Na⁺-H⁺ Exchanger Regulatory Factor 1 (NHERF1) PDZ Scaffold Binds an Internal Binding Site in the Scavenger Receptor Megalin

Craig Slattery¹, Kayte A. Jenkin², Aven Lee³, Anna C. Simcocks², Andrew J. McAinch², Philip Poronnik⁴ and Deanne H. Hryciw²

¹UCD School of Biomolecular and Biomedical Science, UCD Conway Institute, University College Dublin, Dublin, Ireland, ²School of Biomedical and Health Sciences, Victoria University, Melbourne, Australia, ³University of Queensland Centre for Clinical Research (UQCCR), Brisbane, Australia, ⁴Health Innovations Research Institute, School of Medical Sciences, RMIT University, Melbourne, Australia

Key Words

Megalin • NHERF1 • PDZ binding motif • Albumin endocytosis

Abstract

The scavenger receptor megalin binds to albumin in the microvilli of the renal proximal tubule, and transports the ligand to the intravillar cleft for processing by endocytosis. Albumin endocytosis in the proximal tubule is regulated by protein complexes containing a number of transmembrane and accessory proteins including PDZ scaffolds such as NHERF1 and NHERF2. PDZ scaffold proteins bind to class I PDZ binding motifs (S/T-X-Φ) in the extreme C-terminus of targets. Megalin contains a functional PDZ binding motif (SDV) in its distal terminus, however a potential interaction with the NHERF proteins has not been investigated. As megalin associates with NHE3 in the microvilli and NHE3 is tethered to the intravillar cleft via its interaction with NHERF1, we investigated if there is a direct interaction between megalin and NHERF1 in renal proximal tubule cells. Using confocal microscopy we determined that megalin and NHERF1 co-localise in the apical region in proximal tubule cells. Immunoprecipitation experiments performed using rat kidney lysate indicated that megalin bound NHERF1 *in vivo*. Using

fusion proteins and peptides, we determined that PDZ2 of NHERF1 bound to megalin and that this interaction was via the C-terminus of megalin directly and in the absence of any accessory protein. We next investigated which domain in megalin was regulating this interaction. Using GST fusion proteins we determined that the loss of the most distal C-terminus of megalin containing the PDZ binding motif (SDV) did not alter its ability to bind to NHERF1. Significantly, we then identified an internal NHERF binding domain in the C-terminus of megalin. Using peptide studies we were able to demonstrate that NHERF1 bound to an internal PDZ binding motif in megalin and that a loss of a single threonine residue abolished the interaction between megalin and NHERF1. Finally, in proximal tubule cells, silencing NHERF1 increased megalin expression. Therefore, we have identified a novel protein interaction in proximal tubule cells and specifically identified a new internal PDZ binding motif in the C-terminus of megalin.

Copyright © 2011 S. Karger AG, Basel

Introduction

The low-density lipoprotein (LDL) receptor gene family contains a number of cell surface receptors that

are required for many functions including endocytosis and cell signalling [1]. The scavenger receptor megalin, a member of the LDL family, is localised primarily in the apical membrane of epithelial cells, the most abundant expression in proximal tubule cells. In addition, megalin has been localised to a variety of epithelia including epididymis, type II pneumocytes, yolk sac epithelia, labyrinth epithelium of the ear, ciliary body of the eye, lacrimal gland, thyroid, parathyroid, ependymal and choroid plexus in brain [2]. In proximal tubule cells, megalin is the most abundant endocytic receptor and it actively reabsorbs a number of proteins from the glomerular filtrate including vitamin D binding protein [3], haemoglobin [4], insulin [5] and albumin [6]. Importantly, loss of megalin leads to proteinuria associated with a number of renal diseases [7].

Albumin binds to megalin in the microvilli, and is translocated to the intravillar cleft in proximal tubule cells [1]. Subsequent endocytosis of albumin by the proximal tubule is a highly regulated process that is dependent on a number of protein-protein interactions [8]. Specifically, albumin endocytosis requires the formation of a large protein complex in the intravillar cleft containing megalin, the Na⁺-H⁺ exchanger isoform 3 (NHE3), the vacuolar H⁺-ATPase (v-H⁺-ATPase) and the Cl⁻ channel CIC-5 [7, 9, 10]. This complex is likely to include accessory proteins such as the scaffold PSD-95/Dlg/ZO-1 (PDZ) domain proteins Na⁺-H⁺ exchanger regulatory 1 (NHERF1) and Na⁺-H⁺ exchanger regulatory 2 (NHERF2) [9]. These scaffolds bind to PDZ binding motifs (S/T-X-Φ) located in the C-terminus of target proteins, and are thought to play a role in nucleating the protein complex required for albumin endocytosis.

