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New Insights into the Roles of Megalin/LRP2 and the Regulation of its Functional Expression

María-Paz Marzolo[1,2,*] and Pamela Farfán[1,2]

[1] Laboratorio de Tráfico Intracelular y Señalización, Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas.
[2] Millenium Nucleus in Regenerative Biology (MINREB). Pontificia Universidad Católica de Chile.

ABSTRACT

Since the discovery of the low-density lipoprotein receptor (LDLR) and its association with familial hypercholesterolemia in the early 1980s, a family of structurally related proteins has been discovered that has apolipoprotein E as a common ligand, and the broad functions of its members have been described. LRP2, or megalin, is a member of the LDLR family and was initially called gp330. Megalin is an endocytic receptor expressed on the apical surface of several epithelial cells that internalizes a variety of ligands including nutrients, hormones and their carrier proteins, signaling molecules, morphogens, and extracellular matrix proteins. Once internalized, these ligands are directed to the lysosomal degradation pathway or transported by transcytosis from one side of the cell to the opposite membrane. The availability of megalin at the cell surface is controlled by several regulatory mechanisms, including the phosphorylation of its cytoplasmic domain by GSK3, the proteolysis of the extracellular domain at the cell surface (shedding), the subsequent intramembrane proteolysis of the transmembrane domain by the gamma-secretase complex, and exosome secretion. Based on the important roles of its ligands and its tissue expression pattern, megalin has been recognized as an important component of many pathological conditions, including diabetic nephropathy, Lowe syndrome, Dent disease, Alzheimer's disease (AD) and gallstone disease. In addition, the expression of megalin and some of its ligands in the central and peripheral nervous system suggests a role for this receptor in neural regeneration processes. Despite its obvious importance, the regulation of megalin expression is poorly understood. In this review, we describe the functions of megalin and its association with certain pathological conditions as well as the current understanding of the mechanisms that underlie the control of megalin expression.

Key words: Alzheimer's Disease, cubilin, diabetic nephropathy, FXR, gallstone disease, GSK3, Lowe syndrome, megalin, PPAR, RAP, regeneration.

1. MEGALIN STRUCTURE AND INTRACELLULAR TRAFFICKING

Megalin/LRP2, as its name indicates, is a giant membrane glycoprotein of 600 kDa that belongs to the low-density lipoprotein receptor (LDLR) protein family. The members of this family are type I membrane proteins that act mainly at the cell surface, where they efficiently bind and internalize several physiologically relevant molecules, such as the cholesterol carrier apolipoprotein E, which is a common ligand recognized by all LDLR receptors (Willnow et al., 1992; Hussain, 2001) (**Table I**). Megalin is composed of a large extracellular domain consisting of four cysteine-rich complement-type ligand binding repeats, responsible for ligand binding, that binds to the chaperone RAP (receptor associated protein) for its folding in the endoplasmic reticulum (ER) (Bu and Marzolo, 2000). The repeats are separated from each other by β-propeller domains (Saito et al., 1994), structured by repeats of YWTD flanked by EGF-like modules, that are generally important for the proper receptor folding in this family of proteins (Culi et al., 2004; Lighthouse et al., 2010) as well as for the dissociation of their ligands in the acidic endosomal compartment (Jeon et al., 2001) (**Fig. 1**). In addition, megalin contains one transmembrane domain that targets it to membrane domains rich in cholesterol and glycosphingolipids (Marzolo et al., 2003) and is also a substrate for the gamma-secretase complex (Zou Z et al., 2004; Biemesderfer, 2006).

Next to the transmembrane domain, human megalin has a cytoplasmic domain of 209 amino acid residues that contains several motifs that regulate receptor trafficking and function (**Fig. 2A**). Among these are three NPXY motifs that have been linked to LDLR and LRP1 internalization mediated by clathrin, recycling from the endosomal compartment to the plasma membrane and basolateral distribution (Bansal and Gierasch, 1991; Matter et al., 1992; Li et al., 2001; van Kerkhof et al., 2005; Donoso et al., 2009). However, the roles of these motifs have not been clearly defined for megalin/LRP2. In fact, there are evidences linking megalin endocytosis to non-clathrin mediated pathways involving trafficking proteins such as the small GTPase Arf6 (Wolff et al., 2008) and caveolin 1 (Bento-Abreu et al., 2009). Two cytoplasmic proline-rich sequences and a PDZ-binding motif have been implicated in the direct and indirect interaction of megalin with cytoskeletal and cytosolic scaffold and signaling proteins, such as GIPC/synectin, megalin-binding protein, ANKRA, myosin VI, SKIP, Disabled 2 (Dab2) and APPL1 (Rader et al., 2000; Patrie et al., 2001; Larsson et al., 2003; Petersen et al., 2003; Naccache et al., 2006; Erdmann et al., 2007). An unusual feature of megalin among the LRP family of receptors (with the exception of LRP5 and 6, which participate in Wnt/β catenin signaling (Zeng et al., 2005)), is that it is constitutively phosphorylated by GSK3 at a PPPSP motif, contained in a distal proline-rich motif of the cytoplasmic tail (Yuseff et al., 2007). This PPPSP motif is the most significant in terms of basal phosphorylation of the receptor, despite the presence of several other consensus phosphorylation sites for PKC, CK-II and PKA (**Fig. 2A**) and its function is related to the control of megalin recycling from the endosomes (Yuseff et al., 2007) (see section 4.3). Both the PPPSP motif and the PDZ-binding motif are very well conserved in megalin sequences from different species **(Fig. 2B)**.

* Corresponding author: María-Paz Marzolo, Laboratorio de Tráfico Intracelular y Señalización, Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda # 340, Santiago 6513492, Chile, Telephone: 56 2 6762112, Email: mmarzolo@bio.puc.cl

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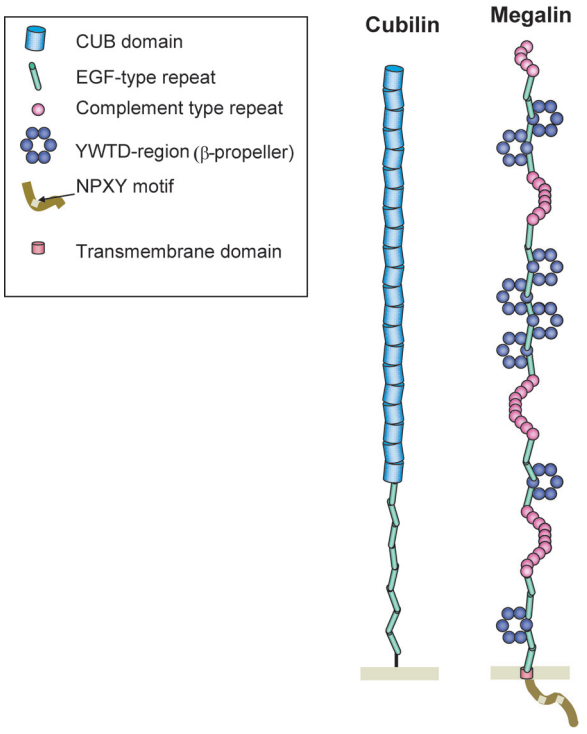

Figure 1. Schematic representation of megalin and its coreceptor cubilin: Megalin shares common structural motifs with other members of the LDLR family, including ligand-binding repeats, epidermal growth factor (EGF) repeats, YWTD spacer domains, a single transmembrane domain and a short cytoplasmic domain containing conserved endocytic motifs. Cubilin is a peripheral membrane protein with 27 cub domains and a sequence with amphipathic helical characteristics located in the amino-terminal region that has been implicated in cubilin plasma membrane anchoring/association. Cubilin requires megalin for its internalization.

