Cell Surface Levels of Organellar Na\(^+\)/H\(^+\) Exchanger Isoform 6 Are Regulated by Interaction with RACK1*

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In mammalian cells, four Na\(^+\)/H\(^+\) exchangers (NHE6–NHE9) are localized to intracellular compartments. NHE6 and NHE9 are predominantly localized to sorting and recycling endosomes, NHE7 to the trans-Golgi network, and NHE8 to the mid-trans-Golgi stacks. The unique localization of NHEs may contribute to establishing organelle-specific pH values and ion homeostasis in cells. Mechanisms underlying the regulation and targeting of organellar NHEs are largely unknown. We identified an interaction between NHE9 and RACK1 (receptor for activated C kinase 1), a cytoplasmic scaffold protein, by yeast two-hybrid screening using the NHE9 C terminus as bait. The NHE9 C terminus is exposed to the cytoplasm, verifying that the interaction is topologically possible. The binding region was further delineated to the central region of the NHE9 C terminus. RACK1 also bound NHE6 and NHE7, but not NHE8, in vitro. Endogenous association between NHE6 and RACK1 was confirmed by co-immunoprecipitation and co-localization in HeLa cells. The luminal pH of the recycling endosome was elevated in RACK1 knockdown cells, accompanied by a decrease in the amount of NHE6 on the cell surface, although the total level of NHE6 was not significantly altered. These results indicate that RACK1 plays a role in regulating the distribution of NHE6 between endosomes and the plasma membrane and contributes to maintaining luminal pH of the endocytic recycling compartments.

Maintenance of the luminal pH of intracellular compartments is fundamental for various cellular events, such as receptor-mediated endocytosis and recycling to the plasma membrane (1, 2). Tight regulation of the pH is accomplished by the coordinated functioning of a subset of ion transporters. The vacuolar H\(^+\)-ATPase (V-ATPase)\(^2\) is a primary mediator of acidification (3). A number of observations have also indicated the existence of proton leak systems. For example, pharmacological inhibition of V-ATPase results in an immediate dissipation of the proton gradient across the organelar membrane (4). The luminal pH along the secretory pathway becomes gradually acidic from endoplasmic reticulum to Golgi and secretory vesicles. A previous fluorescence imaging study using live endocrine cells estimated that proton pump activity increases and, on the contrary, proton leakage decreases along the secretory pathway (5). Although our understanding of the molecular identities responsible for proton leakage is incomplete, it is assumed that organelle-specific pH is controlled by a balance between two major activities (i.e. proton pumping by V-ATPases and proton leakage from the organelar lumen) (6).

To date, nine Na\(^+\)/H\(^+\) exchanger (NHE) isoforms have been identified in mammals. NHE1–NHE5 are localized to the plasma membrane in various cell types (7–11). NHE6 and NHE9 are predominantly localized to sorting and recycling endosomes in several cultured cell lines (12). NHE7 and NHE8 are localized primarily to the trans-Golgi network and mid-trans-Golgi stacks, respectively (12–14). Four organellar NHEs may mediate K\(^+\)/H\(^+\) exchange rather than Na\(^+\)/H\(^+\) exchange under physiological conditions and could be responsible for proton leakage from organelles, contributing to the establishment of a unique organellar luminal pH. The boundaries of such intracellular compartments are not static and not definite, since the organelles are connected by continuous membrane trafficking. Therefore, the steady state localization of NHE isoforms is under a dynamic equilibrium. However, little is known about the mechanisms that regulate the localization and activity of organellar NHEs.

NHEs are composed of a relatively conserved N-terminal transmembrane domain (10–12 transmembrane helices) and a more variable C-terminal hydrophilic domain. In NHE1, the most extensively studied isoform, the C-terminal domain is cytoplasmic (15) and serves as a regulatory domain. The interaction of NHE1 with lipids and with various proteins, including calcium-binding proteins, kinases, and scaffolding proteins, plays an important role in modulating the activity and localization of NHE1 (16–19). Divergent amino acid sequences in the C-terminal domains of NHEs suggest the existence of NHE isoform-specific functions and regulatory mechanisms.

We conducted a yeast two-hybrid screen using the C terminus of human NHE9 as bait to identify interacting proteins that may regulate intracellular NHEs. RACK1, a scaffold protein originally identified from a rat brain cDNA library as an anchoring protein for protein kinase C (20), was identified. Pulldown assays showed that three NHE isoforms (NHE6, -7, and -9)
interact with RACK1. The function of RACK1 in the distribution of NHE6 between endosomes and the cell surface and in the maintenance of endosomal pH was investigated.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Transfection—* COS-7 and HeLa cells were maintained with Dulbecco’s modified Eagle’s medium and minimum essential medium containing 10% fetal calf serum, respectively. The cells were cultured at 37 °C in 5% CO₂. For transfection of plasmid DNA, TransIT LT-1 (Mirus) and Lipofectamine 2000 (Invitrogen) were used according to the manufacturer’s instructions. Three sets of duplex siRNAs targeted against human RACK1 and a control siRNA were purchased from Invitrogen for RACK1 knockdown. Transfection of siRNA was performed with Lipofectamine 2000.

