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Characterization of a Histidine Rich Cluster of Amino Acids in the Cytoplasmic Domain of the Na^+/H^+ Exchanger

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We examined the function of a highly conserved Histidine rich sequence of amino acids found in the carboxyl-terminal of the Na^+/H^+ exchanger (NHE1). A fusion protein containing the sequence HYGHHH (540–545) and the balance of the carboxyl terminal of the protein did not bind calcium but bound to an immobilized metal affinity column and could be used to partially purify the exchanger protein. Mutation of the sequence to either HYGAAA or HYGRRR did not affect activity of the intact protein. Mutation to HHHHHH did not affect proton activation of the Na^+/H^+ exchanger or localization but caused a decreased maximal velocity suggesting that this conserved sequence is important in maximal activity of the Na^+/H^+ exchanger.

KEY WORDS: Na^+/H^+ exchanger; proton sensing; histidine residues.

ABBREVIATIONS: BCECF-AM, 2′,7′-Bis-(2-Carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester; DTT, dithiothreitol; HA, hemagglutinin; NHE1, Na^+/H^+ exchanger isoform 1; pH_i, intracellular pH SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

INTRODUCTION

The Na^+/H^+ exchanger is a ubiquitous plasma membrane protein that regulates intracellular pH (pH_i) by exchanging one intracellular proton for an extracellular sodium (Fliegel and Dibrov, 1996; Orlowski and Grinstein, 1997). Several isoforms of the protein have been identified in mammals that are designated NHE1–6. The NHE1 isoform was the first isoform discovered and is present in all mammalian cells (Sardet *et al.*, 1989). It is involved in pH regulation (Fliegel and Dibrov, 1996; Orlowski and Grinstein, 1997) control of cell volume (Grinstein *et al.*, 1983) and is activated by growth factors (Pouyssegur, 1985). The Na^+/H^+ exchanger family shares a conserved structure with a membrane-associated *N* terminus of approximately 500 amino acids and a large regulatory, hydrophilic cytoplasmic domain of

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approximately 300 amino acids. The cytoplasmic domain can modify the activity of the membrane domain and is subject to phosphorylation mediated by growth factor and protein kinase-dependent pathways (Pouyssegur, 1985; Moor and Fliegel, 1999).

An important feature of the Na^+/H^+ exchangers is their high sensitivity to pH_i. The Na^+/H^+ antiporters are activated allosterically by decreases in pHi leading to rapid increases in activity over relatively small pH changes. Their activation by decreasing pH_i is steeper than can be accounted for by a simple Michaelis–Menten process. Kinetic analysis of the NHE1 isoform of the Na^+/H^+ exchanger has determined that there is an internal proton modified site that is independent of the proton binding site that is used as a substrate for transport (Aronson *et al*., 1982). Early results have also suggested that the *N*-terminal transmembrane region contains the H^+ sensor site and that the *C*-terminal domain modulates the value of set point (Wakabayashi *et al*., 1992; Orlowski and Grinstein, 1997). When the protein is truncated at amino acid 515 the *N* terminus is still subject to allosteric modulation (Wakabayashi *et al*., 1992; Levine *et al*., 1993). However, other more recent studies suggest that a complex and specific interaction between the *C*-terminus and the *N*-terminal membrane domain are involved in modulating the H^+ modifier site and the set point of the antiporter (Yun *et al*., 1995).

One amino acid important in Na^+/H^+ exchanger activity is Histidine. Histidine residues have been shown to be critical to proton translocation ability of a variety of types of Na^+/H^+ exchanger. Early studies (Grinstein *et al*., 1985) showed that histidine-specific reagents produce significant inhibition of the mammalian Na^+/H^+ exchanger. We have also shown that a single membrane associated histidine residue (His 367) is important in the function of sod2, the Na^+/H^+ exchanger of *S. pombe* (Dibrov *et al*., 1998). Similarly His 225 of the bacterial Na^+/H^+ exchanger of *Escherichia coli* (NhaA) was the only histidine important in NhaA function (Gerchman *et al*., 1993). It should be noted that both His 225 of NhaA and His 367 of sod2 are not necessarily predicted to be within transmembrane segments, but may be associated in loops between membrane segments (Fliegel and Dibrov, 1996; Gerchman *et al*., 1999). Only one study has analyzed histidine residues within putative transmembrane domains of NHE1. Mutation of histidine 349 altered amiloride sensitivity of the protein, however there was no change in affinity for Na^+ and no effect on the ability of the transporter to respond to an acid load (Wang *et al*., 1995). The study did not examine any role for His residues that are not directly within the membrane domain of the Na^+/H^+ exchanger.

