

## Soluble Megalin Is Accumulated in the Lumen of the Rat Endolymphatic Sac

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**ABSTRACT.** The endolymphatic sac (ES) is believed to play an important role in maintaining homeostasis in the inner ear by the absorption and endocytosis of endolymph. Megalin is a 600-kDa multiligand endocytic receptor expressed in certain types of absorptive epithelia including kidney proximal tubules. We analyzed the immunoreactivity for megalin in rat ES by immunofluorescence, immunogold electron microscopy, and immunoblotting. With immunostaining, the luminal substances of the ES were strongly stained for megalin. Megalin was also localized in luminal macrophage-like cells and both types of epithelial cell (mitochondria-rich cells and ribosome-rich cells). In these cells, the megalin was localized in the lumen of endosomes, but was not membrane associated. This localization pattern indicates that the megalin in these cells is not a membrane receptor, but merely one of the constituents that are endocytosed from the lumen of the ES. Immunoblotting indicated that the megalin in the ES is a 210-kDa molecule lacking a cytoplasmic domain. This suggests that the megalin in the ES may be a soluble form, different from the 600-kDa membrane-bound receptor expressed in kidneys. Taken together, it is likely that the megalin in the ES lumen is a soluble component and may be endocytosed by the ES epithelial cells. Furthermore, we found that the tectorial membrane, an acellular structure in the cochlea, gave a strong megalin immunoreaction. Since the cochlea is connected to the ES, the megalin may be transported alone or with the components of the tectorial membrane from the cochlea to the ES lumen through longitudinal flow.

**Key words:** megalin/endolymphatic sac/tectorial membrane/cubilin/endocytosis

### Introduction

The endolymphatic sac (ES) is a part of the endolymphatic compartment of the inner ear and constitutes a blind appendage of the membranous labyrinth. In the ES, there are two types of epithelial cell, i.e., the mitochondria-rich cell (also called the light cell or the type-1 epithelial cell) and the ribosome-rich cell (also called the dark cell or the type-2 epithelial cell) (Lundquist, 1965; Fukazawa *et al.*, 1990; Dahlmann and von Düring, 1995). The ES lumen contains a viscous endolymph including macromolecular complexes such as proteoglycans; however, the precise composition of the material is not fully understood (Friberg *et al.*, 1986; Hultcrantz *et al.*, 1997). These macromolecules are taken up and degraded by free-floating cells such as

luminal macrophages and lymphoid cells. The ES is believed to play an important role in maintaining homeostasis in the inner ear, by the absorption and secretion of endolymph (Kimura and Schuknecht, 1965; Lundquist, 1965; Friberg *et al.*, 1986; Erwall *et al.*, 1988; Rask-Andersen *et al.*, 1999), pressure regulation in the endolymphatic compartment (Rask-Andersen *et al.*, 1987), immunodefense of the inner ear (Rask-Andersen and Stahle, 1980; Tomiyama and Harris, 1986), the removal of waste products and foreign materials from the endolymphatic space (Fukazawa *et al.*, 1991), and endocrine function (Qvortrup and Bretlau, 2002).

Megalin, a member of the low-density lipoprotein receptor family, is an endocytic membrane receptor for a 600-kDa transmembrane glycoprotein (Kerjaschki and Farquhar, 1982; Saito *et al.*, 1994) that has a relatively large extracellular domain, a single transmembrane domain, and only a small cytoplasmic domain. Megalin is expressed in several absorptive epithelial cells, including kidney proximal tubules, visceral yolk sac, epididymis, female reproductive tracts, and inner ear epithelia (Moestrup and Verroust, 2001;

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Christensen and Birn, 2002). To date, more than 40 different ligands of megalin have been identified representing a wide variety of substances including lipoproteins, hormones, vitamin-binding proteins, enzymes, drugs, and immune-related proteins. The expression of megalin is most abundant in the kidney proximal tubule cells and the visceral yolk sac epithelial cells. In these absorptive cells, megalin is colocalized with cubilin, a 460-kDa multiligand endocytic membrane receptor (Moestrup and Kozyraki, 2000). Both megalin and cubilin bind ligands on the apical membrane and are internalized and transported with the ligands into the endosomes. In the endosomes, these receptors release the ligands and are then translocated into the apical tubules for recycling from the endosomes to the apical membrane (Hatae *et al.*, 1986). In contrast, the ligands are delivered to the lysosomes for degradation (Le Panse *et al.*, 1997; Ishida *et al.*, 2004).

