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Research Article

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Identification of Rat Yolk Sac Target Protein of Teratogenic Antibodies, gp280, as Intrinsic Factor-Cobalamin Receptor

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Abstract

Previous studies in the rat have shown that antibodies to gp280, a protein > 200 kD and closely associated with the early endocytic system can induce fetal malformations. Although gp280 is thought to act as a receptor, its ligand(s) is not known. In the current study, we report that purified gp280 from rat kidney, like the intrinsic factor-Cobalamin receptor (IFCR), binds to the intrinsic factor-cobalamin (IF-Cbl) complex with an association constant of 0.3×10^9 M^{-1} and mediates its internalization. Furthermore, antibodies raised to purified gp280 and IFCR inhibited the binding of IF-[^{57}Co]Cbl complex to intestinal, renal, and yolk sac apical membranes and revealed a single identically sized protein on immunoblotting of the renal membranes. Both antibodies precipitated a single radiolabeled protein > 200 kD from cellular extract from [^{35}S]methionine-labeled yolk sac epithelial cells, and antibody to gp280 inhibited the uptake and internalization of ^{125}IF-Cbl. Immunoelectron microscopy using the two antibodies revealed that in the kidney, both proteins were colocalized. These observations suggest that IF-Cbl complex is a ligand for gp280 and that gp280 and IFCR are identical proteins. (*J. Clin. Invest.* 1997. 99: 2317–2322.) Key words: intrinsic factor receptor • glycoprotein 280 • teratogenic antibody • endocytosis • cobalamin

Introduction

More than 30 yr ago, Brent et al. (1) showed that antikidney antibodies of undefined specificity induced fetal malformations when administered to pregnant rats before day 10 of pregnancy. Numerous experiments provided evidence that the antibodies did not bind to the fetus but bound to the yolk sac epithelium, which at that time of pregnancy is the only fetal–maternal interface that functions as the placenta. It was thus suggested that the antibodies interfered with a key function of this membrane. A defined target for the antibodies was identified by the isolation of a protein referred to as gp280 (2–4), which was concentrated in clathrin-coated areas of the proximal tubule and the yolk sac epithelia. These observations suggested that the target protein, gp280, is associated with the endocytic apparatus and may act as a receptor. Monoclonal antibodies to gp280 were able to induce the same type of fetal malformations as the antikidney antibodies, whereas antibodies to megalin (also previously designated as gp330 or Heymann nephritis antigen) (2, 5), a high molecular weight multiligand receptor (6–10) with a similar tissue and membrane distribution (2, 11), failed to induce malformations, suggesting that the binding properties of gp280 to unknown ligands were crucial.

Independent of these investigations, Seetharam et al. (12) isolated from rat renal brush borders a protein of > 200 kD that binds with high affinity to intrinsic factor-cobalamin complex. This protein, known as intrinsic factor cobalamin receptor (IFCR)[1] is identical to that expressed in the intestine, where it functions in the transcytosis of cobalamin (Cbl; vitamin B_{12}). In addition to its expression in the kidney, IFCR is also expressed in the yolk sac membranes, where its activity and protein levels are regulated during the gestation period (13), being maximum at the time when anti–gp280 antibodies are teratogenic.

The similarity in the mobility and size on SDS-PAGE and restricted tissue distribution of gp280 and IFCR suggested the possibility that IFCR and gp280 are similar or identical proteins and the results of this study show that the two proteins are functionally and immunologically identical.

Methods

Monoclonal antibodies to gp280 and megalin were prepared as described earlier (2). The specificity of polyclonal rabbit antibodies raised against gp280 or megalin purified from kidney cortex by immunoaffinity chromatography (3) and against IFCR prepared from rat kidney cortex by ligand affinity on solid phase intrinsic factor (IF) Cbl complexes (12) have been reported earlier. ^{57}[Co]Cbl (15 μCi/μg) was purchased from Amersham Co. (Arlington Heights, IL).

Membrane isolation. Brush border membranes were prepared from rat ileum, kidney, and yolk sac as previously reported (14, 15). Rat renal intermicrovillar clefts (400 mg protein) were solubilized at 4°C in Tris-buffered saline containing Triton X-100 (1%) and 5 mM $CaCl_2$.

Determination of IFCR activity. IF-^{57}[Co]Cbl complex was prepared as described (12). The EDTA inhibitable binding of IF-^{57}[Co]Cbl

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1. *Abbreviations used in this paper:* Cbl, cobalamin (vitamin B_{12}); IF, intrinsic factor; IFCR, IF-Cbl receptor.

