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Identification of Cytoplasmic Subdomains That Control pH-Sensing of the Na^+/H^+ Exchanger (NHE1): pH-Maintenance, ATP-Sensitive, and Flexible Loop Domains[1]

Toshitaro Ikeda,* Bernhard Schmitt,*,[2] Jacques Pouysségur,† Shigeo Wakabayashi,*,[3] and Munekazu Shigekawa*

*Department of Molecular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka 565; and †Centre de Biochimie, Centre National de la Recherche Scientifique, Université de Nice, 06108 Nice Cedex 2, France

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To precisely identify the cytoplasmic subdomains that are responsible for the intracellular pH (pH_i)-sensitivity, ATP depletion-induced inhibition and Ca^{2+} activation of the Na^+/H^+ exchanger (NHE1), we generated a set of deletion mutants of carboxyl-terminal cytoplasmic domain and expressed them in the exchanger-deficient cell line PS120. We evaluated pH_i-sensitivity of these mutants by measuring the resting pH_i in cells placed in an acidic medium (pH 6.0) and pH_i-dependence of 5-(*N*-ethyl-*N*-isopropyl)amiloride-sensitive $^{22}Na^+$ uptake. Detailed analysis revealed that the cytoplasmic domain of NHE1 is consists of at least four subdomains in terms of pH_i-sensitivity of the unstimulated NHE1: I, aa 516-590/595; II, aa 596-635; III, aa 636-659; and IV, aa 660-815. Subdomains II and IV were silent for pH_i-sensitivity. Subdomain I had a pH_i-maintenance function, preserving pH_i-sensitivity in a physiological range, whereas subdomain III, overlapping with the high affinity calmodulin (CaM)-binding site, exhibited an autoinhibitory function. Deletion of subdomain I abolished the decrease of pH_i-sensitivity induced by cell ATP depletion, indicating that domain I plays a crucial role in this phenomenon. Deletion of subdomain III rendered the inhibition by ATP depletion less efficient, suggesting the possible interaction between subdomains I and III. On the other hand, tandem elongation of subdomain II by insertion did not affect either the inhibitory function of domain III or the removal of this inhibition by ionomycin or thrombin. However, deletion of subdomain II partially abolished the inhibitory effect of subdomain III. Subdomain II thus seems to function as a mobile "flexible loop," permitting the CaM-binding subdomain III to exert its normal function. These findings, together with our previous data, support a concept that cell ATP, Ca^{2+}, and growth factors regulate NHE1 *via* a mechanism involving direct or indirect interactions of specific cytoplasmic subdomains with the "H^+-modifier site."

Key words: ATP depletion, domain structure, growth factor, intracellular Ca^{2+}, Na^+/H^+ exchanger.

A family of mammalian Na^+/H^+ exchangers was recently cloned (*1-5*). All these exchanger molecules apparently consist of two distinct functional domains, *i.e.*, an N-terminal ion-transport domain containing 10-12 transmembrane spanning segments and a C-terminal cytoplasmic regulatory domain. NHE1 is a ubiquitous plasma membrane transporter that regulates intracellular pH (pH_i) and cell volume in virtually all cells (*6, 7*). Among the known isoforms, NHE1 has most extensively been studied in terms of the structure-function relationship (*8-16*). NHE1 is a phosphorylatable, calmodulin-binding protein (*8, 14*) and highly sensitive to inhibition by amiloride analogues (*10*). It has *N*- and *O*-linked oligosaccarides (*12*) and exists as an oligomer in the membrane (*13*).

The most prominent feature of NHE1 is that it is rapidly activated in response to various extracellular stimuli, including growth factors, Ca^{2+}-releasing agonists, and hyperosmotic stress (*6, 7*). These stimuli enhance activity of NHE1 by shifting its pH_i-dependence to the alkaline side (*17-19*). This mode of activation is different from that for epithelial isoforms NHE2 and NHE3, because activation of the latter exchangers by growth factors was reported to be due to an increase in the maximal activity (V_{max}) (*20, 21*). On the other hand, ATP depletion inhibits NHE1 by shifting its pH_i-dependence to the acidic side (*9, 22*) as well as by reducing V_{max} (*20, 23, 24*). The observed shift in

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[3] To whom correspondence should be addressed. Phone: +81-6-833-5012 (Ext. 2566), Fax: +81-6-872-7485, e-mail: wak@ri.ncvc.co.jp

Abbreviations: aa, amino acid(s); CaM, calmodulin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DOG, 2-deoxyglucose; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NHE1, Na^+/H^+ exchanger isoform 1; pH_i, intracellular pH; pH_o, extracellular pH.

pH_i-sensitivity is usually explained by assuming a regulatory H^+-modifier site in NHE1 that is distinct from the Na^+ or H^+ transport site (*25*). This modifier site is thought to be a key element in the regulation of NHE1.

Our previous studies revealed that deletion of the entire cytoplasmic domain or its subdomain of NHE1 causes a constitutive acidic shift of pH_i-dependence of Na^+/H^+ exchange (*9, 11*), whereas deletion or point mutations of the high affinity calmodulin-binding segment induced a constitutive alkaline shift of pH_i-dependence (*15*). The latter segment was previously identified as one that plays a key role in Ca^{2+}-induced activation of NHE1 (*14-16*). However, subdomains in the cytoplasmic domain have never systematically been examined for their function.

In the present study, we generated NHE1 mutants carrying deletions of the cytoplasmic domain and expressed them in the exchanger-deficient cell line PS120. Using cells expressing these mutants, we attempted to answer several important questions not yet fully studied: (1) which cytoplasmic subdomains affect the pH_i-sensitivity of NHE1 in the quiescent cells?; (2) which region(s) plays an essential role in ATP depletion-induced inhibition of NHE1?; (3) which cytoplasmic region(s), in addition to the CaM-binding domain identified previously (*14*), is required for Ca^{2+}-induced activation? Detailed analyses revealed that the cytoplasmic domain of NHE1 is separated into at least four different subdomains in terms of pH_i-sensitivity. We provide evidence showing that some of these subdomains play an essential role in the Ca^{2+}-induced activation or the ATP depletion-induced inhibition of NHE1.

