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Renal brush border membrane bound intrinsic factor

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A highly active receptor for intrinsic factor (IF)-cobalamin (Cbl) complex has been detected and reported in mammalian kidney earlier (Seetharam, B., et al. (1988) J. Biol. Chem. 263, 4443–4449). The physiological role of this receptor in normal Cbl homeostasis is not known. In addition to binding of exogenously added IF-[^{57}Co]Cbl, the renal apical membranes contain endogenous IF or IF-Cbl. Washing with pH 5/EDTA buffer enhanced the binding of exogenously added IF-[^{57}Co]Cbl to renal apical but not basolateral membranes. The pH 5/EDTA extract from renal apical membranes bound [^{57}Co]Cbl. The complex also bound to rat ileal brush border membrane and promoted ileal transport of [^{57}Co]Cbl. On immunoblots using monospecific antiserum to IF a 62 kDa protein was identified in renal and intestinal apical membranes, serum and in tissue extracts of unperfused rat liver, kidney and heart. The 62 kDa band was eliminated from the renal apical membranes following pH 5/EDTA wash. Rat urine demonstrated unsaturated [^{57}Co]Cbl binding (0.2 to 0.4 pmol/day) of which only 30–40% was immunoprecipitated with anti IF and could be identified on immunoblots. The identification of IF in rat renal apical membranes (160–200 ng/mg protein) and secretion of only traces of IF in urine suggest that the renal IF-Cbl receptor may play a role in sequestering IF/IF-Cbl and prevent urinary loss of Cbl.

Introduction

Dietary cobalamin (vitamin B-12; Cbl) is assimilated in animal tissues following intestinal absorption of Cbl bound to gastric intrinsic factor (IF). The intestinal absorption is mediated by IF-Cbl receptor located in the microvillus pits of ileal apical brush border membrane [1]. In addition to the ileal mucosa a highly active IF-Cbl receptor is also expressed in the mammalian kidney [2]. The physiological role of the renal receptor, if any, in normal Cbl homeostasis is not clear. Although, the renal receptor binds exogenously added IF-[^{57}Co]Cbl complex, it is not known whether the receptor binds IF-Cbl complex in vivo. This uncertainty is due to the long held view that the ligand IF is never found in the circulation [3,4]. Even though, direct evidence for IF entry into circulation is not available, IF and other Cbl binding proteins have been detected in trace amounts in the urine of patients with pernicious anemia [5,6]. The source of urinary IF in these patients is not known. However, since a patient with selective cobalamin malabsorption also excreted IF, the origin was suggested to be from the stomach itself and not due to transcytosis of IF via the intestinal epithelial cells [5].

The present work was undertaken to address the issue of urinary excretion, the presence of endogenous IF in the kidney and possible role of renal IF-Cbl receptor. Studies described herein have identified immunoreactive IF for the first time in rat serum, urine and renal apical brush border membranes. Moreover, the IF identified from these sources had a molecular mass of 62 kDa, a value 12 kDa higher than that for pure gastric IF. The potential regulatory role of kidney in normal Cbl homeostasis by IF-Cbl receptor salvage pathway is discussed in view of the detection of IF bound to renal apical but not basolateral membranes.

Experimental procedures

Materials

Cyano[^{57}Co]cobalamin (15–220 μCi/μg) was purchased from Amersham Corporation (Arlington Heights, IL). ^{125}I-protein A (> 30 μCi/μg) was purchased from ICN Radiochemicals (Irvine, CA). Cellulose Nitrate membranes used for immunoblotting were purchased

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from Schleicher and Schuell (Keene, NH). Male Sprague-Dawley rats (150–200 g) were purchased from Sasco Inc. (Omaha, NE). Human kidney pieces were obtained from unidentified donors from the Nephrology Unit at Froedtert Memorial Lutheran Hospital (Milwaukee, WI). Dog kidney was a generous gift from the Physiology Department of the Medical College of Wisconsin. The tissues were kept frozen at −70 °C and used within 1 to 2 weeks.

Purified proteins and their antisera

Rat stomach IF was purified by affinity chromatography using monocarboxylic acid derivative of Cbl as affinity ligand by the procedure described earlier [7]. R-type Cbl binder from hog stomach was purified according to Allen and Mehlman [8] using nucleotide free Cbl as the affinity ligand. Rat plasma transcobalamin II was partially purified according to Lindemans et al. [9]. Antiserum to rat IF and hog R binder was raised in rabbits essentially as described earlier [10].

Preparation of rat tissue homogenates and subcellular fractions from the kidney

Rat liver, kidney, heart and stomach homogenates were prepared from perfused and non-perfused animals using 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM benzamidine.

