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Short communication

Intrinsic factor covalently bound to Sepharose as affinity medium for the purification of a soluble intrinsic factor receptor from human urine

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Abstract

We have identified a soluble receptor for intrinsic factor (IF) in human urine. The purification of this protein by affinity chromatography required a preliminary purification of IF from hog pyloric mucosal extract. This was achieved by thermolabile cobalamin–ethanol–aminohexane Sepharose affinity chromatography with a 133-fold purification, a yield of 45% and a specific binding activity of 15720 pmol/mg protein. The purified Cbl–IF complex was coupled to epoxy-Sepharose with a yield of 23.8% and a specific activity of 1.2 nmol per mol of gel. The soluble IF receptor was purified form 200 ml of urine concentrate of pregnant women. Desorption was performed at pH 5.0 and in the presence of 5 m*M* EDTA. The soluble IF receptor was purified 17 200-fold with a yield of 52% and a IF binding capacity of 3260 pmol per mg of protein. A single protein with a M_r of 70 000 was found in silver-stained SDS-PAGE.

1. Introduction

The absorption of cobalamin (Cbl, vitamin B_{12}) in man and other mammals requires the binding of Cbl to the gastric intrinsic factor (IF) and the subsequent binding of Cbl–IF complex to an ileal receptor [1]. The ileal receptor has been isolated from intestinal mucosal extract of the pig, guinea pig and dog by affinity chromatography using Cbl-Sepharose gel saturated with IF [2–6]. Guéant et al. [7] have purified the intrinsic factor receptor from pig ileum, using different affinity media including IF covalently coupled to Sepharose and IF coupled to Cbl-Sepharose. The receptor is usually solubilized using detergents such as Triton X-100 [8]. The IF–Cbl receptor is present in the ileal mucosa of several mammalian species. It is also expressed in other organs and tissues such as kidney and liver [9,10]. Its physiological role in Cbl transport across renal cells is not known [9,10]. Recently, we have identified for the first time a soluble IF receptor in urines [11]. In the present work, we have purified this protein by affinity chromatography and determined its electrophoretic physicochemical properties. This purifica-

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tion required a preliminary purification of intrinsic factor from hog pyloric mucosal extract.

Experimental

2.1. Chemicals

Crystalline hydroxocobalamin (OH-Cbl) was obtained from Sigma (St. Louis, MO, USA), crystalline cobinamide from Calbiochem (La Jolla, CA, USA) and cyano[^{57}Co]cobalamin (spec. act. 220 μCi/μg) from the Radiochemical Centre (Amersham, UK). EAH-Sepharose-4B, epoxy-activated Sepharose, Superose 6 and Superose 12 were purchased from Pharmacia (Uppsala, Sweden). Other reagents were obtained from Sigma [12,13].

2.2. Urine samples

Urines were collected from 6 pregnant women at the last week of pregnancy and concentrated 20-fold by ultrafiltration at 4°C. Samples were pooled and used for affinity chromatography 24 h after collection.

2.3. Preparation of hog pyloric mucosal extract

Hog pyloric mucosal extract (2 g) (Mathé, Paris, France) was incubated with 500 ml of 0.1 *M* $NaHCO_3$ (pH 8.2) containing 0.5 *M* NaCl. The extract was filtered through a filter paper (no. 4, Prolabo, Paris, France). Unsatured Cbl binding capacities of IF and haptocorrin were determined using the methods of Gottlieb et al. [14] and Begley and Trachtenberg [15] with hemoglobin-coated charcoal and cobinamide. Haptocorrin was saturated by incubation of hog pyloric mucosal extract with 200-fold excess of cobinamide.

