SA6

The role of NHERF in the regulation of NHE3 and Npt2 in the renal proximal tubule

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Sodium–hydrogen exchanger regulatory factor (NHERF-1) was initially identified as a renal proximal tubule brush border membrane (BBM) adaptor protein that binds to NHE3 (sodium–hydrogen exchanger 3) and ezrin thereby facilitating the formation of a macromolecular signal complex that promotes cAMP-dependent protein kinase (PKA) phosphorylation and inhibition of NHE3 activity. A second member of this family, NHERF-2, has also been cloned. Although sharing a similar modular structure including two tandem highly conserved PDZ (PSD-95/Dlg/ZO-1) protein interaction domains and an ezrin–radixin–moesin–merlin binding domain, NHERF-1 and NHERF-2 are different gene products, have overlapping but not identical protein targets, and may mediate different cell functions. Rat renal proximal tubules express only NHERF-1 whereas human and mouse proximal tubules express both isoforms. Although both NHERF-1 and NHERF-2 have the capacity to bind the apical membrane transporters NHE3 and the sodium-dependent phosphate co-transporter IIa (Npt2) using *in vitro* assays, the precise role of each of the NHERF isoforms in intact tissue is unknown. To define the physiological role for NHERF in the kidney and to differentiate the role of NHERF-1 from NHERF-2, we generated an NHERF-1 null mouse line using homologous recombination techniques. In NHERF-1 (−/−) mice, NHERF-1 was absent from all tissue but the expression and cellular location of NHERF-2 was only minimally different from wild-type animals. Serum electrolytes were normal in the NHERF-1 (−/−) mice except for a modest decrease in the serum phosphate concentration. The glomerular filtration rate and systemic arterial blood pressure was not different from wild-type mice. The morphology of the kidney of the null mice was also normal and the distribution of actin and ezrin was similar to wild-type animals. In wild-type animals, NHERF-1 was expressed predominantly in the brush border membrane (BBM) while NHEFR-2 was expressed at the base of the microvillus membrane. In wild-type animals, however, some co-localization of the two NHERF isoforms was identified using an array of antibodies. Immunoprecipitation experiments in wild-type mice indicated that NHERF-1 and NHERF-2 formed heterodimers, and that each isoforms co-immunopreciptitated NHE3 and Npt2. In null mice, NHE3 was appropriately targeted to BBM of renal proximal tubule cells and BBM abundance of NHE3 was the same as in wild-type animals. Immunoprecipitation of NHERF-2 from the kidney of null animals resulted in recovery of NHE3. Nonetheless, as compared to wild-type animals, there was a significant deficit in cAMP-associated inhibition of NHE3 activity as assayed in isolated BBM from NHERF-1 (−/−) mice despite the presence of functionally active PKA. This clearly demonstrates that while NHERF-2 showed equivalent activity as NHERF-1 in cAMP-mediated inhibition of NHE3 in model PS120 fibroblasts, NHERF-2 could not substitute for the loss of NHERF-1 in the kidney of mutant mice. As compared to wild-type controls, NHERF-1 null mice demonstrated an increase in the urinary excretion of phosphate while on a regular or low phosphate diet. BBM staining for Npt2 was decreased and BBM abundance of Npt2 was significantly less than wild-type animals. Immunoprecipitation of NHERF-2 from null mice kidneys did not result in recovery of Npt2 suggesting that in the mouse, only NHERF-1 interacts with Npt2. In NHERF-1 (−/−) mice, not only was BBM Npt2 abundance decreased, Npt2 appeared to be mistargeted to intracellular vesicular structures. Using confocal microscopy, Npt2 was demonstrated to accumulate in the Golgi complex of proximal tubule cells. These studies demonstrate a unique subcellular distribution of NHERF-1 and NHERF-2 in the proximal tubule of the mouse kidney. While each protein has the capacity to bind to the renal apical membrane transporters NHE3 and Npt2, at least in *in vitro* assays, only NHERF-1 mediates PKA regulated inhibition of NHE3 activity. The role of NHERF-2 is unknown but speculate that it may be required for cellular targeting of NHE3 or its retention in the apical membrane. The NHERF-1 (−/−) mice also demonstrate an increase in the urinary excretion of phosphate associated with a decrease in Npt2 expression in BBM and the accumulation of Npt2 in the Golgi. Thus, NHERF-1 but not NHERF-2 is required for the targeting and/or apical retention of Npt2 in the renal proximal tubule of the mouse. On the other hand, there appears to be little direct interaction between Npt2 and NHERF-2 proteins. Since their initial description, NHERF-1 and NHERF-1 have been found to interact with 30 or more target proteins including other transport proteins and ion channels in addition to NHE3 and Npt2, hormone receptors, signalling proteins, structural elements, and transcription factors. The NHERF-1 (−/−) mouse and the subsequent development of the NHERF-2 (−/−) mouse should prove to be valuable models for study of the integrated function of PDZ proteins in the kidney.

