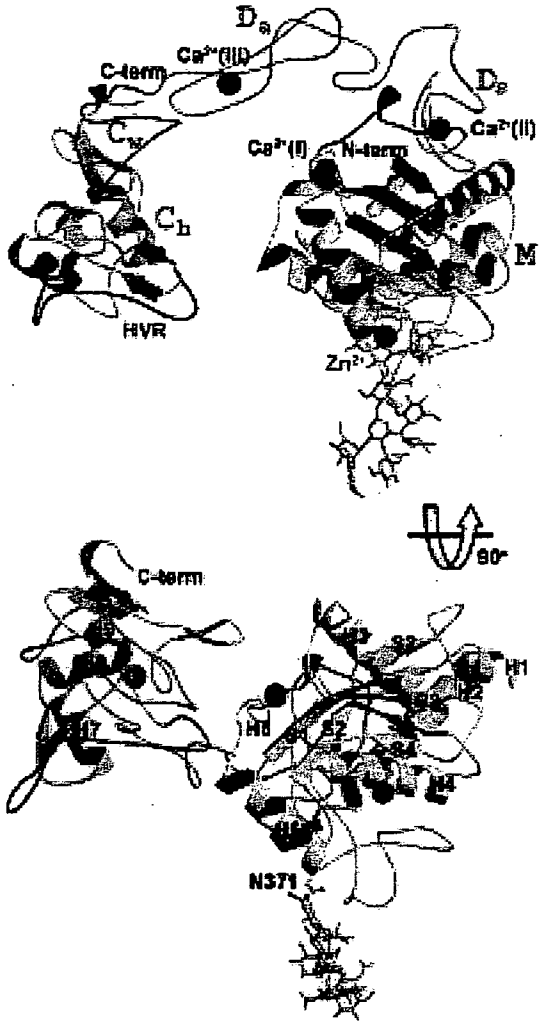

図１：M:メタロプロテアーゼドメイン、D:ディスインテグリンドメイン、C:システインリッチドメイン、HVR: Hyper Variable Region

本研究では3つの異なる結晶系の結晶から独立した計6分子のcatrocollastain/VAP2B分子の構造解析、精密化を行い、それらの構造および既に報告したVAP1構造との重ね合わせによる比較から各サブドメイン間の可動性を見出し、ADAM分子の機能との相関について議論を行った(図２)。

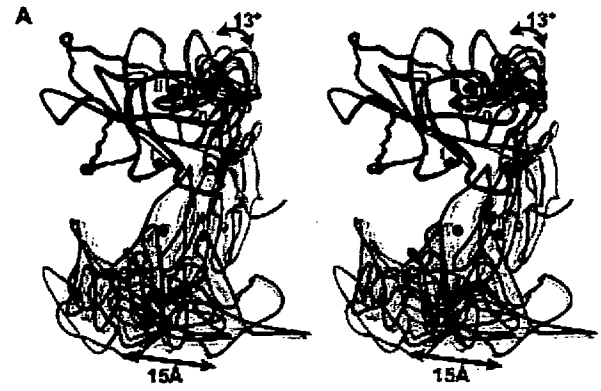

図２：緑：VAP1 M-ドメイン
青・赤：代表的VAP2 M-ドメイン

RVV-XはADAMと相同の重鎖に加え、C型レクチン様の二つの軽鎖を持つ、ヘテロ三量体タンパク質である。結晶構造解析によりこれら3つのポリペプチド鎖の相互作用の詳細を明らかにすることに成功した(図３：Takeda et al. FEBS Lett. (2007))。

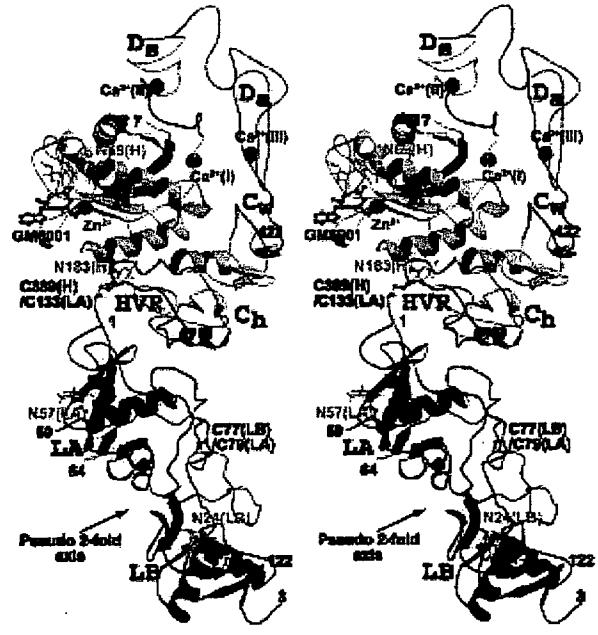

図３：M, D, C, HVR：図１と同じ
LA, LB：レクチン様軽鎖

興味深い事に我々がVAP1の結晶構造からADAMにおけるターゲット認識の可能性部位として同定した超可変領域(Hyper variable region

(HVR)が重鎖と軽鎖の一つの相互作用部位となっていた。さらに2つの軽鎖はFactor X結合因子であるX-bpと非常に高い立体構造の相同性を示し、RVV-Xの軽鎖はFactor XのGlaドメインを特異的に認識するエクソサイトを形成することが示唆された。これを基にドッキングモデルとRVV-XによるFactor X活性化メカニズムを提案した(図４)。

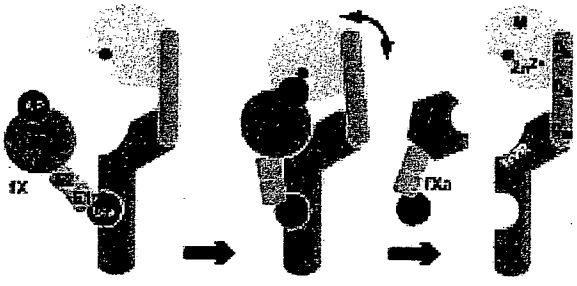

図４：RVV-XによるFactor X　活性化メカニズム

D. 考察

二つの蛇毒ADAMホモログタンパク質の結晶構造解析により、ADAMファミリータンパク質に共通したサブドメイン間の可動性やターゲット認識機構に関する新しい知見を得ることに成功した。特にRVV-Xの結晶構造はHVRを介したタンパク質間相互作用を明らかにした初めての例であると共に、ADAMファミリータンパク質が分子進化的に基質特異性を獲得したことを示唆する興味深い事例である。また、RVV-Xにおいては酵素活性部位と６５Å離れた部位に基質認識のエクソサイトが存在し、このような空間的に離れた部位に存在するエクソサイトが他のADAMファミリータンパク質においてもターゲット特異性の決定に重要である可能性を示唆した。

