Characterization of an Epithelial ~460-kDa Protein That Facilitates Endocytosis of Intrinsic Factor-Vitamin B\textsubscript{12} and Binds Receptor-associated Protein* 

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By using receptor-associated protein (RAP) as an affinity target, an intrinsic factor-vitamin B\textsubscript{12} (IF-B\textsubscript{12})-binding renal epithelial protein of ~460 kDa was copurified together with the transcobalamin-B\textsubscript{12}-binding 600-kDa receptor, megalin. IF-B\textsubscript{12} affinity chromatography of renal cortex membrane from rabbit and man yielded the same ~460-kDa protein. Binding studies including surface plasmon resonance analyses of the protein demonstrated a calcium-dependent and high affinity binding of IF-B\textsubscript{12} to a site distinct from the RAP binding site. The high affinity binding of IF-B\textsubscript{12} was dependent on complex formation with vitamin B\textsubscript{12}. Light and electron microscope autoradiography of rat renal cortex cryosections incubated directly with IF-\textsuperscript{57}Co-B\textsubscript{12} and rat proximal tubules microinjected in vivo with the radioligand demonstrated binding of the ligand to endocytic invaginations of proximal tubule membranes following by endocytosis and targeting of vitamin B\textsubscript{12} to lysosomes. Polyclonal antibodies recognizing the ~460-kDa receptor inhibited the uptake. Immunohistochemistry of kidney and intestine showed colocalization of the IF-B\textsubscript{12} receptor and megalin in both tissues. In conclusion, we have identified the epithelial IF-B\textsubscript{12} binding receptor as a ~460-kDa RAP-binding protein facilitating endocytosis.

Cellular uptake of vitamin B\textsubscript{12} (B\textsubscript{12}),\textsuperscript{1} the cofactor for the intracellular enzymes methionine synthase and methylepoxymethyl coenzyme A mutase, is controlled by cellular receptors facilitating the endocytic uptake of B\textsubscript{12} in complex with its binding proteins, intrinsic factor (IF)\textsuperscript{2} and transcobalamin (TC) (1–3). The low uptake of free B\textsubscript{12} is clearly evidenced by the development of hematological and/or neurological symptoms, when synthesis of IF (4) or TC (5) is impaired.

Whereas the IF and TC carriers have been characterized extensively, our knowledge of the receptors facilitating the uptake of B\textsubscript{12} complexes remains limited. Uptake of IF-B\textsubscript{12} in the small intestine and the existence of saturable and high affinity IF-B\textsubscript{12} binding sites in the mucosa were established nearly 30 years ago (6, 7). Later studies (for review, see Ref. 3) suggested that binding of IF-B\textsubscript{12} to the binding sites is followed by internalization of IF-B\textsubscript{12} and lysosomal degradation of IF, whereas B\textsubscript{12} is transcytosed and secreted to plasma in complex with TC. The receptor, first isolated in small amounts from the ileal mucosa by ligand affinity chromatography, was reported as a 230-kDa protein of unknown structure (8). Later, a protein of a similar size and immunoreactivity was isolated from the kidney cortex (9). Recently, this protein has shown identity (10) with an immunopurified kidney and yolk sac protein, previously referred to as gp280. The latter has been shown previously to be the target of rat teratogenic antibodies (11) and to associate with the endocytic apparatus in rat yolk sac cells (12, 13).

The TC-facilitated uptake of B\textsubscript{12} from plasma and various tissue fluids apparently occurs via more than one receptor. One candidate receptor is a 120–130-kDa membrane protein of unknown structure identified in several human tissues including placenta and liver (14). Another receptor, which we recently identified as a high affinity receptor of TC-B\textsubscript{12} (15), is megalin, the 600-kDa endocytosis-mediating receptor expressed in several absorptive epithelia including renal proximal tubule, yolk sac, and the brain ependyma. The receptor (16–18) belongs to the low density lipoprotein receptor family and binds, in addition to TC-B\textsubscript{12}, a variety of substances with basic regions, including aminoglycosides (19), clusterin (20), lipoproteins (21), and RAP (21–23). The last represents a 40-kDa endoplasmic reticulum protein serving as a novel kind of chaperone or escort protein preventing aggregation of RAP-binding receptors (24, 25). RAP affinity chromatography represents an effective way of purifying megalin (26, 27).