2.4. Purification of IF

Hydroxocobalamin was coupled to EAH-Sepharose-4B by thermolabile Co–N linkage according to the method of Nexo [16] adapted as described [12]. Hydroxo[^{57}Co]cobalamin (OH[^{57}Co]Cbl) in 0.1 *M* $NaHCO_3$ was prepared from cyano[^{57}Co]cobalamin by exposure to light in 8 m*M* HCl. OH-Cbl (4 mmol) and 13 pmol OH[^{57}Co]Cbl were incubated with 4 g swollen EAH-Sepharose 4B in 0.1 *M* $NaHCO_3$ (pH 8.2) containing 0.5 *M* NaCl at 4°C for 24 h under rotative agitation and protection from light. The suspension was subsequently poured into a column (10 × 1 cm I.D.) and washed with 500 ml of 0.1 *M* $NaHCO_3$ (pH 8.2) containing 0.5 *M* NaCl, 500 ml of 0.5 *M* NaCl and 500 ml of 0.02 *M* Tris-HCl buffer (pH 8.0). The coupling efficiencies of OH-Cbl and OH[^{57}Co]Cbl, determined using UV spectrometry and γ-ray counting (LKB, Wallac, Turku, Finland, Model 1261), were about 20 and 14%, respectively. The hog pyloric mucosal extract (saturated with 200-fold excess of cobinamide) was filtered through the column at a flow-rate of 0.5 ml/min, the gel was washed successively with 250 ml of 0.02 *M* Tris-HCl buffer (8.2) containing 0.5 *M* NaCl and 250 ml of 0.02 *M* Tris-HCl buffer (pH 8.4). The total amount of IF coupled to unsolubilized Cbl was estimated from determination of the unsaturated cobalamin binding capacity of hog pyloric mucosal extract before and after elution. The cobalamin intrinsic factor complex was desorbed after incubation of the column at 37°C for 20 h.

2.5. Determination of IF activity by size exclusion in hog gastric mucosal extract

The sample was incubated with a 200-fold excess of cobinamide and 0.74 pmol of CN[^{57}Co]Cbl and made up to a total volume of 1 ml in 0.02 *M* Tris-HCl buffer (pH 7.4)/0.15 *M* NaCl/1 m*M* $CaCl_2$/1.5 m*M* NaN_3. The sample volume was 30 ml. After microfiltration through a 0.40-mm filter, the sample was applied to a Superose 6 B column (50 × 1 cm I.D.), eluted with a Tris-HCl buffer at a flow-rate of 0.5 ml/min, using a Gilson HPLC pump (Model 305, Villiers le Bel, France).

2.6. Preparation of IF–Sepharose gel

Epoxy-actived Sepharose (2 g) (Pharmacia) was swollen and washed with 500 ml of 0.1 *M*

$NaHCO_3$ buffer (pH 8.3) containing 0.5 *M* NaCl. This gel was incubated with 9 nmol of purified saturated IF. The suspension was mixed by slow rotation overnight at 37°C. The gel was washed with a 200-fold volume of 0.02 *M* Tris-HCl buffer (pH 8.0)/0.15 *M* NaCl/1 m*M* $CaCl_2$ in order to eliminate the remaining free IF and to block the free epoxirane groups.

2.7. Purification of the soluble IF receptor

The Cbl-IF-Sepharose gel (8 ml) was incubated with about 200 ml of urine concentrate of pregnant woman at 4°C on a rotating mixer for 48 h. The suspension was washed successively with 500 ml of 0.02 *M* Tris-HCl buffer (pH 8)/0.7 *M* NaCl/1 m*M* $CaCl_2$, 500 ml of 0.02 *M* Tris-HCl buffer (pH 8)/0.15 *M* NaCl/1 m*M* $CaCl_2$ and 500 ml of 0.02 *M* Tris-HCl buffer (pH 8)/0.15 *M* NaCl. The gel was packed into a small column (10 × 1 cm I.D.). Elution of the soluble IF receptor was performed by adding EDTA at a final concentration of 1 m*M* and by decreasing the pH down to 4.5 by dropwise addition of 0.01 *M* HCl. The suspension was gently stirred for 1 h after desorption and the pH was slowly raised up 7 by dropwise addition of 0.01 *M* NaOH.

