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Transfer of cobalamin from intrinsic factor to transcobalamin II

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Abstract

The process is obscure by which cobalamin (Cbl) in the endocytosed intrinsic factor (IF)-cobalamin (Cbl) complex is released and transferred to transcobalamin II (TCII) within the enterocyte. Using recombinant IF and TCII, binding of Cbl to IF at pH 5.0 was 70% of binding at pH 7.0, whereas for TCII alone, the value was only 12%. TCII binding activity was lost rapidly at lower pH, but this was not due to protease action. TCII incubated at pH 5.0 with cathepsin L was degraded and could not subsequently bind Cbl. Thus, transfer from IF to TCII is unlikely to occur within an acid compartment. Only 13–15% of bound Cbl was released at pH 5.0 and pH 6.0 from either rat IF, human IF, or human TCII. The K_a of human or rat IF at pH 7.5 was 2.2 nM; for TCII, the value was 0.34 nM. At pH 7.5, Cbl transfers from IF to TCII, but only to a limited extent (21%), as detected by nondenaturing electrophoresis. Transfer of Cbl from IF to TCII could not be demonstrated at pH values of 5.0 or 6.0. Thus, luminal transfer of Cbl between IF and TCII is likely to be limited, but is possible. The most likely mechanism for intracellular transfer of Cbl from IF to TCII involves initial lysosomal proteolysis of IF, with subsequent Cbl binding to TCII in a more neutral cellular compartment.

Keywords: Human IF; Rat IF; Human TCII; Vitamin B_{12}; Cobalamin binding proteins

1. Introduction

Cobalamin (Cbl; vitamin B_{12}) absorption in mammals is mediated by a series of binding proteins. Dietary Cbl is bound first to haptocorrin (Hc; R protein) in the gastric lumen, but is transferred to gastric intrinsic factor (IF) in the duodenum following degradation of Hc by pancreatic proteases [1,2]. The IF-Cbl complex then travels the length of the small intestine where it enters the ileal mucosal cell by receptor-mediated endocytosis [3] bound to cubilin, the receptor specific for IF-Cbl [4]. After a period of about 4 hr, the Cbl appears in the blood bound to transcobalamin II (TCII; transcobalamin) [5]. The events within the enterocyte that regulate the transfer of IF to TCII are less clearly delineated. The IF-Cbl complex is taken up from the apical border of the cell, and the IF is degraded [6,7]. This degradation is inhibited by leupeptin and by NH_4^+—treatments that alter the function of the acid endosomal compartments within the cell.

Despite a large body of research describing the physiology of Cbl absorption, there remain a number of areas that are not fully understood. About 10% of the TCII receptor is located in the apical microvillus membrane of rat enterocytes, and it has been suggested that some Cbl absorption occurs from endocytosis of the receptor occupied by TCII-Cbl that diffuses into the lumen via the tight junctions [8]. If this occurs, it is not known whether Cbl transfer between IF and TCII could occur in the lumen, or if Cbl is released inside acid vesicles with formation of a TCII-Cbl complex either intracellularly or after TCII secretion [9]. Two possible mechanisms have been reported for the initial release of Cbl from IF; proteolysis by cathepsin L [10], and/or acid pH (5.0) alone [11].

Elucidation of these events within the cell has been hampered (1) by the very low levels of intracellular TCII; (2) by the sensitivity of IF to glutaraldehyde, making high resolution of IF compared to TCII within cellular organelles by immunoelectron microscopy very difficult; and (3) by the very small mass of Cbl uptake even in cell culture systems [12]. IF and TCII are present in gastric juice and serum, respectively, but are accompanied by many other proteins that might complicate the study of Cbl transfer between the proteins, even if Cbl binding due to Hc were blocked by cobinamide. To avoid some of these difficulties, recombinant IF and TCII have been produced in *Pichia pastoris*, and the relative affinity of Cbl for these prepara-

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tions are compared under the same conditions while observing the transfer of Cbl from one protein to the other. The results of these studies demonstrate that direct transfer of Cbl from IF to TCII could take place within the intestinal lumen, although to a limited extent. The results further strengthen the hypotheses that proteolysis must be the initial event that releases Cbl from IF, that binding of Cbl to TCII occurs outside the acid vesicle, and that release of Cbl from endocytosed luminal TCII occurs within the acid endosomal/lysosomal compartments by the action of cathepsin L.