SA7

Compensatory mechanisms in the NHE3 Na^+/H^+ exchanger knockout model of congenital diarrhoea

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Gene-targeted mice lacking the NHE3 Na^+/H^+ exchanger, which is expressed on apical membranes of small intestinal and colonic epithelial cells, have a severe intestinal absorptive defect and are currently the only animal model of congenital diarrhoea. This phenotype in the NHE3 knockout mouse, and the absence of a similar phenotype in mice lacking NHE2, which is also expressed on apical membranes throughout the intestinal tract, indicates that NHE3 is the major Na^+/H^+ exchanger that functions in concert with apical Cl^-/HCO_3^- exchange to mediate electroneutral absorption of NaCl from the lumen of the gut. The epithelial Na^+ channel (ENaC) and colonic H^+,K^+-ATPase were upregulated in the colon of NHE3 null mutants, suggesting that the activity of a coupled system consisting of ENaC, the apical K^+ channel, and the colonic H^+,K^+-ATPase, which together mediate Na^+/H^+ exchange across the apical membrane, is the major mechanism that compensates for the loss of NHE3 in the colon. To test the role of the colonic H^+,K^+-ATPase in this system, ENaC activity in the colon of wild-type and colonic H^+,K^+-ATPase knockout mice was examined during dietary Na^+ depletion, which increases serum aldosterone and shifts the mechanism of Na^+ recovery to electrogenic absorption via ENaC. ENaC activity was sharply depressed in the colonic H^+,K^+-ATPase knockout, demonstrating that the H^+,K^+-ATPase is needed for maximum Na^+ absorption via ENaC. To identify

molecular changes underlying compensatory mechanisms activated in the small intestine, cDNA microarrays and Northern blot analyses were used to compare global mRNA expression patterns in small intestine of NHE3-deficient and wild-type mice. Among the genes identified were members of the RegIII family of growth factors, which may contribute to the increased absorptive area in the NHE3 knockout intestine, and a large number of interferon-γ responsive genes. The latter finding is of particular interest as interferon-γ has been shown to reduce secretion in both cultured intestinal epithelial cells and in the NHE3-deficient small intestine via a mechanism involving down-regulation of the apical CFTR Cl^- channel and the basolateral Na^+–K^+–$2Cl^-$ cotransporter. Serum interferon-γ was elevated fivefold in NHE3-deficient mice; however, there was no evidence of inflammation, and unlike conditions such as inflammatory bowel disease, levels of other cytokines were unchanged. In addition, quantitative PCR analysis showed that upregulation of interferon-γ mRNA was localized to the small intestine and did not occur in colon, spleen, or kidney. These *in vivo* data suggest that elevated interferon-γ, produced by gut-associated lymphoid tissue in the small intestine, is part of a homeostatic mechanism that is activated in response to the intestinal absorptive defect in order to regulate the fluidity of the intestinal tract.

SA8

The CLCAs: proteins with ion channel, cell adhesion and tumour suppressor functions

