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THE HYDROPHOBIC NATURE OF THE PIG INTRINSIC FACTOR RECEPTOR IN THE INTESTINE

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The pig intestinal intrinsic factor receptor has been isolated and dissociated into its α and β subunits. The β subunit was found to be more hydrophobic than the α subunit. In a detergent solution only the α subunit was accessible to digestion with papain. The whole isolated receptor was introduced into artificial single bilayer liposomes where is apparently was randomly oriented. Liposomes containing the receptor were digested with papain and the polypeptide segments that stayed in the lipid fraction were extracted and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Four species were found with M_r values of 23 000, 45 000, 70 000 and 86 000.

Introduction

The intestinal intinsic factor receptor binds the vitamin B-12-intrinsic factor complex. This is followed by transport of the vitamin into the enterocyte. The receptor has been solubilized [1–4] and isolated [5,6] from pig and human small intestine. The pig receptor is known to consist of two carbohydrate-containing subunits, α and β, which have molecular weights of 70 000 and 130 000, respectively [6].

The structure and orientation of the receptor have recently been studied [7]. However, we do not know how the functioning receptor is built up from its subunits. It is thought that the receptor is embedded in the lipid bilayer with a hydrophobic polypeptide segment localized in the β subunit [7], but it is not known whether the β subunit spans the membrane.

In this study the hydrophobic nature of the subunits was examined and the lipophilic segments of the receptor were characterized.

Materials and Methods

Preparation of the receptor extract. The solubilization was performed with detergent (Triton X-100) essentially according to the method of Marcoullis and Gräsbeck [1] with slight modifications as described in Ref. 7. Usually, four fresh pig ilea were used for one extraction.

Isolation of the pig intrinsic factor receptor. To prepare the highly purified receptor, affinity chromatography was used on Sepharose-bound vitamin B-12 gel (Sepharose-B_{12}) to which intrinsic factor was adsorbed [6]. The purified pig intrinsic factor for the affinity gel was isolated from pig gastric mucosa using affinity chromatography as described by Allen and Majerus [8]. The receptor was labelled with ^{125}I using the chloramine-T method [9]. The radioactively iodinated subunits were produced as described earlier [7].

Gel exclusion chromatography. Gel filtrations were performed in a 2.6 × 90 cm column filled with Sephadex G-200. The running buffer was 50 mM Tris-HCl (pH 7.4) containing 0.9% NaCl, 2 mM $CaCl_2$, 0.05% Triton X-100 and 0.01% Merthiolate (receptor buffer). The flow rate was 12.5 ml/h. Runs were performed at +4°C and fractions of 2.3–2.8 ml were collected. The column was calibrated with Blue Dextran 2 000 (M_r 2 000 000), human cobalophilin (Stokes' radius 4.57 nm, M_r 120 000), porcine intrinsic factor (Stokes' radius 3.57, M_r 69 000), human transcobalamin II (Stokes' radius 2.66 nm, M_r

Abbreviation: SDS, sodium dodecyl sulfate.

38 000) and free vitamin B-12 (M_r 1 355).

Production of single bilayer phosphatidylcholine vesicles. 1 g of L-α-phosphatidylcholine (type V-E from egg yolk, Sigma) in 1 ml of chloroform/methanol (9 : 1, v/v) was dried as described earlier [10]. It was dispersed in 5 ml of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl. 240 mg of sodium deoxycholate (Merck) were added to 1 ml of the phosphatidylcholine dispersion. 80 μg of the purified receptor in 3 ml of receptor buffer were added and the clear solution was run through a Sephadex G-50 column (2.5 × 30 cm) in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl. Liposomes were collected from the V_0 fractions and subsequently examined. The success of the preparation procedure was checked by electron microscopy after negative staining with 0.5% uranyl acetate.

Isolation of the hydrophobic regions of the receptor. Phosphatidylcholine vesicles containing the receptor were adsorbed onto a column (0.8 × 6 cm) filled with washed Octyl-Sepharose CL-4B, through which buffer was run at a flow rate of 5 ml/h. The column was washed with 50 ml of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl and the gel was extruded and suspended in 20 ml of buffer containing 100 μl papain solution (from *Papaya latex*, papainase, EC 3.4.22.2; 616 U/ml, Sigma). The gel was incubated at +37°C for 1.5 h on a mechanical mixer and packed again in a chromatography column. The gel was washed with 100 ml of buffer.

