Receptor activity-modifying proteins (RAMPs 1–3) are single transmembrane accessory proteins critical to various G-protein coupled receptors for plasma membrane expression and receptor phenotype. A functional receptor for the vasodilatory ligand, adrenomedullin (AM), is comprised of RAMP2 or RAMP3 and calcitonin receptor-like receptor (CRLR). It is now known that RAMP3 protein-protein interactions regulate the recycling of the AM2 receptor. The major aim of this study was to identify other interaction partners of RAMP3 and determine their role in CRLR-RAMP3 trafficking. Trafficking of G-protein-coupled receptors has been shown to be regulated by the Na+/H+ exchanger regulatory factor-1 (NHERF-1), an adaptor protein containing two tandem PSD-95/Discs-large/ZO-1 homology (PDZ) domains. In HEK 293T cells expressing the AM2 receptor, the complex undergoes agonist-induced desensitization and internalization. However, in the presence of NHERF-1, although the AM receptor (CRLR/RAMP3) undergoes desensitization, the internalization of the receptor complex is blocked. Overlay assays and mutational analysis indicated that RAMP3 and NHERF-1 interact via a PDZ type I domain on NHERF-1. The internalization of the CRLR-RAMP complex was not affected by NHERF-1 when CRLR was co-expressed with RAMP1 or RAMP2. Mutation of the ezrin/radixin/moesin (ERM) domain on NHERF-1 indicated that NHERF-1 inhibits CRLR/RAMP3 complex internalization by tethering the complex to the actin cytoskeleton. When examined in a primary culture of human proximal tubule cells endogenously expressing the CRLR-RAMP3 complex and NHERF-1, the CRLR-RAMP complex desensitizes but is unable to internalize upon agonist stimulation. Knock-down of either RAMP3 or NHERF-1 by RNA interference technology enabled agonist-induced internalization of the CRLR-RAMP complex. These results, using both endogenous and overexpressed cellular models, indicate a novel function for NHERF-1 and RAMP3 in the internalization of the AM receptor and suggest additional regulatory mechanisms for receptor trafficking.

The recent discovery of receptor activity-modifying proteins (RAMPs) has broadened the field of G-protein-coupled receptor (GPCR) regulation. RAMPs were discovered as required accessory proteins to an orphan GPCR, now termed the calcitonin receptor-like receptor (CRLR) (1). The three RAMP isoforms (1–3) are products of three distinct genes and yield unique single transmembrane accessory proteins. RAMPs are required for the plasma membrane expression and determination of receptor phenotype for CRLR (1, 2). RAMPs have recently been found to associate with additional members of the Class II family of GPCRs (3). Co-expression of RAMP1 with CRLR yields a functional calcitonin gene-related peptide (CGRP) receptor, whereas co-expression of RAMP2 or RAMP3 with CRLR produces a receptor responsive to adrenomedullin (AM) (AM1-R and AM2-R, respectively) (4, 5). AM and CGRP are multifunctional peptides with many overlapping functions, ranging from potent vasodilation to proliferation to regulation of salt and water balance (6). The RAMP isoforms have shown differential expression patterns in different organ systems and in different pathophysiological states, suggesting a regulatory role for RAMPs in both physiological and pathophysiological situations. Furthermore, the identification of RAMP interactions with additional members of the Class II GPCR family and RAMP expression in cell lines lacking CRLR have raised the possibility of novel functions for RAMPs in GPCR regulation.

It has been shown in other GPCR systems that interactions with PDZ-95/Discs-large/ZO-1 homology (PDZ) domain proteins are responsible for altering the receptor trafficking after agonist stimulation (7–9). In particular, a protein called Na+/H+ exchanger regulatory factor-1 (NHERF-1) has been shown to regulate the trafficking of the β2-adrenergic receptor, β-opioid receptor, and parathyroid hormone receptor (PTHR) after agonist activation (2, 10, 11). NHERF-1 is an adapter protein that is thought to tether membrane receptors to cytoskeletal proteins through PDZ interactions and interactions with merlin and the ezrin/radixin/moesin (ERM) family of cytoskeletal proteins (12).

Comparable with the C terminus of the β2-adrenergic receptor, PTHR, cystic fibrosis transmembrane conductance regulator, and platelet-derived growth factor receptor, human RAMP3 C terminus contains a type-I PDZ recognition

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Jennifer M. Bomberger‡, William S. Spielman §§, Carolyn S. Hall †, Edward J. Weinmann †, and Narayan Parameswaran ‡

From the ‡Department of Physiology, Michigan State University, East Lansing, Michigan 48824 and the †Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201

Receptor activity-modifying protein (RAMP) isoform-specific regulation of adrenomedullin receptor trafficking by NHERF-1

