Purification by cobalamin–Sephadex affinity chromatography and intrinsic factor-binding activity of an extramembrane proteolytic product from pig ileal mucosa

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We have purified a cobalamin-binding protein obtained by papain digestion of pig intestine by cobalamin–AH–Sephadex affinity chromatography, with a purification factor of 17300, a yield of 63 % and a cobalamin-binding activity of 11 260 pmol/mg of protein. The protein contained 3.8 % carbohydrate and was O- and N-glycosylated. Its molecular mass was 69 kDa on SDS/PAGE and its isoelectric point was 5.1. It had a binding

activity for both [⁵⁷Co]cobalamin and [⁵⁷Co]cobalamin–intrinsic factor in native PAGE autoradiography and it inhibited the binding of intrinsic factor to the intact intestinal receptor with an IC₅₀ of 49.31 nmol/l in a radioisotope assay. In conclusion, the purified protein shared a binding activity for both cobalamin and intrinsic factor–cobalamin complexes and could correspond to the extracellular domain of the ileal intrinsic factor receptor.

INTRODUCTION

Dietary cobalamin (vitamin B-12; Cbl) is assimilated in the distal ileum by a receptor-mediated endocytosis of the intrinsic factor (IF)–Cbl complex. The IF–Cbl receptor complex is located in the microvillus pits of the ileal apical brush-border membrane [1]. It has been isolated from intestinal mucosal extracts of pig, guinea-pig and dog [2–6], by solubilization with Triton X-100 [2,7] and affinity chromatography, using affinity media such as IF non-covalently bound to Cbl–Sephadex gel [8], IF bound to an anti-IF–Sephadex [6], or human IF covalently bound to Sephadex [9]. The pig ileal receptor purified by our group has a molecular mass of 256 kDa in native PAGE [9]. Its α -subunit shares a binding site for IF–Cbl and its incubation with lectin inhibits the binding activity of the receptor [10]. Digestion of the pig ileal mucosa by papain removes the outer part of the IF–Cbl receptor. This fraction of the receptor has been named ‘papain- α ’ and has been purified by affinity chromatography on Cbl–IF–Sephadex gel [8]. Direct binding of Cbl to the IF–Cbl receptor has been suggested by several groups. Mathan et al. found a binding activity for corrinoids in ileal mucosal homogenates of guinea-pig [11] and Kanazawa et al. found that cobinamide (Cbi) and Cbl bind to IF receptor extract from pig ileal mucosa [12]. Ramasamy and Seetharam [13] and Guéant et al. [14] have observed a significant uptake of Cbl (even if lower than that of IF–Cbl) by CaCo-2 and HT29 cells, two human carcinoma cell lines which resemble fetal enterocytes [13,14]. All these results suggest that the extracellular domain of the receptor can recognize both IF–Cbl and Cbl. The present work confirms this hypothesis. We have identified a Cbl-binding protein in a protein extract obtained by papain digestion of pig ileal mucosa. The protein has been purified by Cbl–Sephadex affinity chromatography and its binding activity was tested for both Cbl and IF–Cbl.

EXPERIMENTAL

Chemicals

Crystalline hydroxycobalamin (OH–Cbl) was obtained from Sigma (St. Louis, MO, U.S.A.), crystalline Cbi from Calbiochem (La Jolla, CA, U.S.A.), and ⁵⁷Co-labelled cyanocobalamin (CN[⁵⁷Co]Cbl) (specific radioactivity 8.15 MBq/ μ g) from the Radiochemical Centre (Amersham, Bucks., U.K.). AH–Sephadex-4B, Superose 6 and Superose 12 were purchased from Pharmacia (Uppsala, Sweden). Other reagents were obtained from Sigma.

Preparation of papain extract

The papain extract was prepared as described by Kouvonen [8]. Ten fresh ilea were rinsed three times with 0.9 % NaCl solution and filled with 20 mM Tris/HCl buffer (pH 7.4) containing 18×10^6 units of papain/l and 0.4 mM PMSF. Ileae were plugged up and maintained at 4 °C under a rotative agitation for 24 and 48 h. Then, the intraluminal solution was collected and centrifuged at 27000 *g* for 1 h. The pellet was discarded and the supernatant used as papain extract.