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Chloride conductances that are sensitive to calcium (CaCC) are found in most cells and tissues. However, assigning a molecular identity to a particular conductance has proved challenging, not least because of the variety of observed current phenotypes. The CLCAs are a recently identified mammalian gene family that when heterologously expressed behave as chloride channels when exposed to calcium. They are widely distributed in both excitable and non-excitable tissues including epithelia, endothelia, smooth muscle, neurons and brain. The prototypical CLCA family member was isolated and subsequently cloned from bovine tracheal epithelium (Ran *et al.* 1992). When incorporated into planar lipid bilayers, the purified protein formed channels of approximately 25 pS, had a linear *I–V* relationship in symmetrical solutions and was inhibited by DIDS. Similar properties were found for the cloned protein when transiently expressed in *Xenopus* oocytes or COS-7 cells (Cunningham *et al.* 1995). It is now recognized that four CLCA family members are expressed in mammals, although this family does not seem to be represented in *C. elegans*, *Drosophila*, or birds (Gruber *et al.* 2000). When compared to calcium-sensitive chloride currents recorded from native cells, the heterologously expressed currents have many properties in common, including ion selectivity profile ($I^- > Cl^-$), sensitivity to several agents widely used as chloride channel blockers (e.g. DIDS, NPPB), and an outwardly rectified current–voltage relationship under whole-cell recording conditions. However, other features of heterologously expressed and/or purified CLCAs, such as the lack of a time dependent component to the whole-cell current, sensitivity to the reducing agent DTT, and relatively large single channel conductance suggest that by themselves they do not fully recapitulate the native channel/current phenotype. This may reflect a role for the CLCAs as subunits of a more complex multimer including other as yet unidentified or unrecognized polypeptides. Surprising evidence for such an interaction has recently been presented when current more typical of that found in vascular smooth muscle was reconstructed in HEK 293 cells by co-expression of mCLCA1 and the non-pore forming β subunit of the large conductance BK channel (Greenwood *et al.* 2002). However, the potential functions of members of the CLCA family are not confined to their channel properties; intriguingly, certain members of the CLCA family have also been demonstrated to function both as cell adhesion molecules, and as tumour suppressors. When expressed in an underlying substrate of endothelial cells, one bovine member of this family (LuECAM1, bCLCA2) mediates adherence of metastatic melanoma cells, an interaction that can be blocked by a monoclonal CLCA antibody (Goetz *et al.* 1996). Furthermore, a murine family member, mCLCA1 binds to β_4-integrin, activating downstream FAK/ERK signalling pathways (Abdel-Ghany *et al.* 2002). Additionally both hCLCA1 and hCLCA2 are down-regulated in colon and breast cancer (Gruber & Pauli 1999; Bustin *et al.* 2001), while heterologous expression of the murine homologues in tumour cells reduce colony formation in inoculated animals and promote apoptosis (Elble & Pauli, 2001). A role for the CLCAs is also emerging in the field of asthma research, where the asthma-associated cytokine IL-9 both promotes expression of hCLCA1 and mucus secretion in airway goblet cells (Toda *et al.* 2002). In this context, the localization of the murine homologue of hCLCA1 (mgob5, mCLCA3) on the membrane of mucus granules is highly consistent (Leverkoehne & Gruber, 2002). Given the diverse nature of potential functions of the CLCA family, considerable further studies will be required to determine whether the CLCAs form independent ionic channels, in addition to elucidating the interdependence of conductance, adhesion and tumour suppressor roles.

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SA9

Mechanism and regulation of CFTR-dependent epithelial Cl^- and HCO_3^- transport

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Cl^- absorption and HCO_3^- secretion are central functions of secretory epithelia. CFTR mediates and/or regulate these transport activities as evident from the aberrant Cl^- and HCO_3^- transport is cystic fibrosis (CF). However, the exact role of CFTR in Cl^- absorption and HCO_3^- secretion and its regulation are not well understood. In a previous work we showed that CFTR stimulates Cl^--dependent HCO_3^- transport and this activity is aberrant in CFTR mutants associated with CF. The identity of the protein mediating HCO_3^- transport and the mechanism of this stimulation are obscure. Characterized net Cl^- and HCO_3^- fluxes and Cl^- and HCO_3^- currents in HEK293 cells and *Xenopus* oocytes expressing WT-CFTR show that the bulk of the CFTR-supported Cl^- and HCO_3^- transport are coupled. However, HCO_3^- current could not account for net CFTR-stimulated HCO_3^- transport. In search of this mechanism we found that CFTR does not regulate the activity of any of the anion

exchangers, AE1, AE2, AE3 or AE4. By contrast, CFTR markedly activated Cl^- and HCO_3^- by all members of the luminal SLC26 transporters tested DRA, Pendrin and SLC26A6. Current and voltage measurements revealed that the all SLC26 transporters are electrogenic Cl^- and HCO_3^- transporters with isoform specific stoichiometry. DRA activity occurred at a stoichiometry of $Cl^-/HCO_3^- \geqslant 2$. By contrast SLC26A6 activity occurred at stoichiometry of $HCO_3^-/Cl^- \geqslant 2$. DRA/CLD activity was responsible for the Cl^- and HCO_3^- transport stimulated by CFTR, since mutations in DRA/CLD that are associated with congenital chloride diarrhoea (CLD) had no Cl^-/HCO_3^- exchange activity in either the presence or absence of CFTR. To identify domains in CFTR responsible for regulation of the SLC26 transporters the activity of ΔR-CFTR and ΔC-CFTR from which the PDZ ligand was deleted were examined. ΔR-CFTR was unable to activate DRA/CLD. ΔC-CFTR at low expression levels could not be co-immunoprecipitated with DRA/CLD or activate the transporter. However, at high expression levels ΔC-CFTR and ΔC-DRA interacted with each other and ΔC-CFTR activated Cl^- and HCO_3^- transport by ΔC-DRA, indicating multiple interacting domains between CFTR and the SLC26 transporters. These findings provide a molecular mechanism for epithelial HCO_3^- transport. Expression of one SLC26 transporter and its activation by CFTR will result in electrogenic transport, whereas expression of two SLC26 transporters with opposite stoichiometry in the same membrane domain will result in electroneutral transport. Furthermore, these findings are relevant to the CF-associated aberrant Cl^- and HCO_3^- transport, and reveal a new function of CFTR with implications for CF and congenital chloride diarrhoea.