Kounnas *et al.* (1994) demonstrated that the inner ear epithelia of embryonic mice expressed megalin. Using immunogold electron microscopy, Mizuta *et al.* (1999) demonstrated that megalin was localized in the epithelial cells of the rat cochlear duct, a part of the endolymphatic compartment of the inner ear. It was previously reported that ES epithelial cells could take up several macromolecule tracers introduced from the cochlear duct or by direct injection (Fukazawa *et al.*, 1990; Fukazawa *et al.*, 1991; Furuta *et al.*, 1992; Hoshikawa *et al.*, 1994; Kakigi *et al.*, 2004). Therefore, we expected that megalin, as a membrane receptor, would be localized in the ES, which like the cochlear duct constitutes part of the endolymphatic compartment of the inner ear. However, in the present study we revealed that megalin exists in the lumen of the ES and is taken up by ES epithelial cells via macropinocytosis.

## Materials and Methods

### Animals and antibodies

Sprague-Dawley (SD) rats (body weight, 200–300 g) and BALB/c mice were used as the experimental animals. The animals were anesthetized with ether prior to use in the experiments. All experimental procedures were approved by the Animal Research Committee of Kagawa University. A monoclonal antibody for rat megalin was prepared as previously described (Ishida *et al.*, 2004). Briefly, the rat yolk sac microsomal fraction was used as an antigen to immunize mice. The mice whose sera exhibited specific reactivity to the yolk sac apical cytoplasm on immunohistochemistry were selected for fusion. By fusing the myeloma and spleen cells from the immunized mice, we obtained hybridomas. The hybridomas were initially screened by means of immunohistochemistry. The positive hybridomas were subsequently screened by means of immunoblotting using rat yolk sac or kidney microsomes. A hybridoma cell clone (mAb2E12) that produced an antibody for megalin was selected. The specificity of mAb2E12 is shown in Fig. 1A and

B. Megalin that had been purified from a rat kidney microsomal fraction by affinity chromatography on an anti-megalin antibody Sepharose column and an affinity-purified rabbit polyclonal antibody raised against the megalin cytoplasmic domain were a kind gift from A. Saito, Niigata University, Niigata, Japan (Saito *et al.*, 1994).

### Immunoblotting

Six SD rats were perfused via the left cardiac ventricle with phosphate-buffered saline (PBS) at 4°C for 1 min. After perfusion, the ES were carefully removed from the temporal bone and immersed in sample buffer (62.5 mM Tris, 2% SDS, 42 mM dithiothreitol (DTT), 8 M urea, and 0.01% bromophenol blue) for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were then boiled for 3 min and the solubilized proteins were separated by SDS-PAGE through 5% to 20% gradient gels (Ready Gels J; Bio-Rad, Tokyo, Japan). The bands were transferred to nitrocellulose membranes or polyvinylidene fluoride (PVDF) membranes using a semidry blotting apparatus. For immunoblotting, the membranes were incubated with PBS containing 5% non-fat dry milk, 0.1% Tween 20, and 0.02% sodium azide overnight at 4°C to block the nonspecific binding of antibodies, followed by incubation with primary antibodies for 1 h at room temperature. The membranes were then washed in PBS and incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody. Finally, the immunoblots were visualized with diaminobenzidine as the substrate, or enhanced with a chemiluminescence reagent (ECL; GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and detected with autoradiography film (Hyperfilm-ECL; GE Healthcare Bio-Sciences Corp. NJ, USA). The specificity of the immunoreactions was controlled by application of a normal mouse IgG or a normal rabbit IgG.

### Immunofluorescence microscopy

Ten SD rats were fixed by perfusion via the left cardiac ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) containing 4% sucrose for 5 min. After perfusion, the temporal bone containing the ES and the cochlear was carefully removed, immersed in the same fixative, and maintained overnight at room temperature. The materials were then decalcified for three weeks in 10% EDTA in 0.1 M phosphate buffer (pH 7.3) containing 7% sucrose at 4°C. The decalcified tissues were prepared for cryopreservation by immersing in a 0.1 M phosphate buffer containing 10% dimethyl sulfoxide and 7% sucrose for 1 h at 4°C. Cryostat sections (6 µm) were cut and mounted on silane-coated glass slides. The sections were incubated with PBS containing 10% normal goat serum for 20 min to block nonspecific binding, and then incubated with anti-rat megalin monoclonal antibodies (culture supernatant of a 2E12 hybridoma cell line), the polyclonal antibody against the rat megalin cytoplasmic domain, or anti-rat cubilin monoclonal antibodies (culture supernatant of a 7A3 hybridoma cell line) (Ishida *et al.*, 2004) overnight at 4°C. After washing with PBS, the sections were incubated with Alexa 488-

conjugated goat anti-mouse IgG or Alexa 594-conjugated goat anti-rabbit IgG (1:500; Invitrogen Corp., Carlsbad, CA, USA) for 1 h at room temperature, and then examined under a confocal laser scanning microscope (LSM-GB200, Olympus Co., Tokyo, Japan). The specificity of the immunoreactions was controlled by the application of a normal mouse IgG or a normal rabbit IgG.