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motif, whereas CRLR, RAMP1, and RAMP2 do not contain any PDZ recognition sequences (8, 10, 13–16). We hypothesized that RAMP3, via its interaction with NHERF-1, can regulate the trafficking of the CRLR-RAMP3 complex. We show here that although CRLR-RAMP1 and CRLR-RAMP2 complexes do not interact with NHERF-1, CRLR-RAMP3 complex interacts with NHERF-1 via the PDZ domain of NHERF-1. Moreover, we show here that overexpression of NHERF-1 in HEK 293T cells alters the trafficking pattern of the receptor complex to block the internalization of the receptor by tethering the receptor complex to the actin cytoskeleton via interactions between RAMP3 and NHERF-1 through a type I PDZ domain. Furthermore, we also demonstrate that in primary human proximal tubule cells (which express endogenous NHERF-1 and CRLR-RAMP3), the CRLR-RAMP3 complex does not internalize upon agonist stimulation. Knocking down NHERF-1 or RAMP3 expression with RNA interference causes the receptor to undergo internalization upon agonist treatment, suggesting critical roles for both NHERF-1 and RAMP3 in receptor internalization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Adrenomedullin was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). 125I-labeled adrenomedullin was purchased from Amersham Biosciences. Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin/streptomycin, and trypsin-EDTA were purchased from Invitrogen. RAMP3 antibody was purchased from Santa Cruz Biotechnology, Inc. (Cruz, CA), and NHERF-1 (1:1000) antibody was from Affinity Bioreagents (Golden, CO). Alexa Fluor 488-phalloidin was purchased from Molecular Probes (Eugene, OR). Anti-mouse Cy3 and anti-rabbit Cy5 secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). All other reagents were of the highest quality available.

**Cell Culture and Transfection Protocols**—HEK 293T cells were obtained from ATCC and are maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin. Transfection of HEK 293T cells was performed using Lipofectamine Plus protocol (Invitrogen). Cells were transfected with the DNA and Lipofectamine Plus as per manufacturer's protocol. Cells were collected for assays after 48 h of transfection. Human proximal tubule epithelial (hPTE) cells were acquired from Mediatech and maintained in Dulbecco's modified Eagle's medium low glucose medium containing 10% fetal bovine serum, 1% penicillin-streptomycin. hPTE cells were treated for a desensitization assay and then incubated with serum-free medium overnight before agonist exposure. hPTE cells were transfected using Lipofectamine Plus protocol (Invitrogen). Cells were transfected with hPTE cells were treated for a desensitization assay and then rechallenged with AM (100 nM) for 10 min at 37 °C. Determination of cAMP level was measured using the Biotrap cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in human proximal tubule cells were calculated using a standard curve ranging from 10 to 100 fmol of cAMP. Each experiment was done in triplicates and repeated at least three times. Data is expressed as the percentage of maximal response, percentage of forskolin.

**cAMP Accumulation Assays**—Human proximal tubule cells were seeded on a 24-well plate until reaching 80–90% confluency and then incubated in serum-free medium overnight before agonist exposure. hPTE cells were transfected with hPTE cells were treated for a desensitization assay and then rechallenged with AM (100 nM) for 10 min at 37 °C. Determination of cAMP level was measured using the Biotrap cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP levels in human proximal tubule cells were calculated using a standard curve ranging from 10 to 100 fmol of cAMP. Each experiment was done in triplicates and repeated at least three times. Data is expressed as the percentage of maximal response, percentage of forskolin.

**RNA Interference Analysis**—Gene-specific dsRNA for lacZ (control), NHERF-1, and RAMP3 were generated and purified using the BLOCK-iT Dicer RNA interference kit from Invitrogen. hPTE cells were transfected with dsRNAs using Lipofectamine 2000 as per the manufacturer's instructions (Invitrogen). 48 h after transfection, cells were frozen for mRNA analysis or used for cAMP accumulation assays or for immunofluorescence microscopy.

**Reverse Transcriptase PCR (RT-PCR) Analysis**—RT-PCR analysis was performed as described before (18). Total RNA was isolated from hPTEs using TRIzol reagent (Invitrogen). After sodium acetate-ethanol precipitation and several ethanol washes, RNA was used as a template in a reverse transcriptase PCR amplification procedure. The RT-PCR reaction was carried out using Superscript One-Step RT-PCR with Platinum Taq (Invitrogen), in accordance with the manufacturer's specifications. Reactions were carried out with a PerkinElmer Life Sciences model 9600 thermal cycler in 50 μL of total reaction volumes subjected to the following conditions: 1) 94 °C for 2 min (1 cycle); 2) 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s (30 cycles); and 3) 72 °C for 7 min (1 cycle). Products were separated by gel electrophoresis and subsequently visualized by ethidium bromide staining and ultraviolet illumination. Photographs of the gels were taken and digitalized with a UMAX Astra 2000P flat-bed scanner.

**Mutagenesis Procedure**—Site-directed mutagenesis was performed using a PCR-based strategy that employs the Pfu Turbo polymerase (Stratagene, La Jolla, CA). A pair of complementary oligonucleotides containing the appropriate point mutations in the sequence of RAMP/CRLR or a premature stop codon at position 145 or 147 codon of RAMP3 for deletion mutants were synthesized (Michigan State University Macromolecular Structure Facility). The PCR for the mutation was as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; 68 °C for 5 min; and 3) 68 °C for 7 min (1 cycle). Products were separated by gel electrophoresis and subsequently visualized by ethidium bromide staining and ultraviolet illumination. Mutations were confirmed by automated sequencing (Michigan State University Genomic Technology Support Facility).

**Immunofluorescence Microscopy**—HEK 293T and hPTE cells were transfected as described above and seeded at 24 h after transfection onto collagen type I-coated coverslips. Desensitization assays were performed as described above. Cells were then fixed with 4% paraformaldehyde for 30 min at room temperature. Samples were permeabilized with 0.1% v/v Triton X-100 in phosphate-buffered saline and blocked overnight in 0.1% v/v Triton X-100 in phosphate-buffered saline + 10% goat serum. Samples were incubated in primary antibody in blocking buffer for 2 h at room temperature (NHERF-1 at 1:250 and RAMP3 at 1:200). Appropriate secondary antibodies were applied for 1 h at room temperature (goat anti-mouse Cy3 at 1:500 and goat anti-rabbit Cy5 at 1:500).
anti-rabbit Cy5 at 1:400). Cytoskeletal staining was carried out using Alexa Fluor 488-phalloidin antibody at 1:75 (Molecular Probes). Cover-slips were mounted in Shandon mounting medium, and slides were stored at 2–8 °C until analysis. Cells were visualized on a Zeiss 210 laser confocal microscope at a zoom of 2. Images presented are representative single optical sections of a z-series taken from at least 20 fields/experiment and at least three individual experiments.