Brush border membrane from rat, canine and human kidney were prepared according to Kessler et al. by the Ca^{2+} aggregation method [11]. The IF-Cbl receptor activity was enriched in these membranes 15–20-fold with a 15–20% recovery. Basolateral membranes were prepared according to Molitoris and Simon [12]. The membranes were enriched 12-fold for Na^+/K^+-ATPase with 15% recovery. Golgi-rich membranes were isolated by the sorbitol density centrifugation method of Freedman et al. [13]. The 20/30 fraction was enriched 10-fold for galactosyltransferase activity. Cytosolic fraction of rat intestinal mucosa and kidney were obtained as follows: Fresh rat kidneys and scraped mucosa were cut into small pieces and homogenized in 0.25 M sucrose containing 5 mM K-phosphate buffer (pH 7.4) and 5 mM disodium EDTA. The homogenate was centrifuged for 2 h at 105 000 × *g*. The clear supernatant thus obtained was carefully removed from the center of the tube.

Isolated renal brush border membranes from rat, canine and human kidney (1 ml; 8–10 mg protein) were incubated with 1 ml of (a) 10 mM K-phosphate buffer (pH 7.4), (b) 10 mM K-phosphate buffer (pH 7.4) containing 5 mM EDTA, (c) 10 mM K-phosphate buffer (pH 5.0), (d) 10 mM K-phosphate buffer (pH 5.0) containing 5 mM EDTA. Following incubation for 2 h at 22 °C, the membranes were washed once more and homogenized in 1 ml of 10 mM Tris-HCl buffer (pH 7.4). In a separate experiment renal apical brush border (75 to 100 mg protein) was treated with pH 5/EDTA buffer for 2 h at 5 °C. The mixture was centrifuged at 20 000 × *g* for 30 min. To the neutralized (pH 7.4) supernatant guanidine-HCl (2 M) was added and dialyzed against 1 litre of 2 M guanidine-HCl for 4 h at 5 °C. The dialysis was then continued by dialyzing against 6 l of water for 36 h with a change every 12 h. The dialyzed fraction was used for in vitro binding and in vivo uptake studies.

Immunoblotting for IF and R-proteins

Tissue homogenates or membranes (10–50 μg protein) were incubated in a volume of 100 μl containing 1 mM EDTA, 1 mM PMSF, 1 mM *N*-ethylmaleimide 2 mM benzamidine for 30 min at 37 °C. The reaction mixture was treated with sodium dodecyl sulfate (final concentration 2%), kept in boiling water bath for 5 min and subject to SDS-PAGE electrophoresis on 7.5% gel. The immunoblotting of the separated proteins was carried out essentially according to Brunette [14] and probed with monospecific antisera to rat IF or hog R protein and ^{125}I-protein A. The autoradiography was carried out by exposure to Kodak X-omatic AR film and developed for various periods (days). A standard immunoblot was developed using pure IF (5–40 pmol). The autoradiograph was developed for 24 or 96 h and scanned using a laser densitometer.

Binding of [^{57}Co]Cbl complexed to Cbl binders to the renal plasma membranes

The EDTA inhibitable binding of IF-[^{57}Co]Cbl and R-[^{57}Co]Cbl complexes to washed and unwashed membranes, and association constant (K_a) for IF-[^{57}Co]Cbl were determined according to Hooper et al. [15]. The binding of TC II - [^{57}Co]Cbl (1.84 pmol) to renal membranes was carried out essentially according to Seligman and Allen [16].

Other methods

In vivo uptake studies were carried out using [^{57}Co]Cbl complexed to either gastric or renal extracts. The complex (3.7 pmol; 1.5 ml) was orally administered through a feeding tube. The rats were killed 4 h later and the tissues (liver and kidneys) were washed and counted for [^{57}Co]Cbl to measure the transport of orally administered [^{57}Co]Cbl to the tissues.

Anesthetized adult rats were perfused through the left ventricle of the heart using phosphate-buffered saline. The perfused liver, kidney and heart were removed, cut into small pieces and homgoenized. 10–50 μg of tissue homogenate protein was processed for immunoblotting as described above. Protein in tissue homogenates and isolated cellular fractions were determined according to Lowry et al. [17] using bovine serum albumin as standard. The binding of [^{57}Co]Cbl to

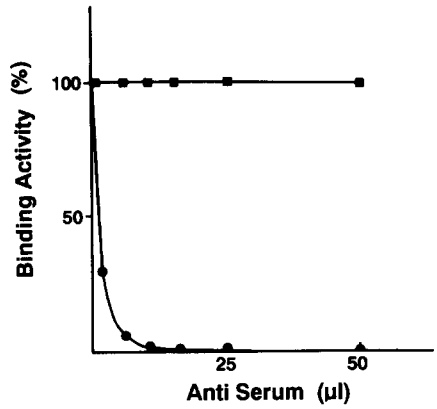

Fig. 1. Effect of anti-IF serum on blocking and binding reactions. The binding of rat IF (0.74 pmol binding ability) to [^{57}Co]Cbl [1.48 pmol] was determined by the charcoal absorption method. The EDTA inhibitable binding of rat IF-[^{57}Co]Cbl (1.48 pmol) to rat ileal brush border membrane (800 µg protein) was determined as described in Methods. Indicated amounts of antiserum to IF were added and preincubated with IF or with ileal membranes for 15 min at 22°C. Following this incubation [^{57}Co]Cbl to IF and IF-[^{57}Co]Cbl to the ileal membranes were added. The binding values [^{57}Co]Cbl to IF (●) and of IF-[^{57}Co]Cbl to ileal membranes (■) shown are average of three separate binding reactions.