**Plasmid Construction for Expression in Escherichia coli and Yeast—** To obtain pGBT9-hNHE9Ct-(494–645), the corresponding DNA fragment was amplified by PCR with the following primers: forward1 (5’-GGGGGATCCGACCTG-GATGAAATCTCAG3’-H11032) and reverse1 (5’-GGGTTGACCTTAATTCGACTGAGATGACGACC3’-H11032). The PCR product was cloned into the GAL4BD-fusion expression vector pGBT9 with BamHI-Sall. For expression of recombinant MBP fusion proteins, the corresponding DNA fragments for hNHE6, hNHE7, hNHE8, and hNHE9 were amplified by PCR from previously constructed NHE plasmids (12). In all constructs, BamHI-Sall PCR fragments were cloned into the pMAL-cRI bacterial expression vector (New England Biolabs). Plasmid Construction for Expression in Mammalian Cells—Expression vectors encoding C-terminally tagged human NHE6.1. To construct an expression plasmid for Myc-VAMP3 (vesicle-associated membrane protein 3)-EGFP, a PCR fragment encoding N-terminal Myc-tagged human VAMP3 was amplified from a human skeletal muscle cDNA library by PCR with the following primers: forward (5’-GGGGGATATCAGGAAACAAAAACT-CATCTCAGAAGAGATCGGTGATTGATGCTCTACAGG-TGCACTACCTG-3’) and reverse (5’-CCTCACTAAAGAAGAGAGA-CAACCACACAAGCA3’). The Myc tag sequence is included in the forward primer. An EcoRI-BamHI fragment was introduced into pEGFP-N3. The plasmid was then further used as a template to construct the VAMP3-EGFP-Myc expression plasmid. A PCR fragment encoding C-terminally GFP- and Myc-tagged VAMP3 was amplified with the following primers: forward (5’-GGGGGATATCAGGAAACAAAAACT-CATCTCAGAAGAGATCGGTGATTGATGCTCTACAGG-TGCACTACCTG-3’) and reverse (5’-CCTCACTAAAGAAGAGAGA-CAACCACACAAGCA3’). The N-terminal Myc tag sequence was omitted from the forward primer and instead included in the reverse primer. The EcoRI-NotI fragment was introduced into pEGFP-N3.

To construct an expression vector for the GFP-fused RACK1 binding region of NHE6 (GFP-NHE6-(537–588)), the corresponding EcoRI-BamHI fragment from the bacterial expression plasmid for MBP-NHE6-(537–588) was cloned into pEGFP-C2.

**Antibodies—** Mouse monoclonal anti-Myc IgG (clone 9E10) and mouse monoclonal anti-HA IgG (clone 12CA5) were purchased from Roche Applied Science. Rabbit polyclonal anti-HA IgG was obtained from Santa Cruz Biotechnology, Inc. Mouse monoclonal primary antibodies against α-tubulin (clone DM1A), NHE1 (clone 54), Na⁺/K⁺ ATPase α (clone C464.6), and transferrin receptor (clone H68.4) were purchased from Sigma, BD Biosciences, Upstate Biotechnology, Inc., and Zymed Laboratories, respectively. Anti-RACK1 mouse IgM (clone 20) was purchased from BD Biosciences. Horseradish peroxidase-conjugated secondary antibodies were purchased from Vector Laboratories. Alexa-488-, Alexa-546-, and Alexa-568-conjugated goat secondary antibodies were purchased from Invitrogen. Rabbit polyclonal anti-NHE6 and anti-NHE9 antibodies were prepared against isoform-specific sequences in the C terminus. Rabbis were immunized with bacterially expressed fragments of NHE6 (83 amino acids) and NHE9 (68 amino acids), respectively. Antisera were collected and affinity-purified. Conjugation of anti-NHE6 antibody with horseradish peroxidase was performed using the Peroxidase Labeling Kit-NH₂ (Dojindo Laboratories).