2.8. Determination of soluble IF receptor activity in size exclusion

The urine sample was incubated with 30 μl of human gastric juice (Cbl binding capacity to IF 32.47 pmol/ml), previously saturated with 0.74 pmol of CN[^{57}Co]Cbl in "receptor buffer" in a 200-fold excess of cobinamide (to block haptocorrin) and made up to a total volume of 1 ml in 0.02 *M* Tris-HCl buffer (pH 7.4)/0.15 *M* NaCl/1 m*M* $CaCl_2$/0.5 m*M* NaN_3/0.05% Triton X-100 (v/v). After microfiltration through a 0.40-μm filter, the sample was applied onto a Superose 12 B column (50 × 1 cm I.D.) eluted with receptor buffer at a flow-rate of 0.5 ml/min, using the Gilson HPLC pump.

2.9. Electrophoresis (SDS-PAGE) of the soluble IF receptor

Electrophoresis was performed using the automated Phast System (Pharmacia) with a 8–25% polyacrylamide gradient gel, in 0.20 *M* Tris-HCl (pH 7.5) 0.20 *M* tricine 0.55% SDS. The separation was carried out at 250 V (10.0 mA) at 15°C. Phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin were used as reference proteins. Each sample contained 2.5% SDS and 5% β-mercaptoethanol. The samples were heated at 100°C for 5 min and bromophenol blue up to about 0.01% was added before loading. The proteins were visualized using the silver staining technique described by Heukeshoven and Dernick [17].

3. Results

Purification of IF receptor from concentrated urines by affinity chromatography required a preliminary purification of IF from hog pyloric mucosal extract for preparing the affinity Sepha-

Table 1
Purification of IF from hog pyloric mucosal extract

	Total protein[a] (mg)	Total (^{57}Co)Cbl-IF binding activity (pmol)	Specific activity[b] (pmol/mg)	Yield (%)	Purification factor
Mucosal extract	503.55	59 304	118		
Affinity chromatography	1.688	26 568	15 721	44.8%	133

[a] Determined using the protein assay described by Smith et al. [18].
[b] The specific activity was assayed by Superose 6 B size exclusion as described under Experimental; it was expressed as pmol of Cbl bound per mg of purified protein.

rose gel. This was achieved by thermolabile cobalamin-EAH Sepharose affinity chromatography with a 133-fold purification, a yield of 45% and a specific binding activity of 15 720 pmol/mg proteins (Table 1). Before purification, a single peak was observed in the mucosal extract in Superose 6 B size exclusion, with a retention time of 34 min (Fig. 1A). The retention time of