IF, R-protein, TC II and urine was determined by the charcoal method of Gottlieb et al. [18]. Urine was collected from rats housed in individual metabolic cages every 24 h and 48 h. Urine collected for 48 h from three separate rats was pooled (50 ml) and dialyzed against 5 mM Tris HCl buffer containing 1 mM PMSF for 72 h with a change of the same buffer at 24 and 48 h. The dialyzed urine was concentrated by lyophilization to dryness and the dried material was reconstituted in 1 ml of water and aliquots (50–100 µl) were used for immunoblotting with anti IF.

Results

Specificity of the IF antiserum

The antiserum against pure rat IF and hog R protein used in these studies have been validated both by immunoprecipitation and by western blots to react only with IF and R protein, respectively [19]. Using these antisera we have described [19] in rats, the detection of IF in the stomach and salivary glands and of R protein in the parietal cells and serous acini of the pancreas and salivary glands. The other properties of the polyclonal antiserum to IF raised in rabbits are shown in Fig. 1. The antiserum to rat IF blocked the binding of [^{57}Co]Cbl to IF, immunoprecipitated IF-[^{57}Co]Cbl [19] but did not block the binding of rat IF-[^{57}Co]Cbl to the rat ileal apical membrane IF-Cbl receptor. Thus, these results suggested that the polyclonal antibody to IF was 'blocking antibody' (type I) and not the 'binding' (type II) antibody.

Urinary excretion of [^{57}Co]Cbl binding activity

In a 24 h period, the rats excreted in urine unsaturated [^{57}Co]Cbl binding of 0.2 to 0.4 pmol (Table I). When [^{57}Co]Cbl complexed to rat urine was tested for immunoprecipitation with anti IF serum approx. 30–40% of the counts was immunoprecipitated.

Urinary excretion of IF in rats suggested that IF is filtered by the glomerulus. Following filtration, IF is either sequestered by the proximal tubular receptor or is secreted in the urine. Thus, in order to test the former possibility, presence of endogenous IF in renal apical membranes was tested.

Binding of IF-[^{57}Co]Cbl to pre and post pH 5/EDTA washed renal apical brush border membranes

The binding of rat IF-[^{57}Co]Cbl complex occurred primarily to the apical brush border membrane but not to the basolateral membrane (Table II). The EDTA inhibitable binding (2400 fmol/mg protein) of IF-[^{57}Co]Cbl increased nearly 2-fold upon washing the membranes with pH 5/EDTA buffer. There was little or no binding of hog R-[^{57}Co]Cbl or TC II-[^{57}Co]Cbl complexes to either unwashed or washed apical membranes. The binding to basolateral membranes of [^{57}Co]Cbl occurred only when complexed with TC II but not with R-protein or IF. The binding of TC II-[^{57}Co]Cbl complex to the basolateral membranes was only 1/40 to 1/70 of the binding of IF-[^{57}Co]Cbl complex to the apical membranes. The enhanced binding of IF-[^{57}Co]Cbl to pH 5/EDTA washed rat renal apical membranes was not unique to this species alone as similar washing treatment enhanced the binding of rat IF-[^{57}Co]Cbl to human (Fig. 2A) and canine (Fig. 2B) renal apical membranes. Washing the membranes with pH 5/EDTA did not change the association con-

TABLE I

Urinary excretion of unsaturated [^{57}Co]Cbl binding activity in rats

Adult rats (30-day-old) were allowed to have free access to food and water. The urine was collected over a period of 24 h. The total [^{57}Co]Cbl binding was determined using [^{57}Co]Cbl (1.5 pmol) by the albumin coated charcoal method. 1–2 ml of charcoal supernatant containing 5000–7000 dpm were used for immunoprecipitation with anti IF serum (1–5 µl). The immune complex was precipitated with formalin fixed Staph A cell suspension. The immune pellet was washed with 1 ml phosphate-buffered saline and counted.

Rat number	Volume of urine/24 h (ml)	[^{57}Co]Cbl bound (pmol)	IF (pmol)
1	10.34	0.42	0.126
2	9.56	0.21	0.084
3	8.67	0.19	0.076
4	6.00	0.15	0.06
5	11.32	0.20	0.06
6	8.10	0.40	0.120

TABLE II

Binding of [^{57}Co]Cbl complexed with Cbl binders to renal plasma membranes

Adult rats (30-day-old) were used to prepare apical and basolateral membranes. The Ca^{2+}-dependent binding was assayed as described in Methods using [^{57}Co]Cbl (1.5 pmol) complexed to rat IF, hog R protein and human TC II. The values represent mean ± S.D. of four assays using two separate membrane preparations.