**Yeast Two-hybrid Screening—** GAL4-based yeast two-hybrid screening was performed using 152 amino acids from the C terminus of human NHE9 as bait. pGBT9-hNHE9Ct-(494–
was used to screen a human skeletal muscle cDNA library cloned into the GAL4AD-fusion expression vector pACT2 (BD Biosciences). The Saccharomyces cerevisiae AH109 strain (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1_UAS::GAL1::TATA-HIS3, GAL2_UAS::GAL2::TATA-ADE2, ura3::MEL1_UAS::MEL1::TATA-lacZ) was co-transformed with pGBT9-hNHE9Ct-494 and the pACT2 cDNA library. Transformants (1.06 × 10⁶) were screened on SD medium containing 0.67% yeast nitrogen base, 2% glucose and lacking appropriate nutrients for selection (Leu-, Trp-, Ade-, and His-). Growth-positive clones were isolated, and pACT2 plasmids were retrieved. The cDNA inserts were amplified by PCR using the following primers designed for the flanking region of the pACT2 MCS: forward (5’-CTATTCGATGATAAGAGTACCCACCAAACCC-3’) and reverse (5’-GTGAACCTTGGGTTTTTTCAGTATCTACGAT-3’). The PCR products were directly subjected to DNA sequencing. Expression and Purification of MBP Fusion Proteins—MBP fusion proteins were expressed in E. coli. E. coli Rosetta-gami transformants were induced with 0.4 mM isopropyl-β-D-galactopyranoside at 30 °C for 4 h. Bacterial cells were harvested, suspended in lysis buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture, and sonicated five times for 30 s. After centrifugation at 20,000 g for 15 min at 4 °C for 30 s. After centrifugation at 20,000 × g for 15 min at 4 °C, the supernatant was incubated with amylose-resin (New England Biolabs) for 2 h at 4 °C. Resin was washed with lysis buffer and bound proteins were eluted with 10 mM maltose.

Microinjection—NHE9-MycHis was transiently expressed in COS-7 cells cultured on collagen coated glass dishes. Mouse monoclonal anti-Myc IgG in PBS (0.17 μg/μl) was injected into the cytoplasm using a semiautomatic microinjection apparatus, Injectman N2 and Femtojet (Eppendorf), equipped with Femtotip-II. After incubation at 37 °C for 15 min, cells were fixed with 4% formaldehyde and subjected to immunofluorescence analysis. In control experiments, either Myc-VAMP3-EGFP or VAMP3-EGFP-Myc was transiently expressed instead of NHE9-MycHis.

Expression and Purification of MBP Fusion Proteins—MBP fusion proteins were expressed in E. coli. E. coli Rosetta-gami transformants were induced with 0.4 mM isopropyl-β-D-galactopyranoside at 30 °C for 4 h. Bacterial cells were harvested, suspended in lysis buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture, and sonicated five times for 30 s. After centrifugation at 20,000 × g for 15 min at 4 °C, the supernatant was incubated with amylose-resin (New England Biolabs) for 2 h at 4 °C. Resin was washed with lysis buffer and bound proteins were eluted with 10 mM maltose.

Immunoprecipitation—For the detection of organellar NHEs, cells were lysed in a syringe equipped with a 26-gauge needle. Boiling or heat treatment of the sample was omitted to avoid aggregation of NHEs. When indicated, samples were treated with PNGase F (New England Biolabs) according to the manufacturer’s instructions, except boiling of the samples was omitted. Protein samples were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in PBS containing 0.1% Tween 20 and 7.5% skim milk, hybridized with the indicated primary antibodies and hors eradish peroxidase-conjugated secondary antibodies. After extensive washing in PBS containing 0.1% Tween 20, immunoreactive signals were visualized with the chemiluminescence-based ECL plus detection system (Amersham Biosciences). Digital images were recorded using an LAS-1000 instrument (Fuji Film) and analyzed by ImageJ software (National Institutes of Health).

Pulldown Assay—COS-7 cells were washed three times with ice-cold PBS and lysed in lysis buffer (25 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture. Lysates were incubated on ice for 30 min and centrifuged at 20,000 × g for 10 min at 4 °C, and the supernatants were incubated at 25 °C for 2 h with 2 μg of recombinant MBP fusion protein immobilized to amylose-resin. After extensive washing with lysis buffer, resin fractions were separated by SDS-PAGE, and bound RACK1 was detected by immunoblotting with anti-RACK1 IgG.

Co-immunoprecipitation—For immunoprecipitation, HeLa cells were washed three times with ice-cold PBS and lysed in PBS containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture. Cells were lysed on ice for 30 min, homogenized in a syringe with a 26-gauge needle (20 strokes), and centrifuged for 10 min at 20,000 × g at 4 °C. The supernatants were collected and mixed with 10 μl of Protein G Plus-agarose (Santa Cruz Biotechnology) preincubated with either 2 μg of anti-NHE6 rabbit IgG or normal rabbit IgG. After incubation at 4 °C for 3 h with gentle rotation, beads were washed four times with lysis buffer, and immunoprecipitates were separated by SDS-PAGE. NHE6 and RACK1 were detected by immunoblotting with horseradish peroxidase-conjugated anti-NHE6 IgG and anti-RACK1 IgM, respectively.

