Na^+-H^+ exchange regulatory factors NHERF-1 and NHERF-2: roles in albumin endocytosis in the proximal tubule

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A key function of the renal proximal tubules is to constitutively reabsorb the several grams of albumin that pass across the glomerular barrier per day. This occurs *via* receptor-mediated endocytosis and requires the formation of a macromolecular complex that involves the scavenger receptor megalin, the Cl^- channel ClC-5 and the Na^+-H^+ exchanger isoform 3 (NHE3). The exact composition of the complex and role of these proteins remains, however, unclear. Patients with Dent's disease (genetic defects in ClC-5) and ClC-5 knockout mice have persistent proteinuria, demonstrating an obligate role for ClC-5 in albumin uptake. We have previously shown that the cytosolic C-terminus of ClC-5 interacts with cofilin and Nedd4-2 to regulate albumin uptake (Hryciw *et al.*, 2003; Hryciw *et al.*, 2004). As ClC-5 contains a potential C-terminal PDZ binding motif, we investigated if ClC-5 interacted with the NHERF-1/2 PDZ scaffolds and the role of this interaction in albumin uptake.

For this study, we used the widely accepted model of renal albumin uptake, the opossum kidney (OK) proximal tubule cell line. Western blotting was used to confirm that these cells expressed NHERF1/2 and electron microscopy was used to confirm subcellular localisation. Co-immunoprecipitation was used to determine whether NHERF1/2 bound to ClC-5 in OK cell lysates. GST-fusion proteins were used to determine which PDZ domain of NHERF-2 bound to ClC-5 and maltose-binding fusion proteins used to identify the binding site for NHERF-2 on the C-terminus of ClC-5. Endogenous NHERF-2 and NHERF-1 were silenced by the use of siRNA transfection plasmids and albumin uptake was measured by standard fluorescent methods. Cell surface biotinylation was also used to monitor changes in ClC-5 under these conditions.

Using electron microscopy we demonstrated that OK cells expressed both NHERF-1 and NHERF-2 with NHERF-1 primarily at the microvilli while NHERF-2 was on intracellular membranes consistent with sites of albumin endocytosis. Co-immunoprecipitation in OK cell lysates showed that NHERF-2 but not NHERF-1 bound to ClC-5 *in vivo*. GST-pulldowns revealed that the C-terminus of ClC-5 bound to NHERF-2 and that this interaction occurred via PDZ-2 of NHERF-2. Further, *in vitro* experiments with maltose-binding protein fusions confirmed that NHERF-2 bound to an internal site on the C-terminus of ClC-5 and not to the terminal PDZ binding motif of ClC-5. Functional analysis of this interaction demonstrated that silencing of NHERF-2 significantly reduced albumin uptake, accompanied by a reduction in cell surface expression of ClC-5. This suggests that NHERF-2 plays a key scaffolding role in the endocytic complex. In contrast, when NHERF-1 was silenced, there was an increase in albumin uptake paralleled by an increase in surface levels of ClC-5.

Our data are consistent with a model in which the efficacy of albumin uptake is dependent on the availability of the components of the macromolecular complex. NHERF-1 is typically responsible for restricting the lateral mobility of NHE3 in the membrane and we propose that knockdown of NHERF-1 may increase the availability of NHE3 to the endocytic complex, resulting in more ClC-5 being recruited into the complex thereby increasing albumin uptake. NHERF-2, on the other hand, plays an integral role in the endocytic complex itself.

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