2. Materials and methods

2.1. Construction of vectors containing recombinant human IF and TCII

Human IF cDNA in pBluescript 12 was transferred by subcloning a *Hind*III-BamH1 fragment into pAlter-1 (Promega, Madison, WI) at the EcoR1-*Hind*III sites after ends were made flush with Klenow enzyme [13]. The 5′ end of the human IF cDNA was then modified by primer-directed polymerase chain reaction (PCR) mutagenesis to allow fusion of the cDNA encoding IF behind the *Saccharomyces cerevisiae* α-mating factor signal sequence in the *P. pastoris* expression vector pPIC9 (Invitrogen, Carlsbad, CA) [14]. PCR reactions, performed using a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA), contained 500 ng of each primer and 35–70 ng of template DNA. Taq polymerase (Fisher Scientific, Pittsburgh, PA) was used for the reactions, which were subjected to 30–35 cycles of 95°, 50°, and 72° for 1 min each. The modified IF cDNA was excised by XhoI/EcoR1 digestion of the pAlter-1/human IF plasmid and subcloned into the yeast vector pPIC9 to create pPIC9/human IF. The IF produced by this vector had one extra glutamic acid residue at the 5′ end, a consequence of the cleavage of the Glu-Lys-Arg-Glu α-mating factor signal peptide cleavage site. This construct had binding characteristics for both cobalamin and for the IF-Cbl receptor, as found in native IF [13,14,15].

Human TCII cDNA was obtained by reverse transcription-PCR reaction from total RNA isolated from human intestine (Clontech, Palo Alto, CA). The primers used were obtained from the 5′ and 3′ translated regions of the cDNA previously isolated from a human umbilical cord (HUVE) cell library [16]. The primer for the 5′ end was CTCGAGAAAAGAGAGGCT ↓ GAAGCTGAGATGTGTGAAATACCAGAGATGGA, where CTCGAG was the XhoI site, ↓ denotes the signal cleavage site (Glu-Lys-Arg-Glu), and the next GAG (underlined) is the first amino acid of mature TCII. The primer for the 3′ end was GAATTCCTACCAGCTAACCAGCCTCAGCTCAA, where GAATTC is an EcoR I site, and CTA (underlined) is a stop codon. The resulting PCR product was ligated into pGEM™-T Easy (Promega, Madison, WI). It was sequenced and shown to be identical to the TCII previously reported [17], except for the replacement of the serine with leucine at position 376. The TCII cDNA was cut from pGEM™-T-Easy with XhoI and EcoRI to expose the restriction sites, and cloned into pPIC9 (Invitrogen, Carlsbad, CA) between the XhoI and EcoRI sites. Unlike recombinant human IF, the expressed recombinant TCII had no extra amino acid residues at the 5′ end. The binding affinity of this construct for Cbl was not different from recombinant TCII produced in Sf9 cells by baculovirus infection, using the method reported previously by Quadros et al [18].

Both recombinant proteins had the binding expected from native proteins. Recombinant IF bound 1 mol of Cbl/mole of protein. Recombinant TCII bound 0.5 mol of Cbl/mole of protein, purifying the protein by dialysis against water in the absence of Cbl. This method of purifying native TCII produced a binding ratio of 0.56 mol Cbl/mole of protein when compared with dialysis against Cbl containing fluid, presumably due to improved folding [19]. We chose to dialyze only against water, so that all the TCII was available as the apoprotein. Accordingly, we estimated the amount of TCII to be added to assays from the Cbl binding capacity.

