Nm23-H2 Interacts with a G Protein-coupled Receptor to Regulate Its Endocytosis through an Rac1-dependent Mechanism*

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G protein-coupled receptors (GPCRs) constitute the largest family of transmembrane proteins involved in signal transduction. They regulate a wide variety of physiological responses such as neurotransmission, inflammation, cell growth and differentiation, and smell and taste perception. For example, the thromboxane A2 receptor (TP) is implicated in the regulation of diverse pharmacological events, including platelet aggregation, constriction of vascular and bronchial smooth muscle cells, as well as mitogenesis and hypertrophy of vascular smooth muscle cells (1). Two TP receptor isoforms were identified that are generated by the alternative splicing of a single gene, TPα (343 amino acids) and TPβ (407 amino acids), which share the first 328 amino acids (2, 3). Previous experiments performed by Parent et al. (4) demonstrated that only TPβ, but not TPα, undergoes agonist-induced and tonic (constitutive) internalization, which are dictated by distinct motifs in the C terminus of the TPβ receptor. Our efforts in the laboratory have been focused on understanding the molecular mechanisms involved in the regulation of TPβ (5, 6). The majority of GPCRs undergo internalization following agonist stimulation. This internalization can participate in receptor desensitization, resensitization, degradation, or activation of signaling cascades such as mitogen-activated protein kinase pathways (7–9). It has long been questioned if GPCRs signaling and endocytosis constitute separate events, and if they are regulated by distinct molecular mechanisms (9). In this regard, we have recently shown that Gαq signaling is directly involved in agonist-induced internalization of the TPβ receptor, suggesting that, in the case of some GPCRs, signaling and endocytosis are tightly connected (6). However, the molecular mechanism by which Gαq protein regulates TPβ agonist-induced internalization is still unclear and is one of the major interests of our laboratory. Growing evidence has shown that endocytosis of GPCRs is governed by signaling molecules (6, 9). Indeed, some studies have demonstrated that members of the Rho family such as RhoA, Rac, and Cdc42 as well as a member of the ARF family, ARF6, play a crucial role in GPCR endocytosis (10–12). Nevertheless, the regulation of these small G proteins and how they are involved in this process is not well understood. It has been suggested that the regulation of Rho/ARF-mediated actin cytoskeleton rearrangement is either directly or indirectly involved in the regulation of GPCR endocytosis (13–20). In addition, several other signaling proteins participating in endocytosis have been identified. Recently, it has been shown that Nm23-H1 regulates dynamin-dependent endocytosis (12, 21, 22). Nm23 (also known as NDPK, NDK, and Awd) was first identified as a nucleoside diphosphate kinase that is involved in the regulation of GPCR endocytosis (13–20). In addition, several other signaling proteins involved in endocytosis have been identified. Recently, it has been shown that Nm23-H1 regulates dynamin-dependent endocytosis (12, 21, 22). Nm23 (also known as NDPK, NDK, and Awd) was first identified as a putative tumor metastasis suppressor and as an essential element of fly development (23). In human, Nm23 represents a family of nucleoside diphosphate kinases (NDPKs) encoded by eight different genes. According to their genomic architecture and phosphotransferase activity, human Nm23 genes were classified into two groups. Group I is represented by the Nm23-H1 to Nm23-H4 isoforms, whereas group II contains the Nm23-H5 to Nm23-H8 isoforms (23). Group I Nm23 proteins possess a highly conserved kinase active site. On the other hand there is no strict conservation of the kinase site motifs for group II, and only Nm23-H6 has been reported to display kinase activity (23). Nm23 proteins are multifunctional and were shown to be involved in a fascinating variety of cellular...
activities, including proliferation, development, and differentiation (24). Several studies demonstrated that regulation of these processes by Nm23 proteins is the result of their ability to modulate diverse transmembrane signaling pathways, including transforming growth factor-β1, nerve growth factor, platelet-derived growth factor, and insulin-like growth factor-1 as reviewed by Otero et al. (25). NDPβ2 (Nm23-H1) was recently shown to promote activation of the Go1, subunit of heterotrimeric G proteins (25). Nm23 was also suggested to function as a nucleoside diphosphate kinase involved in the activation of dynamin by promoting its GTP loading (22). Inactivation of the Nm23-H1 (also known as avd) gene in Drosophila led to the inhibition of endocytosis occurring at nerve terminals (22). Moreover, Palacios et al. (12) reported that ARF6-gTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis of E-cadherin and transferrin receptors during disassembly of adherens junctions. They also proposed that Nm23-H1 promotes adherens junctions disassembly by inactivating the Rac1 signaling pathway. Furthermore, these studies demonstrated that Nm23-H1 inactivates Rac1 by binding Tiam1, a guanine nucleotide exchange factor (GEF) for Rac1 (12, 27). In addition, the same authors showed that expression of Nm23-H1-ΔKpn, a mutant that lacks the killer of prone homology domain involved in the oligomerization and the function of the wild-type Nm23, could reverse ARF6-mediated inactivation of Rac1 signaling, because it could not bind Tiam1 (12). To our knowledge, Nm23 proteins were never demonstrated to interact with a transmembrane receptor and more particularly, their role in GPCR endocytosis remains to be addressed.