In the present study we have identified and characterized the function of a high molecular mass protein (~460 kDa) eluting together with megalin from a RAP-Sepharose column loaded with solubilized renal cortical membranes. Surprisingly, we observed that whereas megalin binds TC-B\textsubscript{12} but not IF-B\textsubscript{12}, the copurifying ~460-kDa protein displays a high affinity for IF-B\textsubscript{12} but not for TC-B\textsubscript{12}. Extended analyses of the molecular ligand-receptor interaction and the in vivo uptake in

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1 Vitamin B\textsubscript{12} is cyanocobalamin. In the organism, cyanocobalamin is converted to the active forms of cobalamin, methyl- and 5'-deoxyadenosine-5'-ylcobalamin. The abbreviation B\textsubscript{12} is employed to cover all forms of cobalamin which can be converted to the active form.

2 The abbreviations used in this paper are: IF, intrinsic factor; TC, transcobalamin; RAP, receptor-associated protein; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonic acid; EDAC, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide; PAGE, polyacrylamide gel electrophoresis; LRP, low density lipoprotein receptor-related protein; α\textsubscript{2}MR, α\textsubscript{2}-macroglobulin receptor.
renal proximal tubules characterize the ~460-kDa protein as a RAP-binding receptor facilitating endocytosis of IF-B_{12} and conveying lysosomal targeting of the vitamin.

**EXPERIMENTAL PROCEDURES**

**Ligands and Receptors—**Human IF was purified from gastric juice (28). Porcine IF was similarly isolated from lyophilized gastric mucosal extract powder from GEA (Denmark). Rat IF was from Sigma (U. S. A.). The IFs were coupled with either ^{57}Co-B_{12} (0.05 μmol/mL, 389 kBq/ml) from Amersham (U. K) or unlabelled B_{12} as described (29). Labeling with ^{15}N was performed using the IODO-GEN (Pierce, Belgium) method. Specific activity was approximately 10^7 Bq/μg of protein. gp280 was immunopurified from rat renal cortex as described (11). Recombinant RAP was produced in transfected Escherichia coli.

**RAP and IF-B_{12} Affinity Chromatography—**Rabbit kidney cortex (100 g) was homogenized on ice in 450 mL of 310 mM sorbitol, 15 mM Hepes, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1,080 g for 10 min, followed by centrifugation of the supernatant at 80,000 × g for 40 min and resuspension of the pellet in 140 mM NaCl, 2 mM CaCl_2, 1 mM MgCl_2, 10 mM Hepes, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride. The suspension was rehomogenized, and the homogenate was centrifuged at 45,000 × g for 40 min. The pellet was solubilized in 1% Triton X-100 (Boehringer, Germany) in 100 mM Tris·HCl buffer. Nonsolubilized material was removed by centrifugation at 30,000 × g for 30 min. The solubilized membranes were then pumped onto columns of CNBr-activated Sepharose coupled with either recombinant RAP (5 mg/mL Sepharose) as described previously (26), human (200 μg/mL Sepharose), or porcine IF-B_{12} (3 mg/mL Sepharose). After extensive wash with 10 mM NaHPO_4, 150 mM NaCl, 2 mM CaCl_2, 0.6% CHAPS, bound protein was eluted by adjusting the pH to 4.0 and adding 5 mM EDTA.

**Antibodies—**Monoclonal antibody 5B-B11, recognizing rabbit megalin, and monoclonal antibody 5A-C12, recognizing rabbit ~460-kDa protein, were raised by fusion of NS-1 myeloma cells with spleen cells from a BALB/c mouse immunized with rabbit megalin and the ~460-kDa protein purified by RAP affinity chromatography. The procedure was as described previously (30). Polyclonal antibodies were produced by Western blotting. Polyclonal antibodies against rat megalin and rabbit ~460-kDa protein were raised in guinea pigs immunized with RAP affinity-purified megalin or ~460-kDa protein (50 μg in 3-week intervals). The antibodies were purified further by protein A affinity chromatography (Immunopure®, Pierce) and by antigen affinity purification. Rabbit polyclonal antibodies against rat gp280 were produced in rabbits immunized with rat gp280 and purified using protein A affinity chromatography.

