, “heterotypic” fusion of clathrin-coated vesicles with early endosomes.
A Rab11-TPβ Interaction Directs TPβ Recycling

and "homotypic" fusion between early endosomes (54–56). Rab11 was shown to be associated with post-Golgi membranes, including the trans-Golgi network and the perinuclear recycling endosome. It is known to regulate transport from early endosomes to recycling endosomes, slow recycling from the perinuclear recycling endosome to the cell surface, and traffic to the trans-Golgi network. On the other hand, Rab4 exhibits overlapping distribution with both Rab5 and Rab11 in early and recycling endosomes and thus regulates traffic from early endosomes and recycling endosomes to the plasma membrane (54–56). Rab proteins organize multiprotein complexes associated with specific vesicle trafficking pathways reflecting the complexity in the regulation of intracellular trafficking.

We previously reported that TPβ could undergo constitutive and agonist-induced internalization (16, 19) which resulted in targeting of the receptor to the Rab11-positive perinuclear recycling endosome (23). We also observed that Rab11, but not Rab4, was a governing factor in TPβ recycling to the cell surface (23). Because of the extensive colocalization of TPβ with Rab11 and the pronounced effects of Rab11 on the recycling of TPβ, we hypothesized that Rab11 could interact with TPβ. Our results showed for the first time that Rab11 interacts directly with a membrane receptor, in this case the G protein-coupled receptor for TXA2 (TPβ), to direct its trafficking to the perinuclear recycling endosome and back to the cell surface. Interestingly, TPβ did not interact with Rab4 demonstrating the specificity of the Rab11-TPβ interaction. Specificity in the interaction between a GPCR and RabGTPases is required for agonist-induced internalization of this TPβ C-tail domain. Recent studies isolated a Rab11-RBD that may mediate Rab11 effects in recycling endosomes. This sequence is not the second and third, intracellular loop of TPβ but is specifically interacted with Rab11. In summary, we have demonstrated for the first time a direct interaction between Rab11 and its effectors occurs when Rab11 in the trafficking of TPβ (A335–344) is not an effector of Rab11 since interaction between Rab11 and its effectors occurs when Rab11 is in its GDP-bound form. On the other hand, one could think that TPβ is an upstream regulator of Rab11. However, agonist treatment of TPβ did not modulate the TPβ-Rab11 interaction. Because we know that TPβ does not interact with GTP-bound Rab11, our data provide evidence that stimulation of TPβ does not result in activation of Rab11. This is in agreement with the role of Rab11 in the trafficking of TPβ following its constitutive internalization (23). Indeed, we previously demonstrated that Rab11 promoted the recycling of TPβ internalized in the absence of agonist. Thus Rab11 does not require TPβ activation to perform its function on TPβ trafficking. Further studies will be needed to elucidate how Rab11 activity is regulated in relation to TPβ internalization. Experiments are underway in our laboratory to identify proteins found in a GDP-bound Rab11-TPβ complex to better understand how Rab11 directs TPβ trafficking.

In summary, we have demonstrated for the first time a direct interaction between Rab11 and a membrane receptor which determines the intracellular trafficking and recycling to the cell surface of the receptor after internalization. It will be interesting to see in future work if other membrane proteins exhibiting trafficking through the recycling endosome also directly interact with Rab11.

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The Intracellular Trafficking of the G Protein-coupled Receptor TPβ Depends on a Direct Interaction with Rab11

Emilie Hamelin, Caroline Thériault, Geneviève Laroche and Jean-Luc Parent

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