EXPERIMENTAL PROCEDURES

Materials—The amiloride derivative, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) was a gift from New Drug Research Laboratories of Kanebo (Osaka). $^{22}NaCl$ and [7-^{14}C]benzoic acid were purchased from Dupont NEN. All other chemicals were of the highest purity available.

Cells, Culture Conditions, and Stable Expression—The Na^+/H^+ antiporter-deficient cell line (PS120) (*26*) and transfectants were maintained in Dulbecco's modified Eagle's medium (Life Technologies) containing 25 mM $NaHCO_3$ and supplemented with 7.5% (v/v) fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37°C in the presence of 5% CO_2. The exchanger-deficient PS120 cells (5×10^5 cells/100-mm dish) were transfected with each plasmid construct (20 μg) by the calcium phosphate co-precipitation technique as described (*9*). Cell populations that stably express NHE1 variants were selected by the "H^+-killing" procedure as described (*9*). All experiments in this study were carried out using the resulting stable transfectants consisting a mixed population expressing each plasmid with different copy number.

Construction of Na^+/H^+ Exchanger Mutants—The plasmid including cDNA coding for the Na^+/H^+ exchanger (NHE1 human isoform) deleted of the 5′-untranslated region was described previously (plasmid designated pEAP-Δ5′) (*9*). Construction of plasmids carrying C-terminal truncation mutants Δ515, Δ566, Δ635, Δ698, and Δ792, and internal deletion mutants lacking aa 515-566 (Δ515-566), and aa 515-635 (Δ515-635) was described previously (Fig. 1) (*9, 11, 14*). For the construction of C-terminal truncation mutants Δ540, Δ575, Δ580, Δ585, Δ590, Δ595, Δ605, Δ615, and Δ625, the oligonucleotide antisense primers were designed to contain a stop codon and an exogenous *Eco*RI site just after the triplet corresponding to the amino acid indicated. Using these antisense primers and an upstream sense primer corresponding to aa 279-285, DNA fragments were generated by PCR with pEAP-Δ5′ as a template (*11, 14*). The generated fragments were digested and inserted into *Bsu*36I and *Eco*RI (cloning site of pECE) sites of pEAP-Δ5′.

For the construction of mutants carrying other C-terminal truncations, internal deletions or insertions, we created two unique restriction sites for *Afl*II and *Nde*I in the positions of plasmid pEAP-Δ5′ corresponding to aa 635 and aa 659 of NHE1, respectively. We created these restriction sites by designing complementary sense and antisense primers in such a way that the original amino acids were not changed. With these mutant primers and two external primers corresponding to aa 279-285 (sense) and the 3′-noncoding region (antisense), we generated DNA fragments including replaced nucleotides by repeating PCR twice using pEAP-Δ5′ as a template (*11*). The DNA fragments were then digested and inserted into *Bsu*36I (aa 358) and *Eco*RI sites of the plasmid pEAP-Δ5′. The resulting plasmid (pEAP-Δ5′AN) did not contain the 3′-noncoding sequence, which is not essential for expression of the wild-type NHE1. In pEAP-Δ5′AN, the *Bsu*36I site (aa 635) in pEAP-Δ5′ was replaced with the *Afl*II site.

For the construction of Δ659, the oligonucleotide antisense primer was designed to contain a stop codon and an exogenous *Eco*RI site just after aa 659. Using this antisense primer and the sense primer corresponding to aa 279-285, a DNA fragment was generated by PCR with pEAP-Δ5′AN as a template. The generated fragment was digested and inserted into the *Bsu*36I and *Eco*RI restriction sites of pEAP-Δ5′AN. For the construction of internal deletion mutant Δ596-634, the antisense primer was designed to contain an exogenous *Afl*II site just after aa 595. Using this antisense and the same sense primer used for Δ659 and pEAP-Δ5′ as a template, a DNA fragment was generated by PCR, digested with *Bsu*36I and *Afl*II, and then inserted into the *Bsu*36I and *Eco*RI sites of pEAP-Δ5′AN. For the construction of insertion mutant +596-636, the sense and antisense primers were designed to contain an exogenous *Afl*II site just before aa 596 and an exogenous *Eco*RI site and a stop codon just after aa 815, respectively. A DNA fragment was produced by PCR using pEAP-Δ5′ as a template, digested with *Afl*II and *Eco*RI, and then inserted into the *Afl*II and *Eco*RI sites of pEAP-Δ5′AN. This insertion mutant contains two aa 596-636s in tandem. DNA sequences of incorporated PCR fragments were determined on a Perkin Elmer ABI Model 373S autosequencer.

Measurement of $^{22}Na^+$ Uptake and pH_i—EIPA-sensitive $^{22}Na^+$ uptake was measured in stable transfectants grown up to confluence in 24-well dishes after cells had been acidified by a NH_4Cl prepulse technique as described (*16*). Stable transfectants were incubated for 5 h in a serum-free, bicarbonate-free culture medium buffered with 20 mM Hepes (pH 7.4) to maintain the Na^+/H^+ exchanger in the resting state. Cells were loaded with NH_4Cl for 30 min at 37°C in NaCl standard solution [20 mM Hepes/Tris (pH 7.4), 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$,

and 5 mM glucose] containing 0-30 mM NH_4Cl. When cell ATP was depleted, NH_4^+ loading was performed for 30 min in choline chloride standard solution containing 0-50 mM NH_4Cl, 5 mM KCl, and either 5 mM 2-deoxyglucose or 5 mM 2-deoxyglucose plus 2 μg/ml oligomycin in order to avoid intracellular Na^+ accumulation caused by ATP depletion. The choline chloride standard solution contained 20 mM Hepes/Tris (pH 7.4), 120 mM choline chloride, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. When NH_4Cl was present, the concentration of choline chloride was reduced to adjust the osmolarity of the medium. After NH_4Cl loading, cells were rapidly washed once with choline chloride standard solution and then incubated in the same medium for 40 s. $^{22}Na^+$ uptake was started by exposing cells to choline chloride standard solution containing $^{22}NaCl$ (37 kBq/ml) (final concentration, 1 mM) and 1 mM ouabain. After 40 s, cells were rapidly washed four times with ice-cold phosphate-buffered saline to terminate $^{22}Na^+$ uptake. For some wells, the choline chloride solution additionally contained 0.1 mM EIPA. When the effect of ionomycin or thrombin was measured, these agents were present in the choline chloride solution during washing and $^{22}Na^+$ uptake. Cells were then solubilized with 0.1 N NaOH, and radioactivity was measured on a γ-counter.