**Fusion Protein Overlays and Western Blotting**—Overlay assays and Western blotting were performed as described before (15). 10 μg of GST-fusion proteins were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose filters. Filters were blocked with 5% w/v fat-free milk powder in Tris-buffered saline with Tween 20 (TTBS: 20 mM Tris, pH 7.4, 500 mM NaCl, 0.1% v/v Tween 20) and incubated overnight at 4 °C in lysates of HEK 293T cells with or without overexpression of NHERF-1. Blots were then washed three times with TTBS buffer and incubated with anti-EBP50 (NHERF-1) polyclonal antibody for 2 h at room temperature. After three washes with TTBS, filters were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Invitrogen), washed again with TTBS, soaked in Supersignal West Pico chemiluminescent substrate (Pierce), and exposed to x-ray film. The same protocol, with the exception of the overnight incubation with cell lysate, was followed for immunoblot analysis of RAMP3.

**Statistics**—Data are presented as mean ± S.E. Single group comparisons exercised a paired Student's t test method. Statistical significance was set at p < 0.05.

**RESULTS**

*Role of NHERF-1 in Internalization of the CRLR-RAMP Complex*—We and others have previously shown that the AM2 receptor (CRLR + RAMP3 complex) undergoes agonist-stimulated desensitization, internalization, and degradation (2, 15, 20). Furthermore, we have recently shown that in the presence of N-ethylmaleimide-sensitive factor (NSF), the AM2 receptor complex undergoes recycling, instead of following a degradation pathway, after agonist-stimulated internalization in HEK 293T cells (15). In this study, we have examined the role of another protein, namely NHERF-1, on agonist-induced trafficking of the CRLR/RAMP3 complex.

Pretreatment of HEK 293T cells expressing CRLR and RAMP3 with 10 nM AM for 1 h resulted in desensitization of the adenylate cyclase response from 50% (of forskolin stimulation) in untreated cells to 28% in AM-treated cells (Fig. 1A). NHERF-1 overexpression with AM2R had no significant effect on receptor desensitization. Experiments were performed in triplicates, and data are expressed as the percentage of maximal stimulation (% Forskolin). *, p < 0.05; n ≥ 4. F, NHERF-1 inhibits the internalization of AM2R in HEK 293T cells. HEK 293T cells were transfected and pretreated with agonist as described in A. Receptor internalization was measured by whole cell competition binding assays using 125I-rAM as ligand (cold rAM served as the competitor), and the number of binding sites/cell was estimated using the GRAPHPAD PRISM software.

No pretreatment represents samples at maximal radioligand binding that were not preincubated with agonist. AM pretreatment represents samples pretreated with AM (10 nM) for 1 h, washed as indicated under “Experimental Procedures,” and tested immediately after the wash steps for radioligand binding. NHERF-1 overexpression in cells expressing AM2R caused altered receptor trafficking to inhibit the internalization of the receptor complex. *, p < 0.05; n ≥ 4 experiments.

In Fig. 1C, NHERF-1 did not alter desensitization of AM2R in transfected HEK 293T cells. HEK 293T cells transiently transfected with CRLR and RAMP3 with or without NHERF-1. At 48 h after transfection, cells were treated for 1 h with AM (10 nM) and then washed, and adenylate cyclase activity was measured. After agonist pretreatments, membranes were extracted, and AC activity in response to 100 nM AM was measured. NHERF-1 overexpression with AM2R had no significant effect on receptor desensitization. Experiments were performed in triplicates, and data are expressed as the percentage of maximal stimulation (% Forskolin). *, p < 0.05; n ≥ 4. If, NHERF-1 inhibits the internalization of AM2R in HEK 293T cells. HEK 293T cells were transfected and pretreated with agonist as described in A. Receptor internalization was measured by whole cell competition binding assays using 125I-rAM as ligand (cold rAM served as the competitor), and the number of binding sites/cell was estimated using the GRAPHPAD PRISM software.

![Graph showing receptor internalization](image_url)
addition, as shown in Figs. 1B and 2, CRLR-RAMP3 complex underwent agonist-induced internalization as determined by receptor binding and immunofluorescence microscopy. These findings are in agreement with those of Kuwasako et al. (2) and those reported by us recently (15). However, overexpression of NHERF-1 with CRLR-RAMP3 resulted in a remarkable change in the agonist-induced receptor complex trafficking in HEK 293T cells. HEK 293T cells transfected with CRLR-RAMP3 and NHERF-1 showed similar levels of adenylyl cyclase activity and desensitization patterns as compared with CRLR-RAMP3 alone (Fig. 1A). However, in the presence of NHERF-1, the receptor complex failed to internalize with agonist pretreatment (Figs. 1B and 2). To determine whether NHERF-1 co-expression had changed the kinetics of internalization, agonist pretreatment was carried out for a time course extending to 4 h. 4 h of agonist pretreatment still yielded a complete inhibition of receptor complex internalization in HEK 293T cells co-expressing NHERF-1 and the CRLR-RAMP3 complex, as compared with a continued internalization of the receptor complex in HEK 293T cells lacking NHERF-1 overexpression (Fig. 1C). Co-expression of NHERF-1 with the CRLR-RAMP3 receptor complex did not significantly change the receptor expression levels. These results indicate that overexpression of NHERF-1 alters the trafficking of the CRLR-RAMP3 receptor complex after AM-stimulated desensitization.

