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Oligomerization of the *Saccharomyces cerevisiae* Na^+/H^+ antiporter Nha1p: Implications for its antiporter activity

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Abstract

The Na^+/H^+ antiporter (Nha1p) from the budding yeast *Saccharomyces cerevisiae* plays an important role in intracellular pH and Na^+ homeostasis. Here, we show by co-precipitation of differently tagged Nha1p proteins expressed in the same cell that the yeast Nha1p l forms an oligomer. In vitro cross-linking experiments then revealed that Nha1p-FLAG is present in the membranes as a dimer. Differently tagged Nha1p proteins were also co-precipitated from *sec18-1* mutant cells in which ER-to-Golgi traffic is blocked under non-permissive temperatures, suggesting that Nha1p may already dimerize in the ER membrane. When we over-expressed a mutant Nha1p with defective antiporter activity in cells that also express the wild-type Nha1p-EGFP fusion protein, we found impaired cell growth in highly saline conditions, even though the wild-type protein was appropriately expressed and localized correctly. Co-immunoprecipitation assays then showed the inactive Nha1p-FLAG mutant interacted with the wild-type Nha1p-EGFP protein. These results support the notion that Nha1p exists in membranes as a dimer and that the interaction of its monomers is important for its antiporter activity.

Keywords: Yeast Na^+/H^+ antiporter; Salinity resistance; Oligomerization; Co-precipitation; In vitro cross-linking; Dominant negative effect

1. Introduction

All living cells maintain intracellular Na^+ concentrations and pH at certain levels [1–3]. Na^+/H^+ antiporters are ubiquitous membrane proteins found in the plasma membrane as well as in the organelle membranes of cells in species ranging from bacteria to humans and higher plants [1–15]. These proteins catalyze the exchange of monovalent cations (Na^+ or K^+) and H^+ across the membranes, thereby regulating cytoplasmic and organelle cation concentrations and pH [1–15]. As such, they play an important role in diverse physiological processes, including the control of the cell cycle and cell proliferation [16,17], transepithelial Na^+ movement [18], salt tolerance [19,20], vesicle trafficking, and biogenesis [21].

The budding yeast *Saccharomyces cerevisiae* has a plasma membrane type Na^+/H^+ antiporter encoded by the *NHA1* gene [22]. It belongs to a family of fungal plasma membrane Na^+/H^+ antiporters that includes Sod2p [7], Zsod2p [23], Cnh1p [24], CtNha1p [25], HaNha1p [25], and AnNha1p [25] from *Schizosaccharomyces pombe*, *Zygosaccharomyces rouxii*, *Candida albicans*, *Candida tropicalis*, *Hansenula anomala*, and *Aspergillus nidulans,* respectively. *S. cerevisiae* Nha1p participates in regulating the intracellular concentrations of Na^+ or K^+ by using the electrochemical H^+ gradient across the plasma membrane; this eliminates the excess cations from the cytosol [26–28]. At higher pH values, Nha1p helps maintain the intracellular pH homeostasis by using the outward K^+ or Na^+ gradient [27]. Interestingly, Nha1p also appears to be involved in cell-cycle regulation [29,30].

Nha1p consists of two domains. One is an integral membrane domain containing 12 transmembrane segments while the other is a hydrophilic tail domain that may be cytosolic [22,25], similar to what has been found for the mammalian NHEs [8–15]. Accumulating evidence suggests that the hydrophilic tail domain in both yeast Nha1p and the mammalian NHEs mediate interactions with other proteins that regulate the antiporter activity of Nha1p and the NHEs [25,31–40].

Abbreviations: Nha1p, Na^+/H^+ antiporter 1 protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DDM, *n*-dodexyl β-D-maltoside; DSS, disuccinimidyl suberate; *o*-PDM, *N,N′*-(*o*-phenylene) dimaleimmide; WT, wild-type

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The Na^+/H^+ antiporter proteins from bacteria, yeast and mammals are functionally the same, although the mutual direction of Na^+ and H^+ Na^+/H^+ current does differ between the organisms. However, despite these functional similarities, the primary sequences of these proteins show relatively little homology. Consequently, the structural basis needed for ion transport is unclear at present, and it is not known whether universal ion exchange mechanisms exist. It has been noted that bacterial and mammalian antiporters form stable dimers [41–46]. However, the functional significance of this dimer formation is not thoroughly understood; moreover, it has not been shown that Nha1p also undergoes dimer formation. Here we show that the yeast Nha1p indeed also forms a dimer; we also show that this dimer formation is important for the antiporter function of the protein. Given that dimerization is also a feature of bacterial and mammalian antiporters, we argue that Na^+/H^+ antiporters universally adopt and require a dimerized structure.

2. Materials and methods

2.1. Yeast strains and media

The *S. cerevisiae* strains used in this study are SK5 (*ena1*::*HIS3*::*ena4*Δ *nha1*Δ::*LEU2*) [25] and Mtsec18 (*sec18-1*). SK5 is a derivative of G19 (a W303-1B derivative: *MATa ade2-1 his3-11/15 leu2-3/112 trp1-1 ura3-1 can1-100 ena1*::*HIS3*::*ena4*Δ) [47] and bears a deletion of the *NHA1* gene; G19 was kindly provided by Dr. A. Rodriguez-Navarro (Ciudad University, Spain). Mtsec18 is derived from YPH499 (*MATa ura3-52 lys2-80 ade2-10 trp1-Δ63 his3-Δ200 leu2-Δ1*) and has a defect in its secretory mechanism due to a temperature-sensitive mutation in its *SEC18* gene; it was a kind gift from Dr. Yoshinori Ohsumi (National Institute of Basic Biology, Okazaki, Japan). All yeast strains were routinely cultured at 30 °C in YPD medium (1% yeast extract, 2% peptone and 2% glucose) [48] or in SCD medium (0.17% yeast nitrogen base without ammonium sulfate and amino acids, 0.5% ammonium sulfate, 0.5% casamino acids, and 2% glucose) [48] supplemented with appropriate nucleotides and amino acids. The *Escherichia coli* strain JM109 [49] was used to propagate the plasmids and to express various proteins. *E. coli* cells were cultured in L broth at 37 °C with an appropriate antibiotic for the selection of transformants, as described previously [50].