pH_i was measured by monitoring the distribution of [^{14}C]benzoic acid (74 kBq/ml) under the same conditions as those used for $^{22}Na^+$ uptake measurement, except that the uptake medium contained [^{14}C]benzoic acid and nonradioactive NaCl (*15, 16*). pH_i at an acidic extracellular pH was measured essentially as described previously (*15, 27*). Briefly, confluent cells in 24-well dishes were serum-depleted for 5 h and then incubated for 1 h in a low pH medium [20 mM MES/Tris (pH 6.0), 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM glucose]. Cells were incubated for 5 min in the same low pH medium containing 7.4 kBq/ml [7-^{14}C]benzoic acid and then washed four times with ice-cold phosphate-buffered saline. ^{14}C-radioactivity was counted on a liquid scintillation counter. pH_i was calculated as described previously (*27*).

In some experiments, we measured pH_i-dependence of $^{22}Na^+$ uptake in cells pH_i-clamped by the K^+/nigericin method (*23, 24*). Serum-depleted cells in 24-well dishes were preincubated for 30 min at 37°C in Na^+-free choline chloride/KCl medium [20 mM Hepes/Tris (pH 7.4), 120 mM choline chloride/KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$] containing 5 μM nigericin and either 5 mM glucose, 5 mM 2-deoxyglucose, or 5 mM 2-deoxyglucose plus 5 μg/ml oligomycin. The KCl concentration in this medium was varied from 1 to 120 mM, while the total concentration of KCl plus choline chloride was maintained at 120 mM. Our preliminary experiment indicated that a relatively long preincubation time (30 min) was required for pH equilibration in response to imposed K^+ gradients. $^{22}Na^+$ uptake was started by adding the choline chloride/KCl solution containing $^{22}NaCl$ (37 kBq/ml) (final concentration, 1 mM), 1 mM ouabain, and 100 μM bumetanide. After 1 min, cells were rapidly washed four times with ice-cold phosphate-buffered saline to terminate $^{22}Na^+$ uptake. For some wells, the choline chloride solution additionally contained 0.1 mM EIPA. pH_i was calculated from the imposed [K^+] gradient by assuming $[K^+]_i/[K^+]_o = [H^+]_i/[H^+]_o$ at equilibrium and an intracellular [K^+] of 120 mM.

Others—Protein concentration was measured by bicinchoninic assay system (Pierce Chemical) using bovine serum albumin as a standard.

RESULTS

Identification of Cytoplasmic Subdomains That Control pH_i-Sensitivity of NHE1—We generated NHE1 mutants carrying a set of deletions of the cytoplasmic domain to identify subdomains that control pH_i-sensitivity of the exchanger (Fig. 1). First, we evaluated the ability of various mutant transfectants placed in an acidic medium to control their resting pH_i. Cells expressing the Na^+/H^+ exchanger possess a significantly higher resting pH_i in acidic media compared to the exchanger-deficient PS120 cells (*15, 27*). Although the resting pH_i is determined by both the pH_i-sensitivity of a particular NHE1 mutant and its activity (V_{max}) expressed in the plasma membrane, this approach has the following advantages: it permits us to evaluate H^+-extrusion *via* mutant exchangers under nearly physiological conditions; it also permits us to assess pH_i-sensitivity of a much larger number of mutant transfectants in a single experiment, compared to the measurement of pH_i-dependence of EIPA-sensitive $^{22}Na^+$ uptake.

In Fig. 2, we plot the resting pH_i for individual mutant transfectants at $pH_o = 6.0$ against their EIPA-sensitive $^{22}Na^+$ uptake activity that was measured separately under conditions where $^{22}Na^+$ uptake nearly reached V_{max}. In truncation mutants Δ792, Δ698, and Δ659, both the resting pH_i and $^{22}Na^+$ uptake activity did not differ significantly from those of the wild type, suggesting that the cytoplasmic C-terminal tail (aa 659-815) is nearly silent for pH_i-sensitivity of NHE1. Further truncation of 24 amino acids (Δ635) increased the resting pH_i in spite of a significant decrease in $^{22}Na^+$ uptake activity, indicating that Δ635 has high pH_i-sensitivity, which is consistent with the previous results (*15, 28*). Δ635 lacks the high affinity CaM-binding domain (aa 635-659) that was previously shown to have an autoinhibitory function against the "pH_i-sensor" of NHE1 (*15*). Upon further truncation to aa 595, high pH_i was maintained without a significant change in $^{22}Na^+$ uptake

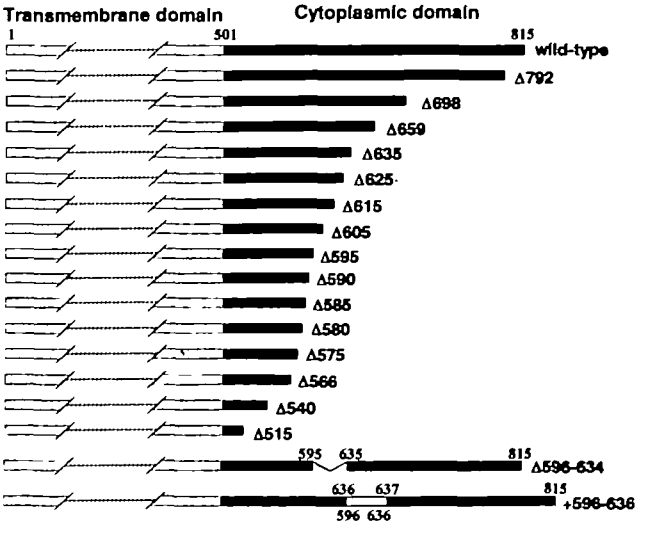

Fig. 1. **Schematic representation of NHE1 deletion mutant constructs.** Numbers indicate amino acid residues.

activity. However, pH_i decreased gradually when amino acids were further deleted down to aa 515. Thus successive deletion of the cytoplasmic C-terminal tail changes the resting pH_i, and the result suggests that the mutants tested can be separated into several groups in terms of pH_i-sensitivity.