**Analyzes of Ligand-Receptor Interactions—**Affinity measurement of the binding of IF-B_{12} to purified 460-kDa protein was performed using a microtiter well assay for ligand-receptor interactions (31) with ^{125}I-labeled human IF-B_{12} and by surface plasmon resonance measurements (1) on a BiACore 2000 instrument (Pharmacia, Sweden). For the surface plasmon resonance analyses, the BiACore sensor chips (type CM5, Pharmacia) were activated with a 1:1 mixture of 0.2% EDC and 0.05 M N-hydroxysuccimide in water. Rabbit megalin, rabbit ~460-kDa protein, and human IF-B_{12} and were immobilized at a concentration of 40 μmol/mL in 10 mM sodium acetate, pH 4.5, and the remaining binding sites were blocked with 1 mM ethanamine, pH 8.5. The surface plasmon resonance signal from immobilized rabbit megalin, rabbit ~460-kDa protein, and IF-B_{12} generated 19,237, 157,999 and 1,905 BIAcore response units (RU) per μg of protein. gp280, or nonimmune rabbit serum IgG was added to the rat IF- ^{57}Co-B_{12} incubation buffer.

**Protein Sequence Analysis—**Approximately 5 μg of the ~460-kDa IF-B_{12}-binding rabbit receptor was electrodissociated from a 4–16% SDS-polyacrylamide gel to a polyvinylidene difluoride membrane (Problot, Applied BioSystems). The electrodissociated band was cut out and subjected to Edman degradation using an Applied Biosystems 477A sequencer equipped with a 120A on-line chromatograph. A cross-flow reaction and a n d1mM phenylmethylsulfonyl fluoride. The suspension was rehomogenized and the homogenate was centrifuged at 45,000 × g for 40 min. The pellet was solubilized in 1% Triton X-100 (Boehringer, Germany) in 100 mM Tris·HCl buffer. Nonsolubilized material was removed by centrifugation at 30,000 × g for 30 min. The solubilized membranes were then pumped onto columns of CNBr-activated Sepharose coupled with either recombinant RAP (5 mg/mL Sepharose) as described previously (26), human (200 μg/mL Sepharose), or porcine IF-B_{12} (3 mg/mL Sepharose). After extensive wash with 10 mM NaHPO_4, 150 mM NaCl, 2 mM CaCl_2, 0.6% CHAPS, bound protein was eluted by adjusting the pH to 4.0 and adding 5 mM EDTA.

**Molecular Characterization of the Rat IF-B_{12} Receptor—**Approximately 5 μg of the ~460-kDa protein was homogenized on ice in 450 mL of 310 mM sorbitol, 15 mM Hepes, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1,080 g for 10 min, followed by centrifugation of the supernatant at 80,000 × g for 40 min and resuspension of the pellet in 140 mM NaCl, 2 mM CaCl_2, 1 mM MgCl_2, 10 mM Hepes, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride. The suspension was rehomogenized, and the homogenate was centrifuged at 45,000 × g for 40 min. The pellet was solubilized in 1% Triton X-100 (Boehringer, Germany) in 100 mM Tris·HCl buffer. Nonsolubilized material was removed by centrifugation at 30,000 × g for 30 min. The solubilized membranes were then pumped onto columns of CNBr-activated Sepharose coupled with either recombinant RAP (5 mg/mL Sepharose) as described previously (26), human (200 μg/mL Sepharose), or porcine IF-B_{12} (3 mg/mL Sepharose). After extensive wash with 10 mM NaHPO_4, 150 mM NaCl, 2 mM CaCl_2, 0.6% CHAPS, bound protein was eluted by adjusting the pH to 4.0 and adding 5 mM EDTA.