Determination of papain extract activity in gel filtration

The IF–Cbl-binding activity of the papain extract was determined by incubating 400 μ l of the freshly prepared extract with 30 μ l of human gastric juice (Cbl-binding capacity 32.47 pmol/ml) previously saturated with a 200-fold excess of Cbi (to block haptocorrin) and 0.74 pmol of CN[⁵⁷Co]Cbl in 20 mM Tris/HCl buffer (pH 7.4) containing 1 mM CaCl₂ and 1.5 mM NaN₃. The incubation volume was 1 ml. The Cbl-binding activity of papain extract was determined by incubating 400 μ l of the sample with 0.74 pmol of CN[⁵⁷Co]Cbl as described above. After filtration

Abbreviations used: IF, intrinsic factor; Cbl, cobalamin; CN[⁵⁷Co]Cbl, ⁵⁷Co-labelled cyanocobalamin; OH–Cbl, hydroxycobalamin; Cbi, cobinamide; GNA, *Galanthus nivalis* agglutinin; SNA, *Sambucus nigra* agglutinin; DSA, *Datura stramonium* agglutinin; PNA, peanut agglutinin; MAA, *Maackia amurensis* agglutinin.

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through a 0.22 μm -pore-size filter, the sample was applied to a Superose 12 column (1 cm $\times$ 30 cm) eluted with the Tris/HCl buffer described above at a flow rate of 0.5 ml/min, using a Gilson HPLC pump, model 305. The same experiment was repeated with 400 μl of papain extract previously saturated with 200 pmol/ml of Cbi.

Affinity chromatography

OH-Cbl was coupled to AH-Sepharose 4B by thermolabile Co-N linkage as described by Nexø and Guéant et al. [15,16]. Hydroxy[^{57}Co]cobalamin (OH[^{57}Co]Cbl) was prepared from CN[^{57}Co]Cbl by light exposure in 8 mM HCl; 4 mmol of OH-Cbl and 14 pmol of OH[^{57}Co]Cbl were incubated with 4 g of swollen AH-Sepharose-4B in 0.1 M NaHCO₃ (pH 8.2) containing 0.5 M NaCl at 4 °C for 24 h under rotative agitation and protection from light. The suspension was washed with 500 ml of 0.5 M NaCl, 500 ml of 0.1 M NaHCO₃ (pH 8.2) containing 0.5 M NaCl, and 500 ml of 0.02 M Tris/HCl buffer (pH 8.0). One litre of freshly prepared papain extract was pooled. Cbi was added at a concentration of 200 pmol/ml. The extract was filtered through a 0.80 μm filter and incubated with affinity gel for 24 h at 4 °C, under rotative agitation. The gel was washed with a 100-fold volume of 20 mM Tris/HCl buffer (pH 7.4) containing 0.15 NaCl and 1 mM CaCl₂. Papain protein was eluted after incubation of the gel at 37 °C for 24 h. The protein concentration was measured in the eluate, using bicinchoninic acid (BCA) as active reagent of the protein assay (Pierce, Rockford, IL, U.S.A.), according to the manufacturer's instructions. BSA was used as standard protein [17].

Physicochemical properties of the radio-iodinated purified 'papain' protein

The purified 'papain' protein was iodinated using the method of Markwell [18], as described for purified IF [19]. The iodinated protein was run in Superose 12 gel filtration as described above. The peak corresponding to the iodinated 'papain' protein was collected and studied by isoelectric focusing. This was performed in a 110 ml LKB column, type 8101 (LKB, Bromma, Sweden), packed with carrier ampholytes of pH values ranging from 2.5 to 8, at a final concentration of 1% in a 0–50% (w/v) sucrose gradient containing 5 M urea.