(5 pmol) to membrane preparations was measured as described (12). The effect of gp280 and IFCR polyclonal antiserum on the membrane binding (25 μg for renal and yolk sac and 200 μg for intestinal membranes) of the ligand was tested by the addition of the ligand, IF-^{57}[Co]Cbl following a preincubation of the membranes for 30 min at 22°C with the antisera (2–50 μl).

Cell culture. Cloned BN/MSV cells, which are derived from a rat yolk sac teratocarcinoma and express gp280 and megalin, were grown in minimum essential medium supplemented with 10% FCS (16).

Immunochemical studies. Solubilized brush border proteins were separated by SDS/PAGE on 4.9% gels (17) and electroblotted onto nitrocellulose sheets that were saturated with nonfat dry milk before incubation with polyclonal antibodies raised against the IFCR, gp280, or megalin. Bound IgG were revealed with alkaline phosphatase–conjugated goat anti–rabbit IgG (Promega Corp., Madison, WI). Cultured BN/MSV cells were labeled with ^{35}[S]Translabel (1,000 Ci/mmol; ICN, Orsay, France) for 120 min and solubilized by Triton X-100 (1%) (18). Immune complexes formed with polyclonal antibodies specific for the IFCR, gp280, or megalin were collected on protein A-Sepharose 4B beads, eluted under nonreducing conditions with Laemmli sample buffer and subjected to SDS-PAGE (17).

Immunolocalization of IFCR and gp280. Ultrathin (70–90 nm) cryosections were obtained from paraformaldehyde (8%)-fixed rat kidneys as previously reported (19). All sections were preincubated in PBS containing 1% BSA and 0.05 M glycine and incubated overnight at 4°C with a mouse monoclonal anti–gp280 (1–3 μg/ml) or with a polyclonal rabbit IFCR antibody diluted 1:6,400 and subsequently with 10 nm goat anti–mouse-gold or 10 nm protein A-gold (Bio Cell, Cardiff, UK) at 4°C for 2 h. The sections were contrasted with methyl cellulose containing 0.3% uranylacetate and studied with an electron microscope (CM 100; Phillips Electronic Instruments Co., Mahwah, NJ). Controls performed in the absence of primary antibody and in the presence of nonspecific monoclonal antibodies or rabbit immunoglobulin did not show any specific labeling.

Binding and internalization of [^{125}I]IF-Cbl complex. IF (10 μg) purified from rat gastric extracts (20) was iodinated by the IODOGEN method according to the manufacturer (Pierce, Rockford, IL) using 7.5 MBq of ^{125}I. The specific activity of ^{125}I-IF was 1 MBq/μg. BN/MSV cells cultured in 24-well plates (Nunc, Inc., Rosklide, Denmark) were incubated for 1–4 h in the presence of [^{125}I]IF-Cbl complex, diluted in serum-free MEM, 0.1% BSA. Cell-associated radioactivity was measured by counting the washed cells after solubilization of the cell layer in 1% Triton X-100 after a wash of the cells with pH 5.5 EDTA buffer to remove any cell surface radioactivity. Degradation of IF was measured by precipitation of the incubation medium in 12.5% TCA. To measure the effects of anti–gp280 antibodies, identical experiments were carried out in which cells were preincubated for 3 h under control conditions, or in the presence of either polyclonal anti–gp280 antibody, monoclonal anti–gp280, or normal rabbit serum.

Purification of IFCR by immunoaffinity on Sepharose-4B covalently coupled with mAb to gp280. The immunoadsorbent was packed into a 1 × 3–cm column and washed extensively with Tris-HCl buffer, pH 7.4, containing 140 mM NaCl, 5 mM $CaCl_2$, and 0.1% Triton X-100. The Triton X-100 (1%)–solubilized intermicrovillar extract (10 ml) was centrifuged (25,000 *g*, 30 min) and the supernatant (8 ml, 300 mg protein), containing 2 nmol of IF-Cbl binding capacity, was passed over the column. The column was sequentially washed with (*a*) 200 ml of the loading buffer, (*b*) 200 ml of Tris buffered saline containing 5 mM EDTA, and (*c*) 0.1 M Na acetate buffer, pH 3.0, containing 0.1% SDS, 0.1% Triton X-100, and 5 mM EDTA. The eluate (20 ml) was immediately neutralized to pH 7.4, and $CaCl_2$ was added to a final concentration of 10 mM. The eluate (20 ml containing 120 μg protein) was assayed for IF-^{57}[Co]Cbl binding activity and by nonreducing SDS-PAGE (5%) (17). The association constant K_a was determined using IF-^{57}[Co]Cbl (0.03–1.8 pmol) as described (21).