2.1.1. Plasmids

To express *NHA1* under the control of the *NHA1* promoter in pRS314 [51], we constructed pRS314-NHA1 by inserting a 4.7-kbp *Xba*I fragment containing the *NHA1* locus derived from the cosmid 2A16 [25] into the *Xba*I site of pRS314. To construct the plasmid pRS314-NHA1-EGFP, which expresses Nha1p tagged at its C-terminus with the enhanced green fluorescent protein (EGFP) [52], PCR was performed using pEGFP-N3 (Clontech) as the template and the oligonucleotide primers #1 and #2, which bear *Sph*I and *Sal*I sites (Table 1). The EGFP fragment between the *Sph*I and *Sal*I sites was then cloned into *Sph*I and *Sal*I sites of pRS314-NHA1 generated by PCR using the oligonucleotide primers #3 and #4. The pKT10-NHA1-FLAG and p520-NHA1-6xHis plasmids were constructed to over-express *NHA1* in yeast under the control of the *GAP* promoter in the yeast expression vectors pKT10 [53] and p520 (a kind gift from Dr. Yoshinobu Kaneko, Osaka Univ.), which are *ura*$^+$ and *trp*$^+$ multi-copy vectors, respectively. For this purpose, the *NHA1* ORF fragment was amplified from yeast genomic DNA by PCR using oligonucleotide primers #1 and #5 and then digested with *Kpn*I and *Sph*I. The PCR product (*Kpn*I–*Sph*I) was then inserted together with the FLAG (#6 and #7) (Table 1) or 6xHis (#8 and #9) (Table 1) tag adaptor (*Sph*I–*Sal*I) between the *Kpn*I and *Sal*I sites of pKT10 and p520. Amino acid substitutions of Nha1p were mainly generated by PCR using oligonucleotide primers #10–#11, #12–#13, and #14–#15 (Table 1); these reactions yielded the Asp-145 to Asn, Asp-266 to Asn and Ser-433 to Ala substitutions, respectively. In addition, a NHA1 mutant bearing the Ser-27 to Tyr substitution was isolated by a random mutagenesis study. The plasmid pYES2-NHA1-EGFP was constructed to express the *NHA1-EGFP* fusion under the control of the *GAL1* promoter. For this, the *NHA1* ORF (*Kpn*I–*Sph*I) and the EGFP fragment (*Sph*I–*Sal*I) were inserted between the *Kpn*I and *Xho*I sites of the yeast expression vector pYES2 (Invitrogen).

Table 1
Oligonucleotide primers used in this study

Oligo DNAs	Sequence (5′–3′)
#1	5′-TCT$_{(i)}$GCATGCCCTTATTGAGACCA-3′
#2	5′-ACGC$_{(ii)}$GTCGACTTTATTAATACACAAAGATATA-3′
#3	5′-ACAT$_{(i)}$GCATGCCCATGGTGAGCAAGGGCGAGG-3′
#4	5′-CC$_{(ii)}$GTCGACTTACTTGTACAGCTCGTCC-3′
#5	5′-TACT$_{(iii)}$GGTACCATGGCTATCTGGGAGCAA-3′
#6	5′-CAGGACTATAAAGACGACGACGACAAATGAG-3′
#7	5′-TCGACTCATTTGTCGTCGTCGTCTTTATAGTCCTGCATG-3′
#8	5′-CATCATCATCATCATCATTAATG-3′
#9	5′-TCGACATTAATGATGATGATGATGATGCATG-3′
#10	5′-CCGCAACAAATCCTATTCTGGCGC-3′
#11	5′-GCGCCAGAATAGGATTTGTTGCGG-3′
#12	5′-GGGTGTGAATGACCTATTGGTATC-3′
#13	5′-GATACCAATAGGTCATTCACACCC-3′
#14	5′-AGTTCATGGCTCGGCTGTTGCAATCAT-3′
#15	5′-ATGATTGCAACAGCCGAGCCATGAACT-3′

Restriction sites of *Sph*I, *Sal*I and *Kpn*I are underlined and marked as (i), (ii) and (iii), respectively.

2.2. Salinity-resistant cell-growth assay on agar plates

Yeast cells carrying various *NHA1* constructs were grown in SD medium at 30 °C to the logarithmic phase and then diluted serially and spotted onto SD (0.17% yeast nitrogen base without ammonium sulfate and amino acids, 0.5% ammonium sulfate, and 2% glucose, 20 mM MES–Tris, pH 5.5) plates supplemented with 0, 0.2, 0.4 or 0.6 M NaCl. The plates were incubated for 4 days at 30 °C.

2.3. Fluorescence microscopy

Yeast cells expressing Nha1p-EGFP were grown in SD medium at 30 °C to the logarithmic phase and observed under a fluorescence microscope (BX51, Olympus, Tokyo, Japan) equipped with a NIBA filter. Images were recorded using an ORCA-ER1394 digital camera (Hamamatsu Photonics, Hamamatsu City, Japan).

2.4. Expression of the Nha1p-EGFP fusion protein

Yeast cells carrying pYES2-NHA1-EGFP were grown in SC+Raffinose medium (0.17% yeast nitrogen base without ammonium sulfate and amino acids, 0.5% ammonium sulfate, 0.5% casamino acids, 2% raffinose) to the early logarithmic phase. The expression of Nha1p-EGFP from the *GAL1* promoter of the pYES2 plasmid was induced by adding galactose at a final concentration of 2%, followed by additional incubation for 4 h.

2.5. Co-precipitation, cross-linking and immunoblotting

For the pull-down assay with Ni-NTA agarose, crude membranes obtained from total cell lysates by centrifugation (100,000×g) were solubilized in buffer containing 1% *n*-dodecyl β-D-maltoside (DDM) (DOJINDO, Japan), 25 mM Tris–HCl, pH 8.0, 250 mM NaCl, 20% glycerol, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin. The solubilized proteins were incubated overnight with Ni-NTA agarose beads (Qiagen) at 4

°C. For immunoprecipitation with anti-FLAG antibody, crude membranes were solubilized with IP buffer (1% DDM, 25 mM sodium phosphate, pH 7.4, 250 mM NaCl, 20% glycerol, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin). The 1% DDM-solubilized extracts were then incubated overnight with anti-FLAG M2 affinity gel beads (Sigma) at 4 °C. After extensive washing of the beads, the precipitates were resuspended in SDS sample buffer and heated at 37 °C for 5 min prior to SDS-PAGE and immunoblotting with anti-penta-His, anti-FLAG M2, anti-GFP, anti-HA, or anti-Gas1p (kindly provided by Dr. Ryogo Hirata, Institute of Physical and Chemical Research (RIKEN), Japan) antibodies, as described previously [54]. Immunoreactive bands were visualized by means of the ECL Plus Western Blotting Detection System (Amersham Biosciences).

For in vitro cross-linking experiments, crude membranes were prepared from yeast cells transformed with pKT10-NHA1-FLAG or its derivatives by centrifugation (100,000×*g*), and then resuspended in 25 mM sodium phosphate, pH 7.4, 250 mM NaCl, 20% glycerol, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin. The membranes (5 mg/mL) were then incubated for 30 min at room temperature with various concentrations of the cross-linker reagents disuccinimidyl suberate (DSS) (PIERCE) or *N*,*N*′-(*o*-phenylene) dimaleimide (*o*-PDM) (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO). The reaction was stopped with Tris–HCl, pH 7.6 (final concentration; 25 mM) or dithiothreitol (DTT) (final concentration; 10 mM). After an additional incubation for 15 min at room temperature, the membranes were subjected to SDS-PAGE and immunoblotting with anti-FLAG antibody.

2.6. Measurement of intracellular Na^+ and K^+ concentrations

Yeast cells were cultured in 25 mL SD medium containing 0.3 M NaCl at 30 °C to the logarithmic phase of growth (OD_{600}=0.6) for approximately 6 h. Harvested cells were trapped on membrane filters (0.45 μm, Advantec) and washed with 10 mM $MgCl_2$ solution containing sorbitol to maintain isotonicity. The cells on the filter were then soaked in distilled water and boiled for 15 min. Cell debris was removed by centrifugation, after which the supernatants were subjected to optical emission spectrophotometry (Perkin Elmer, Optima 3000XL), as described previously [25]. The total amount of protein in the cell extracts after boiling was measured by the method of Lowry et al. [55]. The $Na^+/(Na^+ + K^+)$ ratios (%) were calculated by dividing the total intracellular Na^+ plus K^+ concentration (μmol/μg protein) by the intracellular Na^+ concentration (μmol/μg protein).