Second, we devised a simple method to correct for variation in the activity of individual mutant exchangers expressed in the plasma membrane, which reflects the number of the active mutant molecules expressed. We measured initial rates of $^{22}Na^+$ uptake into the particular mutant transfectants that had been acid-loaded to pH_is of 7.23 ± 0.09 ($n=20$) and <6.2 by 30-min prepulses with 3 and 30 mM NH_4^+, respectively, and the ratio between these activities was calculated for each mutant (Fig. 3). Because cells prepulsed with 30 mM NH_4^+ exhibited $^{22}Na^+$ uptake activity close to V_{max} (see Fig. 4), the ratio should reflect a change in the pH_i-sensitivity if measured in an appropriate pH_i range. As shown in Fig. 3, the ratios for mutants with C-terminal truncation down to aa 659 were not significantly different from that of the wild type. However, the ratio increased significantly upon further truncation of 24 amino acids (⊿635). It remained elevated as deletion proceeded to aa 590, then decreased gradually as deletion approached aa 566. We observed that pH_i-sensitivities of ⊿595 and ⊿590 were similar. However, slight uncertainty remains about this, because the resting pH_i was higher for ⊿595 than for ⊿590, although V_{max} was also lower in the latter (Fig. 2). For the mutants with extensive C-terminal truncation (⊿515-⊿566), which exhibit significantly lower pH_i-sensitivity, we calculated the ratio between $^{22}Na^+$ uptake activities measured after the 30-min prepulses with 7 and 30 mM NH_4^+ (inset to Fig. 3). The ratio decreased as deletion proceeded from aa 566 to 515, suggesting that the

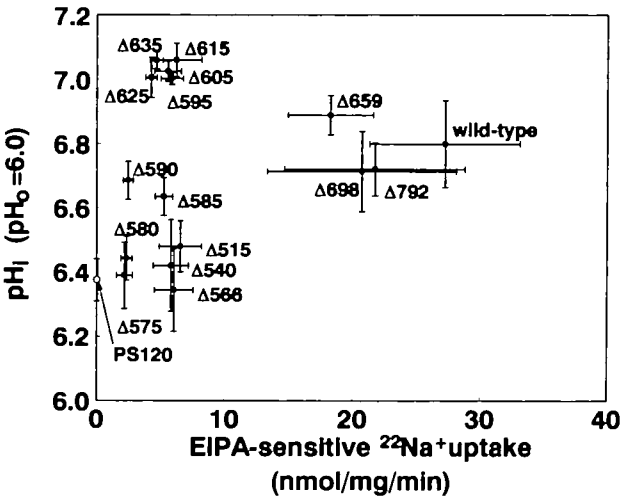

Fig. 2. **Intracellular pH and $^{22}Na^+$ uptake in cells expressing various NHE1 deletion mutants.** pH_i at $pH_o=6.0$ and EIPA-sensitive $^{22}Na^+$ uptake after 30 mM NH_4^+ prepulse were measured as described under "EXPERIMENTAL PROCEDURES." Data are means±SD of four independent experiments. Open circles, PS120 cells.

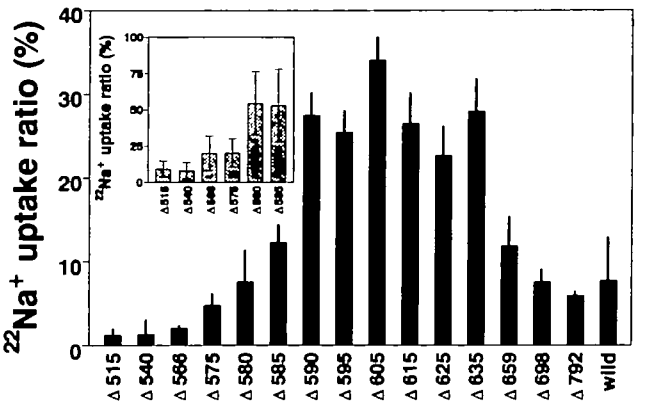

Fig. 3. **pH_i-sensitivity of deletion mutants as estimated by the ratio between $^{22}Na^+$ uptake activities after 3 mM NH_4^+ and 30 mM NH_4^+ prepulses.** EIPA-sensitive $^{22}Na^+$ uptake activities in individual mutant transfectants prepulsed with 3 and 30 mM NH_4^+ were measured as described under "EXPERIMENTAL PROCEDURES" and the ratio of these activities are shown. The inset shows the ratio between EIPA-sensitive $^{22}Na^+$ uptake activities after 7 mM NH_4^+ and 30 mM NH_4^+ prepulses. Data are means±SD of four independent experiments performed on different days.

