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A SIMPLIFIED TECHNIQUE TO ISOLATE THE PORCINE AND HUMAN ILEAL INTRINSIC FACTOR RECEPTORS AND STUDIES ON THEIR SUBUNIT STRUCTURES

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SUMMARY

The porcine intestinal intrinsic factor receptor was isolated with affinity chromatography utilizing vitamin B12-intrinsic factor-Sepharose and pH adjustments. The purification was about 70 000-fold and in sodium dodecyl sulphate electrophoresis it resolved into two carbohydrate-containing 70 000 and 130 000 dalton bands (alpha and beta subunits) indicating purity. The human receptor was similarly purified and radioiodinated for further studies. It was also composed of two subunits (90 000 and 140 000 dalton). The alpha subunits bound to anti-intrinsic factor antisera.

In mammalian ileal mucosa there is a receptor which binds vitamin B_{12}-IF at neutral pH in the presence of Ca^{++}. It has been solubilized using Triton X-100 (1,2) and the porcine receptor was isolated with combined immuno and affinity chromatography (3) in our laboratory. This article describes a simplified technique to isolate the receptor subsequently applied to both porcine and human material and permitting studies on the subunit structures of the isolated receptors.

METHODS AND PURIFICATION

Purification (at 4^{o} unless stated otherwise). For each isolation four ilea were excised immediately after the pigs had been killed. Receptor extract was prepared essentially as described (2) by homogenization and extraction for 3 days

Abbreviations: B12, vitamin B12; IF, intrinsic factor; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; V_o, totally excluded volume in gel filtration.

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with 50 mmol/l Tris-buffer of pH 7.4 containing 0.05 % Triton X-100 and 0.4 mmol/l PMSF (extraction buffer) followed by ultracentrifugation. The pH was adjusted to 4.5 with 0.1 N HCl. The precipitate was removed by centrifuging at 10 000 x g for 30 min. 2 ml of wet B12-Sepharose was suspended in the supernatant, mixed overnight and removed using a porous glass filter. The endogenous B12-binders were adsorbed. The pH of the filtrate, containing unadsorbed receptor, was adjusted to 7.4 and 3.5 ml of wet B12-Sepharose gel to which isolated (4) porcine IF had been bound was suspended in it. 10 mmol/l $CaCl_2$ was added followed by incubation for 2 h at room temperature. The gel was collected on a porous glass filter, transferred to a column and washed with receptor buffer (extraction buffer + $CaCl_2$, flow rate 15 ml/h) until the absorbance at 280 nm was zero. The adsorbed receptor was eluted by washing the gel three times with 5 ml receptor buffer of pH 5.0 and containing 0.15 % Triton X-100.

The eluate from the affinity chromatography was finally ultrafiltered to 3 ml, dialyzed against pH 5.0 buffer containing 0.05 % Triton X-100 and filtered through Sephadex G-200 in the same buffer. The fractions emerging in the V_o were dialyzed and ultrafiltered to 3 ml and dialyzed against receptor buffer. The fractions in the included volume (micelles + protein) were discarded.

Polyacrylamide SDS gel electrophoresis. Dialyzed samples were reduced with 2-mercaptoethanol for 2 min at 100° in the presence of SDS followed by alkylation with iodoacetamide (5). Samples of 10 µl were applied to a 5 % gel slab and electrophoresis was performed for 3-4 h with 0.05 mol/l imidazole running buffer (pH = 7.0). Protein was stained with Coomassie Brilliant Blue and carbohydrate with Schiff's reagent (6). Gels containing [^{125}I] proteins (vide infra) were divided into 1.5 mm wide pieces with a slicer and counted for radioactivity. Molecular weights of proteins were determined (7) using ovalbumin and human serum albumin (monomer and dimer) as reference proteins.

The human receptor was isolated in a similar way three times but starting from single 5-8 cm long pieces of ileum, obtained during operations for colon cancer.

RESULTS

The progress of the purification of the pig receptor is presented in Table I. After the last Sephadex G-200 gel filtration at pH 5.0 performed to remove Triton X-100 micelles, B12-binders and B12 dissociated from the column the material had been purified about 70 000 times. The amount of final product was too small to determine the degree of purification of the human receptor.