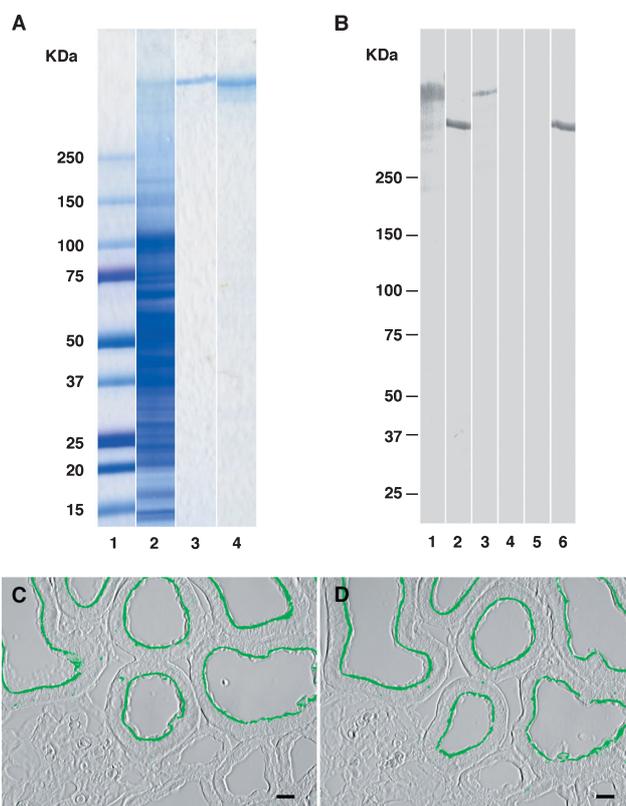
### Immunogold electron microscopy

Five SD rats were fixed by perfusion via the left cardiac ventricle with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) containing 4% sucrose for 5 min at room temperature. After perfusion, the tissue blocks containing the ES were carefully dissected and immersed in the same fixative for 1.5 h at 4°C. The ES was carefully removed from the temporal bones under a dissection microscope, cut into small pieces, washed in 0.1 M cacodylate buffer containing 7% sucrose, and then dehydrated in a graded series of dimethylformamide at room temperature. The tissue was embedded in LR White (London Resin Co., Reading, UK), followed by polymerization by ultraviolet light irradiation for 24 h at -20°C. Ultrathin sections (50–70 nm) were cut with an ultramicrotome and mounted on nickel grids (200 mesh). Each grid was sunk in a droplet of 10% normal goat serum in PBS for 20 min at room temperature, and then incubated in a droplet of anti-rat megalin monoclonal antibody (culture supernatant of a 2E12 hybridoma cell line) overnight at 4°C. After washing with PBS, the sections were incubated with a mixture of 10 nm gold-conjugated goat anti-mouse IgG (1:50; British BioCell International, Cardiff, UK) for 2 h at room temperature. Subsequently, the sections were washed with PBS and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 15 min at room temperature, washed with distilled water, dried, and then stained with 2% uranyl acetate for 3 min and Reynolds' lead citrate for 30 s. The dried sections were then coated with carbon and observed under a JEM-1200EX electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV. The specificity of the immunoreactions was controlled by application of a normal mouse IgG.

## Results

### Identification of mAb2E12 as the anti-megalin antibody

Fig. 1A shows an SDS-PAGE gel stained with Coomassie brilliant blue. The protein (lane 4) bound on the antibody (mAb2E12) Sepharose column ran as a single band and it was very similar in size to the band (lane 3) of the affinity-purified megalin. On immunoblotting (Fig. 1B), the antibody (mAb2E12) reacted with the affinity-purified megalin (approximately 470 kDa) (lane 3) and the megalin in the kidney microsomal fraction (lane 1). In our experimental system, the 600-kDa protein (called megalin or gp330, generally) had an estimated mass of approximately 470 kDa (Fig. 1A and B; lanes 3 and 3, respectively). Furthermore,

