

# Essential aspartic acid residues, Asp-133, Asp-163 and Asp-164, in the transmembrane helices of a $\text{Na}^+/\text{H}^+$ antiporter (NhaA) from *Escherichia coli*

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**Abstract** The importance of negatively charged residues in transmembrane helices of many cation-coupled transporters has been widely demonstrated. Four Asp residues were located in the putative transmembrane helices of the *Escherichia coli*  $\text{Na}^+/\text{H}^+$  antiporter, NhaA. We replaced each of these Asp residues by Asn in plasmid encoded *nhaA* and expressed these constructs in an *E. coli* mutant defective in both *nhaA* and *nhaB*. Substitution of Asp-65 or Asp-282 (in the extramembrane region) had no effect on supporting the host mutant growth in the high NaCl- or LiCl-containing medium, and these two mutants had normal  $\text{Na}^+/\text{H}^+$  and  $\text{Li}^+/\text{H}^+$  antiporter activities. In contrast, substitution of Asp-133, Asp-163 or Asp-164 was detrimental to survival of the host mutant and impaired both  $\text{Na}^+/\text{H}^+$  and  $\text{Li}^+/\text{H}^+$  antiporter activities. These three Asp residues, conserved in the *nhaA* homologs from different species and which are located closely in the 3rd and 4th putative transmembrane helices, appear to play important roles in cation binding and transport.

**Key words:** NhaA;  $\text{Na}^+/\text{H}^+$  antiporter;  $\text{Li}^+$  ion; pH sensor; Transporter

## 1. Introduction

The  $\text{Na}^+/\text{H}^+$  antiporter is a ubiquitous membrane transporter in bacteria, plant and mammalian cells, and is assumed to be essential for regulation of intracellular pH,  $\text{Na}^+$  concentration and cell volume [1–3]. Two distinct  $\text{Na}^+/\text{H}^+$  antiporters, *nhaA* and *nhaB*, have been characterized and their genes were cloned from *E. coli* [4,5]. The NhaA antiporter activity is dependent on the environmental pH such that the activity is greatly enhanced as pH increases; at pH 7.0, NhaA has negligible antiporter activity, while at pH 8.5 the activity is enhanced by three orders of magnitude [6,7]. Unlike NhaA, NhaB antiporter activity is relatively constitutive although the affinity for  $\text{Na}^+$  increases ten times as pH elevates from 7.2 to 8.5 [8]. At alkaline pH, NhaA alone is not sufficient to maintain intracellular pH for proper growth [9]. Indeed, a mutant, HIT-1, defective in the NhaB could not survive in medium adjusted at pH higher than 8.0 [10]. Therefore, NhaB is assumed to be essential for intracellular pH homeostasis under alkaline conditions. A mutant defective in NhaA is not tolerant to elevated salt concentration in the medium. This mutant could not grow not only at high  $\text{Na}^+$  concentration (0.6 M at pH 7.5) [11], but also at low concentration of  $\text{Li}^+$  (0.3 M) [12]. These defects could be overcome by exogenic expression of *nhaA* in the

mutant. Unlike NhaB, the NhaA antiporter has relatively high transport activity for  $\text{Li}^+$ , which may cause tolerance to  $\text{Li}^+$  by extruding the ion into the medium. Therefore, the NhaA antiporter may function primarily in regulating intracellular salt concentration. Interestingly, the expression of *nhaA* is promoted by elevated salt concentration in the medium [13], which is consistent with the possible role of NhaA in regulation of intracellular salt concentration.

Both antiporters were estimated to have 10–12 transmembrane helices by hydropathy plot analyses [4,5,14]. No details regarding the structure-function relationships have yet been elucidated except that His-225 may constitute part of the pH sensor integrated in the antiporter, as revealed by site-directed mutagenesis [14]. Since the importance of negative charges in cation transporters has been widely observed in many membrane transporters [15–21], here we addressed the functional involvement of five Asp residues in the NhaA antiporter (four in the transmembrane and one in the extramembrane domains) by substituting Asn for Asp residues. Three conserved Asp residues, Asp-133, Asp-163 and Asp-164, were revealed to be important for antiporter function.

