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Structural and functional analysis of extracellular loop 4 of the Nhe1 isoform of the Na^+/H^+ exchanger

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ABSTRACT

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitously expressed plasma membrane protein. It regulates intracellular pH by removing a single intracellular H^+ in exchange for one extracellular Na^+. The membrane domain of NHE1 comprises the 500 N-terminal amino acids and is made of 12 transmembrane segments. The extracellular loops of the transmembrane segments are thought to be involved in cation coordination and inhibitor sensitivity. We have characterized the structure and function of amino acids 278–291 representing extracellular loop 4. When mutated to Cys, residues F277, F280, N282 and E284 of EL4 were sensitive to mutation and reaction with MTSET inhibiting NHE1 activity. In addition they were found to be accessible to extracellular applied MTSET. A peptide of the amino acids of EL4 was mostly unstructured suggesting that it does not provide a rigid structured link between TM VII and TM VIII. Our results suggest that EL4 makes an extension upward from TM VII to make up part of the mouth of the NHE1 protein and is involved in cation selectivity or coordination. EL4 provides a flexible link to TM VIII which may either allow movement of TM VII or allow TM VIII to not be adjacent to TM VII.

1. Introduction

A key pH regulatory protein in mammalian cells is the Na^+/H^+ exchanger, type I isoform (NHE1). NHE1 is a ubiquitous plasma membrane protein that catalyzes removal of a single intracellular proton in exchange for a single extracellular sodium ion [1–3]. Aside from regulation of intracellular pH, NHE1 has numerous other indirect physiological roles. It promotes cell growth and differentiation [4], facilitates inward sodium flux in response to osmotic shrinkage [5], and enhances cell motility [6]. Additionally, NHE1 has several pathological roles. Studies in culture have suggested that NHE1 may be important in breast cancer cell invasiveness and have demonstrated that in NHE1 activity enhances invasion by breast cancer cells not only by raising intracellular pH, but also by acidification of the extracellular microenvironment of tumor cells [7–10]. The extracellular acidification may be necessary for protease activation which facilitates the digestion and remodeling of the extracellular matrix [8], critical in metastasis.

In the myocardium it promotes heart hypertrophy and exacerbates the damage that occurs during myocardial ischemia and reperfusion. Inhibitors of NHE1 have proven useful in preclinical trials, for protecting the myocardium in various disease states [11–14]. However clinical trials with NHE1 inhibitors have been disappointing, possibly due to a lack of specificity of the inhibitors [15]. Further knowledge of NHE1 structure and function may facilitate the development of more specific inhibitors for clinical use.

The mammalian NHE1 protein is believed to consist of 12 transmembrane (TM) segments that comprise the N-terminal 500 amino acids. These are connected by a series of extra membrane segments which form intracellular and extracellular loops (EL). Loops between α-helices can influence their arrangements and packing [16] and can modulate protein function [17]. For NHE1, EL5 may be involved in drug sensitivity and cation binding [18,19]. EL2 links TM segments I and II and mutation of residues of EL2 affects both the drug sensitivity and the activity of NHE1 [20,21]. Two models of the topology of NHE1 are in question with two different models, one model is based on cysteine accessibility studies [22] and a second being based on computational comparison with the deduced structure of the *Escherichia coli* Na^+/H^+ exchanger NhaA suggested [23]. Although significant variation in the predictions occurs after amino acid 315, prior to this region the models are essentially identical with the exception of the latter model predicting that the first two transmembrane segments are cleaved free of the protein. Amino acids 278–290 are predicted to form an EL in both models. They form EL4 connecting TM VII and VIII in the model of Wakabayshi et al. [22] and are predicted to form the same loop in the model of Landau et al. [23] connecting the same two transmembrane segments that are numbered V and VI in that model. Little is known about this particular loop connecting

Abbreviations: CHO, Chinese hamster ovary; EL, extracellular loop; HA, hemagglutinin; NHE1, Na^+/H^+ exchanger type 1 isoform; MTSES, ([2-Sulfonatoethyl] methanethiosulfonate); MTSET, ([2-(Trimethylammonium)ethyl] methanethiosulfonate); TM, transmembrane segment

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TM VII (amino acids 251–273) and VIII (amino acids 291–311) in terms of its functional role and contribution by the amino acids present in it. We have earlier examined the structure and function of TM VII. We found that TM VII is a predominantly α-helical segment, that is critical to NHE1 function and that it contained pore lining residues [24,25].

ELs of transporters are often critical to their function. For example in the serotonin transporter, the glutamate transporter and the glycine transporter [26–28] ELs play key roles in function. For Na^+,K^+ ATPase the second EL is a major determinant of extracellular cation affinity [29,30]. Amino acids of the acetylcholine receptor [31,32] and NhaA [33] ELs are also involved in the attraction of cation to the extracellular surface. In this study we examined the role of EL4 (amino acids 274–290) of the NHE1 isoform of the mammalian Na^+/H^+ exchanger. We also used NMR to characterize the structural properties of synthetic peptides representing EL4.

2. Materials and methods

2.1. Materials

Streptavidin–agarose resin was from Sigma-Aldrich (St. Louis, MO, USA). Toronto Research Chemicals, (Toronto, Ontario, Canada) supplied MTSET and MTSES. Lipofectamine™ 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA) and PWO DNA polymerase was obtained from Roche Applied Science (Roche Molecular Biochemicals, Mannheim, Germany). Sulfo-NHS-SS-biotin was from Pierce Chemical Company (Rockford, IL, USA).