**Light Microscope Autoradiography with ^{125}Co-B_{12}-labeled Rat IF—**Semithin cryosections of rat kidney cortex prepared as described above were preincubated in 10 mM NaHPO_4, 50 mM glycine, 150 mM NaCl, 0.1% skim milk followed by incubation with rat IF- ^{57}Co-B_{12} (1.3 × 10^6 cpm/mL) in 10 mM NaHPO_4, 50 mM Tris buffer, 150 mM NaCl, 1% CaCl_2, and 0.1% skim milk. Sections were washed, fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and prepared for light microscope autoradiography using Amersham EM-1 emulsion. After 4–6 days of exposure the sections were developed and observed in a Leitz Laborlux S. For inhibition studies either unlabeled rat IF-B_{12} (244 nmol, porcine IF-B_{12} (3 μL), RAP (7 μL), polyclonal rabbit anti-gp280, or nonimmune rabbit serum IgG was added to the rat IF-B_{12} incubation buffer.

**Rat Kidney Proximal Tubule Uptake of Human ^{125}I-IF-B_{12}, after Intravenous Injection—**Male Wistar rats were anesthetized with sodium thiopental and placed on a thermostatically controlled heated table. The jugular vein was infused with saline (6.9 mL/h). The urinary bladder was catheterized and urine was collected. After 45 min of saline infusion human ^{125}I-IF-B_{12} (5 × 10^6 cpm in 0.3 mL) was injected into the femoral vein. 20 min later the kidneys were fixed by retrograde perfusion through the abdominal aorta with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The cortex was cut into small tissue blocks and fixed in OsO_4 in Veronal acetate buffer, dehydrated in graded alcohols, and embedded in Epon 812. 1-μm sections were cut on a Reichert-Jung Ultracut E ultramicrotome and prepared for light microscope autoradiography using Amersham LM-1 emulsion.

**Microinjection of Single Kidney Proximal Tubules with IF-^{125}Co-B_{12}—**Male Wistar rats (n = 10), 220–240-g body weight, were anesthetized with sodium thiopental. The animals were placed on a thermostatically controlled heated table. A tracheostomy was performed, and the jugular vein was catheterized 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. Perirenal temperature was maintained at 37–38 °C. The urether was catheterized, and urine was collected in counting vials. 100–200 μL of surface prostate secretions were incubated in 65–75 mL of free ^{57}Co-B_{12} or rat IF-^{57}Co-B_{12} in 0.15 μL NaCl, 1 mM CaCl_2, and Lissamine green. Urine was collected for 30 min following each micropuncture. Inhibition studies were performed by double injection of the same proximal tubule with IF-^{57}Co-B_{12} and subsequently IF-^{57}Co-B_{12} with an excess of either unlabeled porcine IF-B_{12} or RAP. This was also performed in reversed order. Uptake was calculated as the differ-
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Fig. 1. RAP affinity chromatography of CHAPS-solubilized rabbit kidney cortical membranes. Nonreducing SDS-PAGE (4–16%) of proteins eluted with 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 10 mM EDTA, pH 4.0. 20 µl of each 1-ml fraction was loaded on the gel. The positions of Pharmacia standard molecular mass markers and the α-M subunit (180 kDa) are indicated. The two lower panels are immunoblots of RAP affinity fractions using the monoclonal antibody 5B-B11 (anti-megalin) and the monoclonal antibody 5A-C12 (anti-460-kDa protein). 20 µl of each fraction was run in a 4–16% SDS-polyacrylamide gel and subjected to immunoblotting using an alkaline phosphatase-labeled anti-mouse antibody as secondary antibody. Lanes a show nonreducing SDS-PAGE (upper panel) or immunoblot (lower panels) of whole cortical membranes with the two monoclonal antibodies 5B-B11 and 5A-C12. Lane b, upper panel, shows reducing SDS-PAGE of whole cortex membranes.

Fig. 2. Binding of <sup>125</sup>I-IF-B<sub>12</sub> to the protein fractions eluted by RAP affinity chromatography of rabbit kidney (see Fig. 1). The protein from 10 µl of fractions similar to those displayed in Fig. 1 was coated to microtiter wells, and binding of human <sup>125</sup>I-IF-B<sub>12</sub> (4,000 cpm) was assayed by incubation in 100 µl of 140 mM NaCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM Hepes, 1% bovine serum albumin, pH 7.8, for 16 h at 4°C. The inset shows reducing (lane a) and nonreducing (lane b) SDS-PAGE of the ~460-kDa protein purified by IF-B<sub>12</sub> affinity chromatography of the protein fractions from the RAP column. Lane c shows immunoblotting with the anti-460-kDa protein monoclonal antibody 5A-C12.

