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Megalin-mediated endocytosis of transcobalamin-vitamin-B_{12} complexes suggests a role of the receptor in vitamin-B_{12} homeostasis

(glycoprotein 330/low density lipoprotein receptor family/surface plasmon resonance/kidney/yolk sac)

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ABSTRACT **Kidney cortex is a main target for circulating vitamin B_{12} (cobalamin) in complex with transcobalamin (TC). Ligand blotting of rabbit kidney cortex with rabbit ^{125}I-TC-B_{12} and human TC-^{57}Co-B_{12} revealed an exclusive binding to megalin, a 600-kDa endocytic receptor present in renal proximal tubule epithelium and other absorptive epithelia. The binding was Ca^{2+} dependent and inhibited by receptor-associated protein (RAP). Surface plasmon resonance analysis demonstrated a high-affinity interaction between purified rabbit megalin and rabbit TC-B_{12} but no measurable affinity of the vitamin complex for the homologous α_2-macroglobulin receptor (α_2MR)/low density lipoprotein receptor related protein (LRP). ^{125}I-TC-B_{12} was efficiently endocytosed in a RAP-inhibitable manner in megalin-expressing rat yolk sac carcinoma cells and *in vivo* microperfused rat proximal tubules. The radioactivity in the tubules localized to the endocytic compartments and a similar apical distribution in the proximal tubules was demonstrated after intravenous injection of ^{125}I-TC-B_{12}. The TC-B_{12} binding sites in the proximal tubule epithelium colocalized with megalin as shown by ligand binding to cryosections of rat kidney cortex, and the binding was inhibited by anti-megalin polyclonal antibody, EDTA, and RAP. These data show a novel nutritional dimension of megalin as a receptor involved in the cellular uptake of vitamin B_{12}. The expression of megalin in absorptive epithelia in the kidney and other tissues including yolk sac and placenta suggests a role of the receptor in vitamin B_{12} homeostasis and fetal vitamin B_{12} supply.**

Vitamin B_{12} and other cobalamins (B_{12})‡‡ form complexes with three mammalian tissue fluid proteins. These include gastric intrinsic factor, transcobalamin (TC) present in plasma, and haptocorrin found in most secretions. Intrinsic factor secreted from the stomach is essential for the intestinal uptake of vitamin B_{12}, whereas TC facilitates the cellular uptake of B_{12} from plasma and various tissue fluids into a wide spectrum of cells (1, 2). Lack of synthesis of either intrinsic factor (3) or TC (4) leads to hematological, gastrointestinal, and/or neurological disorders.

B_{12} is widely distributed in the mammalian organism and high concentrations are seen in the liver and kidney. Animal studies (5–7) have shown that the kidney plays a major role in the regulation of the plasma B_{12} level by maintaining a pool of free B_{12}. On the other hand, liver B_{12} is largely bound to the B_{12}-dependent enzymes, methylmalonyl coenzyme A mutase and methionine synthetase. During deficiency, B_{12} is released from the kidney rather than from the enzyme-bound pool of the vitamin in the liver and other organs (7). Radiolabeled B_{12} taken up in the kidney is localized exclusively in the renal proximal tubules (8). B_{12} is only taken up in the kidney when it is bound to TC, and if administrated B_{12} exceeds the saturation level of plasma TC, free B_{12} is excreted in the urine (9). In agreement with this fact, high- affinity binding sites for complexes, but not for free B_{12}, have been shown on the luminal surface of isolated rat (10) and hog tubule cells (11). Interestingly, the proportional uptake in the kidney of dietary B_{12} or intravenously administrated TC-B_{12} is higher at a high B_{12} load in the organism, and a regulatory mechanism for uptake and storage of B_{12} in the kidney has been suggested (5, 6, 8, 10, 12).

Megalin [previously designated glycoprotein 330 (gp330) or Heymann Nephritis autoantigen] is a 600-kDa endocytosis-mediating membrane protein expressed in the tubule epithelium (13–15) and some other absorptive epithelia, e.g., in yolk sac, lung, retina, and inner ear (16, 17). Megalin belongs to the low density lipoprotein receptor gene family (18) and has been shown to bind different substances including clusterin (19), polybasic aminoglycoside drugs (20), and several of the proteins binding to the homologous 600-kDa α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (α_2MR/LRP)—e.g., the 39–44 kDa receptor-associated protein (RAP) and plasminogen activators (free or in complex with type-1 inhibitor) (for reviews, see refs. 21 and 22). However, in view of the largely epithelial expression of megalin, many of the *in vitro* interactions with large plasma proteins (e.g., lipoproteins) may be of minor biological importance compared with uptake of hitherto unidentified natural ligands in the ultrafiltrate and other tissue fluids. RAP has an outstanding role in the studies of megalin and other low density lipoprotein receptor family proteins due to its antagonizing effect on ligand binding of all known ligands to the receptors (for reviews, see refs. 21–24). The function of RAP is not fully elucidated, but recent studies on α_2MR/LRP have shown a function as a chaperone involved in the processing of the receptors maybe by means of down-regulation of their binding

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Abbreviations: α_2MR, α_2-macroglobulin receptor; B_{12}, vitamin B_{12}/cobalamin; LRP, low density lipoprotein receptor related protein; pro-uPA, pro-urokinase; RAP, receptor-associated protein; SPR, surface plasmon resonance; TC, transcobalamin.