**RAMP Isoform-specific Regulation of CRLR-RAMP Receptor Complex Trafficking**—To determine whether this effect of NHERF-1 is specific for RAMP3, the additional RAMPs (RAMP1 or RAMP2) were tested for their ability to act with NHERF-1 to alter the receptor complex trafficking. Interestingly, in contrast to RAMP3, the presence of NHERF-1 did not alter the internalization pattern of the CRLR-RAMP1 or -RAMP2 receptor complexes. No significant differences were seen in the receptor expression levels from whole cell binding when CRLR was co-expressed with RAMP1, RAMP2, or RAMP3 (with and without NHERF-1). Desensitization patterns in cells transfected with CRLR+RAMP1 or CRLR+RAMP2 also remained unchanged in the absence or presence of NHERF-1 (Fig. 3, A–D). These results indicate that RAMP3 must contain a molecular feature distinct from the other RAMPs that allowed its interaction and action with NHERF-1.

**Role of PDZ Interactions in Trafficking of the CRLR-RAMP3 Complex**—We have observed before that NSF regulated the CRLR-RAMP3 complex recycling by interacting specifically with the PDZ recognition motif present at the extreme C terminus of RAMP3. Neither CRLR nor the other two RAMP isoforms (RAMP1 or RAMP2) contain PDZ recognition motifs. To test the hypothesis that this domain is critical for interaction of the CRLR-RAMP3 complex with NHERF-1, the PDZ motif (-DTLL) on RAMP3 was deleted (RAMP3ΔDTLL), and an internalization assay was performed. Deletion of this domain did not affect basal adenylyl cyclase activity or the desensitization response of the CRLR-RAMP3 complex in response to AM, even in the presence of NHERF-1. In addition, the RAMP3 PDZ motif mutant (RAMP3ΔDTLL) showed no difference in receptor expression levels at the plasma membrane (as measured with whole cell binding), as compared with wild-type CRLR/RAMP3 complex (in the presence/absence of NHERF-1). Unlike CRLR-RAMP3 complex, the CRLR-RAMP3ΔDTLL receptor complex was now capable of AM-induced internalization, as measured by whole cell receptor binding and immunofluorescence microscopy (Figs. 4A and 5).

To further test the hypothesis that the absence of the PDZ motif on the RAMP2 accounts for the lack of ability of NHERF-1 to interact and therefore inhibit internalization, the PDZ motif of RAMP3, the amino acids -DTLL, were substituted on the C terminus of RAMP2, in exchange for its original four C-terminal amino acids -EAQA. The CRLR-RAMP2ΔDTLL mutant receptor complex expression levels and basal adenylyl cyclase activity were comparable with those of the CRLR-RAMP2 complex, as determined by whole cell binding and adenylyl cyclase assays. Additionally, in the absence of NHERF-1, both CRLR-RAMP2 and CRLR-RAMP2ΔDTLL showed similar levels of AM-stimulated internalization. However, co-expression of NHERF-1 with CRLR-RAMP2ΔDTLL was now capable of inhibiting the internalization of the receptor complex with agonist pretreatment, as compared with wild-type CRLR-RAMP2 complex (Fig. 4B). These findings provide...
Fig. 3. The role of NHERF-1 in post-endocytic sorting of CGRP1 (CRLR-R1) and AM1 receptor. A, NHERF-1 overexpression does not alter CGRP receptor desensitization after agonist stimulation. HEK 293T cells were transfected and pretreated as in Fig. 1A, and then membranes were extracted. Adenylate cyclase activity was measured in membranes stimulated with 100 nM CGRP for 15 min. Experiments were performed in triplicates and expressed as the percentage of maximal stimulation (% Forskolin). *, p < 0.05; n = 3. No Pretreat represents samples at maximal receptor stimulation that were not preincubated with agonist. B, NHERF-1 overexpression does not alter the trafficking of the CGRP receptor after agonist stimulation. HEK 293T cells were transfected with CRLR and RAMP1, with or without NHERF-1. 48 h after transfection, cells were pretreated with CGRP (10 nM) for 1 h and washed as described under "Experimental Procedures," and receptor internalization was measured with whole cell competition binding using [125I]-rCGRP as the ligand and cold rCGRP as the competitor. The number of binding sites/cell was estimated with GRAPHPAD PRISM software. *, p < 0.05; n = 3. No Pretreatment represents samples at maximal radioligand binding that were not preincubated with agonist. C, NHERF-1 overexpression does not alter AM1 receptor desensitization after agonist induction. HEK 293T cells were transfected and pretreated as in Fig. 1A, and then membranes were extracted. Adenylate cyclase activity was measured in membranes stimulated with 100 nM AM for 15 min. Experiments were performed in triplicates and expressed as the percentage of maximal stimulation (% Forskolin). *, p < 0.05; n = 3. D, NHERF-1 overexpression does not alter the internalization of AM1 receptor after receptor activation. HEK 293T cells were transfected with CRLR and RAMP2, with or without NHERF-1. 48 h after transfection, cells were pretreated with AM (10 nM) for 1 h and washed as described under "Experimental Procedures," and receptor internalization was measured with whole cell competition binding using [125I]-rAM as the ligand and cold rAM as the competitor. The number of binding sites/cell was estimated with GRAPHPAD PRISM software. *, p < 0.05; n = 3.

additional evidence that the PDZ motif on the RAMP3 interacts with NHERF-1, causing an inhibition of receptor internalization, despite normal desensitization.

