Characterization of the interaction between p100, a novel G-protein-related protein, and rat liver endosomes

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p100 is a recently identified 100 kDa protein which shares a putative receptor-binding sequence with the signal transducing G-proteins G_i and G_o . In liver, p100 immunoreactivity is distributed between the cytosolic and the microsomal fractions [Traub, Evans & Sagi-Eisenberg (1990) *Biochem. J.* **272**, 453–458; Udrisar & Rodbell (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6321–6325]. More specifically, we have localized the membrane-associated form of p100 to an endosomal subfraction of rat liver microsomes. In this study we have investigated the nature of the interaction between p100 and microsomal membranes. p100 was located on the cytoplasmic surface of the microsomal vesicles, and could be released by treatment with 0.5 M-NaCl or 0.5 M-Tris/HCl, pH 7.0. However, p100 was not released by non-ionic detergents, such as Triton X-100. Binding of p100 to the membrane was reversible, as both membrane-released and cytosolic p100 could re-bind stripped (Tris-washed) microsomes. Soluble p100 could not, however, bind to untreated microsomes. Binding to stripped microsomes approached saturation and was inhibited by up to 60% by either heat treatment or mild trypsin treatment of the vesicles. This implies that the interaction between p100 and the microsomal vesicles involves the direct binding of p100 to vesicular proteins. This binding was regulated by both adenine and guanine nucleotides. As p100 contains a region similar to the C-terminal decapeptide of α_i (the α -subunit of G_i) and has a localization that is restricted to an endosomal subfraction, we propose that cytosolic p100 may bind to cytoplasmically exposed domains of internalized receptors. Thus, like the adaptins, p100 may be involved in the process of sorting and receptor trafficking through the endosomal compartment of the cells.

INTRODUCTION

We have previously detected a 100 kDa protein (p100), which is immunologically related to the signal-transducing GTP-binding proteins (G-proteins) G_i and G_o , in the cytosolic fraction of rat basophilic leukaemia (RBL-2H3) cells [1]. The area of similarity between p100 and the G-proteins corresponds to the C-terminal region of the G-protein α -subunit. This was indicated by cross-reactivity between p100 and antibodies directed against a synthetic decapeptide from the C-terminal end of α_i . These antibodies also cross-react with the C-terminal region of α_{i1} and α_{i2} [2,3]. This particular region of the α -subunit plays a vital role in receptor recognition [3–8] and has been functionally assigned as the putative receptor-binding site [9].

In RBL cells, p100 was associated with a guanosine 5'-[γ -thio]triphosphate (GTP[S])-binding activity that was stimulated by acidic phospholipids such as phosphatidylserine [1]. We distinguished p100 from EF-2, a soluble 100 kDa GTP-binding protein [10,11] by several criteria. EF-2, but not p100, is phosphorylated by Ca^{2+} /calmodulin-sensitive protein kinase III and serves as a substrate for ADP-ribosylation by diphtheria toxin. In addition, highly purified EF-2 does not exhibit any immunological cross-reactivity with the anti- G_i antibodies [1].

Recently we demonstrated that p100 is present in several rat tissues and that it is particularly enriched in liver and kidney [12]. In liver, we [12] and others [13] have localized p100 to both the cytosolic and microsomal fractions. More specifically, we found the membrane-associated form of p100 in an endosomal fraction (termed D-R) previously shown to be a ligand-free but receptor-enriched subfraction of liver endosomal vesicles [12].

We have purified p100 from both rat liver cytosol and liver microsomes [13a]. Partial amino acid sequencing of both forms of p100 revealed that p100 is a previously unidentified protein. However, the N-terminus sequence obtained for p100 exhibits significant similarity to the deduced N-terminal amino acid sequences of both α - and γ -adaptins, a group of 100 kDa proteins that are major components of the adaptor polypeptide complexes [14–16]. Adaptors are believed to play a central role in the process of receptor-mediated endocytosis [14–16]. Therefore p100 may be a novel member of the adaptin family. To explore this possibility, in the present study we have investigated in detail the nature of the interaction between soluble p100 and the membrane. In particular, we examined whether p100, like the adaptins, could cycle on and off the vesicle surface and how this membrane association is regulated. Our results indicate that reversible protein–protein interactions are involved in binding of soluble p100 to endosomes present within the microsomal fraction. This implies the existence of an endosomal receptor for p100, and the possible nature of such a receptor is discussed.