Examination of the amino acid sequence of the carboxyl terminal region of the NHE1 isoform of the Na^+/H^+ exchanger revealed an unusual sequence of six amino acids HYGHHH at position 540–545. This histidine rich region was located proximal to the *N*-terminal transport domain of the protein. We noted that this histidine rich sequence is similar to that used as a ''His'' tag for proteins to allow purification of proteins by immobilized metal affinity chromatography (Hochuli *et al*., 1987; Smith *et al*., 1988). It has been reported that Y substitutions are not very disruptive to the metal coordination ability of histidine tags (Kasher *et al*., 1993) suggesting that this region could also chelate metal ions. Because of the presence of this unusual sequence, and because previous results with some Na^+/H^+ exchangers that have shown that histidine residues can be important in Na^+/H^+ exchanger activity, we

examined the role of the HYGHHH sequence in the activity of the antiporter. Our results identified a group of amino acids in the antiporter that can chelate metal ions. This group could influence the maximal activity of the protein however mutation of this group of amino acids did not influence the proton sensing ability of the Na^+/H^+ exchanger.

MATERIALS AND METHODS

Materials

Restriction endonucleases and DNA modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD). The AP-1 cell line was a generous gift of Dr. S. Grinstein (Hospital for Sick Children, Toronto, Canada). The site-directed mutagenesis kit (Transformer™ Site-directed Mutagenesis Kit) was from Clontech Inc. (Palo Alto, CA). Anti-HA antibody was purchased from Boehringer Mannheim (Laval, Quebec, Canada) or Berkeley Antibody Co. (Richmond, CA). All other chemicals not listed were of analytical or Molecular Biology grade and were purchased from Fisher Scientific (Ottawa, Ontario, Canada), Sigma (St. Louis, MO) or BDH (Toronto, Ontario, Canada).

Plasmids and Site-Directed Mutagenesis

The plasmid pYN4 + was used to express the Na^+/H^+ exchanger in AP-1 cells. It contains the full-length sequence of the human NHE1 isoform of the antiporter with a hemagglutinin (HA) tag on the carboxyl terminal of the coding region (Wang *et al*., 1998). pYN4 + contains the RSV-LTR promoter, NHE1-HA tag, TK polyA signal and neomycin resistance gene (aminoglycoside 3′-phosphotransferase).

Three types of mutants of the carboxyl terminal region of the Na^+/H^+ exchanger were made. The sequence HYGHHH at position 540–545 was mutated to HHHHHH (6 His), HYGAAA (3 Ala) and HYGRRR (3 Arg) (underlining indicates the mutated amino acids). The synthetic oligonucleotides used for mutation were TCG AAG ACA TCT GTG GCC AC**C** AC**C** **A**TC ACC ACC ACT GGA AGG ACA AGC TTA ACC GGT TTA ATA AGA AAT ATG TG, ATC TGT GGC CAC TAC GGT G**CT GCA GC**C TGG AAG GAC AAG CTC AAC C, and ATC TGT GGC CAC TAC GGT C**GT CGA CG**C TGG AAG GAC AAG CTC AAC C respectively. (Bold residues indicate mutations, underlined residues indicate restriction sites introduced to select positive clones). The reaction was as described in the Transformer™ Site-Directed Mutagenesis Kit. Mutant constructs were checked by restriction enzyme digestion with the new enzyme sites, followed by DNA sequencing.