3. Results

3.1. EGFP-tagged Nha1p is functional in SK5 cells

SK5 is a *S. cerevisiae* strain that bears a deletion of the *NHA1* gene (*ena1-4*Δ *nha1*Δ). To test whether over-expressed Nha1p and EGFP-tagged Nha1p are functional, they were expressed in SK5 cells. A serial dilution series of each culture was spotted on agar plates and analyzed for salinity resistance in high NaCl conditions (Fig. 1A). SK5 cells expressing wild-type Nha1p grew competently on agar plates with concentrations of NaCl as high as 0.2–0.6 M, whereas yeast cells carrying the empty vector exhibited extensive sensitivity to this level of salinity. The expression of the Nha1p-EGFP fusion protein from either a low- or high-copy vector also reversed the salt sensitivity of the SK5 cells. However, when Nha1p-EGFP was over-expressed from the high-copy vector, there was some cell growth retardation, even on agar plates lacking NaCl, suggesting that over-expression of Nha1p is somewhat toxic for the cells. Fluorescence microscopy revealed that the Nha1p-EGFP fusion proteins expressed from both the low- and high-copy vectors localized to the plasma membrane (Fig. 1B). These results indicate that fusion of EGFP to the C-terminus of Nha1p has essentially no effect on the function of Nha1p.

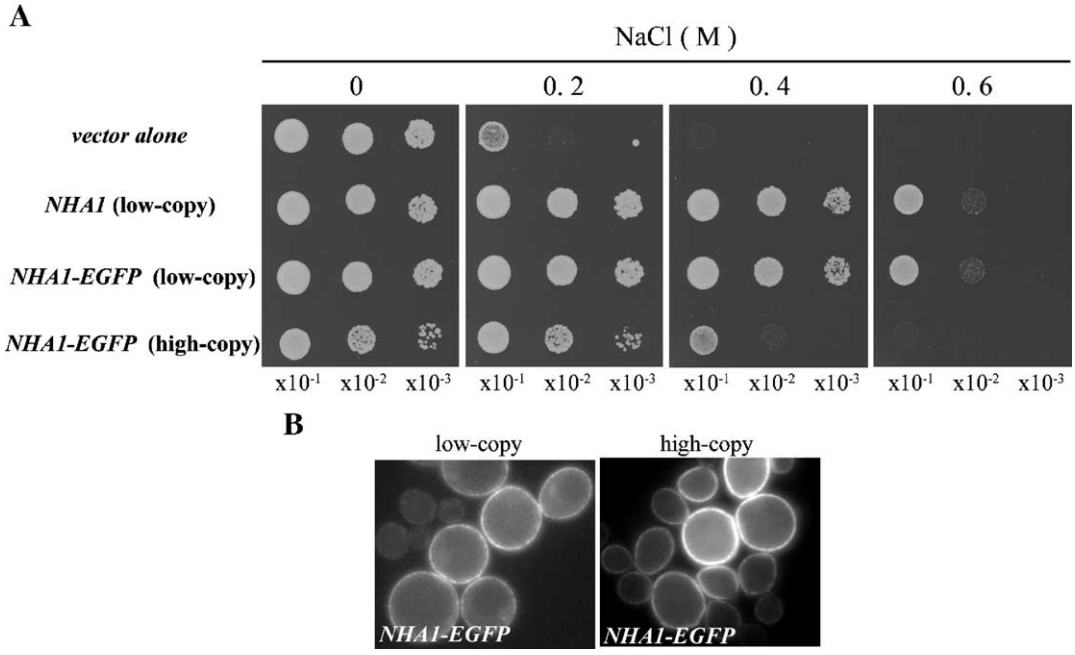

Fig. 1. The EGFP-tagged form of NHA1 is functional in SK5 cells. (A) Salinity-resistant growth of SK5 cells transformed with various *NHA1* constructs. The transformed SK5 cells were grown to the logarithmic phase, diluted serially and then spotted onto SD plates, pH 5.5 supplemented with NaCl as indicated. The plates were incubated at 30 °C for 4 days. (B) Cellular localization of the Nha1p-EGFP fusion proteins. SK5 cells transformed with *NHA1-EGFP* constructs were grown to the logarithmic phase and then observed by fluorescence microscopy.

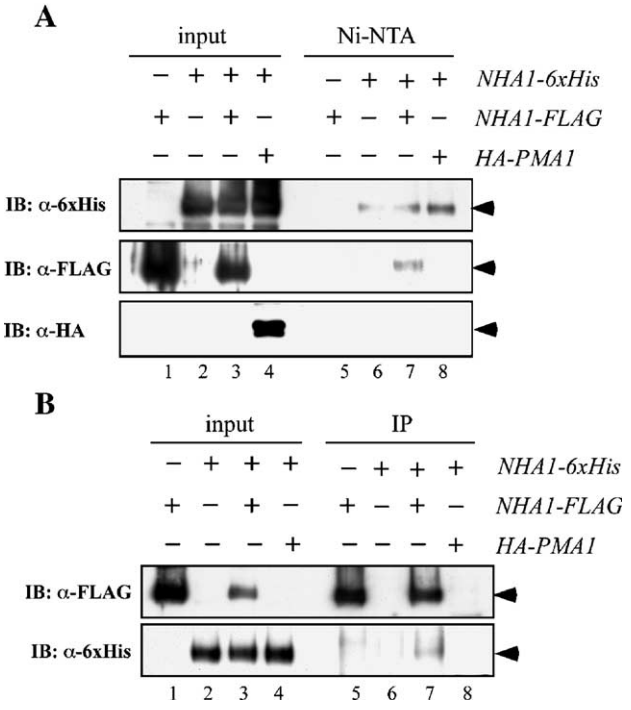

Fig. 2. Detection of Nha1p oligomerization by co-precipitation analyses. (A) The crude membranes from transformed yeast cells were solubilized by DDM and the supernatants (input) after centrifugation were subjected to precipitation by Ni-NTA agarose. The specific Ni-NTA precipitates were then subjected to immunoblotting analysis after separation by SDS-PAGE. The antibodies used were the anti-penta-His, anti-FLAG M2, and anti-HA monoclonal antibodies. (B) The supernatants (input) prepared from DDM-solubilized membranes by centrifugation were subjected to immunoprecipitation with anti-FLAG M2 agarose affinity gels. The specific precipitates (IP) were then subjected to immunoblotting analysis after separation by SDS-PAGE. The antibodies used were the anti-FLAG M2 and anti-penta-His monoclonal antibodies.