MATERIALS AND METHODS

Materials

Bovine retina rod outer segment (ROS) membranes were kindly provided by Dr. A. M. Spiegel (National Institutes of Health). Antisera KE/2 and KE/3 were raised against a synthetic peptide corresponding to the C-terminal decapeptide of α_i , as described previously [12]. These antisera are equivalent to AS/6 and AS/7 in [2,3]. KE/3 antiserum was further purified by

Abbreviation used: App[NH]p, adenosine 5'-[β -imidio]triphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; α_i , α -subunit of the inhibitor G-protein G_i ; α_o , α -subunit of the retinal G-protein G_o ; EC₅₀, concentration producing 50% of maximal response; G-protein, signal-transducing GTP-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; LDM, low-density microsomes; ROS, retinal rod outer segment; TBST, Tris-buffered saline containing 0.05% Tween 20; Tos-Phe-CH₂Cl, *N*-tosyl-L-phenylalanine chloromethyl ketone ('TPCK').

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affinity chromatography on a peptide-Sepharose column. The peptide (Lys-Glu-Asn-Leu-Lys-Asp-Cys-Gly-Leu-Phe) was coupled to CNBr-activated Sepharose-4B, as recommended by the manufacturer. Elution of the peptide-specific antibodies was as described by Harlow & Lane [17], using 100 mM-glycine/HCl, pH 2.5, followed by 100 mM-triethylamine, pH 11.5. Ascitic fluid from a monoclonal antibody directed against rat liver ribophorin I was generously provided by Dr. D. Sabatini and Dr. G. Kreibich (New York University). ^{125}I -labelled second antibodies were prepared by iodination of affinity-purified donkey anti-rabbit or goat anti-mouse IgG (Jackson Laboratories) by the chloramine-T method. *N*-Tosyl-L-phenylalanine chloromethyl ketone (Tos-Phe-CH₂Cl, 'TPCK')-treated trypsin and soybean trypsin inhibitor were purchased from Sigma. ATP, CTP and adenosine 5'-[β -imido]triphosphate (App[NH]p) were from Sigma, and GTP[S], guanosine 5'-[β -thio]diphosphate (GDP[S]) and ATP[S] from Boehringer Mannheim. All other reagents were of analytical grade.

Subcellular fractionation

The basic subcellular fractionation of rat liver was performed essentially by the method of Graham [18]. Briefly, male Sprague-Dawley rats (approx. 2 months old) were killed by cervical dislocation, followed by decapitation. The livers were rapidly excised and placed into ice-cold 250 mM-sucrose. All subsequent procedures were performed at 0–4 °C. After weighing and coarse mincing of the liver with scissors, the tissue was homogenized in a Dounce homogenizer with 5 vol. of buffer A (25 mM-Tris/HCl, pH 7.5, 250 mM-sucrose, 1 mM-EDTA and 1 mM-phenylmethanesulphonyl fluoride). The resulting crude homogenate was centrifuged at 3000 *g* for 10 min and the pellet was discarded. The post-nuclear supernatant was re-centrifuged at 10000 *g* for 20 min, and the resulting pellet was washed twice by resuspension in buffer A and centrifugation at 10000 *g* as above. The post-mitochondrial supernatant was re-centrifuged at 100000 *g* for 60 min. The final high-speed supernatant represented the cytosolic fraction, while the 100000 *g* total microsomal pellet was washed twice in buffer A before final resuspension in buffer A. In the experiments presented in Figs. 7 and 8, the microsomal pellet was used without washing. Rough and smooth microsomes were prepared as described by Kreibich *et al.* [19] and modified by Hortsch & Meyer [20].

Total microsomes were stripped by washing in 500 mM-Tris/HCl, pH 7.0. Routinely, 500 μl of microsomes, containing approx. 7 mg of protein, was adjusted to 500 mM-Tris/HCl, pH 7.0. After incubation on ice for 30 min, the stripped membranes were recovered by centrifugation at 100000 *g* for 60 min. The supernatant fraction containing the extracted proteins was aspirated and diluted 3-fold for use in rebinding experiments. The stripped membrane pellet was resuspended at 4.5 mg/ml in buffer B (25 mM-Tris/HCl, pH 7.5, and 1 mM-EDTA) and either used directly for rebinding experiments or treated as stated in the Figure legends prior to use in the rebinding assay. Protein concentrations of the various fractions were determined by the method of Bradford [21], using BSA as the standard.

Treatment of rat liver microsomes

For the p100 solubilization experiments, washed 100000 *g* microsomes were diluted to a protein concentration of 1.25 mg/ml in buffer B, or buffer B containing salt, acid, base, detergent or nucleotide, to give the final concentrations stated in the Figure legends. After incubation on ice for 30 min, the microsomes were recovered by centrifugation at 100000 *g* for 60 min. The supernatants were recovered and mixed with 4 $\times$ SDS sample buffer, and the pellets were resuspended directly in SDS

sample buffer. Samples derived from 20 μg of microsomes were analysed by SDS/PAGE.

Alternatively, microscale treatment of microsomes was performed using a Beckman Airfuge. Samples containing 250 μg of microsomes were incubated with the indicated reagents in 25 mM-Tris/HCl, pH 7.5, in a final volume of 100 μl . After incubation on ice for 30 min, the samples were centrifuged in the Airfuge at 30 kPa for 30 min. The supernatants were aspirated and mixed with 25 μl of 5 $\times$ SDS sample buffer. Pellets were resuspended directly in 125 μl of SDS sample buffer. Samples were then subjected to SDS/PAGE followed by staining or by transfer to nitrocellulose.