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‡‡Vitamin B_{12} is cyanocobalamin. In the organism, cyanocobalamin is converted to the active forms of cobalamin, methyl-, and 5′-deoxyadenosylcobalamin. The abbreviation B_{12} is employed to cover all forms of cobalamin that can be converted to the active form.

activity in the endoplasmic reticulum/Golgi compartments (25, 26).

The present study shows that megalin is a high-affinity receptor for TC-B_{12} complex. Binding of the complex to megalin leads to cellular uptake of B_{12} and degradation of TC. This observation provides evidence for a novel nutritional function of megalin and suggests a role of the receptor in B_{12} homeostasis.

MATERIALS AND METHODS

Reagents. Rabbit TC was purified from rabbit plasma as described (27). Human TC was partially purified from human seminal plasma and complexed with ^{57}Co-B_{12} or unlabeled B_{12} as described (28). Human intrinsic factor was purified as described (29). Recombinant human RAP in its nonglycosylated 37-kDa form was generated in transfected *Escherichia coli* BL21 cells. Pro-urokinase (pro-uPA) was from Abbott. Rabbit TC-B_{12}, intrinsic factor-B_{12}, and RAP were iodinated by the Iodogen method (Pierce). Ten micrograms of protein was incubated with 5 μg of immobilized Iodogen and 7.4 MBq of ^{125}I for 3 min at 20°C. The iodinated protein was purified on a 4-ml Sephacryl-200 column and analyzed by SDS/PAGE, which showed one protein band with an electrophoretic mobility corresponding to a molecular mass of 39 kDa for ^{125}I-RAP and 43 kDa for ^{125}I-TC. The binding of ^{125}I-TC-B_{12} to megalin decreased 30–40% after 1 week of storage of the radiolabel at −20°C, whereas ^{125}I-RAP remained stable for weeks. The specific activity was approximately 1 MBq/μg of protein. Megalin was purified from rabbit renal cortex as described (30). α_2MR/LRP was purified from solubilized human placental membranes as described (31). Heparin was from Løvens Kemiske (Copenhagen, Denmark). Sheep polyclonal anti-rat megalin antibody (30) and nonimmune sheep immunoglobulin was purified using protein A beads (Immunopure, Pierce). A novel monoclonal antibody 5B-B11 against rabbit megalin was raised by fusing mouse myeloma cells (NS-1) with spleen cells from a BALB/c mouse immunized with purified rabbit megalin.

Ligand and Immunoblotting. Ligand blotting was essentially carried out as described. In short, rabbit renal cortical membranes, purified megalin, and α_2MR/LRP and BN/MSV (32) yolk sac cells were subjected to SDS/(4–16%) PAGE and electroblotted onto Immobilon membranes (Millipore). Membrane strips were incubated with radiolabel (3×10^3 Bq/ml) in 10 mM Hepes, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1% bovine serum albumin (pH 7.8). Similar strips used for immunoblotting were blocked in 2% nonfat dry milk and 0.05% Tween-20 in the Hepes buffer and subsequently incubated with antibody in the Hepes buffer added 0.2% nonfat dry milk. Sheep anti-rat megalin antiserum was diluted 1:10,000 and monoclonal anti-rabbit megalin antibody was used at a concentration of 2 μg/ml.

Kinetic Analyses of Ligand-Receptor Binding. Kinetic analysis of the interaction of ligands with purified megalin and α_2MR/LRP was performed using a microtiter well assay with iodinated ligand as described (30) and by surface plasmon resonance (SPR) measurements on a BIAcore instrument (Pharmacia). For the SPR analyses, the BIAcore sensor chips (type CM5, Pharmacia) were activated with 1:1 mixture of 0.1 M *N*-ethyl-*N'*-(3-diethylaminopropyl) carbodiimide and 0.1 M *N*-hydroxysuccimide in water. Rabbit megalin and human α_2MR/LRP were then immobilized at a concentration of 40 μg/ml in 10 mM sodium acetate (pH 4.5 and pH 3.0), respectively, and the remaining binding sites were blocked with 1 M ethanolamine (pH 8.5). The SPR signal from immobilized receptors generated 9,000–18,000 of the mass equivalent BIAcore response units. Finally, the flow cells were washed using 100 mM of phosphoric acid. The flow buffer was 10 mM Hepes, 150 mM NaCl, and 2 mM $CaCl_2$ (pH 7.4), at 25°C. The flow cells were washed with 100 mM of phosphoric acid regenerating 99% of the binding activity for the ligands studied. The binding data were analyzed using the BIAevaluation program. The number of ligands bound per immobilized receptor was estimated by dividing the ratio $RU_{ligand}/mass_{ligand}$ with $RU_{receptor}/mass_{receptor}$.