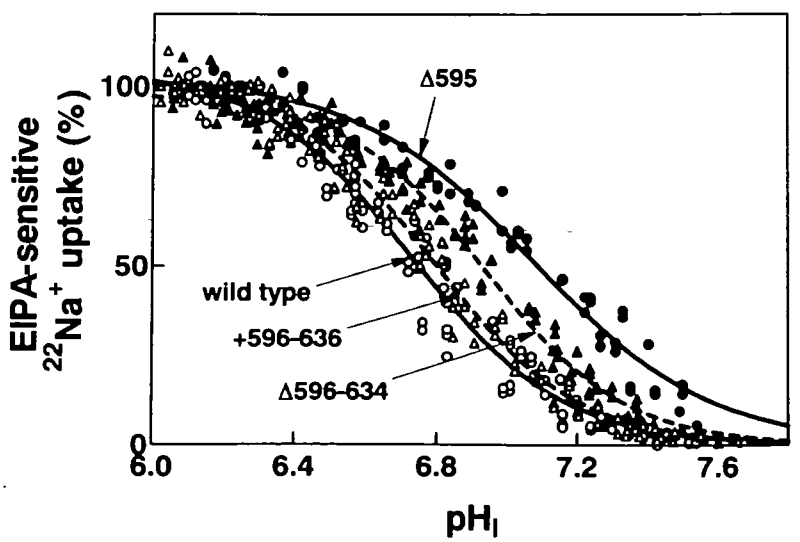

Fig. 4. **pH_i dependence of EIPA-sensitive $^{22}Na^+$ uptake in several NHE1 variants.** The rate of $^{22}Na^+$ uptake and pH_i during uptake were measured in cells transfected with the following NHE1 variants: wild-type (○), ⊿595 (●), ⊿596-634 (▲), and +596-636 (△). $^{22}Na^+$ uptake was measured by the NH_4^+-prepulse method and activity was normalized by the V_{max} value. Data were taken from at least three independent experiments.

overall structure of this region is required for the maintenance of high pH_i-sensitivity.

Finally, in order to confirm these results, we directly measured pH_i-dependence of EIPA-sensitive $^{22}Na^+$ uptake in several selected mutants and compared their pK values (Figs. 4 and 7A). The rank order of pKs for the deletion mutants tested was $\Delta635 \cong \Delta595 >$ wild-type $\cong \Delta698 \cong \Delta659 > \Delta566 > \Delta515$. Based on all these results, we conclude that the cytoplasmic domain of NHE1 consists of at least four subdomains in terms of pH_i-sensitivity: (I) aa 516-590/595, (II) aa 596-635, (III) aa 636-659, and (IV) aa 660-815.

Role of pH_i-Silent Region aa 596-635—As described above, aa 596-635 of the cytoplasmic domain of NHE1 is silent for pH_i-sensitivity. We produced mutants deleted of or inserted with this region (see Fig. 1). It should be noted that in these mutants the autoinhibitory CaM-binding domain (aa 635-659) was preserved but displaced to different positions in the primary structure. We measured pH_i-dependence of EIPA-sensitive $^{22}Na^+$ uptake into cells expressing these mutants (Fig. 4). In the internal deletion mutant (Δ596-634), the pH_i-dependence was shifted to the alkaline side, although the shift was significantly less than that in Δ595. In the tandem insertion mutant (+596-636), however, no shift of pH_i-dependence was not observed. Thus the autoinhibitory function of aa 635-659 was partially lost in Δ596-634, whereas it was kept in the insertion mutant.

In Fig. 5 shows the effect of short incubation with ionomycin or thrombin on EIPA-sensitive $^{22}Na^+$ uptake into mutant transfectants prepulsed by 5 mM NH_4^+. Enhancement of exchanger activity in response to these stimuli was mostly abolished in the internal deletion mutant (Δ596-634) as well as in Δ595, whereas it was preserved in the insertion mutant (+596-636). This activation was due to an alkaline shift of the pH_i-dependence of $^{22}Na^+$ uptake (data not shown). In Δ595, we observed a slight enhancement of $^{22}Na^+$ uptake by ionomycin (Fig. 5). This is probably due to intracellular acidification caused by ionomycin-induced Ca^{2+} mobilization. All these data suggest that the segment immediately N-terminal to the CaM-binding domain needs to be long enough for the CaM-binding domain to exert its inhibitory effect.

ATP Depletion-Induced Decrease in pH_i-Sensitivity—Cell ATP depletion has previously been shown to cause a large acidic shift of pH_i-dependence of $^{22}Na^+$ uptake by NHE1 (*9*). We determined which segment of the cytoplasmic domain is responsible for the inhibition by ATP depletion. ATP was depleted by preincubating cells with either 5 mM DOG or 5 mM DOG plus 2 μg/ml oligomycin during a 30-min NH_4^+ preloading, which reduced cell ATP to 15.3±4.0 and 2.2±1.0% (means±SD, n=3) of control, respectively. These treatments induced a large acidic shift of pH_i-dependence of $^{22}Na^+$ uptake without any change in V_{max} in the wild-type NHE1 (Fig. 6A), consistent with the previous report (*9*). However, this does not agree with other reports (*20, 23, 24*) showing V_{max} to be reduced by ATP-depletion. In some of these previous studies (*23, 24*), pH_i was manipulated using the K^+/nigericin pH-clamp method. Thus the discrepancy could be due to the difference in the methods employed. We thus repeated the same experiment using the K^+/nigericin method (Fig. 6B). The result was essentially the same, except that the acidic shift induced by DOG alone was significantly larger in cells treated with NH_4^+ than in those treated with K^+/nigericin. The latter DOG effects may reflect a difference in the severity of ATP depletion that could have resulted from the different levels of metabolism in cells treated with NH_4^+ compared to those treated with K^+/nigericin. In cells treated with NH_4^+, ATP was depleted at a relatively alkaline pH_i during the 30-min NH_4^+ preloading, whereas

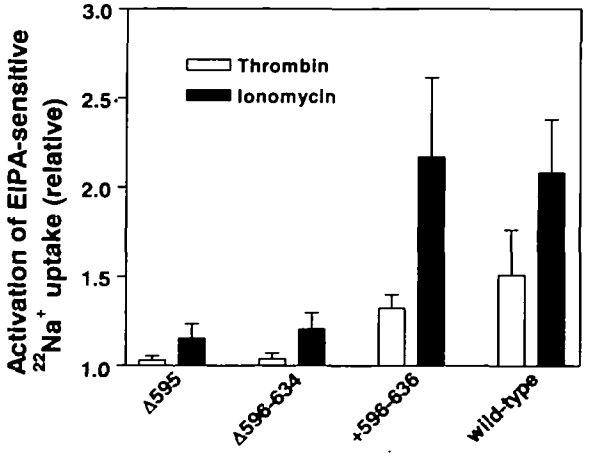

Fig. 5. **Effects of ionomycin and thrombin on the rate of EIPA-sensitive $^{22}Na^+$ uptake.** EIPA-sensitive $^{22}Na^+$ uptake was measured in the absence or presence of ionomycin and thrombin as described under "EXPERIMENTAL PROCEDURES." Stimulation by ionomycin or thrombin is expressed relative to the activity in the absence of these agents (means±SD of four independent experiments).