Binding of p100 to rat liver microsomes

The source of soluble p100 for the binding experiments was either the rat liver 100000 *g* high-speed supernatant fraction, prepared as described above, or the stripped microsome supernatant, diluted 3-fold in buffer B. The binding assay was carried out in a final volume of 1.5 ml containing 25 mM-Tris/HCl, pH 7.5, 1 mM-EDTA and 450 μg of stripped microsomes. Soluble-p100-containing fractions were added at concentrations of 0.5–15 mg/ml, as indicated in the Figure legends. A sample of soluble p100 in the absence of stripped microsomes was always included in the assay. The samples were incubated on ice for 30 min and then the bound protein was separated from the soluble protein by centrifugation at 100000 *g* for 60 min. The pellets were resuspended in SDS sample buffer. Aliquots were analysed by SDS/PAGE, followed by transfer on to nitrocellulose and analysis with KE/2 antiserum.

Electrophoresis and immunoblotting

Discontinuous SDS/PAGE was performed as described by Laemmli [22]. Gels were either stained with Coomassie Blue R250 or transferred to nitrocellulose in 15.6 mM-Tris/120 mM-glycine at 200 mA for 3 h, as described by Gershoni [23]. Blotted proteins were visualized by brief staining in 0.5 % Ponceau S and the positions of the molecular mass standards were marked, followed by complete destaining in blotting transfer buffer. The membranes were then incubated in 1 % BSA in 10 mM-Tris/HCl (pH 8.0)/150 mM-NaCl/0.05 % Tween 20 (TBST) for 4–5 h to block non-specific membrane binding sites. The blots were then incubated with the specified antibodies, as detailed in the Figure legends, for 5–12 h. All antibodies were diluted in 0.1 % BSA in TBST. After washing four times in TBST, the membranes were incubated with ^{125}I -labelled second antibodies (0.2 $\mu\text{Ci}/\text{ml}$) for 1–2 h. After washing four times in TBST and twice in distilled water, the filters were air-dried and the immunoreactive bands were visualized by autoradiography at –80 °C. For quantitative analysis, the regions of the blot corresponding to the labelled bands were excised and counted directly in a γ -radiation scintillation counter.

RESULTS

Subcellular distribution of p100

Rat liver homogenate was fractionated by differential centrifugation and the supernatant and pellet fractions generated were analysed by Western blotting using KE/3 antiserum. KE/3 was raised against a synthetic decapeptide (KENLKDCGLF) corresponding to the C-terminal end of α_i . Therefore these antibodies recognized the α -subunit of G_i , present in ROS membranes (Fig. 1, lane h). Affinity-purified KE/3 also recognized p100 present in the liver homogenate (lane i) [12]. Essentially all of the p100 immunoreactivity in the homogenate was recovered in the 3000 *g* post-nuclear supernatant (lane j).

Fig. 1. Subcellular distribution of p100 immunoreactivity

ROS membranes (5 μ g, lanes a and h) and 20 μ g each of rat liver homogenate (b and i), 3000 g post-nuclear supernatant (c and j), 10000 g mitochondrial pellet (d and k), 10000 g post-mitochondrial supernatant (e and l), 100000 g microsome pellet (f and m) and 100000 g supernatant (g and n) were separated on duplicate 10% gels and stained with Coomassie Blue (a to g) or transferred to nitrocellulose. Lanes h–n are from an autoradiograph of the blot, probed with a 1:100 dilution of affinity-purified KE/3. Molecular mass markers are indicated on the left.

Fig. 2. Distribution of p100 immunoreactivity in total, rough and smooth microsomes

Samples of 20 μ g of total rat liver microsomes (lanes a, d and g), smooth microsomes (b, e and h) and rough microsomes (c, f and i) were resolved on a 10% gel and either stained with Coomassie Blue (a–c) or transferred to nitrocellulose. Lanes d–i are from autoradiographs of duplicate blots probed with a 1:100 dilution of either monoclonal anti-(ribophorin I) (d–f) or KE/2 (g–i). Molecular mass markers are indicated on the left.

Following re-centrifugation at 10000 g , only trace amounts of p100 immunoreactivity were detected in the mitochondrial pellet (lane k), and the bulk of the reactivity remained in the post-mitochondrial supernatant (lane l). After high-speed centrifugation, the immunoreactivity was observed in both microsomal (lane m) and cytosolic (lane n) fractions, but appeared relatively enriched within the microsome fraction.