Uptake of Radiolabeled TC-B_{12} in Cultured Rat Yolk Sac Carcinoma Cells. Rat yolk sac carcinoma cells from the BN/MSV cell line (32) were grown in minimum essential medium (MEM) (GIBCO) containing 10% fetal calf serum. Incubation with ^{125}I-TC-B_{12} was carried out in MEM supplemented with 0.1% bovine serum albumin. Degradation of the proteins was measured by precipitation of the incubation medium in 12.5% trichloroacetic acid. Cell-associated radioactivity was measured by counting the cells after solubilization of the cell layer in 1% Triton X-100.

Renal Uptake of Intravenously Injected ^{125}I-TC-B_{12}. The renal uptake of ^{125}I-TC-B_{12} was analyzed by light microscope autoradiography on sections of rat kidney cortex after injection of ^{125}I-TC-B_{12} into the femoral vein. Fifteen minutes after the injection of 1 MBq of ^{125}I-TC-B_{12}, the kidneys were fixed by retrograde perfusion through the abdominal aorta with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer followed by postfixation, dehydration, and embedding into Epon 812. The urether of one kidney was ligated prior to injection of tracer to decrease filtration, thereby facilitating the detection of any possible basolateral labeling.

Micropuncture of Proximal Tubules with ^{125}I-TC-B_{12}. Male Wistar rats (220–255 g) were anesthetized with sodium thiopental and placed on a thermostatically controlled heated table. A tracheostomy was performed, and the jugular vein was catherized and infused with saline, 3.8 ml/h. The left kidney was exposed by flank incision, placed in a stabilized cup, and covered with paraffin oil maintained at 37–38°C. The urether was catheterized and urine was collected into counting vials. Single-surface proximal tubules were injected with 43–75 nl of ^{125}I-TC-B_{12} in 0.15 M NaCl, 1 mM $CaCl_2$, and lissamine green and urine was collected for 20 min. Inhibition studies were performed by double injection of the same proximal tubule with ^{125}I-TC-B_{12} and subsequently ^{125}I-TC-B_{12} with excess of either unlabeled TC-B_{12} or RAP. This was also performed in reverse order. Inhibition was determined by paired comparison of uptake in the same tubule of injected ^{125}I-TC-B_{12} with and without inhibitor. Some tubules microinjected with ^{125}I-TC-B_{12} were fixed by microinjection of 1% glutaraldehyde and small tissue blocks containing the microinfused tubules were postfixed, dehydrated, and embedded into Epon 812. Sections were processed for electron microscopy autoradiography using Ilford L4 emulsion and observed in a Philips EM208 or CM100 electron microscope.

Light Microscopy Autoradiography with ^{125}I-TC-B_{12}. One-micrometer cryosections of rat kidney cortex, fixed in 8% paraformaldehyde in 0.1 M sodium cacodylate buffer by retrograde perfusion through the abdominal aorta, were placed on gelatin coated glass slides and preincubated in 0.01 M PBS, 0.05 M glycine, 0.15 M NaCl, 0.1% nonfat dry milk and 0.02 M NaN_3 followed by incubation with ^{125}I-TC-B_{12} (0.05 MBq/ml) in 0.01 M PBS, 0.05 M Tris-buffer, 0.15 M NaCl, 1 mM $CaCl_2$, 0.1% nonfat dry milk, and 0.02 NaN_3. Sections were washed, fixed in 1% glutaraldehyde, and prepared for light microscope autoradiography using Amersham LM-1 emulsion.

RESULTS

Ligand Blotting of Renal Cortex Membranes with ^{125}I- and ^{57}Co-Labeled TC-B_{12}. A renal binding protein for TC-B_{12} was initially identified by ligand blotting of rabbit renal cortex membranes separated by SDS/PAGE. As shown in Fig. 1, rabbit ^{125}I-TC-B_{12} (lane 1) exclusively bound to a high molecular weight protein. A similar band is seen with human TC

