Second mutations rescue point mutant of the Na^+/H^+ exchanger NHE1 showing defective surface expression

Shigeo Wakabayashi*, Tianxiang Pang, Xiaohua Su, Munekazu Shigekawa

Department of Molecular Physiology, National Cardiovascular Center Research Institute, Fujishiro-dai 5, Suita, Osaka 565, Japan

Received 22 September 2000; revised 28 November 2000; accepted 29 November 2000

First published online 12 December 2000

Edited by Gunnar von Heijne

Abstract We studied the effect of point mutation within the putative 11th transmembrane domain (TM11) of the Na^+/H^+ exchanger NHE1 on the plasma membrane expression. Of the 19 mutants tested, two mutants (Tyr454 or Arg458 replaced by Cys) were retained in the endoplasmic reticulum. Interestingly, Y454C was expressed on the cell surface when one of the endogenous cysteine residues at position 8, 133, 421, or 477 was substituted with alanine. Random mutagenesis at Cys8 and its surrounding residues in the cytosolic N-tail revealed that replacement of Cys8 with Ala was the only identified single residue mutation that rescued Y454C. These results suggest that the abnormal conformation of the region of TM11 containing the Y454C mutation is compensated by the second mutation within other domains such as the N-tail. This approach may provide evidence for the interdomain interaction in NHE1.

Key words: Na^+/H^+ exchanger; Plasma membrane targeting; Membrane topology; Transmembrane domain; Site-directed mutagenesis; Revertant

1. Introduction

The plasma membrane Na^+/H^+ exchanger (NHE) is an amiloride-sensitive electroneutral transporter that catalyzes H^+ extrusion coupled to Na^+ influx. The first cloned exchanger isoform NHE1 [1] is ubiquitously expressed and plays an important role in the regulation of intracellular pH (pH_i) and cell volume (see [2,3] for review). To date, at least six different NHE isoforms are known to exist in the mammalian tissues [2,3]. All the NHE isoforms share a common structure that is separated into two major functional domains, the N-terminal membrane domain (~500 amino acids (aa)) involved in amiloride-sensitive Na^+/H^+ exchange and the C-terminal regulatory cytoplasmic domain (~300 aa) [4]. The amino acid sequence of the membrane domain except the N-terminus (~100 aa) is well conserved among NHE isoforms, suggesting that it may form essentially the same tertiary structure in different isoforms. However, the precise transmembrane structure of NHE is still largely unknown.

Based on cysteine accessibility analysis, we have recently presented a novel topology model of NHE1 comprising 12 membrane-spanning segments (see Fig. 1A) [5]. We have provided evidence that the short cytosolic N-tail is retained in the mature protein without cleavage by a signal peptidase. Furthermore, we proposed that the 19 aa region from Gln449 to Tyr467, which was mapped to the extracellular loop based on hydropathy analysis, forms a new transmembrane segment (TM11) in NHE1, because cysteines incorporated into this region were not accessible to biotin maleimide from either side of the membrane, whereas those incorporated at positions at aa 447–448 and aa 468–476 were accessible to the same reagent from the internal and the external sides of the membrane, respectively ([5], and unpublished observation). This putative TM11 contains a glycine-rich motif (^{454}YGGLRGA) that is well conserved among all NHE isoforms and could thus be important for the function of the exchanger.

During our analysis of properties of cysteine-scanning mutants, we discovered that single amino acid substitutions at two positions within the putative TM11 prevent export of NHE1 mutant proteins from the endoplasmic reticulum (ER) and thereby block their surface expression. Furthermore, we observed that introduction of several second mutations could rescue the plasma membrane targeting of such expression-deficient mutant proteins, suggesting functional interactions between the first and the second mutation sites.

2. Materials and methods

2.1. Materials

3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6(3)$) was purchased from Molecular Probes Inc. The amiloride derivative 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) was a gift from New Drug Research Laboratories of Kanebo, Ltd. (Osaka, Japan). ^{22}NaCl was purchased from NEN Life Science Products. Rabbit polyclonal antibody (RP-cd) against human NHE1 was described previously [6]. All other chemicals were of the highest purity available.