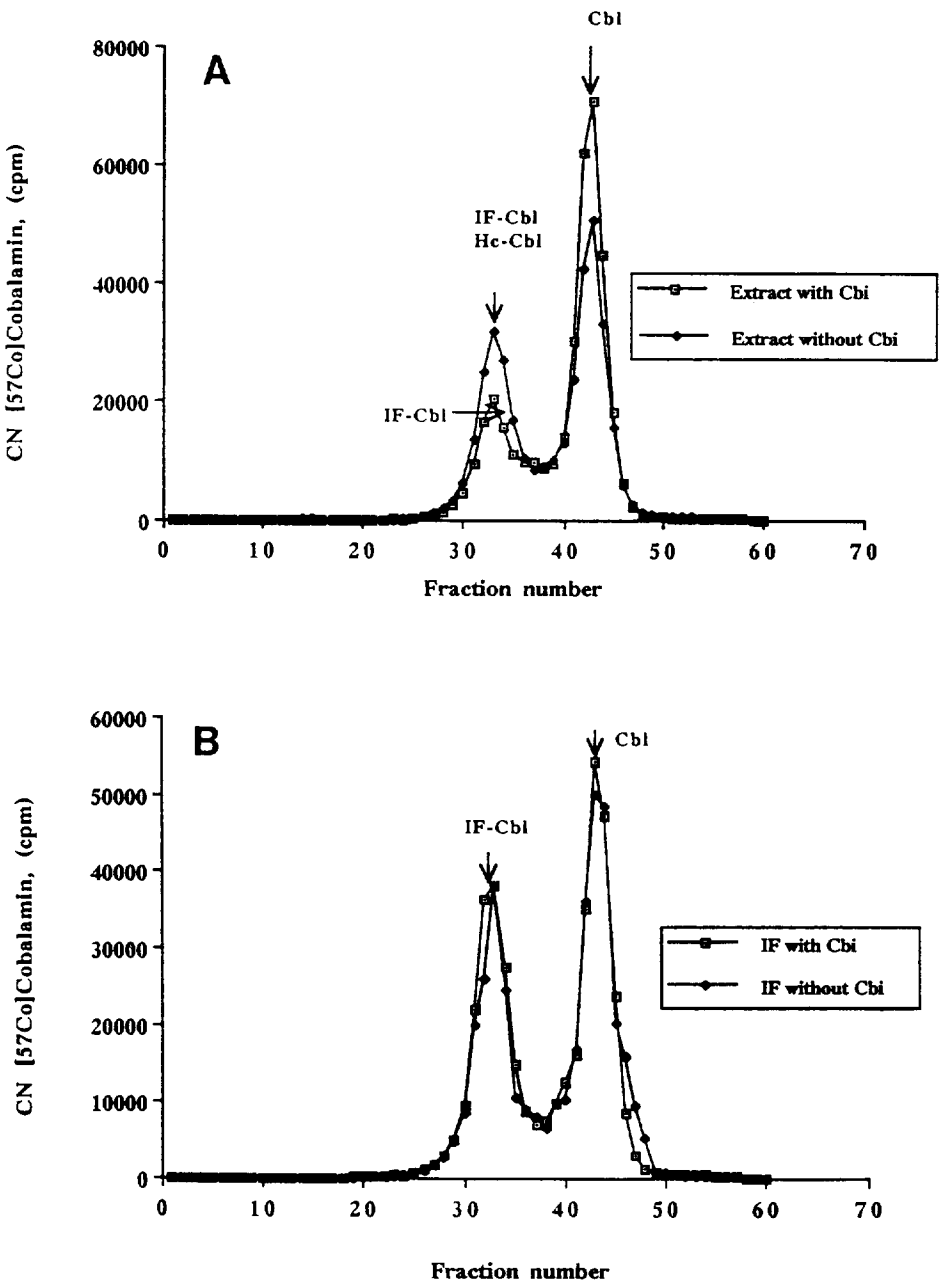

Fig. 1. (A) Superose 6 B size exclusion of IF from hog mucosal extract incubated with labelled cobalamin and a 200-fold excess of cobinamide. The IF–cobalamin complex was eluted at a retention time of 34 min. (B) The purified IF–cobalamin complex was eluted at the same retention in Superose 6 B size exclusion.

the IF peak was not modified when the purified protein was run in gel permeation (Fig. 1B). Its molecular mass was estimated to be 59 000 from SDS-PAGE (Fig. 2). The physicochemical properties of this cobalamin binding protein are identical to those described in the literature [12].

The soluble IF receptor was purified 17 200-fold from 200 ml of urine concentrate of pregnant woman by affinity chromatography, using purified saturated hog intrinsic factor covalently bound to Sepharose. Amounts of total protein (determined using the protein assay described by Smith et al. [18]) in the urine concentrate and of the soluble IF receptor were 234 and 7.2 mg, respectively. The total [^{57}Co]Cbl receptor binding activity of the urine concentrate and of the soluble IF receptor was 44.4 and 23.5 pmol, respectively. The yield of purification was therefore estimated to be 52%. The purified receptor had a IF binding capacity in the order of 3260 pmol/mg. The urine concentrate was preincubated with human gastric juice and with cyano[^{57}Co]Cbl to detect the remaining IF–Cbl binding protein. The peak corresponding to receptor–CN[^{57}Co]Cbl–IF was observed in Superose 6 size exclusion with a retention time between 26 and 31 min (Fig. 3A). After treatment of the urine concentrate of pregnant woman by affinity chromatography, this peak disappeared from the urine eluate (Fig. 3A). The purified receptor–CN[^{57}Co]Cbl–IF complex in Superose 6B size exclusion was eluted at a retention time of 30 min (Fig. 3B). A single protein with a M_r of 70 000 was found in silver stained SDS-PAGE (Fig. 4).