To express the carboxyl terminal of the Na^+/H^+ exchanger the plasmid pQE51 was used. The primers Na/H CTer taa ccc gGG CGT TAC TGC CCC TTG GGG A and the 6 His primer were used with the template described above to amplify a 850 bp product. PCR with the enzyme PWO DNA polymerase (Boehringer Mannheim) was used and the product was blunt end cloned into the *Bam*HI site of

pQE51 that had been filled in. The resulting product coded for a protein (called NX-2) that begins with the sequence MRDLEDICGHHHHHH- and coded for the carboxyl terminal 281 amino acids of human NHE1. (Underlined amino acids indicate the mutations or additions to the amino acid sequence of NHE1.) To produce the carboxyl terminal amino acids of the Na^+/H^+ exchanger without altering the "endogenous histidine tag" of the antiporter a similar strategy was used. An Eco RI-Hind III subfragment of the exchanger was subcloned into pQE51. It originated from NHE1 cDNA that was inserted in the vector pGEX-KG (a generous gift of Dr. B. Berk, Division of Cardiology, Seattle, WA). The fragment contained the carboxyl terminal 299 amino acids of the cytoplasmic domain of the antiporter. The *Eco*RI site was filled with Klenow as described above and cloned into the *Bam*HI (filled in) site and Hind III site of pQE51. The resulting plasmid was called pTEQE and has the deduced *N*-terminal protein sequence of MRDQFR SINEEIHTQFLDHLLTGIEDICGHYGHHH-.

Protein Production and Purification

To produce the cytoplasmic domain of the Na^+/H^+ exchanger (0.8 mM) IPTG was added to log phase cultures of *E. coli* (Topp 2). Cells were grown for 15 hr at 37°C and pelleted. The pellet was suspended in a minimal volume of buffer B containing 8 M urea, 0.1 M NaH_2PO_4, 0.4 M NaCl, 7.5 mM β-mercaptoethanol, 0.5% Tween 20, pH 8.0. The sample was passed through a French press and also sonicated. The lysate was centrifuged at 20,000*g* for 20 min and the supernatant was used for protein purification. It was incubated for 60 min at 4°C with a Ni-NTA Agarose resin (Qiagen). The slurry was then transferred to a column and washed with buffer B. The column was then washed with Buffer B and then some proteins were eluted with buffer B that had a pH of 6.3. Depending on the experiment the column may then have been washed with buffer B at pH 5.9 followed by buffer B at pH 4.5. The eluates were collected and examined by SDS-PAGE. Gels were stained with Coomassie blue or Western blotted with anti-NHE1 antibodies as described earlier (Haworth *et al*., 1993).

Cell Culture and Stable-Transfection

The AP-1 cell line is derived from wild type Chinese hamster ovary cells, that have lost their endogenous Na^+/H^+ exchanger (Rotin *et al*. (1989). These cells were routinely grown, transfected and clones selected with G418 was essentially as described earlier (Wang *et al*., 1998). Individual colonies were isolated without acid selection. For analysis of enzyme activity at least three clones of each Na^+/H^+ exchanger mutant were isolated independently.

Measurement of Wild Type and Mutant NHE1 Activity *in vivo*

The measurement of pH_i was essentially as described earlier (Dyck and Fliegel, 1995; Wang *et al*., 1998). The untransfected and transfected AP-1 cells were grown to 80% confluency on coverslips. Cells were incubated for 20 hr in serum free media

prior to pH_i measurement as described earlier (Wakabayashi *et al*., 1994). ''Normal Buffer'' for pH_i measurement contained 120 mM NaCl, 5 mM KCl 2.0 mM $CaCl_2$, 1 mM $MgCl_2$, 5.0 mM Glucose and 20 mM HEPES, pH 7.4 at 37°C. Intracellular acidosis was induced using 1–50 mM NH_4Cl (Wakabayashi *et al*., 1992; Kapus *et al*., 1994). The pH_i was measured using the dual excitation single emission ratios as described earlier (Dyck and Fliegel, 1995; Wang *et al*., 1998). The activity measurements illustrated come from the measurements of at least two independently isolated clones of each mutant and are typical of at least three clones. The best fit of the data points to the Hill equation was determined by using the curve-fitting routines contained in MLAB (Modeling Laboratory) that equally weighs each point (Knott, 1977). The best fit for J_{max}, n_{app}, and $pH_{Jmax/2}$ (negative logarithm of the $[H_i^+]$ at half-maximum flux) were determined for wild type NHE1 and for each mutant. The $pH_{Jmax/2}$ was determined by solving for the identity between $[H_i^+]_{0.5}^n$ and K′.