2.2. Transformation of P. pastoris

DNA (10 μg) from pPIC9, linearized by SalI digestion, was incubated with KM71 cells (His-) (Invitrogen, Carlsbad, CA) in a 0.2-cm electroporation cuvette and subjected to electroporation at 2500 V, 25 μF, and 400 ohms (Bio-Rad Electroporator, Hercules, CA). The reaction was stopped by the addition of 1 ml of ice-cold 1 M sorbitol. The Mut^s transformants were recovered by plating on minimal glucose plates containing 1.34% yeast nitrogen base with ammonium sulfate but without amino acids, 4×10^{-5}% biotin, and 2% glucose. To identify successful transformation of the *P. pastoris* cell, chromosomal DNA was isolated from the centrifuged cells [20] and the extracted DNA used as a template for PCR. The 5′ primer was provided by Invitrogen (Carlsbad, CA) upstream of the N-terminus of the inserted protein, and the 3′ primer was taken from the sequence of IF or TCII. If a fragment of the correct size was obtained, it was assumed to be the correct protein when the *Pichia* clone also was shown to secrete a Cbl-binding protein.

For expression of the recombinant proteins, these Mut^s transformants in which the alcohol oxidase 1 gene is replaced by the vector were grown overnight at 30°C in 10 mL of BMGY medium (1% Bacto yeast extract, 2% peptone, biotin, and 1% glycerol). Five mL of this culture in 1 L of BMGY medium was grown for 2 days at 30°C with vigorous shaking, the cells were recovered by centrifugation, and were resuspended in BMMY medium (1% Bacto yeast extract, 2% peptone, biotin, and 1% methanol) for induction. The induced culture of Mut^s transformants was incubated at 30°C with vigorous shaking for 1–2 days, with the daily addition of 1% methanol to maintain induction.

Cells were recovered by centrifugation at 3,300 × *g* for 15 min.

2.3. Purification of recombinant proteins

Purification of IF and TCII was performed as previously described [21]. The presence of Cbl binding proteins in culture media and purification steps was detected by the [^{57}Co]Cbl charcoal exclusion assay [22]. Charcoal decolorizing powder (activated, acid washed) was purchased from BDH Chemicals, Ltd. (Poole, England). Radiolabeled Cbl was purchased either from Amersham Life Science, Inc. (Arlington Heights, IL; specific activity = 15 μCi/μg) or from ICN (Costa Mesa, CA; specific activity = 300 μCi/μg). The clarified medium was applied to a 2.5 × 2.0-cm Cbl-agarose column (Sigma Chemical Corp., St. Louis, MO) at 4°C, and the column wrapped in aluminum foil to protect the light-sensitive Cbl. After loading, the column was washed sequentially with 200 mL of 100 mM Tris (pH 7.15) with 2 mM sodium azide, 200 mL of 100 mM glucose, 100 mM glycine, 1 M NaCl, and again with 200 mL of the Tris buffer. IF or TCII was eluted with 4 M guanidine HCl, and the eluant dialyzed extensively for 36 hr against water at 4°C. The protein was concentrated about 50-fold by vacuum dialysis in a collodion membrane (Schleicher and Schuell, Keene, NH), and stored at −80°C until used. The purified proteins demonstrated a single band on SDS-PAGE developed by coomassie blue staining. A few immunoreactive smaller fragments were sometimes seen in the TCII preparations in the absence of a detectable protein band, but the binding characteristics of these preparations did not differ from those without these fragments. The yield of recombinant protein based on Cbl binding (30:1 MW ratio, with known molar ratio of 1:1) was 40–60% for IF and 30–50% for TCII, similar to prior reports [13,18,19].

2.4. Cbl binding studies

2.4.1. pH curves

Binding of Cbl to IF or TCII was performed at pH values from 2.1 to 8.1 (including 3.2, 4.2, 5.2, 6.3, and 7.3) using 200 mM citrate-phosphate buffer, and at pH 8.1 using 0.2 mM phosphate buffer. All buffers contained 25 μg/ml of bovine serum albumin (BSA) to prevent loss of protein on the polypropylene surface. Duplicate tubes were incubated for 15 min at 37°C, and in some experiments for up to 120 min thereafter to assess release of Cbl. Concentrations of IF and TCII used were 44–77 fmol/tube and 32 fmol/tube, respectively. Concentration of binding protein was assessed by amino acid analysis of the purified preparation. Cbl was always added in slight excess at a ratio of 1.05:1 (50–100 fmol/tube) to allow complete saturation of the binding protein. Binding was determined in duplicate samples using the charcoal binding assay described above.