Results

The same protein is identified by immunoblotting of renal brush border membranes and by immunoprecipitation of labeled BN cells. Immunoblotting experiments (Fig. 1 *A*) showed that both gp280 (*left*) and IFCR (*right*) polyclonal antisera recognized a single band (*arrow*) in renal brush borders comigrating with immunoaffinity purified gp280, but failed to recognize pure megalin. To confirm that de novo synthesized IFCR/gp280 is immunoreactive with antisera to both these proteins,

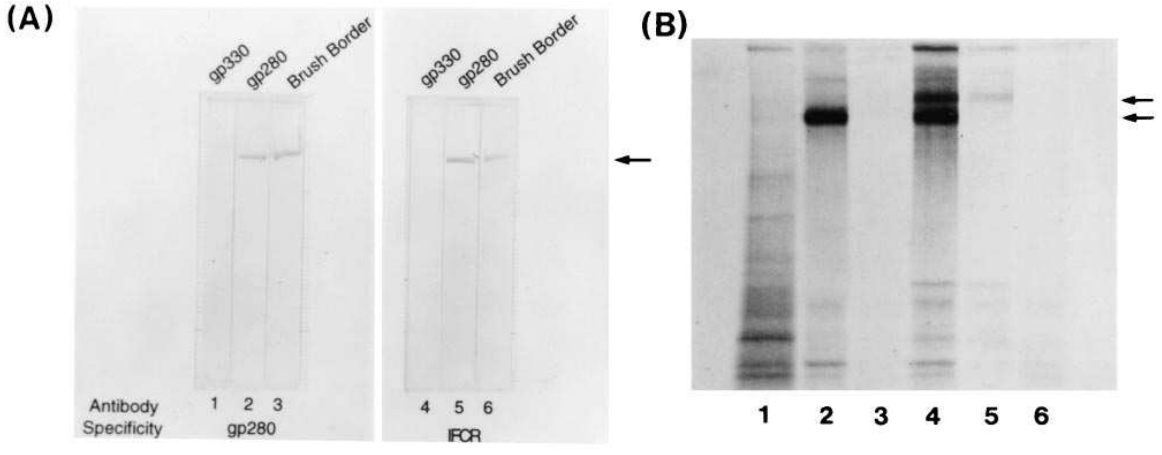

Figure 1. (*A*) Reactivity of solubilized brush border membrane vesicles (100 μg), purified gp280, or megalin (1 μg) with polyclonal anti–gp280 or anti–IFCR antibodies. Immunoblot analysis using anti–gp280 (*right*) or anti–IFCR (*left*) to probe brush border preparation (lanes *3* and *6*), affinity-purified gp280 (lanes *2* and *5*), or affinity-purified megalin (lanes *1* and *4*). The arrow indicates the position of the immunocross-reactive band. (*B*) Immunoprecipitation of gp280/IFCR synthesized de novo by BN/MSV cells. Solubilized proteins were immunoprecipitated using polyclonal anti–gp280 (lane *2*) and anti–IFCR antibodies (lane *4*), and analyzed by 4.9% SDS-PAGE. The supernatants of the immunoprecipitates analyzed in lanes *2* and *4* were further treated with anti–IFCR (lane *3*) or anti–gp280 (lane *5*). Lanes *1* and *2* represent control total labeled proteins in the solubilized cell extract and immunoprecipitation with normal rabbit serum, respectively. The arrows indicate the positions of gp280 (*bottom*) and gp330 (*top*), respectively.

BN cells were metabolically labeled with [^{35}S]methionine. Treatment with polyclonal antibodies (Fig. 1 *B*) to gp280 (lane *2*) or IFCR (lane *4*) precipitated a major labeled protein (*lower arrow*) comigrating with gp280/IFCR isolated from renal brush border. Sequential immunoprecipitations demonstrated that anti–gp280 antibodies removed the protein reactive with anti–IFCR (lane *3*) and vice versa (lane *5*). No labeled proteins were precipitated with preimmune rabbit serum (lane *6*) and the spectrum of labeled proteins in the detergent cell extract before immunoprecipitation with antisera is shown in lane *1*. Some of the lower sized proteins immunoprecipitated with antiserum to either gp280 (lane *2*) or IFCR (lane *4*) could not be further immunoprecipitated from the respective supernatants using antiserum to IFCR (lane *3*) or gp280 (lane *5*), respectively, suggesting that these bands are immunocross-reactive degraded products of gp280 and IFCR. These results clearly prove that IFCR and gp280 are synthesized de novo as a single protein.