Electrophoresis of purified 'papain' protein

All the electrophoreses were performed using an automated Phast System (Pharmacia, Uppsala, Sweden) [20,21]. About 0.5 μg of purified 'papain' protein was used per run. SDS/PAGE was performed with an 8–25% polyacrylamide gradient gel, in 0.02 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% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. The samples were heated at 100 °C for 5 min and Bromophenol Blue was added up to 0.01% (v/v). The proteins were visualized by silver staining as described elsewhere [9,20,21]. Autoradiography of purified 'papain' protein–CN[^{57}Co]Cbl complex was performed by incubating 0.5 pmol of purified protein with the same amount of CN[^{57}Co]Cbl in the presence or absence of 8 pmol of Cbi. The same experiment was done with either 0.5 pmol of IF in the presence of 1 mM of CaCl₂, or 5 mM of EDTA. Non-denaturing PAGE (native PAGE) was carried out with 8–25% polyacrylamide continuous gradient gels, in 0.25 M Tris/HCl (pH 8.8) containing 0.88 M L-

alanine, at 400 V (10.0 mA) and 15 °C, according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden). Albumin, lactate dehydrogenase, catalase, ferritin and thyroglobulin were used as globular reference proteins. The gels were dried and exposed with X-OMAT (Kodak, Eastman, NY, U.S.A.) for 2 days at –80 °C.

Amino acid composition

Samples containing approx. 100 ng of purified 'papain' protein were dialysed against water and dried by lyophilization. The dried sample was hydrolysed in gas phase under 1% phenol/6 M HCl as 150 °C vapours for 1 h. Amino acid analysis was performed in an Applied Biosystem analyser (Foster City, CA, U.S.A.) equipped with a C18 reverse-phase column (220 mm $\times$ 2.1 mm; Brownlee PTC type).

Carbohydrate analysis

The molar carbohydrate content was determined after methanolysis [22] of the native glycoprotein. Carbohydrates were identified by GS as their trimethylsilyl ethers.

Lectin blotting

All the reagents of this experiment were from the glycan differentiation kit of Boehringer (Mannheim, Germany). About 1 μg of control glycoproteins (carboxypeptidase Y, transferrin, fetuin, asialofetuin) and 5 μg of dialysed purified 'papain' protein were directly blotted (in each pit) on to poly(vinylidene difluoride) membrane (Immobilon; Millipore, Bedford, U.S.A.). After impregnation, the proteins were transiently stained with Ponceau S for 5 min. An aliquot (500 μl) of blocking solution was added for 30 min. The membrane was then washed twice for 10 min with 0.05 M Tris/HCl buffer (pH 7.5) containing 0.15 NaCl, and once with 0.05 M Tris/HCl (pH 7.5) containing 1 mM MgCl₂, 1 mM MnCl₂ and 1 mM CaCl₂. The following lectins were added: *Galanthus nivalis* agglutinin (GNA, 0.1 $\mu\text{g}/\text{ml}$), *Sambucus nigra* agglutinin (SNA, 0.1 $\mu\text{g}/\text{ml}$), *Datura stramonium* agglutinin (DSA, 5 $\mu\text{g}/\text{ml}$), peanut agglutinin (PNA, 0.5 $\mu\text{g}/\text{ml}$) and *Maackia amurensis* agglutinin (MAA, 0.5 $\mu\text{g}/\text{ml}$) [23–26]. They were in solution in 0.05 M Tris/HCl buffer (pH 7.5) containing 1 mM MgCl₂, 1 mM MnCl₂ and 1 mM CaCl₂. After incubation for 1 h and washing three times for 10 min with 500 μl of Tris-buffered saline, 10 μl of anti-digoxigenin–alkaline phosphatase (750 units/ml) was added and incubated for 1 h. After washing, the blots were developed with 0.1 M Tris/HCl buffer (pH 9.5) containing 0.05 M MgCl₂, 0.1 M NaCl and 50 μl of 4-Nitro-Blue Tetrazolium chloride. Blots were rinsed several times with water to stop the reaction, dried and photographed.

Inhibition of the binding activity of intact receptor by purified 'papain' protein

The binding of CN[^{57}Co]Cbl–IF/Cbl–IF to 'papain' protein was studied and was compared with intact IF–Cbl receptor from pig homogenate in the presence of bivalent cations, using the IF-receptor phenyl-Sepharose RIA as described by our group [27]. Then, inhibition of the binding of the IF–Cbl complex to intact receptor by 'papain' protein was studied using the same method. Each of a series of test tubes received 100 μl of a tracer solution. This solution was prepared by incubating 45 μl of human gastric juice (32.5 pmol of IF/ml) with 1.10 pmol of CN[^{57}Co]Cbl and a 200-fold excess of Cbi, in respect to Cbl, in 0.02 M Tris/HCl buffer (pH 7.4) containing 0.15 M NaCl in a total volume of 1.5 ml. A volume of 5, 10, 25, 50, 100, or 400 μl of crude extract