The expression of megalin is principally restricted to epithelial cells, specifically at the apical surface (Cui et al., 1996; Morales et al., 1996; Willnow et al., 1996; Nielsen et al., 1998; Zheng et al., 1998; Hermo et al., 1999; Mizuta et al., 1999). Interestingly, despite the presence of some putative basolateral sorting motifs in the cytoplasmic domain of megalin, its apical localization depends mainly on sorting information present in this domain of the receptor because its addition to a reporter protein that lacks sorting information drives trafficking of the reporter to the apical surface of polarized epithelial cells (Marzolo et al., 2003) (**Fig. 3**). Under some specific ligand-triggered conditions, the apical localization of megalin can be transiently modified by transcytosis, a trafficking pathway that drives the internalized receptor to the opposite cell surface instead of allowing the receptor to recycle to the origin membrane domain. Megalin transcytosis has been detected in different epithelial cells and tissues, and is involved in the transport of internalized ligands such as thyroglobulin (Marino et al., 2000), Retinol Binding Protein (RBP) (Marino et al., 2001), leptin (Dietrich et al., 2008), sonic hedgehog (Shh) (Morales et al., 2006) and albumin (Russo et al., 2007) to the opposite plasma membrane and their release to the extracellular environment without degradation.

2. TISSUE DISTRIBUTION AND FUNCTIONS OF MEGALIN

Megalin is expressed in some tissues with cubilin (**Fig.1**), a peripheral membrane protein that requires megalin for its internalization. During mammalian development, megalin and cubilin are already expressed at the 8-cell stage at the time of segregation between the inner cell mass and the throphectoderm (Assemat et al., 2005a). After implantation, megalin and cubilin are expressed in the visceral endoderm (Kalantri et al, 2001) that becomes the visceral yolk sac, which in rodents has a central role in the nutrition of the embryo.

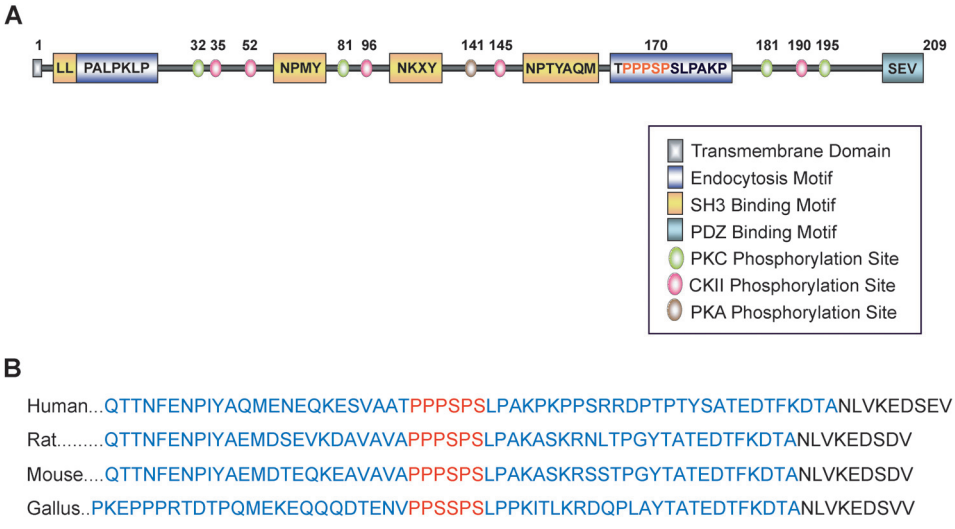

Figure 2. Characteristics of the megalin cytoplasmic domain that determine its trafficking and phosphorylation: a) The 209 amino acid residue cytoplasmic domain of megalin has several putative internalization motifs, including one dileucine and three NPxY motifs. In addition, it contains two proline-rich sequences, one PDZ terminal motif, several putative protein kinase C and casein kinase II phosphorylation motifs as well as one protein kinase A phosphorylation motif. Under basal conditions, these motifs contribute little to the phosphorylation of the megalin cytoplasmic domain. However, within the distal, proline-rich motif there is a PPPSP motif that is responsible for megalin phosphorylation by GSK3β (Yuseff et al., 2007). b) The PPPSP motif (red) is highly conserved in the sequences of megalin cytoplasmic domains from different species, suggesting that the regulation of megalin trafficking and surface expression by GSK3-mediated phosphorylation is a conserved mechanism.

This would allow the capture of nutrients, as apoAI-bearing lipoproteins, for the developing foetus (Assemat et al., 2005a).

2.1. Megalin in the central nervous system

Megalin is expressed early in the development of the neural tube, and it is present in the floor plate (McCarthy et al., 2002)). In the mice neuroepithelium megalin is found at E9.5 (Willnow et al., 1996). The receptor has a role in the formation of brain structures (Willnow et al., 1996; Spoelgen et al., 2005; Wicher et al., 2005). In the adult, megalin expression is restricted in the central nervous system (CNS) to the choroid plexus (Chun et al., 1999; Carro et al., 2005), the ependymal cells of the lateral ventricles (Gajera et al., 2010) and in spinal cord (Wicher et al., 2006). Megalin expression has been reported in the sensory organs, such as

