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Isolation and some properties of a 34-kDa-membrane protein that may be responsible for ribosome binding in rat liver rough microsomes

Tohru Ichimura[1], Tomoya Ohsumi[1], Yukiko Shindo[2], Tamotsu Ohwada[2], Harutaka Yagame[2], Yasunori Momose[3], Saburo Omata[2] and Hiroshi Sugano[2]

[1]*Department of Biosystem Science, Graduate School of Science and Technology and* [2]*Department of Biochemistry, Faculty of Science, Niigata University, 2-Igarashi, Niigata 950-21, Japan* and [3]*Second Research Laboratories, Kissei Pharmaceutical Co., Nagano 399-83, Japan*

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We have isolated, by hydroxyapatite chromatography with a non ionic detergent and a high salt concentration, a non-glycosylated, membrane protein with a relative molecular weight of 34 kDa that had previously been found to be a major constituent of the membrane protein fraction showing ribosome-binding activity derived from rat liver rough microsomes (RM). The isolated 34 kDa protein (p34), when incorporated into a liposome model membrane, exhibited significant binding activity toward ribosomes, its binding properties being similar to those observed with intact RM. Immunochemical analyses using antibodies directed against p34 suggested that it is a membrane-embedded RM surface protein, which is specifically localized in ribosome-attached organelles and widely distributed among mammalian tissues. These results would constitute evidence that p34 is a likely candidate for an RM ribosome-binding protein.

Ribosome-binding protein; Ribosome; Rough microsome; Purification; Liposome

1. INTRODUCTION

Rough and smooth microsomes (RM and SM) form a continuous membrane system, but can be distinguished morphologically due to membrane-bound ribosomes, and separated experimentally on the basis of their different densities. Previous studies have indicated that the ribosome–membrane interaction is affected by mild treatment of RM with a protease, and is markedly decreased in the presence of increasing amounts of monovalent ions, suggesting that RM contain a proteinous factor which can bind to ribosomes through ionic bonds [1–4]. Several RM proteins have each been proposed to be such a ribosome-binding factor [3,5–7]. However, little is known about the direct interaction between ribosomes and the proposed proteins. A 180 kDa protein, recently found in canine pancreatic RM [8], seems to be capable of interacting with ribosomes. Other studies have revealed that ribosome-binding activity quantitatively solubilized from RM is not cofractionated with the 180 kDa protein [9,10].

Recently, we reported that the ribosome-binding activity of stripped (ribosome-depleted) RM was predominantly found in a non-glycosylated, membrane protein fraction obtained on affinity chromatography on a concanavalin A-Sepharose column [11]. Subsequently, we separated the non-glycoproteins into flow-through and bound fractions on a chelating-Sepharose column, and found that the binding activity is concentrated in the flow-through fraction [12]. Although this protein fraction (termed the chelate FT fraction) still consisted of several polypeptides with various molecular weights, we found that a 34 kDa protein (termed p34) was a major component (about 20%; Coomassie blue staining). p34 was found to be highly sensitive to proteolysis and was specifically cross-linked to the 60 S ribosomal subunit with the use of the bifunctional reagent, sulfosuccinimidyl 2-(*m*-azido-*o*-nitrobenzamido)-ethyl-1,3′-dithiopropionate (SAND) [12].

In this paper, we describe the isolation of p34 from rat liver RM and show that it exhibits ribosome-binding activity after reconstitution into liposomes. We also suggest that the protein is a membrane-embedded RM surface protein and is widely distributed among mammalian tissues.

2. EXPERIMENTAL

2.1. *Solubilization and fractionation of microsomal membrane proteins*

Salt-washed RM (350 mg protein, [12]) were suspended in TKM buffer (50 mM Tris/HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.6) and then adjusted to 4% Triton X-100. After incubation for 30 min at 4°C, the suspension was layered over a step gradient consisting of 2 ml of 1.6 M sucrose in 0.1% Triton X-100-containing TKM buffer and 1.5 ml of 0.5 M sucrose in the same buffer, followed by centrifugation for 6 h at 136 000 × *g* (Hitachi RP50 rotor). The pellet was resuspended in

Abbreviations: RM, rough microsomes; SM, smooth microsomes; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Correspondence address: T. Ichimura, Department of Biosystem Science, Graduate School of Science and Technology, Niigata University, 2-Igarashi, Niigata 950-21, Japan. Fax: (81) (25) 262 1175.

