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Cubilin- and megalin-mediated uptake of albumin in cultured proximal tubule cells of opossum kidney

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Cubilin- and megalin-mediated uptake of albumin in cultured proximal tubule cells of opossum kidney.

Background. Reabsorption of albumin from the glomerular filtrate occurs via receptor-mediated endocytosis in the proximal tubule. This process is initiated by binding of albumin in apical clathrin-coated pits, followed by endocytosis and degradation in lysosomes. Although binding sites have been characterized by kinetic studies, the receptors responsible for the binding of albumin have not been fully identified. Two giant glycoproteins, cubilin and megalin, constitute important endocytic receptors localized to the kidney proximal tubule.

Methods. In the present study, we examined the colocalization of cubilin and megalin in the endocytic pathway and the relationship between the uptake of albumin and the expression of cubilin and megalin in opossum kidney (OK) proximal tubule cells by immunocytochemistry and immunoblotting.

Results. OK cells expressed both cubilin and megalin. The light microscope labeling patterns for cubilin and megalin were almost identical and were mainly located at the surface area of the cells. Cubilin and megalin were also shown to colocalize on cell surface microvilli, in coated pits, and in endocytic compartments at the electron microscope level. Endocytosed bovine serum albumin (BSA) was identified exclusively in cells expressing megalin and cubilin. Uptake of BSA-FITC was saturable and inhibited by receptor-associated protein (RAP) and by intrinsic factor-vitamin B_{12} complex (IF-B_{12}) at high concentrations. Significant inhibition was also observed by specific antibodies to cubilin, and megalin and cubilin antisense oligonucleotides likewise significantly reduced albumin uptake. Egg albumin did not affect the uptake of BSA.

Conclusion. The present observations suggest that the two receptors cubilin and megalin are both involved in the endocytic uptake of albumin in renal proximal tubule cells.

Albuminuria is an important indicator and possible pathogenic factor in renal disease, focusing significant interest on the mechanisms behind renal tubular reabsorption of albumin. Although several binding sites of albumin to proximal tubule cells have been characterized by kinetic studies, the receptor(s) responsible for the binding remains obscure.

Our previous micropuncture study demonstrated that in vivo, the reabsorption of albumin in the kidney is achieved by endocytosis and transport into endocytic compartments mediated by megalin [1], a 600 kD multiligand endocytosis receptor [2]. Furthermore, low molecular weight albumin binding proteins (ABPs) have been purified from brush border membranes of rat renal proximal tubule and have been characterized by their distribution at microvilli and in apical endosomes along the proximal tubule by immunocytochemistry [3].

Cubilin, identical to intrinsic factor receptor (IFR) [4], and megalin constitute endocytic membrane glycoprotein receptors in renal proximal tubule epithelium. Megalin is expressed in many absorptive epithelial cells [5, reviewed in 6], whereas cubilin constitutes a slightly smaller, 460 kD endocytic glycoprotein receptor [7], recently cloned [8] and detected so far in only renal proximal tubule [9], yolk sac [9], and ileal enterocytes [10]. Only a few ligands for cubilin have been identified to date, including intrinsic factor-vitamin B_{12} (IF-B_{12}) [4, 7], apolipoprotein A-I [11], high density lipoprotein (HDL) [11], and myeloma light chains [12]. Cubilin and megalin are coexpressed and colocalized in the brush border and the endocytic pathway of the renal proximal tubule epithelium as well as yolk sac epithelial cells [8, 9]. They also copurify from solubilized rabbit renal cortex by using receptor-associated protein (RAP) as an affinity target [7]. The absence of functional cubilin produces proteinuria in patients suffering from hereditary intestinal malabsorption of IF-B_{12} [13, 14] and in dogs with an inherited functional deficiency of cubilin [15], and the absence of megalin produces proteinuria in megalin knockout mice

Key words: renal protein uptake, endocytosis, albumin binding proteins, glycoprotein receptor, vitamin B_{12}, tubular reabsorption.

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[16–19]. The observation that cubilin lacks an obvious transmembrane domain and a cytoplasmic tail [8] suggests that its internalization and intracellular trafficking could be accomplished with the aid of another membrane receptor. Since cubilin calcium dependently and with high affinity binds to megalin [8], these observations together might suggest an interaction between the two receptors in mediating endocytic uptake of different ligands.

Opossum kidney (OK) cells, an established OK cell line, possess similarities to the proximal tubule epithelium in terms of membrane transport and electrical properties [20, 21]. Therefore, this cell line has been widely used as a model system for investigating binding of albumin to the cell membrane, receptor-mediated endocytosis, and regulation of the endocytic pathway [22–26].

To characterize the binding sites for albumin on the plasma membrane of renal proximal tubule and the subcellular colocalization of cubilin and megalin, we studied the relationship between the uptake of albumin and the expression of cubilin and megalin. We suggest that cubilin and megalin are both involved in the tubular reabsorption of albumin.

METHODS

Cell culture

Opossum kidney cells were purchased from American Type Culture Collection (ATCC; CRL-1840, Rockville, MD, USA). Cells (passages 17 to 42) were routinely grown in plastic culture flasks (75 cm^2; catalog no. 430720 Corning Costar, Badhoevedorp, Holland), in Eagle minimum essential medium (EMEM; catalog no. 12-136F; Bio-Whittaker, Walkersville, MD, USA), with 10% fetal calf serum (lot no. 055,54; Biological Industries, Fredensborg, Denmark), 2 mmol/L L-glutamine (catalog no. 17-605E; Bio-Whittaker), and 50 U/mL penicillin and 50 μg/mL streptomycin (catalog no. 17-603E; Bio-Whittaker), at 37°C in a humidified atmosphere containing 5% CO_2. The cells were passaged every third day after plating with a split ratio of 1:5 by 15-minute trypsinization (500 mg/L Trypsin, 200 mg/L Versene; catalog no. 17-161E; Bio-Whittaker). Experiments were conducted with confluent monolayers of OK cells cultured either in eight chamber glass slides (catalog no. 154534; Nalge Nunc International, Naperville, IL, USA) for immunohistochemical and uptake studies, or on Transwell inserts (catalog no. 3450; diameter of 24 mm; pore size of 0.4 μm; Costar, Cambridge, MA, USA) for electron microscope investigations. Cell handling was performed in a laminar flow hood, and cultured cells were observed with an inverted microscope (Leitz, Wetzlar, Germany).

