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Residues important for K^+ ion transport in the K^+-dependent Na^+-Ca^{2+} exchanger (NCKX2)

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ABSTRACT

K^+-dependent Na^+-Ca^{2+} exchangers (NCKXs) play an important role in Ca^{2+} homeostasis in many tissues. NCKX proteins are bi-directional plasma membrane Ca^{2+}-transporters which utilize the inward Na^+ and outward K^+ gradients to move Ca^{2+} ions into and out of the cytosol ($4Na^+$:$1Ca^{2+}$ + $1\,K^+$). In this study, we carried out scanning mutagenesis of all the residues of the highly conserved α-1 and α-2 repeats of NCKX2 to identify residues important for K^+ transport. These structural elements are thought to be critical for cation transport. Using fluorescent intracellular Ca^{2+}-indicating dyes, we measured the K^+ dependence of transport carried out by wildtype or mutant NCKX2 proteins expressed in HEK293 cells and analyzed shifts in the apparent binding affinity (K_m) of mutant proteins in comparison with the wildtype exchanger. Of the 93 residue substitutions tested, 34 were found to show a significant shift in the external K^+ ion dependence of which 16 showed an increased affinity to K^+ ions and 18 showed a decreased affinity and hence are believed to be important for K^+ ion binding and transport. We also identified 8 residue substitutions that resulted in a partial loss of K^+ dependence. Our biochemical data provide strong support for the cation binding sites identified in a homology model of NCKX2 based on crystal structures reported for distantly related archaeal Na^+-Ca^{2+} exchanger NCX_Mj. In addition, we compare our results here with our previous studies that report on residues important for Ca^{2+} and Na^+ binding. *Supported by CIHR MOP-81327.*

1. Introduction

The companion paper [1] introduces in detail the functional roles and structural properties of K^+-dependent Na^+-Ca^{2+} exchangers (NCKX), members of the *SLC24 A* gene family which belongs to the CaCA superfamily of Ca^{2+}/cation antiporters [2,3]. K^+-dependent Na^+-Ca^{2+} exchangers (NCKX) differ from the K^+-independent Na^+-Ca^{2+} exchangers (NCX) by coupling Ca^{2+} extrusion from cells not only to the inward Na^+ gradient but also to the outward K^+ gradient with an exchange stoichiometry of 4 Na^+ against one Ca^{2+} and one K^+ [4–6]. The first NCKX1 cDNA was cloned in 1992 from bovine retinal rod photoreceptors [7]. In 2000, chicken and human retinal cone photoreceptors were found to express NCKX2 [8] which was first identified and cloned from rat brains [9]. As summarized in the introduction of the companion paper [1], we now know that the *SLC24 A* gene family consists of five members found in a wide range of tissues and is important for a wide range of biological processes, yet very little is known about the detailed physiology of NCKX proteins in tissues other than the retina [10]. In rod and cone photoreceptors, light modulates a persistent inward current carried by Na^+ and Ca^{2+} via cGMP-gated channels; the cGMP-gated channels are open in darkness and fully closed by saturating light (reviewed in [11]). In darkness, an inward current via cGMP-gated channels results in a sustained membrane depolarization and likely an elevated cytosolic Na^+ concentration. The role of NCKX proteins is to extrude Ca^{2+} that enters in darkness via the cGMP-gated channels. Upon illumination, reduction of Ca^{2+} influx via cGMP-gated channels and continued extrusion via NCKX causes a lowering of cytosolic free Ca^{2+} concentration which contributes to the process of light adaptation (reviewed in [11]). The combination of depolarized membrane potential and elevated cytosolic Na^+ concentration in darkness might compromise the ability of K^+-independent Na^+-Ca^{2+} exchangers to extrude Ca^{2+} from rod and cone photoreceptors, and, as all Na^+-Ca^{2+} exchangers are bidirectional transporters, might result in a deleterious Ca^{2+} influx [12]. Hence, NCKX proteins might be essential for cells that display sustained periods of membrane depolarization, in part mediated by inward Ca^{2+} currents.