Liposomes were eluted from the column with 94% ethanol. Proteins were separated from the lipid fraction by precipitating them with a biphasic mixture prepared from chloroform, methanol and water (120 : 60 : 45, v/v) according to the method of Brunner et al. [10]. The precipitate forming in the interphase was collected with a Pasteur pipette and washed twice with the same biphasic system. The rest of the chloroform in the last precipitate evaporated in 1% ammonium bicarbonate solution with an air flow at 45°C. The solution was centrifuged and the pellet was solubilized with SDS and prepared for electrophoresis as the other samples. The control experiment was performed in the same manner except that only receptor buffer was used instead of the purified receptor.

SDS-polyacrylamide gel electrophoresis. Samples were reduced with 0.01% dithiothreitol for 2 min at +100°C in the presence of 0.1% SDS. Glycerol and bromphenol blue were added and the samples were introduced into a 10 or 5% polyacrylamide gel slab. Electrophoresis was performed at a voltage of 300 V for 2.5 h with 0.05 M imidazole running buffer (pH 7.0) containing 0.1% SDS. Proteins were stained with an ultrasensitive silver stain [11]. M_r values were determined [12] using as standards hemoglobin (M_r (15 000), ovalbumin (M_r 43 000) and human serum albumin (M_r 65 000).

Hydrophobic interaction chromatography. Samples were introduced into a column (0.8 × 13 cm) filled with Phenyl-Sepharose CL-4B in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl. The flow rate was 20 ml/h. The column was washed with distilled water. Proteins were eluted by lowering the polarity of the buffer by adding ethylene glycol, first up to 50% and than to 100%.

Papain treatment of the ^{125}I-labelled soluble subunits. α and β subunits were prepared by iodination of the receptor as described earlier [7]. The radioactively iodinated subunits were separated from each other by gel filtration through a Sephadex G-200 column. The peaks obtained were pooled and concentrated by ultrafiltration using Visking dialysis tubing. The pooled peaks were separately incubated with 12 U of papain for 1 h at 37°C. After treatment the subunits were run separately through a Sephadex G-200 column.

Results

The purity and activity of the isolated receptor were checked using SDS-polyacrylamide gel electrophoresis and gel filtration together with the intrisic factor-[^{57}Co]vitamin B-12 complex in the presence of Ca^{2+}. In SDS-polyacrylamide electrophoresis the purified material resolved into two bands as before [6]. The addition of the isolated receptor to the intrinsic factor-[^{57}Co]vitamin B-12 complex caused a shift of the elution volume of the latter to the V_0 in the Sephadex G-200 run, indicating that the receptor was able to bind the substrate.

Papain treatment of the subunits

To produce the labelled α and β subunits the radioactive iodination of the purified receptor was performed in a detergent solution for the α subunit and

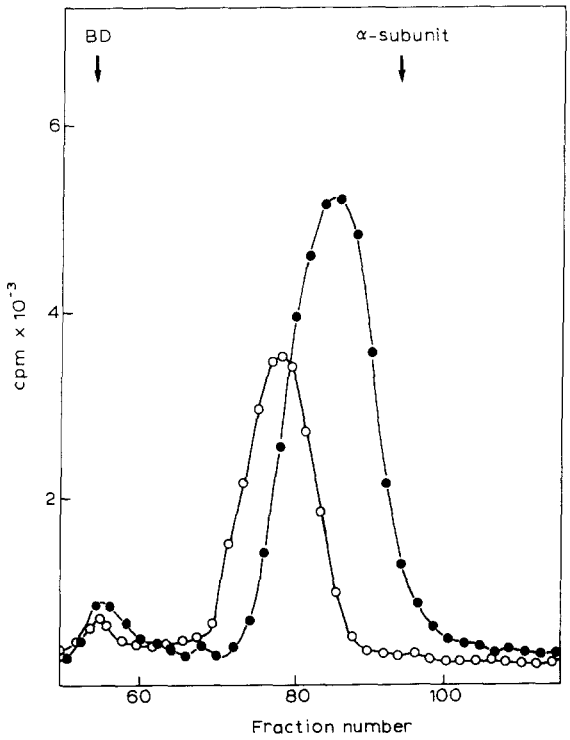

Fig. 1. Sephadex G-200 gel filtration of the radioactively iodinated soluble α and β subunits subjected to papain treatment. The elution volume of the β subunit is unchanged (○——○). The treated α subunit (●——●) emerges earlier than the native one. The arrows indicate the elution volumes of the native α subunit; BD, Blue Dextran 2 000.