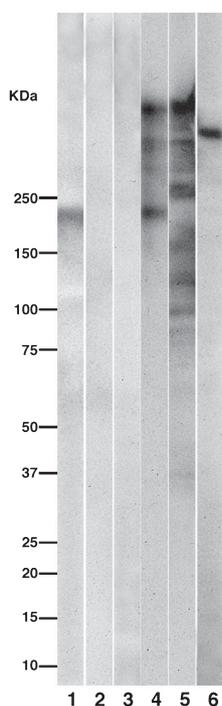


**Fig. 1.** SDS-PAGE and Immunoblot showing the specificity of the anti-megalin antibody (mAb2E12) for megalin. **A.** Kidney microsomal fraction (lane 2), affinity-purified megalin (lane 3), and the protein bound to the monoclonal antibody (mAb2E12) Sepharose column from a rat kidney microsomal fraction (lane 4) were analyzed by 5% to 20% SDS-PAGE and stained with Coomassie brilliant blue (lane 1: molecular marker). **B.** Kidney microsomal fractions (lanes 1, 2), affinity purified megalin (lanes 3, 4), and affinity purified cubilin (lanes 5, 6) were reduced, separated by 5% to 15% SDS-PAGE, immunoblotted with mAb2E12 (lanes 1, 3, 5) and anti-cubilin antibody (mAb7A3) (lanes 2, 4, 6), and then visualized by the diaminobenzidine reaction. Confocal immunofluorescence images of mAb2E12 (**C**) and mAb7A3 (**D**) in the kidney cortex. The figures show the merging of immunofluorescence (green) and electrical pseudo-differential interference contrast images. The staining of both is observed on the apical cytoplasm of the proximal tubules. Scale bar, 10  $\mu$ m.

the immunohistochemical analysis of kidney proximal tubules demonstrated that this antibody strongly stained the apical cytoplasm, containing the endocytic apparatus that is known to express megalin and cubilin (Fig. 1C and D, respectively). Therefore, we used mAb2E12 as the anti-megalin antibody.

### Immunoblot analysis of the endolymphatic sac

mAb2E12 cross-reacted with a single band (210 kDa) in endolymphatic sac preparations (Fig. 2, lane 1), whereas the antibody against the megalin cytoplasmic domain detected no band (lane 2). Immunoblotting of the kidney microsomal

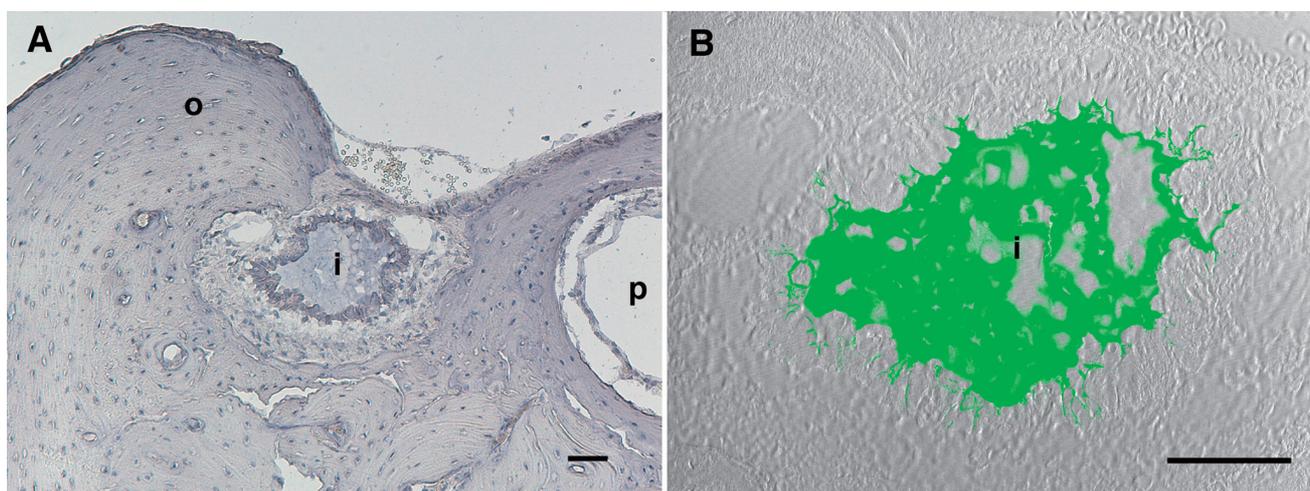


**Fig. 2.** Immunoblot analysis of ES and kidney microsomal fractions. ES (lanes 1, 2, 3) and kidney microsomal fractions (lanes 4, 5, 6) were reduced, separated by 5% to 20% SDS-PAGE, and then immunoblotted with the anti-megalin antibody (mAb2E12) (lanes 1, 4), anti-megalin antibody to the cytoplasmic domain (lanes 2, 5) and anti-cubilin antibody (mAb7A3) (lanes 3, 6). The positive bands were enhanced with a chemiluminescence reagent and detected with autoradiography film.

fraction revealed that mAb2E12 detected some bands in the range 210 kDa to 470 kDa (lane 4). This observation might be explained by the fact that megalin is known to undergo spontaneous degradation into smaller fragments (Kerjaschki and Farquhar, 1982; Makker and Singh, 1984; Jung *et al.*, 1998) and that the sensitivity of the immunoblots was markedly enhanced with the ECL in this experimental system. The antibody against the cytoplasmic domain of the megalin did not detect the 210-kDa band, although it did detect some bands in the range 100 kDa to 470 kDa (lane 5). The anti-cubilin antibody (mAb7A3) failed to detect any band in the ES (lane 3), whereas it detected a single cubilin band (in our experiment system, the 460-kDa protein, called cubilin or gp280 generally, had an estimated mass of approximately 370 kDa) in the kidney microsomal fraction (lane 6).