Immunofluorescence—For immunostaining, cells were fixed with 4% formaldehyde and then permeabilized in PBS containing 0.4% saponin, 1% bovine serum albumin, and 2% normal goat serum. Cells were then incubated with the primary antibody for 1 h, followed by incubation with the secondary antibody for 1 h. In a competition assay, 0.1 μg/μl MBP-NHE6-(511–669) was added to the primary antibody solution. To visualize the localization of internalized Alexa546-conjugated transferrin (Alexa546-Tfn; Invitrogen), cells were incubated at 37 °C for 1 h in serum-free minimum essential medium con-
Cell Surface Biotinylation—HeLa cells were washed three times with ice-cold PBS and incubated with 0.5 mg/ml sulfo-NHS-ss-biotin in PBS at 4 °C for 20 min. After quenching excessive NHS groups with 0.1M glycine in PBS and extensive washing with ice-cold PBS, cells were lysed in PBS containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture. Cell lysates were homogenized in a 1-ml syringe with a 26-gauge needle (20 strokes) and then centrifuged for 10 min at 20,000 × g at 4 °C. Supernatants containing equal amounts of protein were mixed with 100 μl of immobilized Neutravidin beads and incubated at 4 °C for 2 h with gentle rotation. Beads were collected by brief centrifugation and washed three times with lysis buffer. The biotin-labeled surface proteins were separated by SDS-PAGE and analyzed by immunoblotting. The intensity of the immunoreactive bands was quantified using Image J.

Measurement of Endosomal pH—Fluorescence ratio imaging was performed to measure the endosomal pH according to the previously reported procedure (21) with modifications. FITC- and Alexa546-conjugated Tfns were purchased from Invitrogen. HeLa cells were incubated at 37 °C for 1 h in serum-free minimum essential medium containing 75 μg/ml FITC-Tfn, 25 μg/ml Alexa546-Tfn, and 0.1% bovine serum albumin. After extensive washing with Hanks’ balanced salt solution (137 mM NaCl, 5.3 mM KCl, 1.3 mM CaCl2, 0.82 mM MgSO4, 0.34 mM Na2HPO4, 0.44 mM KH2PO4, 4.2 mM NaHCO3, 5.6 mM glucose, and 15 mM HEPES-NaOH (pH 7.4)), cells were observed using an inverted microscope (IX-70; Olympus) equipped with a ×40 UPlanApo objective (numerical aperture 0.85). Images were acquired using an ORCA-MAX digital camera and AQUACOSMOS software. The following interference filter sets were used: band pass 470–495 nm (excitation) and band pass 510–550 nm (emission) for FITC; band pass 530–550 nm (excitation) and long pass >575 nm (emission) for Alexa546. After fixation with 4% formaldehyde, a pH calibration curve was constructed with calibration solutions containing 125 mM KCl, 25 mM NaCl, 5 mM nigericin, and a 25 mM concentration of one of the following buffers: HEPES, pH 7.5 or 7.0, MES, pH 6.5 or 6.0. Fluorescence intensity of the perinuclear recycling endosome was quantified with AQUACOSMOS software, and the ratio of fluorescence intensity (FITC/Alexa546) was calculated after background subtraction. The fluorescence ratio was linear in the range of pH 6.0–7.5. In a single experiment, the endosomal pH values of a random selection of 24 living cells were measured and averaged. Data are shown as the average ± S.E. of six independent experiments.

Statistical Analysis—An unpaired two-tailed Student’s t test was used for statistical comparisons between RACK1 siRNA- and scramble RNA-treated cells in the measurement of endo-

![Image](image-url)
RESULTS

Identification of RACK1 as an NHE9 Binding Partner—To identify novel interacting proteins of NHE9, we performed a yeast two-hybrid screen. A human skeletal muscle cDNA library was screened using the C-terminal 152 amino acids of yeast orthologs faces the organellar lumen or the cytoplasm is controversial (22–27). The orientation is important, because RACK1, which is a cytoplasmic protein, could only associate with the C terminus of NHE9 if that region of the protein extends into the cytoplasm. To determine the orientation of the NHE9 C terminus, we designed an experiment in which immunostaining was combined with microinjection of antibodies against epo-556

tagged VAMP-EGFP-Myc (C). The localization of VAMP3 constructs were visualized by EGFP fluorescence (green). The right column shows merged images. D–E, COS-7 cells transiently expressing NHE9-(494–645) or transfected cells (E) were injected with anti-Myc, incubated at 37 °C for 15 min, and fixed. Localization of microinjected anti-Myc was visualized using an Alexa546-conjugated secondary antibody (red). Localization of VAMP3 constructs were visualized by EGFP fluorescence (green). The right column shows merged images. D–E, COS-7 cells transiently expressing NHE9-(494–645) or transfected cells (E) were injected with anti-Myc, incubated at 37 °C for 15 min, and fixed. Localization of microinjected anti-Myc (red) was visualized as above. Localization of NHE9 (green) was visualized by indirect immunofluorescence microscopy using anti-NHE9 and an Alexa488-conjugated secondary antibody.