In our attempt to identify putative factors involved in TPβ agonist-mediated endocytosis, we performed yeast two-hybrid screening using a HeLa cell cDNA library. Interestingly, we identified Nm23-H2 as a protein capable of interacting with the C-terminal tail of TPβ. Here we report a novel interaction between Nm23-H2 and TPβ that regulates TPβ agonist-induced internalization through a Rac1 mechanism.

EXPERIMENTAL PROCEDURES

Plasmids Construction—The TPβ C-terminal (TPβCT) coding sequences (amino acids 328–407) were isolated by PCR using the Expand High Fidelity System (Roche Applied Science) with the following oligonucleotides TPβCTP (5′-CGAAGGATCCTCATACTTCTTGGCAGAC-GCTGCAC-3′) and TPβCTR (5′-CGAAGGATCCTCACTGCACCTCAGTGCGCCAGC-3′). The PCR fragment was digested with EcoRI and XhoI and ligated into the bait vector pSA2-1 (Clontech) digested with the same enzymes and subsequently used to perform yeast two-hybrid screening as described below. pcDNA3-Myc-Nm23-H2R, pcDNA3-Myc-Nm23-H2H118C, and pcDNA3-Nm23-H2ΔKpn were prepared by PCR using the pGad424-Nm23-H2 plasmid (isolated by yeast two-hybrid screening) and the following primers, respectively, NM23F (5′-GGAGAATTACCTGAGCAACTTGAGCGACCCAGC3′), NM23R (5′-GGCCTGAGTCAGCTCACGTCTCAGATGGATGTCTTTCTTCAT-GAGCCACGTACATG-3′), NM23H118CF (5′-CGGAGAATTACCTGAGCAACTTGAGCGACCCAGC3′), and NM23H118CR (5′-CTGGAATCTCGACCAACCAGATGGATGTCTTTCTTCAT-GAGCCACGTACATG-3′). pcDNA3-Myc-Nm23-H2ΔKpn was an Nm23-H2 plasmid encoding for TPβC in the presence of 100 nM U46619 prior to harvesting. Thereafter, the cells were rinsed with ice-cold PBS and harvested in 800 μl of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM NaPO4, 5 mM EDTA) supplemented with protease inhibitors (9 μM pepstatin, 9 μM antipain, 10 μM leupeptin, and 10 μM aprotinin) (Sigma). Following 60-min incubation of the cells in lysis buffer at 4 °C with rotation, the lysates were clarified by centrifugation for 20 min at 14,000 rpm at 4 °C. One to four micrograms of HA-specific monoclonal were added to the supernatant. After 60 min of incubation at 4 °C, 50 μl of 50% protein A-agarose pre-equilibrated in lysis buffer was added, followed by overnight incubation at 4 °C. Samples were then centrifuged for 1 min in a microcentrifuge and washed three times with TBS. Immunoprecipitated proteins were eluted by addition of 50 μl of SDS sample buffer followed by a 30-min incubation at room temperature. Initial lysates and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting performed by specific antibodies.