### **Immunohistochemical localization of megalin**

The lumen in the intermediate and distal portions of the ES was filled with granular, heterogeneous contents (Fig. 3A), whereas the proximal portion appeared optically empty. The luminal contents in the intermediate and distal portion were strongly stained with mAb2E12 (Fig. 3B). However, the anti-megalin antibody against cytoplasmic domain did not stain the luminal contents and the apical cytoplasm (data not shown). For non-decalcified tissue, the staining pattern of the ES was similar, indicating that the observed staining was not an artifact resulting from the decalcification procedure (not shown). In the cochlear duct, the marginal cells of the stria vascularis, the epithelial cells of Reissner's membrane, and the epithelial cells of the spiral prominence were stained with mAb2E12. Furthermore, the tectorial



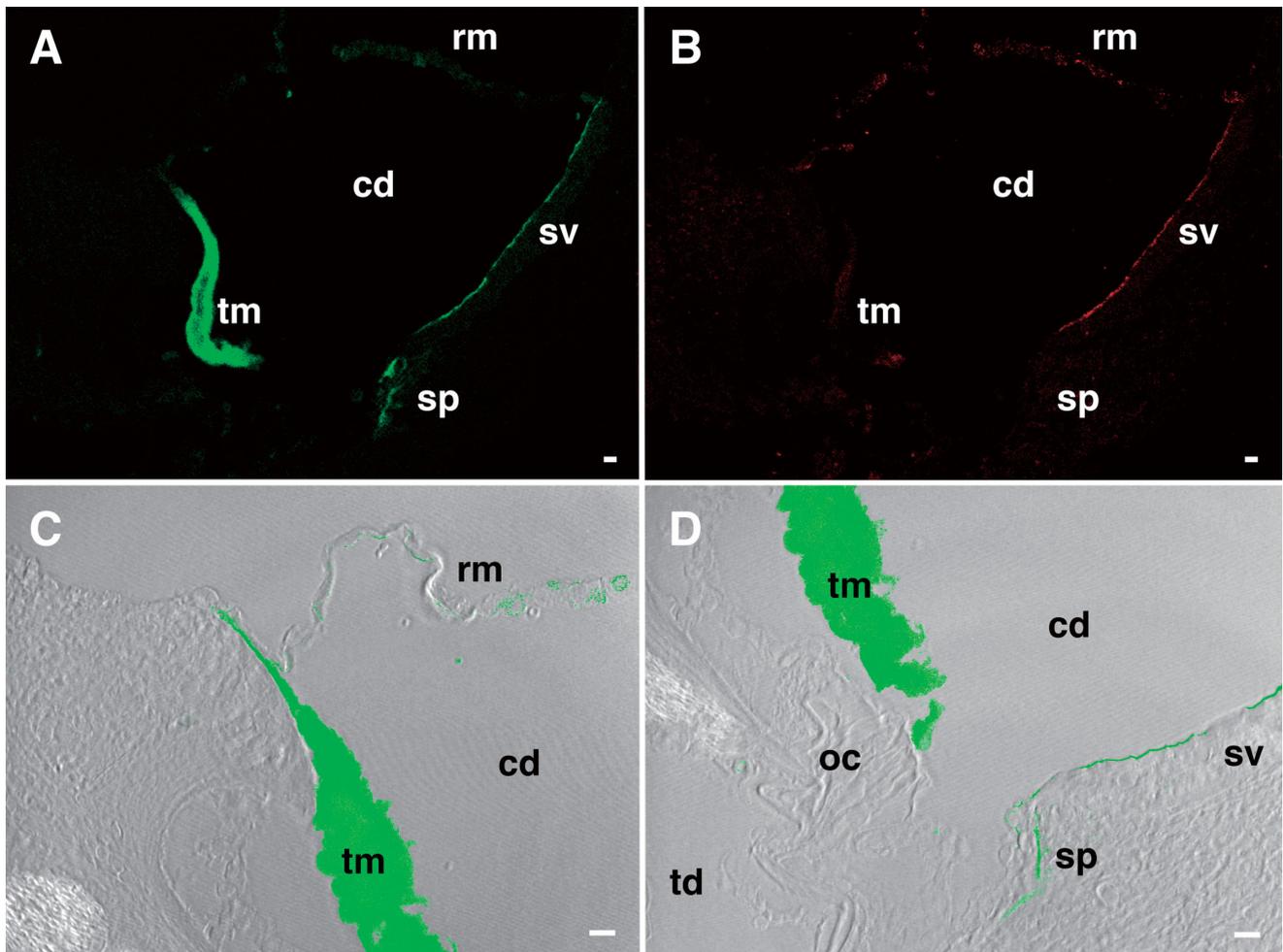
**Fig. 3.** Confocal immunofluorescence images of the anti-megalin antibody (mAb2E12) in the intermediated portion of the ES. **A.** Light micrographs of the intermediated portion are stained with hematoxylin. **B.** Intraluminal substances in this portion are strongly stained with mAb2E12. This figure shows the merging of immunofluorescence (green) and electrical pseudo-differential interference contrast images (*i*, intermediate portion of the ES; *o*, operculum; *p*, posterior semicircular canal). Scale bar, 50  $\mu$ m.