Antibodies and chemicals

Bovine serum albumin (BSA)-FITC, BSA, and egg albumin were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Recombinant RAP [27], polyclonal sheep anti-rat megalin [28], polyclonal rabbit anti-rat cubilin (L242, 403) [29], and monoclonal mouse anti-rat cubilin (mAb 75) [9] were obtained as previously described. Sheep serum (x0503), rabbit anti-sheep serum (z0138), rabbit serum (x0902), swine anti-rabbit-TRITC Ig (R156), rabbit anti-mouse-TRITC Ig (R270), and rabbit anti-sheep-FITC Ig (F135) were from Dako A/S (Copenhagen, Denmark). For inhibition experiments, the immunoglobulins, specific and nonspecific, were purified by protein-A affinity chromatography.

Human IF-B_{12} complexes were kindly provided by Professor Ebba Nexø (Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark).

Immunoblotting/Western blotting

Preparation of opossum kidney cell membranes. OK cells at confluency were detached from the bottom of the flasks by scraping in dissection buffer [0.3 mol/L sucrose, 25 mmol/L imidazol, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.2]. The cells were spun down at 1000 × *g*, 4°C, for five minutes. The pellet containing the cells was resuspended in 1.5 mL dissection buffer, and the cells were homogenized by five strokes at 1250 r.p.m. and centrifuged by 4000 × *g* at 4°C for 15 minutes. The pellet was homogenized and centrifuged again as described previously in this article. The supernatants were pooled and ultracentrifuged (L8-70 M ultracentrifuge; Beckman Instruments, Fullerton, CA, USA) for one hour, 4°C, at 200,000 × *g*. The final pellet containing the cellular membranes was resuspended in sample buffer.

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 4 to 16% polyacrylamide gradient gels and 3% SDS in the sample buffer. The proteins were electroblotted onto nitrocellulose membranes (Hyperbond ECL nitrocellulose; Amersham, Buckinghamshire, UK). After blotting, the membranes were blocked for one hour in phosphate-buffered saline (PBS)/0.1% Tween/5% low fat dry milk, followed by washing three times for 25 minutes totally in PBS/0.1% Tween. Subsequently, the blots were incubated with rabbit anti-rat cubilin antibodies or sheep anti-rat megalin antibodies diluted 1:5000 and 1:20,000, respectively, in PBS/0.1% Tween/1% BSA/2 mmol/L sodium azide, overnight at 4°C. The blots were washed as described previously in this article and incubated for one hour with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit Ig 1:3000 or rabbit anti-sheep Ig 1:9000). After washing, the labeling was visualized by enhanced chemiluminescence (Amersham). As positive controls, affinity-purified cubilin and megalin were used.

Immunofluorescence microscopy

To study the uptake of the BSA-FITC and the expression of cubilin and megalin, direct and indirect double-labeling immunofluorescence techniques were applied to the confluent monolayer of OK cells grown in chambers.

Coexpression of cubilin and megalin. After fixation with 2% formaldehyde for two minutes and preincubation with PBS (0.01 mol/L, pH 7.4) containing 0.1% low fat dry milk for 15 minutes, the confluent monolayers of cells were incubated with a mixture of primary antibodies (monoclonal mouse anti-rat cubilin 0.5 μg/mL and polyclonal sheep anti-rat megalin 1:2000 for one hour) and then incubated with a mixture of secondary antibodies, TRITC coupled to rabbit anti-mouse 1:20, and FITC coupled to rabbit anti-sheep 1:40 for one hour. A mixture of mouse and sheep immunoglobulins was used as control. All steps described previously in this article were performed at room temperature. The slides were mounted in 50% glycerol containing 2% N-propylgallat and 2.4% Tris and were examined in an immunofluorescence microscope (Leica DMR) equipped with a Sony 3CCD color video camera attached to a Sony Digital Still recorder. Images were processed using Adobe Photoshop 4.0 and printed on Epson photo quality paper with an Epson Stylus 740 color printer.

Colocalization of the uptake of BSA and cubilin or megalin. Confluent monolayers of cells were preincubated for 24 hours with serum-free EMEM containing 2 mmol/L glutamine, followed by incubation with 40 μg/mL BSA-FITC for one hour under the same conditions mentioned previously in this article. After fixation with pure acetone for one minute or 2% formaldehyde for two minutes, the cells were incubated with rabbit anti-rat cubilin antibodies 1:500 or sheep anti-rat megalin antibodies 1:2000 for one hour, subsequently incubated with TRITC-coupled swine anti-rabbit Ig 1:20 or rabbit anti-sheep serum 1:10,000 for one hour, followed by incubation with TRITC-coupled swine anti-rabbit Ig 1:20. For uptake of BSA only, incubation with unlabeled BSA was used as control, and for the colocalization studies, nonspecific rabbit and sheep serums were used as controls. The antibody incubations were carried out at room temperature.

Competitive inhibition experiments of binding of BSA to receptors. After preincubation for 24 hours in serum-free medium, OK cells were incubated for 10 minutes with 10 μg/mL BSA-FITC mixed with the following potential competitors of uptake: 0.25 to 1 μmol/L RAP, 30 μmol/L intrinsic factor, 30 μmol/L unlabeled BSA, 30 μmol/L egg albumin, 100 to 400 mg/L purified sheep polyclonal anti-rat megalin IgG, or rabbit polyclonal anti-rat cubilin IgG. The cells were fixed with 2% formaldehyde for two minutes, further processed as described previously in this article, and examined in the microscope for uptake of BSA.

As controls for antibody inhibition experiments, purified nonspecific sheep IgG and rabbit IgG were used.