2.2. Cell culture and cDNA transfection

The NHE-deficient cell line (PS120) [7] kindly provided by Dr. J. Pouysségur (Nice, France) and the corresponding transfectants were maintained as described [5]. All cDNA constructs were transfected into PS120 cells by the calcium phosphate–DNA co-precipitation technique [4] and stable clones were selected by the repetitive H^+-killing selection procedures [4,8]. In some experiments, pSV-neo was

*Corresponding author. Fax: (81)-6-6872 7485.
E-mail: wak@ri.ncvc.go.jp

Abbreviations: NHE, Na^+/H^+ exchanger; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; EIPA, 5-(*N*-ethyl-*N*-isopropyl) amiloride; PBS, phosphate-buffered saline; aa, amino acid(s); TM, transmembrane segment

PII: S0014-5793(00)02348-6

co-transfected with NHE1 mutant plasmids and single stable clones were isolated after selection with G418 (geneticin).

2.3. Construction of NHE1 mutant plasmids

The plasmids including cDNA encoding NHE1 cloned into mammalian expression vector pECE were described previously [4,5]. The cysteine-free form (substituted with Ala) of NHE1 (designated as Cys-less NHE1) and some of its mutant derivatives were also described [5]. All the constructs used in this study were produced by a polymerase chain reaction (PCR)-based strategy as described previously [4] and their sequences were confirmed by DNA sequencing.

2.4. Mutagenesis with random oligonucleotides

For the random mutagenesis within the N-tail of NHE1, we synthesized an antisense primer 5′-GAAGATCCGATGTGGAGAGAG-NNNNNNNNNCGGAGACCGCAGAACCAT-3′ containing mixed nucleotides (N) corresponding to aa 7–9 of NHE1 and a complementary sense primer 5′-CTCTCTCCACATCGGATCTTC-3′ against a portion of antisense primer. DNA fragments produced by PCR were digested and inserted into *Hin*dIII and *Xho*I sites of a mutant cDNA (Y454C). After bacterial transformation, plasmids were prepared from approximately 13 000 colonies of *Escherichia coli*, transfected into PS120 cells seeded on 30 dishes and stable clones were selected over 3 weeks by repetitive H^+-killing selection procedures. Chromosomal DNA was prepared from cells, subjected to PCR reactions using two primers containing appropriate DNA sequences from the vector (pECE) and NHE1 cDNA. Sequences incorporated into chromosomal DNA were confirmed by sequencing PCR fragments.

2.5. Immunofluorescence microscopy

Cells grown on 60-mm dishes were fixed and permeabilized with cold methanol, and blocked as reported previously [9]. Cells were then incubated with polyclonal anti-NHE1 antibody that was preabsorbed with the permeabilized exchanger-deficient PS120 cells, followed by fluorescent staining with the rhodamine-labeled second antibody. After being washed with phosphate-buffered saline (PBS), cells were further stained for 1 min with 1 μM $DiOC_6(3)$. The stained cells were imaged on a fluorescent microscope equipped with CoolSNAP imaging system (RS Photometrics).

2.6. Surface labeling of NHE1 mutants

Surface labeling of NHE1 mutants expressed in the plasma membrane was performed essentially as described previously [5]. Cells were washed with PBSCM (PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 8.3) and incubated for 1 h at room temperature with 1 mM *N*-hydroxysuccinimidobiotin (NHS-biotin, Pierce) in PBSCM. Cells were solubilized with lysis buffer containing 1% Triton X-100 and biotinylated proteins were recovered with streptavidin-agarose beads (Pierce) as described previously [5].

2.7. Others

Giemsa staining was performed essentially as described previously [1]. Cells were washed with PBS, fixed with 10% formaldehyde and incubated for 20 min in Giemsa solution (5% in water). Dishes were washed with water, dried and photographed. Immunoblot analysis of NHE1 protein was performed as described [6]. The EIPA-sensitive $^{22}Na^+$ uptake activity was measured using pH-clamped cells ($pH_i = 5.6$) with K^+-nigericin as in our previous study [10].