TABLE I

PURIFICATION OF PIG ILEAL INTRINSIC FACTOR RECEPTOR BY AFFINITY CHROMATOGRAPHY

purification step	A volume ml	B tot.protein mg[x]	C tot.receptor act. ng[xxx]	D sp.act. C/B	E purif.index	F recovery %
homogenate of ileal mucosa	5000	39800[xx]	191.7	0.0048	1	
receptor extract	220	224	186.4	0.083	17	97.2
supernatant after pH-precipit.	225	157.5	178.9	1.1	229	93.3
eluate from affinity column	5	0.58	116.2	200.3	41730	60.6
V_o-fractions of gel filtration	3	0.32	108.3	338.5	70520	56.5

x) Modified Lowry (13) using HSA as standard.
xx) Dry weight after desalting.
xxx) Capacity to bind vitamin B12-IF expressed as ng B12.

Receptor activity was demonstrated by determining by gel filtration binding of $[^{57}Co]B_{12}$-IF (8). Abnormal IF did not bind to the receptors. In Sepharose 4B runs the typical porcine C I and C II B_{12}-IF-receptor complexes (8) were seen. The largest complex C I dominated.

In SDS electrophoresis the purest porcine material resolved into two bands (70 000 and 130 000 dalton) apparently representing subunits (Fig. 1), named alpha and beta. Both bands contained carbohydrate. Sometimes we also found a third 30 000 dalton band.

The isolated human receptor was radioiodinated (9) followed by purification with Sephadex G-25 and G-200 gel filtration. Sephadex G-200 filtration indicated that iodination caused an about 90 000 dalton component to dissociate from the receptor emerging in the V_o (Fig. 2).

SDS electrophoresis of the iodinated human receptor and the dissociated component indicated that the human receptor

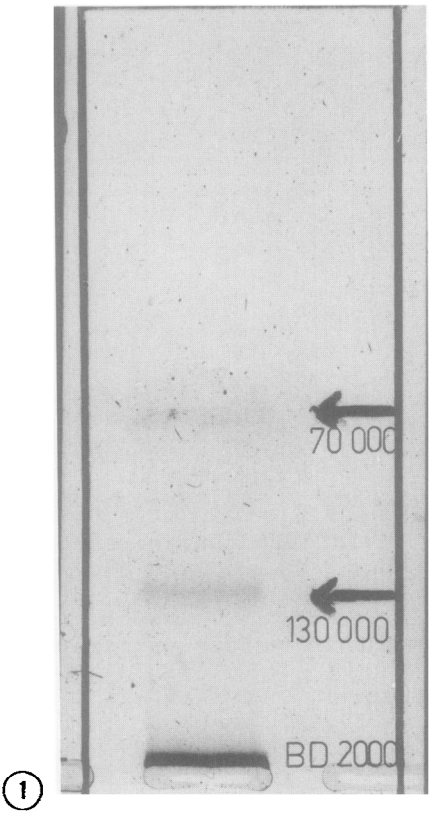

Fig. 1. SDS-electrophoresis of purified pig receptor. The sample resolved into two 70 000 and 130 000 dalton bands (alpha and beta subunits) indicating purity. BD 2000 = Blue Dextran 2000

Fig. 2. Sephadex G-200 gel filtration of ^{125}I-labelled human receptor. Iodination caused dissociation of component b from the receptor (a) which emerged in the V_o with Blue Dextran 2000 (BD).

consists of two subunits (alpha and beta) and that the smaller-molecular species in gel filtration represented dissociated alpha subunit. Sometimes the alpha subunit dissociated further to a 30 000 dalton component (Fig. 3 A, B, C). Following iodination the porcine receptor dissociated in the same way as the human receptor. A 70 000 dalton component was dissociated.

