Vol. 115, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1983 Pages 238-244

Binding of Intrinsic Factor to Ileal Brush Border Membrane in the Rat

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Received July 15, 1983

The rat ileal brush border membrane binds both free [^{125}I]-intrinsic factor (IF) and the IF-[^{57}Co]cobalamin (cbl) complex. This binding is observed with IF isolated from rat stomach, but not from IF isolated from hog, canine and human stomachs. The binding of rat-IF[^{57}Co]cbl can be blocked with free rat IF but not with hog IF. The IF-cbl complex binds at a higher affinity (Ka=0.15 x 10^9 M^{-1}) compared to that of free IF (Ka=0.9 x 10^9 M^{-1}). Rat IF-cbl also binds efficiently to human and canine ileal membranes. While antibody to the canine ileal receptor blocks the binding of rat, human or hog IF-[^{57}Co]cbl to human and canine ileal membranes, it does not affect the binding of rat IF-[^{57}Co]cbl to rat ileal membranes. These findings demonstrate that the rat ileal receptor is different from canine and human ileal receptors.

It has been recognized for a long time that uptake of cobalamin (cbl, vitamin B_{12}) in the rat cannot be mediated efficiently by using either hog (1,2) or normal human gastric juice (3). However Miyata and Inada (4) could demonstrate uptake of [^{57}Co]cbl in the rat by complexing it with a crude extract from rat stomach. The fact that absorption of cbl in the rat is mediated only by rat stomach extract suggested to us that either rat intrinsic factor (IF) or the rat IF-cbl receptor could be unique. Furthermore, if the receptor were unique it might be different from the canine ileal receptor, which has been extensively characterized (5,6).

Our previous studies (5,6) had demonstrated that free IF does not compete for the binding of IF-[^{57}Co]cbl complex to either canine ileal homogenates or purified ileal receptor, even when present in one hundred fold excess. In the present communication, we show that the rat IF-cbl complex appears to bind well to canine and human ileal brush border membranes. Unlike canine and human ileal membranes the rat brush border membranes can bind not only IF complexed with [^{57}Co]cbl, but also free rat [^{125}I]IF. The binding of these two ligands is mediated by Ca^{++} ions, and they compete for the same binding site. We have further shown that the rat receptor is immunologically different from canine ileal receptor.

0006-291X/83 $1.50

Materials and Methods

CN[^{57}Co]cbl and Na[^{125}I] were obtained from Amersham (Arlington Heights, IL). The hog, rat, and dog stomach and canine ileal mucosa were obtained from Pel-Freeze (Rogers, AR). Human ileal mucosa was obtained from autopsy samples within six hours of expiration and the mucosa was frozen at -70° C until used.

Intrinsic factor from human gastric juice and from hog and dog stomachs were isolated according to Allen and Mehlman (7). Purification of IF from 200 rat stomachs was carried out as follows: A 20% (W/V) homogenate in 140 mM NaCl, 5 mM KCl, 5 mM potassium phosphate buffer ph, 7.4, was prepared using a Waring blender at top speed for two minutes. The homogenization buffer contained 1 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged for 60 minutes for one hour at 105,000 x g. The supernatant fraction containing nearly 90% of cbl binding activity was adjusted to pH 5.0 with 1 N acetic acid. The turbid solution was centrifuged at 15,000 x g for 20 minutes and the neutralized supernatant was subjected to affinity chromatography as described by Allen and Mehlman (7). SDS-polyacrylamide gel electrophoresis of IF and [^{125}I]IF was carried out as described earlier (6). With [^{125}I]IF the band was visualized after exposure to Kodak X-omat AR film for 24 hours.

Brush border membranes from ileal mucosa were prepared according to Schmitz et al (8) as modified by Kessler et al (9). The membranes were enriched 8-10 fold for the receptor with a recovery of 8 to 15 percent, and contained less than 0.1 µg trypsin equivalent of protease activity per mg protein. Rat IF was iodinated using Na-[^{125}I] by the Enzymobeads (BioRad Laboratories, Richmond, CA) procedure. Unbound ^{125}I and bound [^{125}I] IF were separated on a column of Sephadex G-150 (1.5 x 30 cm).