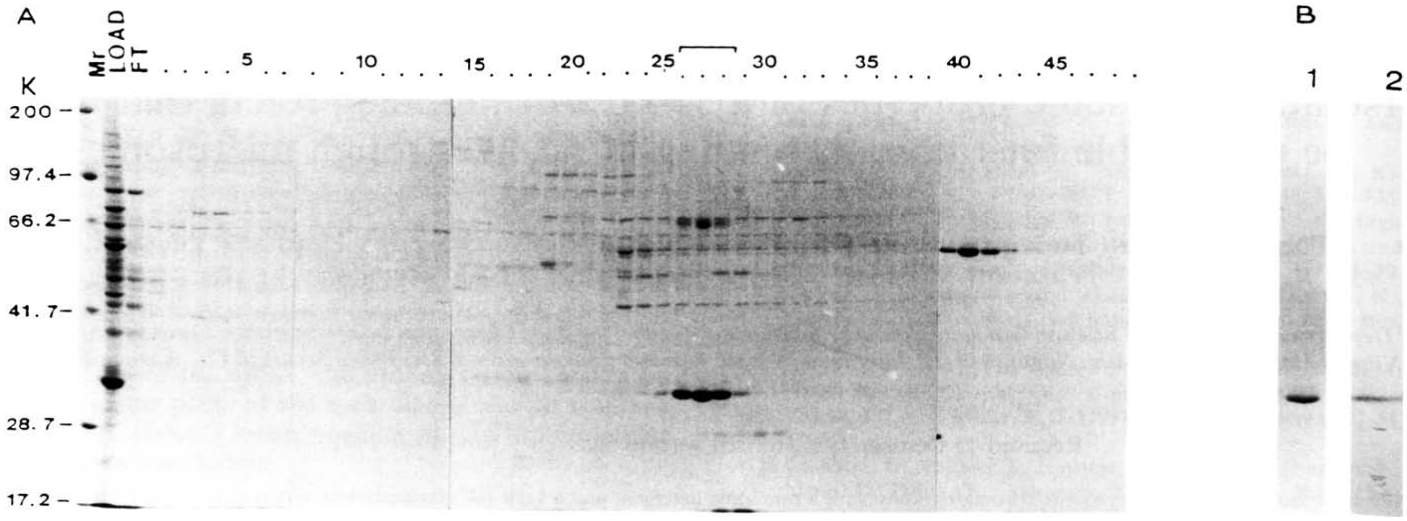

Fig. 1. Isolation of p34 from rat liver rough microsomes. (A) Triton X-100/salt-extracted RM membrane proteins (lane LOAD) were prepared as described in Experimental. The membrane proteins (approximately 5 mg protein) were applied to a TAPS-020810HS ceramic hydroxyapatite column (0.75 × 10 cm) that had been pre-equilibrated with buffer A (20 mM triethanolamine, 500 mM CH_3COOK, 1% Triton X-100 (w/v), 10 mM sodium phosphate, pH 7.6). After collecting the flow-through fraction (lane FT), the column was washed for 10 min with buffer A (lanes 1–10) and then the proteins were eluted with a 60-min linear gradient of phosphate (up to 300 mM) in buffer A. The flow-rate for the entire period was 1 ml/min and 1 ml fractions were collected from the beginning of the buffer A wash. Fractions (indicated along the top) were then analyzed by SDS-PAGE (Coomassie blue staining). Lanes LOAD and FT received 30 μg and 3 μg protein, respectively, and lanes 1–49 received 50 μl of the respective fractions. No protein band was detectable with over 200 mM phosphate (lane 50 onwards). (B) The 34 kDa protein fraction (A, lanes 26–28) was re-chromatographed under the conditions given above and the resulting 34 kDa-fraction was concentrated on a small hydroxyapatite column (0.4 × 3 cm). Fractions were then analyzed by SDS-PAGE (Coomassie blue staining; lane 1, 2 μg protein) and immunoblotting (lane 2, 0.2 μg protein). The molecular mass markers (lane M_r) were myosin (200 K), phosphorylase *b* (97.4 K), bovine serum albumin (66.2 K), actin (41.7 K), carbonic anhydrase (28.7 K) and myoglobin (17.2 K).

