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Structural and functional analysis of critical amino acids in TMVI of the NHE1 isoform of the Na^+/H^+ exchanger

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ABSTRACT

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) resides on the plasma membrane and exchanges one intracellular H^+ for one extracellular Na^+. It maintains intracellular pH and regulates cell volume, and cell functions including growth and cell differentiation. Previous structural and functional studies on TMVI revealed several amino acids that are potentially pore lining. We examined these and other critical residues by site-directed mutagenesis substituting Asn227 → Ala, Asp, Arg; Ile233 → Ala; Leu243 → Ala; Glu247 → Asp, Gln; Glu248 → Asp, Gln. Mutant NHE1 proteins were characterized in AP-1 cells, which do not express endogenous NHE1. All the TMVI critical amino acids were highly sensitive to substitution and changes often lead to a dysfunctional protein. Mutations of Asn227 → Ala, Asp, Arg; Ile233 → Ala; Leu243 → Ala; Glu247 → Asp; Glu248 → Gln yielded significant reduction in NHE1 activity. Mutants of Asn227 demonstrated defects in protein expression, targeting and activity. Substituting Asn227 → Arg and Ile233 → Ala decreased the surface localization and expression of NHE1 respectively. The pore lining amino acids Ile233 and Leu243 were both essential for activity. Glu247 was not essential, but the size of the residue at this location was important while the charge on residue Glu248 was more critical to NHE1 function. Limited trypsin digestion on Leu243 → Ala and Glu248 → Gln revealed that they had increased susceptibility to proteolytic attack, indicating an alteration in protein conformation. Modeling of TMVI with TMXI suggests that these TM segments form part of the critical fold of NHE1 with Ile233 and Leu465 of TMXI forming a critical part of the extracellular facing ion conductance pathway.

1. Introduction

The Na^+/H^+ exchangers (NHE) are membrane proteins present in all known eukaryotic and prokaryotic cells. In mammalian cells, their primary role is to regulate intracellular pH (pH_i), maintaining physiological pH despite acid load from cellular metabolism. Ten mammalian Na^+/H^+ exchanger isoforms have been discovered. The first isoform, NHE1, was cloned in 1989 [1] by Pouyssegur's group and since that time numerous functional studies have led to the understanding of its importance in cell regulation and have identified other NHE isoforms [2,3]. NHE1 is ubiquitously expressed on the plasma membrane, while NHE2–NHE10 are distributed among specific cell types, and have different kinetic characteristics, different membrane localization and are regulated differently [4,5]. NHE1 carries out the pH_i sensitive exchange of one intracellular H^+ for an extracellular Na^+. This is important because variations in pH_i are capable of disrupting normal protein folding, chemical reactions and ion gradients [6]. In addition to pH_i and volume regulation, NHE1 is also essential for cell growth, differentiation, and migration [7–10]. Increased NHE1 levels and activity also have pathological roles in cancer development, cardiac ischemia reperfusion injury, and cardiac hypertrophy [11–15]. Therefore, inhibitors of NHE1 have been investigated for their efficacy of treating cancer and heart disease [15–18].

NHE1 is composed of two domains with 815 amino acids with an apparent molecular weight of 110 kDa in the N-linked and O-linked glycosylated form [4,13,19]. The N-terminal domain consists of approximately 500 residues, which form the 12 transmembrane segments that span the plasma membrane and arrange into a structure for cation translocation. The latter 315 residues of NHE1 form the cytoplasmic regulatory tail that contains phosphorylation sites, and binding sites for PIP_2, ERM (ezrin/radixin/moesin), tescalcin, 14-3-3, carbonic anhydrase, calmodulin, calcineurin homologous protein type 1 and calcineurin homologous protein type 2 [2,3,9,10,20].

