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ORIGINAL ARTICLES

A Membrane-Proximal Region in the C-Terminal Tail of NHE7 Is Required for Its Distribution in the *Trans*-Golgi Network, Distinct from NHE6 Localization at Endosomes

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Abstract Mammalian Na^+/H^+ exchanger (NHE) isoform NHE6 is localized in sorting/recycling endosomes, whereas NHE7 is localized in the *trans*-Golgi network (TGN) and mid-*trans*-Golgi stacks. The mechanism targeting each NHE to a specific organelle is largely unknown, although the targeting is thought to be important for pH control in the lumen of various organelles. NHE6 and NHE7 exhibit distinct localization despite conserved amino acid sequences. To specify the intramolecular region involved in the specific localization, we examined the intracellular localization of chimeric NHE6 and NHE7 constructs. NHEs are composed of an N-terminal transmembrane domain (TM) and a C-terminal hydrophilic tail domain (Ct). Exchange of the Ct between the isoforms suggested that the Ct is required for the specific localization. We further split the Ct into three regions, and chimeras with various combinations of these small regions indicated that the most membrane-proximal region among the three contributes to the specific localization. Mutant forms of NHE7 with sequential alanine substitutions in the most membrane-proximal region, between residues 530 and 589, showed that two regions (residues 553–559 and 563–568) are required for NHE7-like localization. However, NHE6 with alanine substitutions in the membrane-proximal region exhibited no apparent change in localization. These results suggest that two membrane proximal regions (residues 533–559 and 563–568) play an important role in targeting NHE7 to the TGN.

Keywords NHE6 · NHE7 · Localization · Endosome · *Trans*-Golgi network · Chimera · NHE transporter

Introduction

Eukaryotic cells have a well-organized system for trafficking of proteins that are secreted, are endocytosed or need to reach a specific organelle (Perret et al. 2005). Various organelles play key roles in this trafficking. The internal pH value of each organelle is specific for that type of organelle and is important for its functions, such as processing of proteins, incorporation of secretory materials and hydrolysis of biomolecules (Carnell and Moore 1994; Cosson et al. 1989).

Na^+/H^+ exchangers (NHEs), also known as "Na^+/H^+ antiporters," are found in organisms from bacteria to higher eukaryotes and are involved in pH homeostasis and regulation of osmolality in cells. In mammals, NHEs have nine isoforms and are classified into two well-conserved subgroups. Members of first group, NHE1–NHE5, are localized to the plasma membranes of various types of cells, whereas members of the second group, NHE6–NHE9, are found in organelle membranes (Attaphitaya et al. 1999; Baird et al. 1999; Orlowski et al. 1992; Sardet et al. 1989; Tse et al. 1993). NHE6 and NHE9 are predominantly localized to sorting and recycling endosomes (Brett et al. 2002; Nakamura et al. 2005). NHE7 is localized primarily to the *trans*-Golgi network (TGN) (Numata and Orlowski 2001). NHE8 is localized to mid-*trans*-Golgi stacks (Goyal et al. 2003; Nakamura et al. 2005) and is also

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found in intestinal and renal brush border membranes, especially in the intestine in neonatal stages (Becker et al. 2007; Goyal et al. 2005; Xu et al. 2005). The organellar NHEs may mediate the exchange of K^+ for H^+ rather than Na^+ for H^+ under physiological conditions and could be responsible for proton efflux from organelles, contributing to the establishment of the unique organellar luminal pH values (Nakamura et al. 2005). However, the mechanisms underlying the isoform-specific localization of mammalian organellar NHEs and their precise physiological functions are still largely unknown.

Studies to understand how membrane proteins are localized at each compartment, including organelles, have revealed motif sequences that are required for localization. For instance, the binding of PACS1 (phosphofurin acidic cluster sorting protein) to the acidic cluster motif in CIMPR (cation-independent mannose 6-phosphate receptor) is important for the localization of CIMPR to the TGN (Chen et al. 1997; Wan et al. 1998). For NHE1, we have shown that a sequence binding CHP1 (calcineurin homologous protein 1) (Matsumoto et al. 2001) and the formation of an NHE1–CHP1 complex have a dominant role in the localization of NHE1 at the plasma membrane (Matsushita et al. 2007). In this study, we chose NHE6 and NHE7 as a model system to study organelle-specific localization mechanisms because the primary structures of these NHEs are extensively conserved but the proteins show distinct and independent localization. We hypothesized that a small region in the relatively diverse hydrophilic C-terminal halves of these NHEs might contribute to the specific localization. To address this hypothesis, we studied the intracellular localization of chimeric NHE molecules containing regions from both NHE6 and NHE7. These results suggest that a limited region in NHE7 adjacent to the transmembrane domain plays an important role in the distribution of NHE7 in the TGN, distinct from the NHE6 localization at endosomes.

Materials and Methods

Plasmid Construction for Expression in Mammalian Cells

Mammalian expression plasmids for NHE6 tagged with hemagglutinin (HA) at the C terminus and NHE7 tagged with T7 were constructed as described previously (Nakamura et al. 2005). Expression vectors encoding NHE chimeras and mutants were constructed with PCR using appropriate overlapping primers, following methods described previously (Inoue et al. 2001). The expression plasmid for TGN38–EGFP was constructed by inserting EGFP sequence between 58 and 59 amino acids of rat TGN38 into the multiple cloning sites of pEGFP–N3 (Takara Bio, Ohtsu, Japan). The linker sequence between TGN38 and EGFP is TGN38-"PGGG"–EGFP-"GGD"–TGN38.