Recombinant Protein Production and Binding Assay—The cDNA encoding the wild-type (TPβ) and the mutant form (TPβΔC) representing the whole C-terminal domain of TPβ (scambled in the pGEX-4-T1 vector (Amersham Biosciences), and the construct was used to produce a GST-tagged TPβCT fusion protein in Escherichia coli BL21 by following the manufacturer’s instruction. The recombinant TPβCT was purified using glutathione-Sepharose4B (Amersham Biosciences) as indicated and purified fractions were analyzed by SDS-PAGE and was immunoblotted by using a GST-specific polyclonal antibody (Bethyl Laboratories). Glutathione-Sepharose-bound GST-TPβCT was incubated with Myc-NM23-H2 cellular extracts 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 μM pepstatin, 9 μM antipain, 10 μM leupeptin, and 10 μM chymostatin) (Sigma). The binding reactions were then washed three 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.

Results

Nm23-H2 Interacts with TPβ—We have recently shown that the C terminus of TPβ confers specific regulatory mechanisms to this isoform of the thromboxane A2 receptor (4, 26). To identify putative proteins, that could interact with the C terminus of TPβ, we performed yeast two-hybrid screening experiments using the yeast strain pSB9-4a transformed with pAS2.1-TPβCT and a human HeLa cell cDNA library. A total of 1.5 × 106 independent clones were screened yielding over 300 positives. One hundred clones demonstrating a strong growth on selective yeast medium (Trp+, Leu+, His+, and Ade+) were then isolated and characterized by DNA sequencing and then aligned using the NCBI blast alignment search tool. Three clones contained a cDNA fragment coding for the Nm23-H2 protein. Other members of the Nm23 protein family were not isolated in the clones that were sequenced. In this study we report the characterization of Nm23-H2 as a putative protein, which strongly interacts with the C-terminal tail of TPβ. As shown in Fig. 1A, strong growth on Trp+, Leu+, His+, and Ade+ medium was present only in yeast transformed with pAS2.1-TPβCT and pGADGH-Nm23-H2, indicating that Nm23-H2 is interacting with the C terminus of TPβ.

Co-immunoprecipitations and in Vitro Binding Assay—To investigate the interaction of Nm23-H2 with TPβ in a cellular context, we performed immunoprecipitation experiments in HEK293 cells transfected with pcDNA3-Myc-Nm23-H2 and pcDNA3-HA-TPβ in the presence or absence of U46619 stimulation. Cell lysates were incubated with a HA-specific monoclonal antibody and protein A-agarose, and immunoprecipitation reactions were analyzed by immunoblotting with a Myc-specific polyclonal antibody. Our results demonstrate that Myc-Nm23-H2 was co-immunoprecipitated with TPβ following agonist treatment (Fig. 1B). This suggests that the interaction of Nm23-H2 with TPβ is modulated by agonist stimulation. Moreover, to further confirm the interaction of Nm23-H2 with the C-terminal tail of TPβ, we performed an in vitro binding assay using the purified recombinant C-terminal tail of TPβ fused to GST (GST-TPβCT) along with HEK293 cell extracts expressing Myc-Nm23-H2. The results obtained showed that Nm23-H2 could bind specifically to glutathione-Sepharose-bound GST-TPβCT and not to glutathione-Sepharose-bound GST (Fig. 1C). Taken together, our data demonstrate that Nm23-H2 interacts directly with the C-terminal tail of TPβ in an agonist-dependent manner. Interestingly, this constitutes the first study whereby a direct interaction of an Nm23 protein with a G protein-coupled receptor, and to our knowledge to any transmembrane receptor, is shown.