3.2. Oligomerization of Nha1p

To determine whether Nha1p molecules oligomerize in physiological conditions, we performed a co-precipitation experiment with cell lysates that co-expressed Nha1p bearing either of two tags, namely, Nha1p-6xHis and Nha1p-FLAG. Thus, the membranes from co-transformed SK5 cells were first prepared by ultra-centrifugation (100 k×g) and solubilized with DDM. The solubilized extracts (input) were then specifically precipitated with Ni-NTA agarose, after which the precipitates were resolved by SDS-PAGE and analyzed by immunoblotting using anti-FLAG and anti-penta-His antibodies. Nha1p-6xHis and Nha1p-FLAG were appropriately detected in the DDM-solubilized extracts (input) of cells expressing either protein alone (Fig. 2A, lanes 1 and 2) or with the other protein (Fig. 2A, lane 3). When Nha1p-6xHis and Nha1p-FLAG were co-expressed, Nha1p-FLAG was detected in the fraction precipitated with Ni-NTA agarose (Fig. 2A, lane 7). In contrast, Nha1p-FLAG was not found in the precipitates of cells expressing Nha1p-6xHis alone or Nha1p-FLAG alone (Fig. 2A, lanes 5 and 6, respectively). To assess the specificity of the co-precipitation of Nha1p-FLAG with Nha1p-6xHis, cells were transformed with HA-tagged Pma1p, a plasma membrane H^+-ATPase, along with Nha1p-6xHis, and their solubilized extracts were subjected to precipitation with Ni-NTA agarose and immunoblotting analysis using anti-HA antibody. While HA-Pma1p was present in the input fraction (Fig. 2A, lane 4), it was not detected in the Ni-NTA precipitate (Fig. 2A, lane 8). This suggests that the co-precipitation of Nha1p-FLAG with Nha1p-6xHis does not result from incomplete membrane solubilization or non-specific aggregation of membrane proteins. We also found that Nha1p-6xHis was co-immunoprecipitated with the anti-FLAG antibody when it was co-expressed with Nha1p-FLAG (Fig. 2B). These results confirm that Nha1p-6xHis specifically interacts with Nha1p-FLAG, and suggest that Nha1p exists in the membrane as an oligomeric form.

3.3. Nha1p is present in the membrane as a dimer

To determine whether the Nha1p oligomer is a dimer, the crude membranes of yeast cells transformed with pKT10-NHA-FLAG were treated with cross-linkers to obtain covalently bound Nha1p molecules in their oligomeric complex. Either of two chemical cross-linkers were used, namely, DSS, which has a spacer arm length of 11.4 Å, or *o*-PDM, which has a spacer arm length of 7.7–10.5 Å. The cross-linked products were resolved by SDS-PAGE and then

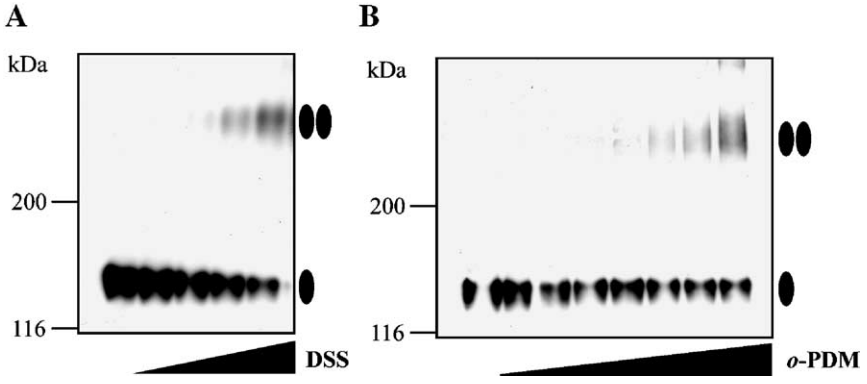

Fig. 3. In vitro Nha1p-FLAG cross-linking experiment. The crude membranes (5 mg/mL) from SK5 cells transformed with pKT10-NHA1-FLAG (high-copy) were incubated for 30 min at room temperature with the cross-linkers DSS (A) or *o*-PDM (B) at 0 (DMSO alone), 0.005, 0.01, 0.02, 0.04 mM and 0, 0.005, 0.01, 0.02, 0.04, 0.1, 0.5, 2 mM, respectively. The cross-linked products were then subjected to SDS-PAGE and immunoblotting analysis using the anti-FLAG M2 monoclonal antibody. The one oval and two ovals indicate the monomeric and dimeric forms of Nha1p, respectively.

analyzed by immunoblotting using anti-FLAG antibody (Fig. 3). In the absence of DSS or *o*-PDM, Nha1p-FLAG was detected as a 130-kDa monomeric protein (one oval). This molecular weight is higher than the predicted molecular weight (110-kDa), which suggests that Nha1p-FLAG has been subjected to several post-translational modifications such as glycosylations and/or phosphorylations. The addition of cross-linkers yielded a broad band at 260-kDa (two ovals) along with the monomer indicated in Fig. 3 by one oval. The intensity of the cross-linked 260-kDa product was dependent on the concentration of the cross-linking reagent. This broad 260-kDa band corresponds to the molecular size expected for the Nha1p homo-dimer. As a control, we subjected the DSS-treated cell extracts to detecting the enodogenous GPI-anchored protein Gas1p; however, the cross-linked product was basically not found on the anti-Gas1p antibody-probed immunoblot (data not shown). Signals at the top area of the immunobotts corresponding to the higher molecular weight materials were observed at the higher concentration of cross-linking reagent (Fig. 3). These may be aggregates of Nha1p with each other or other proteins caused by artificial cross-linking, because at the higher concentration of cross-linker, we observed extensive formation of aggregates of any proteins including Gas1p known as a monomeric protein (data not shown). These results suggest that yeast Nha1p exists in the membrane as a dimeric form rather than as a monomeric form.

3.4. Nha1p oligomerizes before it arrives at the cell surface

We showed previously that Nha1p is transferred to the cell surface via the secretory pathway [30], as is usual for most membrane and secretory proteins [56,57]. To test whether Nha1p oligomerizes before or after it arrives at the cell surface, we performed a co-precipitation experiment using *sec18-1* mutant cells; when these cells are incubated at 37 °C, ER-to-Golgi traffic is blocked [56,57]. Thus, *sec18-1* cells were transformed with vectors expressing Nha1p-6xHis and Nha1p-EGFP, after which the expression of Nha1p-EGFP (which is under the control of the *GAL1* promoter) was induced by shifting the cells to medium containing 2% galactose. The cells were then incubated another 4 h at a permissive temperature (24 °C) or a restricted temperature (37 °C) before their membrane proteins were solubilized and subjected to co-precipitation assays using Ni-NTA agarose. The precipitates were then analyzed by immunoblotting with anti-penta-His monoclonal antibody and GFP-specific antiserum.

We also observed the cells by fluorescence microscopy. At 24 °C, the Nha1p-EGFP fusion protein was localized at the plasma membrane of the *sec18-1* cells (Fig. 4B), similar to what was observed for the control SK5 (*SEC18*$^{+}$) cells in Fig. 1B. However, at the restricted temperature (37 °C), the fluorescence signals of Nha1p-EGFP were mainly observed as punctate structures throughout the cytoplasm, probably in COPII vesicles derived from the ER membrane (Fig. 4B). These observations indicate that at 37 °C, a large proportion of the newly synthesized Nha1p-EGFP fusion proteins accumulate in the intracellular membrane vesicles and do not travel to the cell surface.