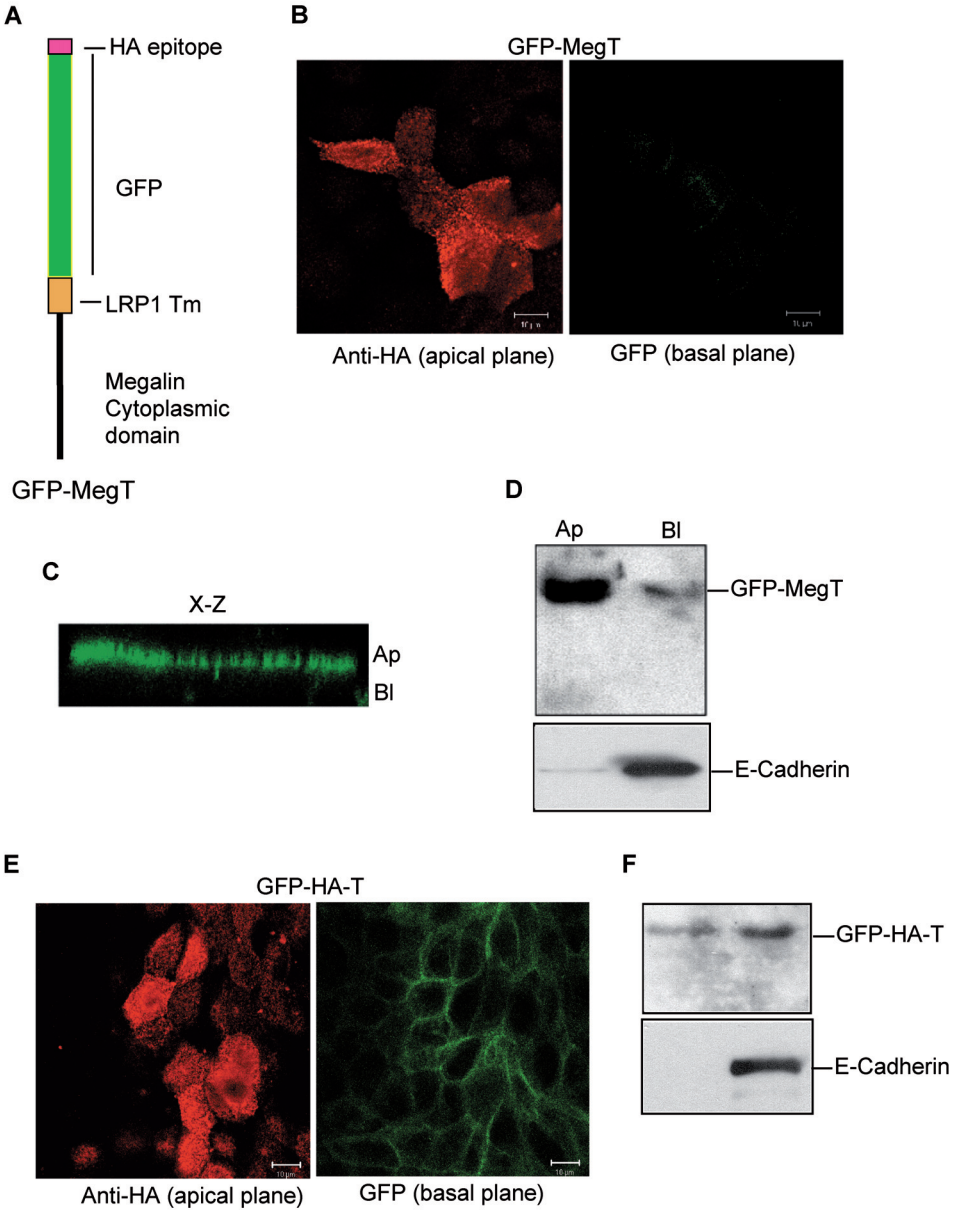

Figure 3. The cytoplasmic domain of megalin contains the information responsible for targeting the protein to the apical surface of polarized epithelial cells (Marzolo et al., 2003). a) Scheme of the chimeric protein consisting of green fluorescent protein (GFP) with an amino-terminal HA epitope, the transmembrane segment of the basolateral receptor LRP1 and the cytoplasmic domain of megalin (GFP-MegT). b) Confocal microscopy of non-permeabilized MDCK cells grown on filters expressing GFP-MegT, showing that it is expressed at the apical surface (detected with an anti-HA antibody), whereas at the basal plane, there is no basolateral GFP signal. c) The X-Z plane shows GFP staining at the apical surface. d) Domain-specific cell surface biotinylation of MDCK cells expressing GFP-MegT shows the apical distribution of the protein, detected with anti-megalin cytoplasmic tail antibody (Marzolo et al., 2003). The monolayer is correctly polarized as the endogenous protein E-Cadherin is localized basolaterally. As controls, the non-polarized distribution of the chimeric protein, GFP-HA-T, containing the cytoplasmic domain of haemaglutinin (with no sorting information) is shown by confocal microscopy (e) and cell surface biotinylation (f).

the inner ear (Mizuta et al., 1999; Konig et al., 2008) and the eye (Lundgren et al., 1997; Assemat et al., 2005a; Fisher and Howie, 2006). The presence of megalin has been also found in non-epithelial cells, such as a subpopulation of neural progenitors in the embryonic mouse spinal cord (Wicher et al., 2005), oligodendrocytes of the mouse spinal cord (Wicher et al., 2006), retinal ganglion cells (Fitzgerald et al., 2007) and cerebellar granule neurons (Ambjorn et al., 2008). To uncover the physiological functions of megalin, complete knock-out (KO) mice (Willnow et al., 1996) and several conditional KOs (Leheste et al., 2003; Rubera et al., 2004; Spoelgen et al., 2005; Gajera et al., 2010) have been generated. Mice completely deficient in megalin have a low survival rate (1/50) and die immediately after birth from respiratory insufficiency (Willnow et al., 1996). These animals exhibit a set of brain malformations known as holoprosencephaly that include forebrain fusion, a common ventricular system and lack of the olfactory bulb (Willnow et al., 1996). Conditional KO mice that lack megalin expression only in the brain show that this receptor has an important role in the development of the ventral telencephalon, which is due to its function as a receptor for signaling proteins and morphogens such as Shh and Bone Morphogenetic Protein 4 (BMP4) (McCarthy et al., 2002; Spoelgen et al., 2005). Megalin also controls BMP4 levels and activity in adult brain neurogenesis (Gajera et al., 2010).

Megalin expression in CNS and the role of several of its ligands in neuronal survival and regeneration have implicated this receptor as a likely mediator of CNS regeneration. Megalin is the receptor for metallothionein (Klassen et al., 2004; Wolff et al., 2006; Fitzgerald et al., 2007), which is neuroprotective and important for regeneration processes (Fitzgerald et al., 2007; Ambjorn et al., 2008; Chung et al., 2008; Pedersen et al., 2009). Cystatin C (Kaseda et al., 2007), clusterin/ApoJ (Bonnard et al., 1997; Min et al., 2003) and apolipoprotein E (White et al., 2001) are other megalin ligands with regenerative and neuroprotective functions in the CNS, but their requirement for megalin as a receptor in regeneration has not been demonstrated. Where and how megalin acts in the adult CNS is still open to debate and requires further study.

Recently megalin has been implicated in the regeneration of the peripheral nervous system because the receptor is responsible for the internalization of transthyretin (Fleming et al., 2009a), a plasmatic and cerebrospinal fluid carrier protein for thyroxine (T4) and vitamin A (Monaco, 2000; Palha, 2002; Fleming et al., 2009b).

2.2. Role of megalin in the kidney and other organs

Megalin is also present in the kidney, lung and thyroid (Willnow et al., 1996; Lundgren et al., 1997; Marino et al., 1999), epydidimis (Hermo et al., 1999), mammary gland (Lundgren et al., 1997; Rowling et al., 2006) and gallbladder (Erranz et al., 2004; Tsaroucha et al., 2008).

In the kidney the receptor has a central and relevant role mediating the recapture of filtered molecules at the level of the proximal tubule (PT) (Leheste et al., 1999; Leheste et al., 2003). Mice that do not express megalin have low molecular weight (LMW) proteinuria, and lose lysosomal proteins in the urine, indicating a role for megalin in the recapture of these enzymes as well as in the biogenesis of lysosomes of the PT cells (Nielsen et al., 2007). Megalin KO mice have important ultrastructural changes in the endosomal compartments of PT epithelial cells, including the absence of apical dense tubules, which correspond to the apical recycling compartment, and other endocytic structures, such as clathrin-coated pits and vesicles (Leheste et al., 1999). Recently, a new function has been discovered for megalin using a mouse strain (line 267) carrying a nonsense mutation in the extracellular domain of the receptor. These mice lose selenium in the urine and also have reduced selenium in brain; on a low-selenium diet, these mice exhibit movement coordination problems (Chiu-Ugalde et al., 2010). This phenotype is due to the role of megalin as a receptor of selenoprotein (Olson et al., 2008).

Other molecules that are bound and internalized by megalin in different tissues include the apolipoproteins apoE and clusterin/apoJ (Kounnas et al., 1995; Morales et al., 1996; Zlokovic et al., 1996; Hammad et al., 2000; Christoffersen et al., 2006), signaling molecules such as angiotensin II and 1-7 (Gonzalez-Villalobos et al., 2005; Gonzalez-Villalobos et al., 2006), leptin (Hama et al., 2004; Dietrich et al., 2008), and insulin (Orlando et al., 1998), albumin (Cui et al., 1996) as well as complexes of vitamins, such as D, B12 and A, with their corresponding vitamin-binding proteins (Christensen and Willnow, 1999) and steroid hormones in complex with sex hormone-binding globulin (**Table I**). Some of these ligands are also recognized by cubilin **(Table II).** Megalin thus plays a key role in the internalization and activation of molecules such as vitamin D and vitamin D-binding protein (DBP), whose activation from the 25-OH form to the active 1,25-OH form occurs predominantly in the kidney. Interestingly, the systemic role of megalin in calcium homeostasis and bone metabolism can be almost completely recapitulated in a mouse model with conditional inactivation of the megalin gene in the kidney (Leheste et al., 2003). Megalin renal functions and its ligands explain why megalin KO mice suffer from extensive proteinuria, low bone density and rickets (Nykjaer et al., 1999). In addition to the kidney, megalin-mediated vitamin D activation also occurs in other tissues, such as the mammary glands (Rowling et al., 2006; Chlon et al., 2008), bone (van Driel et al., 2006) and the liver, specifically in stellate cells (Gressner et al., 2008). Furthermore, megalin may have an antifibrotic role as a receptor for connective tissue growth factor (CTGF) (Gerritsen et al., 2010), which plays a key role in fibrosis in several organs, including the kidney and gallblader. Megalin KO mice lose CTGF in the urine, and the blockage of tubular reabsorption in human healthy volunteers induces an increase of urinary CTGF excretion (Gerritsen et al., 2010).