## 2. Experimental procedures

### 2.1. Plasmids, bacterial strains and culture conditions

The *nhaA* gene encoding a  $\text{Na}^+/\text{H}^+$  antiporter was amplified from chromosomal DNA of W3110, a derivative of *E. coli* K12, and was cloned into pBR322 under control of Tet<sup>r</sup> gene promoter. To minimize the toxic effect of overproduction of NhaA, the initiation codon for NhaA was substituted by CTG, which gave better complementation of *nhaA* mutants (Noumi et al., unpublished result). The *nhaA*-plasmid and its mutants were introduced in the mutant HITΔAB<sup>−</sup> defective in both *nhaA* and *nhaB* [7], and cultured aerobically in LBK medium (NaCl was replaced with the same concentration of KCl in L broth) with ampicillin (50 μg/ml) and kanamycin (25 μg/ml) at 37°C. Growth of mutants was measured turbidimetrically at 600 nm in LBK (LB containing 87 mM KCl for NaCl) adjusted to desired pH with 20 mM Tricine-KOH and supplied with appropriate concentrations of LiCl or NaCl. The *nhaA* with the CTG initiation codon was recloned into pUC18 and used as a template for polymerase chain reaction (PCR) mediated site-directed mutagenesis.

### 2.2. Site-directed mutagenesis of Asp residues in NhaA

Five Asp residues, Asp-65, Asp-133, Asp-163, Asp-164 and Asp-282, were substituted by Asn by PCR-mediated site-directed mutagenesis [22,23]. Mutagenic 21-mer oligonucleotides corresponding to either sense or anti-sense strand of five Asp residues with a single base mismatch in the center, which yield an Asn for Asp residue, were synthesized. In the first PCR reactions, the mutagenic oligonucleotide and a universal primer for pUC18 were used for amplification of a partial fragment of *nhaA*, and an overlapping *nhaA* fragment was amplified with the opposite orientation universal primer and an internal primer for *nhaA*. In the second PCR reaction, two amplified fragments were

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combined and the full-length *nhaA* gene was amplified with two universal primers. The resultant *nhaA* gene was cloned back into pBR322 and the mutation was verified by DNA sequencing.

### 2.3. Preparation of membrane vesicles and measurement of $\text{Na}^+/\text{H}^+$ and $\text{Li}^+/\text{H}^+$ antiporter activities

The mutant HIT $\Delta\text{AB}^-$  carrying an *nhaA* plasmid was cultured in 300 ml of LB containing ampicillin (50  $\mu\text{g}/\text{ml}$ ) and kanamycin (25  $\mu\text{g}/\text{ml}$ ) until the  $\text{OD}_{600}$  reached 0.8–1.0. The cells were harvested, washed and resuspended in 10 mM Tris-HCl (pH 7.2) containing 140 mM KCl, 5 mM  $\text{MgSO}_4$ , 1.5 mM  $\beta$ -mercaptoethanol and 10% glycerol. The cell suspension was passed through a French press, and unbroken cells and cell debris were removed by low-speed centrifugation. Then, the everted membrane vesicles were collected by ultracentrifugation (100,000  $\times g$ , 60 min). The membrane vesicles were resuspended in the same buffer as above and glycerol was added to a final concentration of 50%. The protein concentrations of membrane vesicles were adjusted to 10 mg/ml, then they were stored at  $-20^\circ\text{C}$  and used for assay within a few days. The antiporter activities were assayed with 200  $\mu\text{g}$  of membrane proteins suspended in 2 ml of 10 mM Tricine-KOH (pH 7.0, 8.0 or 8.5) containing 140 mM KCl, and 1  $\mu\text{M}$  quinacrine. The changes in  $\Delta\text{pH}$  were monitored by quenching and de-quenching of quinacrine fluorescence (ex. 420 nm, em. 500 nm) as follows. Initially, respiration was driven by addition of lactate and internal acidic  $\Delta\text{pH}$  was established. Then, the NhaA antiporter was driven by addition of NaCl or LiCl so that the quenched fluorescence was restored by cancelation of  $\Delta\text{pH}$ . The antiporter activities were expressed as percent restoration of initial fluorescence quenching. To preclude endogenous  $\text{K}^+/\text{H}^+$  antiporter activity, 140 mM KCl was included in the assay buffer throughout the experiments.

### 2.4. Other procedures and materials

The protein concentration was determined by the published method [24]. DNA sequences were determined by the dideoxy chain-termination method [25] with T7 DNA polymerase (Pharmacia) and [ $\alpha$ - $^{35}\text{S}$ ]dCTP (DuPont/NEN). Restriction enzymes and T4 DNA ligase were purchased from BRL, and Tth and Pfu thermostable polymerases were from Toyobo (Osaka, Japan).