The 100000 g microsome pellet of rat liver homogenates has been extensively characterized [18,24]. The fraction is composed largely of rough and smooth microsomes, derived from the endoplasmic reticulum following homogenization. Lower amounts of plasma membranes and Golgi, endosomal, lysosomal and peroxisomal membranes are also present. To ascertain whether p100 was associated with endoplasmic reticulum derivatives, we purified rough and smooth microsomes by density

gradient centrifugation. Coomassie Blue staining of the SDS/PAGE-resolved fractions (Fig. 2, lanes a–c) showed that, while clearly similar to the total microsomes, the rough and smooth fractions exhibited different protein profiles. The rough microsomes differed from the smooth primarily by the presence of characteristic low-molecular-mass ribosomal proteins (lane c) [19]. In addition, a protein of 180 kDa, most probably the recently identified ribosome receptor [25], was also highly enriched in the rough microsome fraction (lane c). Antibodies to the rough-microsome-specific marker, ribophorin I [19,20], reacted with the total microsome fraction (lane d) and the rough microsomes (lane f). Only trace levels of ribophorin I immunoreactivity were detected in the smooth microsomes, indicating the purity of our preparations. However, whereas p100 immunoreactivity was easily detectable in crude total microsomes (lane g), we could not detect it in either rough or smooth microsomes (lanes h and i). We concluded that, although p100 immunoreactivity was relatively enriched in total liver microsomes, it appeared to be absent from endoplasmic-reticulum-derived fractions. This finding, together with our previous identification of p100 within a specific subset of rat liver endosomes [12], indicates that p100 appears to be restricted to the endosomal fraction of the 100000 g microsome pellet. This supports our contention that p100 is involved in the process of endocytosis and vesicle trafficking.

Membrane topology of p100

To determine whether the membrane-associated form of p100 was exposed or confined to the interior of the endosomal vesicles, we examined the sensitivity of p100 to proteolysis in the absence or presence of Triton X-100. If p100 is located on the outside of the vesicle, it should be sensitive to protease digestion in both the absence and the presence of detergent. As illustrated in Fig. 3(a), incubation of the microsomes (low-density microsomes, LDM) with trypsin resulted in the degradation of p100 in a dose-dependent manner. Solubilization of the microsomal membranes had no effect on the extent or rate of p100 degradation, indicating that the protein was exposed on the vesicle surface. That the LDM fraction indeed consisted of intact vesicles was revealed by the observation that several other microsomal proteins, with molecular masses of 120, 98, 75 and 35 kDa, were degraded by trypsin in a detergent-sensitive manner. These proteins were resistant to the protease treatment unless Triton X-100 was included, indicating an intracellular location (Fig. 3b).

The cytosolic form of p100 showed a much higher sensitivity to trypsin. At a 100-fold excess of substrates over the enzyme, 50% of cytosolic p100 was degraded, while only 10% of the membrane-associated form was digested (Fig. 3a). In fact, greater than 5-fold more trypsin was required to completely degrade an equivalent amount of membrane-bound p100 when compared with the proteolysis of cytosolic p100 (Fig. 3a). These findings suggest that the antigenic epitope detected by KE/2 and KE/3 may be involved in the binding of p100 to the membrane. Interaction of the epitope with the membrane may result in the observed lower susceptibility to proteolytic digestion.

Nature of interaction between p100 and the membrane

Although p100 was associated with the microsomal fraction, it did not appear to be an integral membrane protein. This was shown by a series of experiments examining the nature of the interaction between p100 and the membrane. The 100000 g LDM pellet was first treated with various reagents and then separated into supernatant and pellet fractions by re-centrifugation at 100000 g . An analysis of the resulting fractions with KE/2 antiserum is presented in Fig. 4(a). Under conditions of low ionic strength and neutral pH (20 mM-Tris/HCl, pH 7.5),

Fig. 3. Tryptic digestion of p100

(a) Samples of 560 μg of 100 000 *g* microsomes (●), microsomes plus 0.5% Triton X-100 (□) or 320 μg of 100 000 *g* liver supernatant (○) were incubated with 0–200 μg of Tos-Phe-CH₂Cl-treated trypsin in a final volume of 100 μl. Incubation was at 37 °C for 15 min, followed by the addition of 200 μg of soybean trypsin inhibitor, and the tubes were chilled on ice. 5 × SDS sample buffer was added and samples were resolved on a 10% gel, transferred to nitrocellulose and probed with a 1:100 dilution of KE/3. p100 immunoreactivity after the treatments was quantified and the values indicated represent the percentage of undigested p100 immunoreactivity present in each fraction. (b) Samples of microsomes (5.6 mg/ml) were treated with 0 (lanes a and b), 50 (c and d) or 100 (e and f) μg of Tos-Phe-CH₂Cl TPCK-treated trypsin/ml in the absence (a, c and e) or presence (b, d and f) of 0.5% Triton X-100. Incubation was at 37 °C for 15 min, followed by addition of soybean trypsin inhibitor and 5 × SDS sample buffer as above. Samples were resolved on a 10% gel and stained with Coomassie Blue. Molecular mass markers are indicated on the left.