Mammalian Cell Culture and Transfection

COS-7 cells (Gluzman 1981) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. NRK cells (Duc-Nguyen et al. 1966) were grown in minimum essential medium (MEM) containing 10% fetal calf serum. All media contained 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air. Transfection of plasmid DNAs was performed using Trans IT LT-1 (Mirus, Madison, WI) in accordance with the manufacturer's instructions.

Antibodies

A mouse monoclonal anti-T7 antibody and a rabbit polyclonal anti-HA antibody were purchased from Novagen (Madison, WI) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The antibodies for transferrin receptor (clone H68.4) and TGN38 (clone 2F7.1) were obtained from Zymed (San Francisco, CA). The antibody for syntaxin6 (clone 30) was purchased from BD Bioscience (San Jose, CA). The NHE6 antibody was previously described (Ohgaki et al. 2008).

Fluorescence Microscopy

Cell fixation and immunostaining were performed as described previously (Nakamura et al. 2005). To visualize the localization of internalized Alexa Fluor 488–conjugated transferrin (A488-Tfn; Invitrogen, Carlsbad, CA), cells were incubated at 37°C for 2 h before fixation in serum-free DMEM containing 0.05 mg/ml A488-Tfn. Samples were observed using a BX-51 microscope (Olympus, Tokyo, Japan) with an ORCA-ER1394 digital camera and AQUACOSMOS software (Hamamatsu Photonics, Shizuoka, Japan).

For comparison of the extent of colocalization, the observed cells were classified into three groups according to the extent of overlapping immunostaining with organelle markers: I, high colocalization, almost complete; II, partial colocalization, in extent between I and III; III, non-colocalization, no colocalization at all. Twenty cells were observed in each batch of experiments (transfection, fixation and observation), and three different batches were analyzed (60 cells in total). Data represent the mean ± standard error of the mean (SEM) of three independent experiments.