Fig. 2. SDS-polyacrylamide gel electrophoresis of the fragments obtained in the papain digestion of the receptor associated with liposomes; 10% polyacrylamide gel slab was used. The run was followed by staining with an ultrasensitive silver stain. (A) Washing solution of the gel after the papain digestion; (B) *Papaya latex* papainase; (C) the fragments of the purified receptor after digestion in the liposome; (D) human serum albumin; (E) ovalbumin; (F) hemoglobin.

as immobilized in the Sepharose-B_{12}-intrinsic factor gel for the β subunit [7]. The separated α and β subunits in the receptor buffer were treated with papain. In the following gel filtration the elution volume of the β subunit was unchanged but the elution volume of the α subunit decreased and corresponded to an M_r of approx. 85 000. Small peaks were also seen in the V_0 in both cases (Fig. 1).

Papain treatment of the receptor associated with liposomes

The collection of the precipitate extracted from the liposomes after papain digestion had to be performed very carefully because of the scarcity of the material. The precipitate appeared as a thin veil in the interphase and had a great tendency to aggregate on the tube walls and around the Pasteur pipette when the organic phase was removed by the air flow through the ammonium bicarbonate solution. The final sediment was solubilized, reduced and analyzed using SDS-polyacrylamide electrophoresis. The sample resolved into four bands corresponding to the M_r values of 23 000, 45 000, 70 000 and 86 000 (Fig. 2). The proportions of the fragments varied from digestion to digestion. However, the two smallest fragments always dominated. The calculated M_r values for the smallest fragments are based on electrophoretic runs in 10% gel and for the others in 5% gel.

The reported M_r for papain is 20 900 [3], very

close to that of the smallest fragment obtained in the sample (the papain used was found to resolve into several bands as seen in Fig. 2). Therefore, the control digestion experiment was performed without the receptor. In SDS-polyacrylamide electrophoresis no band or only a very weak one was seen in the region corresponding to the M_r of papain. On the other hand, the washing solution collected from the column after papain digestion contained in all cases papain, indicating that papain did not bind to the gel.

Hydrophobic interaction chromatography

^{125}I-labelled samples (in 100 μl of receptor buffer) of the α and β subunits pooled after the gel filtration were run separately through a Phenyl-Sepharose CL-4B column in 0.01 Tris-HCl-buffer (pH 7.4) containing 0.9%NaCl. As seen in Fig. 3 both proteins bind to the gel and washing with distillled water did not cause elution of proteins. Decreasing the polarity of the solvent with ethylene glycol (added up to a final concentration of 50%) caused the elution of the α subunit. Elution with pure ethylene glycol also dissociated the β subunit from the gel. The preparation of the α subunit contained a small amount of the β subunit and vice versa, as seen in Fig. 3.

Radioactive iodination of the receptor associated with liposomes

The phophatidylcholine vesicles containing the purified receptor were collected in the V_0 fractions of the Sephadex G-50 gel filtration and concentrated by

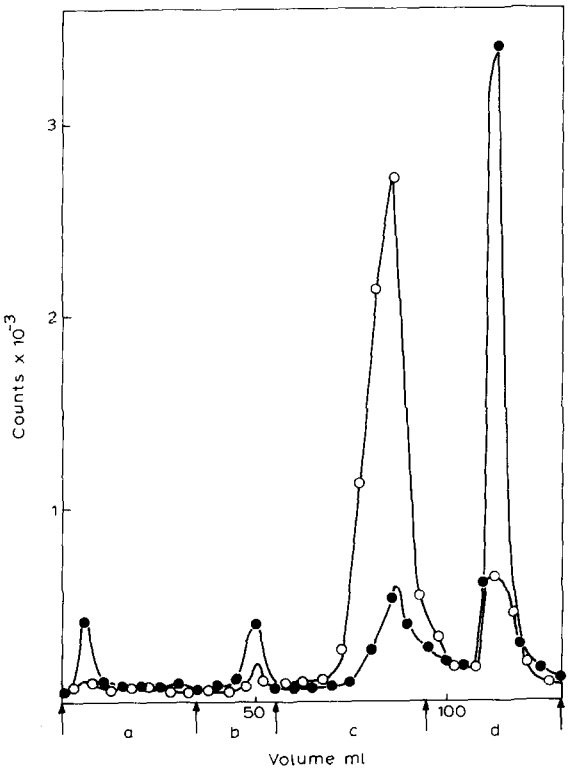

Fig. 3. Hydrophobic interaction chromatography of the iodinated α and β subunits. (a) The samples were introduced into the column in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl. (b) Elution by H_2O. (c) Elution by buffer containing 50% ethylene glycol; the α subunit dissociates (o———o). (d) Elution by 100% ethylene glycol; the β subunit dissociates (•———•).