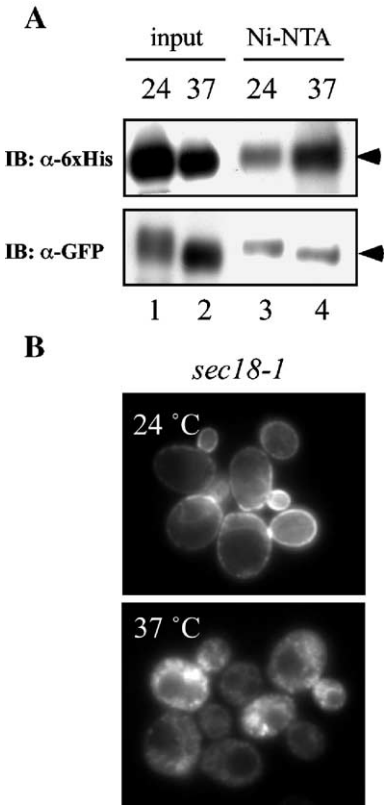

Fig. 4. Detection of Nha1p oligomerization by co-precipitation analyses using *sec18-1* cells. (A) MTsec18 (*sec18-1*) cells co-expressing Nha1p-6xHis and Nha1p-EGFP were grown to the early logarithmic phase at the permissive temperature of 24 °C and then cultured for an additional 4 h at 24 °C (lanes 1 and 3) or 37 °C (lanes 2 and 4) in medium containing 2% galactose to induce the expression of Nha1p-EGFP. Crude membranes were prepared from the yeast cells as described in Materials and methods and solubilized with DDM. The supernatants (input) after centrifugation were subjected to precipitation by Ni-NTA agarose. The specific Ni-NTA precipitates were then subjected to immunoblotting analysis after separation by SDS-PAGE. The antibodies used were anti-penta-His monoclonal antibody and GFP-specific antiserum. (B) MTsec18 (*sec18-1*) cells co-expressing Nha1p-6xHis and Nha1p-EGFP were grown to the early logarithmic phase at 24 °C, cultured for an additional 4 h at 24 °C or 37 °C in medium containing 2% galactose, and then observed by fluorescence microscopy.

As expected, we found that the Ni-NTA precipitates of the *sec18-1* cells incubated at 24 °C contained both Nha1p-6xHis and Nha1-EGFP (Fig. 4A, lanes 1 and 3). Moreover, when the restricted temperature (37 °C) was used, Nha1p-EGFP was again co-precipitated with Nha1p-6xHis (Fig. 4A, lanes 2 and 4). This indicates that Nha1p already exists as an oligomer complex in the intermediate-vesicles, and that Nha1p is largely oligomerized before it arrives at the cell surface (probably within the ER membrane).

3.5. Dominant-negative effect of overexpressing the inactive form of Nha1p on wild-type Nha1p activity

If the dimerization of Nha1p is needed for the antiporter function of this protein, hetero-dimers composed of one active and one inactive monomer would be defective in Nha1p

activity. We used this notion to test whether Nha1p dimerization is needed for the ion transport activity of this protein. Thus, we co-expressed an ion transport-defective (inactive) mutant along with the wild-type Nha1p in SK5 cells. For such mutants, we used Nha1p containing the single amino acid replacements S27Y, D145N, D226N, or S433A, which were previously identified by survey with site-directed or random mutagenesis (K. Mitsui et al., unpublished observation). When SK5 cells were transformed with the plasmids expressing these mutant proteins, their cell growth on agar plates containing various NaCl concentrations was retarded (Fig. 5A); in contrast, the SK5 transformant with wild-type Nha1p grew competently on the plate containing 0.4 M NaCl. The above result suggests that these inactive mutants do not export Na^+ efficiently. The expression levels of these mutant Nha1ps were higher than the level of the wild-type as shown in Fig. 7. The mutant Nha1p fused with EGFP was localized in the plasma membrane for S27Y, D266N and S433A mutations, while D145N mutant Nha1p-EGFP was mainly found in the intracellular membranes (data not shown). Thus, the low or null growth of S27Y, D266N and S433A or D145N mutants should be due to a defect in the Na^+ export mechanism or the plasma membrane targeting, but not to decreased expression of mutant Nha1p. This was further tested by estimating the Na^+ export activity of yeast cells over-producing mutant Nha1p-FLAG proteins. To do so, we measured their intracellular Na^+ concentrations. Since the total concentrations of Na^+ and K^+ in yeast cells have been reported to be kept constant [58], intracellular K^+ concentrations as well as Na^+ concentrations were measured. The intracellular ion ratios designated as $Na^+/(Na^+{+}K^+)$ were calculated by dividing the intracellular Na^+ concentration with the total concentration of intracellular Na^+ plus K^+. The $Na^+/(Na^+{+}K^+)$ value increases in proportion to the extracellular Na^+ concentration (data not shown). Moreover, the $Na^+/(Na^+{+}K^+)$ value decreases in inverse proportion to the Na^+ export activity of the yeast cell. This is shown by comparing the intracellular ion ratios of cells expressing various mutant versions of Nha1p-FLAG when cultured in