Immunostaining, Immunocytochemistry and Ca^{2+}-Binding

Western Blotting was used to determine the presence and expression levels of wild type and mutant NHE1 protein expressed in transfected AP-1 cells. One hundred μg of total cell lysate were resolved on 9% SDS-PAGE and immunoblotted as described earlier (Wang *et al*., 1998). Ca^{2+}-binding by expressed Na^+/H^+ exchanger protein was measured as described earlier (Fliegel *et al*., 1989). A control Ca^{2+}-binding protein calreticulin was run simultaneously (Fliegel *et al*., 1989). Immunocytochemistry was essentially as described earlier (Wang *et al*., 1998).

RESULTS

Protein Expression and Purification

To examine the ''endogenous Histidine tag'' of the Na^+/H^+ exchanger we produced the carboxyl terminal region of the antiporter in *E. coli*. The protein was produced without fusion to other proteins except for a short leader sequence that contained an initiator methionine and a minimal number of other amino acids that are part of a short leading sequence. Induction of *E. coli* to produce the protein resulted in the appearance of a new 43-kDa protein (Fig. 1A). This size was somewhat higher than the predicted molecular weight of 34.5 kDa however we have seen other fusion proteins of this region of the Na^+/H^+ exchanger also possessing a similar anomalous molecular weight (*not shown*). Antibodies against the carboxyl terminal of the Na^+/H^+ exchanger (Haworth *et al*., 1993) reacted against the newly produced protein (*not shown*). The Na^+/H^+ exchanger protein did not bind Ca^{2+} as determined by Ca^{2+}-binding overlay assay however calreticulin a positive control bound Ca^{2+} (*not shown*). To determine if the residues HYGHHH could function as an ''endogenous Histidine tag'' and bind metal ions we attempted to purify the product using immobilized metal (nickel) affinity chromatography. Elution of proteins from the metal affinity column at pH 6.3 yielded a very significant enrichment

of the 43-kDa protein (Lanes 3 and 4, Fig. 1A). Another elution of the column with pH 4.5 buffer yielded a protein of about 30 kDa but did not yield any further full-length product. This protein was not immunoreactive with our antibody against the carboxyl terminal of the antiporter and was only present when cells were induced. This suggested that it was likely a degradation product of the expressed protein that was truncated at the *c*-terminus. Similar results were obtained with an immobilized metal affinity column that was charged with cobalt as opposed to nickel (*not shown*).

To examine if changing the endogenous Histidine tag from HYGHHH to HHHHHH changed the elution profile of the protein we produced the NX-2 protein that contained this mutation. Samples of induced *E. coli* cell lysate were prepared and subject to immobilized metal affinity chromatograph. The results are shown in Fig. 1B. Cells produced the NX-2 protein with an apparent size of 39 kDa. Again this was higher than the predicted molecular weight of 32.5 kDa. Washing the column at pH 6.3 buffer yielded little NX-2 protein but eluted other proteins (*not shown*). Washing the column with pH 5.8 buffer yielded the major 39-kDa protein plus some minor products. A larger protein of approximately double the size was also present and may represent a dimer. The purity of the fusion protein was greater than that obtained by using the endogenous HYGHHH tag.

To examine the effect of altering the HYGHHH sequence on the activity of the Na^+/H^+ exchanger we made three mutants of this sequence in the full-length protein. The sequence was altered to HYGAAA (3 Ala), HYGRRR (3 Arg) and to HHHHHH (6 His). Initially we determined whether alteration of the Histidine rich sequence affected the expression of the Na^+/H^+ exchanger in AP-1 cells. The results (Fig. 2) showed that the 3 Arg, 3 Ala and 6 His mutants were readily expressed. Control (AP-1) cells (Lane 9) showed no detectable NHE1 protein. There was some clone-dependent variation in the levels of expression of the Na^+/H^+ exchanger. We estimated the level of expression of these proteins using scanning densitometry. It was noticeable that the clones for the six His mutant (Lanes 7 and 8) contained an average of approximately two times the level of the unmutated antiporter (Lane 1). In addition both the Arg and Ala mutants contained more protein than the control, this was 52–79% for Arg mutants and between 0 to 56% for the Ala mutants.