E. 結論

ADAMファミリータンパク質をターゲットとした創薬の基盤となる２つの蛇毒ADAMホモログタンパク質の構造決定を行った。計３つの蛇毒ADAMホモログの構造決定により哺乳動物ADAMの基本分子構造が明らかになり、創薬対象であるヒトADAM分子を実際に構造解析する足がかりが掴めた。

研究協力者：五十嵐智子

F. 健康危険情報

特になし。

G. 研究発表

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G. 知的財産権の出願・登録

特願 2007-123841：血管内皮型一酸化窒素合成酵素活性剤、及び一酸化窒素欠乏に起因疾病の予防または治療剤（盛　英三）

.厚生科学研究費補助金　(医療機器開発推進研究事業)

ナノ分子イメージングを活用した次世代創薬アプローチ

分担研究報告書

次期治療標的タンパクの構造解析－２

分担研究者　増田　道隆　　国立循環器病センター研究所循環器形態部室長

研究要旨：循環器疾患、脳神経疾患等の制圧のためにナノテクノロジーを駆使して、治療法の開発を推進することを目的とする。本分担研究では、分子の構造決定に基づく創薬を目指した研究を行う。本年度の標的タンパクは、脂質結合・変形活性をもつ共通のドメイン構造を有する細胞内情報伝達分子タンパク群IRSp53とFerキナーゼの構造を解析する。これを、基盤情報として次世代創薬に貢献する。

A. 研究目的

細胞内情報伝達分子タンパク質の大腸菌による大量発現、精製、結晶化を行い、放射光X線回折法で結晶構造解析を行う。これらは次世代創薬に貢献する基盤情報となる。対象となるタンパク分子ごとにその研究背景と意義を以下に要約する。

(1) 脂質膜結合・変形タンパク質

Rho、Rac、Cdc42などのRhoファミリー低分子GTP結合タンパク質はアクチン細胞骨格の制御に中心的な役割を果たしている分子であり、筋収縮を含む細胞運動や細胞接着の制御に深く関わっている。我々の研究を含め、Rhoファミリー低分子GTP結合タンパク質のエフェクター分子の中に、アクチン細胞骨格制御活性とともに脂質膜結合・変形活性をもつ新規のタンパク質ファミリーがあることが明らかにされ、エンドサイトーシス、膜輸送や食作用など生体膜のダイナミックスを調整する分子として盛んに研究されるようになってきている。これは生体膜のダイナミックスの制御をターゲットとした次世代創薬の可能性を開くものである。これらのタンパク質の分子機能の詳細を明らかにするため、脂質を含んだ結晶構造の解明を初めとして、タンパク結晶構造解析に取り組む。

(2) Ferキナーゼ

FerとFesは２つでファミリーをなす特異な非受容体型のチロシンキナーゼでSH3ドメインを持たない代わりに、アミノ酸配列から（１）で記述した脂質膜結合ドメインを持つことが予測される。Ferはユビキタスに発現して細胞接着制御に関わり、特に内皮細胞では接着分子のリン酸化を介して透過性の制御に関わる。我々は、内皮細胞の細胞間接着分子PECAM-1キナーゼとしてFerを同定している。また、Ferは一部の癌の増悪にも関係することが知られている。FerやFesの脂質膜結合ドメインは活性が負に制御されており、逆にキナーゼ活性は脂質膜結合ドメインにより負に制御されていると考えられる。この制御の詳細を明らかにするため、脂質膜結合ドメインおよび全長のタンパク結晶構造解析に取り組む。

B. 研究方法

RacやCdc42のエフェクター分子IRSp53の脂質膜結合ドメインと、IP3などの脂質ヘッドグループと

の共結晶を作製し、X線回折法により構造を決定する。また、多数のトリプトファン変異体を作成し、FRET解析により、リポソームとの相互作用の部域特異性についての情報を得る。

Ferキナーゼの全長および脂質膜結合ドメインの結晶を作製し、X線回折法により構造を決定する。

C. 研究結果

研究結果のうち、結晶構造解析については心臓生理部・武田室長およびベン-アマー室長との共同研究である。

(1) IRSp53

IRSp53の脂質結合ドメインおよび脂質結合活性部位を欠損した変異体の結晶構造を、2.0Åおよび1.7Åの高分解能で明らかにする事に成功した(図)。IP3との共結晶を2種類作製し、結晶構造解析が進行中である。トリプトファン変異体によるリポソーム結合解析では、これまでの知見と一致しないデータがでており、脂質膜変形の新しい機構を提案すべく研究を進めている。

(2) Ferキナーゼ

脂質結合活性を持つN-端の結晶作製に成功し、結晶構造解析が進行中である。全長については、大腸菌による効率的なタンパク発現に成功し、結晶化条件をスクリーニングしている段階である。

D. 考察

IRSp53の脂質結合ドメインの結晶構造と脂質結合部位の決定は、BARドメインの脂質膜結合の詳細を明らかし、生体膜のダイナミックスの制御をターゲットとする薬剤の開発の基盤情報となる。Ferキナーゼの結晶構造の解明は、Fer−Fesファミリーキナーゼの特異的性質を明らかにするとともに、脂質結合とキナーゼ活性がどのように調節されているかについて示唆を得ることができる。さらに、内皮細胞の透過性制御や機械刺激受容を調節する薬剤の開発につながる可能性がある。またこれらの研究は、新たな細胞生物学の研究領域の創成に資する。

E. 結論

創薬や新研究領域の創成につながる複数のタンパク構造決定に成功した。

F. 研究発表

(研究業績「欧文」)

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H. 知的財産権の出願・登録

特になし

厚生科学研究費補助金　（医療機器開発推進研究事業：ナノメディシン研究）

分担研究報告書

ナノ分子イメージングを活用した次世代創薬アプローチ

分担研究者　国立循環器病センター研究所循環器形態部長　望月直樹

研究要旨　低分子量GTP結合蛋白質が血管新生・血管老化の調節に重要である。このため、新規GTP結合蛋白質Rit, Rinファミリーの機能を解析してあらたな分子標的治療薬の開発に繋げる研究を目指した。Ritが他の癌遺伝子Cdc42の水解促進に作用することを突き止めた。