Plasma membrane	[^{57}Co]Cbl bound (fmol/mg protein)		
	IF-[^{57}Co]Cbl	R-[^{57}Co]Cbl	TC II-[^{57}Co]Cbl
Apical			
unwashed	2400 ± 35	0.04 ± 0.01	0.08 ± 0.02
pH 5/EDTA washed	5100 ± 110	0.05 ± 0.02	0.010 ± 0.03
Basolateral			
unwashed	0.06 ± 0.02	0.04 ± 0.01	55 ± 7
pH 5/EDTA washed	0.07 ± 0.04	0.06 ± 0.01	70 ± 9

stant K_a for IF-[^{57}Co]Cbl. Both washed and unwashed rat renal membranes had a K_a of $(1.2–1.4) \cdot 10^9$ M^{-1} for the binding of IF-[^{57}Co]Cbl.

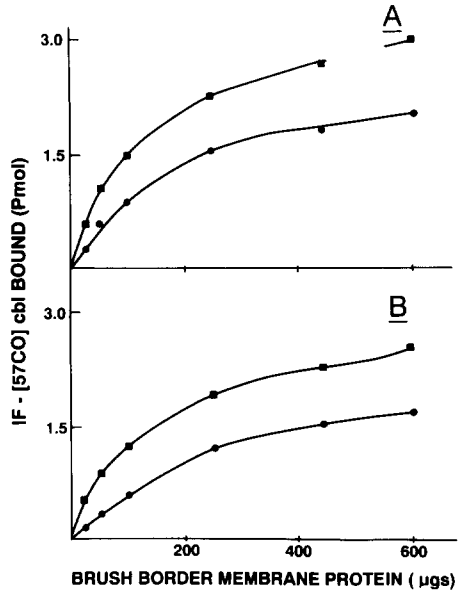

Fig. 2. Effect of pH 5.0/EDTA washing on the binding of IF-[^{57}Co]Cbl to human and canine renal brush border membrane. Renal apical membranes (2–4 mg protein) isolated from human (A) and canine (B) kidneys were incubated for 30 min at 22°C in K-phosphate buffer (pH 5.0) containing 5 mM disodium EDTA. The membranes were pelleted down by centrifugation at 20000 × *g* for 30 min. The membranes were suspended and homogenized in 2 ml of pH 5.0/EDTA buffer. After further incubation, the membranes were recovered by centrifugation. The membrane pellet was suspended and homogenized in 10 mM Tris-HCl (pH 7.4) and used for assay using IF-[^{57}Co]Cbl (3.7 pmol). Control values were obtained by subjecting the membranes to similar treatment except that 10 mM K-phosphate buffer (pH 7.4) with no EDTA was used for washing. The binding of rat IF-[^{57}Co]Cbl to pH 5.0/EDTA treated (■) and control (●) membranes are shown and the values were obtained using duplicate assays with two separate membrane preparations.

TABLE III

Intestinal apical brush border membrane binding and absorption of [^{57}Co]Cbl complexed to renal and gastric extracts

The association constant (K_a) and V_{max} were determined using [^{57}Co]Cbl complexed to the renal and gastric extracts (0.03–1.5 pmol) and rat ileal brush border membrane protein (600 µg). Other details are provided under Methods. Transport of [^{57}Co]Cbl to tissues was evaluated 4 h after the oral administration of [^{57}Co]Cbl complexed to gastric or renal extracts. The animals were sacrificed, the liver and kidney were removed, washed with cold PBS, blotted dry, weighed and cut into small pieces and counted. The kinetic values reported are mean ± S.D. of duplicate assays using brush border preparations from two separate rats. Tissue levels of [^{57}Co]Cbl reported are mean ± S.D. of five separate rats.

Source of Cbl binder	IF-[^{57}Co]Cbl bound (fmol/mg protein)	K_a (10^{10} M^{-1})	Liver and renal [^{57}Co]Cbl (fmol/g)
Renal membrane	55 ± 4	0.8 ± 0.1	340 ± 40
Gastric mucosa	62 ± 7	1.0 ± 0.3	290 ± 20

Binding and uptake of [^{57}Co]Cbl complexed to rat gastric and renal membrane extracts

[^{57}Co]Cbl complexed to either gastric or renal membrane extracts bound equally well to rat ileal brush border membrane (Table III). The IF-[^{57}Co]Cbl bound was between 55 and 60 fmol/mg protein. The association constant K_a was $0.8 \cdot 10^{10}$ M^{-1} and $1 \cdot 10^{10}$ M^{-1} for [^{57}Co]Cbl complexed with renal and gastric extracts, respectively.