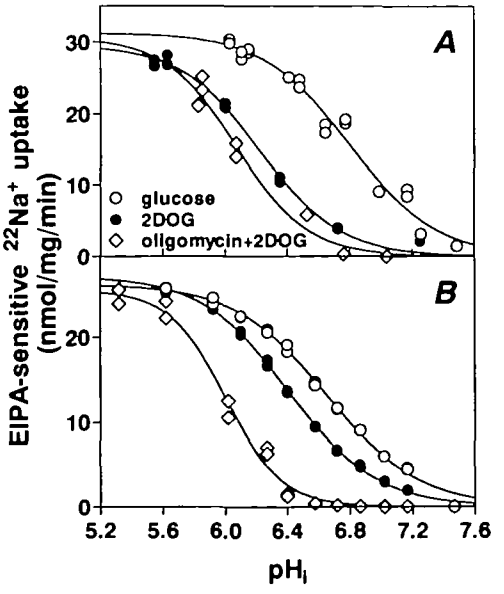

Fig. 6. **Effect of ATP depletion on pH_i-dependence of EIPA-sensitive $^{22}Na^+$ uptake in the wild-type NHE1.** Wild-type transfectants were preincubated for 30 min with either 5 mM glucose (○), 5 mM DOG (●), or 5 mM DOG plus 2 μg/ml oligomycin (△). Then pH_i dependence of $^{22}Na^+$ uptake was measured by the NH_4^+-prepulse (A) or the K^+/nigericin pH_i-clamp (B) method as described under "EXPERIMENTAL PROCEDURES." The slightly more acidic pH_i dependence measured by the latter method may be due to the inhibition of $^{22}Na^+$ uptake by K^+ (*34*).

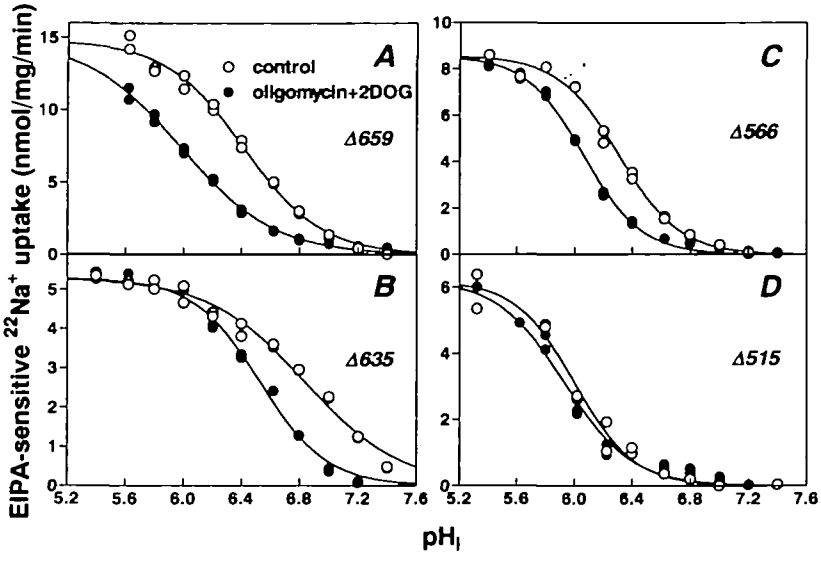

Fig. 7. **Effect of ATP depletion on pH$_i$-dependence of EIPA-sensitive $^{22}Na^+$ uptake by various deletion mutant transfectants.** pH$_i$ dependences of EIPA-sensitive $^{22}Na^+$ uptake into cells transfected with NHE1 deletion mutants were measured under control (○) and ATP depletion (●) conditions by the K^+/nigericin method as described under "EXPERIMENTAL PROCEDURES." ATP depletion was induced by preincubating cells for 30 min with 5 mM DOG plus 2 μg/ml oligomycin.

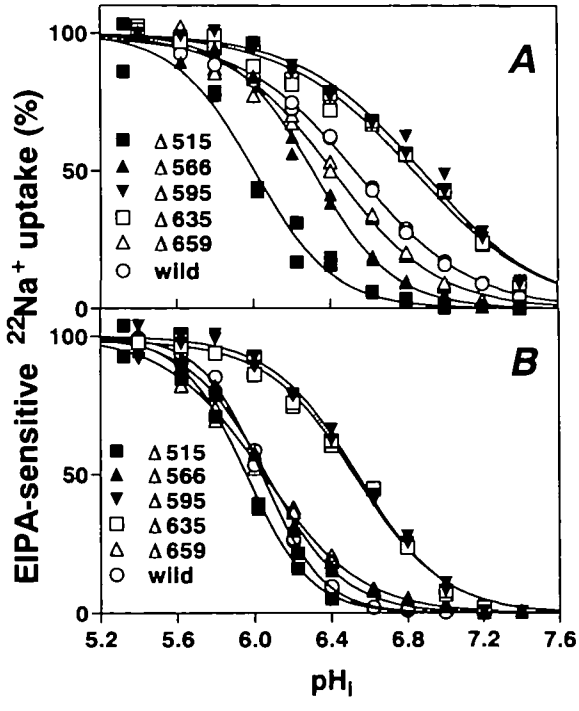

Fig. 8. **Comparison of pH$_i$-dependences of EIPA-sensitive $^{22}Na^+$ uptake in various deletion mutant transfectants under control (A) and ATP depletion (B) conditions.** pH$_i$ dependences of EIPA-sensitive $^{22}Na^+$ uptake into cells transfected with indicated NHE1 variants were measured under control (A) and ATP depletion (B) conditions by the K^+/nigericin method as described under "EXPERIMENTAL PROCEDURES." ATP depletion was induced by preincubating cells for 30 min with 5 mM DOG plus 2 μg/ml oligomycin. Data are represented as the $^{22}Na^+$ uptake activity normalized by the V_{max} value.

in cells treated with K^+/nigericin, ATP depletion was performed for the same period of time but at more acidic pH$_i$s used for the following activity measurements.