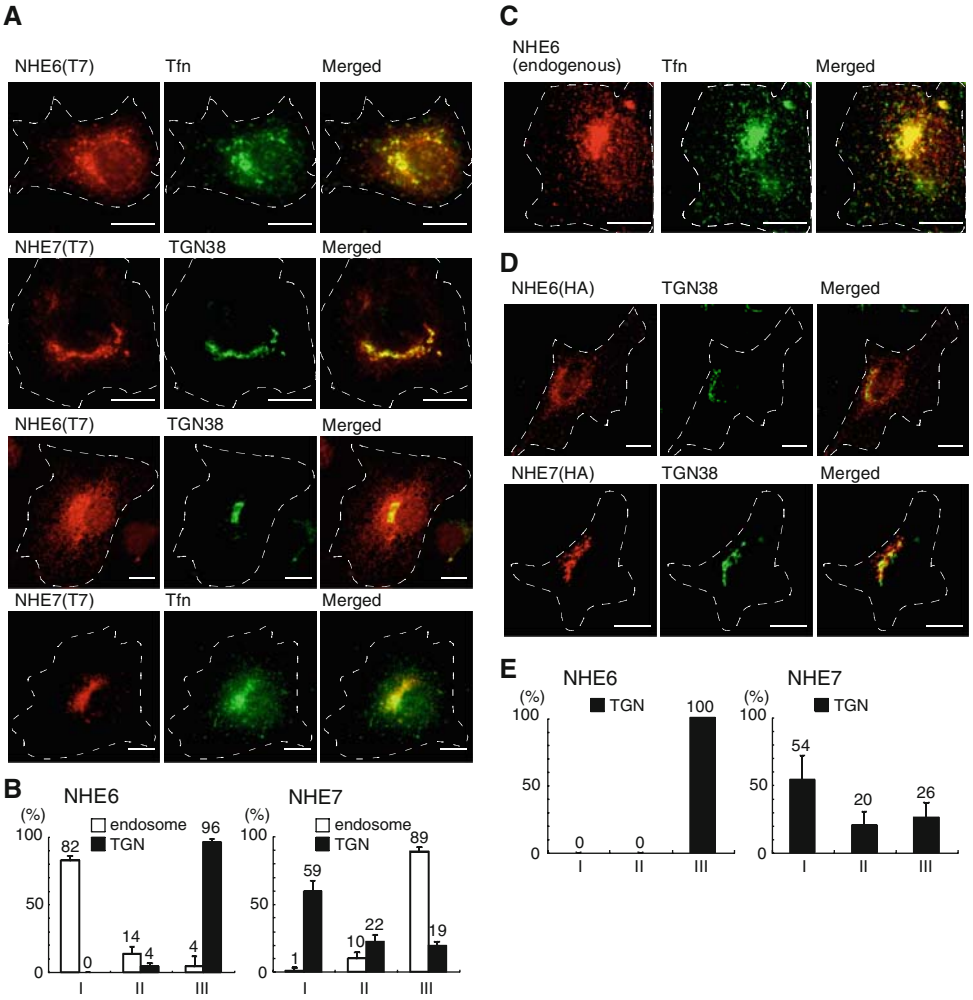

Fig. 1 Intracellular localization of wild-type NHE6 and NHE7. **a** Localization of wild-type NHE6 and NHE7 in COS-7 cells. COS-7 cells were transfected with expression vectors carrying genes for T7-tagged NHE6 or NHE7. Localization of TGN38 and transferrin (Tfn) was visualized with fluorescence microscopy using the fluorescence of transfected TGN38–EGFP and Alexa Fluor 488–labeled Tfn, respectively. T7-tagged wild-type NHEs were detected with an anti-T7 antibody. The shape of each cell is indicated with *dashed lines*. Bars = 10 μm. **b** Cells observed in (**a**) were classified into three groups (I, high colocalization; II, partial colocalization; III, non-colocalization) according to the extent of overlapping immunostaining with endosomes (*white bars*) and TGN (*black bars*). Data represent the mean ± SEM from three independent experiments, where 20 cells were analyzed in each experiment. The mean (%) is also indicated at the top of each bar. **c** Localization of endogenous NHE6. Endogenous NHE6 was detected with an anti-NHE6 antibody. Tfn was visualized as described in (**a**). The shapes of the cells are indicated with *dashed lines*. Bars = 10 μm. **d** Localization of endogenous TGN38 and expressed NHEs in NRK cells. Transiently expressed HA-tagged NHE6 and NHE7 were detected with a rabbit polyclonal anti-HA antibody. The localization of endogenous TGN38 was probed with a mouse monoclonal anti-TGN38 antibody. **e** Colocalization of NHE6 and NHE7 with TGN38. Cells shown in (**c**) were classified into the three groups according to the extent of overlapping immunostaining with TGN (*black bars*). Classification and analysis were performed as described in (**b**)

Results

Intracellular Localization of Wild-type NHE6 and NHE7

We first confirmed the previously reported localization of wild-type NHE6 and NHE7 in recycling endosomes and the TGN, respectively (Brett et al. 2002; Nakamura et al. 2005; Numata and Orlowski 2001). NHEs with a T7 tag at the C terminus were transiently expressed in COS-7 cells, and intracellular localization of the NHE together with a marker protein was analyzed by immunofluorescence microscopy (Fig. 1a, b). NHE6 was located at dot-like structures throughout the cytoplasm and colocalized with fluorescently labeled Tfn, a marker of early and recycling endosomes. NHE7 was found at ribbon-like structures near the nucleus and colocalized with a transiently expressed TGN38–EGFP fusion protein, a TGN marker. These results confirmed the previous reports of localization of these NHEs. Then, we analyzed the colocalization of NHE6 and NHE7 with each

organelle marker. We double-stained cells for an NHE and an organelle marker and categorized the stained cells into the three groups based on the extent of colocalization of the NHE and each marker. In the cells classified as type I NHE and the organelle marker showed almost complete colocalization, while those classified as type III showed none at all. NHE6 was colocalized with Tfn in 82% of the cells (Fig. 1b, white bar in group I) but did not localize with TGN38 in any cells (Fig. 1b, left, black bar in group I). NHE7 was colocalized with TGN38 in 59% of the cells (Fig. 1b, right, black bar in group I), whereas it localized with Tfn in 1% of the cells (Fig. 1b, right, white bar in group I). Thus, NHE6 is predominantly localized at early and recycling endosomes. NHE7 was less specifically localized at the TGN than NHE6 was at endosomes. Some NHE7 might therefore be localized at areas other than the TGN. Using a specific antibody against NHE6, we found that endogenous NHE6 was also located at dot-like structures (Fig. 1c), as exogenously expressed NHE6 was. Therefore, we concluded that exogenous introduction of tagged NHE6 into cells does not affect the intracellular localization. Because we thought that localization of exogenously expressed TGN38 and endogenously expressed TGN38 might differ from each other, we also compared the localization of endogenous TGN38 with the expressed NHEs in NRK cells (Fig. 1d, e). NHE7 was colocalized with TGN38 in 54% of cells, whereas NHE6 was not colocalized with TGN38 in any cells. Thus, we concluded that there is no meaningful difference between the localization of exogenously and endogenously expressed NHE6 and NHE7.

Localization of Chimeric NHE with the Exchange of Transmembrane Domain and Hydrophilic Tail Domain

NHEs are composed of an N-terminal transmembrane domain (TM; ~ 500 amino acids, 10–12 transmembrane helices) and a long C-terminal hydrophilic tail domain (Ct, 150–300 amino acids) (Wakabayashi et al. 2000). The Cts are more variable than the TMs in their amino acid sequences and are thought to be involved in regulating NHE function (Orlowski and Grinstein 2004). Although the organellar-type NHEs (NHE6–9) have shorter hydrophilic domains than the plasma membrane-type NHEs (NHE1–5), the sequences of the hydrophilic domains in NHE6–9 are more divergent (Nakamura et al. 2005). This variety in the Cts of NHE6–9 together with the isoform-specific organellar localization suggests that these hydrophilic domains might be involved in organelle-specific localization of NHEs. Thus, we hypothesized that NHEs contain a sequence in the Ct, but not in the N-terminal TM, which may specify the localization. We designed chimeric proteins by reciprocal replacement of the TM and Ct between the two isoforms