Previous studies have characterised the interactions between NHERF scaffolds and members of the albumin endocytic complex. NHERF1 is localised to the microvilli where it tethers NHE3 to this domain [11], while NHERF2 nucleates the endocytic complex in the intravillar cleft via interactions with NHE3 and CIC-5 [9, 12, 13]. Silencing of NHERF1 increases albumin endocytosis and silencing NHERF2 decreases in albumin endocytosis [9], presumably via an increase and decrease in cell surface availability of CIC-5, respectively [9]. Previously we have hypothesised that the loss of NHERF1 would increase the mobility of NHE3 from the microvilli, resulting in an increased endocytic complex formation in the intravillar cleft [9]. However, as the large C-terminus of megalin contains a PDZ binding motif which has been shown to bind PDZ domain containing proteins MAGI-1 and PSD-95 [14, 15], the role for NHERF1 may be via a direct

association with the scavenger receptor. Given the importance of megalin in regulating albumin uptake, and specifically its ability to translocate albumin to the intravillar cleft [1], the current study was undertaken to determine whether NHERF1 was a direct binding partner for megalin. Further, we aim to determine the binding site in the C-terminus of megalin that could mediate any interaction.

Materials and Methods

Antibodies and immunofluorescence staining

Proximal tubular Opossum Kidney (OK) cells were maintained as described [9]. OK cells were incubated with rabbit anti-megalin (Santa Cruz) and monoclonal anti-NHERF1 (Santa Cruz) and a secondary anti-rabbit antibody conjugated to FITC (Sigma) and an anti-mouse antibody conjugated to TRITC (Sigma). Cells were mounted in FluroSave (Calbiochem) and analysed by confocal microscopy [9].

Protein binding

Western blot analysis of megalin in proximal tubule cells

Antibody control experiments were performed with lysate from rat liver (megalin-negative: [16]), rat kidney and OK cell lysate. Lysates were harvested with lysis buffer described previously [9]. Equal amounts (50 µg) of the samples were resolved on a 4-15% SDS-PAGE gel, and analysed by Western blot with anti-megalin antibody (Santa Cruz). This commercially available antibody is derived against sequence in the C-terminus of rat, mouse and human megalin.

Expression of megalin in response to albumin was assessed as follow: confluent monolayers of OK cells were incubated in serum free media for 48 hr at 37°C, then incubated in the presence or absence of 5 mg/ml albumin for another 48 hours; cell lysates were harvested with lysis buffer described previously [9], and equal amounts (50 µg) were analysed by Western blot with anti-megalin and control anti-β-actin (Sigma-Aldrich).

In vivo interaction

Pre-cleared rat kidney lysate (1 µg), harvested with lysis buffer described previously [9] was incubated with anti-megalin or anti-NHERF1. The complex was immunoprecipitated with Protein-A agarose [9] and the bound proteins analysed by Western blot using anti-megalin or anti-NHERF1.

In vitro interaction

GST-fusion proteins containing the full length NHERF1 and its individual protein binding domains PDZ1, PDZ2 and C-terminus (Cterm) have been previously described [12, 13]. Briefly, the fusion proteins incorporated the following sequences: NHERF1 (amino acids 1 to 348), PDZ1:NHERF1 (amino acids 1 to 133), PDZ2:NHERF1 (amino acids 133 to 260) and C-term: NHERF1 (260 to 348). The C-terminus of megalin was subcloned into pMAL-p2E (New England Biolabs) and a

Fig. 1. Distribution of megalin and NHERF1 in polarized OK cells. X-Y confocal scan showing NHERF1 (red) and megalin (green) at the plane of the apical membrane (scale bars = 10 microns). Overlap in distribution is demonstrated in merged image (yellow). X-Z confocal scan showing that NHERF1 (red) is predominantly at the apical domain and megalin (green) is at the apical domain and cytosol in OK cells. Merger of images clearly indicates areas of co-localisation (yellow) between NHERF1 and megalin.