We then measured the initial rate of recovery from varying degrees of acid load and calculated the proton efflux using the appropriate buffering capacity. There was no difference in the buffering capacity of the cells carrying the wild type or mutant antiporters. Figure 3 shows the results of these experiments. For each cell type, the data were fitted to the Hill equation (solid line) and the J_{max}, the n_{app}, and the $pH_{Jmax/2}$ were solved. The maximum velocities of the different cell types varied from about 8 to 14 mM/min. Some of these variations reflected differences in the level of expression of the protein (Fig. 2). For example, the Arg and the Ala mutants characterized for activity contained approximately 50% more protein than controls in the Western blot analysis and had 50% more activity than the control. However it was noticeable that the 6 His clone contained approximately double the protein of the controls, however the maximum velocity was not increased. There were however no differences in the Hill number or $pH_{Jmax/2}$. The apparent Hill number (n_{app}) was not changed by the mutations varying between 2.1 (3 Ala and 6 His) and 2.4 (3 Arg).

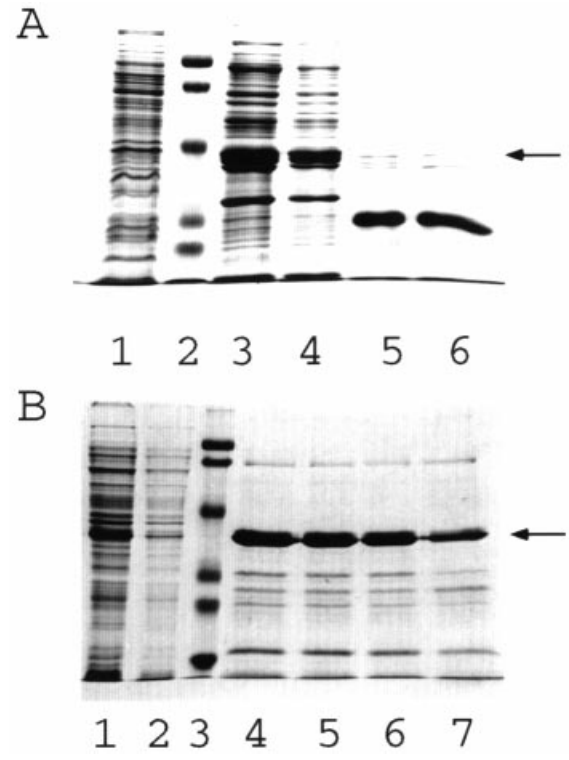

Fig. 1. Purification of the carboxyl terminal region of the Na^+/H^+ exchanger. A. Partial purification using the endogenous HYGHHH sequence. Carboxyl terminal amino acid residues of the NHE1 isoform of the Na^+/H^+ exchanger were expressed in *E. coli* using the plasmid pTEQE as described in the "Materials and Methods". The expressed protein was purified using immobilized metal affinity chromatography and eluted using Buffer of varying pH. Samples were examined by SDS-PAGE. Lane 1, sample applied to column; Lane 2 Bio-Rad prestained markers of size 102, 81, 47, 33, 30, and 24 kDa; Lanes 3 and 4, samples obtained by successive washing of the column with pH 6.3 buffer; Lanes 5 and 6, samples obtained by successive washing of the column with pH 4.5 buffer. Arrow denotes location of the expressed protein. B. Carboxyl terminal amino acid residues of the NHE1 isoform of the Na^+/H^+ exchanger were produced in *E. coli* as described in the "Materials and Methods". The protein included mutation of the HYGHHH sequence to HHHHHH. The expressed protein was purified using immobilized metal affinity chromatography and eluted using Buffer of varying pH. Samples of the fractions were examined by SDS-PAGE. Lane 1, sample applied to column; Lane 2, flow-through of the column; Lane 3 Bio-Rad prestained markers as in "A"; Lanes 4 and 5, samples obtained by successive washing of the column with pH 5.8 buffer; Lanes 6 and 7, samples obtained by successive washing of the column with pH 4.5 buffer. Arrow denotes location of the expressed protein.