In some experiments—after binding to either human or rat IF or to human TCII was complete at pH 7.0—the pH was shifted to either 5.0 or 6.0 by addition of 1 M citric acid, or left at 7.0. Duplicate samples were removed at 15, 30, 45, and 120 min postshift, and free Cbl was removed by the addition of an equal volume (100 μL) of charcoal (25 mg/5 mg BSA/mL of water). The mixture was spun in a microfuge for 8 min, and 180 μL of the supernatant removed and counted to determine the labeled Cbl still bound.

2.4.2. Affinity

Affinity was studied at pH 7.5 and 6.1, using a constant amount (12.5 fmoles) of each binding protein in a final volume of 100 μL. The amount of [^{57}Co]Cbl (15 μCi/μg) added varied from 5.2 to 52 fmoles/assay, and incubation was for 2 hr at 37°C. The assay diluent was 0.25 mM Tris buffer containing 25 μg/mL of BSA. Binding was determined by adding 500 μL of BSA solution (1 mg BSA/mL of 10 mM Tris buffer, either pH 6.1 or 7.5) plus 400 μL of charcoal suspension. After incubating 5 min at room temperature, the mixture was separated by spinning 5 min in a microfuge, and radioactivity was measured in 800 μL of the supernatant solution. K_a was determined by reciprocal plotting of 1/B versus 1/[protein], and the curve drawn by least squares analysis (Cricket Graph). All assays were performed in triplicate.

2.4.3. Cbl transfer

In some experiments, both binding proteins were present simultaneously at a concentration of 67 fmoles of Cbl binding capacity/tube. Either one or the other protein was saturated with [^{57}Co]Cbl (15 μCi/μg) in a final volume of 15 μL, pH 7.2. After incubation for 10 min at 37°C, 15 μL of buffered charcoal was added, incubated for 5 min at room temperature, and centrifuged in a microfuge. After removal of the supernatant containing the original binding apoprotein (IF or TCII) now saturated with Cbl, the other binding protein was added in equal concentration, and the proteins incubated for 2 hr at 37°C. All assays were performed in duplicate. Separation of the two proteins to allow measurement of [^{57}Co]Cbl bound to each one was performed using polyacrylamide gel electrophoresis (PAGE) without SDS [23]. In this system, the Rf of IF and TCII was 0.29 and 0.53, respectively. Autoradiographs were developed from the gel and analyzed by densitometry after scanning the gels using NIH Image version 1.61 (NIH, Bethesda, MD, available at: www:http:rsb.info.nih.gov/nih-image/). In some experiments, recombinant TCII was incubated with cathepsin L or cathepsin B (Calbiochem, La Jolla, CA) using a ratio of 2.8 mU/μg of TCII previously found sufficient for degradation of IF [10].

3. Results

3.1. Cobalamin binding to IF and TCII

To examine the transfer of Cbl from IF to TCII, it was necessary to determine the pH at which Cbl bound to these

proteins. Binding occurred rapidly and was essentially completed by 15 min of incubation. (Fig. 1A) demonstrates that rat IF showed a very broad peak of Cbl binding, from pH 2.1 to 8.1. Human IF, however, showed a 3-fold difference between pH 2.1 and 8.1, gradually increasing Cbl binding as pH rose (Fig. 1B). The curve for Cbl binding to TCII (Fig. 1C) was quite different, in that binding at pH 2–5 was only 20–25% of the maximal binding at pH 7.2. Binding capacity rose rapidly from pH 5–8. The decreased binding at low pH values could not be reversed by addition of lysosomal protease inhibitors, nor by heating for 30 min at 50°C, nor by addition of 1 mg/mL BSA.

One potential mechanism for transfer of Cbl from IF to TCII was suggested by the release of 30% of bound Cbl from purified native rat IF incubated at pH 5.0 [11]. To examine this phenomenon with recombinant proteins, Cbl was bound at pH 7.0 to both rat and human IF and to human TCII to achieve near maximal saturation of the proteins. The solutions containing saturated proteins were adjusted to pH 5.0, 6.0, and 7.0 to determine whether any Cbl was released. Table 1 shows that no more than 8% or 14% of Cbl was released from either human or rat IF, respectively, and that only slightly more Cbl was released at pH 5.0 than at pH 6.0 or 7.0. Cbl release from TCII was also no more than 17%, even at pH 5.0, a condition that does not favor Cbl binding to TCII.