Mammalian NCX and NCKX proteins are now thought to share the same membrane topology [13–16], also observed in the crystal

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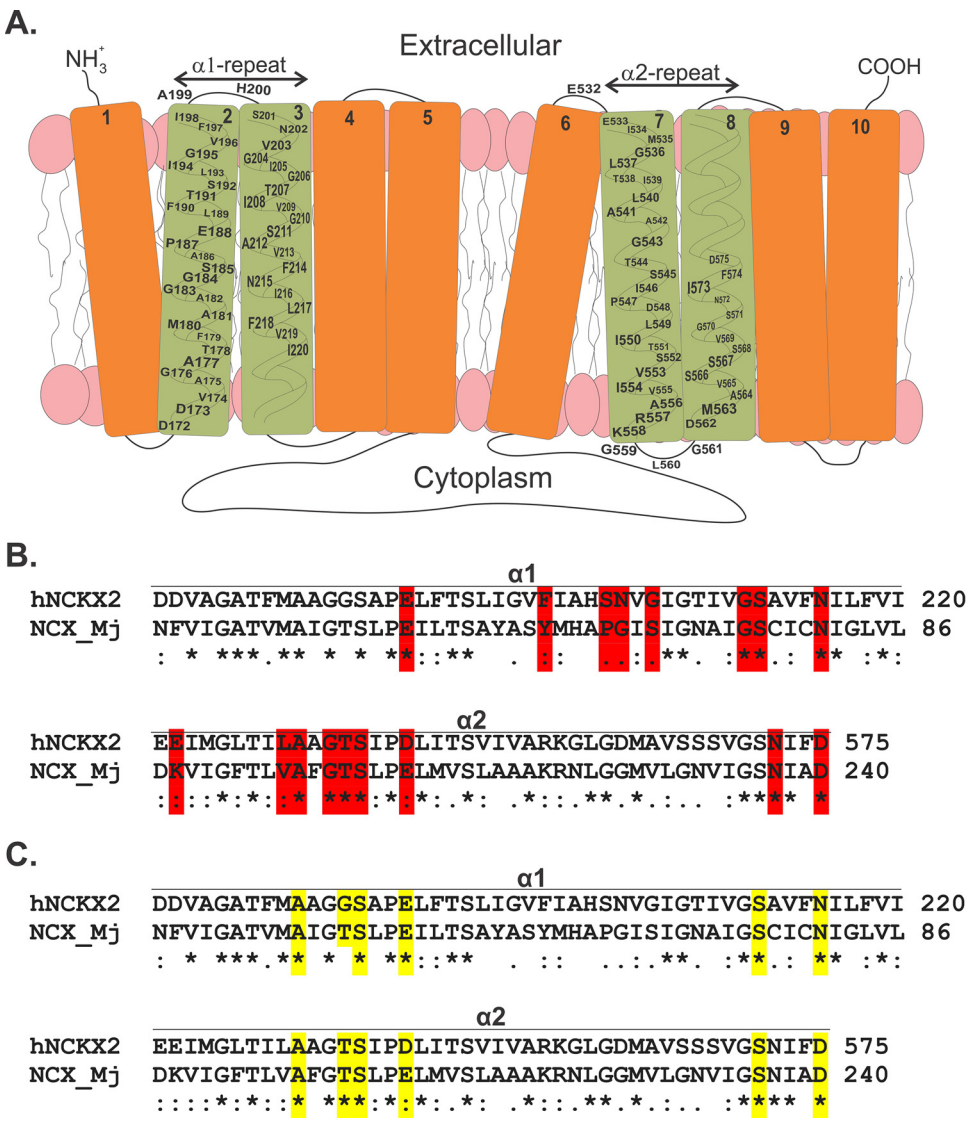