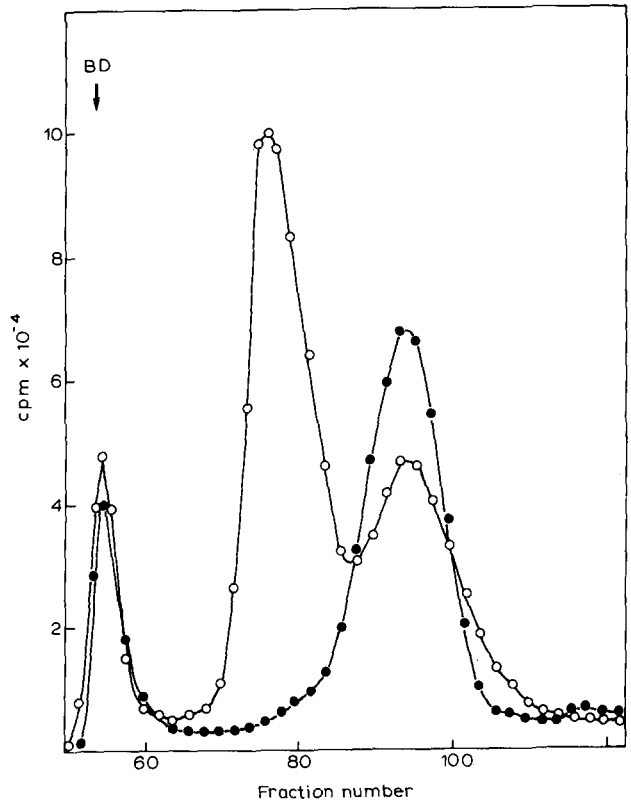

Fig. 4. Sephadex G-200 gel filtration of the purified receptor radioactively iodinated on a phosphatidylcholine vesicle. The iodination caused the dissociation of the proteins from the liposomes (o———o). For comparison purposes the elution profile is presented of the purified receptor radioactively iodinated without liposomes. The α subunit is accessible to iodination as described earlier (•———•) [6].

ultrafiltration through Visking dialysis tubing. 100 μl of the concentrated liposomes were radioactively iodinated using the chloramine-T method. The free iodine was removed by rapid gel filtration and the sample consisting of the V_0 fractions was dissolved in the receptor buffer.

The iodination caused the dissociation of two components as indicated by the results of the following gel filtration run shown in Fig. 4. The dissociated components emerged in the same elution volumes as did the α and β subunits of the receptor.

The isolated β subunit was associated alone with liposomes. The papain digestion was performed in the same manner as in the case of the whole receptor molecule. In the following SDS-polyacrylamide electrophoresis three bands were obtained. The calculated M_r values for them are approx. 15 000 (a broad band), 65 000 and 70 000.

Discussion

A previous study of our group indicates that the β subunit of the pig intrinsic factor receptor carries the bulk of its hydrophobic segments because the β subunit aggregates without detergent, but the α subunit is water soluble [7]. In this study this concept was verified by another technique. Both subunits adsorbed to hydrophobic Phenyl-Sepharose CL-4B gel in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl. This shows that hydrophobic segments are present in the β as well as in the α subunit. However, the α subunit is less hydrophobic because it dissociates from the gel with 50% ethylene glycol whereas the β subunit remains adsorbed.

In the experiment where the radioactively iodinated β subunit in the detergent solution was treated with papain, no change was detected in the size of the molecule. This could be due to the hydrophobicity of the β subunit. The detergents are known to bind to hydrophobic proteins [14]. Especially in the case of a very hydrophobic protein, the protein could be covered by detergent molecules to such an extent that the segments accessible to proteolytic digestion are hidden. Another possibility is that a small hydrophobic segment containing the ^{125}I label is digested off but remains associated with a Triton X-100 micelle, which is known to be eluted from Sephadex G-200 in approximately the same volume as the whole β subunit [1].

Papain treatment of the α subunit causes an apparent increase in M_r to 85 000. I believe there is good evidence that the new molecular species obtained is a dimer of the outermost parts of the α subunit. The treatment of intact mucosa with papain results in liberation of an intrinsic factor-binding component, which we call papain-α and which has an M_r of 45 000. In gel filtration [15] papain α behaves as a dimer.