2.2. Site-directed mutagenesis

For expression and mutagenesis of the human NHE1 protein we used the expression plasmid pYN4+ that contains a fully functional hemagglutinin (HA) tagged human NHE1 isoform of the Na^+/H^+ exchanger with all native cysteines mutated to serine [34]. Mutations to the NHE1 cDNA in pYN4+ changing the indicated amino acids to cysteine (Supplementary Table 1), where indicated, were made using the functional cysteineless NHE1 protein that we described earlier [35]. We have characterized amino acids 271–273 as part of our earlier work on TM VII [25]. Site-directed mutagenesis was as described earlier [35]. Mutations were designed to create or delete a restriction enzyme site. DNA sequencing confirmed the fidelity of DNA amplification and mutations.

2.3. Cell culture and transfections

Stable cell lines were made using AP-1 cells transformed with control and mutant pYN4+Cysless DNA [19]. AP-1 cells lack their endogenous Na^+/H^+ exchanger as described earlier. Transfection was with Lipofectamine™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) [34]. Stable cell lines for experiments were regularly re-established from frozen stocks at passage numbers between 5 and 9. At least two independently made clones of each mutant were made and results are shown from one mutant. Independently made clones all had very similar functional characteristics.

2.3.1. SDS-PAGE and immunoblotting

Cell lysates were made as described earlier [34] and were used for Western blot analysis on 10% SDS/polyacrylamide gels [36]. Identification of the NHE1 protein was via the hemagglutinin (HA) tag on the Na^+/H^+ exchanger using an anti-HA monoclonal antibody. The second antibody was peroxidase-conjugated goat anti-mouse antibody. Chemiluminescence was used to visualize immunoreactive proteins. Quantification was using ImageJ 1.35 software (National Institutes of Health, Bethesda, MD, USA) examining protein expression on X-ray films.

2.4. Cell surface expression

The amount of NHE1 protein that was targeted to the cell surface was measured essentially as described earlier [34]. Briefly, cell surfaces were labeled with sulfo-NHS-SS-biotin and immobilized streptavidin was used to remove plasma membrane labeled NHE1 protein. Equivalent amounts of total and unbound proteins were analyzed by blotting for HA-tagged NHE1 protein. It was not possible to efficiently and reproducibly elute proteins bound to immobilized streptavidin resin. The relative amount of NHE1 on the cell surface was calculated for both the 110 kDa and the 95 kDa (partial or de-glycosylated) HA-immunoreactive species in Western blots of the fractions as indicated in the figures and legends.

2.5. Accessibility of EL4 residues

The accessibility of residues of EL4 was determined based on a modified procedure of [37]. Stable cell lines with mutants of EL4 were grown to confluence and after washing with phosphate buffer saline (PBS), were treated with or without 10 mM MTSET for 20 min at 37 °C in a buffer consisting of 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM glucose and 10 mM HEPES, and pH 7.3 (normal buffer). Cells were washed with PBS three times and then 2 ml of a lysis buffer was added, consisting of 25 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol and 37.5 μM ALLN and a protease inhibitor cocktail described earlier [38]. Cells were scraped off and sonicated two times for 15 s. The solution was spun at 35,000 rpm (100,000 ×*g*) for 1 h and the supernatant was collected. The supernatant was used to immunoprecipitate the HA-tagged NHE1 protein. For this purpose we used the Pierce Crosslink IP kit in which the primary antibody was crosslinked to Protein A/G agarose enabling immunoprecipitation without contamination from the primary antibody. The primary antibody used for immunoprecipitation was a commercially obtained rabbit polyclonal (Santa Cruz, sc-805). After immunoprecipitation, the sample was divided into two. One sample was used to quantify the total NHE1 protein via western blotting. To the second sample IRDye800-maleimide (LI-COR) was added (final concentration 0.2 mM) which would react with any unblocked sulfhydryls of the introduced cysteine. Samples were separated by SDS-PAGE and we examined the protein reacting with the IRDye800-maleimide using the LI-COR system. MTSET accessibility = 100 − %(Fluorescence in the presence of MTSET/Fluorescence in absence of MTSET). Readings were corrected for the amount of NHE1 immunoprecipitated by Western blotting for NHE1 with a monoclonal antibody against the HA tag.

2.6. Na^+/H^+ exchange activity

Na^+/H^+ exchange activity was measured as described earlier [25] using a spectrofluorometer and the pH sensitive dye 2′,7-bis (2-carboxyethyl)-5(6) carboxyfluorescein-AM (Molecular Probes Inc., Eugene, OR, USA). Ammonium chloride induced an acute acid load as described earlier [39]. The initial rate of Na^+ (135 mM)-induced recovery of cytosolic pH was measured as described earlier [25]. Buffering capacities of stable cell lines did not vary and the initial recovery rate measured was from equivalent initial pHs. Wherever indicated, NHE activity was corrected for the level of protein expression and for the targeting of the protein to the cell surface. To determine reactivity with externally reactive sulfhydryl compounds, cells were treated with MTSET or MTSES using a two-pulse acidification assay. Cells were treated with ammonium chloride two times and allowed to recover twice in NaCl containing medium [35]. The first pulse was in the absence of inhibitor and the second pulse was performed after the cells were treated with either 10 mM MTSET or MTSES for 10 min in NaCl containing buffer. The starting pH of recovery was equivalent in the two pulses. The rate of recovery in the absence of inhibitor was