Antireceptor antibody to canine ileal receptor was prepared essentially as described earlier (6). Unlabelled IF and [^{125}I]IF used in these studies were first dialyzed against 15 volumes of 100 mM potassium phosphate buffer, pH 7.4 containing 7.5 M guanidine HCl to ensure removal of any endogenous cobalamin which could be still attached to them. Renaturation of IF was carried out by extensive dialysis against 6L of the phosphate buffer with changes at 24 and 48 hours. The dialyzed protein (5-10 µg) had an absorption of less than 0.02 OD at 340 nm indicating virtual absence of any bound cobalamin.

Binding of [^{57}Co]cbl to IF or [^{125}I]IF, and of [^{125}I]IF or IF-[^{57}Co]cbl to membranes was performed according to Gottlieb et al (10) and Hagedorn and Alpers (11) respectively. The association constant (Ka) was calculated as described earlier (5). Protein was estimated by the method of Lowry et al (12) using bovine serum albumin as a standard.

Results

Purification of intrinsic factor from rat

Table 1 gives a summary of the purification procedure. The IF thus purified gave a single band when electrophoresed in SDS gels under denaturing conditions (Figure 1A) and was purified 260 fold with 33% of the activity recovered. Autoradiography of a 10%

Table I

Purification of intrinsic factor from rat stomach

Fraction	Volume (ml)	Total Protein (mgs)	Total cbl binding ability (nmoles)	Specific activity (pmoles/mg protein)	Recovery (%)
100,000 x g supernatant	1150	19,780	127	6.42	100
pH 5 treatment	1050	10,360	114	11.0	90
Affinity chromatography	30	25	42	1680	33

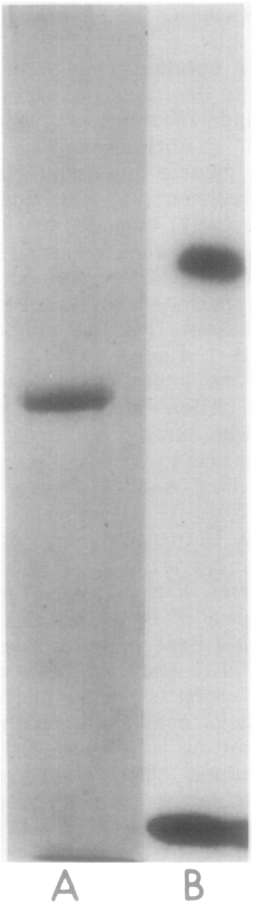


Figure 1 Slab gel electrophoresis of purified rat IF. A) IF (25 µgs protein) was eletrophoresed on a 7% gels. The protein was visualized with Coomassie blue. B) [^{125}I]IF (25,000 cpm) was electrophoresed on a 10% gel, and the radioactive band visualized by exposing the dried gel to x-ray plates for 24 hours. The molecular weight of IF and [^{125}I]IF was calculated using the following marker proteins (25 µgs each), human IgG (Mr = 150,000), muscle phosphorylase b (Mr = 90,000), liver catalase (Mr = 58,000) and yeast alcohol dehydrogenase (Mr = 40,000).

gel SDS polyacrylamide gel also demonstrated only a single rdioactive band (Figure 1B). The molecular weight of IF was 50,000.

Binding of various intrinsic factor-cobalamin complexes to homologous and heterologous brush border membranes

Hog, dog, and human IF purified to homogeneity were used in addition to rat IF for these studies.

Table II shows the results when all four sources of IF were used to form an IF-cbl complex to be bound to brush borders isolated from canine, human and rat ileal mucosa.