membrane was strongly stained, although the organ of Corti, including the interdental cells that are considered to be the cells that secrete the tectorial membrane matrix, was not stained (Fig. 4A, C, and D). The anti-megalin antibody against the cytoplasmic domain stained neither the tectorial membrane nor the epithelial cells of the spiral prominence, although it did stain the marginal cells of the stria vascularis and the epithelial cells of Reissner's membrane (Fig. 4B). The anti-cubilin antibody (mAb7A3) and the normal mouse IgG did not stain all the portions of the ES and the cochlear duct (not shown).

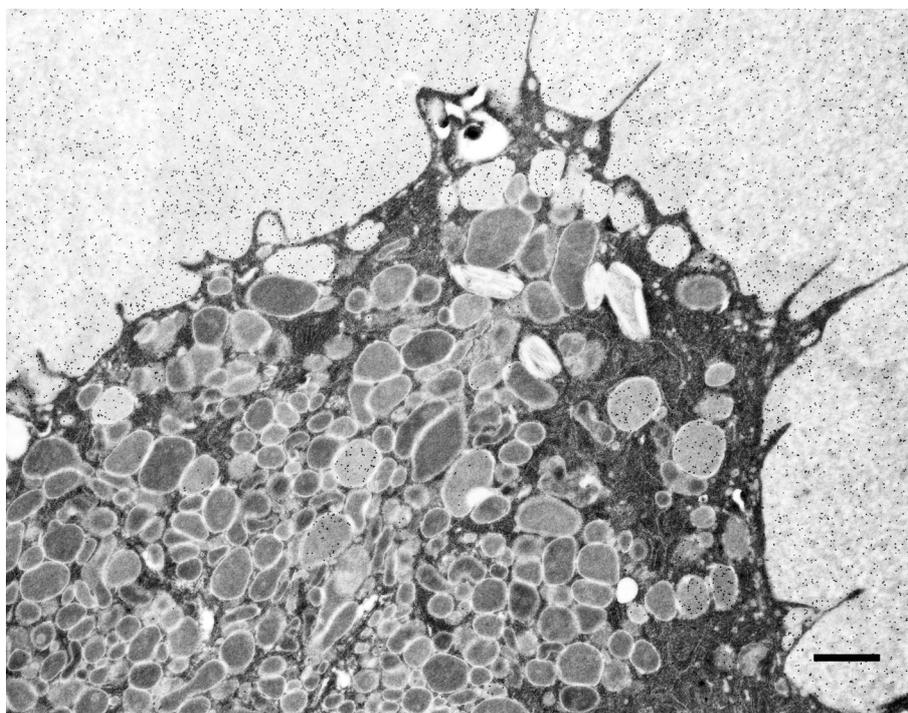
#### Ultrastructural localization of megalin in the ES

The results of the immunogold staining for megalin were

similar to those obtained for the immunofluorescence with cryostat sections. The luminal contents of the ES were strongly immunolabeled. Furthermore, the immunogold staining for megalin strongly stained macrophage-like cells, free-floating cells having invaginations, associated with macropinocytosis in the ES lumen. In these cells, the immunogold staining for megalin stained in the lumen of the apical vesicles (Fig. 5). Two types of ES epithelial cell could be distinguished. The mitochondria-rich cells possessed many long microvilli and mitochondria. On the other hand, the ribosome-rich cells were characterized by a smooth luminal surface with a few shorter microvilli and a few mitochondria that were scattered throughout the cytoplasm. Both types of epithelial cells had membrane protrusions and invaginations associated with macropinocytosis.



**Fig. 4.** Confocal immunofluorescence images of the anti-megalin antibody (mAb2E12) and the anti-cytoplasmic megalin antibody in the cochlear duct. The epithelial cells of Reissner's membrane (*rm*), tectorial membrane (*tm*), marginal cells of the stria vascularis (*sv*), and epithelial cells of the spiral prominence (*sp*) were stained with mAb2E12 (green in **A**, **C**, and **D**). The tectorial membrane (*tm*) and the epithelial cells of the spiral prominence (*sp*) were not stained with antibody against the cytoplasmic domain, although the epithelial cells of Reissner's membrane (*rm*) and the marginal cells of the stria vascularis (*sv*) were stained (red in **B**). The figures (**C** and **D**) illustrate the merging of the immunofluorescence (*green*) images of mAb2E12 and the electrical pseudo-differential interference contrast images (*vd*, vestibular duct; *cd*, cochlear duct; *oc*, organ of Corti; *td*, tympanic duct). Scale bar, 10  $\mu$ m.