3.2. Affinity of Cbl to IF and TCII

The affinity of Cbl for IF has been reported to be about 1 nM, but affinity of Cbl for TCII has been much lower, depending upon the method used [15]. Affinity of Cbl to human IF and TCII was examined, assessing the binding by the charcoal exclusion method. Fig. 2 shows that reproducible binding data was achieved by this method. Table 2 provides the calculated K_a, and shows that the affinity of Cbl for IF did not change from pH 7.5 to 6.1. The K_a of Cbl for TCII was 4–5-fold higher at pH 7.5. At pH 6.1, the affinity of the two proteins was similar, but slightly higher for IF than for TCII. Thus, a complete exchange of ligand would not be expected at any pH value between these two proteins with relatively similar affinity for Cbl.

3.3. Cbl transfer from IF to TCII

Because recombinant IF and TCII elute in nearly the same position in gel filtration and ion chromatography, a nondenaturing polyacrylamide system was used to separate the two molecules with Cbl still bound. When apo-TCII was added to [^{57}Co]Cbl-IF at pH 7.5, 21.6% of the labeled Cbl transferred to TCII (Fig. 3, Lanes 1–4), as would be expected from the 4–5-fold difference in their affinities. The same experiment was performed in the reverse direction to test whether transfer from TCII to IF could occur physiologically in the lumen; only 3.2% of Cbl transferred to IF. This reverse transfer reaction also served to validate the

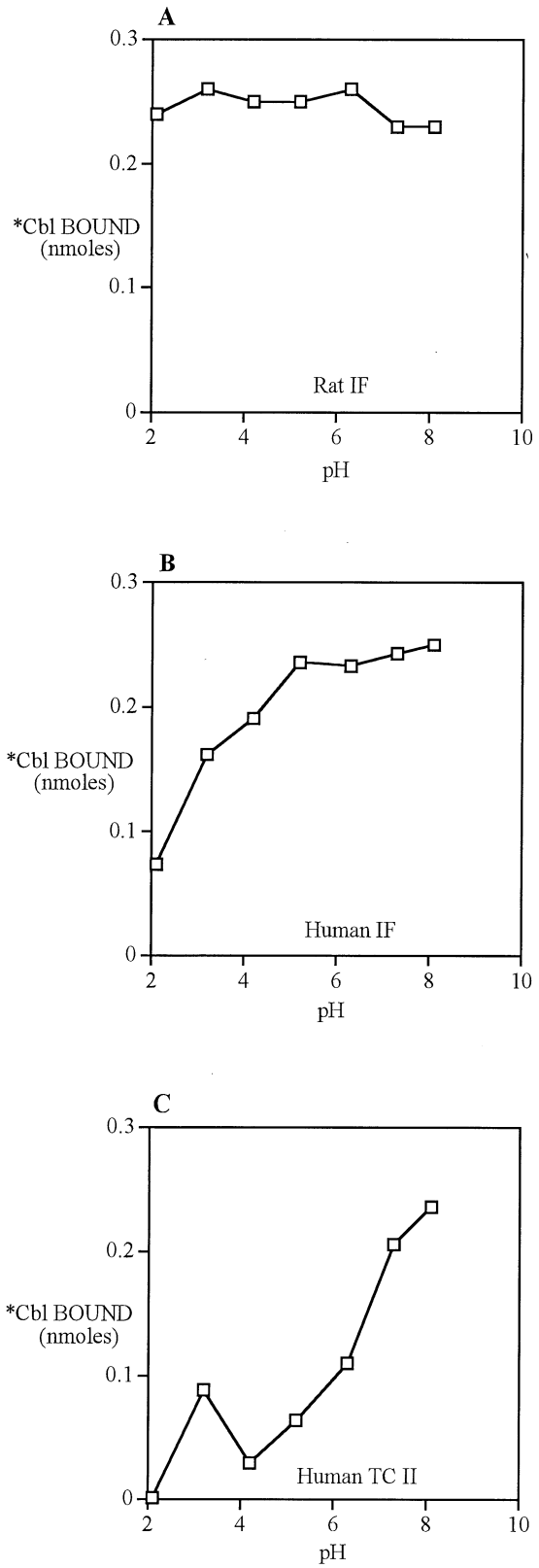

Fig. 1. Binding curves for intrinsic factor (IF) and transcobalamin II (TCII) at various pH values. Binding curves were obtained as described in the Materials and methods section. (A) Recombinant rat IF, (B) recombinant human IF, (C) recombinant human TCII. Each point is the average of duplicate samples. The curves were repeated X2, X3, and X2, respectively, with very similar results.