## 3. Results and discussion

### 3.1. Substitution of Asp residues in the transmembrane helices of *E. coli* NhaA

The importance of negative charges in the transmembrane domains has been well documented in other membrane transporters [15–21]. Since four Asp residues (Asp-65, Asp-133, Asp-163 and Asp-164) were found in the transmembrane helices (Fig. 1 and Table 1), we addressed the functions of these residues. These Asp residues and Asp-282 in the extramembrane region were substituted with Asn by site-directed mutagenesis, and the mutant *nhaA* were expressed in an *E. coli* mutant defective in both *nhaA* and *nhaB* [7]. The mutant host could not grow in medium containing high concentrations of NaCl

(0.65 M, pH 8.0) or LiCl (0.1 M, pH 7.5). Expression of the wild-type *nhaA* conferred resistance to high-salt medium by extruding excess intracellular  $\text{Na}^+$  or  $\text{Li}^+$  through active NhaA antiporter out of the cells. Two mutations, D65N and D282N, were still capable of supporting host mutant survival in high-NaCl or LiCl medium, suggesting that these two substitutions did not impair the antiporter function (Table 1). (The nomenclature of the mutants was designated according to their original residue, position in the NhaA and substituted residue, respectively. For example, D65N indicates that Asp at position 65 of NhaA was substituted by Asn.) In contrast, three other mutations, D133N, D163N and D164N, could not support the growth of the host mutant in high-LiCl medium (Table 1). Prolonged incubation, however, enabled D133N and D163N mutants to grow slightly on the high-NaCl plates suggesting that the two mutations may retain partial but significant antiporter activity. These growth phenotypes were also investigated by measuring the growth of these mutants in liquid cultures with high NaCl or LiCl concentration. Consistent with the results of the plate assay, no growth was observed with D133N, D163N or D164N mutants in the high-LiCl medium (Fig. 2B), while these three mutants grew slowly in the high-NaCl medium (Fig. 2C). We suppose that the mutations did not selectively abolish the  $\text{Li}^+/\text{H}^+$  antiporter activity, because  $\text{Li}^+$  is more toxic than  $\text{Na}^+$  to cells and apparent sensitivity to  $\text{Li}^+$  was more pronounced than to  $\text{Na}^+$  by lowered antiporter activities. Indeed no residual transport activity for  $\text{Na}^+$  was observed for these mutants in an in vitro transport assay as described later. Consequently, Asp-65 and Asp-282 were not essential for antiporter function, whereas Asp-133, Asp-163 and Asp-164 were important for NhaA antiporter activity in vivo.

The temperature-sensitive (ts) phenotype was examined with these mutants, since replacements of certain residues in transporters have been reported to induce ts phenotypes ([26]; Tsuchiya et al., unpublished results). In several cases, the charged residues in transmembrane domains were considered to be involved in maintenance of the stable conformation of transporters by forming a salt-bridge with a counter charge [19,27]. However, none of the five mutants described above showed such a ts phenotype. Neither D133N, D163N nor D164N could support host growth at  $30^\circ\text{C}$ , suggesting that these three Asp residues were directly involved in transport including binding of coupling ions. Both D65N and D282N were still capable of supporting the host growth at  $42^\circ\text{C}$ , suggesting that these two Asp residues did not play a structural role (data not shown).

Table 1

Effects of substitutions of Asp by Asn on the rescue of HIT $\Delta\text{AB}^-$  in medium containing high concentrations of NaCl or LiCl

Mutation in plasmid	Codon substitution	Growth medium			Transmembrane helix
		LBK	0.65 M NaCl	0.1 M LiCl	
Vector (pBR322)		++	–	–	
Wild-type <i>nhaA</i>	none	++	++	++	
Asp-65 $\rightarrow$ Asn	GAC $\rightarrow$ AAC	++	++	++	2nd
Asp-133 $\rightarrow$ Asn	GAC $\rightarrow$ AAC	++	+/-	–	4th
Asp-163 $\rightarrow$ Asn	GAC $\rightarrow$ AAC	++	+/-	–	5th
Asp-164 $\rightarrow$ Asn	GAT $\rightarrow$ AAT	++	–	–	5th
Asp-282 $\rightarrow$ Asn	GAT $\rightarrow$ AAT	++	++	++	extramembrane

The wild-type or mutant *nhaA*-expressing plasmids were introduced in the *E. coli* mutant HIT $\Delta\text{AB}^-$ . Growth of the transformants were examined on LB containing 0.65 M NaCl (pH 8.0) or 0.1 M LiCl (pH 7.5). As a control, NaCl (87 mM) in standard L broth was replaced with the same concentration of KCl (LBK).