Quantitative uptake studies

Quantitative uptake studies were performed as described earlier [25, 30, 31]. Briefly, OK cell monolayers were grown on plastic Petri dishes, and the experiments were performed at day 9 after seeding. Prior to the determination of BSA uptake, the monolayers were washed thoroughly three times at pH 6.0 in order to remove proteins or amino acids that were present during the preincubation period. Subsequently, the monolayers were incubated with Ringer solution containing 10 μg/mL of FITC-labeled BSA (Sigma, 83039 Deisenhofen, Germany), ^{14}C-BSA (ARC, St. Louis, MO, USA), or 20 nmol/L FITC-IF-B_{12} for 15 minutes at 37°C. The IF-B_{12} complexes were labeled by FITC as described by the manufacturer (Pierce, Rockford, IL, USA). As described before, albumin is taken up by endocytosis under these experimental conditions [25, 30, 31]. Uptake was stopped by rinsing the monolayers eight times with ice-cold Ringer's solution. The last rinsing step was controlled routinely for fluorescence, which was not significantly different from background. Nonspecific uptake was determined by including 1000-fold excess of unlabeled albumin. Nonspecific uptake was approximately 10% for FITC-BSA and for ^{14}C-BSA. ^{14}C-BSA was used with high concentrations of inhibitors in order to repeat the experiments, despite the limited availability of some substances. When we compared the inhibitory effects of our maneuvers on FITC-BSA and ^{14}C-BSA uptake, there was no significant difference. Thus, the data were pooled, and we refer to FITC-BSA and ^{14}C-BSA as labeled BSA. Cells were disintegrated by detergent (Triton X-100 0.1% vol/vol in MOPS solution, which guaranteed that all fluorescence measurements were performed at pH 7.4), and the cell-associated fluorescence was measured using a spectrofluorometer according to Gekle et al [30]. Cell-associated radioactivity was determined by β-counting. Protein was determined by the method of Lowry et al [32]. Ringer's solution was composed of (mmol/L) NaCl 122.5, KCl 5.4, $CaCl_2$ 1.2, $MgCl_2$ 0.8, Na_2HPO_4 0.8, NaH_2PO_4 0.2, glucose 5.5, and HEPES 10 (titrated to different pH values with 1 mol/L NaOH or HCl). The inhibition constant (IC_{50}) was calculated according to DeLean, Munson, and Rodbard [33] using the formula, $J = J_0/[1 + ((C/IC_{50})^n)]$, where J_0 is the control uptake, C is the concentration of the inhibitor, IC_{50} is the half-maximal inhibitor concentration, and n is the slope of the curve. Curve fitting was performed according to the least-square method using the Sigma Plot for Windows 2.1 software (Jandel Scientific, Corte Madera, CA, USA).

To gain insight into whether interference with the expression of cubilin may affect albumin uptake, transient transfection with phosphorothioate-modified cubilin antisense or sense oligonucleotides was performed. To select a relevant oligonucleotide sequence, the cDNA produced by reverse transcription of mRNA from OK cells was amplified by PCR using two primers located within the first CUB domain of rat cubilin (upper, TGCCTAC CACAGCCCAAATGA, and lower, AGAGCCACA

ATGACTGCAG). The amplified 0.4 kb fragment was subcloned in PGEM (Promega, Madison, WI, USA) and end sequenced. The 5′ sequence (first 70 bases) was 95% homologous with rat cubilin, including the exact sequence of the upper primer. The 3′ sequence showed 75% homology only with rat cubilin. Consequently, the following OK-cubilin sequences were used (MWG-Biotech AG, Ebersberg, Germany): antisense, 5′-TCATTT GGGCTGTGGTAGGCA-3′, sense, 5′-TGCCTACCA CAGCCCAAATGA-3′.

For these experiments, cells were transfected for 72 hours in serum-free medium using PerFect Lipid (pFx-4) reagent (Invitrogen, Groningen, The Netherlands) as recommended by the manufacturer. Thereafter, albumin uptake was determined in control cells, cells treated with transfection reagent alone, or the oligonucleotides.

Immunocytochemistry

To examine the subcellular localization of cubilin and megalin in OK cells, double gold labeling immunocytochemistry was carried out.

Opossum kidney cells at confluent monolayers were detached from Transwell inserts by scraping and sedimentation. The cells were fixed in 0.1 mol/L sodium cacodylate buffer, pH 7.2, containing 2% formaldehyde, for 10 minutes, and were then spun down at 500 × *g* for 2 minutes at 4°C. The cells were washed three times in cacodylate buffer, had a few drops of 15% gelatin added, and were infiltrated for 30 minutes with 2.3 mol/L sucrose containing 2% paraformaldehyde. The blocks containing the cells were frozen in liquid nitrogen. Cryosections with a thickness of 70 to 90 nm were prepared for electron microscopy with a FCS Reichert Ultracut E ultramicrotome. For EM double labeling experiments, sections were incubated with a mixture of primary antibodies: polyclonal rabbit anti-rat cubilin diluted 1:200 to 1:400 and polyclonal sheep anti-rat megalin diluted 1:2000 to 1:4000 in 0.01 mol/L PBS with 0.05 mol/L glycine and 0.1% nonfat dry milk overnight at 4°C. After probing with primary antibodies, the sections were incubated with 5 nm gold-conjugated goat anti-sheep Ig (1:50; British BioCell International, Cardiff, UK) mixed with 10 nm gold-conjugated goat anti-rabbit IgG (1:50). Finally, the sections were embedded in 2% methylcellulose containing 0.3% uranylacetate and were examined in a Phillips CM 100 electron microscope.

RESULTS

Identification of cubilin and megalin on OK cell membranes

The expression of cubilin and megalin on purified membranes of OK cells was analyzed by Western blotting using polyclonal anticubilin and megalin antibodies. As shown in Figure 1, the specific antibodies identified two high molecular weight proteins at the position corresponding to either cubilin or megalin.

Immunocytochemical colocalization of cubilin and megalin

Coexpression of the two giant glycoprotein receptors was demonstrated by a double-labeling immunofluorescence technique on the confluent monolayers. The labeling patterns for cubilin and megalin were almost identical, that is, expressed in the same cells, and were mainly located at the surface of OK cells. The intensity of labeling among cells varied significantly from area to area (Fig. 2A, B), and in some areas, the cells did not express the two receptors at all. The subcellular distribution of cubilin and megalin was also visualized at the electron microscope level by double-labeling immunocytochemistry. Both receptors were similarly distributed at microvilli areas and often concentrated in clathrin-coated pits and invaginations at the apical cell membrane and colocalized in small and large endocytic vacuoles, as well as in dense apical tubules (Fig. 3).