When [^{57}Co]Cbl complexed to renal and gastric extracts was orally administered to rats, the tissue (liver and kidney) levels of [^{57}Co]Cbl in 4 h was 350 ± 20 fmol/g and 290 ± 20 fmol/g, respectively (Table III).

Immunoblotting studies using anti IF serum

The enhanced membrane binding of IF-[^{57}Co]Cbl upon pH 5.0/EDTA treatment could be due to removal of endogenous IF or IF-Cbl bound to the membrane IF-Cbl receptor. In order to prove this, apical membranes were subjected to immunoblotting following treatment of the membranes with various buffers (Fig. 3). Anti rat IF serum recognized a single polypeptide of 62 kDa (lane 2). This 62 kDa protein band was totally eliminated upon washing the membranes with pH 5/EDTA buffer (lane 5) but not with pH 7.4/EDTA buffer (lane 3). Treatment with pH 5.0 alone (lane 4) resulted in incomplete removal of the 62 kDa protein band. The immunoreactive pure IF was identified with a single band of molecular mass 50 kDa.

The 62 kDa polypeptide identified on renal apical membranes immunoreacted with antiserum to IF and elimination of this band coincided with increased binding of IF-[^{57}Co]Cbl to these membranes. The origin of IF in these membranes is not known, but these observations suggested that IF is filtered by the glomerulus and

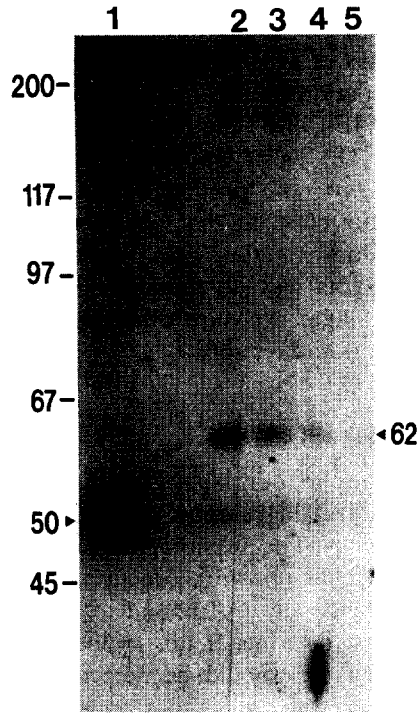

Fig. 3. Immunoblotting of rat renal apical membranes. Rat kidney apical membranes (50 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis (7.5% gel). The separated proteins were transferred to nitro cellulose and probed with anti rat IF (1/100 diluted) and ^{125}I-protein A. Immunoreacting bands shown are, pure IF (lane 1, 2 μg), and membranes washed with buffers pH 7.4 (lane 2), pH 7.4/5 mM EDTA (lane 3), pH 5.0 (lane 4) and pH 5.0/5 mM EDTA (lane 5). The numbers indicated on the side are molecular mass (kDa) of standard markers and/or immunoreactive bands.

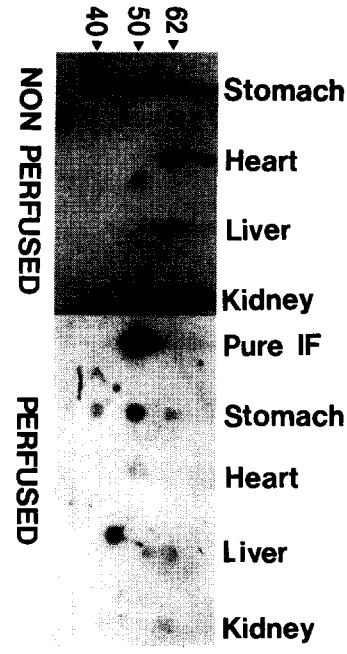

Fig. 4. Immunoblots of rat tissue homogenates. A 10% homogenate in 10 mM Tris-HCl (pH 7.4) containing 1 mM PMSF, 2 mM benzamidine, 1 mM EDTA was prepared from perfused and non perfused rats. Homogenate protein from heart, liver, kidney (50 μg) and stomach (10 μg) were subjected to SDS-PAGE (7.5% gel) and subjected to immunoblotting as described in Methods. The numbers indicated are the calculated molecular mass (kDa) of immunoreactive bands.

is bound to these membranes. In order for IF to be found in the kidney it should be present in blood and perhaps be detectable in other tissues.