Nm23-H2 Inhibits TPβ Agonist-induced Internalization—We were then interested in determining the role of Nm23-H2 in TPβ regulation. Nm23-H1 and Nm23-H2 are 88% identical. Two dominant-negative mutants of Nm23-H1 have been reported. Nm23-H1H118C was characterized as a kinase-defective mutant of Nm23-H1, whereas the Nm23-H1-AKpn mutant was shown to be unable to inactivate Rac1 signaling, because it could not bind and sequester Tiam1 (an Rac1 GEF) (12). The same mutations were thus introduced into Nm23-H2. We first assessed how Nm23-H2 and its two mutants would regulate agonist-induced internalization of TPβ. Fig. 2A shows the results of a TPβ internalization assay following a 2-h incubation with U46619 performed by ELISA (4) in HEK293 cells co-transfected with HA-TPβ and either Myc-Nm23-H2WT, Myc-Nm23-H2H118C, Myc-Nm23-H2ΔKpn, or pcDNA3. Intriguingly, we observed that only Nm23-H2ΔKpn significantly inhibited the agonist-induced internalization of TPβ, whereas expression of Nm23-H2WT or Nm23-H2H118C had no apparent effect on the latter. A time course of receptor internalization...
was performed to further characterize the effect of Nm23-H2-ΔKpn. Data found in Fig. 2B confirmed that only Nm23-H2-ΔKpn significantly inhibited agonist-induced internalization of TPβ with even more drastic effects at earlier time points. These data suggest that the kinase activity of Nm23-H2 is not involved in the process of agonist-induced internalization of TPβ. Failure of Nm23-H2<sup>1118C</sup> to inhibit TPβ internalization signifies that dynamin is not likely involved in modulation of this process by Nm23-H2. On the other hand, the killer of prune homology domain of Nm23-H2, deleted in Nm23-H2-ΔKpn, seems to be important for the progress of the agonist-induced internalization of TPβ, indicating that Nm23-H2 is positively involved in this process. Furthermore, using similar experimental approaches, we determined that Nm23-H2-ΔKpn did

Fig. 1. The TPβ C-terminal tail interacts with Nm23-H2. A, yeast two-hybrid screening was performed using TPβ<sub>CT</sub> and the Human HeLa MATCHMAKER cDNA Library. Nm23-H2 was characterized as a novel molecular partner that interacts with TPβ<sub>CT</sub>. This interaction was confirmed by the use of the selective yeast media (Trp<sup>+</sup>, Leu<sup>+</sup>, His<sup>+</sup>, and Ade<sup>+</sup>). B, co-immunoprecipitation experiments were performed in HEK293 cells transfected with pcDNA3-Myc-Nm23-H2 and pcDNA3-HA-TPβ. The result illustrated here shows that Nm23-H2 co-immunoprecipitated with TPβ in U46619-stimulated HEK293 cells. C, the binding assay was performed using purified recombinant GST-TPβ<sub>CT</sub> protein and Myc-Nm23-H2-expressing HEK293 cell extracts. Nm23-H2 binds to glutathione Sepharose-bound GST-TPβ<sub>CT</sub> but not to glutathione-Sepharose-bound GST. Data presented are representative of three different experiments.
Nm23-H2 Regulates TPβ Agonist-induced Internalization

Fig. 2. Nm23-H2 regulates agonist-induced internalization of TPβ. TPβ agonist-induced internalization was assessed by ELISA in HEK293 cells transiently transfected with pcDNA3-HA-TPβ and either pcDNA3, pcDNA3-Myc-Nm23-H2 WT, pcDNA3-Myc-Nm23-H2H118C, or pcDNA3-Myc-Nm23-H2 ΔKpn and stimulated with 100 nM U46619 for 120 min (A) or for 0–120 min (B). Nm23-H2 ΔKpn caused a significant inhibition in agonist-induced internalization of TPβ.

Not significantly inhibit agonist-induced internalization of the Gaq-coupled DP and IP receptors, as well as of the predominantly Gαq-coupled CRTH2 receptor (Fig. 3). Internalization of the β2-adrenergic receptor was increased by the Nm23-H2ΔKpn mutant. In contrast, Nm23-H2ΔKpn expression resulted in a ~50% inhibition of agonist-induced endocytosis of two other Gαq-coupled receptors, the angiotensin II AT1 and the platelet-activating factor receptors (Fig. 3). This suggests that the role of Nm23-H2 in endocytosis is specific to some GPCRs. These results are in accordance with growing evidence from us and others showing that endocytosis of different GPCRs is regulated by distinct molecular events.