p100 immunoreactivity was recovered in the pellet fraction, together with the majority of the protein (lanes b and b' compared with a and a'). Increasing the salt concentration to moderate levels (150 mM-NaCl) did not affect the association of p100 with the vesicles (lanes c' and d'). However, in the presence of 500 mM-NaCl, the p100 immunoreactivity in the pellet was lost (lane f'), and was quantitatively recovered in the supernatant fraction (lane e'). This effect of salt appeared to be due to an increase in the ionic strength, as similar results were obtained with 500 mM-Tris/HCl, pH 7.5 (lanes g' and h'). Elevation of the pH with 100 mM-NaOH resulted in the solubilization of approximately half of the microsomal protein (lanes i and j), including all of the p100 immunoreactivity. Similar results were obtained with 100 mM-Na₂CO₃ (results not shown), confirming that p100 is not an integral membrane protein [26]. Decreasing the pH by the

Fig. 4. Solubilization of p100 from rat liver microsomes

(a) Equal volumes of the 100 000 *g* supernatant (lanes a, c, e, g, i and k) and pellet (b, d, f, h, j and l) fractions were separated on duplicate 10% gels. The microsomes had been pretreated for 30 min on ice with buffer B alone (a and b), or buffer B supplemented with 150 mM-NaCl (c and d), 500 mM-NaCl (e and f), 500 mM-Tris/HCl, pH 7.5 (g and h), 100 mM-NaOH (i and j) or 500 mM-acetic acid (k and l). Lanes a'–l' are from the Coomassie Blue-stained gel, and lanes a'–l' are from an autoradiograph of a duplicate gel, transferred to nitrocellulose and probed with a 1:100 dilution of KE/2. Only the relevant region of the autoradiograph is shown. Molecular mass markers are indicated on the left. (b) Equal volumes of the 100 000 *g* microsomal supernatant (lanes a, c, e, g and i) and pellet (b, d, f, h and j) fractions were separated on duplicate 10% gels. The microsomes had been pretreated for 30 min on ice with buffer B alone (a and b) or buffer B supplemented with 500 mM-NaCl (c and d), 1% Triton X-100 (e and f), 1% SDS (g and h) or 1% Triton X-100 with 500 mM-NaCl (i and j). Lanes a'–j' are from the Coomassie Blue-stained gel and lanes a'–j' are from an autoradiograph of a duplicate gel, transferred to nitrocellulose and probed with a 1:100 dilution of KE/2. Only the relevant region of the autoradiograph is shown. Molecular mass markers are indicated on the left.

addition of 500 mM-acetic acid left most of the protein in the pellet fraction (lane l). Under these conditions p100 immunoreactivity was recovered in both supernatant and pellet fractions (lanes k' and l'), but with reduced yields. We have obtained similar results by treating the microsomes with urea (results not shown). As both of these are strong denaturing conditions, the poor recovery of p100 probably reflects increased susceptibility to proteolytic degradation under these conditions.

Fig. 5. Binding of p100 to salt-washed microsomes

(a) Microsomes were stripped by treatment with 500 mM-Tris/HCl, pH 7.0, and the salt-extracted soluble p100 was diluted 3-fold and used for the binding assay as described in the Materials and methods section. The assay contained soluble p100 (a), soluble p100 plus 0.3 mg of stripped microsomes/ml (b) or soluble p100 and stripped microsomes plus 500 mM-NaCl (c). Samples of the resulting pellet fractions from the binding assay were resolved on a 10% gel, transferred to nitrocellulose and probed with a 1:100 dilution of KE/2. p100 immunoreactivity was quantified and the values indicated represent the percentage of p100 immunoreactivity in the pellet fractions. (b) The binding assay was performed as described in the Materials and methods section using the 100000 *g* liver supernatant (cytosol) as the source of soluble p100. Stripped microsomes (0.3 mg/ml) were incubated with 0.5 (a), 2 (b), 5 (c), 10 (d) and 15 (e) mg of cytosol/ml and samples of the resulting pellets from the binding assay were resolved on a 10% gel, transferred to nitrocellulose and probed with a 1:100 dilution of KE/2. The blots were quantified as above.

Fig. 6. Inhibition of binding of p100 to stripped microsomes on trypsin treatment or heat treatment

(a) The binding assay was performed as described in the Materials and methods section using salt-extracted and diluted soluble p100. The assay contained soluble p100 (a), soluble p100 and 0.3 mg of stripped microsomes/ml (b), or soluble p100 and microsomes that had been previously treated with 3 µg of Tos-Phe-CH₂Cl-treated trypsin/ml (c) or incubated at 56 °C for 30 min (d). Tryptic digestion was terminated by addition of excess soybean trypsin inhibitor (45 µg) and heat treatment was terminated by chilling the samples on ice. Samples of the resulting pellet fractions from the binding assay were resolved on a 10% gel, transferred to nitrocellulose and probed with a 1:100 dilution of KE/2. Quantification was as in Fig. 5. (b) Samples of stripped microsomes (0.3 mg/ml) were treated with 0 (a), 3 (b), 10 (c) or 100 (d and e) µg of Tos-Phe-CH₂Cl-treated trypsin/ml in the absence (a-d) or presence (e) of 0.5% Triton X-100. Incubation was at 37 °C for 15 min, followed by addition of 30 µg of soybean trypsin inhibitor/ml and then 5×SDS sample buffer. Samples were resolved on a 10% gel and stained with Coomassie Blue. Molecular mass markers are indicated on the left.