mutant that lacked PDZ binding motif (S4633X: MBP- Δ PDZ, respectively) was generated using site-directed mutagenesis as described previously [13]. GST and MBP fusion proteins were generated, and incubated with rat kidney lysate (1 mg) as described previously [9]. Briefly, 50 μ g of the different proteins were incubated with rat kidney lysate (1 mg) for 16 hours at 4°C. The lysate was harvested with lysis buffer described previously [9]. The complexes were added to glutathione sepharose (GST) or amylose (MBP) beads for 1 hour at 4°C [9]. The samples were centrifuged and washed 6 X 15 mins. The bound proteins were analysed by Western blot [9], using antibodies against megalin and NHERF1.

To determine if a C-terminal PDZ binding motif was required for a direct interaction between megalin and NHERF1, 1 μ g of MBP-Megalin, MBP-Megalin Δ PDZ or MBP control were incubated with amylose beads for 3 hrs at 4°C. GST-NHERF1 (4 ng) was then added to the complex and incubated for 1 hour at 4°C. The samples were centrifuged and washed 6 X 15 mins. The complexes were analysed by Western blot using the anti-NHERF1 antibody [9].

Next, to determine if an internal PDZ binding sequence was required for the interaction between megalin and NHERF1, biotinylated peptide sequences containing a conserved (in rat, mouse, human and opossum megalin) potential internal PDZ binding motif peptide were synthesised (Auspep) in addition to a negative control, where the threonine residue was substituted by an alanine residue (Fig. 5B) as this would abolish any interaction with the NHERF1 proteins [17]. 30 nmol of the different peptides were incubated with rat kidney lysate, harvested with lysis buffer described previously [9], for 16 hours at 4°C. The complexes were added to streptavidin coated beads for 1 hour at 4°C [18]. The samples were centrifuged and were washed 6 X 15 mins. Finally the bound proteins were analysed by Western blot analysis using the anti-NHERF1 antibody [9].

Transfections and siRNA

OK cells were transiently transfected with silencing RNA peptides against NHERF1 (Santa Cruz) and a control (random) peptide, following the manufacturers instructions. Protein lysate was harvested with lysis buffer described previously [9], and

50 μ g analysed by Western blot analysis [9], using antibodies against megalin and NHERF1 (Santa Cruz).

Quantification of Results and Statistical Analysis

Statistical analyses of the data were performed using a two-tailed Student's paired T-test or ANOVA where appropriate with a P of less than 0.05 considered significant.

Results

NHERF1 co-localises with megalin in proximal tubule cells

Confocal microscopy was used to determine if NHERF1 is located in similar domains as megalin in proximal tubule cells. OK cells were immunostained with megalin and NHERF1 antibodies (Fig. 1). Megalin immunoreactivity was observed around cell membranes with some cytosolic staining, similar to previously published data [19]. NHERF1 staining was prominent in the plasma membrane (Fig. 1). The merged image shows co-localisation between megalin and NHERF1 near the apical membrane (Fig. 1). X-Z confocal scan demonstrates the area of co-localisation between megalin and NHERF1 is predominantly at the apical membrane of the OK cells (Fig. 1).

Megalin expression is increased in the presence of albumin

In response to the presence of albumin, OK cells must increase their capacity for albumin endocytosis. Exposure to albumin has been previously shown to increase the levels of the proteins involved in albumin uptake including NHE3 [20], and cell surface levels of CIC-5 [21] and NHERF1 and NHERF2 [9] as well as pronounced changes in the underlying cytoskeleton [22].

Fig. 2. Megalin protein levels increase in response to albumin. A: Western blot analysis using the anti-megalin antibody with equal amounts (50 µg) of lysate from rat kidney and OK cells, detected a protein band at ~330 kDa that was not seen in negative (rat liver) control. B: In OK cells exposed to albumin (5 mg/ml) for 48 hours, megalin protein is increased, with loading control (β-actin) unchanged (n = 3). Densitometric analysis of band intensities shows megalin is increased in response to albumin challenge (n = 3, *p<0.01).