Antibodies to gp280 inhibit membrane binding and internalization of IF-Cbl complex. When graded amounts of antiserum to gp280 or IFCR were tested for their ability to inhibit membrane binding of IF-^{57}[Co]Cbl (Fig. 2 *A*), there was a progressive loss of ligand binding to renal, yolk sac, and intestinal membranes. [^{125}I]IF-Cbl complexes bound efficiently to the BN/MSV cells and were rapidly internalized and degraded (Fig. 2 *B*). This process was mediated by IFCR/gp280 since binding of [^{125}I]IF-Cbl and subsequent degradation of [^{125}I]IF were significantly decreased when the cells were preincubated with antiserum to gp280, before the addition of the ligand (Fig. 2 *C*).

Immunolocalization of gp280 and IFCR. The segmental and subcellular distribution determined by immunocytochemical labeling using the monoclonal anti–gp280 antibody and the rabbit polyclonal anti–IFCR antibody were identical (Fig. 3). The labeling was confined to the proximal tubule and no labeling was seen in glomeruli, other nephron segments, or collecting ducts. The labeling was strongest in the first two segments of the proximal tubule as compared with segment 3. As seen in Fig. 3, *a* and *b*, apical labeling was seen in the early endocytic pathway, including coated pits and coated and noncoated endosomes. In addition, there was a certain labeling of the microvilli, mainly in the basal portion, and a strong labeling of the dense apical tubules that are responsible for the membrane recycling in these cells (22, 23). Most lysosomes were not labeled; however, in segment 2 of the proximal tubule there was highly specific labeling of the membrane of the electron-dense lysosomes characteristic of this segment. This labeling was evident with both anti–IFCR (Fig. 3 *c*) and the monoclonal anti–gp280 (Fig. 3 *d*). Since megalin previously has been identified in lysosomes, immunolabeling was also performed with anti–IFCR preabsorbed with purified rat megalin. Except for a slightly weaker reaction, these experiments revealed no differ-