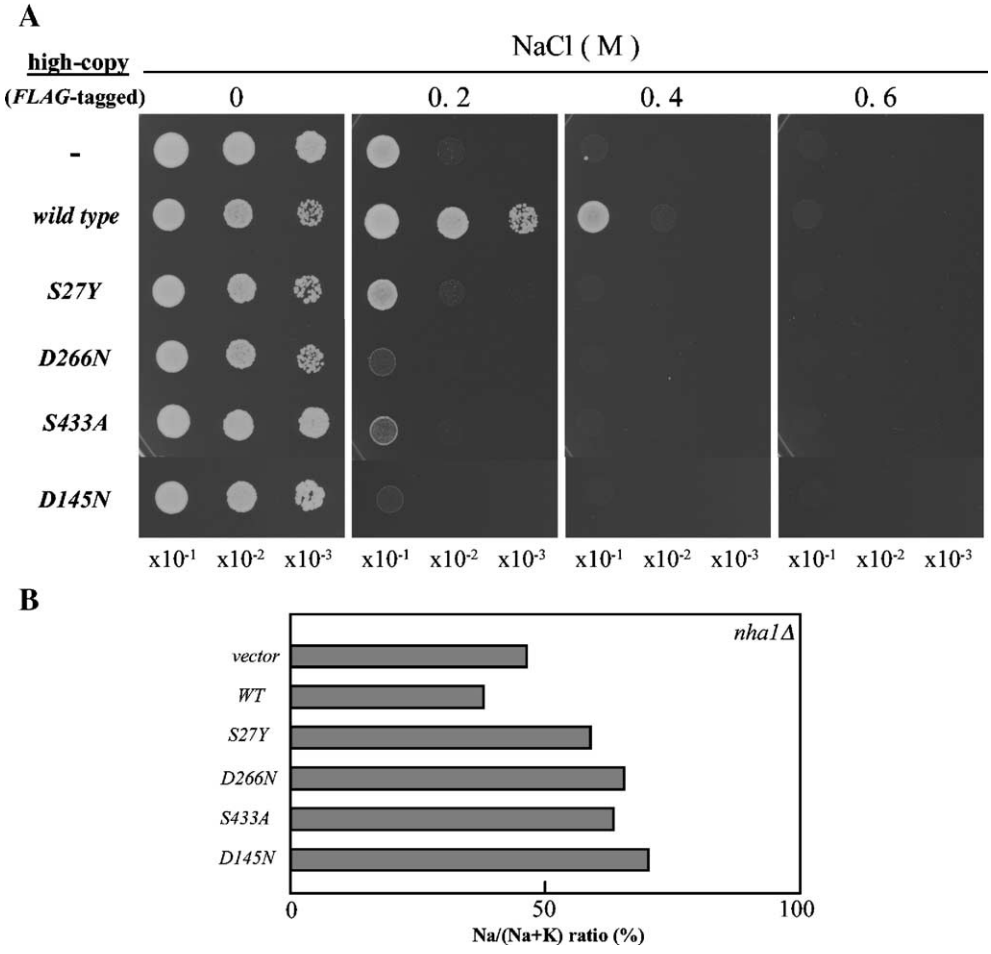

Fig. 5. Salinity sensitivity of SK5 cells transformed with NHA1 mutant constructs bearing single amino-acid substitutions. (A) SK5 cells transformed with high-copy wild-type or mutant pKT10-NHA1-FLAG constructs were grown to the logarithmic phase, diluted serially, and spotted onto SD plates, pH 5.5, supplemented with NaCl as indicated. The plates were incubated at 30 °C for 4 days. (B) Intracellular ion ratios of SK5 cells transformed with high-copy wild-type or mutant pKT10-NHA1-FLAG constructs. The transformed SK5 cells were cultured for 6 h in 25 mL SD medium supplemented with 0.3 M NaCl at 30 °C, after which the cells were trapped on membrane filters (0.45 μm, Advantec Co.), washed, soaked in distilled water, and boiled for 15 min. The supernatants after centrifugation were subjected to optical emission spectrophotometry (Perkin Elmer, Optima 3000XL). The total amount of protein in the cell extracts after boiling was measured by the method of Lowry et al. The $Na^+/(Na^+{+}K^+)$ ratios were calculated by dividing the total intracellular Na^+ plus K^+ concentration (μmol/μg protein) with the intracellular Na^+ concentration (μmol/μg protein) as described in Materials and methods.

medium containing 0.3 M NaCl for more than 6 h (Fig. 5B). The $Na^+/(Na^+ + K^+)$ value of SK5 cells expressing wild-type Nha1p-FLAG was lower (38%) than that of cells carrying the empty vector (47%). As expected, all of the cells expressing the inactive mutants (S27Y, D266N, S433A and D145N) showed higher Na^+ ratios (60%, 67%, 65% and 72%, respectively) than cells expressing wild-type Nha1p-FLAG (38%). These results suggest that SK5 cells expressing an inactive mutant Nha1p cannot discharge the toxic Na^+ out of cytoplasm and therefore accumulate excess Na^+ levels.

Finally, we co-expressed the inactive mutant Nha1p proteins with wild-type (active) Nha1p-EGFP and observed the salinity-resistant cell growth of the resulting cells (Fig. 6A). To ensure that most of the active Nha1p monomers expressed in SK5 cells oligomerized with the inactive Nha1p monomers, active Nha1p was expressed from a low-copy plasmid from the native *NHA1* promoter while the inactive mutant was expressed from a high-copy plasmid under the control of the *GAP* promoter (the level of expression from the *GAP* promoter is known to be high). Under this condition, the level of Nha1p overexpressed from a high-copy plasmid is approximately a few hundred times higher than that of Nha1p expressed from a low-copy plasmid (data not shown). As shown in Fig. 6A, SK5 cells expressing wild-type Nha1p-EGFP only were highly resistant to high concentrations of NaCl, unlike cells carrying the empty vector. However, when SK5 cells over-expressed one of the three inactive mutant Nha1ps S27Y, D266N or S433A together with wild-type Nha1p-EGFP, they had the same salt sensitivity at 0.2 M NaCl as cells carrying the empty vector (Fig. 6A). These results suggest that the inactive mutants completely inhibit the ion transport activity of the wild-type Nha1p when they are co-expressed in the same yeast cells. Moreover, the intracellular ion ratios of the cells expressing wild-type Nha1p along with the S27A, D266N or S433A mutant were higher (41%, 43% and 49%, respectively) than the ratio of cells expressing wild-type Nha1p-EGFP only (29%) (Fig. 6B). Thus, the inactive mutant Nha1p-FLAG proteins bearing the S27Y, D266N or S433A mutations have an extensive dominant-negative effect on wild-type Nha1p-EGFP and inhibit its ion transport activity.

SK5 cells co-expressing wild-type Nha1p-EGFP with another mutant Nha1p molecule, D145N, were more resistant

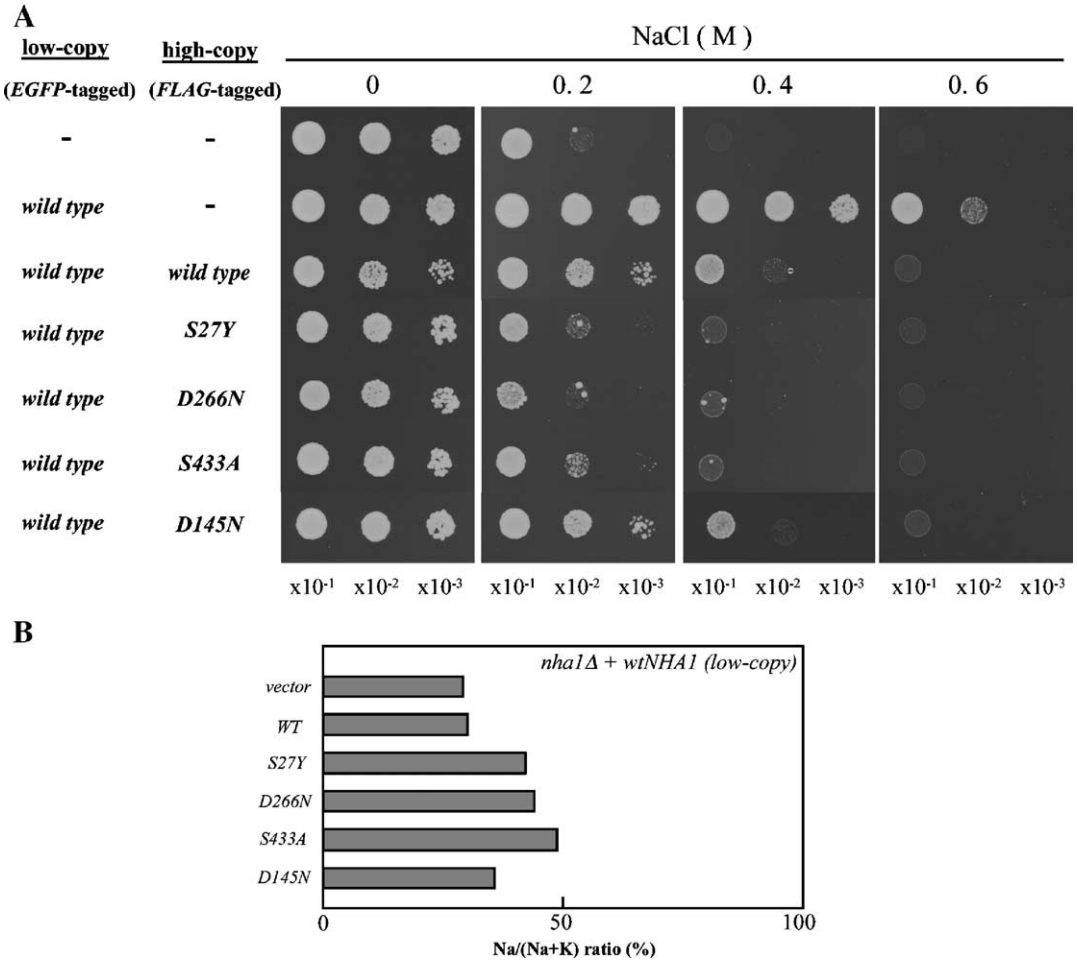

Fig. 6. Dominant-negative effect of inactive Nha1p-FLAG mutants on the cell growth of wild-type Nha1p-EGFP-expressing cells (A) SK5 cells co-expressing the low-copy wild-type Nha1p-EGFP fusion protein with various high-copy mutant Nha1p-FLAG proteins were grown to the logarithmic phase, diluted serially, and spotted onto SD plates, pH 5.5 supplemented with NaCl as indicated. The plates were incubated at 30 °C for 4 days. (B) Intracellular ion ratios of SK5 cells co-expressing the low-copy wild-type Nha1p-EGFP fusion protein with various high-copy mutant Nha1p-FLAG proteins were measured as described in the legend to Fig. 5B.