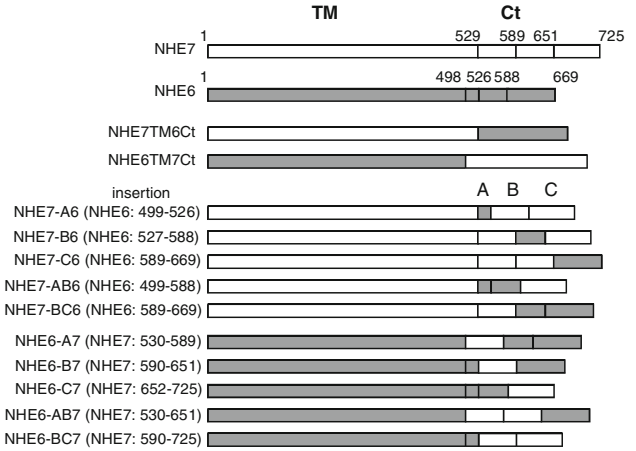

Fig. 2 Schematic illustration of NHE6, NHE7 and chimeric constructs. For TM–Ct chimeras, the transmembrane domain (TM) and the carboxyl terminal domain (Ct) of NHE7 and NHE6 were exchanged (7TM6Ct and 6TM7Ct). The hydrophilic Ct was further divided into three domains (A, B and C) based on the similarity of sequences between NHE6 and NHE7 (see Supplementary Fig. 1). Chimeras were constructed by replacing the indicated region in one isoform with the counterpart region from the other isoform. The regions derived from NHE7 and NHE6 are indicated with *white* and *gray*, respectively

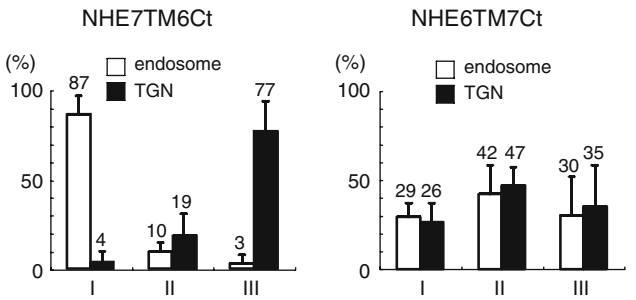

Fig. 3 Intracellular localization of TM–Ct chimeras. COS-7 cells were transfected with the expression vectors for T7-tagged TM–Ct chimeras (NHE7TM6Ct and NHE6TM7Ct). The observed cells were classified into the three groups according to the extent of overlapping immunostaining with endosomes (*white bars*) and TGN (*black bars*). Classification and analysis were performed as described in Fig. 1b

(Fig. 2, NHE7TM6Ct and NHE6TM7Ct). NHE7TM6Ct showed intracellular distribution, like NHE6, and was colocalized with Tfn in 87% of cells and with TGN38 in 4% of cells (Fig. 3). These data suggest that the presence of the NHE6 Ct caused NHE6-like distribution of NHE7TM6Ct. NHE6TM7Ct was colocalized with Tfn and TGN38 in 29% and 26% of cells, respectively, and thus showed distribution at both the TGN and endosome. Although the results were not as drastic as those of NHE7TM6Ct, the presence of the NHE7 Ct increased the localization in the TGN from 0% (seen with wild-type NHE6) to 26% (NHE6TM7Ct) of cells. On the basis of these results, we concluded that the Cts of NHE6 and NHE7 contain structures that determine their intracellular localization.