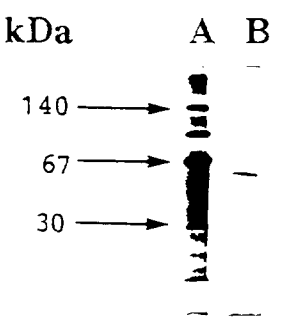

Fig. 2. SDS-PAGE of intrinsic factor in 8–25% continuous gradient gel (silver stained). Lane A: standard proteins. Lane B: purified IF (0.8 μg).

Discussion

The purification of urine soluble IF receptor required the covalent coupling of purified IF to Sepharose. We were faced with the difficulty of knowing if the saturated IF, prepared using thermolabile affinity chromatography and coupled with the Sepharose, bound the soluble urine receptor. Two hypotheses, with respect to the IF–Cbl receptor binding site can be put forward. The first one was suggested by Tang et al. [19], who showed that the receptor binding site is distant from the cobalamin binding site. The second hypothesis (supported by Gräsbeck and Kouvonen [20] and Guéant et al. [7]) suggests that the receptor binding site is common to the cobalamin binding site. If this last hypothesis is true, we may assume than IF bound non-covalently to a cobalamin-Sepharose gel cannot be used for purification of the receptor. There are two possible strategies for binding IF to Sepharose independent of its cobalamin binding site: (1) the use of anti IF-Sepharose bound with the IF-Cbl complex [6], (2) the covalent coupling of saturated IF to epoxy Sepharose [7]. The latter method was used by us for the purification of the IF receptor in the case of pig ileum. We have adapted this second approach for purification of the soluble urine receptor. It has a affinity comparable to that of the receptor purified from pig ileum, but its elution position in Superose 6 suggests that the soluble receptor forms with the IF a smaller complex than the ileal receptor. As a matter of fact, it was eluted in 30 min, whereas the ileal receptor was eluted at high-molecular-mass retention time in Superose 6 B size exclusion. The soluble receptor was adsorbed on to the affinity gel, since the receptor peak disappeared after elution of urine from the affinity column. Moreover, the receptor activity recovered from the affinity gel eluate was eluted at the same retention time as the receptor activity of native urine in Superose 6 B size exclusion. The present affinity procedure allowed to purify the