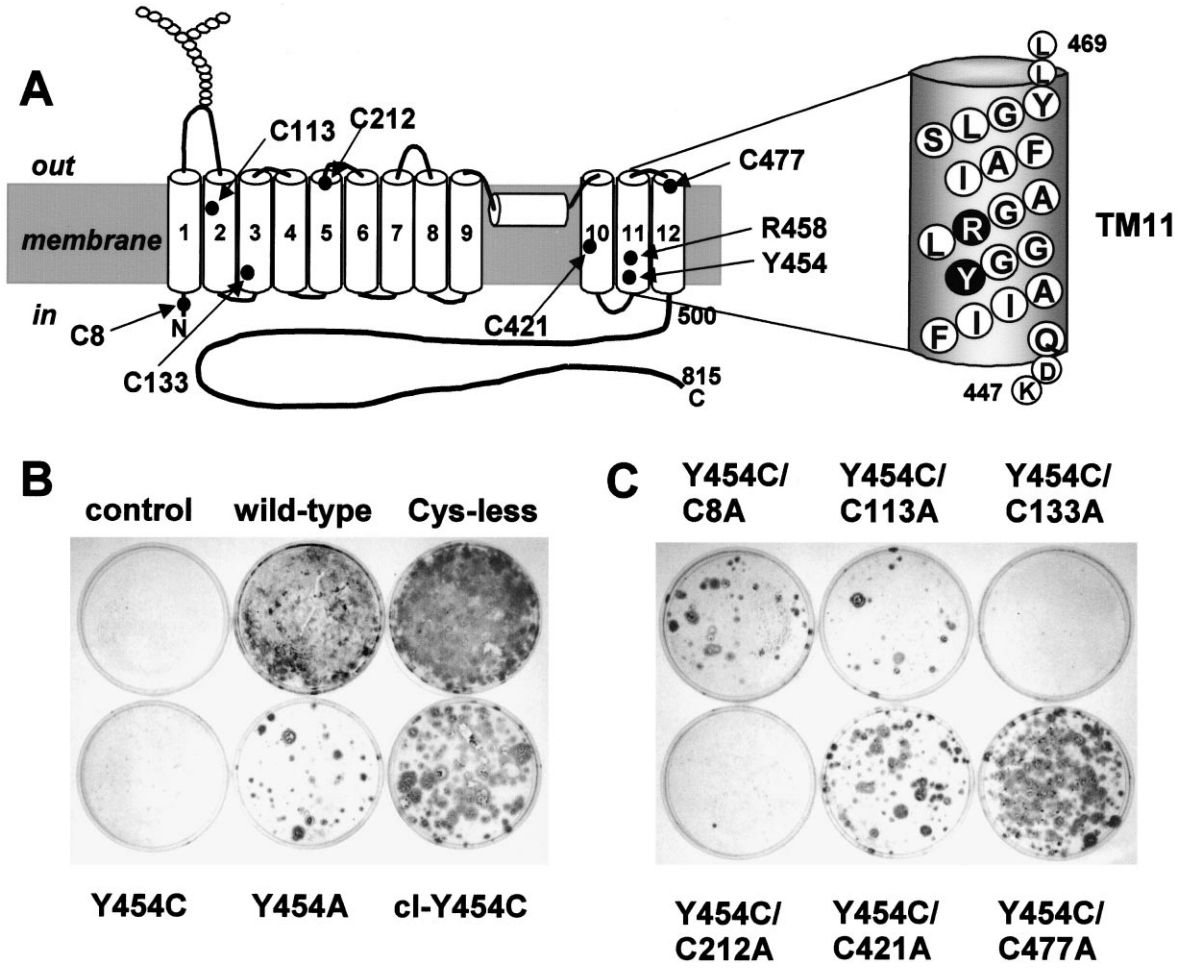

Fig. 1. Stable expression of various mutant NHE1s. A: A topology model of NHE1 reported previously [18]. B and C: Cells seeded on 60-mm dishes were transfected with 10 μg each plasmid and selected over 3 weeks by repetitive H^+-killing selection procedures. Cells were visualized by Giemsa staining. Control: no transfection.

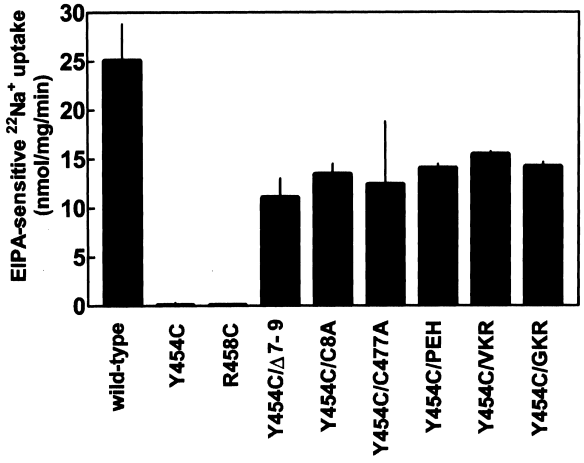

Fig. 2. Na^+/H^+ exchange activity of cells expressing various mutant NHE1s. EIPA-sensitive $^{22}Na^+$ uptake was measured as described under Section 2. Data are means ± S.D. of three determinations.