Attempts are underway to determine the high resolution crystal structure of NHE1. However, several avenues of study have used

Abbreviations: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6) carboxyfluorescein-acetoxymethyl ester; DPC, dodecylphosphocholine; HA, hemagglutinin; Intracellular pH, pH_i; MTSET, ([2-(Trimethylammonium)Ethyl]Methanethiosulfonate); NhaA, E. coli Na^+/H^+ antiporter type A; NHE, Na^+/H^+ exchanger; NHE1, Na^+/H^+ exchanger isoform one; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; TMVI, transmembrane segment VI; TMXI, transmembrane segment XI; WT, wild type

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homology model analysis, mutational analysis, NMR spectroscopy and electron microscopy to reveal significant insights into the secondary and tertiary structure of NHE1 [21–24]. As part of a series of studies analyzing the structure and function of transmembrane regions of NHE1 [21,25,26] we recently analyzed the peptide structure of TMVI (N227–I249) of NHE1 in DPC micelles, and analyzed the functional contribution of amino acids of that segment by using the cysteine scanning accessibility method [27]. Many amino acid residues of TMVI (transmembrane segment VI) were sensitive to mutation to Cys and reaction with external sulfhydryls indicating that TMVI was a critical pore lining segment. Interestingly, TMVI had a structure consisting of a helix, extended region, followed by a helix. This was similar in structure to the critical TMIV of the *Escherichia coli* Na^+/H^+ exchanger NhaA and we proposed that it serves a similar critical role in NHE1 activity. In this study we further characterized this key transmembrane segment. Of the residues in TMVI that maintained sufficient NHE1 activity when substituted to Cys, the key pore lining amino acids of TMVI were Asn227, Ile233 and Leu243. Several other residues had partially depressed activity when substituted to cysteine including, Glu247 and Glu248. Moreover, the Glu248→Cys mutant was partially inhibited by MTSET treatment [27]. Here we further characterized the functional importance of critical amino acids in TMVI. Applying site-directed mutagenesis methods, Asn227, Ile233, Leu243, Glu247, and Glu248 of TMVI were substituted into Asn227→Ala, Asp, Arg; Ile233→Ala; Leu243→Ala; Glu247→Asp, Gln; Glu248→Asp, Gln. These mutant NHE1 proteins were assessed for their protein expression, activity, and surface localization. Limited digestion with trypsin was used to examine protein conformational changes caused by mutations. Additional structural modeling was performed based on previous NMR studies of TMVI and XI to investigate their assembly in the cation translocation pore. Our results demonstrate that TMVI forms a critical part of the cation access pathway along with TMXI. Asn227 and Ile233 form part of the extracellular facing pore while Leu243, Glu247 and Glu248 form critical parts of the intracellular fold of the coordination pathway.

2. Materials and methods

2.1. Materials

PWO DNA polymerase was purchased from Roche Applied Science (Roche Molecular Biochemicals, Mannheim, Germany). Restriction enzymes were obtained from New England Biolabs, Inc. (Mississauga ON, Canada). Lipofectamine™ 2000 reagent was purchased from Invitrogen Life Technologies (Carlsbad CA, USA). 2′,7′-bis (2-carboxyethyl)-5(6) carboxyfluorescein-acetoxymethyl ester was purchased from Molecular probes, Inc. (Eugene OR, USA). Sulpho-NHS-SS-Biotin was from Pierce Chemical Company (Rockford IL, USA). Immobilized streptavidin resin, nigericin and trypsin (phenylalanyl chloromethyl ketone-trypsin) were from Sigma-Aldrich (St Louis MO, USA). Geneticin G418 antibiotics were purchased from American Bioanalytical (Natick MA, USA). Cell culture MEM alpha modification medium was from Thermo Scientific Hyclone (Logan UT, USA).

2.2. Site-directed mutagenesis

Silent mutation restriction enzyme sites were introduced for selection as described earlier [23]. We used the plasmid pYN4+ as a template, which encoded for the entire human NHE1 and a hemagglutinin (HA) tag at the C-terminal end [23]. The HA-tagged NHE1 functions normally as the wild type NHE1 [21]. The oligomers for site directed mutagenesis are indicated in Table 1. Site-directed mutagenesis was performed using PWO DNA polymerase from the Stratagene (La Jolla, CA, USA) QuikChange™ site directed mutagenesis kit. DNA sequencing was performed at the University of Alberta, Department of Biochemistry DNA Core Services Lab.