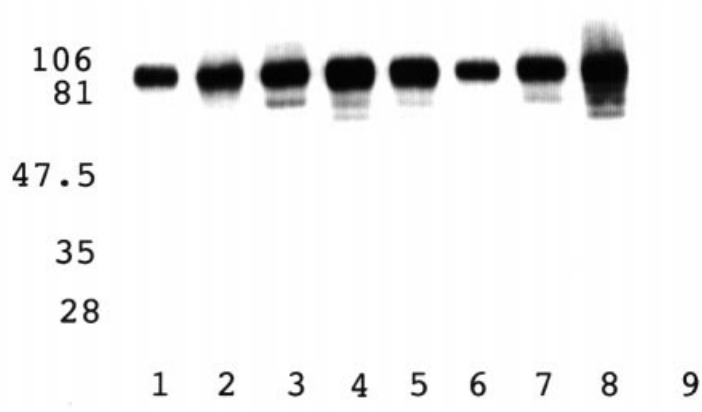

Fig. 2. Western blot analysis of cell lysates of whole AP-1 cells transfected with control and mutated NHE1 cDNA with HA tag. Cells were transfected and prepared for SDS-PAGE and Western blotting with anti-HA antibody as described in the "Materials and Methods". Lane 1, wild type Na^+/H^+ exchanger; Lanes 2–4, independent isolates of the 3 Arg mutant; Lanes 5 and 6, independent isolates of the 3 Ala mutant; Lanes 7 and 8 independent isolates of the 6 His mutant; Lane 9 AP-1 cells not transfected with NHE1 cDNA.

Likewise, the $pH_{Jmax/2}$ was not changed by the mutations, varying between 6.5 (wild type) and 6.8 (3 Ala and 3 Arg).

Figure 4 shows the immunocytochemical localization of the Na^+/H^+ exchanger. Figure 4A shows that the wild-type Na^+/H^+ exchanger is localized in large majority to the plasma membrane. Figure 4B shows that the 6 His mutant is also localized to the plasma membrane. The 3 Ala and the 3 Arg mutants also localized to the plasma membrane (*not shown*). Figure 4C shows untransfected AP-1 cells that showed only a weak background staining and no plasma membrane staining.

DISCUSSION

We have examined the structure and function of the histidine rich region of the carboxyl terminal of the NHE1 isoform of the Na^+/H^+ exchanger. Histidine residues have been shown to be important in function of the *E. coli* Na^+/H^+ exchanger NhaA (Gerchman *et al.*, 1993) and for the yeast *S. pombe* (Dibrov *et al.*, 1998). In the mammalian NHE1 protein, mutation of histidine residues did not alter the transport ability of the protein but one histidine residue affected amiloride sensitivity of the protein (Wang *et al.*, 1995). We noted the presence of a histidine rich region (HYGHHH) at position 540–545 close to the membrane domain that is predicted to terminate near residues 500 (Fliegel and Dibrov, 1996). Initial experiments examined the ability of the histidine rich region to function as an endogenous Histidine tag for the cytoplasmic domain. Our results showed that the HYGHHH region could function as a useful endogenous tag for partial purification of the cytoplasmic domain of the antiporter. The tag could bind to and chelate nickel and cobalt ions.