Colocalization of uptake of BSA-FITC, cubilin, and megalin

The relationship between the uptake of BSA-FITC and expression of cubilin or megalin is shown in Figure 2C and D. Cells showing punctuate labeling with BSA-FITC throughout the cytoplasm representing endocytosed BSA were also peripherally labeled with TRITC coupled to antibodies against cubilin or megalin. Conversely, in areas where the cells were not labeled with BSA-FITC, there was also no labeling for cubilin and megalin, indicating an association between these two receptors and the endocytic uptake of albumin. Although the intensity of BSA-FITC varied from area to area, the labeling pattern was the same. On control slides substituting nonspecific serum for specific serum, the cells labeled with BSA-FITC were not labeled with TRITC.

Qualitative and quantitative evaluation of competitive inhibition of BSA uptake

The uptake of BSA-FITC was significantly reduced by the presence of different inhibitors. As shown in Figure 4, uptake of BSA-FITC was inhibited strongly by RAP and IF-B_{12} complex, was partially inhibited by unlabeled BSA (discussed later in this article), and was not inhibited at all by egg albumin. Uptake was also significantly inhibited by specific antibodies and to a certain extent by nonspecific IgG, especially the rabbit IgG (discussed later in this article).

These morphologic results are consistent with those from the quantitative analysis of competitive inhibition of uptake of albumin (Fig. 5). The addition of the megalin-ligand RAP resulted in a dose-dependent reduction

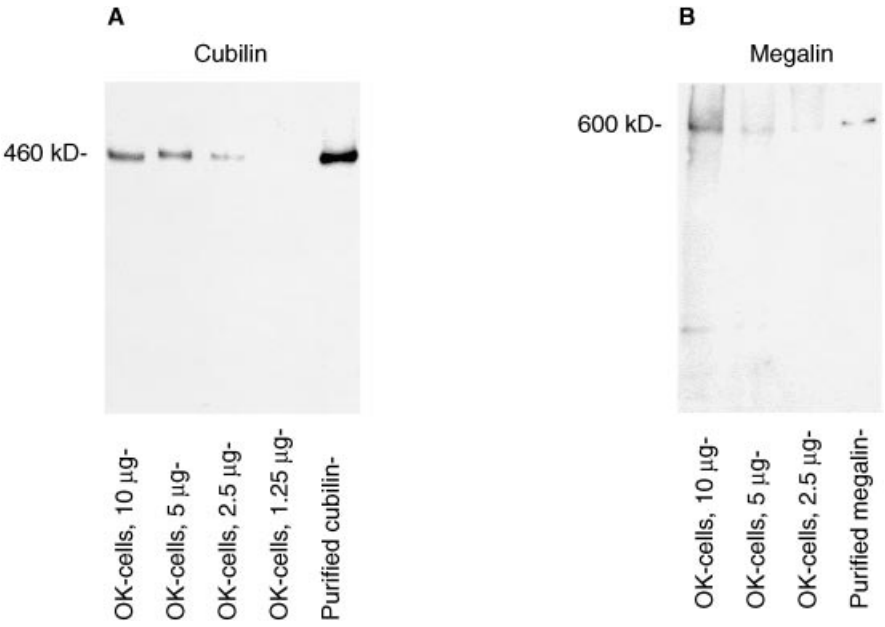

Fig. 1. Detection of cubilin (*A*) and megalin (*B*) on opossum kidney (OK) cellular membranes by Western blotting. From right to left, the lanes represent purified cubilin (*A*) or megalin (*B*) and increasing concentrations of membrane proteins. Details are discussed in the **Methods** section.