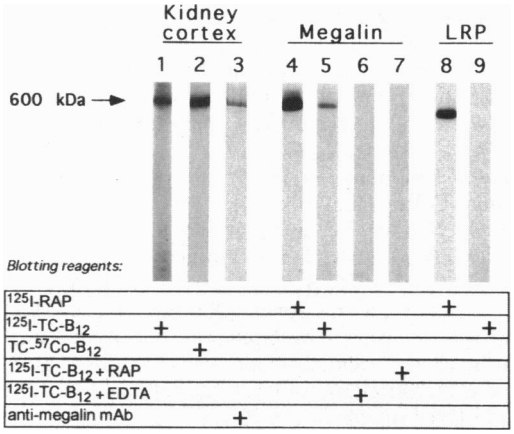

FIG. 1. Ligand blotting of rabbit kidney megalin with radiolabeled rabbit TC-B_{12}. Blotting (4–16% SDS/polyacrylamide gels, nonreducing conditions) of rabbit renal cortex (approximately 500 μg per lane) with ^{125}I-TC-B_{12} (lane 1), TC-^{57}Co-B_{12} (lane 2), and the monoclonal mouse anti-rabbit megalin antibody 5B-B11 (lane 3). Ligand blotting of purified rabbit megalin (0.5 μg per lane, lanes 5–7) and human α_2MR/LRP (0.5 μg/lane, lanes 8 and 9) with ^{125}I-RAP (lanes 4 and 8) and ^{125}I-TC-B_{12} (lanes 5 and 9). Lanes 6 and 7 show the inhibitory effect by EDTA (10 mM) and RAP (1 μM) on ^{125}I-TC-B_{12} binding. The blots were incubated with 35,000 Bq of ^{125}I-ligand/ml at 4°C for 20 h and exposed on Amersham Hyperfilm at −70°C for 3 days (lane 1), 7 days (lane 2), 2 h (lanes 4 and 8), and 6 h (lanes 5–7 and 9).

complexed with ^{57}Co-B_{12} (lane 2). The protein colocalizes with the 600 kDa megalin as visualized by blotting with an anti-rabbit megalin monoclonal antibody (lane 3). Binding to megalin was verified by ligand blotting of ^{125}I-TC-B_{12} to RAP affinity-purified megalin (lane 5). Addition of 1 μM of RAP or 10 mM of EDTA abolished binding (lanes 6 and 7). No binding of human ^{125}I-TC-B_{12} (lane 9) or rabbit TC-^{57}Co-B_{12} (not shown) to the megalin homologue α_2MR/LRP was seen. Neither α_2MR/LRP nor megalin bound ^{125}I- or ^{57}Co-labeled intrinsic factor-B_{12} complex as tested by the ligand blotting assay or by measuring binding to receptors immobilized to microtiter wells (data not shown).

Characterization of the Binding of TC-B_{12} to Purified Megalin. The interaction between TC-B_{12} and megalin or α_2MR/LRP was next evaluated by SPR analysis (Figs. 2 and 3) of the binding of purified TC-B_{12} passed through a BIAcore flow cell coated with purified rabbit megalin or human α_2MR/LRP. For comparison, similar receptor binding data were recorded for the ligands RAP and pro-uPA. In accordance with the ligand blotting experiments (Fig. 1), TC-B_{12} bound only to megalin (Fig. 2*A*) and not to α_2MR/LRP (Fig. 2*B*), whereas RAP rapidly associated to both receptors (Fig. 2 *C* and *D*). Approximately one-half of the associated RAP readily dissociated, whereas the other one-half was tightly bound. A 1:1.4–1.5 ratio for binding of the 44-kDa TC-B_{12} complex to megalin and a similar ratio for binding of the 37-kDa recombinant RAP molecule to both receptors was estimated by comparing the SPR signal from the ligands and immobilized receptors (see *Materials and Methods*), thus suggesting more than one and probably two binding sites for both ligands. Data fitted to a receptor model assuming two binding sites (see legend to Fig. 2). The K_d at 25°C of the high-affinity component was approximately 42 nM for RAP binding to megalin and α_2MR/LRP and about 4–5 times higher for TC-B_{12} binding to megalin. In additional experiments performed in microtiter wells at 4°C (data not shown) ^{125}I-TC-B_{12} bound to immobillized megalin with a half maximal binding at about 12.5 nM. A similar concentration dependence was obtained with TC depleted for B_{12} by dialysis against 8 M urea (data not shown).

The BIAcore sensorgram of the binding of TC-B_{12} to megalin showed inhibition of TC-B_{12} binding when megalin