Table 1
Oligonucleotides used for site-directed mutagenesis of TMVI. Nucleotides mutated are indicated in lower case, and restriction sites introduced are indicated in bold.

A

Mutation	Oligonucleotide sequence	Restriction site
N227A	5′-CAACAACATCGGCCT**tCTaGA**CgcCCTGCTCTTCGGCAGC-3′	*Xba I*
N227D	5′-CAACAACATCGGCCT**tCTaGA**CgACCTGCTCTTCGGCAGC-3′	*Xba I*
N227R	5′-CAACAACATCGGCCT**tCTaGA**CcgCCTGCTCTTCGGCAGC-3′	*Xba I*
I233A	5′-CTGCTCTTC**GGatcC**gcCATCTCGGCCGTGGAC-3′	*Bam HI*
L243A	5′-GACCCCGTGGCGGTTg**ccGCgG**TCTTTGAGGAAATT-3′	*Sac II*
E247D	5′-GTTCTGGCTGTCTTTGAcGA**gAT**TCAC**ATC**AATGAG-3′	*Bsa BI*
E247Q	5′-GTTCTGGCTGTCTTTcAGGA**gAT**TCAC**ATC**AATGAG-3′	*Bsa BI*
E248D	5′-TCTGGCTGTC**TTcGAa**GAcATTCACATCAATGAG-3′	*Bst BI*
E248Q	5′-TCTGGCTGTC**TTcGAacAA**ATTCACATCAATGAG-3′	*Bst BI*

2.3. Cell culture and transfections

Mutant plasmids were transfected in the AP1 cell line, which does not express endogenous active NHE1. AP-1 is a mutant cell line derived from Chinese hamster ovarian cells (CHO) by proton suicide technique [1]. Stably transfected cells were established using LIPOFECTAMINE™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) as described earlier [21]. pYN4+ plasmid encoded a neomycin resistance gene, which allowed the selection of transfected cells using geneticin (G418) antibiotics. Stable cell lines for experiments were regularly re-established from frozen stocks at passage numbers between 5 and 11.

2.4. SDS-PAGE and immunoblotting

Cells were cultured in 60 or 35 mm dishes until 80–90% confluent, and then were harvested as described earlier [26]. Growth medium was removed by aspiration and cell monolayers were washed with 4 °C phosphate-buffered saline. Plates were kept on ice to reduce protein degradation. RIPA Lysis buffer (1% NP-40, 0.25% sodium deoxycholate, 0.1% Triton X-100, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, protease inhibitor cocktail) was added to AP-1 cells for 1–3 min. Cell debris were removed by centrifugation at 14,000 rpm for 5 min at 4 °C. Supernatants were kept in clean eppendorf tubes and they were either frozen for −80 °C storage or prepared for subsequent trypsin treatment and SDS-PAGE. Cell lysates containing NHE1 were resolved on 10% SDS/polyacrylamide gels. Proteins were transferred onto nitrocellulose membrane (BioRad) and detected using anti-HA monoclonal antibody. The secondary antibody was peroxidase-conjugated goat anti-mouse antibody. NHE1 was visualized by enhanced chemiluminescence, and X-ray films (Fuji medical X-ray film) were processed by Kodak X-OMAT 2000 M35 processor. ImageJ 1.35 software (National Institutes of Health, Bethesda, MD, USA) was used to quantify band intensities.

2.5. Cell surface expression

Targeting of NHE1 to the cell surface was examined essentially as described earlier [21,26]. Cells were grown to 50–70% confluence in 60 mm dishes. The plates were placed on ice, washed once with 4 °C PBS followed by a second wash with 4 °C borate buffer pH9 (154 mM NaCl, 7.2 mM KCl, 1.8 mM $CaCl_2$, 10 mM boric acid). 3 ml of freshly made sulpho-NHS-SS-Biotin (Pierce Chemical Company, Rockford, IL, USA) at a concentration of 0.5 mg/ml in borate buffer was added to each plate and cells were incubated for 30 min at 4 °C. Cells were then washed 3 times with cold quenching buffer pH 8.3 (192 mM glycine,