To identify the critical amino acids in the PDZ binding sequence that regulate the RAMP3/NHERF-1 interaction, site-directed mutagenesis was performed to mutate the individual amino acids of the PDZ motif to alanine. Mutations of the individual amino acids in the PDZ motif of RAMP3 did not affect the basal levels of receptor expression and function, as measured by whole cell binding experiments and adenylate cyclase assays, respectively. In addition, the desensitization and internalization in the absence of NHERF-1 were also similar between the wild-type and mutant CRLR-RAMP3 complexes. However, in the presence of NHERF-1, the CRLR-RAMP3T146A complex underwent agonist-stimulated internalization, similar to when expressed without NHERF-1 (Fig. 4C). The other point mutant RAMP3/CRLR complexes behaved like wild-type in the presence of NHERF-1, indicating that Thr146 in the PDZ domain is critical for the PDZ interaction between RAMP3 and NHERF-1.

As described before, NHERF-1 contains two PDZ domains through which it interacts with numerous proteins (21). To determine which PDZ domain of NHERF-1 is responsible for the interaction with RAMP3, the two PDZ domains of NHERF-1 were deleted individually, and agonist-induced internalization assays, employing whole cell receptor binding, were performed. Cells expressing wild-type or mutant NHERF-1 with CRLR-RAMP3 receptor complex showed comparable levels of receptor expression and function, as assessed by whole cell receptor binding and adenylate cyclase assays, respectively. In addition, they showed similar desensitization patterns. However, internalization assays (with whole cell binding) showed that the first PDZ domain of NHERF-1 is responsible for the interaction with RAMP3. HEK 293T cells expressing the mutant NHERF-1ΔPDZ1 (lacking only the first PDZ domain) and CRLR-RAMP3 complex underwent AM-stimulated internalization (Fig. 4D). Deletion of the second PDZ domain of NHERF-1 (NHERF-1ΔPDZ2) had no effect on the internalization pattern of CRLR-RAMP3, similar to wild-type NHERF-1 (Fig. 4D). These findings further confirm that RAMP3 and NHERF-1 are interacting via a PDZ type I domain to inhibit the internalization of the CRLR-RAMP3 receptor complex.

To examine whether the PDZ domain on RAMP3 is physically interacting with NHERF-1, overlay assays were performed. This was accomplished using GST-RAMP3 fusion proteins in an overlay assay with cell lysates of HEK 293T cells overexpressing NHERF-1. Control experiments run with GST protein showed no detectable bands when incubated with NHERF-1 lysates and probed with an NHERF-1 antibody (Fig. 6A). Importantly, GST-RAMP3 fusion proteins showed significant interaction with NHERF-1 in the cell lysates of HEK 293T cells overexpressing NHERF-1 in the overlay assay (Fig. 6A). As a control, lysates of HEK 293T
cells not overexpressing NHERF-1 showed no detectable bands when run with GST-RAMP3 in the overlay assay (Fig. 6B). Additionally, when GST-RAMP3Δ145-8 fusion proteins were tested for interaction with NHERF-1 using the above described overlay assay, no bands were detected, in contrast to wild-type GST-RAMP3 (Fig. 6A). When blots used in the overlay assay were stripped and probed for RAMP3, a band for RAMP3 was detected at the exact location as that of the NHERF-1 band detected in the overlay assay (Fig. 6C). These data demonstrate a physical interaction between RAMP3 and NHERF-1, an interaction that is dependent on PDZ domain interactions of the two proteins and is capable of regulating

FIG. 4. The effect of RAMP3 PDZ motif deletion on the trafficking of the AM2R (CRLR-RAMP3). A, deletion of RAMP3 PDZ motif allowed the internalization of the CRLR-RAMP3 complex when co-expressed with NHERF-1. HEK 293T cells were co-transfected with CRLR, wild-type RAMP3 or RAMP3Δ145-8, and NHERF-1. 48 h after transfection, cells were pretreated with AM (10 nM) for 1 h and washed as described under “Experimental Procedures,” and receptor internalization was measured with whole cell binding using 125I-rAM as the ligand and cold rAM as the competitor. The number of binding sites/cell was estimated with GRAPHPAD PRISM software. *, p ≤ 0.05; n = 3. B, the effect of PDZ motif substitution on the C terminus of RAMP2 on the internalization of CRLR-RAMP complex. Substitution of the PDZ motif (-DTLL) on the C terminus of RAMP2 caused a change in receptor trafficking to inhibit the internalization of the receptor complex when co-expressed with NHERF-1. HEK 293T cells were co-transfected with CRLR, wild-type RAMP2 or RAMP2ΔDTLL, and NHERF-1. 48 h after transfection, cells were pretreated with AM (10 nM) for 1 h and washed as described under “Experimental Procedures,” and receptor internalization was measured with whole cell binding using 125I-rAM as the ligand and cold rAM as the competitor. The number of binding sites/cell was estimated with GRAPHPAD PRISM software. *, p ≤ 0.05; n = 3.