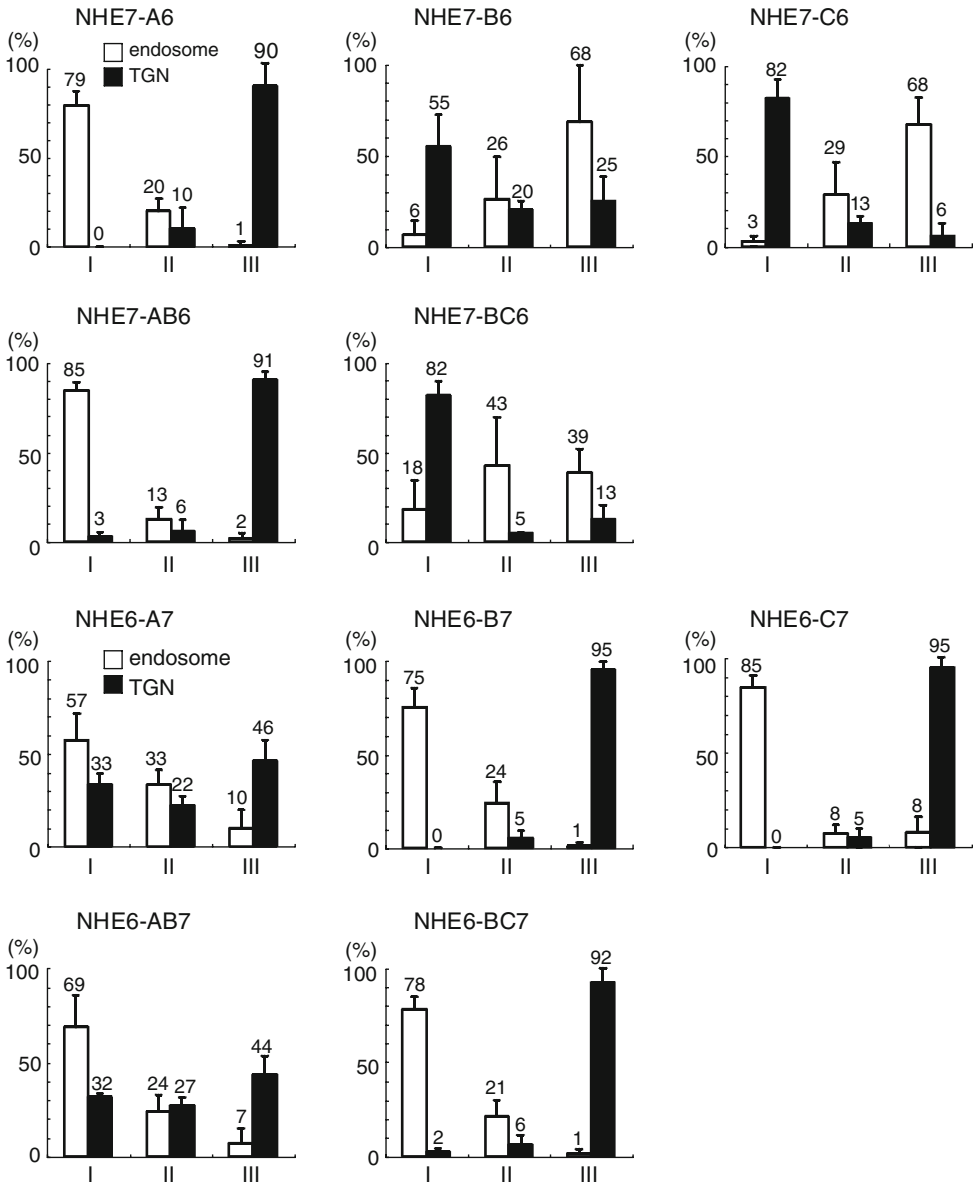

Fig. 4 Intracellular localization of Ct-region chimeras. NHE6–NHE7 chimeras were expressed, and colocalization with TGN38 and transferrin was observed. The observed cells were classified into the three groups according to the extent of overlapping immunostaining with endosomes (*white bars*) and TGN (*black bars*). Classification and analysis were performed as described in Fig. 1b

Localization of Chimeric NHE with Partially Exchanged Hydrophilic C-Terminal Tail

We next tried to narrow down the region within the Ct required for localization. Sequence comparison between the Ct sequences of NHE6 and NHE7 revealed both highly conserved regions and less conserved regions. We divided the Ct into three domains, A, B and C (Supplemental Fig. 1), on the basis of amino acid similarity. The A, B and C domains have 39%, 77% and 41% amino acid identity, respectively. We constructed chimeras in which the A, B or C part from one NHE isoform was exchanged with the corresponding part of the other.

First, we analyzed the NHE7 chimeras containing one or more regions from NHE6. NHE7-B6 (B region derived from NHE6), NHE7-C6 and NHE7-BC6 exhibited similar localization to that of wild-type NHE7. These chimeras were colocalized with TGN38 in 55%, 82% and 82% of cells and with Tfn in 6%, 3% and 18% of cells, respectively (Fig. 4). In contrast, NHE7-A6 and NHE7-AB6 showed similar localization to that of wild-type NHE6. These chimeras were colocalized with Tfn in 79% and 85% of cells, respectively.

Next, we analyzed the NHE6 chimeras containing part of NHE7. NHE6-B7, NHE6-C7 and NHE6-BC7 exhibited similar localization to that of wild-type NHE6. These chimeras were colocalized with Tfn in 75%, 84% and 78% of cells and with TGN38 in 0%, 0% and 2% of cells, respectively (Fig. 4). NHE6-A7 and NHE6-AB7 still colocalized with Tfn in 57% and 69% of cells, respectively,

but showed a marked increase in colocalization with TGN38 (33% and 32% for NHE6-A7 and NHE6-AB7, respectively). Thus, these chimeras were distributed in both TGN and endosomes, similar to NHE6TM7Ct. Together, all of these results suggested that the A regions of these two NHEs are predominantly involved in the localization of each NHE.

Contribution of the Sequences in the Membrane-Proximal Region to Localization of NHE

We found that the A region in the Ct is involved in the localization of NHE6 and NHE7. However, the chimeric experiments have the limitation that the presence of a region from one isoform is always accompanied by the loss of the corresponding region of the original isoform so that two possible explanations (gain of function and loss of function) cannot be distinguished. For instance, although NHE7-A6 showed clear NHE6-like localization, we could not conclude whether the absence of the NHE7-A region or the presence of the NHE6 A-region was the primary factor determining the localization of this chimera. To circumvent this intrinsic problem and to further map the sequence within the A region required for localization, we conducted alanine mutagenesis within the A regions of NHE6 and NHE7 (Fig. 5). First, we constructed four NHE7 mutants, NHE7 (542–547A), NHE7 (569–575A), NHE7 (576–581A) and NHE7 (583–589A), and expressed them in COS-7 cells. These mutant NHE7 s colocalized with TGN38 in 94%, 65%, 71% and 59% of cells, respectively; and none showed substantial colocalization with Tfn (6%, 5%, 6% and 3%, respectively; Fig. 6). We therefore concluded that these mutants are localized mainly in the TGN, as NHE7 is. In contrast to these mutants, two NHE7 mutants, NHE7 (553–559A) and NHE7 (562–568A), were distributed in dot-like structures that were also labeled with Tfn in 86% and 88% of cells, respectively. However, these mutants showed little colocalization with TGN38 (0% and 19%, respectively). Therefore, we concluded that these mutants are localized in the endosome. These results suggest that residues 553–559 and 562–568 of the NHE7 A region are involved in the localization of NHE7 to the TGN. Next, we analyzed eight mutants of NHE6, NHE6 (503–505A), NHE6 (511A), NHE6 (513–515A), NHE6 (515–517A), NHE6 (521–523A), NHE6 (524–526A), NHE6 (511–517A) and NHE6 (518–526A). These mutants colocalized with Tfn in 79%, 88%, 90%, 92%, 90%, 82%, 90% and 82%, respectively, but showed no prominent colocalization with TGN38 (1%, 1%, 1%, 0%, 0%, 2%, 0% and 0%, respectively). These results suggest that the A region of NHE6 does not contribute directly to endosomal localization. Taken together, these mutagenesis analyses suggested that residues 553–559 and 562–568 of the NHE7 A region act as localization motifs that localize NHE7 to the TGN and enable its segregation from NHE6, which is mainly located in early and recycling endosomes.