A. 研究目的

血管新生、血管老化における低分子量GTP結合蛋白質の制御機構についての研究、さらに薬剤スクリーニング系の構築を目指した。これまでcAMP依存性にRap1が活性化されるが、Ritも同様に活性化されることが報告されていた。今回Ritの活性化にともない誘導される細胞内情報伝達系の検討をまず行った。これまでにRitの活性化による細胞内情報伝達系の調節機構はまったく解明されておらず、この分子の機能が明かになれば、血管新生・老化における同分子のかかわりを明かにすることができると考えた。これまでにRasファミリーGTP結合タンパク質の活性化の可視化を行って来た実績から、Ritの活性化可視化プローブの作成も試みる。またRitの可視化プローブの作成により、この分子の活性化抑制薬のスクリーニング系の構築にまで発展させることが可能と考え、本研究を行う。

B. 研究方法

細胞：HEK293細胞、COS7細胞は、通常のDMEM 10%FBSで培養をおこなった。ヒト臍帯静脈の血管内皮細胞(HUVEC）はクラボウ社のHumedia-2を用いて継代培養を行ってpassage が８回以下の細胞を実験に用いた。

蛍光タイムラプスイメージング

HUVEC細胞にRit－GFPを発現させて細胞の運動をモニターリングした。またCOS細胞にRitの優勢活性化型、野生型、優勢劣性型のRitを発現させて、細胞の形態変化を検討した。

活性化型Rit結合蛋白質の検討

これまでRas分子の下流で機能するといわれているRal活性化因子RalGEF3に結合するか否かを調べた。ほかにRas associationドメインを有する複数の分子についてこれらの分子とRitとの結合をプルダウンアッセイで検討した。

Ritの胞膜移行の検討

RitはRasファミリー分子のなかでカルボキシ末端にCAAXモチーフを有さない分子であり、その局在もこれまではっきりしていなかったが、GFP蛍光蛋白質をアミノ末端に付加してその細胞内局在を検討するとともに、細胞膜への局在化モチーフを調べた。

C. 研究結果

Ritの細胞膜局在化：

RitはGFPとの融合蛋白質としてCOS7細胞に発現させた時には、細胞膜に局在し、これはR他のRas分子とは違って、細胞膜のみに局在化して、ほかの細胞内小器官の膜(Golgiなど)には局在しなかった。カルボキシ末端にLysが反復して存在しこのPoly basic領域を欠損させると細胞膜への局在が阻害された。つまり、この部位が細胞膜局在に重要であることがわかった。

さらに、Thin layer chromotographyによってRitのGTP, GDPフォームが細胞内でどちらが優位であるかを検討したところGTP結合型Ritとして発現していることがわかった。つまり、GTPase活性が弱いGTPaseであることもわかった。

Rit結合蛋白質

RAドメインをもつ分子SNX27, AF6, PI3K, RalGEF, RGLなどとRitの結合を検討したが、RGL3のみ結合して、これはGTP依存的であった。RGL3-RAドメインを用いたPull-down assayではRIT-QL(活性化型)が強く沈降してきたために、RGL3がRitの活性化を細胞内で検討するツールとして有用であることがわかった。

Ritの細胞での機能

Ritを発現させるとRhoファミリー分子のCdc42のGAP活性が増加して、Cdc42が不活性化されることがわかった。このために、Cdc42のGAP　ドメインを持ち、かつRAドメインを含む分子をRitの標的あるいは結合蛋白と予想して、これらの分子とRitとの結合を調べたところKIAA1391分子との弱い結合を見出すことができた。RitによるCdc42の不活性化とHUVECの細胞運動の阻害結果は矛盾しなかった。

薬剤スクリーニング系

これまでのPI3Kの活性化可視化プローブをHEK293細胞に発現させて、この活性阻害をみるスクリーニング系を構築したが、96 wellのプレートでは、スクリーニングの効率が悪くまた、十分なSensitibity, specificityがえられなかった。

D. 考察

血管発生時には血管内皮細胞間接着の促進が重要であり、これまでにcAMP-Epac-Rap1の活性化がCadherin接着を増強させることでこれを制御していると考えて研究を行ってきた。cAMPの下流でRitも活性化されると報告されているために、本研究では血管内皮細胞でのRitの機能を検討するとともに、Rit可視化プローブの作成により、このcAMP-Rit系を調節しうる薬剤のスクリーニングまで発展させるために本研究を行った。

これまでYFP-smallG-effectoe-CFPというプローブが非常に有効であることから、まずRitのeffectorを探すことから本研究を開始した。RGL3がGTP結合型Ritと相互作用することが判明した。次年度以降には、Rit-RGL3を骨格とした分子プローブの作成を計画したい。

また、Ritの細胞内での機能がCdc42の水解促進作用であることも突き止めた。COS7細胞でRitを発現させたときにGST-CRIBによるプルうダウンアッセイで上記を示すことができた。さらに血管内皮細胞を用いたcell tracking assayで　GFP-Rit発現細胞が有意に細胞運動が抑制されていた。これもCdc42の抑制効果として矛盾しない結果であると考えられた。

さらに薬剤スクリーニング系の構築を目標にPI3Kの活性阻害、活性増強薬をスクリーニングするために９６穴細胞培養系を確立を試みたが、細胞へ

のプローブの導入効率などの問題で、今後さらに改良が必要であると考えられた。

D. 結論

血管構築細胞の細胞間接着に重要であると予想する低分子量GTP結合タンパク質Ritの機能について検討し、RitがCdc42のGTPaseの活性化にかかわることを示唆する結果を得た。

F. 健健康危険情報

なし

G. 研究発表

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2. 学会発表

特になし。

H. 知的財産権の出願・登録状況

特になし。

別紙４

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Functional importance of charged residues within the putative intracellular loops in pH regulation by Na^+/H^+ exchanger NHE1

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Keywords
charge-reversal mutation; Na^+/H^+ exchanger; pH regulation; surface labeling; transport kinetics

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The plasma membrane Na^+/H^+ exchanger 1 is activated in response to various extrinsic factors, and this process is regulated by an intracellular pH-sensing mechanism. To identify the candidate residues responsible for intracellular pH regulation, we analyzed the functional properties of engineered Na^+/H^+ exchanger 1 mutants with charge-reversal mutations of charged residues located in the intracellular loops. Na^+/H^+ exchanger 1 mutants with mutations at 11 positions were well expressed in the plasma membrane, but that with E247R was not, suggesting that Glu247 is important for the functional expression of Na^+/H^+ exchanger 1. Charge-reversal mutations of Glu131 (E131R, E131K) and Arg327 (R327E) resulted in a shift in the intracellular pH dependence of the exchange activity measured by $^{22}Na^+$ uptake to the acidic side, and it abolished the response to growth factors and a hyperosmotic medium; however, mutations of Asp448 (D448R) and Arg500 (R500E) slightly shifted it to the alkaline side. In E131R, in addition to the change in intracellular pH dependence, the affinities for extracellular Na^+, Li^+ and the inhibitor 5-(*N*-ethyl-*N*-isopropyl)amiloride significantly increased. Furthermore, charge-conserved mutation of E131 (E131D) was found to have no effect, whereas charge neutralization (E131Q) resulted in a slight acidic shift of exchange. These results support the view that the multiple charged residues identified in this study, along with several basic residues reported previously, participate in the regulation of the intracellular pH sensing of Na^+/H^+ exchanger 1. In addition, Glu131 may also be important for cation transport.