3. IMPAIRMENT OF MEGALIN EXPRESSION AND FUNCTION IN DISEASE

Megalin expression, trafficking and/or its ligands are likely involved in some disease conditions that compromise the functioning of organs such as kidney, brain, mammary gland, thyroid and gallbladder. In this context it is important to understand the mechanisms that regulate megalin expression and availability.

TABLE I

Megalin Ligands

Ligands	Reference
Albumin	(Cui et al., 1996)
Aminoglycosides	(Moestrup et al., 1995)
α Amylase	(Birn et al ., 2000a)
Angiotensin II	(Gonzalez-Villalobos et al., 2005)
Angiotensin 1-7	(Gonzalez-Villalobos et al., 2006)
Apolipoprotein B	(Stefansson et al., 1995b)
Apolipoprotein E	(Willnow et al., 1992)
Apolipoprotein H	(Moestrup et al., 1998)
Apolipoprotein J/Clusterin	(Kounnas et al., 1995)
Apolipoprotein M	(Faber et al ., 2006)
Aprotinin	(Moestrup et al., 1995)
Bone morphogenetic protein 4	(Spoelgen et al., 2005)
Ca2+	(Christensen et al., 1992)
Cathepsin b	(Nielsen et al., 2007)
Coagulation factor VIII	(Ananyeva et al., 2008)
Connective tissue growth factor (CTGF)	(Gerritsen et al., 2010)
Cytochrome C	(Orlando et al ., 1998)
Cystatin C	(Kaseda et al., 2007)
Epidermal growth factor	(Orlando et al ., 1998)
Folate binding protein	(Birn et al., 2005)
α Galactosidase A	(Christensen et al., 2007)
Gelsolin	(Vargas et al., 2010a)
Hemoglobin	(Gburek et al., 2002)
Insulin	(Orlando et al ., 1998)
Insulin-like growth factor I (IGF-I)	(Bolos et al., 2010)
Lactoferrin	(Willnow et al., 1992)
Leptin	(Hama et al., 2004; Dietrich et al., 2008)
Lipoprotein lipase	(Kounnas et al., 1993)
Liver type fatty acid binding protein	(Oyama et al., 2005)
Lp(a)	(Niemeier et al., 1999)
Lysozyme	(Orlando et al ., 1998)
Metallothionein	(Klassen et al., 2004)
β1 Microglobulin	(Leheste et al., 1999)
α_2 Microglobulin	(Orlando et al ., 1998)
Myoglobin	(Gburek et al., 2003)
Neutrophil gelatinase associated lipocalin	(Hvidberg et al., 2005)
Odorant binding protein	(Leheste et al., 1999)
Parathyroid hormone	(Hilpert et al., 1999)
Pancreatitis associated protein 1	(Leheste et al., 1999)
Plasminogen	(Kanalas and Makker, 1991)
Plasminogen activator inhibitor type 1	(Stefansson et al., 1995a)
Plasminogen activator inhibitor type 1 urokinase	(Moestrup et al., 1993)
Plasminogen activator inhibitor type 1 tissue plasminogen activator	(Moestrup et al., 1993)
Polymyxin B	(Moestrup et al., 1995)
Prolactin	(Orlando et al ., 1998)
Pro urokinase	(Stefansson et al., 1995a)
Recombinant activated factor VIIa (rFVIIa)	(Seested et al., 2010)
Retinol binding protein	(Christensen et al., 1999)
Seleno protein P	(Olson et al., 2008)
Seminal vesicle secretory protein II	(Ranganathan et al., 1999)
Sex hormone binding globulin	(Hammes et al., 2005)
Sonic hedgehog protein	(McCarthy et al., 2002)
Thyroglobulin	(Zheng et al., 1998)
Transcobalamin vitamin B12	(Moestrup et al., 1996)
Transthyretin	(Sousa et al., 2000)
Trichosantin	(Chan et al., 2000)
Vitamin D binding protein	(Nykjaer et al., 1999)

TABLE II

Cubilin Ligands

Ligands	Reference
Albumin	(Birn et al., 2000b)
Aminoglycosides	(Tauris et al., 2009)
Apolipoprotein Al	(Hammad et al., 1999; Kozyraki et al., 1999)
Apolipoprotein AII	(Dugue-Pujol et al., 2007)
Clara cell secretory protein	(Burmeister et al., 2001)
Hemoglobin	(Gburek et al., 2002)
High density lipoprotein	(Hammad et al., 1999; Kozyraki et al., 1999)
Inmunoglobulin light chains	(Batuman et al., 1998)
Intrinsic factor vitamin B12	(Birn et al., 1997)
Myoglobin	(Gburek et al., 2003)
Recombinant activated factor VIIa (rFVIIa)	(Seested et al., 2010)
Transferrin	(Kozyraki et al., 2001)
Vitamin D binding protein	(Nykjaer et al., 2001)

3.1. Mutations of megalin gene associated with human diseases

Two related, extremely rare conditions directly associated with megalin/LRP2 mutation are Donnai-Barrow syndrome and facio-oculo-acoustico-renal (FOAR) syndrome (Kantarci et al., 2007; Kantarci et al., 2008; Pober et al., 2009). Both conditions are caused by mutations in *lrp2* and are characterized by agenesis of the corpus callosum, developmental delay, proteinuria, hearing loss and ocular abnormalities. These defects highlight the importance of megalin during development in organs such as brain, eye, ear and kidney.

3.2. Kidney disease and the role of megalin

Megalin ligands such as albumin, insulin, leptin, PTH and angiotensin II have been implicated in pathological conditions, including diabetes, hypertension and obesity (Mezzano et al., 2003; Tojo et al., 2003; Vio and Jeanneret, 2003; Saito et al., 2005; Zhang et al., 2005; Wolf and Ziyadeh, 2006; Pollock and Poronnik, 2007), many of which affect renal function. Similarly, megalin-interacting proteins may be involved in disease pathology. For example, the activity of Na(+)/H(+) exchanger isoform 3 (NHE3) is negatively modulated by megalin association (Biemesderfer et al., 1999; Biemesderfer et al., 2001), and its activity is increased in models of diabetes mellitus, which partially explains the Na(+) retention observed in diabetic patients with kidney disease (Hryciw et al., 2004). As a consequence of these disease-related conditions, megalin expression can be severely compromised, accounting for some common features observed in them, specifically the low molecular weight proteinuria and albuminuria. The mechanisms underlying megalin downregulation under these conditions are not known, but they could be related to the activation of the renin-angiotensin system (Tojo et al., 2003), increased TGFβ signaling and increased albumin overload in the lumen of the PT (see also section 4.1).

The cell surface expression of megalin is altered, possibly due to membrane trafficking defects, in two genetic diseases linked to the X-chromosome, Lowe syndrome and Dent disease. In both conditions, protein and salt resorption are affected in kidney proximal tubular cells, ultimately leading to progressive renal failure in these patients (Bockenhauer et al., 2008; Cho et al., 2008). Lowe syndrome, also known as Oculo-cerebro-renal Lowe syndrome, is characterized by congenital cataracts, mental retardation and renal Fanconi syndrome. The latter consists of LMW proteinuria and proximal tubular acidosis. The gene responsible for the disease is OCRL, a phosphatidylinositol 4,5-bisphosphate (PIP(2))-5-phosphatase located on the X chromosome. Dent Disease is also a rare X-linked, recessively inherited proximal renal tubular disorder characterized by LMW proteinuria, hypercalciuria and nephrocalcinosis/nephrolithiasis, but only renal function is affected in these patients. Dent-1 disease is mainly caused by mutations in *CLCN5*, the gene encoding the chloride channel ClC-5 (Marshansky et al., 2002), and Dent-2 is caused by mutations in OCRL (Hoopes et al., 2005; Utsch et al., 2006; Sekine et al., 2007; Cho et al., 2008). Reduced excretion of megalin normally found in the urine has been reported in both Lowe syndrome and Dent disease (Norden et al., 2002; Watanabe, 2004), suggesting that megalin expression at the PT cells apical surface could be impaired in these diseases. The absence of ClC-5 in some patients is related to a significant reduction in megalin expression in PT cells. Some the characteristics of these diseases are found in megalin KO mice, such as LMW proteinuria, as well as the loss of retinol binding protein (RBP), DBP and 25-hydroxy (OH) vitamin D, explaining the rickets. In addition, mouse models for Dent disease have altered expression of megalin in the PT cells (Guggino, 2007). However, it is currently unclear how mutations in the OCRL and ClC-5 genes may affect receptor expression and/or trafficking. It has been suggested that the lack of ClC-5 activity could be related to a failure in endosome acidification and therefore impair megalin recycling to the plasma membrane (Marshansky et al., 2002; Hryciw et al., 2006). OCRL was shown to be able to bind the adaptor protein APPL1, which interacts with megalin cytoplasmic domain (Erdmann et al., 2007). Some effects of the absence of OCRL on endosome dysfunctions and megalin expression at the cell surface in Lowe syndrome have been recently proposed (see