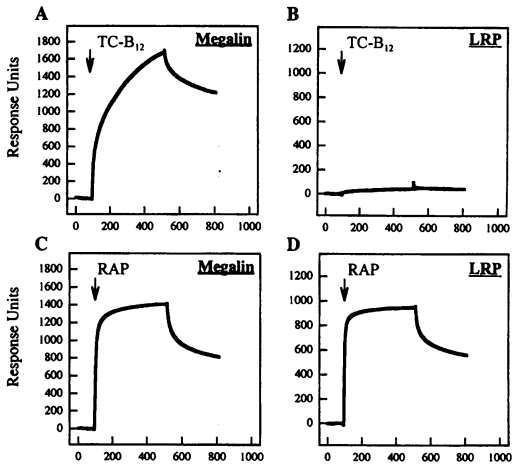

FIG. 2. SPR on BIAcore of ligand binding to megalin and α_2MR/LRP. Dilutions of TC-B_{12} and RAP (40 μg/ml) were passed over two different sensor chips containing purified rabbit megalin (17,179 response units) and human α_2MR/LRP (9,514 response units), immobilized to matrix, and sensorgrams recorded. The sensor chips were subjected to a constant flow with 10 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$ (pH 7.4) and the arrows indicate the start of injection with buffer plus ligand for 400 s. (*A*) The association and subsequent dissociation phase in buffer alone of TC-B_{12} binding to megalin. (*B*) Absence of binding of TC-B_{12} to α_2MR/LRP. (*C* and *D*) The association and dissociation phases for the RAP binding to megalin and α_2MR/LRP. The ratios of bound ligand/receptor after 400 s flow of ligand were calculated to 1.4 mol RAP, 1.4 mol TC-B_{12}, and 1.4 mol RAP and <0.1 mol TC-B_{12} per mol α_2MR/LRP. Fitting of the curves to a two-receptor model gave the following constants: TC-B_{12} (megalin): $k_{on1} = 2.1 \times 10^4\ M^{-1}\cdot s^{-1}$; $k_{on2} = 3.5 \times 10^3\ M^{-1}\cdot s^{-1}$; $k_{off1} = 0.03\ s^{-1}$; $k_{off2} = 6.4 \times 10^{-4}\cdot s^{-1}$; $K_{d1} = 1.4\ \mu M$, $K_{d2} = 182$ nM. RAP (megalin): $k_{on1} = 9.4 \times 10^4\ M^{-1}\cdot s^{-1}$; $k_{on2} = 1.5 \times 10^4\ M^{-1}\cdot s^{-1}$; $k_{off1} = 0.026\ s^{-1}$; $k_{off2} = 6.5 \times 10^{-4}\cdot s^{-1}$; $K_{d1} = 274$ nM, $K_{d2} = 42.5$ nM. RAP (α_2MR/LRP): $k_{on1} = 1.0 \times 10^4\ M^{-1}\cdot s^{-1}$; $k_{on2} = 1.55 \times 10^4\ M^{-1}\cdot s^{-1}$; $k_{off1} = 0.025\ s^{-1}$; $k_{off2} = 6.5 \times 10^{-4}\cdot s^{-1}$; $K_{d1} = 240$ nM, $K_{d2} = 41.7$ nM

was complexed with RAP (not shown). A minor residual TC-B_{12} binding to megalin was seen after low affinity-bound RAP was washed off, which may reflect the binding of TC-B_{12} to a binding site not inhibited by high affinity-bound RAP. In contrast, high affinity-bound RAP completely inhibited the binding of 55-kDa pro-uPA (not shown), a ligand for megalin and α_2MR/LRP. A 0.7:1 ratio was shown for the binding of this ligand to megalin and α_2MR/LRP (not shown), thus suggesting only a single pro-uPA binding site on the two receptors.

Uptake of Radiolabeled TC-B_{12} in Rat Yolk Sac Carcinoma Cells. Receptor-mediated uptake of TC-B_{12} was studied in the rat yolk sac carcinoma cell line, BN/MSV, which expresses megalin (32) as verified by Western blotting and ligand blotting with ^{125}I-TC-B_{12} and RAP (Fig. 3*A*, *inset*). Incubation of the cells with ^{125}I-TC-B_{12} resulted in an efficient RAP-inhibitable uptake and degradation (Fig. 3*A*). A similar RAP-inhibitable uptake of TC-^{57}Co-B_{12} was observed (not shown), thus indicating that the entire complex including the vitamin component is internalized. The uptake of ^{125}I-TC-B_{12} was strongly inhibited by polyclonal anti-megalin antibody and unlabeled rabbit TC-B_{12} (Fig. 3*B*). Heparin inhibited the uptake by approximately 40% (Fig. 3*B*).

Uptake of Intravenously Injected ^{125}I-TC-B_{12} in Rat Kidney. Intravenously injected ^{125}I-TC-B_{12} was localized within the kidney by light microscope autoradiography to identify sites of uptake. Twenty minutes after the injection of ^{125}I-TC-B_{12}, grains were predominantly located to the brush border and apical cytoplasm of proximal tubules indicating that luminal uptake is the predominant pathway of TC-B_{12} uptake in the kidney (Fig. 4*A*).