somal pH and the quantification of cell surface transferrin receptor and NHE6 amount. A p value of <0.05 was considered to be statistically significant.
FIGURE 4. Pulldown assay of RACK1 and four organellar NHE isoforms. A, secondary structure model of NHE9 and the multiple sequence alignment of the C termini of organellar NHE isoforms. B, MBP alone and MBP fusions of organellar NHEs (MBP-NHE6-(511–669), -NHE7-(542–725), -NHE8-(481–581), and -NHE9-(494–645)) were purified, and pulldown assays were performed as described in the legend to Fig. 2.
cytoplasm (Fig. 3D). The signals overlapped those of staining by anti-NHE9 antibody, indicating that the microinjected anti-Myc antibody recognized the Myc tag at the C terminus of NHE9. When anti-Myc was injected into untransfected cells, the signal was observed uniformly throughout the cytoplasm, as in Fig. 3C (Fig. 3E). The anti-NHE9 antibody used in this experiment is only capable of visualizing exogenously overproduced NHE9 and not endogenous expression in COS-7 cells. These results strongly indicate that the NHE9 C terminus is cytoplasmically oriented; thus, interaction with RACK1 is topologically allowed.

**RACK1 Binds Other Organellar NHEs in Vitro and in Vivo**
The amino acid sequence of the RACK1 binding region (residues 528–577) of NHE9 is highly homologous to those of the corresponding regions in NHE6 and NHE7 but not NHE8 (Fig. 4A, underlined). This led us to hypothesize that RACK1 also binds NHE6 and NHE7. To investigate this possibility, pull-down assays were performed using MBP fusions of the full-length C terminus of these organellar NHEs (Fig. 4B). Binding of RACK1 to MBP-NHE6-(511–669) and MBP-NHE7-(542–725) as well as MBP-NHE9-(494–645) was detected by immunoblotting. However, binding to MBP or to MBP-NHE8-(481–581) was not detected, supporting the sequence specificity of the interaction. These results indicate that RACK1 may be a common binding partner of NHE6, NHE7, and NHE9.

To confirm an endogenous association between RACK1 and organellar NHEs in cells, we conducted co-immunoprecipitation assays. For these assays, we tried to generate isoform-specific antibodies against NHE6, -7, and -9. We obtained a rabbit polyclonal anti-NHE6 antibody with the desired specificity and reactivity, but antibodies capable of detecting endogenous expression of NHE7 and NHE9 were not obtained. The performance of the anti-NHE6 antibody was assessed by immunoblotting. HA-tagged NHE6, NHE7, and NHE9 were transiently expressed in COS-7 cells, and the expression was confirmed with an anti-HA antibody. Among these equally expressed isoforms, only NHE6 was detected when the same membrane was probed with the anti-NHE6 antibody (Fig. 5A). There are at least two NHE6 splicing variants, designated NHE6.0 and NHE6.1. NHE6.1 contains an insertion of 32 amino acid residues between Leu143 and Val144 of the originally reported NHE6.0 (30), which alters its mobility on SDS-PAGE (Fig. 5B). The molecular size of endogenous NHE6 was compared with those of transiently overexpressed NHE6.0 and NHE6.1 on the SDS-polyacrylamide gel (Fig. 5C). A previous study reported that NHE6 is an N-glycosylated protein (30). Therefore, we also examined the effect of PNGase F treatment to exclude a possible effect of N-glycosylation. In the absence of the PNGase F treatment, endogenous NHE6 was detected as two diffuse bands (i.e., a major band of 110–94 kDa (Fig. 5C, b) and a faint band of 75–67 kDa (c)). The higher molecular weight form seems to correspond to the highly N-glycosylated, mature form of NHE6, and the smaller forms seem to correspond to the less glycosylated, immature form (30). The two bands of endogenous NHE6 were identical in size to NHE6.1 on the gel, suggesting that NHE6.1, the larger variant, is dominantly expressed in COS-7 cells. Identical results were obtained with HeLa cells (data not shown). We also observed an additional weak band of 58 kDa; however, this band was not detected when NHE6 was overexpressed and was found only in the sample of endogenous NHE6 (filled arrowhead), suggesting that it was nonspecific. Alternatively, it could be a specific form of NHE6. It is noteworthy that overproduction of NHE6 was accompanied by a pronounced increase in smaller proteins (c), suggesting an increased production of immature NHE6 forms under these conditions. Additionally, there was an increase in the intensities of bands larger than 200 kDa (a) when NHE6 was overexpressed. These bands are likely to be oligomers or aggregates of NHE6, as previously reported (15). After PNGase F treatment, the major bands of 110–94 kDa (b) disappeared almost completely, and this was accompanied by an increase in the intensities of the larger (a) and smaller NHE6 forms (c). Also, these bands were shifted to faster migrating forms (a to d and c to e, respectively). Even after the removal of N-glycosylation, endogenous NHE6 migrated to nearly the same position as NHE6.1, suggesting that endogenous NHE6...
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contains an insertion of 32 amino acids that is specific for NHE6.1. Thus, the difference in the apparent molecular size between NHE6.0 and endogenous NHE6 does not reflect N-glycosylation but a difference in the size of the protein moiety.