500 mM CH_3COOK, 1.5% Triton X-100 (w/v) in 20 mM triethanolamine (pH 7.6), followed by centrifugation for 6 h at 100 000 × *g* (Hitachi RP40T2 rotor). The supernatant (approximately 6 mg protein) thus obtained was then subjected to hydroxyapatite chromatography (see text).

2.2. *Preparation of liposomes and the ribosome-binding assay*

To 1 mg of phosphatidylcholine dissolved in chloroform/methanol (2:1, v/v) was added a suitable amount of [^{14}C]phosphatidylcholine (Amersham), and then the mixture was dried to a thin film in a glass tube. The dried phosphatidylcholine was dissolved in 0.25 ml of 20 mM triethanolamine, 500 mM CH_3COOK, 1% Triton X-100 (pH 7.6) containing ~0.2 mg of p34 and 3 mM dithiothreitol. Liposomes were prepared according to the Bio-beads SM-2 method of Wolosin [13] and then dialyzed overnight against a large amount of 50 mM Tris/HCl, 25 mM KCl (pH 7.6). The binding mixture comprised liposomes (7 μg of p34) and 2.3 μg of RNA of ^{3}H-labeled ribosomes [12] in 60 μl of TKM buffer. After incubation at 4°C for 30 min, the mixture was analyzed on a sucrose gradient (a linear 0.3–1.5 M sucrose gradient (3.4 ml) on top of 0.5 ml of 1.7 M sucrose and 0.5 ml of 2.4 M sucrose) as described previously [11,12].

2.3. *Preparation of antibodies*

Approximately 0.5 mg of the chelate FT proteins [12] was subjected to SDS-polyacrylamide (10%) gel electrophoresis (SDS-PAGE). After electrophoresis, the gel band (~0.5 ml) corresponding to p34 (0.1 mg protein) was cut out, homogenized in a small mortar, and then emulsified with phosphate-buffered saline (pH 7.2) and Freund's adjuvant (1:1:2, v/v). The mixture was injected subcutaneously into multiple sites along the back of a rabbit. Three injections were given at 2-week intervals. Serum samples were taken 1 week later, after the second and third injections, and were pooled.

2.4. *Analyses*

SDS-PAGE was performed according to Laemmli [14], except that 100 mM dithiothreitol was substituted for 2-mercaptoethanol. Immunoblot analysis was carried out as described [12]. Protein and RNA concentrations were determined as described previously [12].

3. RESULTS

In previous studies, we used 2 affinity columns for the fractionation of RM membrane proteins; however, the procedure involved long exposure of the proteins to a detergent and the resulting preparation (chelate FT fraction) still contained several proteins with various molecular weights, although p34 was a predominant component [12]. Therefore, in this work we devised conditions for more efficient isolation of p34.

The Triton X-100/high salt-extracted membrane proteins (Fig. 1A, lane LOAD), prepared using a 2-step procedure that was a modification of that of [12] (see section 2) were used as a starting material. The membrane proteins were directly loaded onto the hydroxyapatite column and then chromatographed under the conditions given in the legend to Fig. 1. Fig. 1A shows the polypeptide profile obtained with this chromatography procedure, as analyzed by SDS-PAGE. As shown in Fig. 1A, this procedure allowed effective separation of the 34 kDa protein from several RM membrane proteins (lanes 26–28).

The fractions containing the 34 kDa protein was subjected to re-chromatography. The 34 kDa preparation was then concentrated by stepwise elution on a small