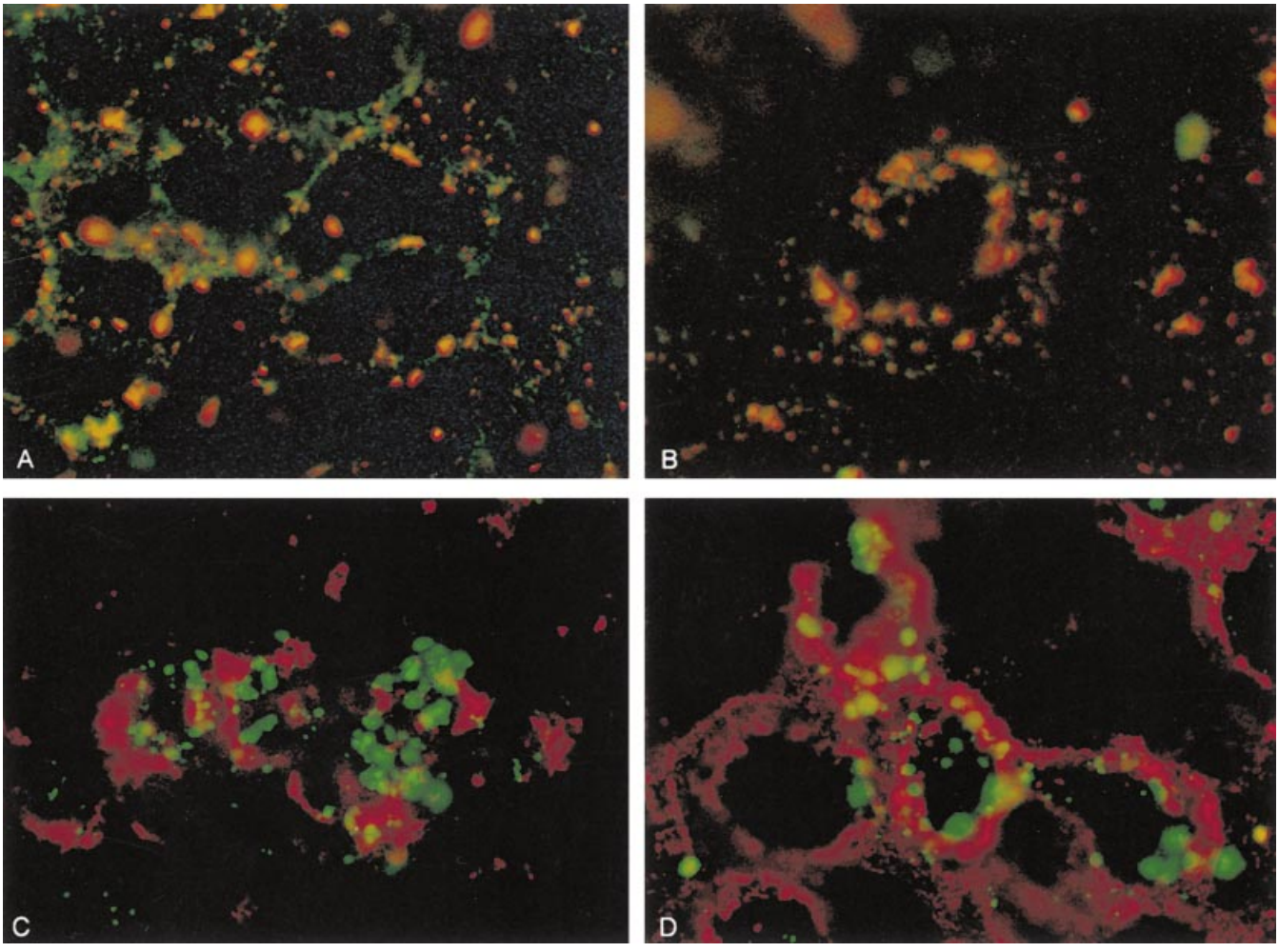

Fig. 2. (*A* and *B*) Immunofluorescent localization of cubilin and megalin illustrating colocalization of cubilin (TRITC) and megalin (FITC) in confluent OK cells. The central part in (*B*) represents one cell. The yellow color illustrates exact colocalization of the two receptors (*A* ×1100, *B* ×1800). (*C*) Immunofluorescent colocalization of BSA-FITC and cubilin or megalin (*D*) illustrated by TRITC labeling. The yellow color represents exact colocalization of endocytosed BSA and the receptor (*C* and *D* ×1400).

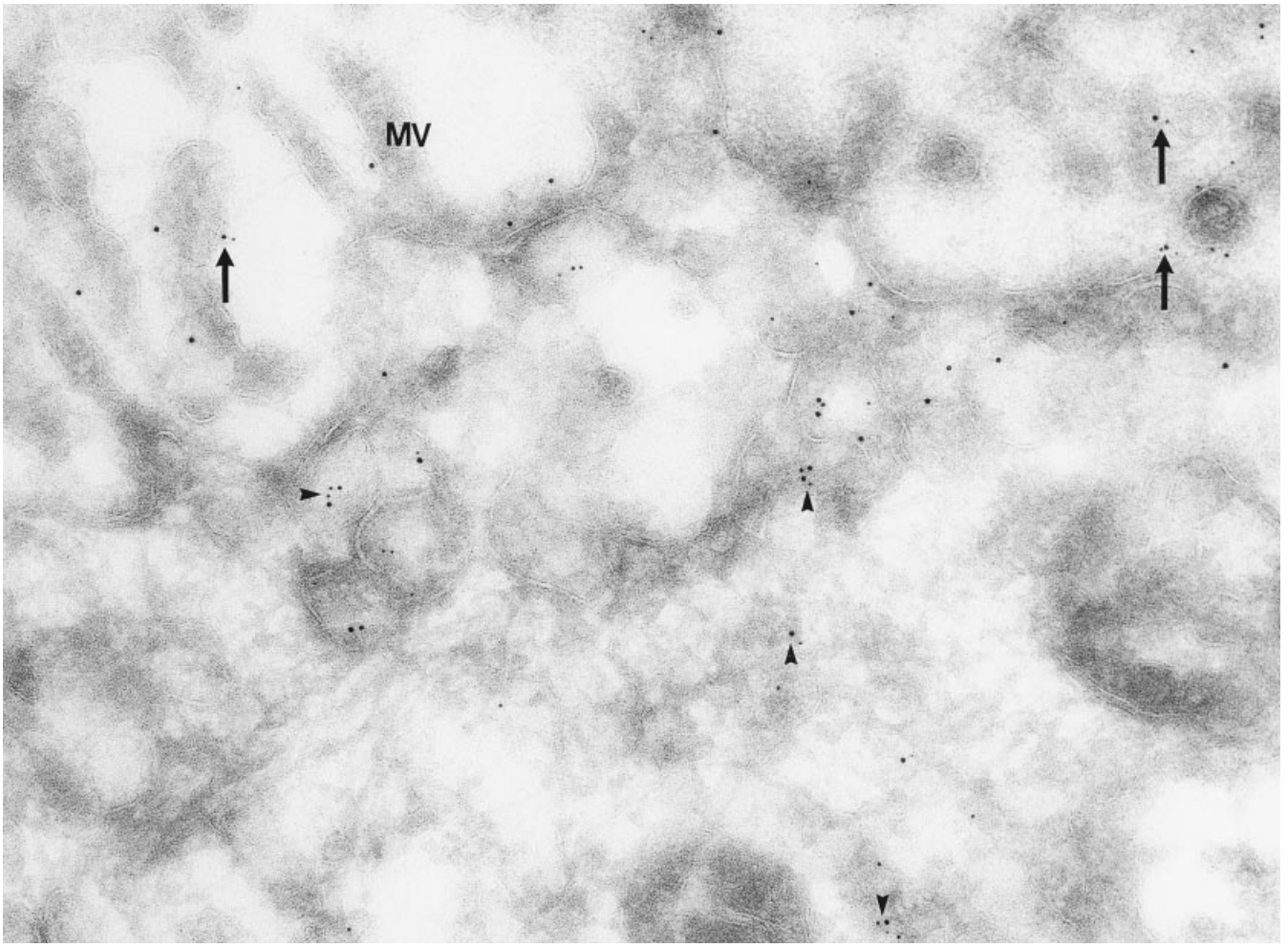

Fig. 3. Electron microscope immunocytochemical double labeling for megalin and cubilin. Megalin, 5 nm gold particles, and cubilin, 10 nm gold particles, are distributed together at the apical plasma membrane (arrows) and in endocytic vacuoles (arrowheads) in OK cells. Apical microvilli (MV) are seen in top of the micrograph (×86,000).