Isoelectric focusing (10). The [^{125}I] labelled porcine receptor gave a single peak at pH 4.4. The human IF-receptor

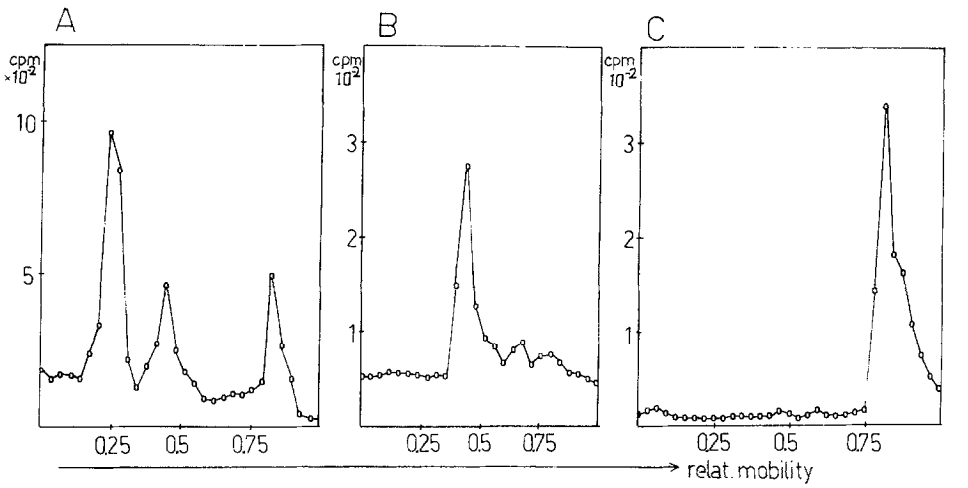

Fig. 3. SDS-electrophoresis of ^{125}I-labelled human receptor
A) Peaks a and b (in Fig. 2) together. The reduced and alkylated preparation was preserved for 2 days at -20°C before the run.
B) Peak b (in Fig. 2) was run immediately after preparation of the sample.
C) Same as B, but the sample was stored 2 days at -20°C before the run.

was focused at pH 4.0 but contained minute quantities of an alkaline component (pI 8.5). The component dissociated by iodination from the porcine and human receptors focused between pH 2.5 and 3.5.

Relation to IF. The components dissociated during iodination from the porcine and human receptors were incubated with two anti IF-sera containing binding-type antibodies, one from a patient with pernicious anemia, the other a goat antiserum against human B_{12}-IF. Both decreased the elution volumes of the dissociated components in Sephadex G-200.

DISCUSSION

The present isolation based on different pH-dependence of binding of components to B_{12}-Sepharose is suitable for

large-scale work and avoids use of antibodies and especially EDTA, difficult to remove from the receptor. Evidence for purity of the isolated porcine receptor includes only two bands in SDS electrophoresis, one peak in electrofocusing, production of typical B_{12}-IF complexes and previous (3,11) degree of purification. Considering the isolation technique and the analogous results of SDS electrophoresis the human receptor may be assumed to be very pure, too.

Both receptors appear to consist of two subunits, alpha and beta, which differ somewhat in size in the two species. The porcine subunits contain carbohydrate.

The experiments described in Fig. 3 and analogous studies on the porcine receptor suggest that the alpha subunit is the component dissociated by iodination. Following reduction with mercaptoethanol it dissociated further into a 30 000 dalton "sub-subunit". The dissociated component reacted with two different anti-IF immune sera. Our interpretation is that the receptor contains a subunit structurally related to IF, similarly as the transcobalamin (II) receptor is related to transcobalamin (12), and that IF may have developed as the result of a duplication of a membrane protein gene. However, other explanations are possible: It represents IF dissociated from the affinity medium or IF being absorbed into the mucosa. Evidence against its IF nature is: It did not bind added B_{12}, the last purification steps took place at pH 5 which dissociates IF from the receptor, and its pI was lower than that of IF. However, it could be chemically altered IF.

It is slightly uncertain whether the component dissociated by iodination is truly identical with the alpha subunit seen in SDS electrophoresis. Strong evidence for their identity is

that in the latter procedure they had the same mobility and both dissociated into a 30 000-dalton component (Fig. 3).

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