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Membrane Topology and Function of the Secretory Na^+-K^+-$2Cl^-$ Cotransporter (NKCC1)

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There is now convincing evidence that the secretion of fluid by many exocrine tissues is due to the transepithelial secretion of Cl^- from interstitium to lumen. In a number of earlier studies we and others have documented that in the salivary glands of several species, including humans, much of this Cl^- secretion is driven by a bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter located in the acinar basolateral membrane.

The salivary Na^+-K^+-$2Cl^-$ cotransporter is a member of a family of electroneutral cation-chloride cotransporters (1, 2) that carry out many important functions in mammalian cells. This family includes the Na^+-K^+-$2Cl^-$ cotransporters (two isoforms have been identified, the "secretory isoform" found in exocrine epithelia and a number of other cell types, and the "absorptive isoform" found in the apical membrane of the renal thick ascending limb of Henle's loop), a Na^+-Cl^- cotransporter (found in the apical membrane of the distal nephron) and the K^+-Cl^- cotransporters (four isoforms have been identified thus far). The physiological roles of the members of this transporter family include transepithelial salt and water transport, cell volume regulation, control of intracellular $[Cl^-]$, and the transport of ammonium (and thereby acid base equivalents; ammonium can substitute for K^+ on the Na^+-K^+-$2Cl^-$ cotransporters). Representative members of all of the above transporters have now been cloned and sequenced (1-3). Hydropathy analyses predict that all of these proteins share a common topology consisting of large hydrophilic N- and C-termini (15-35 kDa and ~50 kDa, respectively) on either side of a central hydrophobic transmembrane region (~50 kDa). The transmembrane region is predicted to consist of 10-12 membrane spanning domains (presumed be α-helices) connected by relatively short intracellular and extracellular loops. The greatest divergence in these transporters is in their N-termini which can be poorly conserved among species even for sequences representing the same cotransporter isoform (3).

Members of the electroneutral cation-chloride cotransporter family have also been identified by sequence homology in lower vertebrates, worms, plants, insects and yeast (3), however, the function of these (putative) proteins remains to be established. No significant homology of the members of this cotransporter family with other membrane transport proteins has been found, suggesting that the electroneutral cation-chloride cotransporters belong to a distinct gene family.

To date, most of the experimental information concerning the structure and function of cation-chloride transporters has come from studies of NKCC1, the "secretory" isoform of the Na^+-K^+-$2Cl^-$ cotransporters. Evidence that the N- and C-termini of NKCC1 are cytosolic has come from antibody accessibility studies (4) and experiments that localize regulatory phosphorylation sites to these domains (1, 5, 6). Also, in an elegant series of experiments, Isenring, Forbush and collaborators have used chimeras and point mutations to explore the role of various regions of NKCC1 in ion translocation and inhibitor binding (7-9). These studies show that the predicted central transmembrane region codes for the information associated with the ion transport properties of NKCC1 while the cytosolic N- and C-termini appear to have little effect on these parameters. These intracellular regions instead may be mainly involved in the regulation of transport activity.

In a recent series of experiments we have tested the topological predictions for the transmembrane region of NKCC1 from various theoretical methods. The prediction methods considered included the classical hydropathy analysis of Kyte and Doolittle (10), the PHDhtm neural network method developed by Rost et al. (11) (http://dodo.cpmc.columbia.edu/pp/predictprotein.html), the TopPred program of von Heijne and collaborators (12) (http://www.biokemi.su.se/~server/toppred2/toppredServer.cgi) and a hidden Markov model recently proposed by Tusnady and Simon (13) (http://www.enzim.hu/hmmtop/). Although the predictions of these methods

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agreed on the general positions of most (but not all) putative membrane spanning domains, there were significant differences in the total number (predictions varied from 10 to 12) and the orientation of individual membrane spanners.

Our approach was to express putative membrane spanning regions of the rat NKCC1 in vitro in the presence of canine pancreatic microsomes. The expression vectors we used were developed by Bamberg and Sachs (14) who generously provided them to us. These vectors append a portion of the β-subunit of the H^+/K^+-ATPase containing multiple glycosylation sites onto the C-terminal end of cotransporter sequence being tested. Since the glycosidation of the β-subunit can be readily detected by a shift in the apparent molecular weight of the resulting recombinant peptide, the integration and orientation of the putative membrane spanning region in the microsome can be easily determined by SDS-PAGE electrophoresis and [^{35}S]-methionine autoradiography. The system consists of two vectors which allow the testing of either the "signal anchor" or "stop transfer" activity, respectively, of the putative membrane spanning domain. Briefly stated our results favor a topology scheme of 12 transmembrane spanners that is most closely predicted by the Kyte-Doolittle (10) and neural network methods (11).

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