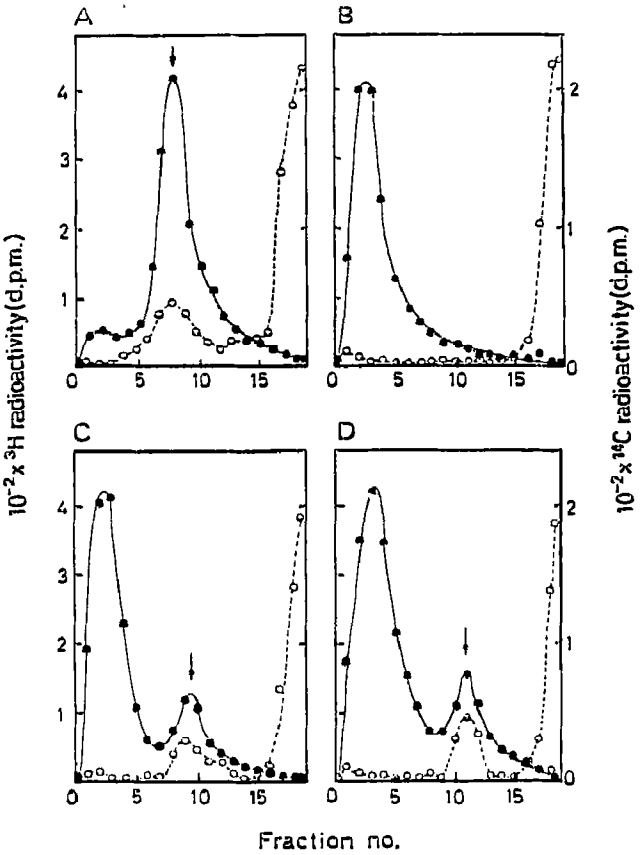

Fig. 2. Binding of liposomes reconstituted with p34 to ribosomes. (A) ^{14}C-labeled liposomes containing p34 were prepared as described in Experimental. The assay mixture, comprising 7 μg of protein of the reconstituted liposomes and 2.3 μg of RNA of ^{3}H-labeled ribosomes in 60 μl of TKM buffer (50 mM Tris/HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.6), was centrifuged through a sucrose gradient (100 000 × g, 10 h), and then the resulting distribution of liposomes and ribosomes was determined. The direction of sedimentation was from right to left. (B) Liposomes composed of lipids alone were subjected to the ribosome-binding assay as in (A). (C) Liposomes (7 μg protein) were incubated at 0°C for 1 h in the presence of trypsin (5 μg/ml), followed by the addition of 30 U of Trasylol/μg trypsin as described [12]. The ribosome-binding assay was performed as in (A). (D) The ribosome-binding assay was performed as in (A) except that the binding mixture and sucrose gradient contained 200 mM KCl. Arrows indicate the positions of the complex between p34-containing liposomes and ribosomes. ●, ^{3}H-radioactivity; ○, ^{14}C-radioactivity.

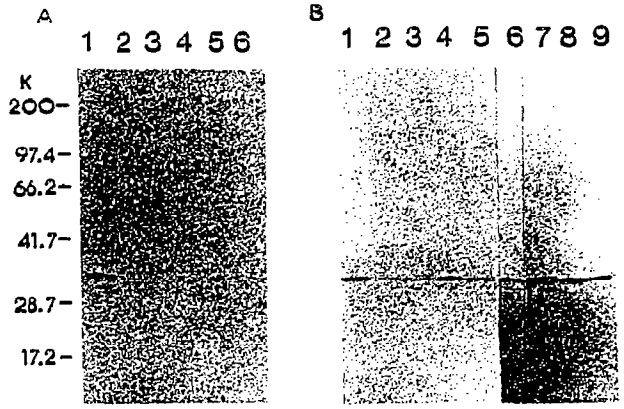

Fig. 3. Distribution of p34. (A) The cellular extracts (20 μg each) were separated by SDS-PAGE, transferred to nitrocellulose, and then probed with the p34 antiserum (1:500 dilution). (Lane 1) RM; (lane 2) SM; (lane 3) cytosol; (lane 4) mitochondria; (lane 5) nuclear envelope; (lane 6) nucleoplasm. The extracts were prepared according to the procedures of [18,19]. (B) The RM extracts (10–50 μg) were probed with the p34 antiserum as in (A). (Lanes 1–4) rat tissues (1, liver; 2, kidney; 3, brain; 4, testis); (lane 5) dog pancreas; (lane 6) hamster liver; (lane 7) mouse liver; (lane 8) pig liver; (lane 9) bovine liver. The molecular mass markers were as in Fig. 1.

column of hydroxyapatite. The preparation thus obtained gave a single protein band (M_r=34 000) on SDS-PAGE (Fig. 1B, lane 1) and antibodies directed against the chelate FT p34 showed immunochemical cross-reaction with the corresponding protein band (Fig. 1B, lane 2). Approximately 250 μg of protein could be obtained by this procedure from 350 mg RM proteins within 2 days. Since p34 represents 0.4% of the total microsomal protein [12], this implies that approximately 20% of the p34 molecules were recovered.