FIG. 5. Localization of CRLR, RAMP3Δ145-8, and NHERF-1 in HEK 293T cells during an internalization experiment. CRLR and RAMP3Δ145-8 co-expressed with NHERF-1 after 1 h of AM (10 nM) pretreatment internalize similarly to the wild-type CRLR-RAMP3 complex in the absence of NHERF-1. Experiments performed as described in the legend for Fig. 2. Fixed cells were stained with anti-RAMP3 antibody (1:200) and anti-NHERF-1 antibody (1:250) with Cy5 anti-rabbit secondary antibody (1:400, in blue) and Cy3 anti-mouse secondary antibody (1:500, in red), respectively; CRLR-GFP is shown in green; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least 20 fields imaged from at least three experiments. Bar scales on all images represent 100 μm.
**Mechanism of Inhibition of CRLR/RAMP3 Internalization by NHERF-1**—The ezrin/radixin/moesin (ERM) domain of NHERF-1 is known to interact with MERM cytoskeletal proteins, allowing NHERF-1 to tether proteins to the actin cytoskeleton (22). Thus, we hypothesized that the CRLR-RAMP3 receptor complex internalization is regulated by NHERF-1 through interactions with NHERF-1 with cytoskeletal proteins. To test this hypothesis, we employed a mutant of NHERF-1 with a deletion of its ERM domain (23). Control experiments with the ERM domain mutants of NHERF-1 co-transfected with CRLR-RAMP3 showed similar levels of receptor expression and function, as measured by whole cell binding and adenylate cyclase. As hypothesized, the ERM domain mutant of NHERF-1, when co-expressed in HEK 293T cells with CRLR-RAMP3 complex, was now capable of internalization. The cytochalasin D experimental data further support that the mechanism of inhibition of the internalization of the CRLR-RAMP3 complex by NHERF-1 by tethering of the receptor complex to the plasma membrane by the interaction of NHERF-1 with the actin cytoskeleton.

The Role of NHERF in Receptor Trafficking in Primary Human Proximal Tubule Epithelial Cells—To examine whether our observations using the overexpressed system in HEK 293T cells could be translated to a more physiological cell type, we chose human proximal tubule cells to test our hypothesis. Because of the many roles of adrenomedullin and NHERF-1 in the kidney, we chose a human primary proximal tubule cell line to perform these studies. The hPTE cells were determined to express CRLR, RAMP2, RAMP3, and NHERF-1 and show high levels of specific adrenomedullin binding and receptor stimulation by 100 nM AM (Fig. 8, A and B). When pretreated with AM for a period of 1 h, desensitization was observed with adenylate cyclase assays (Fig. 8A). However, as we would predict, internalization was not observed (by whole cell receptor binding assays) (Fig. 8B). Internalization was not observed when hPTE cells were pretreated with 10 nM AM for up to 4 h, indicating that desensitization and internalization were not simply showing different kinetics. To test our hypothesis that RAMP3 and NHERF-1 were critical to the inhibition of the internalization of the CRLR-RAMP3 receptor complex, RNA interference technology was employed to individually knock down RAMP3 and NHERF-1. In both mRNA and protein expression studies, RAMP3 and NHERF-1 dramatically decreased in d-siRNA-treated samples, whereas control experiments using lacZ knockdown showed no significant alteration in RAMP3 or NHERF-1 expression as compared with wild-type hPTE cells (Fig. 9, A and B; data not shown). Internalization assays and whole cell receptor binding assays were performed to determine the effect of RAMP3 and NHERF-1 RNA interference on receptor internalization in hPTE cells. Strikingly, when pretreated with agonist, hPTE cells with RAMP3 or NHERF-1 RNA interference showed a regained ability to internalize the receptor complex, unlike the wild-type hPTE cells in which internalization was inhibited (Fig. 9C). This finding demonstrates that RAMP3 and NHERF-1 are both critical for the receptor trafficking of the CRLR-RAMP3 complex in hPTE cells, an unaltered cell line absent the issues of overexpression.

To determine whether the mechanism of inhibition of internalization in hPTE cells was similar to that in the HEK 293T cells, actin cytoskeletal tethering was examined in the hPTE cells. hPTE cells were treated with cytochalasin D, as was used in HEK 293T cells to disrupt the actin cytoskeleton, and internalization assays were performed. Cytochalasin D treatment did not alter the receptor expression levels in the hPTE cells, as measured by whole cell receptor binding, from the levels in untreated cells (data not shown). Interestingly, when cells were treated with cytochalasin D and pretreated with AM for 1 h, the receptor complex was now capable of internalization (Fig. 10). These data support the proposed model that NHERF-1 inhibits the internalization of the CRLR-RAMP3 receptor complex after agonist stimulation by tethering the receptor complex to the actin cytoskeleton via the ERM domain interactions of NHERF-1 with the cytoskeleton and PDZ domain interactions with RAMP3.
NHERF-1 Differentially Targets AM Receptor Subtypes