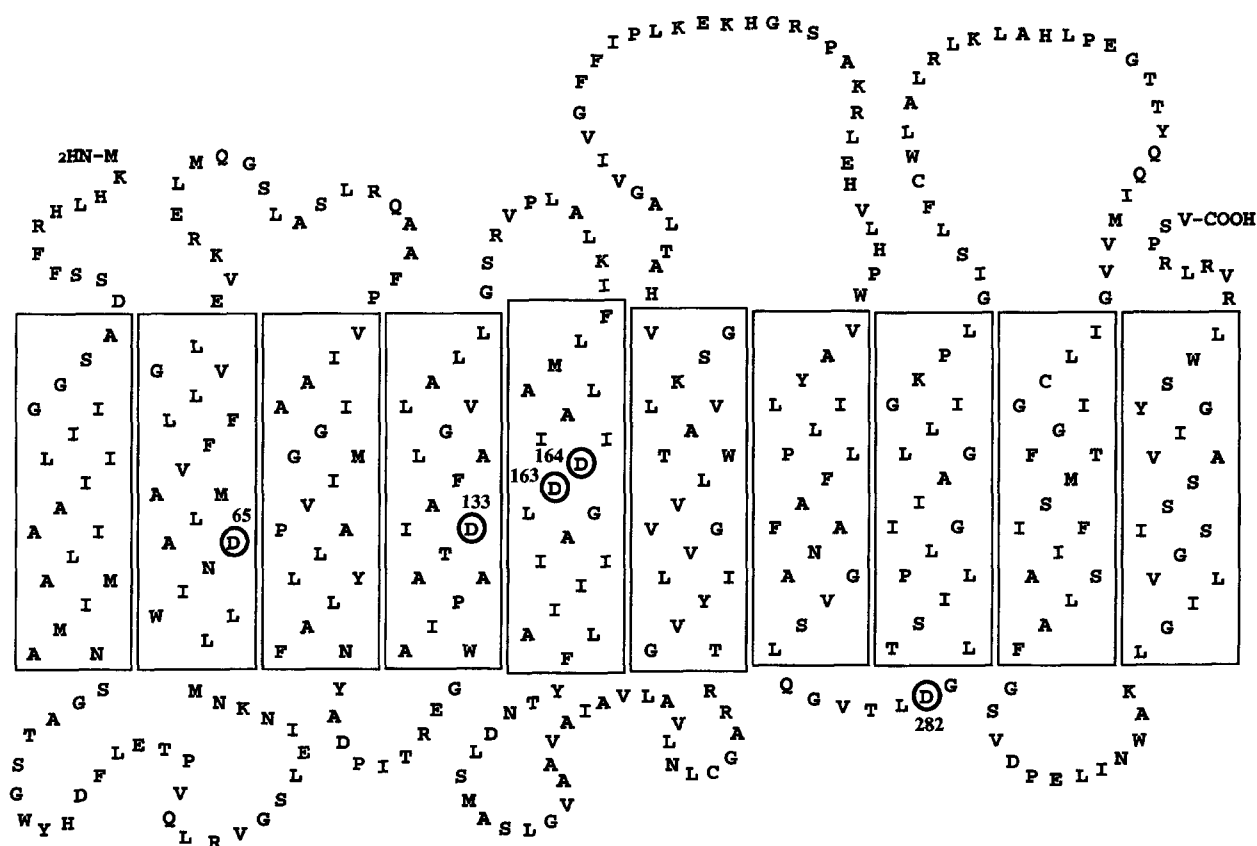


Fig. 1. Location of essential Asp residues in the possible transmembrane domains of *E. coli* NhaA. The possible transmembrane structure of the *E. coli* NhaA is depicted. We tentatively estimated 10 transmembrane helices based on the results of hydropathy analysis [31], amino acid conservation and charge distributions [32] (details for model construction will be described elsewhere). The five Asp residues mutated in the present study are circled and numbered.