Na^+/H^+ exchangers (NHEs) belong to a solute carrier family (SLC9) that is involved in catalyzing the electroneutral exchange of Na^+ and H^+ and regulating pH homeostasis, cell volume, and transepithelial Na^+ absorption [1–6]. Various extrinsic factors, including hormones, growth factors, pharmacologic agents, and mechanical stimuli, control the activity of the ubiquitous exchanger isoform NHE1 [1–6]. NHE1 activation is occasionally a risk factor involved in the pathogenesis of various diseases. For example, it plays a critical role in the onset of cardiac hypertrophy and heart failure during ischemia and reperfusion, as confirmed by the significant reduction in heart damage observed on administration of NHE1-specific inhibitors [7,8]. Various extrinsic factors have been shown to enhance NHE1 activity by shifting the intracellular pH (pH_i) dependence of the Na^+/H^+ exchange to the alkaline side, and this is presumed to occur via interactions between regulatory factors and the cytoplasmic domain of NHE1 and/or by the post-translational

Abbreviations
EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; HA, hemaglutinin; IL, intracellular loop; NHE, Na^+/H^+ exchanger; NHS-LC-biotin, succinimidyl-6-(biotinamide)hexanoate; pH_i, intracellular pH; TM, transmembrane-spanning region.

modification of NHE1 or its accessory factors. This phenomenon is usually explained by the assumption that NHE1 possesses an allosteric regulatory site for intracellular protons that is distinct from the Na^+ or H^+ transport site, and that external stimuli increase the H^+ affinity of this H^+-regulatory site [1,9–11].

Members of the NHE family possess similar general structures, comprising a C-terminal cytoplasmic regulatory domain (~ 300 amino acids) and an N-terminal transmembrane domain (~ 500 amino acids) that contains 12 membrane-spanning segments [1–3]. In the plasma membrane, NHE1 is known to exist as a dimer [12,13], and our recent study provided evidence that dimerization is essential for this molecule to maintain physiologic pH_i sensitivity [14]. We previously demonstrated that deletion of the cytoplasmic subdomains of NHE1 shifted the pH_i dependence of the Na^+/H^+ exchange either to the acidic or the alkaline side [15], implying the importance of the cytoplasmic domain in regulating pH sensitivity. Recently, we also presented evidence that calcineurin homologous protein, an obligatory binding partner of NHE1, is one of the key molecules involved in regulating pH sensitivity [16–18]. Furthermore, we [19] and others [20] reported that mutation of Arg residues, namely Arg440 in intracellular loop 5 (IL5) and Arg327 in IL4, largely shift the pH_i dependence of the exchange to the acidic side. These latter observations raised the interesting possibility that a cluster of charged residues in the ILs along with the calcineurin homologous protein-bound cytoplasmic subdomain may regulate the function of NHE1 as a pH sensor. In this study, in order to further identify the critical residues responsible for regulating pH sensitivity, we analyzed the effect of charge-reversal mutation of charged residues in putative ILs or in transmembrane-spanning region (TM)/IL boundaries on the kinetics of the Na^+/H^+ exchange. Of the 12 mutations examined in this study, we identified Glu131 in IL1 as an important residue involved in regulating pH_i sensitivity and Na^+ transport.

Results

In this study, we focused on the mutation of charged residues in the intracellular loops of NHE1. Figure 1A shows the previously reported membrane topology model of human NHE1 [22]. As the first search, we introduced charge-reversal mutations (Arg, Lys or His to Glu, Glu or Asp to Arg) in 12 charged residues, namely: Arg14 in the N-tail; Glu131 in IL1; Glu247 and His250 in IL3; Arg321 and Arg327 in IL4; Lys438, Lys443, Lys447 and Asp448 in IL5; and Arg500 and Asp504 in the C-tail of hemaglutinin

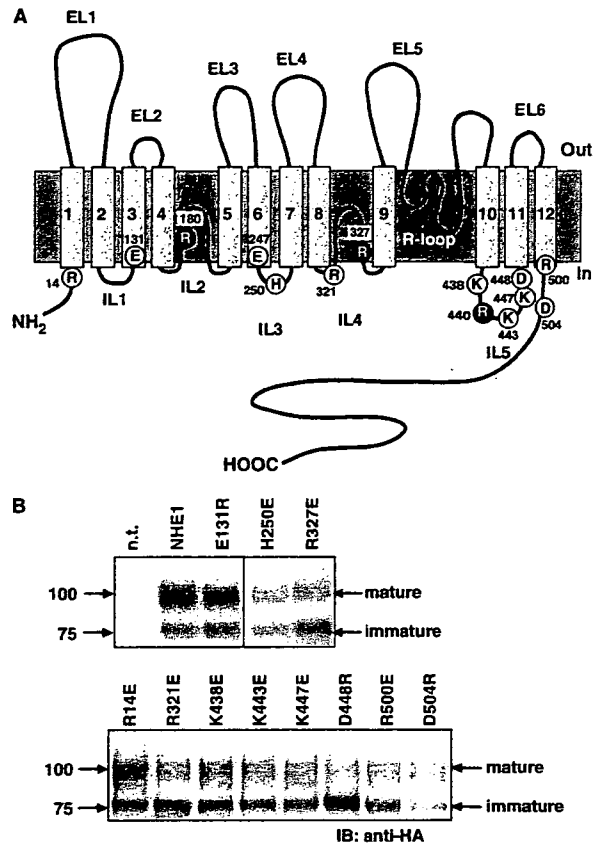

Fig. 1. Expression of charge-reversal mutant NHE1s. (A) Secondary structure model of NHE1. Relative positions of engineered residues are indicated in the figure. R-loop, re-entrant loop. Arg440 [19] and Arg327 and Arg180 [20] were previously reported to be critical residues responsible for pH_i dependence of NHE1. (B) Immunoblot of proteins obtained from cells stably expressing the wild-type or other mutant NHE1s. Cell lysate proteins (20 μg per lane) were subjected to 3–8% SDS/PAGE and immunostained with antibody to HA. n.t., nontransfected PS120 cells.