**FIG. 7.** Mechanism of NHERF-1 inhibition of internalization of CRLR/RAMP3 receptor complex. A, ERM domain mutant of NHERF-1 blocks the inhibition of the internalization of the CRLR-RAMP3 complex seen with wild-type NHERF-1. HEK 293T cells were transfected with CRLR, RAMP3, and wild-type NHERF-1 or ERM domain mutant NHERF-1. 48 h after transfection, cells were pretreated with AM (10 nM) for 1 h and washed as described under “Experimental Procedures,” and receptor internalization was measured with whole cell binding using 125I-rAM as the ligand and cold rAM as the competitor. The number of binding sites/cell was estimated with GRAPHPAD PRISM software. *, p ≤ 0.05; n ≥ 3. B, localization of RAMP3, NHERF-1 (or NHERF-1ΔERM), and actin cytoskeleton in HEK 293T cells during an internalization experiment. Untreated cells show RAMP3 and wild-type NHERF-1 distributed at the plasma membrane and decrease the endocytosis rate of the receptor. After AM pretreatment, all components remain in the same distribution. ERM domain mutant of NHERF-1 shows poor colocalization with the actin cytoskeleton in both untreated and AM-pretreated conditions, and therefore, shows the internalization of RAMP3 with agonist pretreatment. HEK 293T cells were transfected with CRLR, RAMP3, and wild-type or ERM domain mutant NHERF-1. Experiments were performed as described in the legend for Fig. 2. Fixed cells were stained with anti-RAMP3 antibody (1:200) and anti-NHERF-1 antibody (1:250) with Cy5 anti-rabbit secondary antibody (1:400, in blue) and Cy3 anti-mouse secondary antibody (1:500, in red), respectively; actin cytoskeleton is stained with Alexa Fluor 488-phalloidin and is shown in green; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least 20 fields imaged from at least three experiments. Bar scales on all images represent 100 μm. C, cytochalasin D treatment blocks the inhibition of the internalization of CRLR/RAMP3 complex. HEK 293T cells were transfected with CRLR, RAMP3, and wild-type NHERF-1. 48 h after transfection, cells were pretreated with cytochalasin D (10 μM) for 15 min and then with AM (10 nM) for 1 h and washed as described under “Experimental Procedures,” and receptor internalization was measured with whole cell binding using 125I-rAM as the ligand and cold rAM as the competitor. The number of binding sites/cell was estimated with GRAPHPAD PRISM software. *, p ≤ 0.05; n ≥ 3.

**DISCUSSION**

Like other GPCRs, the adrenomedullin and CGRP receptors mediate various physiological actions in different cell types using a variety of mechanisms (24, 25). The receptor undergoes classical life cycle wherein it is phosphorylated upon agonist stimulation, leading to desensitization, internalization, and either recycling or degradation depending on the cell type. However, unlike other GPCRs, the AM and CGRP receptors are regulated by single transmembrane accessory protein called RAMPs (RAMP1–3). In addition to the original observation by Foor’s group (1) that RAMPs are necessary for the cell surface expression and specificity of CRLR to the ligands CGRP and AM, our recent observations demonstrated that RAMPs (particularly RAMP3) have other roles that may regulate the life cycle of the receptor complex (15). Specifically, RAMP3 interaction with NSF was found to be important for recycling the receptor after internalization. The absence of RAMP3 or the inhibition of NSF resulted in receptor complex degradation (15). The present study was undertaken to investigate other binding partners and additional roles for RAMP3 in the CRLR-RAMP life cycle. We show here, using both heterologous expression as well as endogenous systems, that RAMP3 interaction with NHERF-1 is essential for regulating the internalization of the CRLR-RAMP3 complex. In addition, similar to our NSF study, the PDZ motif in RAMP3 is critical for the interaction with NHERF-1. In particular, we have found that the Thr146 in RAMP3 is critical for this interaction. Whether RAMP3 is phosphorylated at this site by any of the kinases and whether this is essential for NHERF-1 interaction are not known.

Several studies have shown NHERF-1 to play differing roles in various cellular processes, including receptor trafficking (12, 21). NHERF-1 has been demonstrated to bind the extreme C terminus of several GPCRs, namely the β2-adrenergic receptor, the κ-opioid receptor, and the P2Y purinergic receptor (8, 13). Agonist exposure promotes NHERF-1 association with the β2-adrenergic and the κ-opioid receptors. Unlike our study, NHERF-1 association with these receptors enhances the recycling of the receptors after agonist stimulation (8, 10). The DSLL motif in the C terminus of the β2-adrenergic receptor was found to be critical for NHERF-1 interaction. In the present study, we also found that the DTLL motif in RAMP3 is essential for the interaction with NHERF-1. Similar to the Thr in the DTLL motif, the Ser in the DSLL motif was found to be essential for the interaction with NHERF-1 (10). In addition to GPCRs, NHERF-1 has also been shown to associate with the epidermal growth factor receptor, a receptor tyrosine kinase. In this case, NHERF-1 association acts to stabilize the receptor at the plasma membrane and decrease the endocytosis rate of the receptor (26). Using an endogenous system, we have also shown here that NHERF-1 is essential to “hold” the receptor-complex at the membrane, the absence of which leads to the internalization of the receptor complex. Although utilizing different mechanisms, collectively these data suggest that NHERF-1...
FIG. 8. Trafficking of AM-R in human proximal tubule cells (hPTE cells). A, in hPTE cells, AM-R signaling desensitizes with AM pretreatment. hPTE cells seeded in 24-well plates were pretreated with 10 nm rAM for 1 h and were washed extensively to remove residual agonist, and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's instructions. cAMP accumulation was measured in membranes stimulated with 100 nm AM for 15 min and was calculated using a standard curve ranging from 10 to 10^5 fmol of cAMP. Each experiment was done in duplicate and repeated at least three times. Data are expressed as the percentage of maximal response, % forskolin. *, p ≤ 0.05; n = 4 experiments. B, AM-R fails to internalize when incubated in AM for varying time points. hPTE cells were grown as described in A. Receptor internalization was measured by whole cell competition binding assays using 125I-rAM as ligand (cold rAM served as the competitor), and the number of binding sites/cell was estimated using the GRAPHPAD PRISM software. No pretreat represents samples at maximal radioligand binding that were not preincubated with agonist. 1, 2, and 4 hr pretreat represent samples pretreated with AM (10 nM) for according time period, washed as indicated under “Experimental Procedures,” and tested immediately after the wash steps for radioligand binding. *, p ≤ 0.05; n = three experiments. In cells endogenously expressing AMR, internalization is inhibited, but desensitization is unaffected.