Figure 1 Superose 12 gel filtration of the 'papain' protein solubilized from pig intestine

(a) The papain extract (400 μ l) was incubated with 0.74 pmol of IF- ^{57}Co]Cbl complex as described in the Experimental section. The 'papain' protein-Cbl-IF complex was eluted with a retention time of 27 min ($\circ$). Adding 10 mM of EDTA to the incubation resulted in a significant decrease (92%) of 'papain' protein- ^{57}Co]Cbl-IF complex ($\square$). (b) When the papain extract was incubated with 0.74 pmol of ^{57}Co]Cbl alone, the peak corresponding to papain protein- ^{57}Co]Cbl complex was eluted with a retention time of 33 min ($\circ$). Filtration of the extract through the Cbl-Sepharose affinity gel resulted in the disappearance of both IF-Cbl (a) ($\bullet$) and Cbl-binding activities (b) ($\bullet$). (c) Superose 12 gel filtration of the purified 'papain' protein incubated with 0.74 pmol of ^{57}Co]Cbl-IF. The papain protein- ^{57}Co]Cbl-IF complex peak was eluted with a retention time of 27 min ($\circ$). This peak was abolished by preincubation of the sample with 10 mM EDTA ($\square$). (d) Superose 12 gel filtration of the purified 'papain' protein incubated with ^{57}Co]Cbl. The 'papain' protein- ^{57}Co]Cbl complex was eluted with a retention time of 33 min ($\circ$).

or purified 'papain' protein was pipetted into each tube together with 10 μ l of solution containing 1 M CaCl_2 /0.1 M MgCl_2 . The incubation volume was made up to 950 μ l by adding 0.02 M Tris/HCl buffer (pH 7.4) containing 0.15 M NaCl. After 1 h of incubation with shaking, intestinal receptor extract (50 μ l) was

added and the final volume was made up to 1 ml. The test tubes were incubated again as previously for 1 h before adding consecutively 200 μ l of a suspension of phenyl-Sepharose and 300 μ l of 0.02 M Tris/HCl (pH 7.4) containing 3.3 M NaCl and 0.5% (w/v) of BSA. After 30 min, the tubes were centrifuged at 1500 g

for 10 min and the pellet was washed three times by repetitive addition of 3 ml of the Tris/HCl buffer described above. The amount of IF-CN^[57Co]Cbl receptor was then estimated from total radioactivity per tube and radioactivity of the pellet. The percentage inhibition was determined from the decreasing binding (fmol/assay) of IF-Cbl to ileal receptor in the presence of increasing quantities of 'papain' protein.

RESULTS

When the papain extract (preincubated with 200 pmol/ml of Cbl) was incubated with [^{57Co}]Cbl-IF, 'papain' protein formed a molecular complex with [^{57Co}]Cbl-IF which was eluted with a retention time of 27 min in Superose 12 gel filtration (Figure 1a). When the papain extract was incubated with CN^[57Co]Cbl alone, a smaller protein-[^{57Co}]Cbl complex was observed at a retention time of 33 min (Figure 1b). No 'papain' protein-[^{57Co}]Cbl peak was observed in gel filtration when the experiment was performed either with non-labelled Cbl or with Cbl-IF instead of Cbl.

The 'papain' protein was purified 17300-fold from 1 litre of papain extract using Cbl-Sepharose affinity chromatography. The activity of the purified 'papain' protein, measured by gel filtration, was of the order of 11260 pmol/mg of protein (Table 1). Filtration of the papain extract through the Cbl-Sepharose affinity gel resulted in the disappearance of both IF-Cbl- and Cbl-binding activities (Figures 1a and 1b). The eluate from affinity chromatography contained a single protein in silver-stained SDS/PAGE, with a molecular mass estimated at 69 kDa (Figure 2, left-hand panel). A proteolytic product of 30 kDa was also observed when the incubation of pig mucosa with papain was extended up to 48 h (results not shown). The purified protein was incubated with [^{57Co}]Cbl-IF in the presence of CaCl₂ and subjected to gel filtration. A 'papain' protein-[^{57Co}]Cbl-IF peak was obtained at a retention time of 27 min (Figure 1c). This peak was abolished when EDTA was present in the place of CaCl₂.