Table II

Specificity of IF-[^{57}Co]cbl binding to rat brush border membrane

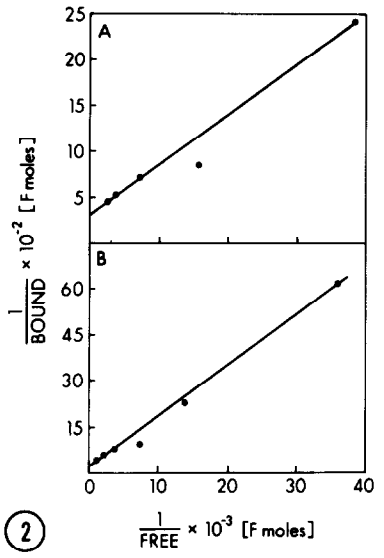
Source of IF-[^{57}Co]cbl	Brush border membrane binding (fmoles/mg protein)					
	Canine		Human		Rat	
	Control	+Antibody	Control	+Antibody	Control	+Antibody
Canine	30 ± 3	0	33 ± 2	0	0	0
Hog	36 ± 4	0	32 ± 2	0	0	0
Human	40 ± 6	0	33 ± 3	0	0	0
Rat	33 ± 3	0	34 ± 4	0	41 ± 4	39 ± 3

IF-^{57}[Co]cbl [0.75 pmoles] complexes from dog, rat, human and hog were incubated with brush border membranes (1.5 to 2 mgs protein) isolated from the ileal mucosa of rat, dog and human, both in the presence, and absence of 2.5 µl of antisera to pure canine ileal receptor. Other assay conditions are given in Materials and Methods. The value represents the mean ± SD of three experiments, using two separate brush border preparations, and refers to that of total binding which is inhibited by EDTA.

Only rat IF mediated binding of cbl to rat ileal membranes. Human, hog and canine IF-cbl complexes bound equally well to human and canine ileal membranes. Antibody directed against pure canine receptor blocked the binding of IF-cbl complexes derived from canine, hog, human, and rat stomachs, when the source of the membranes was dog or human ileum. However, antibody to canine receptor did not inhibit the binding of rat IF-cbl complexes to rat brush border membranes.

Binding of rat [^{125}I]IF and IF [^{57}Co]cbl to rat brush borders

Free IF isolated from dog, hog and human stomach did not bind to homologous or heterologous ileal membrane preparations (5). Figure 2 shows that when a homologous system is used from rat (rat IF and rat membranes), both the IF-[^{57}Co]cbl complex (Figure 2A) and free [^{125}I]IF (Figure 2B) bind to membranes. Both ligands require calcium for binding, Figure 2 records the EDTA inhibitable ligand binding, which accounts for over 80% of total binding. The V_{max} for binding was similar, being 50 fmoles bound/mg protein



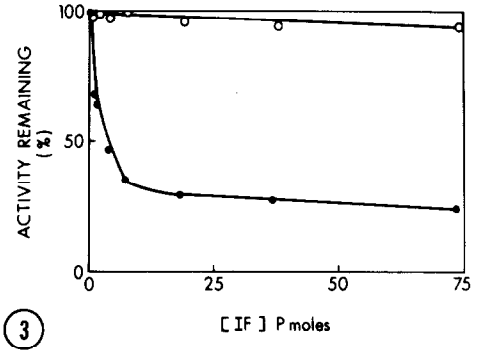


Figure 2 Determination of association constant (Ka) for rat IF-[^{57}Co]cbl (A) and [^{125}I]IF (B). Rat ileal membranes (1.2 mg protein) were used for the binding assay. Other details are provided under Materials and Methods.

Figure 3 Inhibition of the binding of rat IF-[^{57}Co]cbl by IF. Rat ileal brush border membrane (1.2 mgs protein) was preincubated with (0.75-7.5 pmoles) rat IF (•—•) and hog IF (o—o). After 60 min of incubation, rat IF-[^{57}Co]cbl complex (0.75 pmole) was added and further incubated for 60 min at 22° C. Ca^{2+} dependent binding was measured as described earlier (6).