Table 1
Release of [^{57}Co]Cbl from cobalamin binding proteins as a function of pH

Binding protein	pH	% Release at 30 min
Human intrinsic factor	7.0	2 (0, 4)
	6.0	0 (0, 0)
	5.0	8.7 (6.6, 10.8)
Rat intrinsic factor	7.0	13.8 (7.1, 20.4)
	6.0	12.3 (8.6, 16)
	5.0	14.3 (4.6, 24)
Human transcobalamin II	7.0	0
	6.0	17.7
	5.0	15.2

Release of labeled Cbl was performed as described in the Materials and methods section. Two separate experiments were performed using different preparations of rat and human intrinsic factor. The first value in parentheses represents the mean of quadruplicate incubations, and the second value is the average of the two determinations.

assay system, as no transfer would have been expected considering the relative affinities for Cbl. The same result was found when IF was added in 4–5-fold molar excess of TCII. Although the intracellular concentrations of IF and TCII are not known, this result suggests that transfer is not driven by mass effects, but by relative affinity. Thus, some Cbl moves from IF to TCII in the absence of proteolytic release of Cbl from IF, but this was observed only at pH 7.2–7.5, which is similar to that found in the ileal lumen. The experiment could not be performed successfully at pH values below 7.0, because of the much lower Cbl binding by TCII.

Human TC was incubated with cathepsin L for 60 min at 37°C at the mid-range of the concentration (2.8 mU/μg protein) that degraded IF [10]. Under these conditions, over 90% of the binding activity of TCII was destroyed. On denaturing PAGE, a major band of 30 kDa was found, with very little 43-kDa TCII remaining (data not shown). No similar effect was found using cathepsin B.