The genes for NhaA type  $\text{Na}^+/\text{H}^+$  antiporters have been cloned and sequenced from *E. coli* [4], *S. enteritidis* [28], *V. alginolyticus* [29] and *V. parahaemolyticus* [30]. The average amino acid sequence identity over the entire sequence among these species was 57.5% based on the *E. coli* NhaA. All of the four Asp residues Asp-65, Asp-133, Asp-163 and Asp-164 in the

putative transmembrane helices were conserved in the homologs from these species, while Asp-282 in the putative extramembrane region was not conserved. These findings stress the importance of these charged residues in the membrane-spanning domain and are consistent with the functional necessity of Asp-133, Asp-163 and Asp-164 in the antiporter activity, although

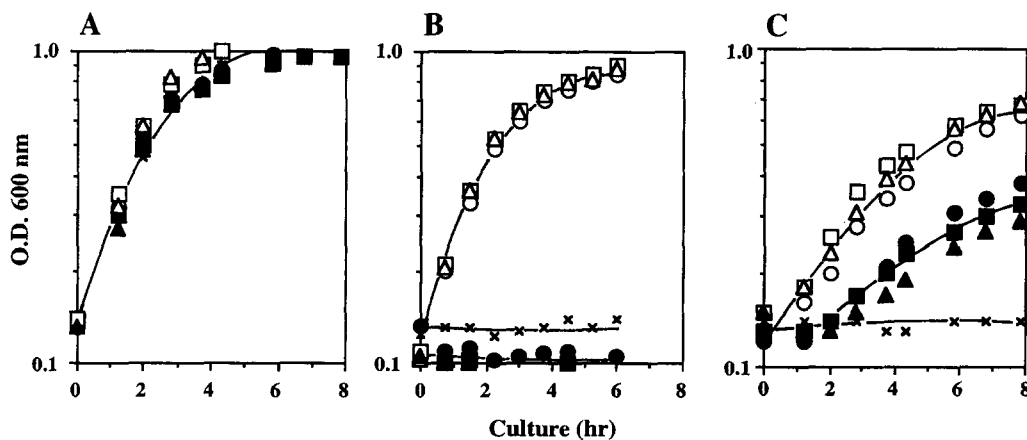


Fig. 2. Growth of NhaA mutants with an Asn for Asp substitution in high-NaCl or LiCl containing medium. The mutant HIT $\Delta$ AB $^-$  carrying the vector only (negative control), wild-type (positive control) or mutant *nhaA*-containing plasmid were grown aerobically in LBK (A), or LBK containing 0.65 M NaCl (pH 8.0) (B) or 0.15 M LiCl (pH 7.5) (C) at 37°C. Cell growth was measured turbidimetrically at 600 nm. × = vector; ○ = wild-type; □ = D65N; ▲ = D133N; ● = D163N; ■ = D164N; △ = D282N.