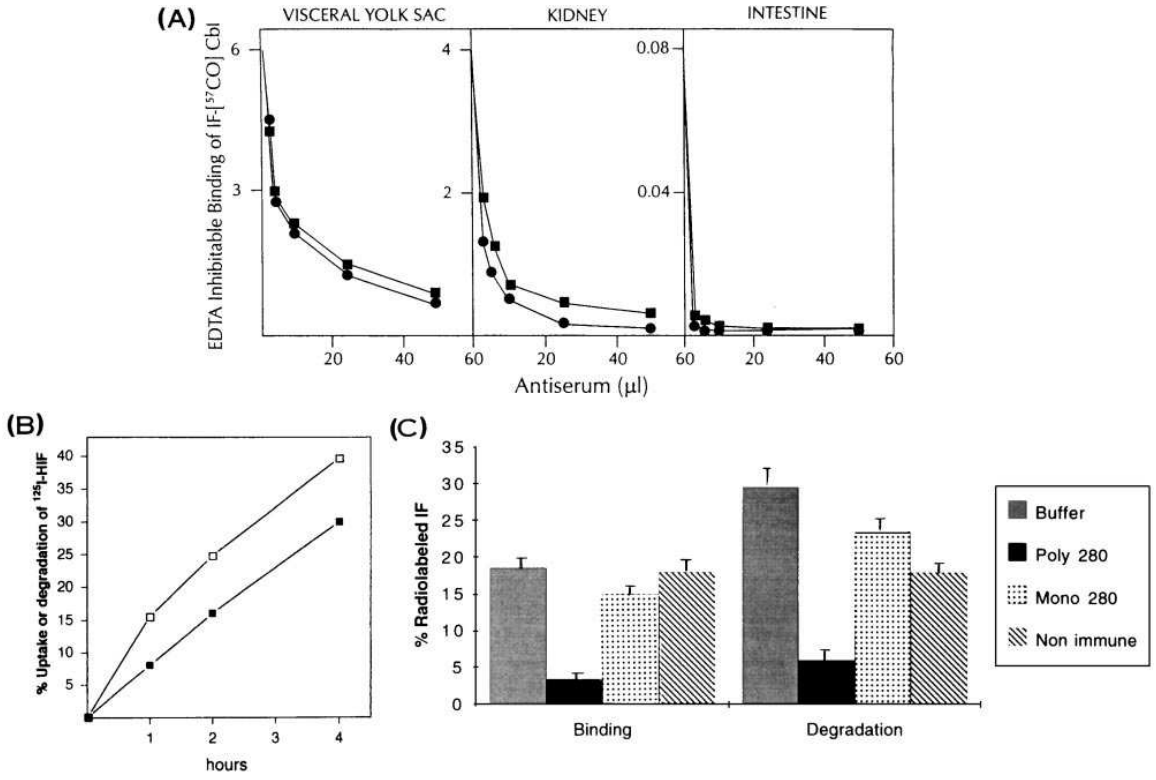

Figure 2. gp280 binds to and mediates the internalization of IF-[^{57}Co]Cbl. (*A*) Inhibition of binding to visceral yolk sac, kidney, and intestinal membranes by polyclonal antiserum to gp280 (●) or anti–IFCR (■). (*B*) Binding and degradation of ^{125}I-IF-Cbl of BN/MSV cells. Confluent cell layers were incubated with ^{125}I-IF-Cbl (10,000 cpm) for 0, 1, 2, or 3 h at 37°C in 400 μl MEM and 0.1% BSA. Degradation (■) was measured as the increase in TCA-soluble radioactivity in the medium. The uptake (□) was measured as the cell-associated radioactivity plus the degraded fraction. (*C*) Antibodies to gp280 prevent internalization and degradation of IF. The values are the mean of duplicates.

