Protein complexes - organising transport and function in space and time

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Introduction: Recent progress in understanding how membrane proteins function and in defining the mechanisms of their regulation indicate that they often exist in highly dynamic macromolecular complexes. These complexes may consist of multiple receptors and ion channels/transporters as well as effector molecules and scaffold proteins. The dynamics of the entire complex provides spatial constraint, defined specificity and maximized functional capacity. In this context, the renal proximal tubule epithelial cell is a case in point. These cells are responsible for the recovery of filtered solutes and proteins from the primary urine and contain a myriad of transporters and receptors to carry out these functions. Our studies over the last decade have focussed on the receptor mediated uptake of albumin by the proximal tubule. This process is dependent on the coordinated regulation of the scavenger receptor megalin/cubulin, the Na^+-H^+ exchanger isoform 3 (NHE3), the vacuolar H^+-ATPase and the Cl^- channel ClC-5 as well as a number of accessory proteins including the PSD-95/Dlg/ZO-1 (PDZ) scaffold proteins, Na^+-H^+ exchanger regulatory 1 and -2 (NHERF1/2). We have previously shown that ClC-5 binds to NHERF-2, an interaction that can regulate albumin uptake. It has been known for some time that megalin has a canonical C-terminal PDZ binding motif. The purpose of the current study was to determine if megalin could interact with NHERF1/2.

Methods: We investigated the interaction between megalin and NHERF1/2 *in vitro* and *in vivo* using standard pull-down and co-immunoprecipiation experiments. We then determined the motifs required for the interactions using inhibitory peptides. The functional role was established using standard fluorescent albumin uptake and cell surface biotinylation techniques in the OK proximal tubule cell culture model.

Results: Co-immunoprecititations from kidney lysate demonstrated that megalin could bind to both NHERF-1 and NHERF-2. Interestingly, this interaction did not occur *via* the C-terminal PDZ binding motif, however, peptides designed against a known internal PDZ binding motif prevented megalin binding to the NHERF-1 and -2. NHERF-1/2 have 2 PDZ modules and using GST fusion proteins we found that megalin bound to the PDZ2 module of NHERF-1/2. In the case of NHERF-2, megalin also interacted with the C-terminal ezrin binding domain. In order to prove conclusively that there was a direct interaction between the two proteins, MBP-fusion proteins of the C-terminus of megalin were incubated *in vitro* with either GST-NHERF-1 or NHERF-2. The resulting complexes were then pulled down and subsequent Western blots confirmed the direct interaction. We next investigated if NHERF-2 could form a complex with megalin and ClC-5 *in vitro*. We found that the megalin C-terminal peptide could only associate with ClC-5 in the presence of NHERF-2, a result that confirmed the central role of NHERF-2 as a scaffold for the 2 proteins. In terms of functional relevance, we found that silencing NHERF-2 with siRNA did reduce albumin uptake and the level of interaction of megalin with ClC-5.

Conclusion: NHERF-1 and NHERF-2 have different spatial localizations in the proximal tubule epithelial cell; NHERF-1 is located primarily at the microvillus, while NHERF-2 is in the intravillar cleft, the site of albumin uptake. The role of these scaffolds is to anchor membrane proteins and restrict their lateral mobility as well as recruiting specific signalling molecules. The data presented here provide the first direct demonstration that megalin and ClC-5 exist in a complex and indicate the molecular basis for this interaction. This is fundamental information required to understand how the myriad of functions and regulatory pathways are compartmentalized in an epithelial cell.