Nm23-H2 Does Not Regulate TPβ-mediated Gαq Signaling—Interestingly, Nm23-H1ΔKpn, lacking the killer of prune homology domain involved in the binding of Tiam1 (a Rac1 GEF), acts as a dominant-negative mutant toward the endogenous Nm23-H1 and as such interferes with the ARF6-mediated decrease of Rac1-GTP (12). Thus, we hypothesized that Nm23-H2 could modulate the agonist-induced internalization of TPβ by mediating the inactivation of Rac1 signaling. However, to investigate this hypothesis, we first had to verify, on one hand, the effect of Rac1 signaling on the agonist-induced internalization of TPβ and, on the other hand, the modulation of Rac1-1 activation following TPβ agonist stimulation. To this end, we studied the effect of expression of Rac1(V12), a constitutively active form of Rac1, on the agonist-induced internalization of TPβ. As seen in Fig. 4, Rac1(V12) significantly inhibited the agonist-induced internalization of TPβ. This suggests that continuous activation of the Rac1 signaling pathway interfered with agonist-induced internalization of TPβ. Rac1(N17), a dominant-negative mutant of Rac1, showed no effect at 120 min of

Nm23-H1 was shown to form a complex with the Gβγ dimers and promote G protein activation by increasing the high energetic phosphate transfer to GDP (30). Kikkawa et al. (31) published that Nm23-H1 also enhances Gαq signaling. It is the histidine kinase activity of Nm23-H1 that is involved in this phosphate transfer (31). Thus, we were next interested in verifying if Nm23-H2 and its two mutants would alter TPβ signaling. Our data indicated that none of the Nm23-H2WT, Nm23-H2ΔKpn, or Nm23-H2H118C constructs affected TPβ-mediated Gαq or Gαs signaling as determined by measuring total inositol phosphates and cAMP generation (data not shown). Similar results were obtained with constitutive forms of Gαq and Gαs (data not shown). We previously demonstrated that Gαs signaling was necessary for TPβ internalization. The fact that Nm23-H2H118C is not affecting Gαq signaling concurs with its lack of effect on TPβ agonist-induced internalization. Similarly, this also suggests that Nm23-H2ΔKpn inhibition of TPβ agonist-induced internalization is accomplished by a mechanism that is not related the Gα protein signaling.

Rac1 Signaling Regulates TPβ Endocytosis—Interestingly, Nm23-H1ΔKpn, lacking the killer of prune homology domain involved in the binding of Tiam1 (a Rac1 GEF), acts as a dominant-negative mutant toward the endogenous Nm23-H1 and as such interferes with the ARF6-mediated decrease of Rac1-GTP (12). Thus, we hypothesized that Nm23-H2 could modulate the agonist-induced internalization of TPβ by mediating the inactivation of Rac1 signaling. However, to investigate this hypothesis, we first had to verify, on one hand, the effect of Rac1 signaling on the agonist-induced internalization of TPβ and, on the other hand, the modulation of Rac1-1 activation following TPβ agonist stimulation. To this end, we studied the effect of expression of Rac1(V12), a constitutively active form of Rac1, on the agonist-induced internalization of TPβ. As seen in Fig. 4, Rac1(V12) significantly inhibited the agonist-induced internalization of TPβ. Thus, it appears that continuous activation of the Rac1 signaling pathway interfered with agonist-induced internalization of TPβ. Rac1(N17), a dominant-negative mutant of Rac1, showed no effect at 120 min of
Nm23-H2 Regulates TPβ Agonist-induced Internalization

TPβ agonist-induced internalization. This will be further addressed below. The activation state of Rac1 was determined by PBD pull-down assays on cell lysates of TPβ-expressing cells following a time-course stimulation of the receptor. The N-terminal regulatory region of (p21)-activated kinase PAK1 referred to as the p21-binding domain (PBD) binds specifically to the Rac1-GTP-activated form. Samples from the GST-PBD pull-down assays (32) were then analyzed by immunoblotting with a Rac1-specific monoclonal antibody. Fig. 5A shows that TPβ stimulation produced a rapid activation of Rac1 at 15 min, which peaked at 30–45 min, followed by a decrease in activated Rac1 at 60 min and complete Rac1 inactivation at 120 min. Noteworthy, the progressive inactivation of Rac1 after 60 min of stimulation coincides with the time period where the maximal agonist-induced internalization of TPβ is observed (Fig. 2B and Ref. 4). Interestingly, expression of Nm23-H2-HΔKpn resulted in a sustained activation of Rac1 over the time interval that we studied (Fig. 5B). This result suggests that Nm23-H2-HΔKpn interferes with the inactivation of Rac1 mediated by an endogenous Nm23 protein. We then speculated that the lack of effect of the dominant-negative Rac1(N17) mutant in Fig. 4 was due to the time point at which the experiment was performed. Because there is no activated Rac1 at 120 min (Fig. 5A), then Rac1(N17) could not have any effect. A time course of TPβ internalization in presence of Rac1(N17) over periods of time where we observed Rac1 activation was then carried out. As can be seen in Fig. 6, Rac1(N17) increased TPβ internalization over the time frame of Rac1 activation by TPβ, indicating that blocking Rac1 signaling promotes internalization of the receptor.