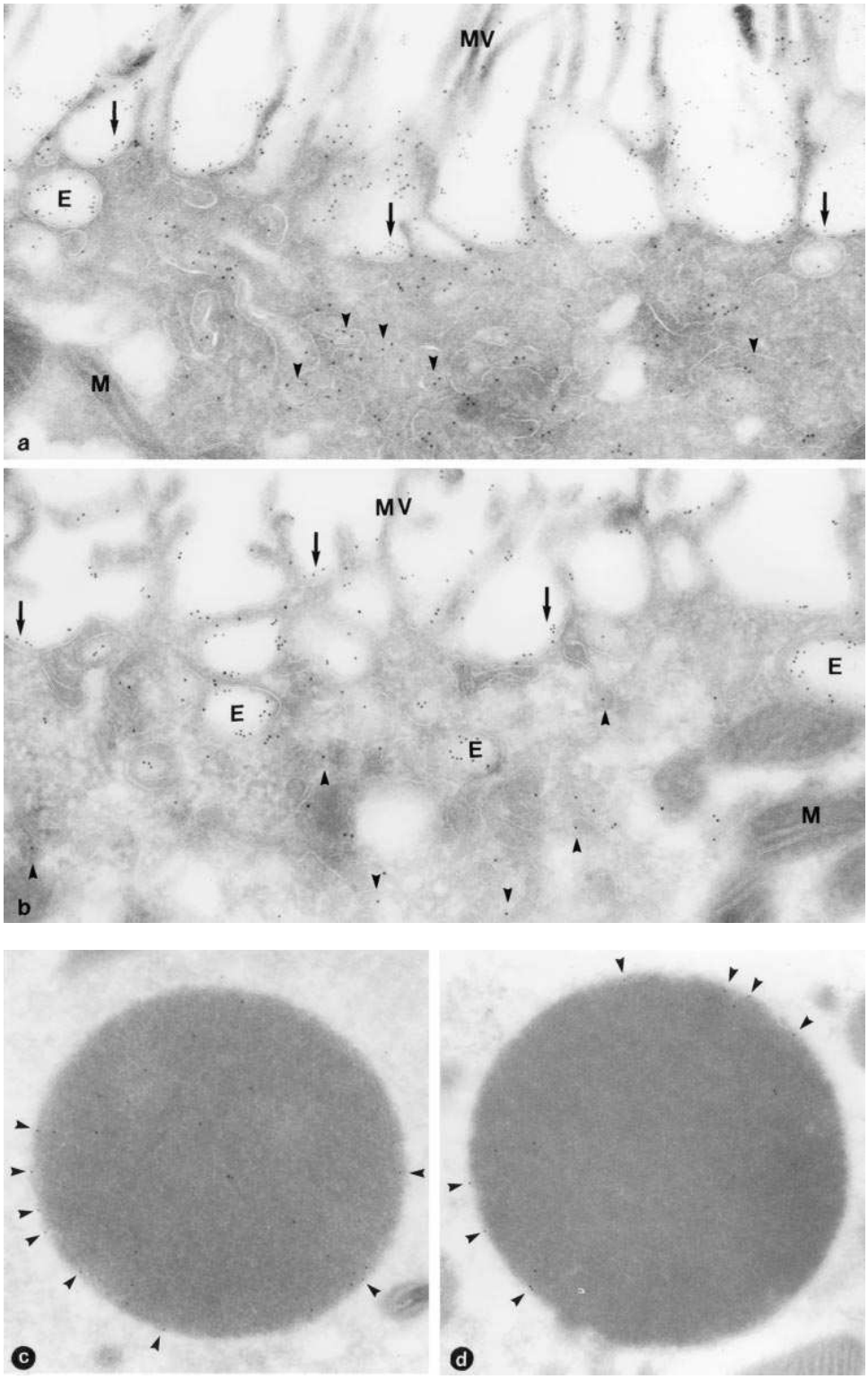

Figure 3. Electron microscope immunocytochemistry of cryosections from rat renal proximal tubules (*a* and *b*) and lysosomes (*c* and *d*) using, in *a* and *c*, rabbit polyclonal anti–IFR antibody and 10 nm protein A gold or, in *b* and *d*, monoclonal anti–gp280 antibody and 10 nm goat anti–mouse gold. Note identical labeling profiles with the two antibodies in the apical part of the proximal tubule cells. Intensive membrane labeling is seen in apical-coated pits (*arrows*), in small and large endosomes, some of which may be membrane invaginations and large endosomes (*E*), in dense apical tubules (*arrowheads*), and on microvilli (*MV*), mainly in the basal part. Virtually no background labeling is seen; e.g., the mitochondria (*M*). *a*, 49,000×; *b*, 57,000×. The electron-dense lysosomes are from segment 2 proximal tubule cells and both show intensive membrane labeling (*arrowheads*). There is also certain labeling of the lysosomal matrix, particularly in *c* (42,000×).

ences in the subcellular distribution of label, whereas tissue binding was abolished by preincubation with affinity-purified gp280.

gp280 purified from immunoabsorbent affinity chromatography binds IF-[^{57}Co]Cbl with high affinity. gp280, eluted from Sepharose beads coupled to gp280-specific mAb, bound IF-[^{57}Co]Cbl (1,800 pmol/mg protein) with high affinity. The purified gp280 was enriched for IFCR-specific activity by $\sim$ 270-fold from an initial value of $\sim$ 6.7, with a recovery of $\sim$ 11%. The association constant K_a determined by using pure rat IF-[^{57}Co]Cbl was 0.3×10^9 M^{-1} (Fig. 4 *A*). Nonreducing SDS-PAGE of the eluate revealed a single high molecular mass protein $>$ 200 kD (Fig. 4 *B*). The initial IFCR activity in the intermicrovillar cleft extract, 2 nmol, was completely retained by

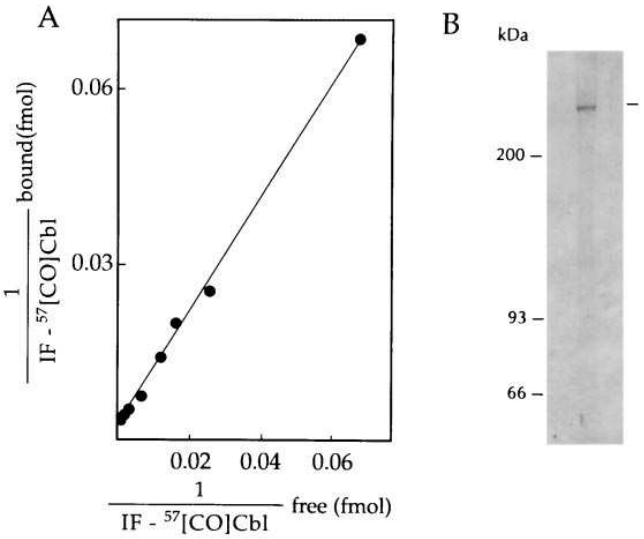

Figure 4. Binding of IF-[^{57}Co]Cbl and SDS-PAGE from the gp280 eluted from Sepharose 4B coupled to monoclonal anti–gp280 antibodies. (*A*) The eluted fraction was used to determine K_a from saturation kinetics obtained using rat IF-[^{57}Co]Cbl (0.03–1.85 pmol). (*B*) Protein (1 μg) was subjected to nonreducing SDS-PAGE (5%) and the band was visualized by silver nitrate staining.