to highly saline conditions than SK5 cells co-expressing S27Y, D266N or S433A mutants (Fig. 6A). Moreover, these cells showed higher antiporter activity, as estimated by the intracellular Na^+ levels (Fig. 6B). This indicates that the dominant-negative effect of D145N is much weaker than that of the other mutations.

3.6. The dominant-negative effect is due to heterodimerization between the wild-type and inactive mutant Nha1p proteins

To determine whether the dominant-negative effect of overexpressing mutant Nha1p is due to its heterodimeric association with the wild-type Nha1p, co-immunoprecipitation experiments were performed. Thus, DDM-solubilized extracts from cells co-expressing mutant Nha1p-FLAG and wild-type Nha1p-EGFP (input) were incubated with an anti-FLAG affinity gel to obtain a specific immunoprecipitate (IP). The input and IP fractions were then resolved by SDS-PAGE and analyzed by immunoblotting using anti-FLAG and anti-GFP antibodies (Fig. 7). The wild-type Nha1p-EGFP fusion protein was detected in the IPs along with the wild-type, S27Y, D266N or S433A Nha1p-FLAG proteins (Fig. 7, upper panel). In contrast, the GPI-anchored protein Gas1p was not observed in the IPs (Fig. 7, upper panel). Notably, the Nha1p-EGFP fusion was not precipitated with the Nha1p(D145N)-FLAG mutant (Fig. 7, bottom panel), which indicates that the wild-type Nha1p-EGFP does not interact with Nha1p(D145N)-FLAG.

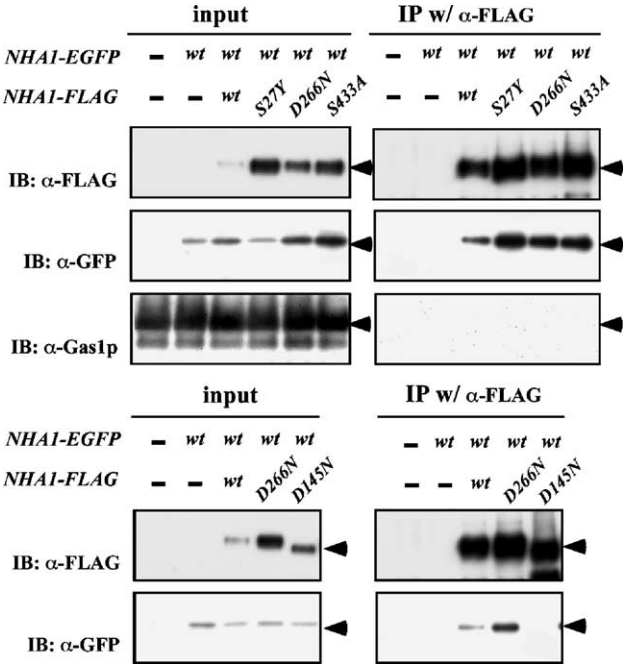

Fig. 7. Interaction of wild-type Nha1p-EGFP with the inactive Nha1p-FLAG mutants. Crude membranes prepared by centrifugation from yeast cells co-expressing the low-copy wild-type Nha1p-EGFP fusion protein and various high-copy Nha1p-FLAG proteins were solubilized with DDM. The supernatants (input) of the centrifuged DDM-solubilized membranes were subjected to immunoprecipitation by incubation with anti-FLAG M2 agarose affinity gel. The specific precipitates (IP) were separated by SDS-PAGE and subjected to immunoblotting analysis with the anti-FLAG monoclonal antibody, the GFP-specific antiserum, and the anti-Gas1p polyclonal antibody.

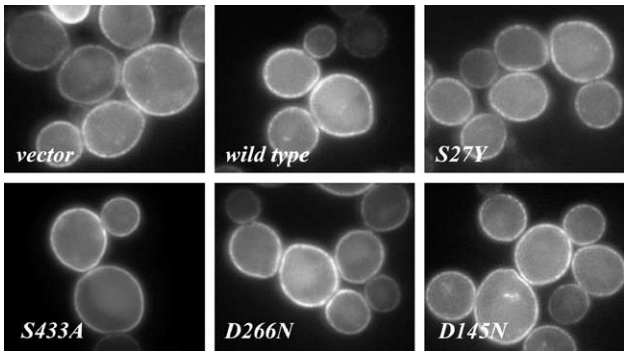

Fig. 8. Cellular localization of the wild-type Nha1p-EGFP fusion protein upon over-expression of various inactive mutant Nha1p-FLAG proteins. SK5 cells co-expressing the low-copy wild-type Nha1p-EGFP fusion protein with various high-copy mutant Nha1p-FLAG proteins were grown to the logarithmic phase at 30 °C and observed by fluorescence microscopy.

This is consistent with the previous observation that this mutation did not have such a marked dominant-negative effect on the antiporter activity of wild-type Nha1p as the other mutations.

3.7. Intracellular localization of Nha1p-EGFP when it is coexpressed with defective mutant Nha1p proteins

That the inactive mutant Nha1ps bearing the S27Y, D266N, or S433A mutations associate with wild-type Nha1p is consistent with their dominant-negative effect on wild-type Nha1p activity. However, it remains possible that the dominant-negative effect is due to the poor localization to the plasma membrane of the heterodimers. Therefore, we tested this possibility by fluorescence microscopy. The cell surface destination of the wild-type Nha1p-EGFP fusion was not affected, when coexpressed with S27Y, D266N and S433A mutant Nha1ps (Fig. 8). We found previously that the S27Y, D266N and S433A mutant Nha1ps localized at the cell surface (K. Mitsui et al., unpublished observation), also supporting that these mutant Nha1ps do not interrupt the plasma membrane targeting of wild-type Nha1p-EGFP fusion. However, the

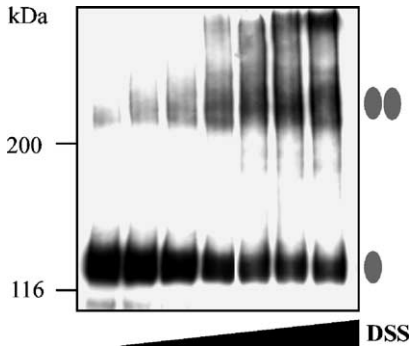

Fig. 9. Oligomerization of the Nha1p (D145N) mutant as detected by SDS-PAGE and immunoblotting. The crude membranes (5 mg/mL) from SK5 cells transformed with pKT10-NHA1(D145N)-FLAG (high-copy) were incubated with the cross-linker DSS (final concentrations; 0, 0.005, 0.01, 0.02, 0.04, 0.1, 0.5 mM) for 30 min at room temperature. The cross-linked products were then subjected to SDS-PAGE and immunoblotting using the anti-FLAG M2 monoclonal antibody. The one oval and two ovals indicate the monomeric and dimeric forms of the D145N Nha1p mutant, respectively.