Fig. 1. Schematic representation of the 2-dimensional topology of the human NCKX2 proteins and the sequence alignment of the α-1 and α-2 repeat regions of the human NCKX2 and NCX_Mj proteins. *Panel A* illustrates that the topology is comprised of two sets of 5 TMS segments separated by a large cytoplasmic loop. Highlighted in *green* on the topology are the α-1 and α-2 repeats residues (TMS2-3 and TMS7-8). *Panel B.* shows the sequence alignment between hNCKX2 and NCX_Mj α-repeats highlighting in *red* Group III residues (residues for which substitution resulted in a large decrease in the apparent affinity for K^+). *Panel C.* shows the sequence alignment between hNCKX2 and NCX_Mj α-repeats highlighting in *yellow* the 12 residues that comprise the cation-coordinating sites described for the NCX_Mj crystal structure (S_{int}, S_{Ca}, S_{mid} and S_{ext}). The sequence alignment was prepared using Clustal Omega online platform (http://www.ebi.ac.uk/Tools/msa/clustalo/). An asterisk (*) denotes conserved residues, a colon (:) denotes conserved substitutions, and a period (.) denotes semi-conserved substitutions. The sequences used can be obtained from the NCBI using the following accession numbers: NCKX2 (NM_001193288), and NCX_Mj (NC_000909 Region: 85705–86613).

structure of the distantly related archaeal Na^+-Ca^{2+} exchanger NCX_Mj [17]. However, sequence homology between NCX and NCKX proteins is limited to two relatively short stretches making up the centre of two sets of five α-helical transmembrane segments that are the core structural component of all NCX and NCKX proteins and are referred to as the α-1 and α-2 repeats (Fig. 1). These two short segments of ~40 amino acids each were shown to contain the cation-coordinating residues of NCX_Mj [17,18] and the cation-coordinating residues in a homology model of NCKX2 that was based on the NCX_Mj crystal structure [19]. In earlier mutagenesis studies of a select number of α-repeat residues, several residues were identified for which substitution to other amino acids resulted in significant shifts in the apparent affinity of NCKX2 for K^+ [20,21] although these two studies report significantly different values for the K^+ Michaelis Menten constant (K_m) of Wild-type (WT) human NCKX2. One NCKX2 residue (Asp575) was identified for which substitution to either Cys or Asn resulted in a complete loss of K^+ dependence, suggesting that this residue is a critical contributor to the K^+ binding site [22]. As reported in the companion paper [1] on residues important for Ca^{2+} affinity, we have now advanced our analytical procedures using sodium-loaded HEK293 cells and more appropriate Ca^{2+}-indicating dyes (Fluo4 and Fluo4 F F) to permit accurate measurements of K_m values for external K^+, even in mutant NCKX2 proteins that have only 5% of WT NCKX2 maximal transport activity [23]. Here, we report on shifts in K^+ ion K_m values for mutant NCKX2 proteins representing all α-1 and α-2 repeat residues (ninety-three residues in total). This work was carried out to address four questions: 1) Obtain experimental support for our homology model of NCKX2 and the residues proposed to contribute to the K^+ binding site; 2) do residues other than those contributing to cation coordination cause changes in the observed K_m for K^+ as observed in our companion paper [1] for residues important for Ca^{2+} affinity; 3) identify other resides whose substitution results in a (partial) loss of K^+ dependence as observed for the Asp575 Asn and Asp575Cys substituents (the corresponding residue in NCX_Mj is Asp240); 4) does any correlation exist in shifts in K_m for K^+ with shifts in K_m for Ca^{2+} (companion paper) [1] and shifts in K_m for Na^+ reported earlier [24]. We will discuss our results in the context of a homology model of NCKX2 [19] and on several crystal structures of archaeal Na^+-Ca^{2+} exchanger NCX_Mj [17,18].

2. Methods

All tissue culture reagents were purchased from ThermoFisher Scientific and all other chemicals were purchased from Sigma-Aldrich unless stated otherwise.