On immunoblotting and probing with anti rat IF a protein with a molecular mass of 62 kDa was identified in rat stomach, heart, liver and kidney homogenates (Fig. 4). In the stomach, two more immunoreactive polypeptide with a lower mass of 50 kDa and 40 kDa were also identified. Upon whole body perfusion, the levels of the 62 kDa polypeptide diminished in the heart, liver, and kidney (Fig. 4). These results demonstrated that the 62 kDa species of IF exists in the somach as well as in other tissues. In order to ascertain the species of IF important in intestinal and possibly renal uptake of Cbl and to study the intracellular membrane association of IF, the plasma and intracellular membranes were subjected to immunoblotting (Fig. 5). IF was associated with renal (lane 9) and intestinal (lane 5) apical but not basolateral membranes (lanes 2 and 6). No IF could be detected in Golgi-enriched membranes (lane 3) and in cytosolic fraction (lane 4) from intestinal mucosa. Very low amount of IF could be detected in renal Golgi membranes (lane 7) and cytosol (lane 8). The 62 kDa and 50 kDa polypeptides that reacted with anti IF serum in the stomach extracts were also detected in the rat serum (lane 10). When concentrated rat urine was subjected to immunoblotting faint but distinct bands of 57 kDa and 40 kDa could be identified (lane a and b).

The origin of circulatory IF could be from the stomach or from accessory organs that have been shown histochemically to contain IF [19]. Homogenates of accessory digestive secretory organs like rat salivary glands and pancreas were subjected to immunoblotting. The 62 kDa species of IF and other polypeptides of

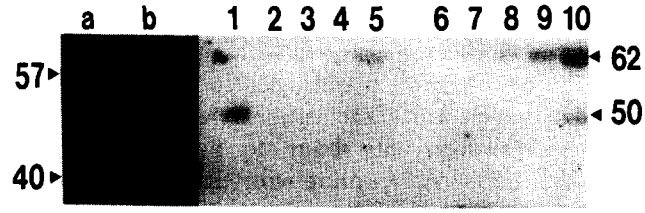

Fig. 5. Immunoblotting of rat intestinal, renal membranes, serum and urine. Isolated apical, basolateral, and Golgi membrane proteins (50 μg), cytosol (50 μg), rat serum (10 μg) were subjected to 7.5% SDS-PAGE and immunoblotted with anti IF serum and ^{125}I-protein A. The marker pure IF (2 μg) (lane 1), intestinal basolateral (lane 2), Golgi (Lane 3), cytosol (lane 4) and apical membranes (lane 5), renal basolateral (lane 6), Golgi (lane 7), cytosol (lane 8) apical membranes (lane 9), rat serum (10 μg) and urine concentrate 50 μl (lane a) and 100 μl (lane b). The numbers indicated are the calculated molecular mass (kDa) of immunoreactive bands.

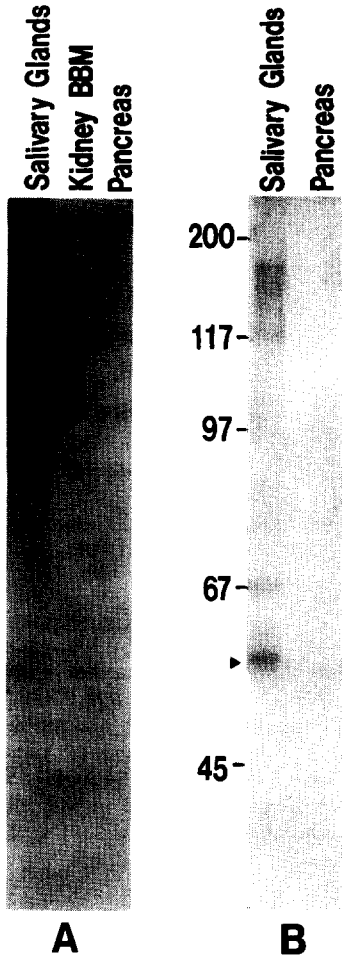

Fig. 6. Immunoblotting of digestive accessory tissues homogenates. (A) Rat salivary gland and pancreatic homogenates and renal brush border membrane protein (50 μg) were separated on SDS-PAGE (7.5%) and subjected to immunoblotting using monospecific antisera to hog R-type binder. (B) Salivary gland and pancreatic extract (50 μg protein) similarly separated was probed with anti rat IF. The numbers indicated on the side are molecular mass (kDa) of standard markers and/or immunoreactive bands.

higher molecular weight (67, 117, 130 and 140 kDa) were identified in salivary glands (Fig. 6B). Under similar conditions, rat pancreatic extract (50 μg protein did not reveal any immunoreactive band with anti IF. When the immunoblots were probed with antisera to anti hog R (Fig. 6A) salivary glands yielded several immunoreactive polypeptides the major one being 117 kDa species. However, anti serum to R protein did not identify 62 kDa polypeptide with either salivary, pancreatic extracts or in renal brush border membrane. Anti R-protein seurm recognized several faint polypeptides (117, 130 and 180 kDa) from pancreatic extracts (Fig. 6A).