The purified protein-[^{57Co}]Cbl complex was eluted at a retention time of 33 min in gel filtration (Figure 1d). A shoulder of the elution profile of the [^{57Co}]Cbl-protein peak was also observed at a retention time of 35 min. This was systematically observed in repetitive runs and could correspond to an oligomerization of the [^{57Co}]Cbl-protein complex as the peaks corresponded to molecular masses of about 140 and 70 kDa respectively (Figure 1d). Autoradiography of native PAGE was performed with the purified protein incubated with either [^{57Co}]Cbl or [^{57Co}]Cbl-IF. The [^{57Co}]Cbl-'papain' protein migrated as a 69 kDa radioactive band (Figure 2; right-hand panel, lane 3). Incubation of the protein with [^{57Co}]Cbl-IF resulted in the formation of a 130 kDa complex (Figure 2; right-hand panel, lane 2). This complex dissociated into IF and [^{57Co}]Cbl-'papain' protein in the presence of EDTA (Figure 2; right-hand panel,

Figure 2 Denaturing SDS/PAGE and autoradiography of native PAGE of purified 'papain' protein

Left-hand panel: the purified 'papain' protein-[^{57Co}]Cbl complex had a molecular mass of 69 kDa in the denaturing SDS/PAGE (lane B). Phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α -lactalbumin were used as reference proteins (lane A). The corresponding molecular masses (in kDa) are indicated by arrows. Right-hand panel: autoradiography of native PAGE was performed with the purified 'papain' protein incubated either with [^{57Co}]Cbl or [^{57Co}]Cbl-IF. The 'papain' protein-[^{57Co}]Cbl-IF complex migrated as a 130 kDa radioactive band (lane 2). This complex dissociated into IF and 'papain' protein-[^{57Co}]Cbl in the presence of EDTA (lane 3). In lane 4, the 'papain' protein-[^{57Co}]Cbl complex migrated as a 69 kDa radioactive band. Albumin, lactate dehydrogenase and catalase were used as reference molecules. The corresponding molecular masses (in kDa) are indicated by arrows (right-hand side).

lane 4). The amino acid composition of the purified protein is given in Table 2. It corresponded to a calculated molecular mass of 68.7 kDa. The carbohydrate content of the purified protein was 3.8 % (Table 3). Lectin-blotting showed a positive reaction with SNA only. By comparison, transferrin reacted with SNA, carboxypeptidase with GNA, fetuin with SNA, MAA and DSA, and asialofetuin with PNA and DSA.

The purified 'papain' protein labelled with ¹²⁵I₂ was run in Superose 12 gel filtration (Figure 3a) and the corresponding peak was collected and studied in column isoelectrofocusing. A main isoprotein was observed at pI 5.1 (Figure 3b). A small peak was also observed which might correspond to an acidic aggregate.

By contrast with the intact solubilized IF receptor, no adsorption of the [^{57Co}]Cbl-IF-'papain' protein complex was observed on phenyl-Sepharose with our radioisotope binding assay (Figure 4a). The 'papain' protein competed with the solubilized intact IF receptor to bind IF-Cbl: a 100 % inhibition of the binding of IF-CN^[57Co]Cbl to the intact IF receptor was reached by pre-incubating the IF ileal receptor with an increasing amount of purified 'papain' protein. A 50 % inhibition was observed at a concentration (IC₅₀) of 49.31 nmol/l of the purified 'papain' protein (Figure 4c). On the other hand maximum inhibition of only 53 % was reached when using the crude papain extract instead of the purified protein (Figure 4d).

Table 1 Purification of the 'papain' protein from papain-solubilized pig intestine using Cbl-Sepharose affinity chromatography

	Volume (ml)	Protein content (mg/ml)	Total protein* content (mg)	Total vitamin B-12 (Cbl) binding capacity (pmol)	Specific activity (pmol/mg)†	Yield (%)	Purification factor
Primary extract	1000	1.675	1675	1087	0.65	—	—
Purified 'papain' protein	2.76	0.022	0.061	687	11260	63.2	17300

* Total protein was determined using the assay described by Smith et al. [17].