(HA)-tagged NHE1. We transfected expression vectors carrying these individual NHE1 point mutants into NHE-deficient PS120 cells and selected the population of cells expressing the NHE variants by activity-dependent selection procedures, i.e. by H^+-killing selection [23]. Of the 12 different mutants, E247R was not expressed, indicating that the substitution of Glu247 with Arg causes a severe functional defect (catalytic inactivity and/or a membrane-expression defect) in the NHE1 molecule. The other 11 NHE1 mutants were well expressed in PS120 cells, as observed in the immunoblot analysis (Fig. 1B). First, we assessed the surface expression of these mutant NHEs by surface biotinylation. To semiquantitatively evaluate this surface expression, we estimated the

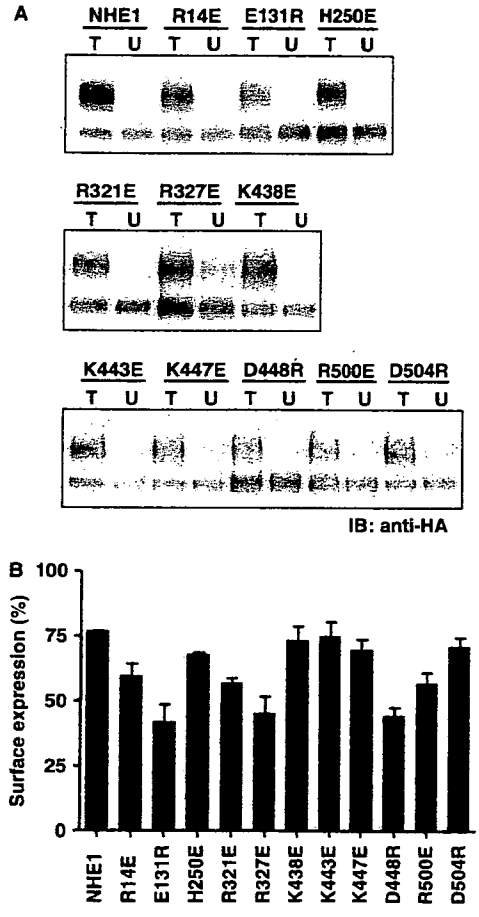

Fig. 2. Surface biotin labeling of various NHE1 variants. (A) Cells expressing various NHE1 variants were treated with 1 mM NHS-LC-biotin, and the cell lysate was incubated with streptavidin-agarose beads. An aliquot of cell lysates before (total, T) and after (unbound, U) incubation with beads was subjected to immunoblot analysis with an antibody to HA. (B) Density of mature and immature protein bands was measured by densitometric scanning and determining the amount of NHE1 proteins absorbed to streptavidin-agarose beads by subtracting the amount of unbound NHE1 from that of total NHE1 (including all four forms of NHE1). Data are represented as a percentage of bead-bound NHE1.

amount of NHE1 protein adsorbed onto streptavidin-agarose beads by subtracting the amount of unbound NHE1 (including immature NHE1) from the amount of total mature NHE1 expressed (Fig. 2A). On the basis of this analysis, we concluded that approximately 75% of the total wild-type NHE1, including the nonspecifically bound proteins that accounted for 5–10%, were adsorbed onto the beads, i.e. expressed on the cell surface (Fig. 2B). Charge-reversal mutations did not alter the surface expression of NHE1 to a large extent, except for the mutations of Glu131, Arg327, and Asp448, which slightly reduced it (Fig. 2B). Essentially the same results were obtained using two different batches of cell populations derived from different transfectants.

Next, we measured the pH_i dependence of the 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA)-inhibitable $^{22}Na^+$ uptake in cells expressing the individual charge-reversal mutants (Fig. 3A). The maximal exchange activity at pH_i 5.4 (V_{max}) was normalized with regard to the amount of surface NHE1, and these normalized values are summarized in Table 1. Whereas the Glu131 (E131R) mutation reduced the normalized activity to ~50%, most other mutations did not alter it to a great extent (Table 1). Interestingly, the pH_i dependence of the $^{22}Na^+$ uptake greatly shifted to the acidic side in the E131R and R327E mutants (Fig. 3B). It should be particularly noted that the steepness of the pH_i dependence curve was highly reduced in E131R, i.e. the Hill coefficient reached approximately 1.0; this result was in sharp contrast to those of all other NHE1 variants, which yielded a higher Hill coefficient of more than 1.0 (Table 1). Furthermore, the pH_i dependence slightly shifted to the alkaline side in D448R and R500E (Fig. 3B). Thus, we identified several charge-reversal mutations (E131R, R327E, D448R, and R500E) that affected the pH_i dependence of the Na^+/H^+ exchanger.

We further constructed some NHE1 mutants at Glu131 (E131Q, E131D and E131K) and measured the pH_i dependence of $^{22}Na^+$ uptake in cells expressing these mutant exchangers. These mutant proteins were well expressed in cells, although the surface expression levels appeared to be slightly lower than that of the wild-type NHE1 (data not shown). We found that charge-preserved mutation (E131D) had no effect on pH_i dependence, suggesting that mutation itself does not much influence the function of NHE1. In contrast, charge-reversal substitution of E131 with another positive residue, Lys, similar to Arg, resulted in a large acidic shift of pH_i dependence as well as a decrease in the normalized V_{max} (Table 1). Thus, the charge of E131 appears to be important for the normal function of NHE1. Furthermore, we found that charge neutralization (E131Q) also resulted in a slight decrease in p*K*a.

Various extrinsic factors are known to activate NHE1 and bring about long-lasting cytoplasmic alkalinization, particularly in the absence of bicarbonate [1–6]. As shown in Fig. 4, thrombin, the protein kinase C activator 4β-phorbol 12-myristate 13-acetate and hyperosmotic stress (200 mM sucrose) all induced a high level of cytoplasmic alkalinization in cells expressing wild-type NHE1, D448R, or R500E. In contrast, such alkalinization was completely absent in cells expressing E131R or R327E (Fig. 4), suggesting that