TGN Localization of NHE7 Mutants Carrying Substitutions in Conserved Residues Putatively Essential for Ion Transport

Previous studies have shown that the forced expression of NHE at a high level perturbs the luminal pH of organelles (Nakamura et al. 2005), raising the possibility that overexpression of NHE localization might affect the luminal pH of organelles. We analyzed the localization of endogenously expressed NHE6 and confirmed that overexpression does not detectably alter its localization (Fig. 1). However, no NHE7 antibody is available that can detect endogenous protein by immunostaining, and we therefore performed all the experiments above with overproduced NHE7. To assess whether the overexpression may have

Fig. 5 Alanine mutagenesis in the juxtamembrane regions of the Cts of NHE6 and NHE7. The juxtamembrane region (A region) in the hydrophilic carboxyl terminal half of NHE7 and NHE6 was partially substituted with alanine residues in regions that differ between NHE7 and NHE6. Six and eight sets of mutated NHEs were constructed for NHE7 and NHE6, respectively. Gaps in sequence alignment and unchanged residues are indicated as "–" and "." respectively

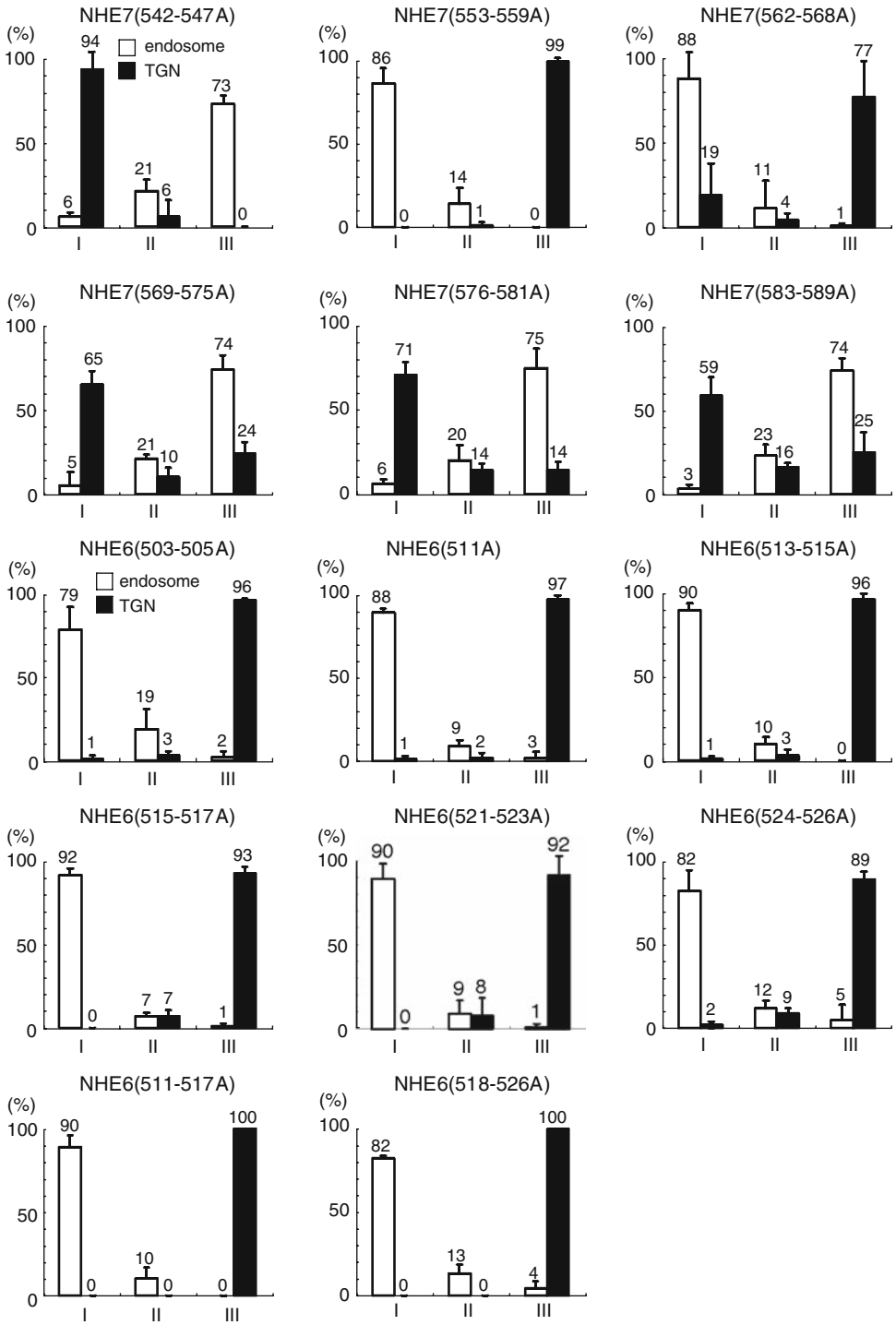

Fig. 6 Intracellular localization of Ct-alanine mutants. Alanine mutants were expressed in COS-7 cells, and colocalization with TGN38 and transferrin was observed. The observed cells were classified into the three groups according to the extent of overlapping immunostaining with endosomes (*white bars*) and TGN (*black bars*). Classification and analysis were performed as described in Fig. 1b