D145N mutant Nha1p was mainly observed in the ER membrane (data not shown), which is consistent with the poor level of association between D145N and the wild-type and the limited ability of this mutant to impose a dominant-negative effect on wild-type protein activity. Moreover, Fig. 9 shows that the D145N mutant Nha1p dimerizes at a lower concentration of cross-linker DSS in SDS-PAGE than the wild-type (compare with Fig. 3), suggesting that the conformation of D145N Nha1p differs from that of the wild-type protein. The altered conformation may increase the affinity between the mutant Nha1p monomers but decrease their affinity for the wild-type Nha1p.

4. Discussion

It has been reported that the Na^+/H^+ antiporters from bacteria and mammals form oligomers [41–46], but it remains to be determined whether this oligomerization plays an important role in the antiporter activity of these proteins. Here, we demonstrated by co-precipitation of two differently tagged Nha1p proteins that the yeast plasma membrane type Na^+/H^+ antiporter Nha1p exists in the membrane as an oligomer. We then showed by cross-linking experiments that Nha1p is present in the membrane as a dimer. Moreover, over-expression of functionally defective Nha1p proteins had a dominant-negative effect on the antiporter activity of the wild-type protein. All of these findings support the notion that dimerization of the monomers affects the ion transport activity of yeast Nha1p. Also, it was suggested that Na^+/H^+ antiporter proteins from all organism are universally and evolutionary conserved as dimeric structures.

For the *E. coli* Na^+/H^+ antiporter NhaA (ECNhaA), combining two mutant NhaA proteins with different patterns of pH-dependent activity caused the pH dependency to shift to the wild-type pattern, which differed from the original mutant phenotype [43]. Thus, the interaction of the monomers was suggested to have a functional effect on the pH dependency of the antiporter. However, this observation did not necessarily indicate that oligomer formation by the antiporter is important for the functions of this protein. For the mammalian Na^+/H^+ exchanger NHE1, Fliegel and collaborators have reported that NHE1 in the placental brush border membranes exists as a dimer with a larger molecular mass (~205 kDa) [45]. Kinetic studies have also suggested that NHE1 functions as an oligomer [59,60]. In contrast, Pouyssegur and collaborators have shown that there are no alterations in the amiloride-sensitive Na^+ flux of NHE1 when the active NHE1 was coexpressed with functionally inactive mutant NHE1, suggesting that NHE1 functions as a monomer within the oligomeric state [44]. Therefore, at present, the functional significance of oligomerization of NHE1 is not well clarified. However, our present results show that expressing an inactive Nha1p mutant has a dominant-negative effect on wild-type protein activity, as it resulted in total loss of antiporter activity. This clearly shows that the dimerization of Nha1p plays an important role in its ion transport function.

The two-dimensional structure of the bacterial antiporter ECNhaA suggests that it forms a dimer structure [41]. Although the resolution of the NhaA structure is relatively low, the structure suggests that the ion transport pathway does not seem to be between the two oligomers. Further, ECNhaA crystal structure was solved very recently [61], although a dimeric structure has not been shown. The crystal structure also supported that the ion pathway is within each monomer, and specifically may involve the four transmembrane domains of the monomer [61–64]. Whether the same is true for Nha1p is not clear, as the primary structures of NhaA and Nha1p share little similarity. However, we have shown previously that most of the C-terminal hydrophilic region of Nha1p is not essential for its antiporter activity [25,31]. Moreover, we have shown in this study that four residues in the putative membrane integral domain of Nha1p are important for its antiporter activity. These observations support the notion that the ion pathway of Nha1p exists in the membrane integral domain of its monomers. If so, the dominant-negative effect imposed by the mutant Nha1p proteins suggests (i) the dimeric state generates a conformational change in the integral membrane domain of each monomer that is required for its ion transport function, and (ii) this mutual transmission of the putative conformational change may be blocked by a single mutation in the membrane integral domain of one of the monomers. In this connection, it should be noted that *Helicobacter pylori* NhaA undergoes a conformational change upon the binding of Li^+, as determined by FRET analyses [65].

Requirement of oligomerization in the transport activity has been described not only for the Na^+/H^+ antiporters, but also for several other transporters including glucose transporter GLUT1 [66,67] and serotonine-Na^+ symporter SERT [68] as well as Na^+/K^+ ATPase [69,70]. Thus, oligomerization of monomers might be one of important features of membrane transport machineries. However, for any cases including Nha1p and bacterial NhaA, further detail of transport mechanism depending on oligomerization is not clarified. More precise biochemical analyses of transport mechanism are required to reveal the significance of oligomerization. One of the required steps to address this issue for Nha1p will be to establish an in vitro reconstitution of the antiporter activity from the purified Nha1p.

We have previously shown that the binding of the membrane protein Cos3p to the juxta-membrane domain in the hydrophilic region of Nha1p activates the latter's antiporter activity [32]. Moreover, it was recently revealed that the protein kinase Hog1p interacts with the hydrophilic tail of Nha1p and activates its antiporter activity [71]. Therefore, it is also possible that the dominant-negative effect imposed on wild-type Nha1p antiporter activity by co-expressing functionally defective Nha1p could be due to their inability to interact with proteins like Cos3p and Hog1p. Arguing against this notion, however, is that the D145N mutant Nha1p did not extensively decrease the antiporter activity of wild-type Nha1p.

Since the crystal structure of Nha1p is not available, the structural basis for its dimerization is not clear. However, it should be noted that the D145N mutant Nha1p failed to

oligomerize with wild-type Nha1p (Fig. 7) and that the monomers of this mutant interacted more tightly with each other than the wild-type monomers did with each other (Fig. 9). These results suggest that the conformation of Nha1p induced by the D145N mutation resulted in a preference for binding to its own form rather than to the wild-type. Therefore, Asp-145 may be involved in the formation of the dimer, either directly or indirectly.

We showed that the oligomerization of wild-type Nha1p occurs before its localization to the plasma membrane and probably occurs in the ER membrane (Fig. 4). It should be noted that while the D145N mutant Nha1p formed a dimer with itself, it did not localize to the plasma membrane. This suggests that dimerization per se is not sufficient for the membrane localization of Nha1p. It may be that the D145N mutation interferes with the transport process in some way. In this connection, it was found that when the plasma membrane proteins Hxt1p and Gap1p, which are a glucose transporter and an amino acid permease, respectively, are exported from the ER, they require the chaperone-type proteins Gsf2p and Shr3p, respectively [72–75]. This was shown by the fact that functional defects in Gsf2p and Shr3p cause Hxt1p and Gap1p to accumulate at the ER. Although it is not known whether the export of Nha1p from the ER also requires such a chaperone-type protein, if it does, it is possible that the D145N mutation in Nha1p may affect its interaction with this putative protein. It is also unclear as yet whether Nha1p dimerization is actually essential for its plasma membrane localization. Further study is required to resolve this issue.

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