Fig. 3. Purification of the ~460-kDa protein by IF-B<sub>12</sub> affinity chromatography of CHAPS-solubilized rabbit kidney cortical membranes. Reducing SDS-PAGE (4–16%) of proteins eluted with 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 10 mM EDTA, pH 4.0. 10 µl of each 1-ml fraction was loaded on the gel. The positions of Pharmacia standard molecular mass markers and the α-M subunit (180 kDa) are indicated. Lane a (nonreducing conditions) is immunoblotting with the anti-460-kDa protein monoclonal antibody 5A-C12.

Fig. 4. Concentration dependence of the IF-B<sub>12</sub> binding to the 460-kDa protein. The ~460-kDa protein was immobilized to microtiter plate by incubation of 100 fmol of receptor protein/well. Binding of <sup>125</sup>I-IF-B<sub>12</sub> in the presence of various concentrations of unlabeled ligand was measured as described in the legend to Fig. 2. ■ indicates the abrogating effect on binding by the addition of 5 mM EDTA to the incubation medium. The inset shows a Scatchard plot of the same data.

RESULTS

Purification of a ~460-kDa Protein from Rabbit Renal Cortex by RAP Affinity Chromatography—Fig. 1 shows the renal cortex membrane proteins bound to and eluted from a RAP-Sepharose column. In the early eluted fractions preceding the bulk of the 600-kDa endocytic receptor, megalin, a predominant protein with an estimated size of ~460 kDa, is seen. SDS-PAGE of whole cortex membranes (lanes a and b) demonstrates megalin and the ~460-kDa protein as the predominant high molecular mass proteins in the cortical membranes. The elution profiles suggest that megalin has the highest affinity for RAP. Amino-terminal sequencing of the 460-kDa protein gave the sequence NTDPQ, which has no homology to known mammalian amino-terminal sequences, indicating that the protein has a yet unknown primary structure. Monoclonal antibodies recognizing either megalin or the ~460-kDa protein were raised and used for Western blotting (Fig. 1, lower panels). The two antibodies showed binding exclusively to the two proteins, except that the antibody against the ~460-kDa protein also recognizes a >800-kDa band.

The ~460-kDa Protein Binds IF-B<sub>12</sub>—Recently, RAP affini-
ty-purified megalin has been shown to bind TC-B12 complexes but not IF-B12 complexes (15). However, when the eluted fractions from the RAP column were assayed for binding of 125I-IF-B12, binding activity was observed in the fractions containing the ~460-kDa protein (Fig. 2). Binding of IF-B12 to the ~460-kDa protein was confirmed by IF-B12 affinity chromatography of the eluted material from the RAP column (Fig. 2 inset, lanes a and b) and by IF-B12 affinity chromatography of whole cortex membranes (Fig. 3). In both cases the ~460-kDa protein was purified. IF-B12 affinity chromatography of human renal membranes yielded a protein of exactly the same size (not shown). In addition to the ~460-kDa protein a >800-kDa protein band is seen as a weak band under nonreducing conditions (Fig. 2 inset, lane b) and by immunoblotting with the monoclonal antibody against the ~460-kDa protein (Fig. 2 inset, lane c, and Fig. 3, lane a). Furthermore, a 40–45-kDa protein of unknown identity was seen in some of the IF-B12 affinity preparations from whole cortex (Fig. 3). The electrophoretic mobility of this protein is slightly slower than RAP (not shown). Amino-terminal sequencing of the electrophoretically 40–45-kDa protein did not, in contrast to rabbit RAP (26), produce any readable sequence, probably because of an amino-terminal modification. The >800-kDa band was not visible under reducing conditions (Fig. 2 inset, lane a), and the protein probably represents a disulfide-dependent dimerization of the ~460-kDa protein. A slightly slower mobility of the reduced ~460-kDa protein suggests the presence of internal disulfide bridges.