Figure 7 shows the effect of the DOG plus oligomycin-induced ATP depletion on pH$_i$-dependences of EIPA-sensitive $^{22}Na^+$ uptake in mutants with various C-terminal truncations by using the K^+/nigericin method. In all these mutants except Δ515, ATP depletion caused an acidic shift of pH$_i$-dependence without a change in V_{max}, as in the wild-type NHE1. In Δ515, neither the acidic pK-shift nor the V_{max} change was observed, which indicates that the cytoplasmic domain is required to confer ATP-sensitivity. Figure 8 shows pH$_i$-dependences of $^{22}Na^+$ uptake normalized by V_{max} in control (A) and ATP-depleted cells (B). Interestingly, in ATP-depleted cells, the wild type, Δ659, and Δ566 all exhibited essentially the same acidic pH$_i$-dependences as that for Δ515, whereas Δ635 and Δ595 exhibited significantly more alkaline pH$_i$-dependences (Fig. 8B). In contrast, pH$_i$-dependences of these mutants under control conditions were generally shifted to the alkaline side (except for Δ515) and the magnitude of the shift varied in the order of pKs described above (Fig. 8A). These results were confirmed when $^{22}Na^+$ uptake was measured by the NH_4^+-prepulse method in a pH$_i$ range from 6.0 to 7.6.

DISCUSSION

In this study, we analyzed the effect of successive C-terminal truncation of the cytoplasmic domain of NHE1 on the pH$_i$-dependence of exchange activity, and found that the cytoplasmic domain of NHE1 consists of at least four subdomains in terms of pH$_i$-sensitivity: I, aa 516-590/595; II, aa 596-635; III, aa 636-659; IV, aa 660-815 (Fig. 9). Truncation of subdomain IV did not exert a significant effect on the pH$_i$-sensitivity of NHE1 (see Δ659 and Δ698 in Figs. 2, 3, and 8A). Further truncation of subdomain III (Δ635) induced an alkaline shift (~0.3 pH unit) of pH$_i$-dependence of the exchange (Figs. 3 and 8A). Subdomain III was characterized previously to be a CaM-binding domain that has an autoinhibitory function against "pH$_i$-sensor" of NHE1 (*15, 16*). Subdomain II, on the other hand, was apparently silent for the pH$_i$-sensitivity (Figs. 2, 3, and 8A), but it plays an important role in the function of subdomain III as discussed below. Further truncation of subdomain I to produce Δ515 resulted in a remarkable

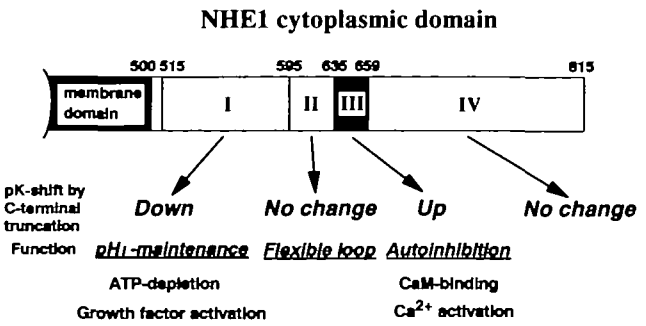

Fig. 9. **Schematic representation of cytoplasmic subdomains identified in this study.** The NHE1 cytoplasmic domain can be separated into at least four subdomains (I to IV) in terms of pH_i-sensitivity. Observed changes in pH_i-sensitivity caused by C-terminal truncation of these subdomains and their proposed functions are shown in the figure. The N-terminal small region (aa 500-515) was not analyzed.

acidic shift of pH_i-dependence (~0.8 pH unit relative to Δ595) (Figs. 3 and 8A), indicating that subdomain I is required for the maintenance of high pH_i-sensitivity of NHE1.

The cytoplasmic domain of Na^+/H^+ exchanger shows the greatest structural divergence among four NHE isoforms (*1-5*). However, subdomain I (aa 516-595) of NHE1 has a relatively high sequence homology with the corresponding regions of NHE2 and NHE4 (45% for NHE1 *vs.* NHE2, 45% for NHE1 *vs.* NHE4, and 70% for NHE2 *vs.* NHE4). This suggests the functional importance of this subdomain in these exchanger isoforms. Subdomain I of NHE1 is relatively rich in α-helical structures according to Chou-Fasman probabilities (*29*). The C-terminal segment of this subdomain (aa 566-590/595), consisting of a relatively well-preserved, single α helix, appears to play an important role in the maintenance of high pH_i-sensitivity, because its truncation (Δ566) induced a large acidic shift of pH_i-dependence (Fig. 8A). However, internal deletion mutants Δ515-566 and Δ515-635 lacking the N-terminal two-thirds of subdomain I, and subdomains I and II, respectively, exhibited low pH_i-sensitivity comparable to Δ515 (data not shown). Thus the overall structure of subdomain I seems to be required for its pH_i maintenance function.

In this study, we showed that ATP depletion primarily affects pH_i-sensitivity of NHE1, but not V_{max}. This response to ATP depletion was preserved in Δ566, but disappeared completely in mutant Δ515 (compare Fig. 7, C and D), suggesting that a region of subdomain I present within aa 516-566 confers ATP-sensitivity. Subdomain I is thus involved in the decrease in pH_i-sensitivity by ATP depletion. This location of an ATP-sensitive site is consistent with a recent report in which the region in or adjacent to the membrane domain confers the sensitivity to ATP depletion in NHE3 (*30*). However, these results are not consistent with those of other investigators who reported that ATP depletion induces a change in V_{max} (*20, 23, 24*) and that the ATP-sensitive site is located in the cytoplasmic tail (aa 635-815) of NHE1 (*22*). At present, we have no explanation for these differences. However, they could be due at least in part to the difference in the experimental conditions used: in these previous studies, relatively narrow pH_i ranges were used for the measurement of Na^+/H^+ exchange (*20, 23, 24*), which do not appear to be sufficient to clearly separate the effects of ATP depletion on V_{max} from that on p*K*.