Here we investigated whether exposure to albumin also upregulated the receptor itself. First we confirmed the specificity of the anti-megalin antibody using Western blots on rat liver lysate (negative control; [16]), rat kidney (megalin positive) and OK cells. We were able to detect a band of predicted size (~330 kDa), which was not present in rat liver samples (Fig. 2A.).

We next exposed OK cells to albumin (5 mg/ml) for 48 hours and equal amounts of lysate were analysed by Western blot. There was no significant difference in the levels of β-actin between the control and albumin treated cells, however, there was a significant increase in the levels of megalin compared to levels in control ($161.9 \pm 26\%$ of control, n = 3, *P < 0.01; Fig. 2B). Thus, similar to NHE3, CIC-5 and NHERF1 and NHERF2, the presence of the ligand albumin, induces an upregulation of the receptor megalin. This demonstrates that albumin regulates the spatio temporal distributions of proteins in the endocytic macromolecular complex.

NHERF1 interacts with megalin

To assess whether NHERF1 binds megalin under endogenous conditions, co-immunoprecipitation experiments were performed on rat kidney lysate. First, NHERF1 antibody was used to immunoprecipitate protein complexes from rat kidney lysate and probed with the anti-megalin antibody. This revealed that megalin was present in the NHERF1 immunoprecipitates (Fig. 3A). In the reverse experiment, rat kidney lysate was probed with anti-megalin antibody and NHERF1 was detected in the immunoprecipitate (Fig. 3A). These results indicate that endogenous megalin and NHERF1 interact *in vivo*.

Identification of the protein binding domains in NHERF1 and megalin

To assess which PDZ modules and domains were required for the interaction between megalin and NHERF1, rat kidney lysate was incubated with GST fusion proteins containing the PDZ modules and

Fig. 3. Megalin binds to PDZ2 of NHERF1. A: Immunoprecipitation indicates that megalin bind NHERF1 *in vivo* and that NHERF1 binds megalin *in vivo*. B: PDZ domains (PDZ1 and PDZ2) and C-terminus (C-term) of NHERF1 were expressed as GST-fusion proteins. The fusion proteins incorporated the following sequences: NHERF1 (amino acids 1 to 348), PDZ1:NHERF1 (amino acids 1 to 133), PDZ2:NHERF1 (amino acids 133 to 260) C-term: NHERF1 (260 to 348). C: Megalin binds to GST-PDZ2 of NHERF1.

Fig. 4. The C-terminus of megalin binds to NHERF1 independently of its distal PDZ binding motif. A: Sequence alignment of megalin C-terminus from a number of species, with the PDZ binding motif highlighted. B: MBP, MBP-megalin and MBP-ΔPDZ (megalin C-terminus lacking PDZ binding domain) were incubated with rat kidney lysate. Both MBP-megalin and MBP-ΔPDZ bind to NHERF1. MBP, MBP-megalin and MBP-ΔPDZ were incubated with GST-NHERF1. Both MBP-megalin and MBP-ΔPDZ bind to GST-NHERF1, indicating megalin binds to NHERF1, independently of the PDZ binding motif or accessory proteins.

C-terminus of NHERF1 (Fig. 3B). These experiments showed that megalin binds the PDZ2 module of NHERF1 (Fig. 3C).

We next performed experiments using MBP-megalin and rat kidney lysate as well as using GST-NHERF1 constructs to demonstrate that the two proteins were capable of directly interacting in the absence of other proteins. Typically, binding to PDZ modules is mediated by the C-terminal three amino-acid PDZ binding motif, a motif that is highly conserved in megalin (Fig. 4A). We then made MBP-fusion proteins expressing the C-terminus of megalin and the C-terminus of megalin with the three terminal amino acids deleted (MBP-ΔPDZ similar to [9]). Experiments were first performed in which the MBP-megalin constructs were incubated with rat kidney lysate and probed for NHERF1 (Fig. 4B left panel) or incubated *in vitro* with GST-NHERF1 (Fig. 4B right panel). In both cases the C-terminus of megalin bound to NHERF1 and this was not dependent on the three terminal amino acids (SDV). This data clearly show that the C-terminus of megalin can bind to NHERF1 in the absence of any other proteins and that the interaction does not require the megalin C-terminus PDZ binding motif.