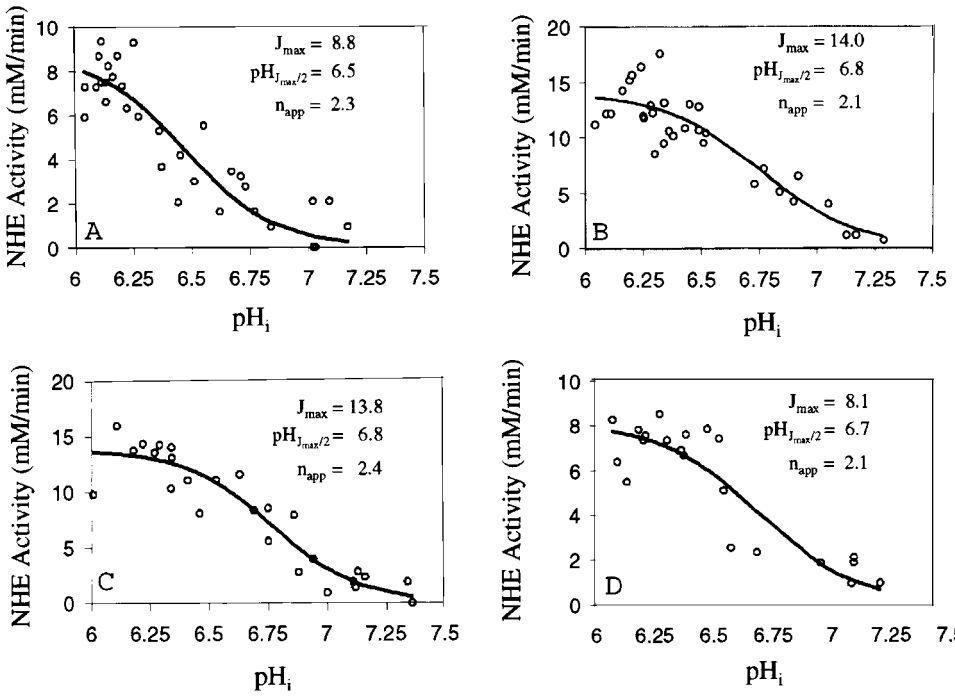

Fig. 3. Dependence of proton efflux on intracellular pH in AP-1 cells transfected with wild type or mutant Na^+/H^+ exchanger. Cells were preincubated for varying concentrations and times with NH_4Cl. A, wild type Na^+/H^+ exchanger; B, 3 Ala; C, 3 Arg; D, 6 His. Recovery was determined in a Na^+-containing (120 mM) at $pH_0 = 7.4$. Efflux was then calculated from the initial rate of recovery times the buffering capacity as described in the "Materials and Methods" and earlier (Silva *et al.*, 1997). J_{max}, K and H were determined by a non-linear regression fit of the data to the Hill model equation ($J = J_{max}[H^+]^n / K_H^n + [H^+]^n$) (Segal, 1975).

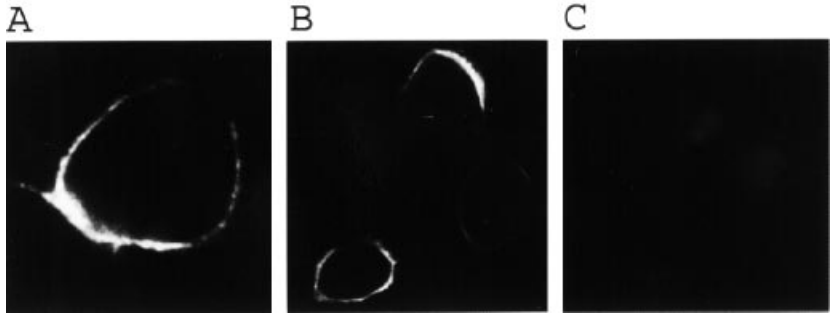

Fig. 4. Immunocytochemical localization of the Na^+/H^+ exchanger protein in transfected AP-1 cells. Cells were transfected and collected and prepared for immunocytochemical analysis with anti-HA antibody as described in the "Materials and Methods". A, B: immunofluorescent images of AP-1 cells transfected with wild type and 6 His mutant respectively. C. immunofluorescent image of non-transfected AP-1 cells prepared with anti-HA antibody as with A and B.

The protein was greatly enriched after passage through and subsequent elution from an immobilized metal affinity column (Fig. 1). A smaller protein was also eluted from the column under more stringent elution conditions. This was likely a degradation product of the larger protein shortened at the *C*-terminus. The antibody used to react with the proteins was made against the most distal 178 amino acids of the carboxyl terminal (Haworth *et al*., 1993). It did not detect this smaller protein (*not shown*) suggesting that the truncation was in the *C*-terminal region.