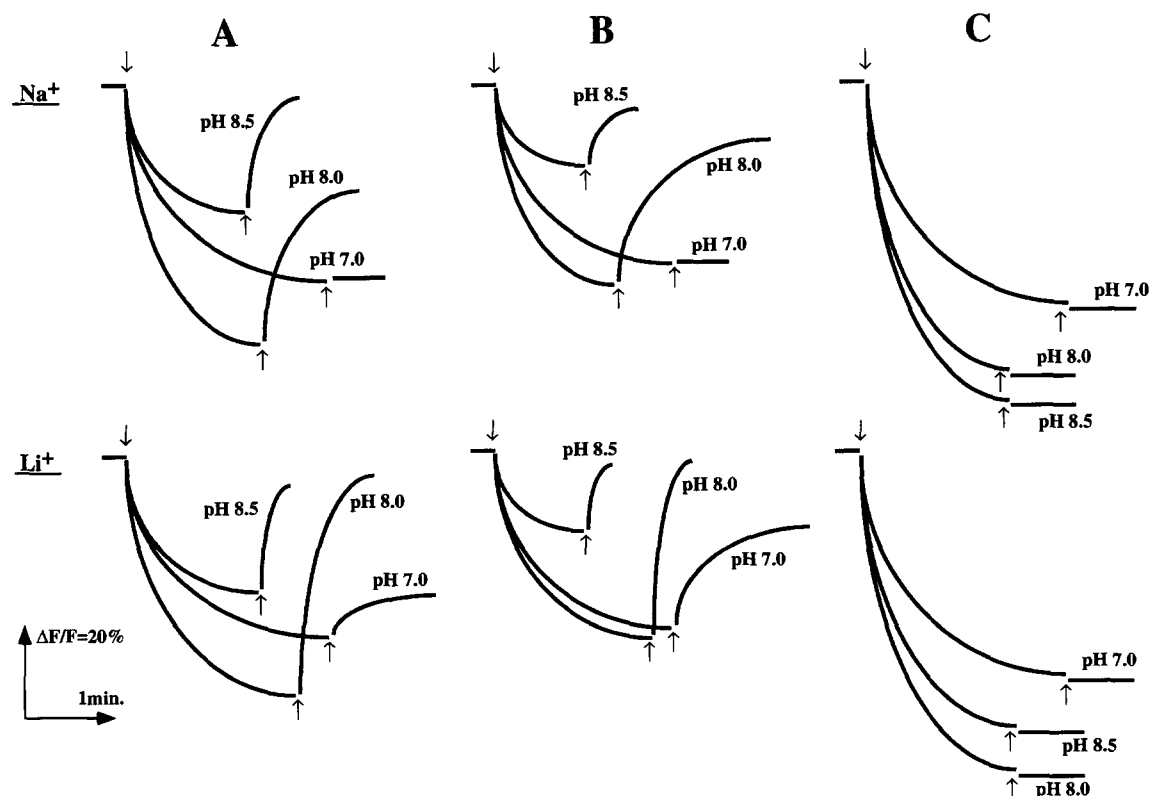


Fig. 3.  $\text{Na}^+/\text{H}^+$  and  $\text{Li}^+/\text{H}^+$  antiporter activities in the everted membrane vesicles from NhaA mutants with Asn for Asp substitutions. Everted membrane vesicles were prepared from the mutant  $\text{HIT}\Delta\text{AB}^-$  carrying an expression plasmid for the wild-type (A) or mutant *nhaA* with Asn for Asp substitutions (B, D65N; C, D164N) as described in section 2. Respiration was initially driven by addition of Tris-lactate (5 mM) to the assay mixture (downward arrows). After fluorescence quenching reached the plateau level, NaCl or LiCl (5 mM) was added (upward arrows) to initiate  $\text{Na}^+/\text{H}^+$  or  $\text{Li}^+/\text{H}^+$  antiport by NhaA. The upper and the bottom panels show  $\text{Na}^+/\text{H}^+$  and  $\text{Li}^+/\text{H}^+$  antiporter activities, respectively. The D282N, and D133N and D163N mutants showed similar results to those of D65N and D164N, respectively.

we cannot explain why Asp-65 is functionally dispensable at present.

### 3.2. $\text{Na}^+/\text{H}^+$ and $\text{Li}^+/\text{H}^+$ antiporter activities of Asp→Asn mutants

Everted membrane vesicles were prepared from the  $\text{HIT}\Delta\text{AB}^-$  mutant, which is defective in both *nhaA* and *nhaB*, carrying a vector plasmid (negative control), wild-type (positive control) or mutant *nhaA*-containing plasmid. The  $\text{Na}^+/\text{H}^+$  and  $\text{Li}^+/\text{H}^+$  antiporter activities were measured as cancellation of  $\Delta\text{pH}$  established by respiratory  $\text{H}^+$  pumping. The  $\text{HIT}\Delta\text{AB}^-$  mutant transformed with the vector only had no  $\text{Na}^+/\text{H}^+$  or

$\text{Li}^+/\text{H}^+$  antiporter activities under any pH conditions examined (Table 2). Transformation with the wild-type *nhaA*-plasmid restored both antiporter activities as revealed by restoration of fluorescence quenching after addition of NaCl or LiCl (Fig. 3A and Table 2). We confirmed the lack of antiporter activity at pH 7.0, while substantial antiporter activities were observed at pH 8.0 and 8.5. The two mutations D65N and D282N gave similar antiporter activities to those with the wild-type *nhaA*, and their activities were enhanced at alkaline pH (Fig. 3B and Table 2). Retention of antiporter activities in these two mutants in vitro was compatible with the in vivo rescue of  $\text{HIT}\Delta\text{AB}^-$  mutant in high-NaCl or -LiCl medium by extruding excess  $\text{Na}^+$