section 4.2). A mouse model for Lowe Syndrome was recently developed (Bothwell et al., 2010) and will help to elucidate how OCRL affects megalin *in vivo*.

3.3. Role of megalin in cholesterol homeostasis

As a lipoprotein receptor, megalin plays a relevant role in cholesterol transport. This is particularly clear during development (Willnow et al., 1996), functioning along with its coreceptor cubilin (Assemat et al., 2005b). In addition to apoE, megalin is the receptor for apoJ/clusterin, which is associated with HDL particles (Kounnas et al., 1995; Calero et al., 1999), and also for Lp(a), an atherogenic particle (Niemeier et al., 1999; Willnow, 1999). Apolipoprotein M is a lipocalin with antiatherogenic properties that is present in pre-β-HDL particles, chylomicrons, VLDLs and LDLs secreted by the liver and the kidney that use megalin as a receptor (Dahlback and Nielsen, 2006; Faber et al., 2006; Dahlback and Nielsen, 2009). In addition to apoM, megalin with its coreceptor cubilin, internalizes apoA-I and apoA-II, structural components of HDLs (Hammad et al., 2000; Dugue-Pujol et al., 2007). Taken together, these data indicate that megalin contributes to the regulation of HDL metabolism. In fact, plasma cholesterol levels and LDL cholesterol have been associated with genetic variations in the megalin gene in the Japanese population (Mii et al., 2007), further suggesting that this receptor has a systemic function in cholesterol homeostasis.

3.4. Megalin in Gallstone Disease

The presence of megalin and cubilin in gallbladder epithelial cells (**Fig. 4A**) (Erranz et al., 2004), but not in liver cells, also suggests the participation of these receptors in biliary cholesterol management. The bile is mainly composed of water, free cholesterol, phospholipids and bile salts secreted by the hepatocytes. In addition, bile contains many proteins that are megalin and/or cubilin ligands, including albumin and apolipoproteins that can act as factors that promote or inhibit gallstone formation. Gallbladder epithelial cells express NHE3 at the apical membrane (Colombani et al., 1996; Silviani et al., 1996; Narins et al., 2004), which could be regulated by megalin as it is in PT cells (Biemesderfer et al., 1999; Biemesderfer et al., 2001). In this way, megalin could regulate bile pH, which is a factor to consider in lithogenicity and gallstone formation. The *lrp2* gene is present in the Lith1 locus of mice, suggesting its relationship to cholesterol gallstone susceptibility (Bouchard et al., 1999). Gallstone disease is a multifactorial condition of the gallbladder, which involves genetic, sex, age and environmental variables (Lammert and Miquel, 2008). A study of a small number of gallstone patients in the Greek population found that both megalin and cubilin were downregulated in gallstone patients at the mRNA and protein levels (Tsaroucha et al., 2008). On the other hand, we have found that gallstone-susceptible mice fed a cholesterol-rich lithogenic diet showed a reduction in megalin mRNA expression (**Fig. 4B**) even before they developed cholesterol crystals or gallstones. Our data also indicate that megalin protein expression is not regulated by cholesterol but is under the control of bile acids (Erranz et al., 2004). This regulatory role of bile acids, have prompt us to test the role of the bile acid receptor FXR in megalin expression (see section 4.1). How the presence of megalin in the gallbladder affects the susceptibility to gallstone development, is a subject of our ongoing studies. Considering the roles of megalin in other tissues and the factors that control bile lithogenicity, megalin could control bile composition by participating in the transport of cholesterol, the regulation of protein bile protein content and/or the activity of proteins such as NHE3. In addition, in gallstone disease there is fibrosis of the gallbladder, induced by the production of TGFβ and the megalin ligand CTGF (Koniniger et al., 2005). A reduction of megalin protein could further promote the fibrotic environment in the gallbladder, increasing the organ dysmotility.

3.5. Megalin in Alzheimer´s Disease

In the brain, the presence of megalin has been implicated in neurodegenerative conditions such as Alzheimer's disease (AD), especially in terms of its protective role in the choroid plexus. Megalin facilitates the clearance of the Aβ peptide that is produced by amyloidogenic processing of the amyloid precursor protein (APP), which can form complexes with different megalin ligands such as clusterin/apoJ (Zlokovic, 1996; Zlokovic et al., 1996; Hammad et al., 1997; Nuutinen et al., 2009) and apoE (Zlokovic, 1996; Zlokovic et al., 1996; Bell et al., 2007). In addition to these ligands, a role for the Aβ-binding protein gelsolin (Ji et al., 2010; Li et al., 2010), a protein that is produced in the epithelial cells of the choroid plexus (Vargas et al., 2010a), was recently demonstrated. Gelsolin has neuroprotective functions in controlling the Aβ-induced production of NO and apoptosis as well as cytoskeletal disruptions in the epithelial cells of the cerebrospinal fluid barrier (Vargas et al., 2010a). The neuroprotective roles of gelsolin were also demonstrated *in vivo* in an AD mouse model and probably require megalin as a receptor (Antequera et al., 2009). Recently, a genetic study found a G/A polymorphism in the megalin promoter that reduced the expression of megalin by 20%, whose association with AD is dependent on the apoE genotype (Vargas et al., 2010b). In addition to Aβ peptide, the protective role of megalin in AD and neurodegenerative conditions is suggested by its role in the incorporation of the neurotrophic factor IGF-I from the serum to the brain through a mechanism that is negatively regulated by GSK3-mediated phosphorylation of the megalin cytoplasmic domain (see section 4) (Bolos et al., 2010). As mentioned, megalin also regulates the transport of leptin in the choroid plexus by transcytosis (Dietrich et al., 2008). Leptin levels have been related to a decrease in the activity of β-secretase or BACE (Marwarha et al., 2010), the limiting step in the formation of Aβ from APP. Decreases of megalin expression with age and in AD patients (Odera et al., 2007; Dietrich et al., 2008) would thus reduce the protective effects of the receptor on brain function.

4. MECHANISMS REGULATING MEGALIN μRNA AND PROTEIN LEVELS AND CELL SURFACE EXPRESSION

Despite current knowledge regarding the different roles of megalin during development and in adult tissues, very little is known about the mechanisms that control its expression and availability at the cell surface. There are several possible mechanisms that could control megalin abundance, including the regulation of its mRNA levels, protein synthesis and protein half-life as well as trafficking of megalin to the cell surface.