In co-immunoprecipitation assays, anti-NHE6 specifically precipitated NHE6 from a HeLa cell lysate (Fig. 6). RACK1 was co-immunoprecipitated with NHE6, suggesting that RACK1 and NHE6 interact in vivo. Densitometric quantification showed that less than 3% of total RACK1 was co-immunoprecipitated with NHE6. Therefore, only a minor fraction of total RACK1 in the cells seems to be associated with NHE6. Although we originally identified RACK1 as a binding partner of NHE9, subsequent studies were exclusively performed with NHE6.

RACK1 Co-localizes with NHE6 in HeLa Cells—We determined the subcellular localization of endogenous NHE6 in HeLa cells using immunofluorescence microscopy. The specificity of NHE6 staining was confirmed by competition between endogenous NHE6 and recombinant MBP-NHE6-(511–669) (Fig. 7A). NHE6 co-localized with the transferrin receptor (TfR), which is consistent with localization at the sorting and recycling endosomes, as previously reported (13) (Fig. 7B). In comparison with transiently overproduced NHE6, which localized predominantly at the sorting endosomes in the cell periphery (12), endogenous NHE6 accumulated primarily in the perinuclear recycling endosomes, although some endogenous NHE6 was detected in the punctate sorting endosomes. Co-localization of RACK1 and NHE6 was further investigated by double immunofluorescent staining. The reported subcellular localization of RACK1 differs depending on the cell line (31–37). As shown in Fig. 7C, RACK1 was distributed throughout the cytoplasm and partial co-localization of NHE6 and RACK1 was detectable at punctate structures in the cell periphery. RACK1 did not accumulate in the perinuclear region; therefore, co-localization was not observed there.

Knockdown of RACK1 Elevates the Endosomal pH—To elucidate the functional importance of the interaction between NHE6 and RACK1, we performed an RNA interference knockdown of RACK1. The expression of RACK1 protein was successfully reduced at 48 h after transfection of siRNA (Fig. 8A). In contrast, expression of α-tubulin was not affected, supporting the specificity of the knockdown. Since organellar NHEs are hypothesized to be responsible for proton leakage, we investigated the influence of RACK1 RNA interference on the pH of recycling endosomes, where the majority of endogenous NHE6 is located. We performed a fluorescence ratio imaging analysis using transferrins labeled with two different fluorescent probes (FITC- and Alexa546-Tfn) (Fig. 8B). In cells treated with a scrambled RNA, the luminal pH of Tfn-containing recycling endosomes was pH 6.45 ± 0.08 (average ± S.E., n = 6), whereas in the RACK1 knockdown cells, endosomal pH was elevated to pH 6.79 ± 0.09 (average ± S.E., n = 6). The difference was significant, because the p value was less than 0.05. Previous studies have shown that alkalinization caused by treatment with
bafilomycin A1, a vacuolar V-ATPase inhibitor, or with some ionophores results in decreased Tfn uptake accompanied by the down-regulation of cell surface TfR and a reduced rate of recycling of internalized receptors (38, 39). Consistent with such observations, Tfn uptake significantly decreased in RACK1 knockdown cells, although the morphology and distribution of the endosomal compartments were not significantly affected (Fig. 8C). Additionally, surface levels of TfR, analyzed by surface biotinylation, decreased to 73 ± 3.5% of the control (average ± S.D., p < 0.05) in RACK1 knockdown cells, although the total amount of TfR was only slightly affected, 90 ± 8.0% of control (average ± S.D.) (Fig. 8D). These results are also consistent with RACK1 depletion causing alkalization of the recycling endosomes. We next examined whether overproducing the NHE6 fragment directly involved in binding RACK1 would mimic the results of RNA interference. A dominant negative effect on Tfn uptake was observed in cells expressing the GFP-fused RACK1 binding region of NHE6 (GFP-NHE6-(537–588)) (Fig. 9). In cells transfected with GFP-NHE6-(537–588), the fluorescence intensity of internalized Tfn was clearly weaker than in neighboring untransfected cells. Such a decrease in the Tfn uptake was not observed in cells expressing GFP alone. These results indicate that the endosomal alkalization observed in RACK1 knockdown cells is due to disruption of the association between RACK1 and NHE6.