Taken together, our findings show that activation of Rac1 signaling interferes with TPβ agonist-induced internalization. Maximal receptor internalization occurs when Rac1 is inactivated. Our data also indicate that Nm23-H2-HΔKpn inhibited TPβ agonist-induced internalization through maintenance of activated Rac1 protein.

Rac1 Signaling Antagonizes the Goq-induced Internalization of TPβ Receptor—We recently reported that Goq signaling is necessary in the agonist-induced internalization of TPβ. In this previous study, we have demonstrated that Goq-R183C (a constitutive form of Goq) was sufficient to induce the internalization of TPβ in absence of agonist stimulation (6). Here, we were thus interested in investigating the effect of the activated Rac1(V12) mutant on Goq-R183C-induced internalization of TPβ. In this experiment, Goq-R183C-induced internalization is assessed by measuring cell surface receptor expression by ELISA as we described previously (6). The results obtained show that Goq-R183C induced a drastic endocytosis of TPβ receptor as its cell surface expression decreased significantly, as expected (6). However, Rac1(V12) expression interfered with Goq-R183C-induced internalization of TPβ (Fig. 7). This suggests that Rac1 signaling antagonizes the function of Goq signaling in the process of TPβ endocytosis.

TPβ-mediated Recruitment of Nm23-H2 to the Plasma Membrane—To assess the intracellular localization of Nm23-H2 and its regulation by TPβ stimulation, we performed immunofluorescence microscopy using HEK293 cells transfected with pcDNA3-HA-TPβ and pcDNA3-Myc-Nm23-H2. Using Myc-specific polyclonal and HA-specific monoclonal antibodies, double labeling of transfected cells was then performed. In the absence of TPβ stimulation, Nm23-H2 has a predominantly cytoplasmic and nuclear distribution while TPβ has both a cytoplasmic and membrane repartition (Fig. 8, upper panel). However, following U46619 stimulation, Nm23-H2 undergoes translocation to the plasma membrane resulting in its significant co-localization with TPβ (Fig. 8, bottom panel). Co-localization of both proteins is also detected in intracellular compartments.