The affinity of IF-B12 was evaluated by a 125I-IF-B12 binding assay on immobilized ~460-kDa protein (Fig. 4), and a Kd of 2 nM at 4 °C was estimated. The binding was abolished completely when a Ca2+-saturating concentration of EDTA was added to the medium. The binding was not inhibited by 1 μM RAP (not shown). The ligand binding to the ~460-kDa protein was characterized further by surface plasmon resonance analysis (Fig. 5), resolving the association and dissociation parameters of the two ligands. Both RAP (Fig. 5A) and IF-B12 (Fig. 5B) bind to the immobilized ~460-kDa protein, whereas only RAP binds to megalin (5D). The BIAevaluation program estimated a 5-fold higher affinity for the binding of human (not shown) and porcine IF-B12 to the ~460-kDa protein compared with the binding of RAP. Furthermore, the data of Fig. 5 show that the binding of RAP to the ~460-kDa protein has a >5 fold lower affinity than the high affinity component of RAP binding to megalin, which binds several RAP molecules (15). Comparison of the surface plasmon resonance response signals (see “Experimental Procedures”) suggests only one IF-B12 and one RAP binding site/ ~460-kDa molecule. Additional surface plasmon resonance analyses (not shown) showed simultaneous binding of RAP and IF-B12 to the ~460-kDa protein, indicating that the two ligands bind to distinct and nonoverlapping sites. Analysis of diluted and dialyzed porcine gastric mucosa extract rich in apo-IF (57Co-B12 binding capacity > 750 nM) showed that binding to the ~460-kDa protein was dependent on the addition of B12. Maximum binding and a curve similar to the one for pure IF-B12 were seen when a saturating concentration of B12 was added (Fig. 5C). Consistent with these data, we found that pure porcine IF depleted for 60% of B12 by dialysis against 8 M guanidine HCl (24 h) and 6 M urea (24 h) has a 60%
decrease in binding to the ~460-kDa protein (data not shown). The binding was restored by resaturation with B\textsubscript{12}.

To examine if the purified ~460-kDa protein is the rabbit counterpart to the rat IF-B\textsubscript{12}-binding protein with a reported size of 230–280 kDa (9–11), we compared the electrophoretic mobility and immunoreactivity of the immunopurified rat protein with that of the rabbit ~460-kDa IF-B\textsubscript{12}-binding protein. As shown in Fig. 6 both proteins migrate identically with faster mobility than both megalin and LRP/\alpha\textsubscript{2}MR, and both are recognized by a guinea pig polyclonal antibody raised against the rabbit ~460-kDa protein.

The ~460-kDa Protein Facilitates Endocytosis of IF-B\textsubscript{12} Which Leads to Targeting of B\textsubscript{12} to Lysosomes—Immunohistochemistry (Fig. 7, A and B) and electron microscope immunocytochemistry (Fig. 7, C and D) on rabbit kidney and terminal ileum with the monoclonal antibody 5A-C12 against the rabbit ~460-kDa protein revealed an apical localization related to the brush border and apical vesicles. In proximal tubule there is an additional labeling of the electron-dense recycling apical vesicles (Fig. 7D). The strongest staining was observed in the kidney. Immunohistochemistry of similar sections with the monoclonal antibody against rabbit megalin revealed an apical staining of both tissues (Fig. 7, E and F).

The potential role of the ~460-kDa IF-B\textsubscript{12}-binding protein as an endocytic receptor was then examined by ligand binding and in vivo uptake experiments in rat proximal tubules. To trace the vitamin component, 57Co-B\textsubscript{12}-labeled IF was used in these studies. Sections of rat kidney were incubated with human or rat IF-57Co-B\textsubscript{12}, and the binding sites for the labeled complexes were localized by light microscope autoradiography (Fig. 8). Autoradiographic grains were concentrated at the base of the brush border (arrowheads), which is confirmed by immunocytochemistry at the electron microscope level showing labeling of brush border (BB) and vesicles (V) of both terminal ileum (arrowheads, panel C) and kidney proximal tubules (arrow, panel D). In proximal tubule there is additional labeling of the membrane recycling compartment dense apical tubules (arrows, panel D). Immunohistochemistry using a monoclonal antibody against megalin (5B-B11) shows similar labeling of epithelial cell brush border on cryostat sections of intestinal mucosa (panel E, enhanced using ABCComplexes) as well as labeling of proximal tubule segment 1 apical cytoplasm (P\textsubscript{1}) and segment 2 brush border (P\textsubscript{2}) on cryostat sections of kidney cortex (panel F, no enhancement). Controls using a nonspecific mouse IgG were negative (inset in panel A). Magnification × 1,240 (panels A and B), × 65,000 (panels C and D), × 580 (panel E), and × 825 (panel F).
on the binding of IF-\(^{57}\)Co-B\(_{12}\) to the renal cortex sections in accordance with the results on the purified \(\sim 460\)-kDa protein.