The purified p34 was reconstituted into liposomes and then its ribosome-binding activity was examined (Fig. 2). This analysis revealed that liposomes containing the p34 protein bound to ribosomes, demonstrating the formation of a complex between the p34-containing liposomes and ribosomes (Fig. 2A, arrow). In contrast, no significant binding of ribosomes was observed with liposomes composed of lipids alone (Fig. 2B). The ability of p34-containing liposomes to bind ribosomes decreased by about 80% on mild treatment with trypsin (5 μg/ml), 0°C, 60 min) or with increasing salt (200 mM KCl) concentrations in the binding mixture (Fig. 2C,D). The sensitivities to protease and salt of the ribosome binding to the p34-containing liposomes closely resembled those in the case of stripped RM [1–4].

It should be noted that ribosome-unbound liposomes appear in Fig. 2A as a slow-sedimenting liposome fraction (fractions 17–19). We examined all fractions in Fig. 2A by means of SDS-PAGE and subsequent immunoblotting with p34 antibodies. The p34 protein was not detected in the slow-sedimenting fraction, being only found with the complex (fractions 7–9) (data not shown), suggesting that ribosome-unbound liposomes are deficient in the p34 protein. These results show that p34 certainly exhibits ribosome-binding activity.

Fig. 3A shows the intracellular distribution of p34, as analyzed by immunoblotting. The protein was detected in RM (lane 1), while none was found in other organelles except the nuclear envelope, the established location of bound ribosomes (lane 5), indicating that p34 is specifically localized in the ribosome-attached organelles. Immunoelectron microscopic analysis revealed that the p34 protein is present on RM membranes and the nuclear envelope (data not shown).

The p34 protein was not released from the mem-

branes on the sonication of RM at various salt concentrations or on treatment at alkaline (11–11.5) pH (sodium carbonate), indicating that it is an integral membrane protein. The p34 protein is likely to be exposed on the RM surface since it was destroyed, as analyzed by immunoblotting, on the treatment of RM with trypsin (8 μg/ml, 0°C, 60 min; this trypsin treatment did not affect most RM proteins, but inhibited the re-binding of ribosomes to stripped RM [4].

Fig. 3B shows that p34 was detected on immunoblotting in all preparations examined; several rat tissues (lanes 1–4), dog pancreas (lane 5), and hamster, mouse, pig and bovine liver (lanes 6–9), suggesting that p34 is distributed widely among mammalian tissues, in line with its assumed general important role.

4. DISCUSSION

Since the finding of p34 in rat liver RM membranes [12], its biological significance has been the subject of our investigations. We determined a portion of the amino acid sequence of this protein, which was then subjected to computer-assisted analysis in a search for homology with other known proteins using the PIR-NBRF database. However, no significant homology was detected with the computer programs we used. We anticipated, therefore, that p34 would be unique in its amino acid sequence and also in its biological function.

In the present work, we have shown that p34 exhibits ribosome-binding activity after reconstitution into liposomes. The binding properties observed were similar to those in the case of intact RM [1–4]. In addition, we showed that p34 is a membrane-embedded RM protein exposed on the RM surface and is widely distributed among mammalian tissues. From these results, p34 seems to be a likely candidate for a membrane protein responsible for ribosome binding in RM, although the possibility that other protein(s) in the RM membrane could also have such ribosome-binding properties has not been excluded entirely.

It should be added that our p34-containing liposomes exhibited a binding stoichiometry of 1 ribosome for every 61 molecules of the p34 protein. This low stoichiometry could be due to the following factors: (i) denaturation of the protein due to the presence of a detergent and the absence of phospholipids during preparation; (ii) aggregation and/or incorrect orientation of the p34 molecules in the lipid bilayer; and (iii) a lack of protein(s) necessary for optimum arrangement of the p34 protein in the membranes of liposomes, which may be present in the native RM membrane.

Recently, in chemical cross-linking studies, RM proteins in the 34 kDa range (termed imp34 [15] and P37 [16] were identified, which are located adjacent to a nascent polypeptide traversing the membrane bilayer of RM and are not identical with the signal sequence receptor [17]. In addition, these proteins were shown to be non-glycosylated, integral membrane proteins [15,16]. The relationship between these proteins and the present one, p34, remains to be investigated. The availability of p34 in a purified form will greatly facilitate studies on the role of this protein in the protein translocation system.

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