† The specific activity was assayed by gel filtration. It was expressed as pmol of Cbl bound/mg of purified protein.

Table 2 Amino acid content of the purified 'papain' protein obtained by Cbl-Sepharose affinity chromatography of papain-solubilized ileum mucosa

Abbreviation: ND, not determined.

Amino acid	Percentage of molecule	
	Present study	Canine ileal receptor solubilized by papain*
Asp	12.56	10.88
Glu	24.39	11.12
Ser	5.43	8.00
Gly	8.53	10.76
His	0.06	2.81
Arg	2.54	3.40
Thr	5.47	6.28
Ala	12.56	8.07
Pro	5.96	6.34
Tyr	0.80	3.11
Val	5.02	4.78
Ile	3.00	4.06
Leu	6.73	7.77
Phe	2.37	4.18
Lys	4.60	5.38
Trp	ND	ND
Met	ND	ND
Cys	ND	ND

* Data from Seetharam et al. [34].

Table 3 Carbohydrate content of 'papain' protein purified from pig ileum

Carbohydrate residue	Content (mol per 3 mol of mannose)
Fucose	—
Galactose	2.10
Mannose	3.00
N-Acetylglucosamine	6.80
N-Acetylgalactosamine	0.89
Neuraminic acid	0.66
Total glycosylation	3.8%

DISCUSSION

The present study shows that a Cbl-binding protein can be removed by papain digestion of pig ileum. The protein was purified by Cbl-Sepharose affinity chromatography and was identified as a 'papain' proteolytic product of the IF receptor since (i) it bound to [^{57}Co]Cbl-IF in a calcium-dependent way (Figures 1 and 2), and (ii) it inhibited very efficiently the binding of IF-Cbl to the intact solubilized receptor (Figure 4). The Cbl-binding activity of the purified protein was observed in both gel-filtration and autoradiography studies (Figures 1 and 2). This protein was not haptocorrin as it had no binding activity for Cbi and its amino acid composition was different to that of haptocorrin (Table 2).

Previous data from our and other groups reported that only IF-Cbl, and not free IF, was recognized by the IF-Cbl receptor from pig intestine [2,6,9]. It can therefore be assumed that the extracellular domain of the receptor shares a binding site which involves both IF and Cbl in a discriminant way, as free cobalamin, but not free IF, is able to bind to the receptor. Two different regions of IF could be involved in its binding with the receptor. The first one would correspond to the Cbl-binding site of IF, whereas the other one would be distant from the former. Tang et

Figure 3 Superose 12 gel filtration of the purified 'papain' protein labelled with $^{125}\text{I}_2$

(a) The iodinated protein eluted at a retention time of 35 min (○). (b) The corresponding peak was collected and studied in column isoelectrofocusing. A single isoprotein isoelectric point was observed at pI 5.1 (●). A smaller acidic peak corresponded to an aggregate.

al. [28] have produced a recombinant peptide from the N-terminal region of IF which recognizes the IF-Cbl receptor, but has no Cbl-binding activity.

The molecular mass of our 'papain' protein in SDS/PAGE (69 kDa) was higher than that of the 'papain α ' fragment obtained by Kouvonen (45 kDa) [8] and lower than that obtained by Seetharam et al. (186 kDa) [29]. This can be explained by differences in the hydrolysis and in the purification methods. We have used Cbl-Sepharose when others authors have used IF non-covalently bound to Cbl-Sepharose gel [8] or IF bound to an anti-IF-Sepharose [29]. In our experience, digestion of the ileal mucosa for two different times of incubation (24 and 48 h), produced a Cbl-binding protein of 69 kDa or of 30 kDa respectively. One hypothesis could be that hydrolysis for 48 h permits the breaking of the extracellular domain of the IF-Cbl receptor into several compounds including 45 and 30 kDa fragments. The 30 kDa fragment would be the one which contains the Cbl-binding site. The IF-Cbl-binding activity of the 'papain' protein was observed in Superose 12 gel filtration and in the phenyl-Sepharose assay. The 'papain' protein inhibited com-