of albumin endocytosis. The calculated inhibitory constant (IC_{50}) was 17 nmol/L, a value close to the affinity constant of RAP binding to megalin [34]. The addition of the cubilin ligand IF-B_{12} complex also resulted in a dose-dependent reduction of albumin uptake. The IC_{50} value for IF-B_{12} was 1.7 μmol/L and was therefore higher as compared with the affinity constant of IF-B_{12} [7]. This suggests that the binding site for albumin is different from the high affinity binding site for IF-B_{12} in CUB domain 5 to 8 of cubilin [35]. To test whether RAP and IF-B12 interact with albumin endocytosis at the same site, we added 3 μmol/L IF-B_{12} in the presence of 300 nmol/L RAP (that is, at maximum RAP inhibition). Albumin uptake in the presence of 300 nmol/L RAP + 3 μmol/L IF-B_{12} was significantly lower (42 ± 3% of control, $N = 4$) as compared with uptake in the presence of 300 nmol/L RAP alone (60 ± 2% of control, $N = 4$). These data indicate that RAP and IF-B_{12} inhibit albumin uptake, at least in part, at different sites. Egg albumin (10 μmol/L) did not affect albumin endocytosis at all (albumin uptake in the presence of egg albumin was 96 ± 5% of control, $N = 6$). By contrast, 10 μmol/L unlabelled albumin reduced the uptake of labeled albumin significantly to 30 ± 5% of control ($N = 8$), and 100 μmol/L reduced uptake to approximately 10% of control [25].

The addition of specific antibodies to megalin or cubilin led to a significant inhibition of albumin uptake as compared with nonspecific IgG (Fig. 6), about 30 and 40%, respectively. However, the nonspecific IgG also exerted an inhibitory action on albumin uptake at concentrations of ≥200 mg/L. The inhibitory effect of nonspecific IgG was not surprising because cubilin has been reported to bind light chains [12]. Because of this nonspecific effect of IgG, we did not test higher antibody concentrations or the combination of the two specific antibodies.

Incubation of OK cells with cubilin antisense oligonucleotides led to a significantly reduced rate of albumin uptake, as compared with cubilin sense oligonucleotides

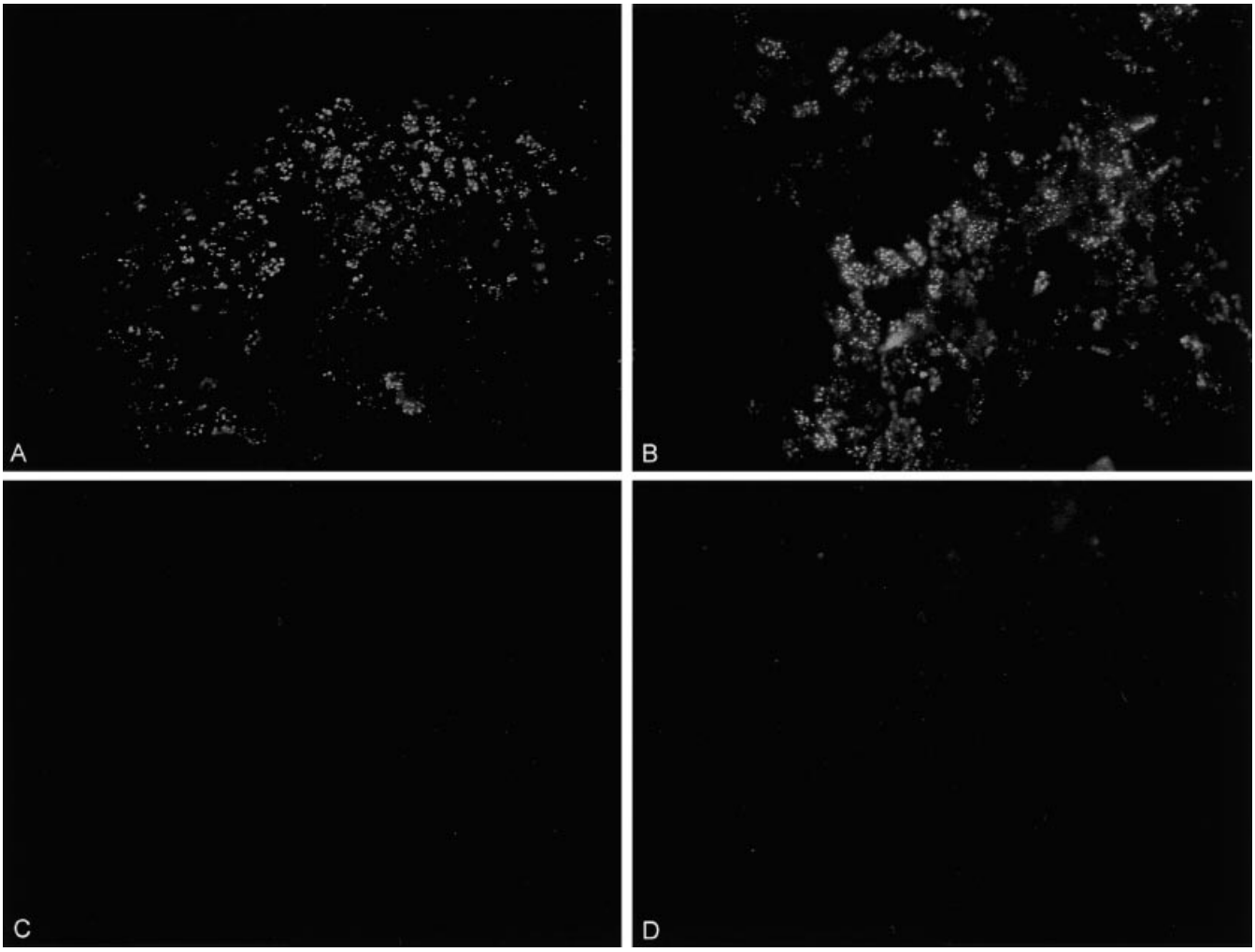

Fig. 4. Immunofluorescence microscopy of inhibition experiments on confluent OK cell monolayers. (*A*) The uptake of BSA-FITC in control experiments. (*B*) No inhibitory effect of 30 μmol/L egg albumin. (*C*) Almost complete inhibition of uptake by 1 μmol/L RAP. (*D*) Very strong inhibition by 30 μmol/L human intrinsic factor (*A, B* and *D* ×300; *C* ×800).