To specifically characterize the potential NHERF binding domain, we next used biotinylated peptides against a potential internal PDZ binding motif in megalin. The C-terminus of rat megalin contains 3 potential PDZ binding domains (Fig. 5A). Of these, only the first potential PDZ binding domain (residues 4498 to 4501 in the rat) and the most distal C-terminus (Fig. 4) were conserved across human, opossum, rat and mouse proteins (Fig. 5B). A peptide corresponding to the first potential PDZ binding domain from rat was used in a pull down analysis. These residues were able to bind to NHERF1 *in vitro* (Fig 5C). Further, incubation with a mutant peptide, where the threonine was mutated to an alanine (Fig. 5B), abolished the interaction between megalin and the NHERF1 protein. Therefore we have identified a novel internal NHERF1

Fig. 5. NHERF proteins bind to an internal PDZ binding site in megalin. A: The C-terminus of rat megalin with 3 potential PDZ binding domains underlined. B: Alignment of opossum, human, rat and mouse potential PDZ binding domains which corresponds to residues 4498-4501 in rat, with the potential PDZ domain underlined. Megalin wildtype (rat/mouse) and mutant PDZ binding domain peptide sequences from rat were synthesised and used in pull-down experiments. Mutant PDZ domain contains threonine at 4499 mutated to an alanine. C: Western blot analysis of the pull-down experiment with wildtype and mutant (see Fig. 5B) megalin peptides showed that NHERF1 binds to the megalin peptide, but not to the mutant peptide.

binding domain in megalin, which is similar to the internal binding domain between NHERF2 and CIC-5 [23].

Functional analysis of the interaction between megalin and NHERF1

Previously, we have demonstrated that silencing NHERF1 increases albumin endocytosis [9]. Using a

Fig. 6. Silencing NHERF1 increases megalin expression *in vitro*. A: NHERF1 protein levels were reduced in cells transfected with a NHERF1 siRNA peptide, but not in untransfected cells, or cells transfected with control siRNA. B: Densitometric analysis indicated that NHERF1 protein levels were significantly decreased (* $P < 0.05$, $n = 3$) when cells were transfected with NHERF1 siRNA peptide, but not untransfected cells or cells transfected with control siRNA. C: Megalin protein levels were increased in cells transfected with a NHERF1 siRNA peptide, but not in untransfected cells, or cells transfected with control siRNA. D: Densitometric analysis indicated that megalin protein levels were significantly increased (* $P < 0.05$, $n = 3$) when cells were transfected with NHERF1 siRNA peptide, but not untransfected cells or cells transfected with control siRNA.

siRNA peptide approach (Fig. 6A-6D), we demonstrated that silencing NHERF1 increased megalin expression in proximal tubule cells (Fig. 6C and 6D). Therefore, the increase in albumin endocytosis in proximal tubule cells where NHERF1 is silenced [9] is likely to be directly due to the increased protein levels of the receptor.

Discussion

It is now clear that the NHERF1 scaffold plays an essential role in regulating proximal tubular physiology via an interaction with the scavenger receptor megalin. Further, this interaction is via a novel internal PDZ binding domain in the intracellular C-terminus of megalin. In the proximal tubule, megalin is located in the microvilli where it binds albumin and then translocates to the endocytic vesicles of the intravillar cleft to facilitate uptake [1]. In addition, megalin associates with NHE3 in this domain [24]. We demonstrate that megalin co-localises with NHERF1 in a predominantly apical distribution. Further, we have shown a direct interaction between NHERF1 and megalin, in the absence of any accessory protein. Previous experiments by others have demonstrated that NHERF1 can tether NHE3 to the microvilli, which serves to limit the lateral mobility of the transporter within the membrane [11]. Our recent studies have also shown that silencing NHERF1 in OK cells increases albumin uptake [9] and increases megalin expression (data shown here). Therefore, our current hypothesis is that the availability of megalin in the endocytic complex is regulated via an interaction with NHERF1 in the microvilli. Further, similar to NHE3 [11], NHERF1 tethers megalin to the microvilli, and upon binding to albumin, megalin then translocates to