the immunoabsorbent with no activity in either the flow through or the initial washes (data not shown). These results clearly demonstrate that purified gp280 contains intrinsic IFCR activity.

Discussion

The results presented in this paper show that gp280 first identified as the yolk sac target of teratogenic antibodies is capable of binding IF-Cbl complex and is most likely identical to the IFCR expressed in kidney, intestine, and yolk sac. This conclusion is supported by the following lines of evidence: (*a*) solid phase monoclonal antibodies to gp280 selectively deplete cortical intermicrovillar clefts of IFCR activity, (*b*) binding of IF-[^{57}Co]Cbl can be demonstrated with affinity-purified gp280, (*c*) anti–gp280 antibodies immunodeplete the protein reactive with the anti–IFCR antibodies, and (*d*) anti–gp280 and anti–IFCR equally inhibit the binding of IF-[^{57}Co]Cbl complex to intestinal, renal, and yolk sac membranes. These observations together with the fact that gp280 isolated from adult rat kidney has very similar K_a (nM) values for IF-[^{57}Co]Cbl binding as rat renal (12) or intestinal (21) IFCR strongly indicate that gp280 is a high affinity receptor for IF-Cbl.

The most popular hypothesis to account for the teratogenic effects of antibodies is derived from the observation that, until the establishment of the allantoic placenta, the amino acids used for the embryonic growth of the fetus are derived from the maternal proteins. The internalized proteins are degraded by the yolk sac epithelial cells and it has been proposed (24) that the teratogenic antibodies decrease the free amino acid pool available to the fetus by inhibiting the internalization of maternally derived proteins. This hypothesis essentially rests on the observation that the teratogenic antibodies reduce fluid phase endocytosis. However, there is no direct link between altered pinocytosis and fetal malformations.

The above hypothesis viewed in light of current observation raises the question of a role of Cbl deficiency in antibody-induced fetal malformation, especially since Cbl is crucial for the rapidly dividing cells of the yolk sac and the embryo. Due to very low levels of Cbl needed, abnormalities of fetal development have only been reported (25) in severe maternal Cbl deficiency, such as those in strict vegetarians (26). However, it is possible that inadequate maternal–fetal transfer of Cbl by the gp280 antibody could create severe Cbl deficiency in the developing embryo even with normal Cbl status of the mother. The observation that peak IFCR activity coincides with the time frame of teratogenic activity of gp280 antibody supports this hypothesis. The main uncertainty regarding this hypothesis is that the availability of IF in circulation is extremely low (27) and it is not known whether the yolk sac epithelial cells synthesize IF locally and secrete it apically.

An alternative hypothesis for the teratogenic effects of gp280 antibodies is that it could bind to gp280/IFCR, causing massive disruption of the endocytic apparatus, which then could prevent not only Cbl transcytosis to the basal pole, but also other events such as membrane fusion and recycling. This hypothesis is supported by the observation that Grasbeck-Immerslund (28, 29) syndrome patients who have loss of IFCR function develop both Cbl deficiency and proteinuria. The latter persists even after correction of Cbl deficiency and the related hematological disorders with Cbl treatment. This observation suggests that loss of surface IFCR due to a potential genetic defect (30) or to administration of antibody may have consequences other than (and including) Cbl deficiency.

It is interesting to note that gp280/IFCR shares similarities with another high molecular weight receptor, megalin, on at least two points. First, the cellular and subcellular distribution of the two proteins are quite similar (2, 3). Second, it has recently been shown (31) that megalin targets Cbl to the lysosomes via transcobalamin II and that it does not bind the IF-Cbl complex. Interestingly, antibodies to megalin are not teratogenic (3). These observations underscore the importance of specific binding and targeting properties of gp280 for Cbl and perhaps other ligands. In summary, the results from this study have identified for the first time that IF-Cbl acts as a ligand for gp280, a protein target of teratogenic antibodies.

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