affected the localization by changing luminal pH, we analyzed the localization of a mutant NHE7 with amino acid substitutions in conserved residues that are putatively essential for ion transport. Negatively charged residues involved in the ion transport activity of NHE have been reported for some organellar NHEs (Fafournoux et al. 1994; Murtazina et al. 2001; Nakamura et al. 2005). Previously, we showed that substitution of corresponding residues in NHE8 (NHE8[E220Q/D225 N]) drastically abolishes its transport activity (Nakamura et al. 2005). Also, recently, we observed that overexpression of NHE6 altered pH in NHE6/Tfn-localized endosomes but that NHE6[E255Q/D260 N] did not (Ohgaki et al. 2010). Taken together, it is strongly suggested that the importance of these residues was highly conserved not only in plasma membrane NHE but also in organelle-type NHE, and it is

highly possible that the function of NHE7(E287Q/D292 N) is also abolished. We designed two different types of NHE7 mutant in which these residues were replaced by uncharged residues. In one of the mutants, Glu-287 and Asp-292 were replaced by Gln and Asn, respectively (NHE7[E287Q/D292 N]). In the other mutant, three residues, Asp-263, Glu-287 and Asp-292, were replaced simultaneously (D263 N/E287Q/D292 N). NHE7(E287Q/D292 N) and NHE7(D263 N/E287Q/D292 N) colocalized with TGN38 in 66% and 59% of cells, respectively, whereas these mutants showed no prominent colocalization with Tfn (1% and 12%, respectively; Fig. 7). We therefore concluded that NHE7(E287Q/D292 N) and NHE7(D263 N/E287Q/D292 N) were localized in the TGN, like wild-type NHE7. These results suggested that the localization of NHE7 was not affected by NHE7 function, which was mediated by the conserved charged residues.

Discussion

Various organelle functions depend on proteins integrated into the organelle. Although how the proteins are localized at specific organelles is an important question, the molecular mechanisms underlying this localization after protein synthesis are not well described, especially for proteins integrated into the organelle membranes (Bonifacino and Traub 2003; Hillerehfeld 1995). In this study, we focused on a family of organelle-type NHEs that share extensive similarity in their primary sequences but localize at different organelles. For example, NHE6 and NHE7 localize at recycling endosomes and the TGN, respectively (Brett et al. 2002; Nakamura et al. 2005; Numata and Orlowski 2001). We confirmed these localizations with fluorescence microscopy (Fig. 1). Although surface labeling analysis showed that a minor fraction of NHE6 localized in the plasma membrane of HeLa cells in a previous study (Ohgaki et al. 2008), we could not detect such low-level expression of NHE6 in the plasma membrane of HeLa cells with fluorescence microscopy. Thus, our microscopic observation in this study with COS-7 cells did not conflict with our previous results. In PC12 cells, NHE7 has been reported to be partly distributed in recycling endosomes (Lin et al. 2005) but was not strongly codistributed with recycling endosomes in our assay with COS-7 cells as well as NRK cells (Fig. 1) and the other group's study with CHO cells (Numata and Orlowski 2001). This difference may be due to the difference of cell lines used in the current and previous studies. We had hypothesized that amino acid sequences that differ between these NHEs might be involved in their localization to specific organelles and further studied NHE6 and NHE7 as models of the family. We found that a small portion of the C-terminal half of these NHEs is involved in their localization: 33 residues between residues 535 and 568 in NHE7 (the A region) are required for NHE7 localization to the TGN. How does the