or transfection reagent alone. Incubation with 1 μmol/L antisense oligonucleotide reduced albumin uptake to 79 ± 4% of control ($N = 12$, $P < 0.05$), whereas 1 μmol/L sense oligonucleotide or transfection reagent did not exert significant effects (102 ± 2% and 98 ± 5% of control, respectively, $N = 12$). At 10 μmol/L, the oligonucleotides exerted toxic effects, as seen by a decrease in cell number (data not shown).

Furthermore, in similar experiments, the uptake of FITC-IF-B_{12} in sense-treated cells was 86 ± 8% of control ($N = 8$). Uptake in antisense-treated cells as compared with sense-treated cells was 71 ± 7% ($N = 8$, $P < 0.05$).

DISCUSSION

In the present study, we have identified cubilin and megalin in OK cell membranes and demonstrated coexpression and colocalization of both glycoproteins by immunocytochemistry and Western blotting. We therefore used the OK cell line to reveal the qualitative and quantitative relationship between the expression of both glycoprotein receptors and the uptake of albumin.

Previous kinetic studies on binding of albumin to brush-border membrane vesicles from renal proximal tubules [36] and to the cell membrane of OK cells [23, 24, 26] suggested that the reabsorption of albumin along the renal proximal tubule lumen occurs by receptor-mediated endocytosis and that the glycoprotein scavenger receptor(s) is involved in this saturable binding process [22]. Furthermore, there are several indications that cubilin and megalin are both involved in the process of the reabsorption of protein and possibly albumin, including (*1*) a similar subcellular distribution at microvilli areas of the cell surface and endocytic compartments in rat renal proximal tubule and yolk sac epithelial cells [8, 9]; (*2*) both receptors mediate binding and subsequent inter-

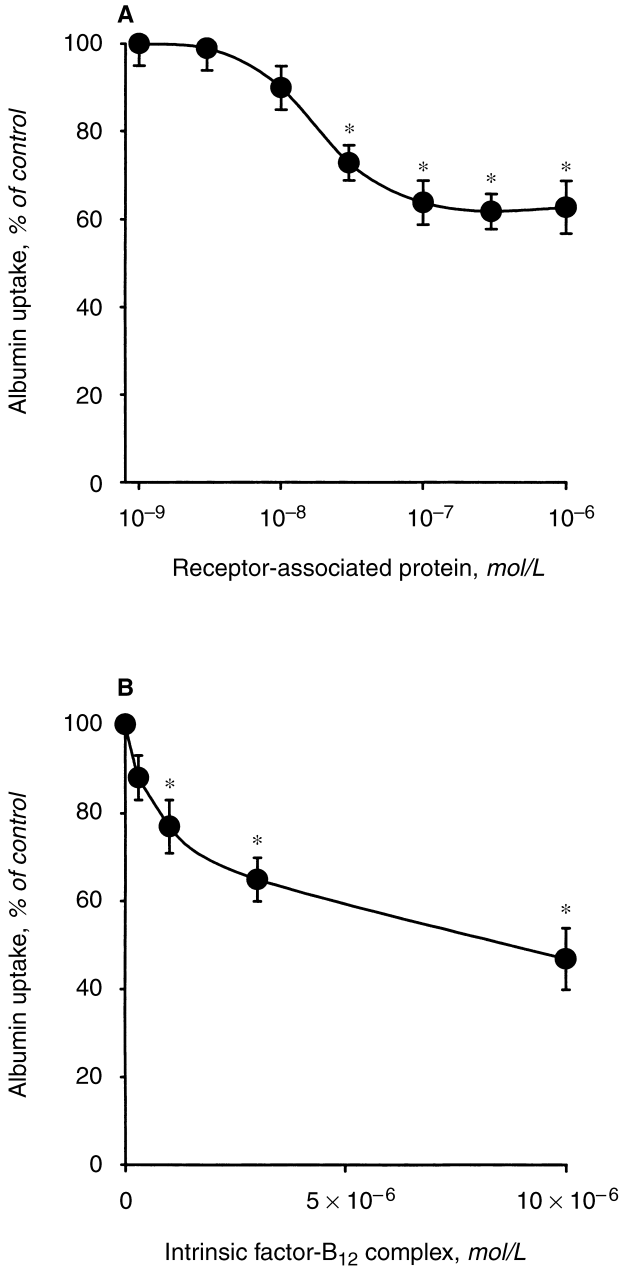

Fig. 5. Concentration-dependent inhibition of albumin uptake by receptor-associated protein (*A*) or by intrinsic factor-B_{12} complex (*B*). $N = 6$ to 9; $^*P < 0.05$.

nalization of proteins in proximal tubule epithelium and probably during fetal development in yolk sacs [6]; (*3*) functional defects of both receptors cause proteinuria [16–19, 37]; (*4*) studies from our lab have shown that albumin can bind to megalin [1]; and (*5*) cubilin has no apparent molecular signal for internalization of the receptor-ligand complex and does bind to megalin with a calcium-dependent high affinity, suggesting megalin to mediate the internalization of cubilin in complex with IF-B_{12} [7, 8].