3. Results and discussion

We introduced 19 cysteine substitutions (one at a time) at positions of aa 447–465 in the putative TM11 of NHE1 (Fig. 1A). We transfected these mutants into exchanger-deficient PS120 cells and subjected them to H^+-killing selection to eliminate cells lacking the Na^+/H^+ exchange activity [8]. We found that cells transfected with Y454C or R458C did not survive this selection procedure (Fig. 1B, data for R458C not shown), indicating that these mutants were not functionally expressed in the plasma membrane. In contrast, cells expressing mutants carrying substitution of Tyr454 with hydrophobic aromatic residues Phe and Trp survived the selection efficiently (data not shown), while those transfected with Y454A survived less efficiently (Fig. 1B). On the other hand, mutants with substitution of Arg458 with basic residues Lys and His survived well (data not shown). Since H^+-killing selection did not produce stable transfectants with Y454C or R458C, we isolated single clones expressing these proteins after subjecting cells to G418 selection. Indeed, these cloned cells did not exhibit any EIPA-sensitive $^{22}Na^+$ uptake (see Fig. 2).

Intriguingly, when Y454C was constructed from the Cys-less NHE1 plasmid, its transfectants survived H^+-killing selection efficiently (Fig. 1B, cl-Y454C), indicating that this mutant is functionally expressed in the plasma membrane. Since all endogenous cysteines had been replaced by Ala in the Cys-less NHE1, we introduced single Cys to Ala substitutions at six positions of endogenous cysteines in the N-terminal membrane domain of the Y454C mutant derived from the wild-type NHE1. As shown in Fig. 1C, Y454C was rescued by substitution of Cys477 with Ala, but less efficiently by the Ala substitution of Cys8, Cys113, or Cys421. In contrast, Y454C was not rescued by the same Ala substitution of Cys133 or Cys212 (Fig. 1C).

The rescue of Y454C by the second mutation C8A is particularly interesting, because Cys8 is far from Tyr454 in the primary structure of NHE1. We introduced random mutations into the DNA segment of Y454C corresponding to positions 7–9 of NHE1 (Ile-Cys-Gly) and transfected the resultant constructs into PS120 cells. We isolated 36 single stable clones by H^+-killing selection and analyzed DNA sequences of 30 plasmids incorporated into chromosomes of these transfectants. Interestingly, 21 clones were found to contain an alanine codon (GCT) at position 8 (Table 1). We did not detect any other single amino acid substitutions, suggesting that rescue of Y454C may occur through replacement of Cys8 specifically with Ala, although we do not know why other alanine codons were not detected. In seven other rescued clones, aa 7–9 were simultaneously replaced with three exogenous amino acids (Table 1). Of note, six of seven clones contained charged amino acids (Glu, Lys, Arg, or His). In order to confirm the roles of these exogenous amino acids in the rescue of Y454C, we produced several Y454C mutants carrying these amino acid substitutions by site-directed mutagenesis (see Fig. 2). We also deleted aa 7–9 (Y454C/Δ7–9) from the Y454C mutant. As expected, these transfectants were able to exhibit relatively high EIPA-sensitive $^{22}Na^+$ uptake activity (Fig. 2). In addition, EIPA concentration dependence of $^{22}Na^+$ uptake was not significantly different between the wild-type NHE1 and a rescued mutant Y454C/C8A; 83.8 ± 8.8 and 102.0 ± 8.9 nM (IC_{50} ± S.E.M. obtained from

Table 1
Results from the random mutagenesis experiment

Nucleotide sequences of recovered clones	Amino acid change	The number of clones
ATC TGT GGC (wild-type)	ICG	
ATC GCT GGC	IAG	21
CCC GAG CAT	PEH	1
GTT AAG AGA	VKR	1
AGG GGA CGC	RGR	1
GGT CCT AAT	GPN	1
GGT AAG CGC	GKR	1
AAA GAG ACC	KET	1
CGG GAG CAG	REH	1
ATC TGT GGC	No change	2

Nucleotide and amino acid sequences for aa 7–9 of the wild-type NHE1 are shown in the second column.