**Levels of NHE6 at the Cell Surface Are Decreased in RACK1 Knockdown Cells**—Either an activation of ion transport activity or an increase in the amount of NHE6 in the recycling endosomes could account for the endosomal alkalization in RACK1 knockdown cells. To elucidate the molecular basis of the endosomal alkalization in RACK1 knockdown cells, the intracellular localization of NHE6 was characterized by immunofluorescence.
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Identification of a Novel NHE9 Binding Partner RACK1—In this study, we identified an interaction between the C terminus of NHE9 and RACK1. RACK1 is a scaffold protein originally identified as an anchor for activated protein kinase C (20). RACK1 is highly homologous to the β subunit of heterotrimeric G proteins (over 40% identity in amino acid sequence) and consists of seven tandem WD motifs, which are protein-protein interaction modules. Comparative structural modeling based on the three-dimensional structure of bovine transducin Gβ (40) suggests that the seven WD repeats are arranged to form a seven-blade β propeller structure, which presumably enables the protein to associate with multiple binding partners through each blade of the β propeller (41). Two independent clones obtained by two-hybrid screening in this study encoded the C-terminal half of RACK1, suggesting that WD5–WD7 are sufficient for binding. RACK1-binding proteins identified to date include key signaling molecules (20), cell adhesion receptors (42), other scaffolding protein that associates ion channels (32), and ion exchangers (31), suggesting that RACK1 plays roles in coordinating protein-protein interactions in diverse intracellular events. This is the first report showing an interaction of RACK1 with an ion transporter that resides primarily in intracellular compartments. It is noteworthy that RACK1 binding partners include a plasma membrane Na\(^{+}/\)H\(^{+}\) exchanger, NHE5 (31). However, the regions on RACK1 that bind to NHE5 and organellar NHEs are thought to be different, as are the functional significances of these two interactions.

Interaction of NHE9 with RACK1 Is Topologically Possible—RACK1 is localized to the cytoplasm (31–37). Therefore, interaction with NHE9 would be possible only if the C terminus of NHE9 is exposed to the cytoplasm. The orientation of the C terminus of mammalian organellar NHEs, as well as their plant and yeast homologs, has remained controversial (22–26, 31). Thus, we verified that the C terminus of NHE9 is in the cytoplasm. For mammalian organellar NHE homologs, several studies indirectly support the cytoplasmic orientation of the C terminus, because the proteins associate with other cytoplasmic proteins via the C terminus (22, 23, 31).

RACK1 Associates with NHE6, -7, and -9 through a Conserved Region in the C Terminus—The interaction with RACK1 appears to be mediated by the central region of the hydrophilic NHE9 C terminus (residues 528–577). The amino acid sequence of this region in NHE9 is highly conserved in NHE6 and NHE7. In vitro pulldown assays indicate that RACK1 also associates with these two organellar isoforms, but not with the more distantly related NHE8 isoform. The RACK1-NHE6 interaction in vivo was confirmed by co-immunoprecipitation. The stretch of 50 amino acid residues constituting the RACK1 binding region of organellar NHEs does not show any significant similarity to previously reported RACK1 binding sequences. However, the RACK1 binding modules in known binding partners show great diversity in their amino acid sequences (reviewed in Ref. 41). We tested whether there is a region within the RACK1 binding region of NHE9 (residues 528–577) that is essential for binding by testing smaller fragments (10–20 amino acid residues) of this region. However,
each fragment was capable of associating with RACK1 in vitro (data not shown). Thus, the interaction may be mediated by multiple sites in the NHE9 C terminus (residues 528–577) rather than by a single or a few critical residues. Further delineation of the RACK1 binding region and co-immunoprecipitation assays with NHE mutants lacking the binding region would be required to address detailed questions about binding (e.g. whether regions other than the C terminus contribute to binding).