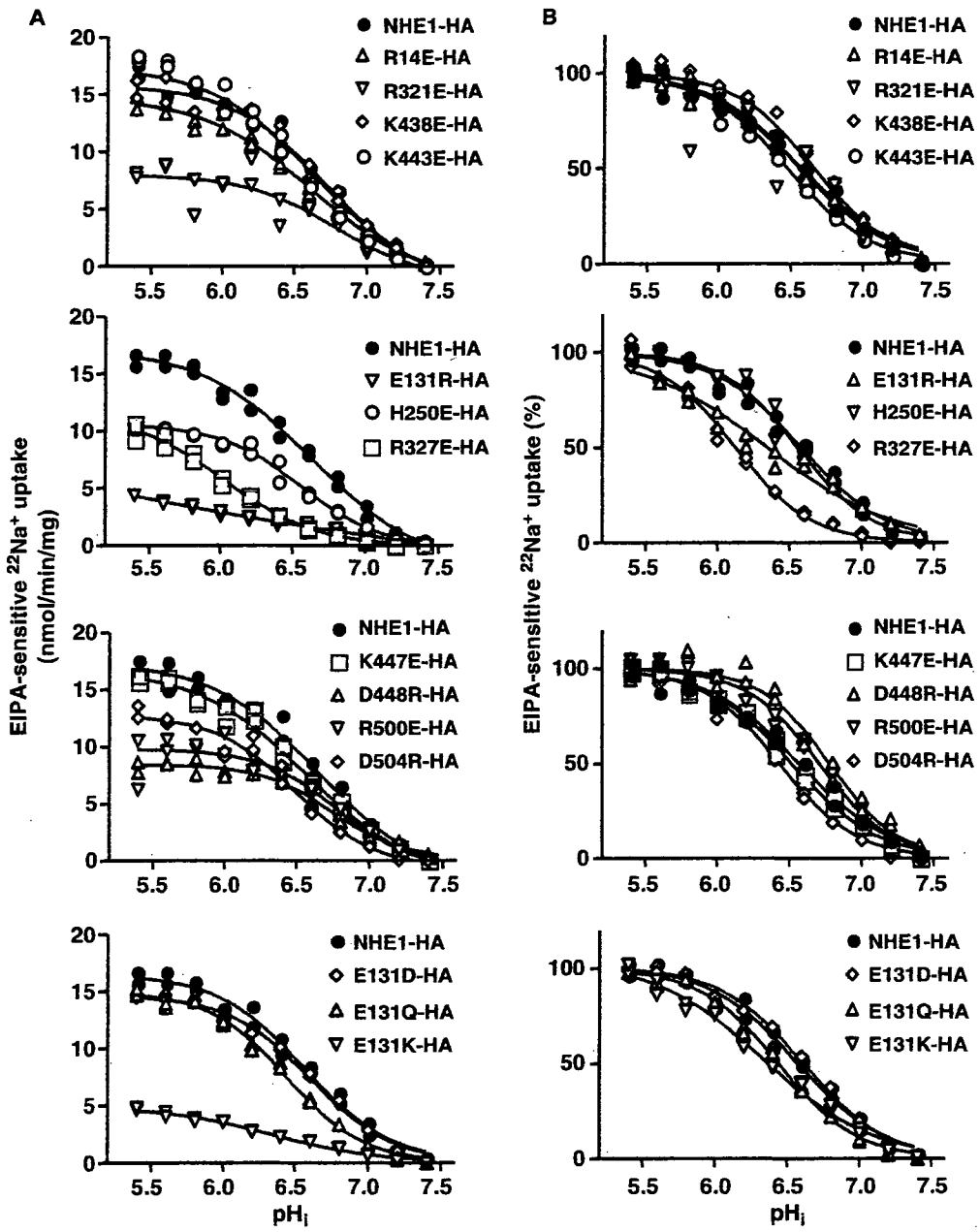

Fig. 3. The pH_i dependence of EIPA-sensitive $^{22}Na^+$ uptake. (A) $^{22}Na^+$ uptake was measured in cells expressing the wild-type or mutant exchangers in the presence or absence of 0.1 mM EIPA after pH_i clamping at various values with K^+/nigericin. The wild-type or mutant exchangers exhibited high maximal $^{22}Na^+$ uptake activities (4–20 nmol·mg^{-1}·min^{-1} at pH_i 5.4). Data were fitted to the sigmoidal dose dependence equation as described in 'Experimental procedures'. (B) Data are normalized to the maximal uptake activity at pH_i = 5.4.

these residues are important for the physiologic response of the exchanger. NHE1 is thought to be activated in response to external stimuli via alkaline shift of a 'pH set-point', i.e. pH_i dependence [1]. The lack of alkalization in cells expressing these NHE1 mutants would be due to their low exchange activity in the neutral pH_i range, resulting from the acidic shift of pH_i dependence, even in the case that extracellular stimuli had slightly activated these mutants via an alkaline shift. In previous studies, we identified many mutations resulting in a large acidic shift of pH_i dependence and in a lack of cytoplasmic alkalinization [15,19,23].

Table 1. Kinetic parameters for the pH_i dependence of $^{22}Na^+$ uptake. The pH_i dependence of EIPA-sensitive $^{22}Na^+$ uptake (Fig. 3) was fitted to a sigmoidal dose-dependent equation. The best fitted value with standard error is represented for p*K* and the Hill coefficient. The V_{max} value is the $^{22}Na^+$ uptake activity at pH_i 5.4 (means ± SD, $n = 3$). The relative amount of surface-expressed NHE1 was calculated from the amounts of total and streptavidin bead-absorbed proteins, and the V_{max} value was normalized (means ± SD, $n = 3$).

NHE1 variants (%)	p*K* for pH_i	Hill coefficient	V_{max} (nmol·mg^{-1}·min^{-1})	Normalized V_{max}
NHE1	6.57 ± 0.02	1.40 ± 0.08	16.19 ± 0.55	100.0
R14E	6.56 ± 0.02	1.40 ± 0.07	13.77 ± 1.04	103.7 ± 8.1
E131R	6.35 ± 0.03	1.00 ± 0.07	4.24 ± 0.32	55.0 ± 9.6
H250E	6.56 ± 0.02	1.68 ± 0.09	10.42 ± 0.12	85.5 ± 1.2
R321E	6.57 ± 0.05	1.30 ± 0.17	8.39 ± 0.70	89.7 ± 3.2
R327E	6.13 ± 0.02	1.68 ± 0.10	9.93 ± 0.72	151.6 ± 22.0
K438E	6.66 ± 0.02	1.60 ± 0.12	15.85 ± 0.96	100.8 ± 8.0
K443E	6.48 ± 0.02	1.51 ± 0.07	18.25 ± 0.20	118.4 ± 9.7
K447E	6.52 ± 0.01	1.48 ± 0.07	16.09 ± 0.30	111.1 ± 7.0
D448R	6.78 ± 0.02	1.84 ± 0.16	8.64 ± 0.07	146.0 ± 11.6
R500E	6.71 ± 0.02	1.73 ± 0.10	10.17 ± 0.50	100.1 ± 7.4
D504R	6.43 ± 0.02	1.65 ± 0.10	12.88 ± 0.62	82.7 ± 4.4
E131Q	6.44 ± 0.02	1.62 ± 0.10	14.66 ± 0.65	126.4 ± 8.1
E131D	6.61 ± 0.02	1.53 ± 0.08	14.63 ± 0.12	135.6 ± 16.4
E131K	6.34 ± 0.05	1.16 ± 0.10	4.68 ± 0.20	57.7 ± 2.9

As E131R exhibited an unusual pH_i profile, we were interested in the other kinetic properties of this NHE1 mutant. Figure 5A shows the dependence of $^{22}Na^+$ uptake on the external Na^+ concentration ($[Na^+]_o$). Interestingly, E131R was observed to have a higher affinity for Na^+ as compared to the wild-type NHE1 (K_m, 1.6 mM versus 6.0 mM; Table 2). Similarly, it also exhibited a higher affinity for Li^+. However, the extracellular pH dependence did not differ between wild-type NHE1 and E131R (Table 2). Furthermore, the NHE1 affinity for the inhibitor EIPA also increased slightly following this mutation (Fig. 5B and Table 2). These results suggest that Glu131 is important for transported cation binding as well as pH_i-sensitivity regulation.