Some of the quantitative aspects of this study were obtained from a standard blot using pure rat IF (data not shown) or from assays. The difference in IF-[^{57}Co]Cbl bound prior to and following pH 5/EDTA wash of renal membranes (approx. 2700 fmol/mg protein) corresponds to endogenous IF or IF-Cbl equivalent to 170 ngs of IF protein/mg of membrane protein. This calculation is based on mole/mole binding of Cbl by IF and IF-Cbl by the receptor and that 1 mg of pure IF binds 27.1 μg of Cbl. From standard immune blots, 1 mg membrane protein bound 200 ng of IF which is a value close to that obtained from the IF-[^{57}Co]Cbl binding kinetics. Quantitation of IF from other immunoblots was not possible due to technical difficulties of longer exposure time needed to observe the bands on autoradiographs.

Discussion

Earlier studies in our laboratory have shown that high levels of IF-Cbl receptor activity is present in mammalian kidney. The physiological role of this receptor is not known. One possible function of this receptor could be to salvage any Cbl bound to IF which might otherwise be lost in urine. If this hypothesis is correct, then, endogenous IF should be detected in the renal tissue.

In the current work we have identified IF in renal brush border membranes. The following evidence supports that the protein identified is IF and not other Cbl binders such as R-type or TC II. First, washing with pH 5/EDTA buffer enhanced the binding of exogenously added IF-[^{57}Co]Cbl (Table II) to these membranes. Second, [^{57}Co]Cbl complexed to IF but not to TC II or R-protein bound to the apical membranes (Table II). Third, a 62 kDa polypeptide was identified in the renal apical membranes that reacted with anti IF and this band was completely eliminated following washing with pH 5/EDTA buffer (Fig. 3). Fourth, the 62 kDa polypeptide could not be detected in renal apical membranes using antiserum to hog R protein (Fig. 6A). Fifth, antiserum to rat IF used in this study did not cross react with R protein from hog stomach [19]. Finally, [^{57}Co]Cbl complexed to renal apical membrane extracts was able to bind *in vitro* to rat ileal membranes with high affinity and *in vivo* promote Cbl absorption (Table III). It is possible but unlikely under the conditions that the absorption of [^{57}Co]Cbl bound to the renal binder is due to in vivo transfer of [^{57}Co]Cbl from the renal binder to endogenous IF.

The size of IF identified in these membranes is 12 kDa higher than pure rat IF isolated from rat stomach extracts. During purification of IF from either gastric juice [20] or gastric mucosa [7,8] the most important step is affinity chromatography on Cbl-sepharose and elution using high (3–5 M) chaotropic salts such as guanidine HCl. This rather harsh treatment could result in a proteolytic modification of IF during purification. When rabbit gastric fundic mucosa was labelled with [^{35}S]methionine for 24 h, antiserum from a pernicious anemia patient recognized 3 bands of molecular mass

53, 46 and 27 kDa [21]. However, cold IF preincubation resulted in the elimination of only 53 kDa protein. The possibility that the patients antiserum recognized other parietal cell antigens was proposed by these authors. In the current study, the polyclonal antiserum used was raised to IF, a single 50 kDa purified protein from rat gastric mucosa. The antiserum to IF, though polyclonal was able to inhibit the binding of IF to [^{57}Co]Cbl but not of IF-[^{57}Co]Cbl to ileal brush border membrane (Fig. 1). Similar results were also observed with renal apical membranes (data not shown). This observation is interesting in view of earlier studies which have demonstrated that serum from most patients with pernicious anemia effectively blocked both reactions [22]. Monoclonal antibodies to pure human IF [23] have identified six antibodies to IF of which only two blocked the binding of IF-[^{57}Co]Cbl to the ileal receptor. Except one antibody, the remaining five which includes the two that blocked the binding of IF-Cbl to the ileal receptor were also effective in hibiting the binding of Cbl to IF. Thus, the polyconal antibody to rat IF behaved like the blocking, type I species. The reason for this unusual property of the anti IF serum is not yet apparent. Possible explanations include different species or proteolytic modification of rat IF during purification. Such proteolytic modification would remove from IF only those sites which are not important for receptor binding, but important in providing some antigenic epitopes close to receptor binding domains. This certainly appears to be true as pure IF (50 kDa) is both able to bind to the receptor [10] and able to promote Cbl absorption [24]. Furthermore, the rat IF-Cbl receptor recognizes or binds IF from rat but not IF from other species [7]. Thus, the receptor binding sequence of rat IF to rat receptor could be different from that of IF and receptor from other species. Based on predicted sequence from cDNA for rat IF and *in vitro* translation using gastric mRNA, the size of IF is between 44 to 46.8 kDa [25]. Thus, the changes in the molecular weight of rat IF could occur during post translational modification. Even though IF is known to be a glycoprotein, the details of carbohydrate processing or other modifications of IF is not known. Despite these uncertainties regarding intracellular processing of IF, the current results suggest that the 62 kDa species of IF is active in vivo as this species of IF was detected in the apical membranes of the gut (Fig. 5) and kidney (Figs. 3 and 5).