Figure 4 Binding kinetics of the IF-CN⁵⁷Co]Cbl complex to the IF receptor from pig ileal extract

(a) Saturation curve of the binding of increasing concentrations of IF-CN⁵⁷Co]Cbl complex to IF receptor from 50 μ l of pig ileal extract. The saturated receptor was separated from the IF-Cbl complex by adsorption to phenyl-Sepharose. The concentration of receptor-IF-Cbl in the assay (○) was calculated by the difference between the concentration of IF-[⁵⁷Co]Cbl adsorbed to phenyl-Sepharose in the presence of CaCl₂ to that in the presence of 10 mM EDTA (non-specific binding). The same experiment was performed with purified 'papain' protein (●); no adsorption of the 'papain' protein-[⁵⁷Co]Cbl-IF was observed on to phenyl-Sepharose. (b) Scatchard plot of the experiment performed with pig ileal receptor (○), corresponding to an association constant at 5.7 nM⁻¹ ($r = 0.99$) and a receptor concentration of 196 fmol/ml of pig ileal homogenate. (c) Inhibition of the binding of 74 pmol of IF-CN⁵⁷Co]Cbl to IF receptor from pig ileal homogenate by increasing the concentration of purified 'papain' protein. The saturated receptor was separated from the [⁵⁷Co]Cbl-IF complex by adsorption to phenyl-Sepharose. The concentration of receptor-IF-Cbl in the assay was measured by γ -ray-counting of total radioactivity and radioactivity adsorbed to phenyl-Sepharose gel. A 50% inhibition was reached in the presence of 49.31 nmol/l of purified 'papain' protein (○, ●). (d) By comparison, only 53% inhibition was observed with the crude papain extract (○, ●).

petitively the binding of the intact solubilized receptor to IF-Cbl using our phenyl-Sepharose radioisotope assay of the receptor activity. No adsorption of the 'papain' protein-IF-Cbl complex to the phenyl-Sepharose solid phase was observed (Figure 4). This showed that the purified protein was not hydrophobic, as previously reported by Kouvonon for 'papain α ' [30]. Recently, we have identified a soluble IF-Cbl receptor in urine. Its excretion was decreased in the case of Gräsbeck-Imerslund disease and increased in the case of tubular nephropathy [31]. It has a molecular mass estimated at 70 kDa in SDS/PAGE [32], it was concluded that this soluble receptor corresponded to a proteolytic cleavage of the extracellular domain of the receptor in the epithelium of kidney tubules [33,34]. By contrast with the present 'papain' protein, the IF uro-receptor was hydrophobic as the IF-Cbl receptor was adsorbed to phenyl-Sepharose [31].

The amino acid composition of our protein was significantly correlated to that of the canine ileal receptor, the highest correlation being observed with the papain proteolytic product of the canine receptor ($r = 0.83$; $P < 0.001$; Table 2) [29]. However, the amino acid composition of the 'papain' protein was not similar to that of the papain product of the canine receptor. One reason may be that these proteins do not have the same animal origin. In addition, the molecular size of the canine protein was much higher than that of our 'papain' protein [29]. A much lower correlation was found with the amino acid composition of the rat receptor and with the digestive and blood Cbl-binding proteins [6,19,29,35,36]. The 'papain' protein was a glycoprotein (Table 3), as previously reported for the α -subunit of the pig intestine receptor [10,37]. We have previously found that the α -subunit of the IF receptor recognizes wheat-germ

agglutinin. In the present study, the 'papain' protein has reacted with SNA. The carbohydrate composition (Table 3) as well as lectin-blotting experiments indicate that the 'papain' protein possesses both O- and N-glycans. The molar ratio of the different monosaccharides and especially the high level of GlcNAc are in favour of the presence of external GlcNAc residues, as found in hybrid-type glycans.

In conclusion, we have purified a 'papain' protein which corresponds to a proteolytic product of the IF-Cbl receptor from pig intestine. This fragment was able to bind free Cbl as well as IF-Cbl complex and its amino acid composition resembled that of the 'papain'-cleavable domain of the canine ileal receptor. The present data provide the first description for a Cbl-binding activity of the IF-Cbl receptor. The role of such a binding activity in the internalization and cellular metabolism of Cbl in enterocytes remains to be elucidated.

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