The effects of detergents on the association between p100 and the microsomal vesicles are illustrated in Fig. 4(b). As noted above, p100 remained membrane-bound at low ionic strength (lane b'), but was solubilized in the presence of 500 mM-NaCl (lane c'). Treatment of the microsomes with the non-ionic detergent Triton X-100, at low ionic strength, resulted in the solubilization of the majority of the proteins (lane e). However, several proteins, including p100, were recovered in the pellet fraction (lane f'). SDS effectively solubilized the microsomes completely (lane g), and accordingly p100 immunoreactivity was recovered in the supernatant (lane g'). A combination of Triton X-100 and 500 mM-NaCl also resulted in almost quantitative solubilization and recovery of p100 in the supernatant fraction (lane i'). Taken together, then, these results show that the interaction between p100 and the microsomal membrane appears to be peripheral and ionic in nature.

We have noticed that p100 displayed slight differences in electrophoretic mobility under some conditions (Fig. 4b, lower panel). However, the changes were not correlated with association of p100 and the membrane, and the precise reason for these differences in mobility remains to be determined.

Reversibility of p100 binding to microsomes

We used the ability of elevated salt concentrations to quantitatively remove p100 from the microsomal membranes to analyse the reversibility of p100 binding. LDM were stripped of bound p100 by washing with 0.5 M-Tris/HCl, pH 7.0. Upon dilution of the solubilized p100 with low ionic strength buffer

and remixing, p100 re-bound to the washed LDM (Fig. 5a, lane b compared with lane a). This binding occurred at 0 °C and was inhibited by the presence of salt (Fig. 5a, lane c compared with lane b).

Stripped LDM also bound cytosolic p100 in a concentration-dependent fashion (Fig. 5b). At a concentration of 0.3 mg of stripped LDM/ml, binding was linear with cytosol concentrations up to 10 mg/ml and approached saturation at 10–15 mg/ml. In contrast, native (unstripped) LDM did not bind additional p100 from the cytosolic pool (results not shown).

To test whether membrane-associated proteins were required for the binding of soluble p100 to the membrane, stripped LDM (0.3 mg/ml) were pre-incubated with Tos-Phe-CH₂Cl-treated trypsin (3 µg/ml) for 15 min at 37 °C. Soybean trypsin inhibitor (30 µg/ml) was then added, followed by salt-extracted and diluted p100, and the incubation was continued for a further 30 min on ice. Trypsin treatment of the stripped LDM fraction decreased (by 40%) the ability of these membranes to bind soluble p100 (Fig. 6a, lane c). At this concentration, trypsin did not have any major effect on the protein profile of the stripped LDM (Fig. 6b, lane b compared with lane a). The vesicular nature of the microsomes was also preserved in the stripped LDM preparation, as proteolysis of some of the vesicle-associated proteins occurred only in the presence of 0.5% Triton X-100 (Fig. 6b, lane e compared with lane d). Increasing the trypsin concentration further (up to 100 µg/ml) decreased the binding by up to 60% (results not shown).

Inhibition of binding of soluble p100 to the stripped LDM was

Fig. 7. Solubilization of p100 from liver microsomes by nucleotides or nucleotide analogues

Equal volumes of the 100000 *g* microsomal supernatant (lanes a, c, e, g, i, k, m and o) and pellet (b, d, f, h, j, l, n and p) fractions were separated on duplicate 10% gels. The unwashed microsomes had been pretreated with 25 mM-Tris/HCl, pH 7.5 (a and b), or 25 mM-Tris/HCl, pH 7.5, supplemented with 1 mM-ATP (c and d), -GTP (e and f), -CTP (g and h), -ATP[S] (i and j), -App[NH]p, (k and l), -GTP[S] (m and n) or -GDP[S] (o and p). Lanes a–p are from the Coomassie Blue-stained gel and a'–p' from an autoradiograph of a duplicate gel which was transferred to nitrocellulose and probed with a 1:100 dilution of KE/2. Only the relevant region of the autoradiograph is shown. Molecular mass markers are indicated on the left.