Table 2  
 $\text{Na}^+/\text{H}^+$  and  $\text{Li}^+/\text{H}^+$  antiporter activities in mutants with Asn for Asp substitutions

Mutation in plasmid	$\text{Na}^+/\text{H}^+$ antiporter activity			$\text{Li}^+/\text{H}^+$ antiporter activity		
	pH 7.0	pH 8.0	pH 8.5	pH 7.0	pH 8.0	pH 8.5
Vector (pBR322)	0	0	0	0	0	0
Wild-type <i>nhaA</i>	2	58	88	23	91	80
Asp-65 → Asn	0	73	69	56	91	76
Asp-133 → Asn	0	0	0	0	0	0
Asp-163 → Asn	0	0	0	0	0	0
Asp-164 → Asn	0	0	0	0	0	0
Asp-282 → Asn	0	67	91	44	98	94

The  $\text{Na}^+/\text{H}^+$  and  $\text{Li}^+/\text{H}^+$  antiporter activities were measured with everted membrane vesicles prepared from the mutant  $\text{HIT}\Delta\text{AB}^-$  carrying the wild-type or a mutant *nhaA*-expressing plasmid. The activities were expressed as percent recovery from the respiration-dependent fluorescence quenching after addition of NaCl or LiCl.

or  $\text{Li}^+$  through active NhaA antiporter. Interestingly, these two mutants had elevated  $\text{Li}^+/\text{H}^+$  antiporter activities at pH 7.0 relative to that of the wild-type (Fig. 3B and Table 2). Although the reason for this is not clear at present, there are two possible explanations as follows: (i) the active pH range might be expanded; and/or (ii) the  $V_{\text{max}}$  of  $\text{Li}^+$  transport might be increased at pH 7.0 by the mutations. Since their activities at pH 8.0 and 8.5 were not changed, the alteration might be achieved by a local conformational change induced by substitution of Asn for Asp.

Three substitutions, D133N, D163N and D164N, resulted in loss of both  $\text{Na}^+/\text{H}^+$  and  $\text{Li}^+/\text{H}^+$  antiporter activities under all pH conditions examined (Fig. 3C and Table 2). These results indicated that these three Asp residues are important for antiporter activities of NhaA. Again, their losses of activity in vitro were consistent with their failure to rescue  $\text{HIT}\Delta\text{AB}^-$  in vivo. Despite the low but significant complementation activity of D133N and D163N for growth of  $\text{HIT}\Delta\text{AB}^-$  on high-NaCl containing plates in vivo, no  $\text{Na}^+/\text{H}^+$  antiporter activity was observed in vitro. This discrepancy may be explained as follows: (i) the fluorescence assay employed here may not have been sensitive enough to detect very low activities, whereas the prolonged incubation accumulated the positive effect of residual activities; and/or (ii) the residual activities were highly labile and were lost during preparation of membrane vesicles.

In the *E. coli* melibiose carrier, four Asp residues are located similarly in the transmembrane helices to those in the NhaA: Asp-31 (1st transmembrane helix), Asp-51 and Asp-55 (2nd helix), and Asp-120 (4th helix). Substitution of each of these Asp residues, except Asp-31, with Asn resulted in loss of transport activity [15–17]. These results were very similar to the observations regarding NhaA presented here. In the melibiose carrier, three Asp residues, Asp-51, -55 and -120, were indicated to be involved in  $\text{Na}^+$  binding. The topological localization and inactivation results strongly suggested that Asp-133, Asp-163 and Asp-164 are involved in  $\text{Na}^+$  recognition and binding in the NhaA antiporter. Our preliminary finding that D133A mutant showed selective loss of  $\text{Na}^+/\text{H}^+$  antiporter activity but retained substantial  $\text{Li}^+/\text{H}^+$  antiporter activity (Noumi et al., unpublished result), also supported the possible involvement of Asp-133 in  $\text{Na}^+$  recognition. It is interesting to note that the importance of three Asp residues in the transmembrane helices was also reported with the metal-tetracycline/ $\text{H}^+$  antiporter [18]. Therefore, it may be a general rule that three Asp residues participate in cation-coupled transport in various transporters. It will be important to classify the roles of these Asp residues including cation recognition and substrate binding in future study.

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