It is important to note that the pH_i-sensitivity (pH giving 1/2 of V_{max}) of the wild-type exchanger in ATP-depleted cells was very low and almost equivalent to that of Δ515, a mutant deleted of subdomain I (Fig. 8B). It is likely that ATP depletion induces a p*K* shift in NHE1 through a structural derangement of subdomain I in a manner mimicked by complete deletion of this subdomain. A very similar low pH_i-sensitivity was also observed in ATP-depleted cells expressing mutants deleted of the C-terminal tail but still retaining subdomain III (Δ698 and Δ659). Under the same conditions, however, the mutants deleted of subdomain III (Δ635 and Δ595) exhibited significantly higher pH_i-sensitivity (Fig. 8B), indicating that the inhibition by ATP depletion becomes much less efficient in these mutants. We hypothesize that subdomain I contains an "acceptor" site for subdomain III and that the interaction between these subdomains is required for such ATP depletion-induced structural derangement to occur. In Δ566, a C-terminal truncation mutant of subdomain I, we again observed a low pH_i-sensitivity similar to Δ515, although this mutant do not retain subdomain III (Fig. 8B). This is probably due to loss of the pH_i-maintenance function of subdomain I caused by its truncation (see above).

In our previous study, we suggested that activation of NHE1 in response to growth factors is mediated *via* two different regions of the cytoplasmic domain: aa 636-656 (subdomain III), which is involved in Ca^{2+}/CaM-dependent, rapid activation of NHE1 and the other being aa 567-635 involved in the slow and lasting activation induced by growth factors (Fig. 9). This was based on the findings that the cytosolic alkalinization induced by a long (10-20 min) stimulation with thrombin is reduced only by 50% in cells expressing mutant Δ635 and that the remaining alkalinization is abolished in cells expressing mutants deleted of aa 567-635 (Δ566). In this study, we found that the thrombin-induced cytoplasmic alkalinization still occurs in cells expressing Δ595 (data not shown). Thus, aa 566-595 in subdomain I seems to be critical for the slow and lasting activation of NHE1 in response to thrombin. Previously, we suggested that thrombin activates NHE1 through a phosphorylatable cytosolic factor(s) (*11*). The latter may interact with this portion of subdomain I.

Subdomain I may have a stimulatory function on the putative H^+-modifier site (see "Introduction"), increasing its proton affinity, and ATP depletion may blunt such interaction. Growth factors, on the other hand, would enhance the stimulatory influence of subdomain I, probably through a cytosolic regulatory factor, in such a way that the H^+-modifier site increases its proton affinity. Alternatively, subdomain I may itself contain the H^+-modifier site, and a conformational change in this subdomain could modulate the proton affinity of the latter. At any rate, convergence of the growth factor- and the ATP-dependent regulations to subdomain I can easily explain the finding that ATP depletion abolished or greatly reduced growth factor-induced activation of NHE1 (*9, 31*). The underlying mechanism for the observed effect of ATP depletion is unclear, although dephosphorylation of the NHE1 protein does not appear to be involved (*11, 23*). Besides protein dephosphorylation, cell ATP depletion would cause cellular

changes such as reorganization of cell cytoskeleton, activation of stress-sensitive cellular reactions, and intracellular acidosis. Interestingly, a heat-shock protein has recently been reported to bind to the cytoplasmic domain of NHE1 in an ATP-dependent manner (*32*). However, it is not known how this protein is involved in the regulation of NHE1.

Truncation of the C-terminal tail including subdomain II (aa 596-635) did not produce any effect on pH_i-dependence of the exchange activity (Figs. 2, 3, and 7A). This domain is predicted to contain a β-sheet, a β-turn, and a random coil, and its homology with the corresponding regions of NHE2 and NHE4 is not so high as in subdomain I (23% for NHE1 *vs.* NHE2 and 20% for NHE1 *vs.* NHE4). Tandem elongation of subdomain II by insertion did not significantly affect pH_i-dependence of Na^+/H^+ exchange or its activation in response to short stimulation with ionomycin or thrombin (see Figs. 4 and 5). Thus the autoinhibitory CaM-binding domain (subdomain III) functions normally in this mutant, in which subdomain III was displaced by 40 amino acid residues from its normal position in the primary structure. In contrast, cells expressing internal deletion mutant Δ596-634, deleted of subdomain II, exhibited a significant alkaline shift of pH_i-dependence under basal conditions and did not fully respond to ionomycin or thrombin (see Figs. 4 and 5), indicating that the function of the autoinhibitory CaM-binding domain is impaired. These results are consistent with the view that the autoinhibitory CaM-binding domain has its "acceptor" within the NHE1 molecule and that interaction of this domain with the acceptor requires the presence of an optimal "spacer" on its N-terminal side. We propose that subdomain II may function as a mobile "flexible loop" required for the interaction of inhibitory subdomain III with the putative H^+-modifier site (Fig. 9).

Finally, it should also be mentioned that the exchange activity decreased significantly as the cytoplasmic domain was truncated to its N-terminus (aa 515) (Fig. 2). A large drop in V_{max} occurred in Δ635, indicating that subdomain I is not involved directly in this process. We suspect that such V_{max} decrease may be due to decreased expression of truncated mutants in the plasma membrane, although this possibility could not be tested in our hands because of the lack of a specific antibody recognizing the N-terminal portion of the NHE1 protein. Similar large decreases in exchange activity were observed in NHE2 and NHE3 mutants as the cytoplasmic domains of these isoforms were truncated to their N-terminal side (*33*).

In summary, we performed the functional mapping of the cytoplasmic subdomains that control the H^+-modifier site of NHE1. The data obtained in this study should be important as the structural basis for the analysis of multiple mechanisms for the regulation of NHE1 because all the regulatory mechanisms are thought to be attributable to change in pK (for pH_i) in the case of NHE1. Further analysis of localization of the H^+-modifier site, interaction of cytoplasmic subdomains with the H^+-modifier site and the ion-transport domain, as well as the function of regulatory cofactors are required to clarify the molecular dynamics of NHE1 regulation.

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