Fig. 7. Hypothetical role for NHERF1 in endocytosis in proximal tubule cells. Megalin, via an interaction with NHERF1, is associated with NHE3 in the microvilli. Albumin binds to megalin in the microvilli of proximal tubule cells. Receptor complex translocates to the intravillar cleft. At this site, the formation of a macromolecular complex containing megalin, NHE3 and ClC-5 occurs via NHERF2. The mature endosome is internalized which allows for the processing of the ligand.

the intravillar cleft to allow for the endocytic complex to form (Fig. 7). Therefore, we further hypothesise that the reduction in NHERF1 alters the mobility of megalin to increase its availability in the endocytic pool. Interestingly, our data presented here only shows a modest (but significant) reduction in NHERF1 expression in cells transiently transfected with NHERF1 siRNA. However, this level of reduction is sufficient to significantly increase megalin protein levels in these cells. This further supports the importance of the levels of NHERF1 protein in OK cells in maintaining the activity of the receptor, with minor variations in NHERF1 levels causing significant changes in megalin protein expression.

The formation of a macromolecular complex is essential for albumin endocytosis. Previous studies have demonstrated that albumin endocytosis plateaus at 0.04 mg/ml in OK cells [7], and increases in albumin concentrations reduce expression of other essential components of the endocytic complex (e.g. CIC-5: [21, 25]). Importantly, our data suggest a key role for NHERF1 in albumin endocytosis in proximal tubule cells via a direct interaction with its receptor megalin. Interestingly, only the PDZ2 domain of NHERF proteins is involved in binding to CIC-5 [9], NHE3 [12] and megalin (data shown here). This leaves the PDZ1 module free to potentially form homo- or heterodimers [26] thereby linking the different membrane proteins together in dynamic complexes in a spatio-temporal manner [11, 27, 28] (Fig. 7). In terms of protein complexes, it is interesting to note that, like CIC-5 [22] and NHE3 [9, 12], an internal PDZ binding motif occurs in the C-terminus of megalin (data shown here). This means that these proteins aggregate via NHERF scaffolds with their most distal C-terminal residues available for interactions with other PDZ domain proteins. Further, interactions with NHERF1 and megalin are predicted to be tethered to the cytoskeleton via the ezrin-actin interaction, which occurs via the C-terminus of NHERF1 [4]. Specifically, based on this data, and work from our group [9] and others [11], we hypothesise that in the microvilli, megalin and NHE3 are complexed via NHERF1 PDZ1 homodimers and tethered to the cytoskeleton via the ezrin binding domain in NHERF1. As this complex moves to a NHERF2/CIC-5 enriched pool, heterodimerisation between NHERF1 and NHERF2 PDZ1 modules could transition the assembly of endocytic complexes that are not tethered to the cytoskeleton (Fig. 7). Therefore, it is likely that the correct spatio-temporal interactions that give rise to a macromolecular endocytic protein complex [29] are nucleated by NHERF scaffolds and are essential for albumin endocytosis.

Previous studies have investigated the role of different protein binding domains in the C-terminus of megalin using a mini-receptor [30]. Using this protein, the loss of the distal PDZ binding motif does not alter the trafficking of megalin and endocytosis in distal tubule Mardin Darby Canine Kidney (MDCK) cells. However, these cells normally lack megalin and the key proteins required for the specific trafficking and activity of megalin may also not be present. For example, in the proximal tubule a loss of CIC-5, significantly reduces the expression of megalin [25]. It is somewhat surprising, given the identified roles for NHERF1 and NHERF2 in albumin uptake in OK cells [9], that NHERF1 and NHERF2 knockout mice show no signs of albuminuria [31]. Possible explanations for these observations may be that other NHERF PDZ scaffold proteins (including NHERF3 and NHERF4) in proximal tubule cells [32] may compensate for the loss of a NHERF1 and NHERF2 in the protein uptake pathway [31].

Nonetheless, members of the LDL receptor gene family, including megalin, play important roles in diverse cellular types and the NHERF proteins are widely expressed in a variety of cell types. Our present study showing megalin binds to the NHERF PDZ scaffolds provides important mechanistic insights into how this important scavenger receptor may be regulated in cells that express these proteins. Importantly, these findings will provide a framework for the development of future renal therapies.

Abbreviations

NHERF1 (Sodium–hydrogen exchanger regulatory factor isoform 1); NHERF2 (Sodium–hydrogen exchanger regulatory factor isoform 2); PDZ (PSD-95/ Discs large/ZO-1); LDL (Low-density lipoprotein).