Discussion

In this study, we analyzed the effect of charge-reversal mutations that were introduced in charged residues located in the intracellular loops of NHE1 on the function of this molecule. The surface expression levels and normalized exchange activity (V_{max}) of NHE1 were not drastically altered for molecules with 11 tested mutations, suggesting that these mutant exchangers exhibit normal protein folding, proper membrane targeting, and relatively high transport activity. However, we were unable to obtain the stable expression of E247R by using an activity-dependent selection procedure. Among the NHE isoforms, Glu247 located in TM6 or IL3 is highly conserved and may play a critical role in cation transport and/or membrane expression. We observed that the substitution of Glu131 with Arg shifted the pH_i dependence of the Na^+/H^+ exchange to the acidic side, whereas mutations at Asp448 and Arg500 induced a slight alkaline shift. In addition, we confirmed a previous result [20] that charge-reversal mutation of Arg327 brought about a large acidic shift. Thus, the present study provides reasonable evidence that the side chains of charged residues located in the intracellular loops of NHE1 may play an important role in pH_i regulation by this molecule.

Although NHE1 catalyzes electroneutral Na^+/H^+ counter-transport, it is also known to be a highly proton-dependent transporter [1]. Whereas NHE1 is activated with an increase in the cytosolic H^+ concentration, it is completely inactivated at $pH_i > \sim 7.4$ (pH set-point). There is substantial evidence that this fascinating function of NHE1 can be attributed to the existence of cytosolic H^+-modifier site(s) distinct from its cation transport site [1,9–11]. For example, we previously reported that $^{22}Na^+$ efflux from cells expressing NHE1 can be activated by mild cytosolic acidification only from pH 7.4 to 7.2, implying the existence of an H^+-regulatory site(s) that is required for H^+-induced activation of NHE1 [11]. Furthermore, the steep activation curve for $^{22}Na^+$ efflux suggested that protonation of multiple amino acid side chains may be involved in the regulation of pH_i sensing by NHE1 [11]. The involvement of multiple protons was also suggested in a recent kinetic study on

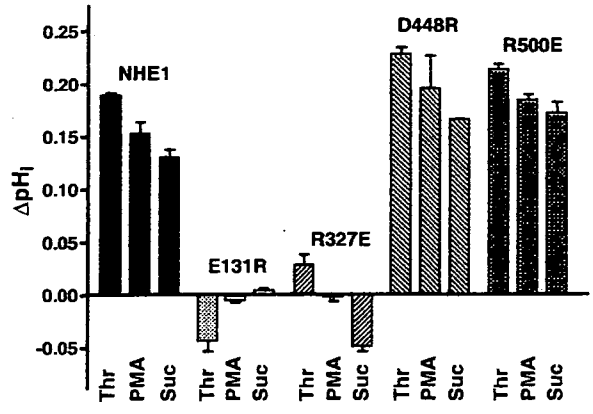

Fig. 4. Change in pH_i in response to extrinsic stimuli. Changes in pH_i were measured as described in Experimental procedures using the [^{14}C]benzoic acid equilibration method. Cells expressing the wild-type or other NHE1 mutants were stimulated for 15 min at 37 °C with 2 U·mL^{-1} thrombin, 1 μM 4β-phorbol 12-myristate 13-acetate, or 200 mM sucrose (hyperosmotic stress). Data are represented as the difference in pH_i before and after stimulation of cells with external agents. The resting pH_i before stimulation was in the range 7.2–7.4.

NHE3 expressed in MDCK cells, although the activation kinetics in this case were slower when compared with NHE1 [24,25]. In general, a His residue is considered to be a probable candidate involved in pH sensing by NHE1, because its imidazole moiety is the only side chain that ionizes in solution within a physiologic pH range. Indeed, His225 and His367 were reported to be important residues in the Na^+/H^+ antiporters of *Escherichia coli* [26,27] and *Schizosaccharomyces pombe* (Sod2) [28,29]. In addition, the mutations of His479 and His499 in rabbit NHE3 were reported to shift the pH_i dependence of Na^+/H^+ exchange to the acidic side [30]. However, recent studies suggest the importance of residues other than His in regulating pH sensitivity. For example, in *Helicobacter pylori* NhaA, several non-His residues (Lys347, Asp172, etc.) were identified as critical residues for pH regulation [31]. In human NHE1, Arg440, Arg327 and Arg180 were previously identified as important residues involved in regulating pH_i dependence [19,20]. Our present study provides additional information about the critical role of charged residues located at the regions of NHE1 facing the cytoplasm. We found that charge-preserved mutation (E131D) had no effect on pH_i dependence, whereas charge-reversal substitution of E131 with another positive residue, Lys, similar to Arg, resulted in a large acidic shift of pH_i dependence. Furthermore, we found that charge neutralization (E131Q) also resulted in a slight decrease in p*K*a. These results suggest that negatively charged residues at position 131, but not positively charged ones, are required for the