Intravenous injection of \(^{125}\)I-IF-B\(_{12}\) was followed by renal accumulation of the radioligand. Twenty min after injection, 2% of the dose was recovered in the kidneys. Light microscope autoradiography (Fig. 9A) showed labeling of only the brush border and the apical cytoplasm of the proximal tubules thus indicating glomerular filtration of IF-B\(_{12}\) and subsequent uptake in proximal tubules.

In \textit{vivo} microinjection of IF-\(^{57}\)Co-B\(_{12}\) into single rat kidney proximal tubules revealed an uptake of 76 ± 3% (16 tubules in six animals) of the injected radioactivity. Electron microscope autoradiography (Fig. 9B) of tubules fixed 20 min after the microinjection of the radiolabel showed labeling of endocytic invaginations, vacuoles, and lysosomes in proximal tubules. When fixation was performed after 45 min the radiolabeling of lysosomes was predominant (Fig. 9, C and D). Thus, IF-B\(_{12}\) is internalized by endocytosis and B\(_{12}\) transported to lysosomes. Uptake of IF-\(^{57}\)Co-B\(_{12}\) was inhibited significantly by unlabeled IF-B\(_{12}\) and anti-rat gp280/IF receptor antibody but not by RAP (Fig. 10). Control experiments with \(^{57}\)Co-B\(_{12}\) alone showed no significant uptake (Fig. 10).

**DISCUSSION**

In the present study we have identified and characterized a RAP-binding \(\sim 460\)-kDa protein as a high affinity IF-B\(_{12}\) receptor and shown its colocalization in renal and intestinal epithelium with the RAP- and TC-B\(_{12}\)-binding giant receptor, megalin. Furthermore, the present data visualize for the first time the \textit{in vivo} receptor-facilitated endocytosis of IF-B\(_{12}\) and the targeting of the vitamin to lysosomes.

Affinity chromatography of solubilized rabbit renal cortex and surface plasmon resonance analysis showed that the \(\sim 460\)-kDa protein binds RAP with lower overall affinity than megalin. RAP has no measurable effect on the binding of IF-B\(_{12}\) to the receptor, which is in contrast to its strong inhibition of TC-B\(_{12}\) binding to megalin. The biological relevance of the binding of RAP to the IF receptor remains to be established. One tempting possibility is that RAP is involved in the processing of newly synthesized receptors in analogy with its suggested role as an escort protein (24, 25) preventing aggregation of LRP/\(\alpha_2\)MR and megalin.

The surface plasmon resonance analysis demonstrated that IF only binds efficiently to the \(\sim 460\)-kDa protein when it is in complex with B\(_{12}\). Measurable binding activity in IF-rich por-
Cine gastric mucosa extract was only observed when B12 was added to the extract. Furthermore, affinity-purified IF-B12 depleted for B12 loses binding activity equal to the loss of B12. Binding was restored by adding B12. These data are in good agreement with studies on IF-B12 binding to intestinal membranes of various species (33, 34, and references therein). The $K_d$ for IF-B12 as estimated by BIAcore analysis was 10-fold lower than the affinity measured by the microtiter plate assay. Generally, we have observed a difference in the $K_d$ of the binding of RAP and other ligands to megalin and LRP/a2MR similar to that measured by the two methods (15). The difference may rely on the difference in immobilization and/or that the flow on the BIAcore chip decreases the association rate of ligand.

The 460-kDa protein characterized here is most likely the rabbit homolog to the IF-B12 affinity-purified rat protein with a reported size of 230 kDa (9), which recently has shown identity (10) to the rat teratogenic target antigen, designated gp280 (11). In this study, RAP and IF-B12 affinity-purified rabbit; 460-kDa protein as well as immunoaffinity-purified rat gp280 migrated identically in an reducing and nonreducing SDS gel. The size was estimated using the receptors LRP/a2MR α-chain (515 kDa) and megalin (600 kDa) as high molecular mass standards.