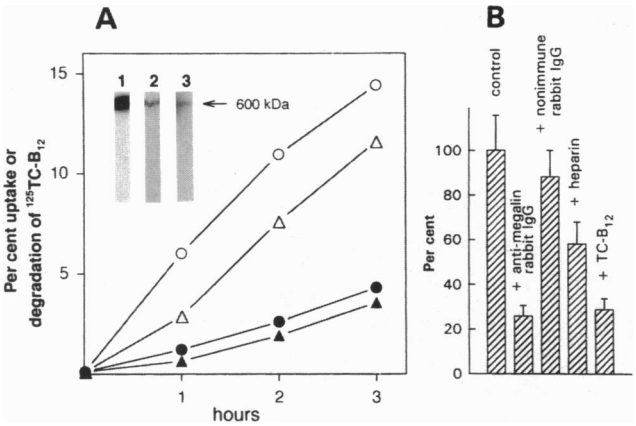

FIG. 3. (*A*) Uptake and degradation of ^{125}I-TC-B_{12} in the rat yolk sac carcinoma cell line, BN/MSV. Confluent cell layers in 24-well plates were incubated with ^{125}I-TC- for 0, 1, 2, and 3 h at 37°C in 400 μl of MEM and 0.1% bovine serum albumin. Degradation (△) was measured as the increase in trichloroacetic acid-soluble radioactivity in the medium. Uptake (○) was measured as the cell-associated radioactivity plus the degraded fraction. The values represent the mean of duplicates. The solid symbols represent degradation (▲) and uptake (●) of ^{125}I-TC-B_{12} in the presence of 1 μM of RAP. (*Inset*) Blotting of solubilized BN/MSV cells (≈500 μg per lane) with ^{125}I-RAP (1) and polyclonal anti-rat megalin antibody (2) and ^{125}I-TC-B_{12} (3). The blots were incubated with 35,000 Bq ^{125}I-ligand/ml at 4°C for 20 h and exposed on Amersham Hyperfilm at −70°C for 6 h (lane 2) and 5 days (lane 3). (*B*) Effect of purified polyclonal sheep anti-megalin antibody (1.4 mg/ml), sheep nonimmune antibody (1.4 mg/ml), heparin (500 units/ml), and unlabeled TC-B_{12} (100 nM) on the 2-h uptake of ^{125}I-TC-B_{12}.

Uptake of ^{125}I-TC-B_{12} Microinjected in Proximal Tubules *in Vivo*. Microinjection of rat kidney proximal tubules resulted in 67 ± 4% (n = 14 tubules in 6 animals) uptake of ^{125}I-TC-B_{12}, thus showing efficient uptake of the vitamin/TC complex in kidney tubules. Tubules fixed 15 min after the microinjection of ^{125}I-TC-B_{12} and processed for electron microscopy revealed autoradiographic labeling of endocytic invaginations and vacuoles in proximal tubules only (Fig. 4 *B* and *C*). A significant inhibition of the proximal tubule uptake of ^{125}I-TC-B_{12} by unlabeled TC-B_{12} and RAP was observed in tubules injected with and without inhibitor. The uptake was inhibited 84 ± 8% (n = 5, P = 0.01) by unlabeled TC-B_{12} and 38 ± 10% (n = 8, P = 0.01) by RAP.

Binding of ^{125}I-TC-B_{12} to Sections of Rat Kidney Cortex. Sections of rat kidney cortex were incubated with ^{125}I-TC-B_{12} followed by light microscope autoradiography to localize binding sites within the proximal tubule. Grains were observed in relation to the brush border and the apical cytoplasm of proximal tubules, and concentrated at the base of the brush border (Fig. 5 *A* and *B*). No other segment of the nephron revealed any significant labeling. Thus, ^{125}I-TC-B_{12}-binding sites have the same localization as megalin (13–15). The binding of ^{125}I-TC-B_{12} was inhibited by unlabeled B_{12}-TC, RAP (Fig. 5*A*, *inset*), EDTA (10 mM, not shown), and polyclonal anti-megalin antibody (Fig. 5*C*).

DISCUSSION

It is well established that the cellular uptake of B_{12} depends on the binding to the carrier proteins, intrinsic factor and TC, and the subsequent transport of the vitamin-carrier complexes by receptor-mediated endocytosis, but little is known about the membrane structures functioning as receptors. In this study we demonstrate that the endocytic receptor, megalin, is a high-affinity receptor mediating uptake of TC-B_{12} complexes. These data, together with the previous demonstrations of the dense expression of megalin in the renal proximal tubule

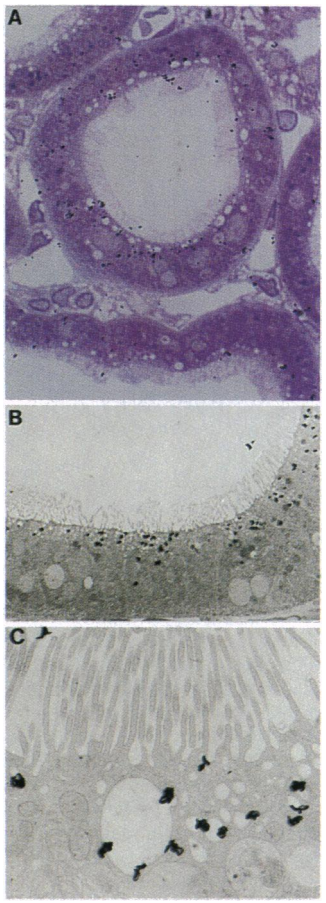

FIG. 4. Uptake of ^{125}I-TC-B_{12} in rat kidney proximal tubules. (*A*) Light microscopic autoradiography of cortex 15 min after intravenous injection of ^{125}I-TC-B_{12}. Most grains are located over the apical part of the cells, in particular brush border and vacuoles. (×335.) (*B* and *C*) Electron micrographs of proximal tubule fixed 20 min after tubular injection of ^{125}I-TC-B_{12} by micropuncture. Grains are located over the apical part of the cell (*A*) predominantly in relation to endocytic invaginations (I) and vacuoles (V). (*B* ×750 and *C* ×5000).