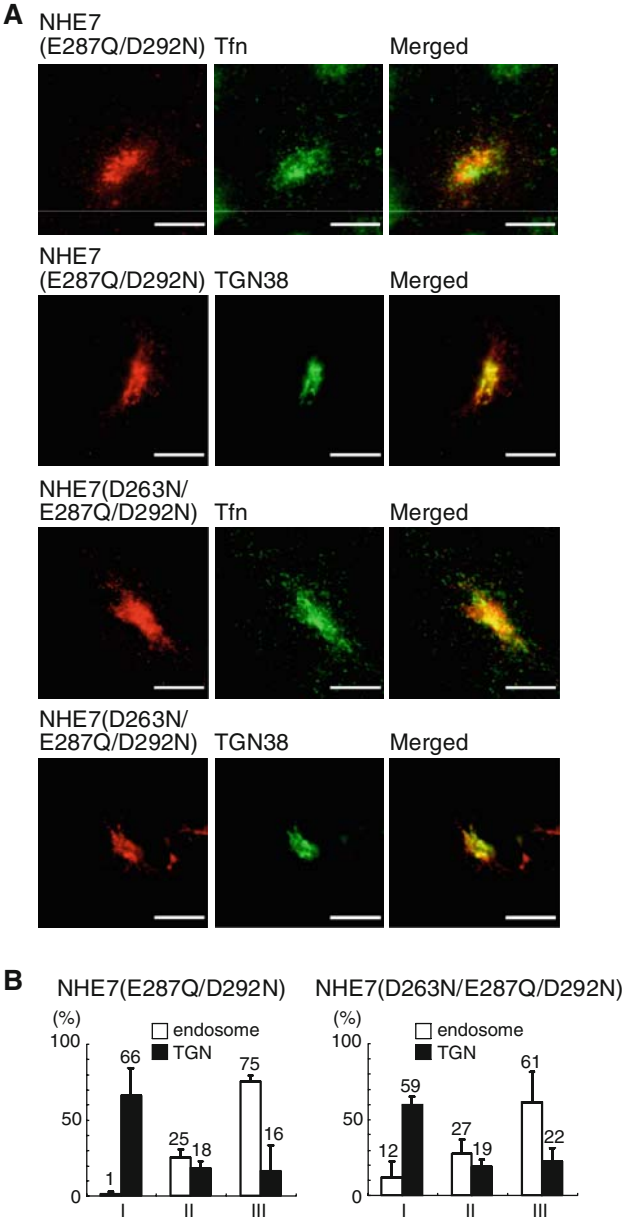

Fig. 7 Intracellular localization of NHE7 with amino acid substitutions in conserved negatively charged residues. The indicated NHE7 mutants with amino acid substitutions in conserved residues putatively necessary for ion transport activity were constructed and expressed in COS-7 cells. **a** COS-7 cells were transfected with the expression vectors for T7-tagged mutant NHE7(E287Q/D292 N) and NHE7(D263 N/E287Q/D292 N). Localization of TGN38 and transferrin (Tfn) was visualized by fluorescence of transfected TGN38-EGFP and Alexa Fluor 488–labeled Tfn, respectively. T7-tagged NHE7 mutants were detected using an anti-T7 antibody. Bars = 10 μm. **b** Cells were observed as indicated in (**a**) and then classified into the three groups according to the extent of overlapping immunostaining with endosomes (*white bars*) and TGN (*black bars*). Classification and analysis were performed as described in Fig. 1b

sequence determine the different localization of NHE7 from NHE6? The result of alanine substitutions on NHE6 suggested that the NHE6 A region did not contain any sequences that directly play a role in the endosomal localization. Thus, we hypothesize that the sequence required for delivery to the endosome is contained in the whole amino acid sequence of NHE6 and, in the case of NHE7, in the sequence outside of the A region. We suggest that the A region of NHE7 has an additional function in segregating NHE7 in the TGN from NHE6 in the endosome, perhaps because it is required for retrieval from the endosome to the TGN. Thus, NHE6 stays in the endosome because it does not contain the A-region sequence.

The sequence of the A region does not exactly match that found in other proteins in databases such as NCBI BLAST, WoLF PSORT and SWISS-PSORT. However, we did find sequences similar to those reported to be involved in protein endocytic trafficking: the YXXΦ-like motif ("YFRV") in residues 556–559 and a sequence possessing many acidic residues in residues 562–568. The YXXΦ motif binds adaptor protein complexes (AP complexes) for endocytosis of receptor proteins (Bonifacino and Traub 2003). This sequence has been found in mannose-6-phosphate receptor protein (M6PR), which is localized at the late endosome and TGN and cycles between the two compartments (Hillerehfeld 1995). The YXXΦ sequence is required for endocytosis, and mutations in the YXXΦ motif of M6PR block cycling and keep the protein in the late endosome (Bonifacino and Traub 2003). Disruption of the YFRV sequence in NHE7 by amino acid replacement might perturb the TGN localization mediated by the AP complex and cause the accumulation of NHE7 in the endosome. The acidic cluster motif contains several consecutive acidic amino acid residues that are recognized by PACS1 (Wan et al. 1998). PACS1 binds to the motif when a serine residue in the motif is phosphorylated by casein kinase II. However, because no essential serine residue was found in residues 553–568 of NHE7, this sequence was not likely to function as an acidic cluster motif and may instead have another, as yet unknown, mechanism.

NHE7 binds SCAMP2, a secretory carrier membrane protein; and shuttling of NHE7 between the Golgi and endosomes may be regulated by this protein (Lin et al. 2005). The sequence of the SCAMP binding site is separate from the A-region sequence of NHE7 (Supplemental Fig. 1). Therefore, the regulatory system involving SCAMP2 may differ from the putative mechanism for TGN localization involving the A region. We found previously that NHE6 binds RACK1 (receptor of activated C kinase) and that knockdown of RACK1 causes accumulation of NHE6 in the endosomes (Ohgaki et al. 2008). The sequence required for RACK1 binding is in the middle of the Ct, separate from the A-region sequence (Supplemental Fig. 1). The RACK1 binding site is conserved in NHE7. Because these binding partners contribute to the TGN/endosome localization, the chimeric proteins containing the NHE6 TM and NHE7 A region (NHE6TM7Ct, NHE6-A7 and NHE6-AB7) still exhibit endosomal localization as well as remarkably increased TGN localization. The increased TGN localization with some NHE7 mutants, including NHE7-B6/BC6/C6, might result from altered binding affinity for RACK1 and SCAMP2, although the precise mechanism is not clear. A recent study showed that NHE7 binds to caveolin in the plasma membrane and endocytoses with clathrin (Lin et al. 2007). These observations suggest that putative mechanisms for cycling among the plasma membrane, TGN and endosome exist for NHE7, although it remains an open question whether the sequence we identified in this study plays this functional role. Although putative binding partners were suggested above, the presence of other binding partners is possible. A survey of putative binding partners will be a crucial step in clarifying the sorting mechanisms involving the A-region sequence.

In this study, we used NHE6–NHE7 chimera formation to identify the sequence required for the specific distribution of these NHEs. We identified the juxtamembrane region as important for the localization. This approach has the advantage of excluding sites required by both NHE6 and NHE7 for localization; in fact, the binding region of RACK1 (Ohgaki et al. 2008) was not detected using this approach. Thus, the approach of using chimeras to detect the sequence required for the specific localization of NHE6 and NHE7 may be useful and applicable for other sets of NHE family members as well.

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