**Fig. 5.** Immunocytochemical localization of megalin (10 nm gold) in a macrophage-like cell. The macrophage-like cell has many dense granules and vesicles in the cytoplasm. Some of these exhibit immunogold staining for megalin. Megalin is localized in the lumen of the vesicles. Scale bar, 1  $\mu$ m.

In these cells, megalin localized in the lumen of the apical vesicles, as well as the macrophage-like cells, although labeling intensities were lower than for the macrophages (Fig. 6A and B). The Golgi compartments and the deep cytoplasmic granules surrounding the Golgi exhibited extremely low-level immunolabeling for megalin. All control sections were also immunostained at extremely low levels (not shown).

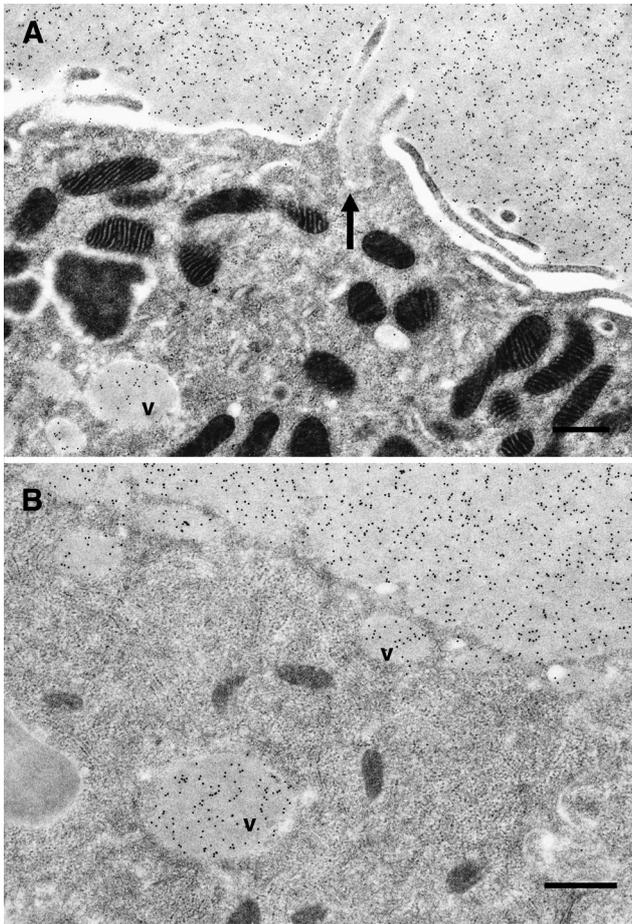
### Discussion

The present study revealed that megalin is localized in the ES, whereas cubilin is not. An unexpected finding was that both the localization and the molecular weight of the megalin were different from those in other absorptive tissues such as kidney proximal tubules and yolk sacs. In the ES, the megalin was demonstrated to be mainly accumulated in the lumen of the ES, and its molecular weight was smaller than the megalin found in the kidney. These results indicated that the megalin in the ES epithelium does not function as a membrane receptor for receptor-mediated endocytosis, but is merely one of the cellular components that are endocytosed from the lumen of the ES.

Our immunogold electron microscopic observations revealed that the megalin is localized in luminal macrophage-like cells and two types of epithelial cell. In the ES epithe-

lial cells, the megalin was localized in the lumen of the endosomes and was associated neither with the endosome membrane nor with the plasma membrane. Furthermore, structures suggestive of macropinocytosis were observed in the ES epithelial cells, as well as in the macrophage-like cells. Hence, the megalin in the lumen could be endocytosed through macropinocytosis by the macrophages and the ES epithelial cells. We believe that the endocytosed megalin is degraded in lysosomes. These results were different from the membrane-bound form of megalin and cubilin that function as membrane receptors in kidney proximal tubules and in yolk sac epithelial cells. In these tissues megalin and cubilin were localized in the membrane of endocytic compartments including coated pits, apical tubules, and apical vesicles for receptor-mediated endocytosis (Christensen *et al.*, 1998; Ishida *et al.*, 2004).

The immunoblotting demonstrated that the megalin in the ES has a molecular weight of 210 kDa and is smaller than the 600 kDa megalin expressed in the kidney. Furthermore, the ES megalin was lacking a cytoplasmic domain, although both the 210-kDa and 600-kDa megalins possess a domain that was stained with the anti-megalin antibody (mAb2E12). Previous studies have indicated that megalin exists in two forms: a membrane-bound form and a soluble form (Bachinsky *et al.*, 1993). Soluble forms of megalin have been demonstrated to be released into the medium of a rat yolk sac carcinoma cell line (Orlando and Farquhar,



**Fig. 6.** Immunocytochemical localization of megalin (10 nm gold) in the apical cytoplasm of ES epithelial cells. Megalin is localized in the lumen of the vesicles (v), in a mitochondria-rich cell (A) and in a ribosome-rich cell (B). Megalin is also localized in the lumen and the pseudopodia-like structures (arrow). Background gold labeling is extremely low in the cytoplasm. Scale bar, 500 nm.