epithelium (13–15), suggest megalin to be relevant for the renal retention of B_{12}. The renal TC-facilitated uptake of B_{12} from the ultrafiltrate has been calculated to about 1.5 μg per day (10) based on a TC-B_{12} sieving coefficient of 10–15% and a TC-B_{12} plasma concentration of 80 pM. This amount equals the requirement of dietary B_{12} taken up by means of the intrinsic factor and its receptor in the terminal ileum. In addition to this crucial role of megalin in the kidney, the expression of the receptor in different absorptive epithelia (16, 17) suggests uptake of TC-B_{12} from the tissue fluids lining, e.g., central nervous system ependyma, inner ear epithelia, retina and lung epithelium. Furthermore, the expression of megalin in the yolk sac, placenta (17), and trophectoderm (33) suggests an important nutritional role of megalin for the fetal supply of B_{12}.

Binding sites for TC-B_{12} have previously been described (10, 11, 34–39) on several different cell types including fibroblasts, hepatocytes, renal tubule cells, and syncytiotrophoblasts. The Ca^{2+}-sensitive binding sites previously described (10, 11) on renal proximal tubule cells may be identical to megalin, whereas TC-B_{12} binding sites on several nonepithelial cell types not expressing megalin must be of different origin. A protein with unknown primary structure has been purified by ligand-affinity chromatography (35, 37, 38). It is suggested to be a homodimer with an estimated size of 120–130 kDa (35) as judged by SDS/gel electrophoresis. The protein has also been detected by immunoblotting of isolated rat Mg^{2+}-precipitated kidney basolateral membranes (40), and this

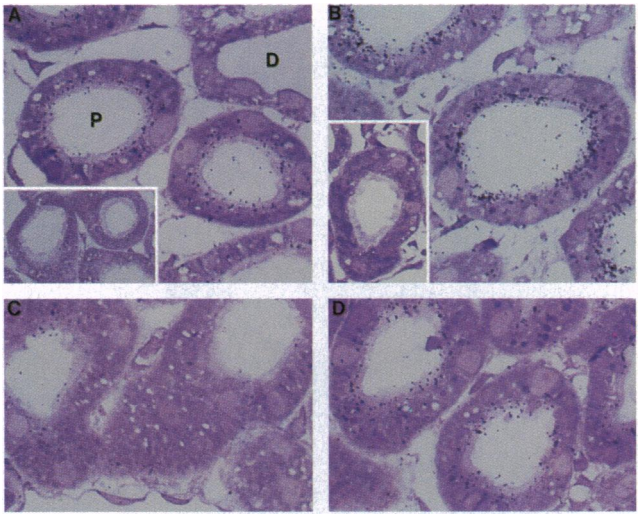

FIG. 5. Cryosections of rat kidney proximal tubule incubated with ^{125}I-TC-B_{12} and processed for light microscope autoradiography. (*A*) Incubated with ^{125}I-TC-B_{12} and exposed for 4 days reveals grains located in relation to the brush border and the apical part of proximal tubule cells (P). Grains are concentrated at the base of the brush border. There is no labeling of neighboring distal tubule (D). Control section (*Inset*), incubated with an excess of unlabeled TC-B_{12} (26 μM), shows almost no grains. (*B*) Incubated with ^{125}I-TC-B_{12} and exposed for 6 days shows a more intense, but similarly located, autoradiographic labeling of proximal tubules. Section (*Inset*) incubated with ^{125}I-TC-B_{12} and RAP (7 μM) shows no grains. *C*, incubated with ^{125}I-TC-B_{12} and purified polyclonal sheep anti-megalin (0.2 mg/ml), shows a significant decrease in autoradiographic labeling when compared with *D*, incubated with ^{125}I-TC-B_{12} and nonimmune sheep IgG (0.2 mg/ml; 5 days of exposure). [*A–D* ×335 (*Inset* in *A*, ×110; *Inset* in *B*, ×165).

observation may suggest some basolateral uptake uptake of TC-B_{12} into the nephron. However, the localization of the renal binding sites for the vitamin complex, as well as the present functional studies of filtration and uptake of TC-B_{12}, suggest that luminal uptake by means of megalin in the proximal tubules accounts for a main uptake in the kidney. Furthermore, a net luminal uptake implies a net transport of B_{12} from the tubule cells across the basolateral membrane. The high synthesis of TC, as suggested from Northern blotting (41), may assist such a transport of B_{12} into plasma.