Acknowledgements

The authors would like to thank Assoc Prof Darren Kelly, for donation of tissue samples. This work was supported by grants from the National Health and Medical Research Council of Australia (to P. P.) and Victoria University Researcher Development Grants Scheme (to D.H.H.). C.S is a Government of Ireland Research Fellow funded by the Irish Research Council for Science, Engineering and Technology. K.A.J. and A.C.S. have Australian Postgraduate Awards.

References

- Christensen EI, Birn H: Megalin and cubulin: synergistic endocytic receptors in renal proximal tubule. *Am J Physiol Renal Physiol* 2001;280:F562-F573.
- Saito A, Pietromonaco S, Loo AK, Farquhar MG: Complete cloning and sequencing of rat gp330/"megalin," a distinctive member of the low density lipoprotein receptor gene family. *Proc Natl Acad Sci USA* 1994;91:9725-9729.
- Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI, Willnow TE: An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D₃. *Cell* 1999;96:507-515.
- Young SP: Inhibition of endosome fusion in primary hepatocytes prevents asialoglycoprotein degradation but not uptake of transferrin iron demonstrating that intracellular iron release occurs from early endosomes. *FEBS Lett* 2000;466:135-138.
- Orlando RA, Rader K, Authier F, Yamazaki H, Posner BI, Bergeron JJ, Farquhar MG: Megalin is an endocytic receptor for insulin. *J Am Soc Nephrol* 1998;9:1759-1766.
- Cui S, Verroust PJ, Moestrup SK, Christensen EI: Megalin/gp330 mediates uptake of albumin in renal proximal tubule. *Am J Physiol* 1996;271:F900-907.
- Gekle M: Renal tubule albumin transport. *Ann Rev Physiol* 2005;67:573-594.
- Pollock CA, Poronnik P: Albumin transport and processing by the proximal tubule: physiology and pathophysiology. *Curr Opin Nephrol Hyperten* 2007;16:359-364.
- Hryciw DH, Ekberg J, Ferguson C, Lee A, Wang D, Parton RG, Pollock CA, Yun CC, Poronnik P: Regulation of albumin endocytosis by PSD95/Dlg/ZO-1 (PDZ) scaffolds. Interaction of Na⁺-H⁺ exchange regulatory factor-2 with CIC-5. *J Biol Chem* 2006;281:16068-16077.
- Sakamoto H, Sado Y, Naito I, Kwon TH, Inoue S, Endo K, Kawasaki M, Uchida S, Nielsen S, Sasaki S, Marumo F: Cellular and subcellular immunolocalization of CIC-5 channel in mouse kidney: colocalization with H⁺-ATPase. *Am J Physiol Renal Physiol* 1999;277:F957-F965.
- Cha B, Kenworthy A, Murtazina R, Donowitz M: The lateral mobility of NHE3 on the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. *J Cell Sci* 2004;117:3353-3365.
- Yun CC, Lamprecht G, Forster DV, Sidor A: NHE3 kinase A regulatory protein E3KARP binds the epithelial brush border Na⁺/H⁺ exchanger NHE3 and the cytoskeletal protein ezrin. *J Biol Chem* 1998;273:25856-25863.
- Yun CH, Oh S, Zizak M, Steplock D, Tsao S, Tse CM, Weinman EJ, Donowitz M: cAMP-mediated inhibition of the epithelial brush border Na⁺/H⁺ exchanger, NHE3, requires an associated regulatory protein. *Proc Natl Acad Sci USA* 1997;94:3010-3015.
- Larsson M, Hjalmar G, Sakwe AM, Engström A, Höglund AS, Larsson E, Robinson RC, Sundberg C, Rask L: Selective interaction of megalin with postsynaptic density-95 (PSD-95)-like membrane-associated guanylate kinase (MAGUK) proteins. *Biochem J* 2003;373:381-391.
- Patrie KM, Drescher AJ, Goyal M, Wiggins RC, Margolis B: The membrane-associated guanylate kinase protein MAGI-1 binds megalin and is present in glomerular podocytes. *J Am Soc Nephrol* 2001;12:667-677.