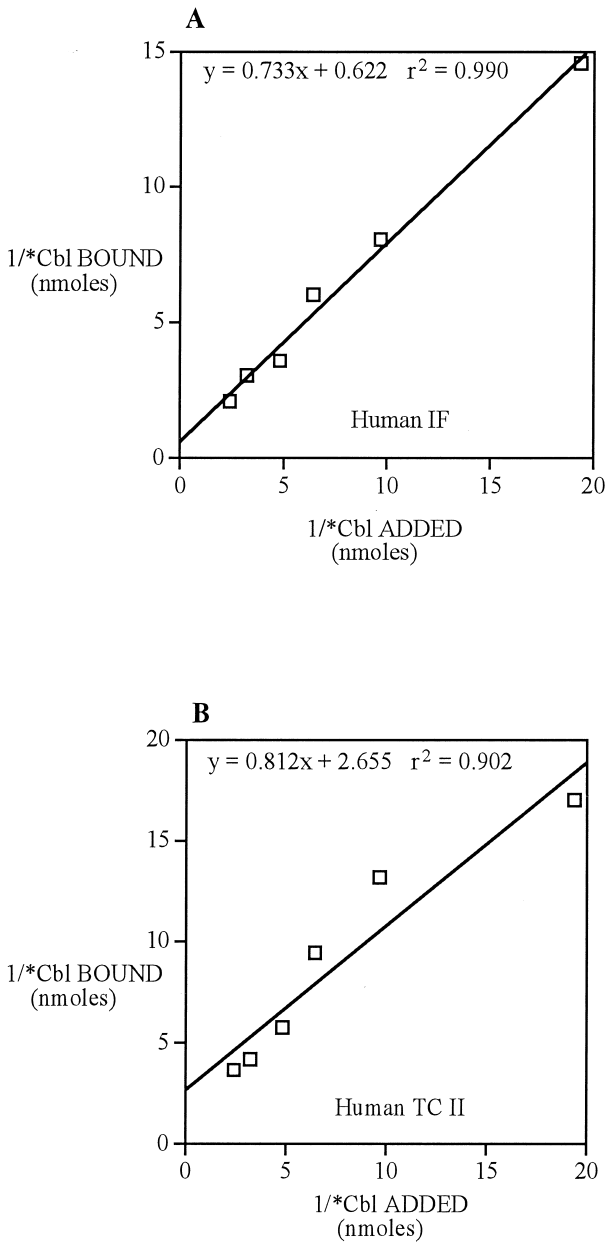

Fig. 2. Reciprocal plots of Cbl binding to intrinsic factor (IF) and transcobalamin II (TCII) at pH 7.5. Binding curves and calculation of the affinity (K_a) of Cbl for each binding protein was performed as described in the Materials and methods section and Table 2. (A) recombinant human IF, and (B) recombinant human TCII. Each point is the average of duplicate samples. Each curve was repeated, with very similar results.

4. Discussion

Cbl is transferred from Hc to IF in the duodenum [1,2], and the resulting IF-Cbl complex travels to the ileal lumen where it binds to its receptor and is absorbed by receptor-mediated endocytosis. In the lumen, the IF-Cbl complex is also exposed to apo TCII and/or the TCII-Cbl complex probably derived from the enterocyte [8,24]. In fact, TCII has been proposed to play a role in the absorption of ileal Cbl where it is largely bound to IF. The affinity of Cbl for TCII has been reported to be 1–2 logs stronger than for IF [15], suggesting that undegraded IF could transfer its Cbl to TCII. Degradation of IF would then occur after Cbl transfer. However, comparative affinities of Cbl for IF and TCII have not been examined using the same methods and comparing preparations of similar purity. The release of about 30% of Cbl from purified rat IF in the absence of proteolysis appeared to be consistent with such a direct transfer [11]. In such a scenario, release of Cbl prior to degradation of the binding protein would be unlike the transfer of Cbl from Hc to IF in which Hc proteolysis initiates the process [1]. When absorbed, whether as IF-Cbl alone or partially as TCII-Cbl, Cbl is released in enterocytes either by low pH (~5.0) alone or by degradation at acid pH, as transcytosis and degradation are inhibited by the addition of chloroquine or leupeptin [5,7,10].

Table 2
Binding affinity of recombinant human IF and TCII for [^{57}Co]Cbl

pH	K_a	
	IF (nM)	TCII (nM)
7.5	2.2	0.34
6.1	1.0	1.8

Affinity for intrinsic factor (IF) and transcobalamin II (TCII) was measured as described in the Materials and methods section. Each curve was generated from two samples and an average of duplicate determinations at every concentration point.

The results of the current experiments support the possibility of limited lumenal transfer of Cbl from IF to TCII. They also favor initial degradation of IF in the lysosome (or perhaps another closely related acid vesicle), with movement of the Cbl either by vesicle fusion, diffusion, or both to a site of neutral pH where it binds to TCII, and do not support Cbl release by pH 5.0 alone. The affinity of Cbl for TCII is tighter than for IF, but it is relatively similar, more so than previous estimates not based on direct comparisons. Thus, direct transfer from IF to TCII occurs only to a small degree. Cbl binding at pH 5.0 is poor for TCII, presumably due to conformational changes in the protein, and TCII is degraded by cathepsin L; thus, acid compartments that would degrade IF are an unlikely site for the transfer of Cbl to TCII. Finally, there is little release of Cbl from IF in the absence of proteolysis; thus, degradation of IF must be the initial event in the transfer of Cbl.