Fig. 8. Dose-dependence of nucleotide-mediated solubilization of p100 from microsomes

Unwashed microsomes in 25 mM-Tris/HCl, pH 7.5, were incubated on ice for 30 min with increasing concentrations of ATP (○), ATP[S] (●), App[NH]p (△), GTP (□) or GTP[S] (■). After centrifugation at 100000 *g*, samples of the resulting supernatant and pellet fractions were resolved on 10% gels, transferred to nitrocellulose and probed with a 1:100 dilution of KE/2. p100 immunoreactivity was quantified and the values indicated are percentages of total p100 immunoreactivity in the supernatant fractions.

also observed following heat treatment of the microsomes. For these experiments, stripped LDM were incubated at 56 °C for 30 min and then cooled on ice prior to the addition of salt-extracted and diluted p100. Binding activity was again decreased

by 60% (Fig. 6a, lane d). These data suggest the involvement of microsomal protein(s) in the cycling of cytosolic p100 on and off LDM.

Nucleotide-sensitivity of p100 membrane association

Both self-association and subcellular distribution of members of the adaptin family have recently been shown to be regulated by purine nucleotides [27–29]. For example, a 110 kDa Golgi-associated protein [30], termed β -COP [31,32], has recently been shown to exhibit sequence similarity to the β -adaptins [31]. β -COP dissociates from the Golgi on depletion of intracellular ATP levels [28]. In addition, p100 appears to be associated with a GTP-binding activity [1,13]. Therefore we analysed whether the association of p100 with the microsomal membrane was affected by nucleotides. The results of these experiments are shown in Fig. 7. Incubation of the microsomal vesicles with ATP (1 mM) released only a few additional proteins into the supernatant (lane c compared with lane a). This treatment did, however, result in the quantitative solubilization of membrane-associated p100 (lane c' compared with lane a'). GTP (1 mM) was less effective and released approx. 30% of membrane-associated p100 (lanes e' and f'), while CTP (1 mM) was ineffective (lanes g' and h'). To test whether nucleotide hydrolysis was required, the effects of non-hydrolysable analogues of both adenine and guanine nucleotides were studied. At a concentration of 1 mM, ATP[S] (lanes i' and j') and App[NH]p (lanes k' and l') caused the complete dissociation of p100. GTP[S] appeared less effective, and at a concentration of 1 mM-GTP[S], 50% of p100 was released (lanes m' and n'). In contrast, GDP[S] (1 mM) had no effect (lanes o' and p').

The combined data from a series of dose-response experiments are presented in Fig. 8. Nucleotide-induced dissociation of p100 from the microsomes was dose-dependent. ATP[S] and App[NH]p were most effective, releasing 50% of membrane-bound p100 at a concentration of 50 μ M (EC_{50}). ATP, GTP and GTP[S] were less effective, with EC_{50} values of 200, 1500 and 900 μ M respectively. It is noteworthy that the effect of the nucleotides was independent of divalent cations. No differences in the ability of the various nucleotides or analogues to solubilize p100 were observed in the presence of either 10 mM-MgCl₂ or 1 mM-EDTA (results not shown). Although this is surprising, it should be noted that the effect of ATP on AP-2 adaptor self-association is also Mg²⁺-independent [27].

DISCUSSION

p100 was initially identified as a 100 kDa protein that cross-reacted with anti-peptide antisera directed against the α -subunit of G_i [1,12,13]. The region of similarity corresponds to the C-terminal domain of both α_i and α_o , a region that plays a vital role in receptor–G-protein coupling [3–8]. In liver, p100 is found in both the cytosolic and microsomal fractions. More specifically, we have localized the membrane-associated form of p100 to a receptor-enriched subfraction of liver endosomal vesicles, but could not detect it in purified rat liver plasma membranes or in the Golgi [12]. In the present study we show that p100 is also absent from both rough- and smooth-endoplasmic-reticulum-derived vesicles (Fig. 2), supporting the notion that the membrane-associated form of p100 is specifically restricted to endosomes.

Endocytic vesicles are highly specialized organelles that play a central role in receptor recirculation and down-regulation.

Proteins that specifically associate with these endocytic vesicles are therefore likely candidates to participate in the process of endocytosis. One such candidate is p100. This is supported by our recent studies showing that the *N*-terminal amino acid sequence of p100 shows significant similarity to the *N*-terminal ends of both α - and γ -adaptins [13a]. Therefore in the present study we investigated the nature of the interaction between p100 and endosomes present within the microsomal fraction.

The topology of p100 membrane association was studied by protease protection experiments. These experiments revealed that on treatment of LDM with trypsin, p100 was degraded to the same extent independent of the presence of Triton X-100 (Fig. 3). This observation indicated that p100 was cytoplasmically orientated. It also demonstrated that the vesicles present within the microsomal pellet retained their original polarity and were not formed during homogenization under conditions that could have resulted in the entrapment of cytosolic p100.