1993), into the medium of an immortalized rat proximal tubule cell line expressing membrane-bound megalin (Jung *et al.*, 1998), and into normal human urine (Norden *et al.*, 2002). These soluble forms are fragments of approximately 200 to 220 kDa, suggesting they are truncated forms of the membrane-bound megalin. The similarity of the molecular weights of these megalins suggests that the megalin in the ES is also a soluble form, as opposed to a membrane-bound form. Soluble forms of various integral membrane receptors have previously been described, including a low-density lipoprotein receptor and an interleukin-6 receptor (Peters *et al.*, 1996; Begg *et al.*, 2004). Some soluble forms are produced by the release of the ectodomain via a single proteolytic cleavage of the membrane receptors, whereas others are secretory products (Ehlers and Riordan, 1991). Megalin possesses an RXRP sequence that constitutes a probable proteolytic processing site for precursor processing endo-

proteases of the furin family (Saito *et al.*, 1994). Such processing could be responsible for the generation of the soluble form of megalin (Farquhar *et al.*, 1995). Recent studies have shown that the ectodomain shedding of megalin is mediated by a metalloprotease in an opossum kidney proximal tubule cell line (May *et al.*, 2002; Zou *et al.*, 2004). The physiological significance of most soluble forms of receptor is poorly understood, although it is known that many soluble forms have ligand-binding capacity.

From where does the soluble megalin originate? We found no evidence that the soluble megalin originated from the ES epithelium. The Golgi compartments and the deep cytoplasmic granules surrounding the Golgi exhibited an extremely low-level immunolabeling for megalin. In rat embryos after gestational day 14, the endolymphatic duct did not stain for megalin, although the cochlear expressed megalin (Assemat *et al.*, 2005). Since the ES is connected to the cochlea, it is possible that the soluble megalin is transferred from the cochlea to the ES via the endolymphatic duct. In fact, we found that the tectorial membrane overlying the organ of Corti in the cochlea was particularly heavily stained with mAb2E12, although it was not stained with anti-cytoplasmic megalin antibody. It is likely that the tectorial membrane, which is an acellular superstructure, may contain the soluble form of megalin, although the molecular weight of this megalin remains undetermined. In addition to the tectorial membrane, the marginal cells of the stria vascularis, the epithelial cells of Reissner's membrane, and the epithelial cells of the spiral prominence were also stained with mAb2E12. These results are consistent with those described previously for cochlea epithelial cells obtained using immunogold electron microscopy, in which megalin was localized in the apical vesicles of these cells, and its localization pattern resembled that in kidney proximal tubules (Mizuta *et al.*, 1999). Previous studies have suggested that the ES promotes homeostasis in the membranous labyrinth by the regulation of endolymph volume and the elimination of endolymph debris (Thalmann and Thalmann, 1999). Furthermore, it has been proposed that endolymph is secreted from both the stria vascularis and the vestibular dark cell area, and is then reabsorbed in the ES (the longitudinal flow theory) (Lundquist, 1965; Kimura *et al.*, 1980; Manni and Kuijpers, 1987). Manni and Kuijpers (1987) demonstrated the existence of longitudinal flow under physiological conditions; i.e., the macromolecules are secreted into the endolymph, subsequently passed into the cupula and otolithic membrane, which has the same basic structure as the tectorial membrane, and after being released the macromolecules and/or their derivatives gradually move toward the ES. Therefore, we speculate that megalin is secreted by the marginal cells of the stria vascularis and/or the epithelial cells of Reissner's membrane and is incorporated into the tectorial membrane. Then, after being released, it is transported to the ES by means of longitudinal flow, and is eliminated by macropinocytosis. However, pre-

vious studies have suggested that the ES epithelial cells may be capable of secreting proteinaceous substances (Kimura and Schuknecht, 1965; Lundquist, 1965; Friberg *et al.*, 1986; Erwall *et al.*, 1988; Rask-Andersen *et al.*, 1999). Hence, we cannot rule out the possibility that the soluble megalin is generated by alternative RNA splicing and is exocytosed into the lumen of the ES.

To our knowledge, this is the first report that demonstrates the localization of megalin in the ES. This localization and the structure of the megalin in the ES are different to those in other absorptive tissues. Further studies are required to define the exact structure of the megalin in the ES, to determine its ligand-binding potential, and to ascertain the functional importance of the soluble megalin.

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