FIG. 9. Effect of RAMP3 and NHERF-1 RNA interference (RNAi) on internalization of AM2-R in human proximal tubule cells. RAMP3 and NHERF-1 RNA interference disrupts the inhibition of the internalization of AM-R after agonist pretreatment. hPTE cells seeded in 48-well plates were transfected with d-siRNA of RAMP3 or NHERF-1 and incubated for 48 h to allow RNA knockdown. A, mRNA analysis by RT-PCR showed dramatically decreased levels of RAMP3 and NHERF-1 mRNA in RAMP3 and NHERF-1 RNA interference samples (respectively) as compared with wild type (WT), whereas lacZ knockdown had no effect on RAMP3 or NHERF-1 message levels (data not shown). RNA isolation and RT-PCR performed as described under “Experimental Procedures” section. n = three experiments. B, immunofluorescence microscopy of hPTEs with RAMP3 or NHERF-1 RNA interference demonstrated greatly decreased levels of RAMP3 and NHERF-1 protein expression in RAMP3 and NHERF-1 RNA knockdown cells, as compared with wild-type hPTE cells. lacZ knockdown had no effect on RAMP3 or NHERF-1 protein expression in hPTEs (data not shown). Cells were prepared as described under “Experimental Procedures” for immunofluorescence microscopy. Fixed cells were stained with anti-RAMP3 antibody (1:200) or anti-NHERF-1 antibody (1:250) and detected with a Cy3 anti-rabbit secondary antibody (1:500). Images shown are representative of at least three experiments. C, AM-R is capable of internalization when RAMP3 or NHERF-1 is knocked down with RNA interference. hPTE cells were grown as described in the legend for Fig. 8. Receptor internalization was measured by whole cell competition binding assays using 125I-rAM as ligand (cold rAM served as the competitor), and the number of binding sites/cell was estimated using the GRAPHPAD PRISM software. No pretreat represents samples at maximal radioligand binding that were not preincubated with agonist. AM pretreat represents samples pretreated with AM (10 nM) for 1 h, washed as indicated under “Experimental Procedures,” and tested immediately after the wash steps for radioligand binding. *, p ≤ 0.05; n = 3.
interaction with receptor tyrosine kinases and G protein-coupled receptors and RAMP3 is mandatory to enhance the portion of receptors present at the cell surface.

Recent data suggest that RAMP3 can interact with receptors other than CRLR. It is important to determine whether this novel role of RAMP3 in receptor trafficking is specific for CRLR or also for the other receptors that RAMP's interact with, namely vasoactive intestinal peptide receptor, PTH1-R and PTH2-R, and glucagon receptors (3). NHERF-1 and NHERF-2 have been reported to interact with the PTH1R in a scaffolding capacity and tether the receptor to phospholipase C-β and the actin cytoskeleton (11, 16). In opossum kidney cells, NHERF-1 mediates PTH-stimulated entry of extracellular calcium by a mechanism that is apically localized, phospholipase-dependant, and pertussis toxin-sensitive and that requires an intact actin cytoskeleton. NHERF-1 was also shown to inhibit the activation-independent internalization of the PTH1R in kidney distal tubule cells (when stably expressed) (28). Given the ability of RAMP2 and RAMP3 to interact with the PTH1R and PTH2R, respectively, RAMP3 may be interacting with NHERF-1 to play a trafficking role in these systems as well.

In addition to AM, Roh et al. (27) have reported that intermediate, a newly discovered peptide from the calcitonin gene peptide superfamily, can also bind the CRLR-RAMP3 complex. It remains to be determined whether this reported function for RAMP3 is specific for AM or whether another peptide like intermediate could yield similar results. In our studies of interaction of RAMP3 with NSF and NHERF-1, the PDZ motif of RAMP3 was found to be critical for the interaction. Whether this motif binds additional proteins in a cell type-specific manner remains to be examined. One could hypothesize that these predicted cell type-specific interactions would lead to regulation of the various events in the receptor life cycle. In addition to RAMP3, RAMP1 and RAMP2 also regulate the expression of CRLR at the plasma membrane. Whether RAMP1 and RAMP2 bind other proteins similar to RAMP3 also remains to be examined.

CONCLUSIONS

This study has shown that one of the heterodimeric partners of CRLR, RAMP3, is capable of altering the trafficking of the receptor complex after agonist stimulation by interacting with NHERF-1 via its PDZ domain and thus the actin cytoskeleton. We have demonstrated here for the first time that NHERF-RAMP3 interaction dissociates receptor desensitization from the internalization of the CRLR-RAMP complex. Additionally, this reveals a novel function for the RAMP accessory proteins in receptor trafficking and an additional difference between the AM1R and AM2R. With recent reports of RAMP's complexing and regulating GPCRs other than CRLR, future studies will focus on additional binding partners of RAMPs and how they regulate the various events in the GPCR life cycle.

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