The present study shows that megalin and cubilin are both involved in albumin uptake in OK cells. First, the colocalization of megalin and cubilin was established in OK cells both at the light microscopic and the ultrastructural level. Furthermore, a clear correlation between the expression level of the two receptors on individual cells was identified by immunofluorescence. Double labeling using fluorescent albumin and antibodies against either of the two receptors always showed a strong correlation between the expression of both receptors and the uptake of albumin. The variation in receptor expression between individual cells is most probably due to cellular developmental differences. Thus, mature cells with a high level of receptor expression were also most effective in albumin uptake. Second, inhibition of albumin uptake was observed with antibodies against both megalin and cubilin as well as with RAP and IF-B_{12}. RAP serves as a chaperone for members of the low-density lipoprotein receptor family, of which only megalin has been identified in renal proximal tubule cells. Binding of virtually all ligands to megalin [6, 38–41], including cubilin [8], is significantly inhibited by RAP, and as we have previously shown, in vivo RAP is a strong inhibitor of albumin uptake in the proximal tubule [1]. RAP also binds with low affinity to cubilin; however, it does not affect binding of IF-B_{12} to cubilin [7]. Thus, the inhibitory effect of RAP on albumin uptake may be explained by different mechanisms, including a decreased binding of albumin to megalin or possibly cubilin and/or a decreased binding of cubilin to megalin. The present study does not allow for distinguishing between these possible modes of action. The significant reduction in albumin uptake induced by IF-B_{12} supports the involvement of cubilin, since this is the only identified IF-B_{12} binding protein. An additive inhibitory effect was observed with RAP and IF-B_{12} at RAP concentrations above that which gave maximal inhibition. This indicates that RAP and IF-B_{12} do not act at a single, identical site, as also demonstrated previously by plasmon resonance analysis [7]. The difference in the results between the IC_{50} for IF-B_{12} found in this study and the previous observed affinity constant of IF-B_{12} [7] rather suggests that the observed inhibition of albumin uptake by IF-B_{12} is due to additional low affinity binding sites shared by the two proteins. Accordingly, two distinct binding sites or two functional uptake components might exist in the proximal tubule. Indeed, it has been shown that albumin reabsorption in rabbit proximal tubule can be described by the combination of a low- and a high-affinity transport mechanism [42]. The authors tentatively ascribed these two processes to the fluid phase and adsorptive endocytosis without ruling out the possibility of binding sites. However, the low-affinity high capacity uptake of albumin is not likely to be due to fluid phase endocytosis, since this would only account for a minor uptake, but is rather due to low-affinity binding sites. The

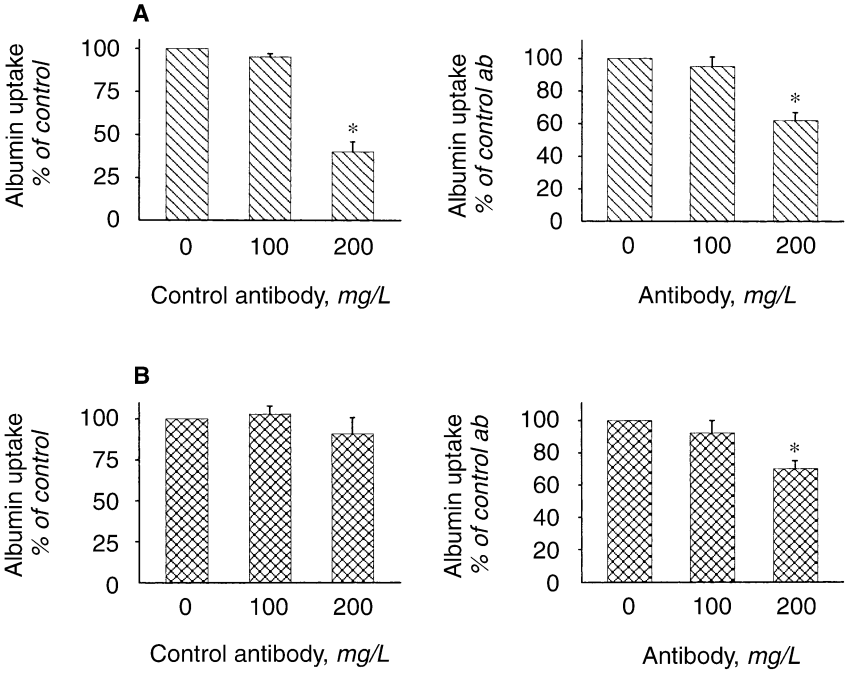

Fig. 6. (*A*) Inhibition of albumin uptake by cubilin antibody as compared with nonspecific IgG. Notice that also the nonspecific rabbit IgG at 200 mg/mL significantly reduces the uptake of albumin. Left, rabbit; right, α-cubulin. (*B*) Inhibition of albumin uptake by megalin-antibody as compared with nonspecific IgG at each appropriate concentration. Left, sheep; right α-megalin. $N = 9$. $^*P < 0.05$.

observed inhibition of albumin uptake by nonspecific immunoglobulin can be explained by the recent finding of cubilin acting as a scavenger receptor for light chains [12]. Recent studies have shown that specific anticubilin antibodies were effective in decreasing uptake and degradation of HDL, whereas there was no effect of nonspecific immunoglobulins [11]. This apparent discrepancy with the present study may simply be explained by the fact that the high-affinity ligand HDL does not share binding sites with nonspecific IgG, whereas the low-affinity ligand albumin does. The possibility of a nonspecific inhibition by just any protein can be excluded, since egg albumin was without effect. Finally, the involvement of cubilin was substantiated by the use of cubilin antisense oligonucleotides. Incubation with cubilin antisense led to a significantly reduced albumin uptake as compared with sense or transfection reagents alone. Thus, the contribution of cubilin to albumin uptake has been demonstrated by three independent approaches. As mentioned in the **Methods** section, the OK cubilin sequence is not 100% homologous to rat cubilin. The precise functional consequences of these sequence differences, if any, are not known at present. It is conceivable that the affinity constants for IF-B_{12} or albumin may be shifted as compared with rat cubilin. However, the antibody and antisense data make it unlikely that there are fundamental qualitative differences in the behavior of OK cubilin as compared with rat cubilin.

The present data do not allow for the identification of specific binding sites on either megalin or cubilin, and do not exclude the possibility that there are additional binding proteins for albumin. However, the data suggest a significant role of these two receptors in albumin uptake. In addition, the present study demonstrates membrane-associated cubilin and megalin in OK cells and establishes their subcellular colocalization, thereby identifying a suitable model system to study the relationship between uptake of protein and the expression of cubilin and megalin.

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