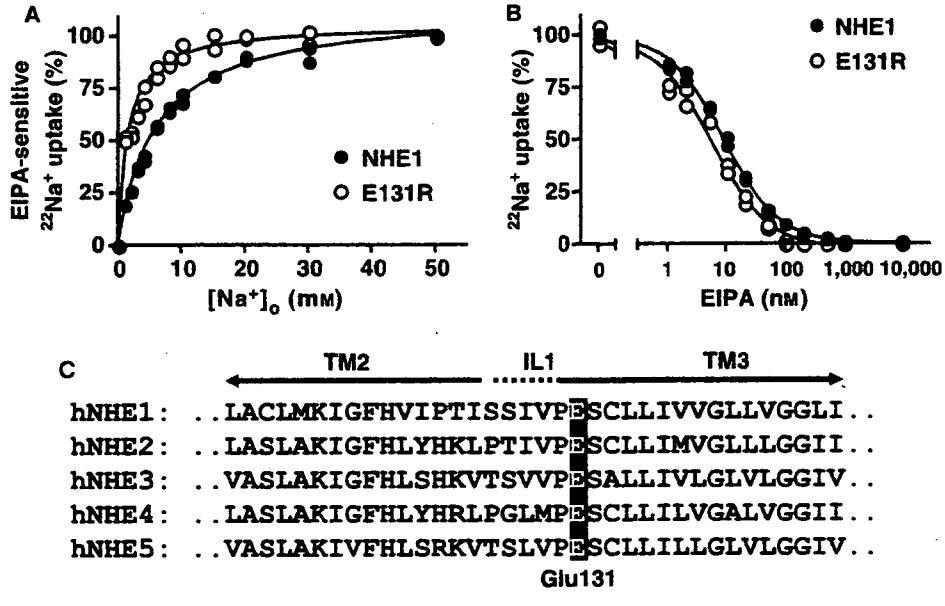

Fig. 5. External Na^+, pH or EIPA dependence of $^{22}Na^+$ uptake. (A) EIPA-sensitive $^{22}Na^+$ uptake in cells expressing the wild-type NHE1 or E131R was measured as a function of Na^+ concentration. (B) EIPA-sensitive $^{22}Na^+$ uptake in cells expressing the wild-type NHE1 or E131R was measured as a function of EIPA concentration. Data were fitted to the dose dependence equation as described in Experimental procedures. (C) Sequence alignment of the TM2/IL1/TM3 boundary regions of human NHE1–NHE5 (GenBank accession numbers: NM_003047, NM_003048, NM_004174, NM_177084, and NM_004594, respectively).

Table 2. Kinetic parameters of $^{22}Na^+$ uptake in cells expressing the wild-type NHE1 or E131R. The data for the extracellular Na^+ were fitted to the steady-state kinetic equation, and the extracellular Li^+, EIPA or pH dependencies of $^{22}Na^+$ uptake were fitted to the one-site competition equation. Data are the best fitted values with standard error. IC_{50} is the concentration of Li^+ or EIPA giving half-maximal inhibition of $^{22}Na^+$ uptake.

Parameters	NHE1	E131R
K_m for Na^+_o (mM)	6.04 ± 0.28	1.60 ± 0.16
IC_{50} for Li^+_o (mM)	5.28 ± 0.57	2.66 ± 0.17
IC_{50} for EIPA (nM)	9.27 ± 0.45	5.89 ± 0.70
pK for pH_o	7.57 ± 0.09	7.47 ± 0.09

normal function of NHE1. We also showed that in addition to decreasing the pKa value, charge-reversal mutation of Glu131 also reduces the Hill coefficient (~ 1.0), and consequently, the pH_i profile becomes nonsigmoidal. As the Hill coefficient would generally reflect the number of protons involved in the pH_i dependence of Na^+/H^+ exchange, Glu131 might serve as an H^+-accepting side chain. There is substantial evidence that the pKa values of charged residues such as Glu and Arg existing in proteins are often considerably different from those of amino acids in an aqueous solution, and consequently, these residues are capable of undergoing protonation/deprotonation in the physiologic pH_i range. For example, a recent electrostatic analysis based on the crystal structure of the bacterial Na^+/H^+ antiporter NhaA suggests that there exists a set of charged residues with unusual pKa values that may be involved in the pH-induced activation of NhaA [32]. Thus, in the present study, we demonstrated the importance of charged residues of NHE1. However, we do not exclude the possibility that mutation might cause local structural distortion, in addition to charge displacement. For example, it should be noted that mutants E131R and E131K, but not E131D or E131Q, exhibited reduced V_{max} activity normalized by the surface expression level, suggesting that the introduction of a positive charge may also inhibit the rate-limiting step of transport reaction, which could be related to the local structural change.

We observed that, as compared to wild-type NHE1, E131R shows a higher affinity for Na^+/Li^+ and a slightly higher affinity for EIPA, exhibiting an acidic and nonsigmoidal pH_i dependence. On the other hand, such a large increase in Na^+ affinity was not observed in R327E (data not shown) and in R440E, which is reported to exhibit a similar large acidic shift in pH_i dependence [19]. There is increasing evidence that the binding site for cation transport in NHE1 is located within its membrane-spanning region. For example, previous studies have shown that mutations of residues within TM4 and TM9 of NHE1 reduce the Na^+ affinity or the cation transport activity of this molecule [33–36]. TM4 and TM9 are also suggested to be involved in the binding of amiloride derivatives, i.e. NHE inhibitors [33,34,37–39]. Furthermore, a recent study reported that some cysteine residues engineered in TM4 are accessible to SH-modifying reagents from the aqueous phase, suggesting that these residues may form the pore-lining region that permits cation translocation [40]. In addition, mutations of Glu262 and Asp267 in TM7 and E391 in the re-entrant loop (Fig. 1A) were reported to abolish or greatly reduce the exchange activity of these exchangers, suggesting that these residues may play a critical role in cation binding [5,13]. Thus, TM4 and TM9 along with reported critical regions contain probable candidate residues involved in the cation transport pathway. Glu131 is highly conserved among the NHE isoforms (Fig. 5C) and is considered to be located in the TM3/IL1 boundary. Our observations suggest that TM3/IL1 may be structurally linked to the cation transport pathway, and that a Glu131 mutation may significantly alter the structure of the cation translocation pore.

At present, the precise mechanism by which ion exchange by NHE1 is activated via the protonation of modifier sites remains unclear. Two-dimensional crystal analysis of the *Methanococcus jannaschii* Na^+/H^+ antiporter MjNhaP1 that showed its homology with eukaryotic exchangers revealed that the density distribution within bundles of transmembrane helices is significantly altered in a pH-dependent manner [41]. Furthermore, the recently reported crystal structure of the bacterial Na^+/H^+ antiporter NhaA predicted that the modification of charged residues within cytoplasmic loops may activate NhaA by exposing its cation transport site by reorienting the transmembrane helices [42]. These studies raise the possibility that a pH-dependent conformational change in the transmembrane helices from the inactive to the active form commonly occurs in various Na^+/H^+ antiporter family members. It is possible that several critical charged residues identified in NHE1 may control the opening/closing of the cytoplasmic gate of the exchanger in a pH-dependent manner. In this context, it is of interest to note that in the Na^+/Cl^--dependent neurotransmitter transporter ($LeuT_{Aa}$), some salt bridges between Arg and Glu residues are suggested to control substrate transport via the opening/closing of cytoplasmic and extracellular gates [43]. Although further experiments will be necessary, we consider that Glu131 would be