- Zheng G, Bachinsky DR, Stamenkovic I, Strickland DK, Brown D, Andres G, McCluskey RT: Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, gp330 and LRP/alpha 2MR, and the receptor-associated protein (RAP). *J Histochem Cytochem* 1994;42:531-542.
- Wang S, Raab RW, Schatz PJ, Guggino WB, Li M: Peptide binding consensus of the NHERF-PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR). *FEBS Lett* 1998;427:103-108.
- Short DB, Trotter KW, Reczek D, Kreda SM, Bretscher A, Boucher RC, Stutts MJ, Milgram SL: An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. *J Biol Chem* 1998;273:17979-179801.
- Lou X, McQuistan T, Orlando RA, Farquhar MG: GAIP, GIPC and Galphai3 are concentrated in endocytic compartments of proximal tubule cells: putative role in regulating megalin's function. *J Am Soc Nephrol* 2002;13:918-927.
- Kliscic J, Zhang J, Nief V, Reyes L, Moe OW, Ambuhl PM: Albumin regulates the Na⁺/H⁺ exchanger 3 in OKF cells. *J Am Soc Nephrol* 2003;14:3008-3016.
- Hryciw DH, Ekberg J, Lee A, Lensink IL, Kumar S, Guggino WB, Cook DI, Pollock CA, Poronnik P: Nedd4-2 functionally interacts with CIC-5: involvement in constitutive albumin endocytosis in proximal tubule cells. *J Biol Chem* 2004;279:54996-55007.
- Hryciw DH, Pollock CA, Poronnik P: PKCα mediated remodelling of the actin cytoskeleton is involved in constitutive albumin uptake by proximal tubule cells. *Am J Physiol Renal Physiol* 2005;288:F1227-F1235.
- Wellhauser L, Penmatsa H, Naren AP, Bear CE: Identification of the NHERF2 binding site for the Chloride/Proton Transporter CIC-5. *Biophys J* 2009;96:445a-446a.
- Biemesderfer D, DeGray B, Aronson PS: Active (9.6S) and inactive (21S) oligomers of NHE3 in microdomains of the renal brush border. *J Biol Chem* 2001;276:10161-10167.
- Piwon N, Gunther W, Schwake M, Bosl MR, Jentsch TJ: CIC-5 Cl⁻-channel disruption impairs endocytosis in a mouse model for Dent's disease. *Nature* 2000;408:369-373.
- Shenolikar S, Minkoff CM, Steplock DA, Evangelista C, Liu MZ, Weinman EJ: N-terminal PDZ domain is required for NHERF dimerization. *FEBS Lett* 2001;489:233-236.
- Lee A, Rayfield A, Hryciw DH, Ma TA, Wang D, Pow D, Broer S, Yun C, Poronnik P: Na⁺-H⁺ exchanger regulatory factor 1 is a PDZ scaffold for the astroglial glutamate transporter GLAST. *Glia* 2008;15:119-129.
- Naren AP, Cobb B, Li C, Roy K, Nelson D, Heda GD, Liao J, Kirk KL, Sorscher EJ, Hanrahan J, Clancy JP: A macromolecular complex of β2 adrenergic receptor, CFTR, and ezrin/radixin/moesin-binding phosphoprotein 50 is regulated by PKA. *Proc Natl Acad Sci USA* 2003;100:342-346.
- Hryciw DH, Ekberg J, Pollock CA, Poronnik P: CIC-5: A chloride channel with multiple roles in renal tubular albumin uptake. *Int J Biochem Cell Biol* 2005;38:1036-1042.
- Takeda T, Yamazaki H, Farquhar MG: Identification of an Apical Sorting Determinant in the Cytoplasmic Tail of Megalin. *Am J Physiol Cell Physiol* 2003;284:C1105-1113.
- Cunningham R, Esmaili A, Brown E, Biswas RS, Murtazina R, Donowitz M, Dijkman HB, van der Vlag J, Hogema BM, De Jonge HR, Shenolikar S, Wade JB, Weinman EJ: Urine electrolyte, mineral, and protein excretion in NHERF-2 and NHERF-1 null mice. *Am J Physiol Renal Physiol* 2008;294:F1001-1007.
- Donowitz M, Cha B, Zachos N, Brett CL, Sharma A, Tse CM, Li X: NHERF family and NHE3 regulation. *J Physiol* 2005;567:3-11.