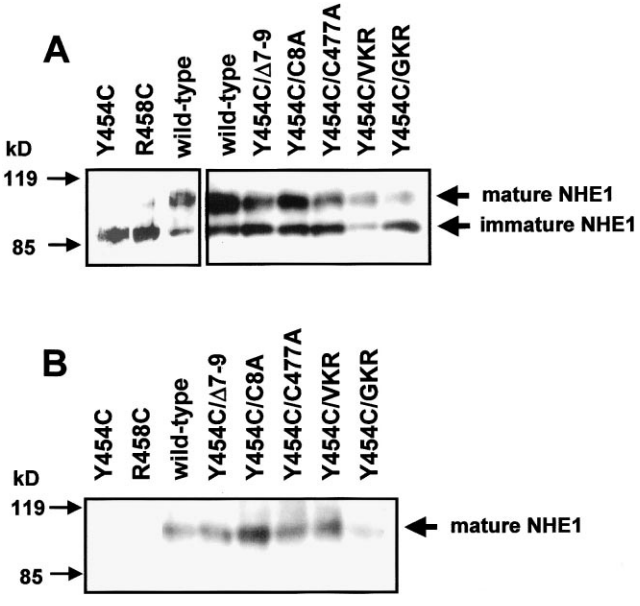

Fig. 3. Immunoblotting and surface labeling of various mutant NHE1s. Cells expressing NHE1 variants were labeled with NHS-biotin, solubilized with the lysis buffer, and then treated with streptavidin-agarose. Total proteins (50 μg) (A) or streptavidin-recovered proteins (B) were separated on 8.5% polyacrylamide gel electrophoresis and visualized by immunoblot with anti-NHE1 antibody.

curve fitting), respectively, suggesting that the structural integrity of EIPA-binding site was preserved upon rescued expression.

Fig. 3A shows immunoblot of expressed proteins stained with anti-NHE1 antibody. We observed two forms of the wild-type exchanger with a high (~110 kDa) or low (~90 kDa) apparent molecular weight, which are thought to be N- and O-linked glycosylated mature protein or high mannose-containing immature protein, respectively [11]. We detected only the immature form in cells expressing Y454C and R458C, whereas we detected both forms in cells expressing mutants carrying additional substitutions (Fig. 3A). Fig. 3B shows results obtained from surface labeling of exchanger proteins with NHS-biotin. We observed the biotinylation of expressed proteins for mutants other than Y454C and R458C.

Fig. 4 shows immunofluorescence staining with anti-NHE1 antibody for cells expressing several NHE1 variants. Most of Y454C and R458C proteins were localized in the intracellular membranes that were stained with the ER marker $DiOC_6(3)$, but they were not detected in the plasma membrane (Fig. 4). In contrast, the wild-type NHE1 and the rescued mutants Y454C/C8A and Y454C/C477A were expressed in the plasma membrane as well as in the ER. As reported previously [12], we often observed the accumulation of NHE1 in the peripheral regions of cells that are probably focal adhesions.

In this study, we found that single cysteine substitution at Tyr454 or Arg458 strongly inhibits the surface expression of NHE1 by apparently preventing its export from the ER. These residues are located within a segment corresponding to Gln449–Tyr467 which was predicted to form a membrane-spanning segment (TM11) based on our topological analysis of NHE1 as briefly discussed in Section 1 (see also Fig. 1). This putative TM11 is predicted to contain a non-α-helical glycine-rich motif (^{454}YGGLRGA) and the C-terminal α-helix (^{461}IAFSLGY). The former non-α-helical region in the putative TM11 might be important for ion transport by the NHE, as similar helix-unwound regions near Gly residues in the membrane have been shown to form Ca^{2+}-binding sites in the recently reported crystal structure of the SR Ca^{2+}-ATPase [13]. Consistent with this view, this glycine-rich motif is highly conserved among mammalian NHE isoforms and NHE homologs from lower animals and appears to be important for the exchanger function as suggested by our recent finding that several point mutations incorporated into this region greatly influence pH_i dependence of Na^+/H^+ exchange (Wakabayashi, S. et al., unpublished observation).