The purified receptor associated with phosphatidylcholine vesicles was labelled with ^{125}I. In subsequent gel filtration the sample resolved into three peaks. The peaks in the included volume correspond to the α and β subunits. Radioactive iodination of the purified receptor in detergent solution always caused the α subunit to become labelled and to dissociate [6,7,16]. We have been able to produce the ^{125}I-labelled β subunit in two ways: by labelling either the receptor-intrinsic factor-vitamin B-12 complex or the purified receptor adsorbed to Sepharose B_{12}-intrinsic factor gel in the absence of detergent. In the former case the reason for the β subunit becoming labelled is most likely a conformational change occurring upon the binding of the intrinsic factor-vitamin B-12 complex to the receptor so that the residues of the β subunit accessible to iodination are exposed. In the latter case the β subunit faces out from the gel and is therefore accessible to iodination [7]. In the present case both explanations are possible, but the latter seems to be more likely. It is very probable that when the receptor is incorporated into liposomes the molecules are randomly orientated and some receptor molecules are located so that the β subunit faces outward and is accessible to iodination.

The method developed for papain digestion of immobilized liposomes appeared to be both practical and simple. However, there is one critical point to which attention must be paid. The digestion must be performed under circumstances where papain (or another proteolytic enzyme) does not bind to the gel by hydrophobic interaction. This can be arranged using a suitable buffer system and/or such small amounts of the gel that its capacity to bind substances by hydrophobic interaction is totally blocked by the liposomes.

In the experiments presented papain was not found to bind to the gel. According to the results of

SDS-polyacrylamide electrophoresis the washing solution after the digestion contained papain. The control experiment performed without receptor indicated that papain did not run through the system to the solution analyzed by SDS-polyacrylamide electrophoresis.

Papain digestion of the purified receptor incorporated in the phosphatidylcholine vesicles produced four molecular species having the M_r values 23 000, 45 000, 70 000 and 86 000. Due to the extraction system only hydrophobic proteins should exist in the sample introduced into the electrophoresis. However, it is possible for a water-soluble protein to coextract if it is noncovalently bound to a hydrophobic molecule. The substances would dissociate from each other in SDS-polyacrylamide electrophoresis. In fact, it is very likely that in the intact receptor molecule the two subunits are associated in this way. It is thus theoretically possible for the α subunit to be present in the SDS-polyacrylamide electrophoretic run in spite of its water solubility.

Because in the liposomes the orientation of the receptor molecules evidently is random, papain is able to digest both ends of the molecule. The data presented above provide several alternatives to build up the jigsaw puzzle in which the pieces are the fragments of the receptor. In the following scheme the β subunit is suggested to span the lipid bilayer several times in a snake-like manner. Such a S-shaped folding has been shown to exist inter alia in the case of the anion-exchange protein from the erythrocyte membrane [17].

In the schematic representation (Fig. 5) the β subunit is suggested to be orientated symmetrically in the lipid bilayer. The calculated M_r for the molecule constructed from the sum of the M_r values of the digested segments is over 100 000. The M_r values for the segments which are embedded in the lipid bilayer are all estimated to be about 10 000. The M_r for the whole β subunit is 130 000 [6]. However, these calculated values are not very relevant because in SDS-polyacrylamide electrophoresis the membrane proteins may behave in an anomalous fashion depending on the binding of different amounts of detergent [18].

To detect if the hydrophobic molecular species all originate from the β subunit, this subunit was introduced alone into liposomes followed by papain digestion and extraction as described above. Subsequent SDS-polyacrylamide electrophoresis produced three bands corresponding to the M_r values: 70 000, 65 000 and approx. 15 000 (a very broad band) as indicated in Results. Thus, digestion of liposome-bound whole receptor and β subunit alone produced very different fragments. It is obvious that the α subunit and the whole tertiary structure of the receptor influence the orientation of the β subunit in the liposomes. The conformation and orientation of the single β subunit are thus different from that of the β subunit contained in the complete receptor structure and cannot be used to describe the spatial structure of this subunit in the intact receptor.

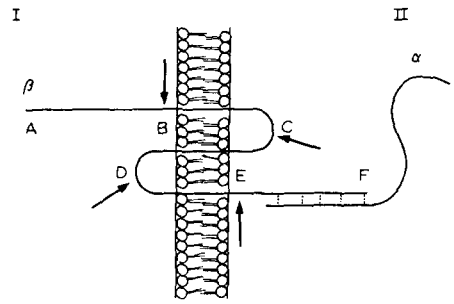

Fig. 5. A schematic view of the following of the polypeptide chains of the intrinsic factor receptor. The arrows indicate the sites broken by papain. When digested on side I, the chains B–D (M_r 23 000), D–F (M_r 45 000) and the α subunit (M_r 70 000) stay in the lipid bilayer. When digested on side II, the chains A–C (M_r 45 000) and C–E (M_r 23 000) remain intact.

To reveal the detailed structure of the β subunit in the lipid bilayer it is planned to use hydrophobic and hydrophilic labelling of the polypeptide chain before enzymatic digestion.

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