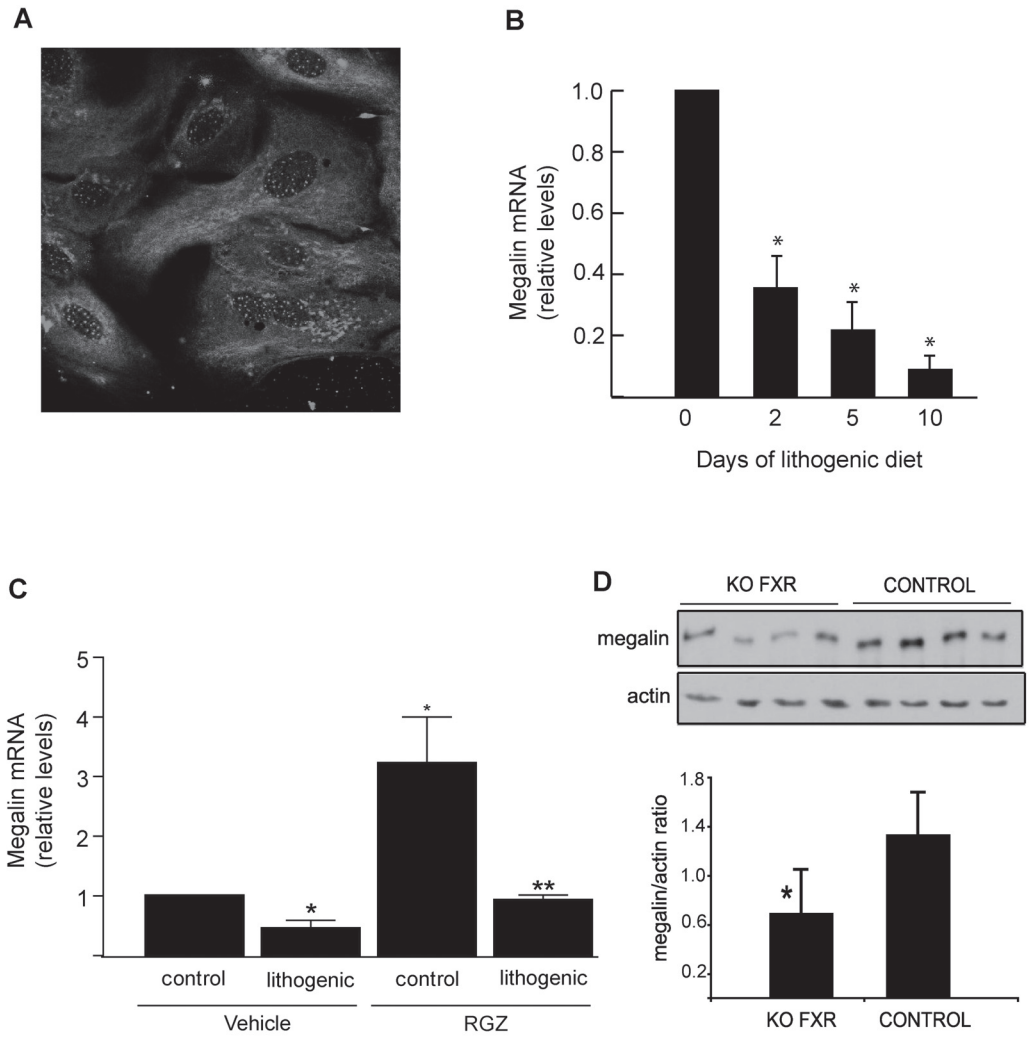

Figure 4. Megalin expression is regulated by a lithogenic diet and the nuclear receptors PPARγ and FXR. a) The presence of megalin in gallbladder epithelial cells in primary culture, detected by an antibody against the cytoplasmic domain of the receptor with confocal microscopy. b) In 8-week-old mice fed a lithogenic diet, the expression of gallbladder megalin mRNA determined by qPCR (relative to GAPDH as a housekeeping gene) was significantly downregulated from the second day (n = 4 mice per group). Values are average ± SD. c) Pretreatment of mice with the PPARγ agonist rosiglitazone (20 mg/kg/day) for two days, followed by either control diet or lithogenic diet for 10 days in the presence of rosiglitazone or vehicle, shows that rosiglitazone treatment significantly increased megalin mRNA expression, determined by qPCR, in control animals. In mice fed the lithogenic diet for 10 days, the PPARγ agonist rescued the reduction in megalin mRNA (n = 4 mice per group). Values are average ± SD, $*P < 0.05$ vs. megalin control, $**P < 0.05$ vs. megalin under lithogenic diet. d) Detection of megalin protein by western blot from mouse kidney cortex. The expression of megalin in kidney of mice null for the nuclear receptor FXR is significantly reduced compared to the controls. $*P < 0.05$

4.1. Regulation of megalin mRNA and protein expression

In terms of megalin mRNA expression, there have been several studies investigating the megalin promoter. The transcription factor Sp1 has been shown to bind a non-consensus TATA box in the human promoter and play a role in the basal transcription of megalin in parathyroid cells (Knutson et al., 1998). In addition, a sequence from position +5 to +11, downstream of the transcription initiation site, is important for the topology of the binding site of the transcription factor IID to the megalin promoter in the TATA box region (Knutson et al., 2000a). Megalin expression is also regulated by the methylation of a CpG island identified in the human promoter, which is correlated with the lack of megalin expression in some cell lines, in contrast to the parathyroid, in which its promoter is unmethylated and megalin is expressed (Knutson et al., 2000b). In the rat megalin promoter, a proximal Sp1 site and a JCV repeat are important for activating transcription (Zhao et al., 2001).

Our group has been working on understanding the regulation of megalin expression at transcriptional level under different stimulus. Considering the protective roles that PPARs and their agonists have, for example, in renal disease we started analyzing if megalin could be a target of these nuclear receptors. Through analyzing the megalin promoter, we identified three consensus sites for peroxisome proliferator-

activated receptor (PPAR) that directly bind PPARα and PPARγ but not PPARβ/δ. Moreover, the treatment of PT cells with PPARα and γ agonists induces significant increases in megalin mRNA and protein expression, an effect that is counteracted by specific antagonists for the nuclear receptors. Finally, PPARs also regulate the expression of megalin *in vivo* in mouse and rat kidney (Cabezas et al., 2011). In addition, the treatment of mice with rosiglitazone, a PPARγ agonist, induced a significant increase in megalin expression in the gallbladder and, interestingly, this treatment also rescued the reduction in megalin expression that results from feeding animals a lithogenic diet (**Fig. 4B-C**). PPARα and γ and their agonists are important players in several systems during development and in the adult, including lipid metabolism, adipose tissue and bone differentiation; they also exert protective effects against pathologies such as diabetic nephropathy, obesity and hypertension (Kostadinova et al., 2005; Zhang and Guan, 2005; Chetty and Sharma, 2006; Drew et al., 2006; Kepez et al., 2006; Semple et al., 2006). As previously noted, megalin is known to play a role in several of the above systems, which makes the receptor an important element to consider when analyzing the broader functions of PPARs.

Bile acids can modulate the expression of megalin at the protein level in PT cells *in vitro* as well in gallbladder *in vivo* (Erranz et al., 2004). Recent unpublished data from our laboratory indicate that the nuclear receptor FXR could be responsible for mediating the activating effects of bile acids on megalin expression, as receptor levels in the kidneys of FXR KO mice were significantly decreased compared to controls (**Fig. 4D**) and the specific FXR activator GW4064 upregulated megalin mRNA expression in gallbladder epithelial cells cells in culture as well *in vivo* in mice.

Although there are few known regulators of megalin expression, it is notable that several of the molecules that affect megalin mRNA and/or protein levels also are ligands of the receptor, and their levels and availability are regulated by megalin itself. At the transcriptional level, megalin mRNA expression is augmented by retinoic acid (vitamin A) and vitamin D treatment in cells from different origins and species (Liu et al., 1998). Both vitamins A and D carried by their specific vitamin binding proteins, RBP and DBP, are endocytosed by megalin. In breast cancer cells, the expression of megalin, its coreceptor cubilin and the adaptor protein Dab2 are induced by retinoic acid (Chlon et al., 2008). The differentiation state of cells, such as rat PT cells and F9 embryonal carcinoma cells, also positively regulates the expression of megalin (Liu et al., 1998). For example, the differentiation of F9 cells with retinoic acid and cAMP induces the expression of megalin protein and of the RAP (Czekay et al., 1995). Clusterin/apoJ provides another example of regulation of megalin by its own ligand (Ammar and Closset, 2008). This glycoprotein can act as an apoptosis inducer or inhibitor and is involved in pathologies including cancer and Alzheimer's disease (Zlokovic, 1996; Ammar and Closset, 2008; Nuutinen et al., 2009). The antiapoptotic role of clusterin in prostatic cells is achieved through a megalin-mediated signaling process involving the activation of PI3K/AKT (Ammar and Closset, 2008). Interestingly, clusterin increases megalin mRNA and protein expression (Ammar and Closset, 2008).