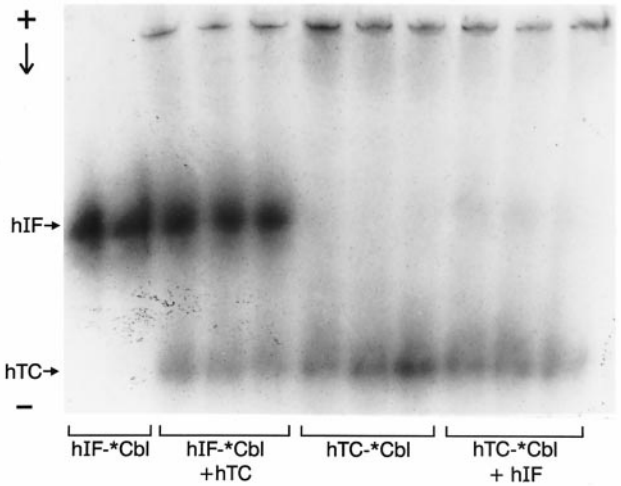

Fig. 3. Autoradiograph of binding of [^{57}Co]Cbl to recombinant intrinsic factor (IF) and transcobalamin II (TCII). The binding proteins were mixed with labeled Cbl, and equal amounts of Cbl binding capacity were added and separated on a nondenaturing acrylamide gel, as described in the Materials and methods section. The migration of IF and TCII alone are shown in Lanes 1 and 2, and Lanes 6–8, respectively. Note that some Cbl transfers to TCII when bound initially to IF (Lanes 3–5), but very little transfer occurs when Cbl is bound first to TCII (Lanes 9–11). The groups of samples represent the results of duplicate or triplicate incubations.

The affinity of Cbl for IF and TCII has been reported to range from 0.15–13 nM and from 1–30 pM, respectively [15,25]. The wide range noted has been thought to reflect the variations in the methods employed. The method used here (charcoal exclusion) has been used by our group previously [1], and the value for IF confirms the most commonly observed K_a of about 1 nM. The relatively low Cbl binding to TCII at acid pH is consistent with the reversible dissociation of Cbl from TCII reported at pH 2 [15]. The release of 30% of Cbl from native rat IF was not reproducible using recombinant rat or human IF. This difference from our earlier report [11] may be due to the difference between native and recombinant proteins, or may reflect some minor contamination of proteolytic enzymes with the native rat IF. It seems less likely that the different glycosylation of the two preparations could account for the difference, as deglycosylated recombinant IF demonstrates the same affinity for Cbl as the intact protein [26]. Cbl has been reported to reversibly dissociate from IF, but at pH 12.5 [27].

After Cbl is released from IF in lysosomes [7,10,28], it is subsequently bound to TCII in a different cellular compartment. As binding of Cbl to TCII is rapid and it takes about 4 hr from the time of transcytosis of IF-Cbl to secretion of TCII-Cbl, one would predict that nonprotein-bound Cbl be found within the cell. As predicted, Quadros et al. [29] reported in guinea pig loops in vivo that 3 hr following uptake of Cbl, 44% of the vitamin was bound to IF, 26% was free, and 16% was bound to TCII. Dan and Cutler [6] found that internalized IF was degraded with a half-time of 4 hr, and that free Cbl was present during transcytosis in Caco-2 cells. These authors postulated that the compartments for release of Cbl from IF and for binding to TCII would be separate. On the other hand, Ramanujam et al. [12] noted in Caco-2 cells that Cbl was secreted bound to TCII from cells without serum present as a source of TCII, but that all intracellular Cbl was bound to either IF or TCII. The present data are consistent with the hypothesis that transfer of Cbl from IF to TCII takes place in different compartments, and should be unbound to protein at some time during transcytosis. The site for Cbl transfer presumably is in the basolateral secretory pathway because most TCII secreted from cultured guinea pig ileum moves to the serosal side of the tissue [23].

The transfer to TCII probably occurs to locally produced TCII and not outside the cell to serum TCII. Dix et al. [30] found that in the absence of serum proteins, Caco-2 cells secreted a protein with the properties of TCII. These findings were confirmed by Ramanujam et al. [7] in opossum kidney cells. Some evidence in guinea pig ileum suggests that Cbl derived from the enterocyte binds to TCII that arises from endothelial cells in the lamina propria [23]. However, diffusion to the correct extracellular site for TCII binding seems unlikely, as Cbl is a large molecule and the transfer is efficient, with very little free Cbl secreted basolaterally [6,12]. The most likely hypothesis currently seems

to involve intracellular Cbl tansfer to TCII, although the precise location of the transfer is not known. These data are most likely to be obtained by immunoelectron microscopy when the problem of loss of IF and TCII antigenicity by oxidizing cross-linking reagents is overcome.

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