DISCUSSION

In the past few years, the relation between GPCRs signaling and their endocytosis has been the subject of debate. We have recently shown that the first step of regulation of TPβ agonist-mediated endocytosis involves the activation of Goq signaling (6). However, the exact molecular mechanisms and partners involved in this regulation are still unknown. To identify such molecular partners we performed yeast two-hybrid screening using the C-terminal tail of TPβ and a human HeLa cell cDNA library. Here we report that Nm23-H2 interacts with the C-terminal tail of TPβ. Nm23, also known as NDP kinase (NDPK), is a 17-kDa histidine kinase involved in the regulation of a wide variety of cellular functions. Nm23 was shown to regulate cell growth, differentiation, tumor metastasis, kinase signal transduction, GTPase activation, and very recently endocytosis events (12, 22, 24). Nm23-H2 is one of the eight members of the Nm23 family. Co-immunoprecipitation results demonstrated that Nm23-H2 interacts with TPβ in a cellular context. Interestingly, this interaction seemed to be triggered by the activation of TPβ, suggesting that Nm23-H2 could be involved in the regulation of the molecular events subsequent to TPβ agonist stimulation. Thus we first investigated the effect of Nm23-H2 on TPβ agonist-induced internalization. Co-expression of TPβ and Nm23-H2WT in HEK293 cells did not change the agonist-induced internalization of TPβ. Two dominant-negative mutants of Nm23-H1 are known in the literature (12). Nm23-H1H118C is a kinase-deficient mutant shown to interfere with dynamin-mediated endocytosis (12). On the other hand, Nm23-H1-ΔKpn, which lacks the killer of prune homology domain, is unable to bind Tiam1 (an Rac1 GEF) (12). The corresponding mutations were introduced into Nm23-H2, because Nm23-H1 and Nm23-H2 are highly identical (100% identity in the regions of the mutations). The ability of the two mutants to modulate TPβ agonist-induced internalization was then evaluated. Nm23-H2H118C had no effect on the agonist-promoted internalization of TPβ suggesting that the kinase activity of Nm23-H2 is not involved in this process. We previously reported that TPβ internalization is dynamin-dependent (4). In this case however, the Nm23-H2H118C data indicate that Nm23-H2 would regulate TPβ internalization through a mechanism not involving dynamin. Surprisingly, Nm23-H2-ΔKpn expression significantly inhibited the agonist-mediated inter-
nalization of TPβ identifying the killer of prune homology domain of Nm23-H2 as a regulator of this process. There is specificity of Nm23-H2-ΔKpn toward TPβ, because this mutant failed to prevent internalization of the G\textsubscript{q}-coupled β\textsubscript{2}-adrenergic, DP, and IP receptors. Nm23-H2-ΔKpn inhibited by 50% the endocytosis of the G\textsubscript{q}-coupled angiotensin II AT\textsubscript{1} and platelet-activating factor receptors. On the other hand, internalization of the CRTH2 receptor was not significantly affected by Nm23-H2-ΔKpn expression. The CRTH2 receptor is predominantly a G\textsubscript{i}-coupled receptor but was also reported to be coupled to G\textsubscript{q} (33). Thus our data suggest that regulation of GPCR endocytosis by Nm23-H2 is a pathway specific to at least some, but not all, G\textsubscript{q}-coupled receptors. Differences in the complement of proteins expressed or localized at particular subcellular sites (compartmentalization) between cell types could be a determinant of this pathway for a given receptor. More studies will be necessary to fully understand the specificity of Nm23-H2 toward various classes of GPCRs. Our preliminary data indicate that agonist-induced endocytosis of TPβ is not modulated by expression of Nm23-H1, Nm23-H1\textsubscript{H118C}, or Nm23-H2-ΔKpn constructs in our system (data not shown), further indicating specificity in the TPβ/Nm23-H2 mechanism.