In contrast to clusterin, the signaling molecule angiotensin II, which signals through the ATR1 receptor but also binds to and can be internalized by megalin (Gonzalez-Villalobos et al., 2005), negatively regulates receptor expression at both the mRNA and protein levels (Hosojima et al., 2009). Interestingly, the deleterious effect of angiotensin II can be counteracted by insulin, another megalin ligand (Orlando et al., 1998), partly through the inhibition of the ERK pathway by ATR1 activation, downstream of the IRS/PI3K signaling pathway (Hosojima et al., 2009). We also have evidence of the protective role of ATR1 antagonists on megalin expression *in vivo*. The treatment of rats with losartan, an ATR1 antagonist, can protect against the reduction of megalin induced by daily injections of BSA (1g/100 body weight) for one week (**Fig. 5**). It has been suggested that the reduction of megalin in diabetic nephropathy, which is also characterized by albuminuria and fibrosis, could be mediated by toxic levels of albumin (Caruso-Neves et al., 2006) and/or by the cytokine TGFβ, which can be induced by albumin treatment *in vitro* (Gekle et al., 2003; Diwakar et al., 2007). Whether the megalin promoter is directly regulated by this cytokine through the Smad2/3 pathway is not known.

4.2 Regulation of megalin protein levels and cell surface expression

Besides the regulation of megalin mRNA levels, there are other mechanisms controlling the availability of the receptor. Various molecules and processes also regulate the levels of megalin protein and its availability at the cell surface. As previously mentioned, megalin folding requires the assistance of RAP (Farquhar et al., 1995), a chaperone that acts in the lumen of the ER, specifically in the ligand binding repeats. This action probably functions to avoid premature binding of ligands that are synthesized in the same cell, as has been

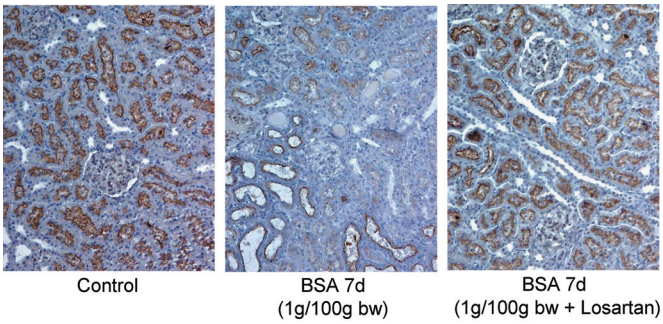

Figure 5. The angiotensin II receptor I inhibitor losartan protects against the reduction of megalin in kidney of rats subjected to BSA-induced tubulointerstitial damage and proteinuria. Sprague- Dawly rats were divided in three groups of four animals each. The control group was intraperitoneally injected with saline for seven days. The BSA group received BSA (1g/100 gr BW/day; intraperitoneal injections) for seven days. The last group received Losartan (40 mg/kg/day) administered by gavage for four days and then losartan was given simultaneously with BSA (1g/100 gr BW/day; intraperitoneal injections) for seven days. At the end of this period, the animals were euthanized, and kidneys were extracted and fixed to perform inmunohistochemistry to detect megalin, as previously described (Erranz et al., 2004; Cabezas et al., 2011). BSA injections alone reduced megalin immunostaining, and this reduction was rescued by simultaneous treatment with losartan.

described for LRP1 (Bu and Marzolo, 2000). The importance of RAP in megalin protein expression is clearly illustrated by the phenotype of RAP KO mice (Birn et al., 2000). These animals express 23% of normal levels of megalin in the renal PT and exhibit other phenotypes consistent with a lack of megalin, such as LMW proteinuria and the urinary excretion of α-amylase and DBP (Birn et al., 2000; Lisi et al., 2008). The absence of RAP also results in reduction of megalin levels in the thyroid, resulting in defects in thyroglobulin secretion into the colloid and moderate increases in the levels of thyroid-stimulating hormone (Lisi et al., 2006). In addition to the ligand binding domains, the folding of the β-propellers/EGF domains are also assisted by a chaperone, MESD. MESD is an ER-resident protein that facilitates the correct folding and surface expression of the LRP5/LRP6 receptors, which are responsible for the Wnt/β-catenin pathway (Koduri and Blacklow, 2007), as well as megalin (Lighthouse et al., 2010). Accordingly, mice KO for MESD exhibit a reduction in megalin at the surface of the visceral endoderm and an accumulation of the receptor in intracellular compartments (Lighthouse et al., 2010).

The cytosolic adaptor protein Dab2, which binds to the cytosolic domain of megalin (Oleinikov et al., 2000), is also important in the expression and function of megalin during development (Maurer and Cooper, 2005) and in adult tissues (Nagai et al., 2005). This protein is critical for embryo development, as it is required for nutrient internalization by the visceral endoderm, where megalin is expressed (Morris et al., 2002). The loss of Dab2 is also associated with a loss of cell surface polarity of megalin and E-cadherin and the formation of the primitive endoderm outer layer in embryoid bodies (Yang et al., 2007). Adult Dab2 conditional KO mice show excretion of proteins such as DBP in the urine, a reduction in total megalin expression and the redistribution of the receptor from intracellular vesicles and endosomal compartments to microvilli at the apical surface of PT cells (Morris et al., 2002; Nagai et al., 2005).

Megalin expression and trafficking are also dependent upon modifications in the cytoplasmic domain of the receptor. Specifically, the phosphorylation of the megalin cytoplasmic domain by GSK3 (**Fig. 6)** is able to decrease its cell surface expression by a negative regulation of megalin recycling without changing its apical distribution or endocytosis (Yuseff et al., 2007). The serine within the PPPSP motif constitutes the main phosphosite in the megalin cytoplasmic tail. This motif seems not to act as a priming site for other phosphorylation events, as the phosphomimetic PPPDP mutant receptor is not as highly phosphorylated as the wild type and shows a level of phosphorylation similar to the PPPAP mutant **(Fig. 6B)**. GSK3 binds directly to megalin tail (**Fig. 6C**) and phosphorylates the receptor cytoplasmic domain independently of the presence of the megalin ectodomain (**Fig. 6D**). This modification of megalin could enable signaling pathways that have GSK3 as target for activation or inactivation to regulate the activity of the receptor by affecting its cell surface expression. Among these pathways, some are triggered by signaling molecules that are megalin ligands, such as insulin, IGF-I and Shh, and others by pathways that are related to megalin, such as Wnt/β-catenin, which requires LRP5/6 and MESD. Moreover, as previously discussed, the adaptor protein APPL1 which has a role in signaling endosomes related to AKT activation (Schenck et al., 2008; Zhao and Guan, 2008; Cheng et al., 2009), links the megalin cytoplasmic domain to OCRL, which is mutated in patients with Lowe Syndrome. The apical distribution of megalin is not affected by the downregulation or mutation of OCRL, but its cell surface expression and recycling are significantly decreased (Vicinanza M et al, manuscript in preparation). This opens the possibility that the phosphorylation of megalin may be also affected in Lowe Syndrome and Dent-2 patients, which could partially account for the disease phenotypes. Finally, the ligand clustering/apoJ also modulates the receptor's phosphorylation, possibly on a tyrosine residue of the cytoplasmic tail (Ammar and Closset, 2008). How this phosphorylation regulates megalin function and/or trafficking is as yet unknown.