The current results viewed in the context of IF mediated transport of Cbl has the following implications. Even though it is widely accepted that ileal transport of Cbl is mediated by IF, the cellular sorting pathways for IF-Cbl within the enterocytes are not clear. Studies using cultured enterocytes and ileal explants in culture [26,27] have suggested that IF-Cbl is internalized via receptor mediated endocytosis. Following this uptake Cbl is transferred from IF to TC II [24]. Transcytosis of IF and entry into circulation via the ileal cell has never been directly demonstrated. The difficulty in proving this is compounded by the fact that it is difficult to internalize large amounts of labeled IF due to the small amounts of receptor expressed on the ileal cell surface [28]. Highly labelled recombinant IF is not yet available for these studies. Immunocytochemical studies [29] have shown IF to be localized in apical microvillus pits and endosomes although, more recently IF has been shown to be present in the inner leaflets of basolateral membranes [30] and based on this finding it was concluded that IF might enter circulation via the intestine. In the current study no IF could be detected on immunoblots of intestinal and renal basolateral membranes (Fig. 5). Although IF could not be detected in the mucosal cytosol and Golgi-rich membranes, IF could be detected in the renal cytosol and Golgi. These results suggest that IF is probably handled differently in these tissues. Furthermore, in the intestinal mucosa, endogenous IF might have undergone degradation or modification to be recognized by the anti IF serum. When injected into portal or femoral vein both [^{125}I]IF and [^{125}I]IF-[^{57}Co]Cbl were rapidly cleared by an unknown receptor in the liver. The uptake was rapid; however, excretion into bile occurred slowly and in 60 min about 5% of IF was degraded and free ^{125}I radioactivity was excreted in bile (unpublished results). Thus, it is unlikely that the source of renal membrane IF is via intestinal transit.

Whatever the origin of IF in circulation is, (via intestine or from tissues) the amount is too small to be detected by methods other than immunoblotting. In the rat kidney apical membrane, endogenous IF represents about 160–200 ng IF/mg protein. Based on immunoprecipitation of [^{57}Co]Cbl complexed to rat urine about 80–170 pg of IF is secreted per day and the rest is probably sequestered by the renal IF-Cbl receptor. The results in Fig. 2 show that both human and canine kidney apical membranes contain endogenous IF bound to it. These results and earlier studies [5,6] detecting IF in concentrated human urine suggest that trace amounts of IF may escape renal receptor and is excreted in all species.

The physiological importance of renal utilization of Cbl via IF-Cbl receptor is not known. Under normal physiological conditions the renal tissue may merely salvage filtered IF and Cbl. The renal IF-Cbl receptor mediated utilization of Cbl may be important to prevent urinary loss of Cbl and to maintain the body stores for some period of time when dietary Cbl is not assimilated via the intestine. Gastrectomized rats maintain their body stores of Cbl up to several weeks [31] and this has been attributed to TC II mediated resupply of free Cbl known to accumulate in the kidney [32,29]. The intracellular site of accumulation of [^{57}Co]Cbl in the kidney appears to be in the lysosomes [33,34]. Thus, the

presence of endogneous IF and IF-Cbl receptor in mammalian kidney suggest that this ligand/receptor system could play an additional role in normal Cbl homeostasis separate from TC II/TC II receptor system noted by Scott et al. [32].

Another important aspect of this study is the finding that binding of [^{57}Co]Cbl bound to IF and TC II occurred to opposite membranes of the kidney. The significance of this finding is not yet apparent. Much needs to be learned regarding the mechanism of entry and exit of Cbl in mammalian cells in general and polarized cells like renal and ileal epithelial cells in particular [35]. While very little is known regarding the intracellular sorting and targetting of TC II-Cbl receptor, the IF-Cbl receptor has been clearly identified by immunocytochemical studies in apical but not in the basolateral membranes [1,2]. Moreover, in the current studies the ligand IF was identified in the apical membranes of both intestine and kidney (Fig. 5). However, the location of TC II receptor is not known. If the TC II receptor is targeted exclusively to the basolateral membranes then it would mean that entry into and exit out of these polarized epithelial cells is mediated by two separate receptor-ligand systems. Transcytosis experiment of [^{57}Co]Cbl in renal epithelial cells show that [^{57}Co]Cbl bound to IF is taken up apically but not basolaterally and the exit of Cbl bound to TC II occurs exclusively via basolateral membranes (Seetharam, B., et al., unpublished observations). The physiological role of IF-Cbl receptor in the kidney and processing of IF-Cbl complex in the tissue needs further attention. Towards this end, we are studying the polarized delivery of IF-Cbl and TC II receptors and transport of IF-Cbl using proximal tubule derived renal epithelial cells.

In summary, we have identified endogenous functional IF in renal apical brush border membranes. Minute amounts of IF from the circulation is sequestered by renal IF-Cbl receptor to minimize urinary loss of Cbl. This salvage pathway may contribute to the Cbl stores of the kidney and help in the reutilization of Cbl via TC II by the tissues.

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