Go\textsubscript{q} signaling by TPβ is necessary for the activation of the machinery responsible for internalization of this receptor, and interfering with this signaling pathway results in a drastic decrease in receptor internalization (6). We observed that Nm23-H2-ΔKpn did not affect Go\textsubscript{q} signaling, as determined by inositol phosphate measurement. This indicated that Nm23-H2-ΔKpn was not exerting its effect at the level of Go\textsubscript{q} activity. Interestingly, Palacios et al. (12) recently published that ARF6, which is involved in membrane trafficking, facilitated the process of membrane endocytosis by recruiting Nm23-H1. The same study showed that the killer of prune homology domain of Nm23-H1 mediated the inactivation of Rac1 signaling by binding Tiam1 and interfering with its GEF activity on Rac1. Because our results demonstrated that Nm23-H2-ΔKpn inhibited TPβ agonist-induced internalization, we hypothesized that
Nm23-H2 could play a positive role in TPβ internalization by promoting the inactivation of Rac1 signaling. This hypothesis implied first that Rac1 signaling could interfere with TPβ agonist-induced internalization and second that Rac1 signaling has to be inactivated for the internalization process to occur. Thus, on the first hand, we investigated the effect of Rac1 signaling on TPβ internalization with the use of Rac1(V12), a constitutively active mutant of Rac1. This revealed that Rac1(V12) expression led to a significant inhibition of TPβ agonist-induced internalization. This result suggested that the Rac1 signaling pathway negatively affected the agonist-induced internalization of TPβ. Rac1 activation was then assessed using a well characterized Rac1-GTP p21-binding domain assay (PBD assay) (32) to monitor the Rac1 activity during the process of agonist-induced internalization of TPβ. We have seen that TPβ agonist stimulation promoted a transient increase in the cellular amount of active Rac1-GTP, which peaked at 30–45 min and steadily declined afterward. Interestingly, we demonstrated that, in the presence of Nm23-H2-ΔKpn, a sustained activation of Rac1 was observed during TPβ stimulation. This suggests that Nm23-H2 is involved in TPβ-induced modulation of Rac1 signaling. These findings are in accordance with the hypothesis postulated above. It is interesting to note that constitutively active Rac1(V12) blocked TPβ agonist-induced internalization and that maximal TPβ internalization coincided with the moment when Rac1 was inactivated. This indicates that Rac1 activation by TPβ would slow down its internalization, which would pick up once Rac1 gets inactivated. This statement is supported by the fact that Rac1(N17), a dominant-negative mutant of Rac1, promoted a significant increase in the rate of agonist-induced internalization of TPβ at 30, 45, and 60 min, which coincides with the activation of Rac1 signaling. Rac1(N17) had no effect on TPβ agonist-induced internalization at 120-min U46619 stimulation, a result that can be explained by the absence of Rac1-GTP at this time point. Furthermore, sustained activation of Rac1 signaling mediated by either Nm23-H2-ΔKpn or Rac1(V12) expression led to a significant decrease in the agonist-induced internalization of TPβ. It has been proposed that Rac1 could regulate receptor endocytosis by modulating phosphatidylinositol 4,5-bisphosphate turnover at the plasma membrane (10). In addition, Lamaze et al. (34) demonstrated that the active mutant of Rac1 blocked the internalization of the transferrin receptor. However, the molecular mechanism involved in this regulation is still unclear. Moreover, an increase in the localization of Nm23-H2 to the plasma membrane was shown following TPβ receptor agonist stimulation by immunofluorescence microscopy suggesting that TPβ recruited Nm23-H2 to the plasma membrane. This recruitment of Nm23-H2 is probably necessary for Tiam1 binding and inactivation of Rac1 signaling, both latter processes being present at the plasma membrane (12, 23, 24).

Growing evidence demonstrates the crucial role played by GPCRs signaling in their endocytosis process (6, 9). We recently reported that Goα signaling is strongly involved in the regulation of agonist-induced internalization of Goα-coupled GPCRs (6). Here we have shown that, contrary to Goα signaling, Rac1 activation interferes with the agonist internalization.

**Fig. 7.** Rac1 signaling interferes with Goα-induced internalization of TPβ. Goα-induced internalization of TPβ was assessed by measuring the cell surface expression of the receptor by ELISA. Cell surface expression of TPβ was compared between HA-TPβ-expressing HEK293 cells transfected with either pcDNA3, pcDNA3, and pcDNA3-Goα-R183C or pcDNA3-Goα-R183C and pHOOK-Rac1(V12).

**Fig. 8.** Immunofluorescence microscopy of TPβ co-localization with Nm23-H2 in HEK293 cells. Immunofluorescence experiments were performed to determine the intracellular localization of Nm23-H2 and TPβ using HEK293 cells transiently transfected with pcDNA3-HA-TPβ and pcDNA3-Myc-Nm23-H2 constructs. The transfected cells were incubated with 500 nM U46619 or not for 90 min. HA-specific monoclonal as well as Myc-specific polyclonal antibodies were used to label HA-TPβ and Myc-Nm23-H2, respectively. U46619 stimulation promotes extensive colocalization of TPβ with Nm23-H2 at both the cell membrane and in intracellular compartments.
of TPβ. Gαq and Rac1 signaling appears to play an antagonist role in the internalization process of TPβ. Indeed, Rac1(V12) inhibited Gαq-R183C-induced internalization of the receptor. Rac1 signaling has been shown to regulate the actin cytoskeleton and membrane organization. It is therefore possible that agonist-induced internalization of TPβ and perhaps other GPCRs involves a tight coordination of different signaling pathways.

Briefly, we reported here the first demonstration of an interaction of a member of the Nm23 proteins with a membrane receptor, in this particular case, a GPCR. This interaction regulates internalization of the receptor through a Rac1-dependent mechanism. Specificity was seen in the GPCRs affected, indicating that our findings constitute a novel, distinct molecular regulatory mechanism of GPCR internalization.

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