As stated above, we found that the Y454C and R458C mutant proteins were mostly retained in the ER membrane (Fig. 4). This suggests that these mutant proteins may be properly translocated to the ER membrane. The ER has the ability to recognize, retain and degrade misfolded or incom-

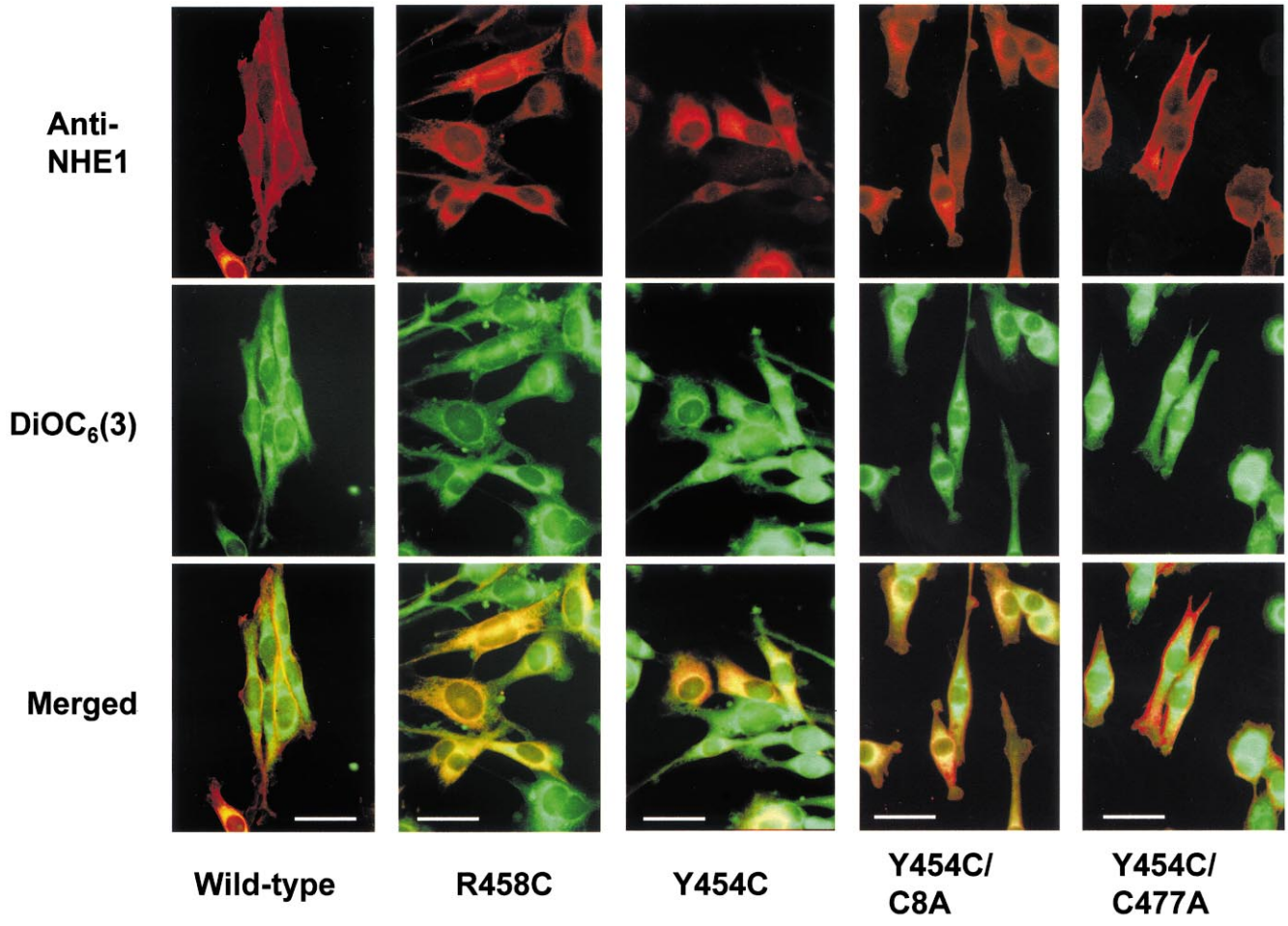

Fig. 4. Immunofluorescence microscopy of cells expressing various mutant NHE1s. Cells grown on 60-mm dishes were stained with anti-NHE1 antibody followed by incubation with rhodamine-labeled second antibody as described under Section 2. Cells were further stained for 1 min with the ER marker $DiOC_6(3)$. Two images for rhodamine and $DiOC_6(3)$ were merged with the aid of imaging software. Bars: 30 μm.