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FIG. 10. Uptake of microinjected $^{57}$Co-B$_{12}$-IF in rat proximal tubules. No significant uptake was observed with $^{57}$Co-B$_{12}$ (three punctures in two rats). Coupling of $^{57}$Co-B$_{12}$ to IF increased uptake of radioactivity to 76% (16 punctures in six animals). Uptake was inhibited significantly by unlabeled B$_{12}$-IF (eight double punctures in five animals) but not by RAP (seven double punctures in five animals). Anti-gp280 antibody showed a significant inhibition compared with control IgG (22 punctures in seven animals). Double punctures, which are microinjections of the same single tubule with and without potential inhibitor, were performed to avoid bias caused by the different length of the tubule distal to the injection site.

Microinjection of 460-kDa protein into rat proximal tubules revealed uptake by luminal endocytosis in proximal tubules. Panel A, no other segment of the nephron showed any significant labeling. Only the first part of the proximal tubules ($P_1$) reveals heavy labeling, suggesting that the filtered $^{125}$I-IF-B$_{12}$ is reabsorbed efficiently, as illustrated by a neighboring, scarcely labeled, later segment of the proximal tubule ($P_2$) in panel A. In addition, proximal tubules were microinjected with IF-$^{57}$Co-B$_{12}$ and fixed 20 min after microinjection followed by electron microscope autoradiography (panel B). Autoradiographic grains were localized to the brush border (BB), endocytic invaginations (EI), and vacuoles (EV). When fixation was performed 45 min after microinjection most grains were localized to lysosomes (L), showing targeting of the endocytosed $^{57}$Co-B$_{12}$ to lysosomes (panels C and D). Magnification $\times$ 990 (panel A), $\times$ 18,000 (panels B and D), $\times$ 580 (panel C).

3 S. K. Moestrup and C. Jacobsen, unpublished results.
standard markers. Furthermore, both are recognized by the same polyclonal guinea pig antibody. Amino-terminal sequencing of the ~460-kDa protein identified a unique sequence. Ongoing cDNA cloning of the protein will elucidate whether the protein has structural homology to other receptors, e.g., the low density lipoprotein receptor family proteins, which also bind RAP.

The in vivo studies of the uptake of IF.55Co-B12 in the rat proximal tubule showed a ~460-kDa protein-facilitated uptake very similar to the megalin-mediated uptake of TC-B12 described recently (15). 20 min after uptake 55Co-B12 was largely found in endocytic vacuoles and later in lysosomes. The same may be the case in the intestine, where B12 is known to be transcytosed and secreted basolaterally into blood in complex with TC (35). Studies on polarized human Caco-2 intestinal cells (36) and a renal opossum cell line (37) suggest that proteolysis of IF is a prerequisite for transcytosis of B12. A similar transcytosis and secretion of B12 in complex with TC in the kidney are also likely because the kidney is the organ with the highest TC synthesis as estimated by Northern blotting of various tissues (38).

IF is mainly present in the gastrointestinal tract, but minor amounts of IF have also been reported elsewhere in the organism including plasma, urine (39), and bound to renal brush-border membranes (40). The renal uptake of filtered IF-B12 and TC-B12 (via megalin; Ref. 15) might therefore help conserve the transcytosis and secretion of B12 in complex with TC in the gastrointestinal system may suggest that the ~460-kDa protein has ligand specificities other than RAP and IF-B12 in analogy with the other RAP-binding giant receptors, megalin and LRP, which both display an unusual versatility in ligand specificity (41–44). An additional role of renal IF-B12 receptor is also suggested by the fact that patients with inherited pernicious anemia caused by an autosomal recessive defect in intestinal binding and uptake of IF-B12 (Imerslund-Gräbeck disease) (45, 46) have proteinuria. A similar inherited B12 malabsorption disease combined with proteinuria has been described in dogs (47). We are presently screening proteins for binding to the purified ~460-kDa IF receptor to evaluate further its ligand binding properties.

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