for IF-[^{57}Co]cbl, and 33 fmoles bound/mg protein for [^{125}I]IF. However, the affinity of receptor for IF-cbl was six times greater than for IF alone (K_a of 0.15 x 10^9 M^{-1} for the complex vs 0.9 x 10^9 M^{-1} for IF). With both ligands the optimal binding of rat IF-[^{57}Co]cbl (0.75 p moles) could be inhibited (Figure 3) by a prior incubation of the membranes with free cold rat IF (0.75 to 75 p moles). 75-80% of IF-cbl binding could be inhibited by IF alone, presumably by competition for the same sites on the membrane. However, when rat IF was replaced by hog IF, there was no inhibition to the binding of the rat IF-[^{57}Co]cbl complex. Other divalent cations such as Mg^{2+} or Mn^{2+} could replace Ca^{2+}. At a concentration of 10 mM for Mg^{2+} or Mn^{2+} very similar binding to membranes was observed with both [^{125}I]IF and the IF-[^{57}Co]cbl complex (data not shown).

Discussion

Although many aspects of cobalamin metabolism have been well studied, less is known about the interaction of IF-cbl with the receptor and its subsequent uptake by the ileal enterocyte. As receptors from different species are purified and characterized, these gaps in our knowledge will be filled. Purification of human, guinea pig (13,14) and rat

(15) receptors have been achieved using IF-cbl bound to Sepharose beads. The ileal receptor from dog could not be purified with IF-cbl as a ligand, but was successfully isolated when antibody to IF was bound to Sepharose (6). Because of this difference we wondered if the receptor in some species might demonstrate different properties from the traditional requirements for IF-cbl.

In the present paper, we have used pure IF (Figure 1) isolated from rat stomach. The IF isolated is free from contaminating proteins, and hence iodination labels only a single polypeptide (Figure 1B), allowing binding to be detected by measuring radioactivity. We have demonstrated that both IF-cbl and [^{125}I]-IF bind to rat brush border membranes (Figure 2). The Ca^{++} specificity and competition (Figure 3) for binding sites between free IF and the IF-cbl complex suggests that the same binding sites are involved. However, this competition for binding of rat IF-cbl is not observed with hog IF, suggesting that rat receptor recognizes only rat IF, with or without cbl bound to it.It seems unlikely that binding of free [^{125}I]-IF is due to coupling with endogenous free cbl either on the IF or on the membrane. First, the same washing procedure used to prepare IF and membranes have been used in other species where free IF does not bind to the membranes (5). Second, purified IF does not demonstrate an absorption maximum at 340 nm. Even though absorption measurements at 340 nm are not sensitive enough to detect cbl contamination at very low (fmole) levels, the equilibrium dialysis method has been effectively used by Allen and Mehlman (7) to remove > 99% of endogenous cbl bound to IF. Finally, addition of trace amounts of labelled cbl increased the K_a by six fold. If there were a small contamination of IF by endogenous cbl, one might expect the same binding characteristics as a larger amount of IF-cbl, but the V_{max} should be lower. In fact, the opposite results were obtained.

The properties of the rat brush border receptor suggest that it is unique compared with other IF-cbl receptors. Only IF prepared from rat stomach recognizes the rat receptor, which is different immunologically from dog receptor (Table II). Moreover, free rat IF can bind to the rat receptor. The detection of unique characteristics could suggest that other receptors differ in ways not yet determined. Therefore, the discrepancies observed between the structure of canine (6), human and guinea pig receptors (14,15)

could represent real differences. It may be simplistic to assume that receptor derived from different sources should be more or less the same.

The demonstration of free IF binding could represent a scavanging mechanism whereby free IF could be bound to the surface of the cell after discharging its cbl inside the enterocyte. In this way free lumenal cbl, if any exists, could be recaptured for absorption. Such a mechanism was postulated by Hines et al (16). The binding of free IF to brush borders could explain the results of these investigators, although their studies were performed in guinea pigs.

It is of interest to note that transferrin receptor also binds apotransferrin (17) and that transferrin is recycled back to the membrane after the delivery of iron into the cell, without undergoing degradation by the lysosomal enzymes. Whether a similar mechanism exists for the ileal absorption of cbl needs further study.

ACKNOWLEDGMENTS: This work was supported by grant AM26638 from the National Institutes of Health. We thank Ms. Marlene Jimenez for her efforts in the initial phases of this work, and Catherine Camp for assistance in the preparation of this manuscript.

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