pletely folded proteins and thereby ensures the structural integrity of synthesized proteins [14,15]. As suggested from the study of the processing of several proteins, the quality control system in the ER appears able to recognize subtle structural differences in proteins [15]. Although it is currently not known whether the Y454C and R458C proteins are still able to catalyze Na^+/H^+ exchange, the quality control mechanisms in the ER clearly recognized abnormal conformations of these mutant proteins. However, the scope and nature of the structural alteration in the NHE1 protein caused by Y454C or R458C mutation are not clear at present, although many misfolded proteins have been shown to inappropriately expose hydrophobic surfaces that are normally buried in the protein's interior or at the interface with other subunits [17,18]. The heat shock protein 70 may possibly be involved in the processing of NHE1 protein in the ER, because these proteins have been shown to interact with each other [16].

We found that several second point mutations (C8A, C113A, C421A, and C477A) at least partially rescued the surface expression of Y454C, suggesting that these mutations somehow rearrange the structure of Y454C. An example for such revertants was reported in CFTR in which expression of Δ508 was rescued by the second mutation at a nearby residue (R553M or R553Q) [19]. According to our topology model of NHE1, Cys421 and Cys477 are mapped to TMs 10 and 12 neighboring TM11, respectively, while Cys113 is mapped to TM2 (see Fig. 1). These endogenous cysteines are not accessible to biotin maleimide from the aqueous environment [5]. The simplest interpretation of the present results would be that the regions of TMs 2, 10 and 12 containing the second mutation sites may interact directly with the region of TM11 containing Tyr454, thereby suppressing the effect of the Y454C mutation. However, it is also possible that the correction of misfolding in the mutant protein may occur through a long-range structural alteration involving interactions between distant TMs.

In contrast to cysteines within putative TMs, Cys8 is mapped to the cytosolic N-tail which is far from Try454 in the primary structure of NHE1. Cys8 is accessible to biotin maleimide from the cytosolic side of the plasma membrane [5]. Random mutagenesis revealed that a change of Cys to Ala is the only single amino acid substitution at position 8 in the N-tail that permits the rescue of plasma membrane targeting of the Y454C mutant. These data suggest that misfolding of NHE1 protein caused by the Y454C mutation was corrected at least partially by a relatively specific structural alteration in the N-tail, allowing some of this mutant protein to escape the ER quality control mechanisms. Thus, the C8A mutation in the N-tail of NHE1 induces a change in the conformation of the Y454C mutant. Recently, the short N- or C-tail region has been reported to be important for the correct folding and proper membrane targeting of the SR Ca^{2+}-ATPase [20] or the Na^+/Ca^{2+} exchanger [21], respectively. In the crystal structure of the SR Ca^{2+}-ATPase [13], a significant portion of the N-tail appears to be closely associated with the actuator domain of the ATPase that is considered to be involved in the transmission of major conformational changes. On the basis of these findings and the specific requirement of Ala at position 8 for the rescue of the plasma membrane targeting of the mutant protein (Table 1), we speculate that the N-tail of NHE1 may interact directly with the region of TM11 containing Tyr454. However, we do not exclude the possibility that two regions only interact randomly during protein folding. The amino acid sequence of the N-tail is highly variable among NHE isoforms (see [2,3] for review) and the N-tails of NHE isoforms except NHE1 may be cleaved off during protein processing, suggesting that the interaction between N-tail and TM11 could be specific for NHE1.

In summary, we found that the surface expression of NHE1 is prevented by single amino acid substitutions within the putative TM11, which is rescued by second amino acid point mutations within other TMs and at the cytosolic N-tail. The second mutations corrected the abnormal conformations induced by the first mutation, thereby permitting NHE1 mutant protein to at least partially escape quality control in the ER. Although the underlying mechanism for this phenomenon remains unclear, this work provides the first evidence supporting the interdomain interaction in the membrane domain of NHE1.

Acknowledgements: This work was supported by Grants-in-Aid for Scientific Research 09680642 and 10470013 from the Ministry of Education, Science, and Culture of Japan, and grants from CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST), and the Uehara Memorial Foundation.

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