As has been described for other members of the LDLR family and for APP (Marzolo and Bu, 2009), megalin is also a substrate for metalloproteases or *sheddases,* which induce ectodomain shedding that produces a carboxy-terminal fragment, which is then subject to receptor intramembrane proteolysis by the γ-secretase complex (Zou Z et al., 2004; Biemesderfer, 2006). It has been suggested that after processing by the γ-secretase complex, the resultant megalin intracellular domain, or MICD, could travel to the nucleus and play a transcriptional regulatory role, decreasing the expression of megalin and other megalin-regulated proteins in PT cells, such as NHE3 (Li et al., 2008). The transcriptional role of the MICD has been recently challenged by *in vivo* data from transgenic mice expressing MICD (Christ et al., 2010). These animals did not show decreases in megalin protein or effects on the endocytic function of renal PT. However, it is important to note that the construct used in this report has three Flag epitopes that could affect the nuclear expression of the MICD, which, in fact, was not detected in the nuclei of PT cells. Therefore, regarding the production of MICD that has been reported *in vitro*, whether it is produced *in vivo* and the potential roles of this fragment are still controversial aspects to solve. However, the kidney is not the only organ in which this type of processing may occur. In fact, megalin nuclear staining in the oligodendrocytes in the spinal cord white matter of embryonic and adult mice has been reported (Wicher et al., 2006). In addition, megalin can be found in several fragments of human gallbladder epithelial cells, suggesting proteolysis (Erranz et al., 2004), and nuclear staining can be seen using an antibody that recognizes the human cytoplasmic domain (**Fig. 4A**). Together, these findings suggest that megalin processing could be physiologically relevant in, for example, the regulation of megalin half-life. The next steps in this line of investigation should focus on the mechanisms that regulate proteolytic processing of megalin, the roles of its ligands that have signaling properties and megalin´s intracellular adaptor or interacting proteins, as have been studied for APP and other receptors of the LDLR family (Marzolo and Bu, 2009).

It has been shown that the urine normally contains microvesicles or exosomes, membrane particles of 40 to 100 nm secreted from renal epithelial cells after the fusion of multivesicular bodies with the luminal plasma membrane (Pisitkun et al., 2004; Keller et al., 2006; Knepper and Pisitkun, 2007; Dimov et al., 2009). Megalin and cubilin are among the several components contained in urine-derived exosomes (Pisitkun et al., 2004). In some disease conditions, including chronic renal diseases, the exosome protein profile is altered

(Zhou et al., 2008; Gonzales et al., 2009). However, until now, there has been no report indicating changes in the presence of megalin in exosomes in different diseases, such as Dent Disease or Lowe Syndrome, diabetic nephropathy or megalin KO mouse models. Dent and Lowe patients are reported to have lower levels of urine megalin (Norden et al., 2002), but the method used in this study did not discriminate between megalin associated with exosomes and megalin derived from protease-mediated shedding of the extracellular domain (Fernandez-Llama et al., 2010). It is therefore possible that under some pathophysiological conditions, the levels of megalin secreted in exosomes could be affected and could serve a diagnostic biomarker. Because exosomes are secreted from almost all cells, it is also possible that in other organs, as well as the kidney, exosomes containing megalin could be a physiological mechanism to regulate the availability of ligands and/or to transport ligands from one cell to another.

5. CONCLUSIONS

Since the discovery of megalin/LRP2 as an important endocytic receptor present in the epithelial cells of the kidney PT several new ligands and functions for this protein have been uncovered. In addition to its role in the internalization of different molecules, the possibility that megalin is a target and/or regulator of signal transduction pathways, as well as the emergence of its roles in neurodegeneration and regeneration processes and in chronic and genetic diseases, underscores the importance of understanding the mechanisms that regulate megalin expression and functions.

At mRNA level megalin expression is regulated positively by retinoic acid, vitamin D, bile acids and ligands of PPAR α and γ. The presence of the receptor at the cell surface is under the control of the ER chaperones RAP and MESD and membrane trafficking mechanisms including megalin internalization

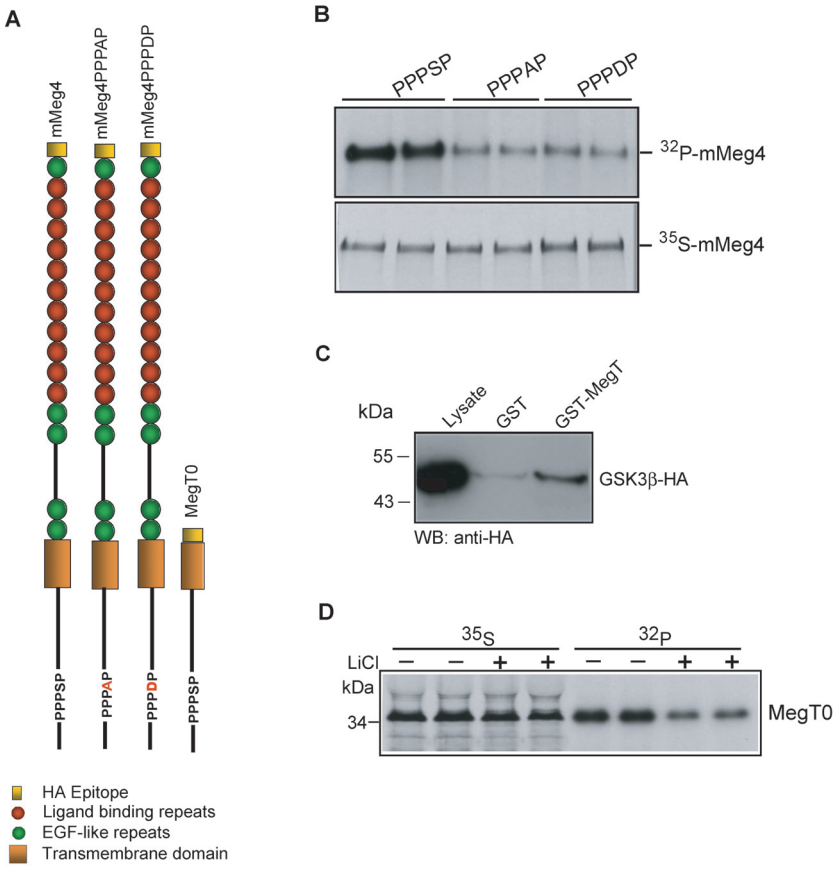

Figure 6. Megalin phosphorylation in the PPPSP cytoplasmic motif is mediated by GSK3 and does not require the receptor ectodomain. a) Schematic representation of megalin minireceptors containing the fourth ligand binding, transmembrane and cytosolic domains of human megalin, with an amino-terminal HA epitope (mMeg4); MegT lacks the ectomain. b) Minireceptors with wild-type or mutant (PPPAP or PPPDP) cytoplasmic domains were expressed in MDCK cells. Cells were metabolically labeled either with ^{35}S-methionine/cysteine or ^{32}P-orthophosphate for 4 or 2 h respectively. After cell lysis, proteins were immunoprecipitated and resolved by SDS-PAGE and autoradiography. The presence of the serine residue within the PPPSP motif was critical for the phosphorylation of the minireceptor. c) HEK-293 cells were transfected with GSK3-HA, and the cell lysates were subject to pull-down assays with GST-megalin cytoplasmic domain (GST-MegT) or GST alone as a control. The megalin cytoplasmic domain interacts with GSK3. d) MegT was transfected in MDCK cells and the cells were metabolically labeled as in b) but in the presence or in the absence of LiCl, to inhibit GSK3. Phosphorylation of this construct indicated that this modification does not require the megalin ectodomain and is mediated by GSK3 because it decreased significantly in the presence of LiCl compared to the control (NaCl).

and recycling dependent on the interaction of its cytoplasmic domain with adaptor/cytosolic proteins and the kinase GSK3. Finally, two still poorly understood processes, megalin´s proteolytic processing and the secretion of the receptor in exosomes could be also relevant mechanisms controlling the availability of the receptor and its function at the cell surface.

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