When we modified the HYGHHH to a six His sequence, we obtained a better purification of the Na^+/H^+ exchanger protein. Using the HYGHHH sequence, the full-length product was eluted at pH 6.3. Further elution at lower pH yielded only the shorted protein. By changing the HYGHHH to a six histidine tag, the full-length protein now remained bound to the column at pH 6.3 and a pH of 5.8 was required for removal from the column. These results show that we had increased the affinity of the endogenous histidine tag for the nickel column. This change also allowed a higher degree of purification of the protein since a wash at pH 6.3 removed more of other contaminating proteins.

We examined the physiological effects of mutation of the endogenous Histidine tag of the antiporter. Mutations were made in the Histidine rich region (AA 540–545) that changed the sequence to HHHHHH, or to HYGAAA, or to HYGRRR. The apparent Hill number (n_{app}) was not changed by the mutations varying between 2.1 (3 Ala and 6 His) and 2.4 (3 Arg). Likewise, the $pH_{i\,max/2}$ was not changed by the mutations varying between 6.5 (wild type) and 6.8. (3 Ala and 3 Arg). We noted however an effect on the V_{max} of the protein. The V_{max} of the 3 Ala and 3 Arg mutations was increased compared to that of the control, however this was certainly the result of a somewhat higher level of expression of the protein in these cells lines (Fig. 2). However, the V_{max} of the 6 His mutation was the same as the control, even though almost double the level of protein was present in these cells (Fig. 2). This suggests that the change to the 6 His mutant, had an inhibitory effect on the maximal velocity of the Na^+/H^+ exchanger. The exact nature of the inhibition is not clear at this time. It is unlikely that there was an effect on the conformation of the membrane domain. It is possible that the endogenous Histidine tag interacts with metal ions *in vivo*, and that the increased affinity of the mutant leads to an inhibitory effect though lack of release of the ion.

The HYGHHH sequence of the NHE1 isoform of the Na^+/H^+ exchanger is well conserved. It is present in the human (Sardet *et al*., 1989) rat (Orlowski *et al*., 1992) mouse (Dewey, M. J. and Bowman, L. H. unpublished, Genebank No. U51112), rabbit (Fliegel *et al*., 1991) and other mammalian NHE1 isoforms in mammals. It is also conserved in many other NHE1 isoforms of the protein in more unrelated species such as *Xenopus* (Busch, 1997), the salamander *Amphiuma* (McLean *et al*., 1999) in the carp (*Cyprinus carpio*) and in the European flounder *Platichthys flesus* (unpublished observations, Wright, C and Cossins, A. R. Genebank accession numbers AJ006916 and AJ006918, respectively). This high degree of conservation confirms an important role for the HYGHHH sequence in the function of the protein. It should be noted however that the HYGHHH sequence is not conserved in the other Na^+/H^+ exchanger isoforms (NHE2–NHE6) though NHE5 contains a similar sequence HHGYHY (Attaphitaya *et al*., 1999). The HYGHHH

sequence is also found on the rubella virus membrane glycoprotein E1 (Vidgren *et al*., 1987) where an additional histidine follows the sequence, however, its role has not been studied there. Similar histidine rich sequences including some with six adjacent Histidine residues are found in a variety of proteins. The histidine proline rich glycoprotein has a histidine dependent interaction with heparin that is both pH and metal ion dependent (Borza and Morgan, 1998). Some histidine rich proteins have been shown to bind metals ions (Nair and Robinson, 1999) or calcium (Ridgeway *et al*., 1999). In the case of the Na^+/H^+ exchanger, our results showed that this histidine rich region could bind Nickel or cobalt (Fig. 1), but could not bind calcium (*not shown*). In addition the results suggest that this region did not affect protein function in a pH dependent manner.

While we found that mutation of the sequence resulted in a decreased activity of the protein, the protein was still functional overall. These results are similar to the early results of Grinstein *et al*. (Grinstein *et al*., 1985) that showed inhibition of the antiporter with histidine-specific reagents. Our results localize the region of the protein in which histidines are important. We also found that in these mutants, the activation by protons was unchanged when comparing mutated to unmutated antiporter. Therefore it is clear that unlike His 225 of *E. coli* and His 367 of sod2, the histidine residues of NHE1 are not involved in proton sensing by the mammalian Na^+/H^+ exchanger. Future experiments will attempt to determine the residues involved in proton sensing in the mammalian Na^+/H^+ exchanger.

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