The existence of different receptors for TC-B_{12} may reflect different mechanisms for maintenance of B_{12} homeostasis. Fig. 6 is a simplified hypothetical model of the putative carrier- and receptor-controlled supply and transport of plasma B_{12} as deduced by combining the present data and previous literature. This model, including three different receptors (the intrinsic factor–B_{12} receptor, megalin, and a putative ubiquitously expressed TC-B_{12} receptor), may help in explaining early *in vivo* data (5, 6, 8, 10, 12) showing that the proportional uptake in the kidney of dietary B_{12} or intravenously administrated TC-B_{12} is higher at a high B_{12} load in the organism (5, 6, 8, 12). The mechanism for this regulation is unknown, but it is intriguing to speculate that a high B_{12} load may induce down-regulation of the nonrenal TC-B_{12} receptors. This would indirectly increase the plasma TC-B_{12} concentration and the renal filtration of the complex leading to an increased megalin-mediated uptake of TC-B_{12}.

We did not observe any difference in the binding of TC and TC-B_{12}, thus suggesting that both the apo- and the holoform of TC are taken up in the kidney. Our observations are in agreement with the results of Yamada *et al.* (11) who described an EDTA-sensitive binding of TC-^{57}Co-B_{12} to a high molecular weight protein in the brush border membranes isolated from homogenates of hog kidney cortex. Both apo- and holo TC

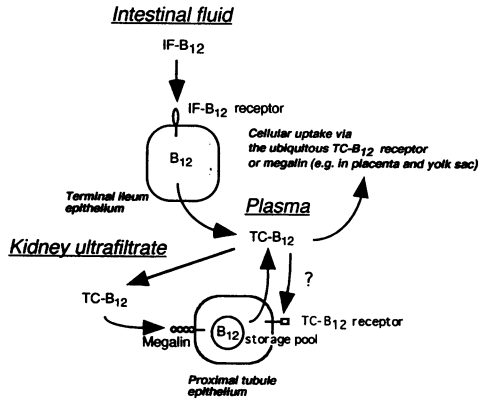

FIG. 6. Hypothetical model of important pathways for B_{12} as controlled by its binding proteins and three different receptors for B_{12}-binding protein complexes. Intestinal uptake of B_{12} occurs after complex formation with intrinsic factor (IF) and uptake via the specific receptor in the terminal ilium epithelium. The vitamin is subsequently delivered to the plasma in complex with TC. From plasma, TC-B_{12} is either filtered in the kidney and subsequently endocytosed via megalin and stored predominantly as free B_{12} or taken up in other tissues via megalin or the alternative ubiquitously expressed TC-B_{12} receptor. A basolateral expression of the TC-B_{12} receptor in renal proximal tubules might also contribute to the uptake of B_{12} in the kidney.

were reported to inhibit the binding to this receptor, which might be identical to megalin. The binding of both the apo- and holo TC seems biological meaningful, because binding of only the holoform would result in loss of TC in the urine.

Positively charged regions are important for the binding of ligands to low density lipoprotein receptor family receptors (20) and heparin binding to the ligands, RAP, and uPA-plasminogen activator inhibitor-1, completely inhibits binding to megalin (15, 30). Heparin has previously been reported to bind to TC (42) and, accordingly, we observed a heparin-binding consensus sequence (43) at residues 217–222 (Gly-Arg-Arg-Gln-Arg-Ile) within TC. A high dose of heparin inhibited the uptake of TC-B_{12} partially (40%), thus indicating that the heparin-binding site participates in receptor recognition.

SPR analysis on megalin suggested more than one and probably two binding sites for TC-B_{12} and RAP, whereas only a single pro-uPA-binding site on megalin and α_2MR/LRP was detected. The complex binding kinetics may be accounted for by heterogeneity of the binding activity of the immobilized receptors and/or involvement of more than one region in the ligands participating in receptor interaction. This has been shown to be the case for RAP binding to megalin (44) and α_2MR/LRP (45). A spectrum of different affinities for one ligand may therefore exist, and the affinity constants obtained by fitting the binding curves from SPR analyses or conventional receptor binding analyses may therefore represent only rough estimations of the avidity of the various ligand-receptor interactions.

Cross competition between RAP and TC-B_{12} suggests that the TC-B_{12} binding sites on megalin are identical to, or in the vicinity of, the suggested two RAP binding sites. In contrast to TC-B_{12}, RAP displayed a very rapid dissociation of one-half of the maximally associated amount, thus indicating that one of the two RAP molecules binds with very low affinity as compared with the other RAP molecule. Furthermore, this suggests a very low affinity inhibition of one of the TC-B_{12} binding sites by RAP, which is a likely explanation for the residual uptake of ^{125}I-TC-B_{12} in proximal tubules and BN/MSV cells in the presence of high concentrations of RAP.

The present new insight into the receptor-mediated uptake of B_{12} may have implications for B_{12} deficiency disease. Reported inherited defects causing selective B_{12} deficiency include lack of intrinsic factor synthesis (46), synthesis of abnormal intrinsic factor (47), TC deficiency (4), and a selec-

tive intestinal malabsorption known as Imerslund–Gräsbeck syndrome (48, 49) caused by defective receptors for IF-B_{12} in the ileal mucosa (50). It is conceivable that an impaired function of megalin also is a contributing cause of B_{12} deficiency.

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