As demonstrated by electrophoretic mobility in SDS-PAGE, the endogenous NHE6 in HeLa and COS-7 cells appears to be NHE6.1, a longer variant containing an insertion of 32 amino acid residues, although we have not yet examined the effect of O-glycosylation. There has been no prior description of which form of NHE6 is endogenously expressed, and this is the first evidence indicating that NHE6.1 is endogenously expressed in some cell lines. When transiently expressed in COS-7 cells, both variants localized to the endosomes (data not shown). Therefore, the insertion of 32 amino acid residues does not appear to affect the localization of NHE6, and the significance of the insertion is not currently clear.

**Functional Significance of Interaction between NHE6 and RACK1**—Nhx1p, a yeast homolog of NHE6, plays an important role in the regulation of endosomal pH. Deletion of the NHX1 locus results in excess acidification of intracellular compartments and causes secretion of a vacuolar protease, carboxypeptidase Y, and impaired exit of an endocytosed G-protein coupled receptor, Ste3, from endosomes to the vacuole (43, 44). Additionally, our previous study showed that overexpression of organellar NHEs results in the alkalization of intracellular compartments in mammalian cell lines (12). These data are consistent with intracellular NHEs being components of the proton leak system in post-Golgi compartments. In the present study, knockdown of RACK1 resulted in the alkalization of the endosomal lumen. The markedly decreased surface expression of NHE6, which results in an increase in intracellular NHE6, may contribute to the pH elevation by increasing the proton efflux from endosomes. We emphasize that the total expression of NHE6 was not altered in RACK1 knockdown cells, indicating that RACK1 is not involved in translation, maturation, or stability of NHE6. Adequate acidification of endocytic compartments is essential for receptor-mediated endocytosis and recycling (38, 39, 45). These results suggest that association with RACK1 is involved in localization of NHE6 at the plasma membrane and in efficient receptor-mediated endocytosis of Tfns, probably by regulating the pH of the recycling endosomes. There are two mechanisms by which RACK1 could modulate organellar pH by its interaction with NHE6: 1) interaction with
RACK1 suppresses endocytosis of NHE6 and maintains NHE6 at the cell surface, or 2) RACK1 association accelerates exocytosis of NHE6. These two possibilities are not mutually exclusive, and further studies are necessary to understand the molecular basis by which RACK1 influences the distribution of NHE6. Although a subtle co-localization of NHE6 and RACK1 was observed by immunostaining, the precise location at which the interaction takes place remains to be identified. Co-localization was generally observed at small punctate structures in the cell periphery rather than at the perinuclear recycling endosomes. Therefore, we postulate that the interaction takes place at sorting endosomes or at subdomains in the plasma membrane (e.g. clathrin-coated pits, where RACK1 was previously reported to localize in HEK-293 cells) (34).

There also remains the important question of whether the altered intracellular distribution of NHE6 alone could account for the observed endosomal alkalinization. The results in this study do not exclude the possibility that RACK1 also regulates the function of other proteins, such as V-ATPase and functionally associated anion channels, which would affect the ion homeostasis in the endosomes independently of NHE6. So far, there have not been any reports about such an interaction. However, it will be worthwhile to estimate the effect of RACK1 knockdown on the expression and proton pumping activity of V-ATPase in a future study. We are thinking that the loss of RACK1 binding could up-regulate the ion transport activity of NHE6 as well as altering its intracellular localization. Activating the transport activity might be attributed to post-translational modifications, such as phosphorylation by other proteins, because RACK1 does not have any functional domains other than WD repeats, and its known function is to scaffold other proteins, such as protein kinases C. At present, it seems difficult to dissect the effect of RACK1 binding on the subcellular localization and ion transport activity, because altered Na\(^+/H^+\) (or K\(^+/H^+\)) exchange activity would directly affect the endosomal pH, and the altered endosomal pH would affect both the intracellular membrane trafficking and the enzymatic activity of NHEs. Identification of other components in the NHE6-RACK1 complex and kinetic studies of ion transport will further elucidate the effect of RACK1 on the ion transport activity of NHE6. In future studies, we also need to verify whether the functional consequences of the interaction are the same in the three organelar NHE isoforms.

Regulation of Na\(^+/H^+\) exchangers is principally achieved by interactions with various proteins or lipids through their isoform-specific C terminus. We have identified RACK1 as a novel binding partner of NHE6, -7, and -9. An in vivo interaction between RACK1 and NHE6 was confirmed by co-immunoprecipitation and by immunostaining. RACK1 influences the surface distribution of NHE6, and it is interesting that the physiological function of ion transporters like NHE6, which are localized predominantly at post-Golgi compartments, could be regulated by changes in the distribution between intracellular compartments and the plasma membrane. As a scaffold protein, RACK1 could regulate NHEs in response to a variety of signals. It is tempting to speculate that the function and localization of NHE6 is regulated through protein kinase C-dependent signaling in some situations. Further studies will provide insights into the functions of organelar NHEs in cell physiology.

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