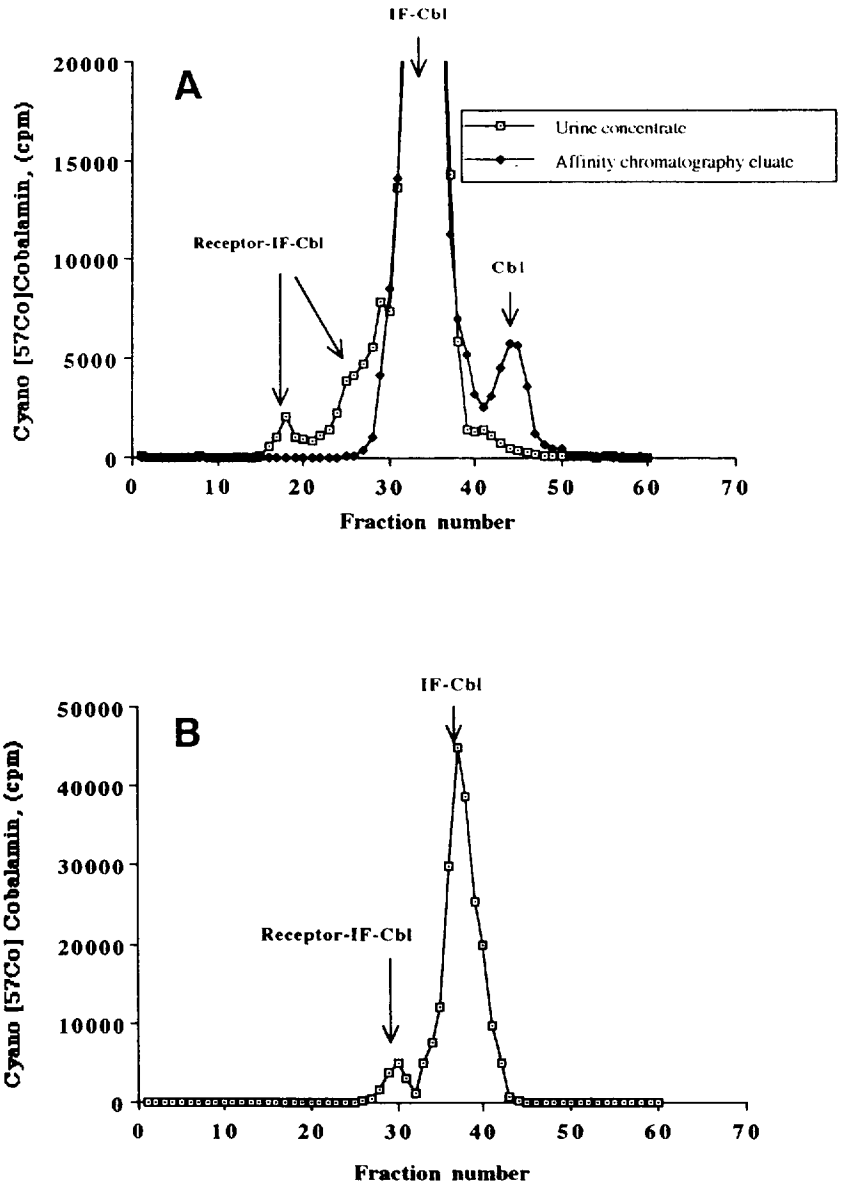

Fig. 3. (A) Superose 6 B size exclusion profile of IF receptor binding activity in urine concentrate from pregnant women before and after treatment by affinity chromatography. The urine samples were incubated with human gastric juice, labelled cobalamin and a 200-fold excess of cobinamide. (B) Superose 12 B size exclusion of purified soluble IF receptor incubated with human gastric juice and with labelled cobalamin. The purified soluble IF receptor was eluted at a retention time of 30 min.

receptor in a single step. Indeed, a single protein band was observed with an apparent molecular mass of 70 000, which is slightly lower than that of the subunit α. Several authors, including our group, have previously shown that the subunit α contains the receptor binding site. We may, therefore, assume that the soluble purified receptor is a proteolysis product of the α-subunit. The use of affinity chromatography, in which the IF is not covalently adsorbed onto the support, presents the risk of introducing a contaminated IF into the preparation of the purified IF. The

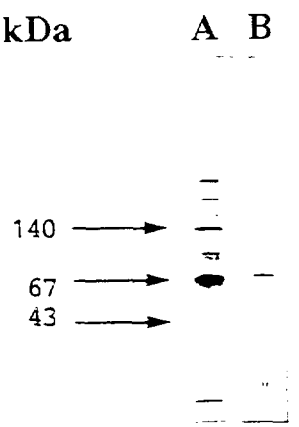

Fig. 4. SDS-PAGE of the purified soluble IF receptor in 8–25% continuous gradient gel (silver stained). Lane A: standard proteins. Lane B: purified soluble IF receptor (0.4 μg).

presence of this contaminated protein should be difficult to detect in electrophoresis. This method reported here may be used for purification of the soluble receptor at a micro-preparative scale in order to determine its microsequence and the physico-chemical properties of the soluble IF receptor. The interest is considerable since no one has, until now, managed to purify the human IF receptor.

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