Treatment of the microsomal vesicles with a variety of extraction procedures revealed that p100 is not an integral membrane protein (Fig. 4). Rather, p100 was associated with the microsomal fraction through ionic interactions. This could reflect an interaction between soluble p100 and a protein component of the microsomal vesicles. To analyse this possibility further, we studied the mechanism of attachment of soluble p100 to the membrane, by examining the ability of stripped microsomes to rebinding soluble p100. These studies indicated that p100 bound to stripped, but not untreated, microsomes in a saturable manner (Fig. 5). This suggests the presence of a fixed number of binding sites for p100 on the microsomes. That microsomal protein(s) served as specific recognition and binding sites for p100 was also indicated by the finding that the binding of p100 to the membranes was sensitive to heat and protease treatment of the membranes (Fig. 6). These results imply that p100 cycles between cytosolic and membrane-associated pools, and that cytoplasmically orientated domains of endosomal proteins, which are protease-sensitive, are the binding sites for soluble p100.

The nature of these putative binding proteins is at present unknown. However, because of the occurrence of a putative receptor-binding site within p100 and the restricted localization of the membrane-associated form of p100 to the receptor-enriched endosomal fraction [12], it is tempting to speculate that the cytoplasmically exposed domains of internalized receptors contribute to the specific binding of p100. Moreover, since p100 shares an antigenic epitope with G_i and G_o that has been implicated in receptor binding, those receptors coupled to G_i are candidates for association with p100 through this G-protein-like region. Indeed, it has been suggested that within the endosomal compartment, following the dissociation of receptors from ligands, some sorting mechanism must allow for the pinching off of the membrane-bound receptors into vesicles which re-cycle back to the plasma membrane [33,34]. Such a sorting mechanism may involve the interaction of the cytoplasmic domains of the internalized receptors with a protein that mediates the appropriate targeting of the receptor-enriched vesicles. We propose that p100 may function in this manner. This hypothesis is supported by the following observations. First, the retinal protein arrestin, like p100, exhibits similarity to the C-terminal decapeptide of α_i [35]. Arrestin functions in the desensitization process of visual transduction, and is believed to operate, at least in part, by competition with G_i for rhodopsin binding by virtue of its shared sequence with α_i [36]. Secondly, recent studies by us [37,38] and others [39] have revealed that a cluster of positive charges within the G-protein-coupled receptors is important in both the recognition and the activation of G-proteins. This suggests that an ionic interaction between the G-protein and its

respective receptor occurs. Finally, β -adrenergic receptor mutants that fail to couple to G_s do not undergo agonist-mediated sequestration [40]. Furthermore, mutant receptors which exhibit impaired down-regulation have a decreased ability to couple to G_s [41]. Therefore it appears that similar regions of the receptor are involved both in G-protein coupling and in the process of receptor down-regulation. Internalized receptors could thus comprise the only binding sites for p100 on the endosomal membrane. Alternatively, this interaction may follow or facilitate the binding of cytosolic p100 to another vesicular protein. At present we cannot distinguish between these two possibilities.

The interaction between p100 and the microsomal membrane was regulated by purine nucleotides. ATP[S] and App[NH]p were more potent than ATP in releasing p100 from the microsomes. Furthermore, GTP[S] and GTP, but not GDP[S], promoted p100 dissociation, albeit to a lesser extent. This suggests that neither hydrolysis of the nucleotide nor phosphorylation was required. Therefore it appears that it is the binding of the nucleotide triphosphate that results in the release of p100 from the membrane. The mechanism underlying these nucleotide effects requires further investigation. It is interesting, however, that ATP inhibits self-association of the AP-2 clathrin assembly protein complex [27]. This effect of ATP, like that on p100 membrane association, is independent of Mg^{2+} [27]. These data further support the notion that p100 may be related to the adaptins and is involved in receptor trafficking.

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REFERENCES

1. Sagi-Eisenberg, R., Traub, L. M., Gat-Yablonski, G. & Aridor, M. (1989) In *The Guanine-Nucleotide Binding Proteins* (Bosch, L., Kraal, B. & Parmeggiani, A., eds.), pp. 349–357, Plenum Publishing Corporation, New York
2. Goldsmith, P., Gierschik, P., Milligan, G., Unson, C. G., Vinitzky, R. Malech, H. I. & Spiegel A. M. (1987) *J. Biol. Chem.* **262**, 14683–14688
3. Simonds, W. F., Goldsmith, P. K., Codina, J., Unson, C. G. & Spiegel, A. M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7809–7813
4. Hamm, H. E., Deretic, D., Mazzoni, M. R., Moore, C. A., Takahashi, J. S. & Rasenick, M. M. (1989) *J. Biol. Chem.* **264**, 11475–11482
5. Cerione, R. A., Kroll, S., Rajaram, R., Unson, C., Goldsmith, P. & Spiegel, A. M. (1988) *J. Biol. Chem.* **263**, 9345–9352
6. McKenzie, F. R., Kelly, E. C. H., Unson, C. G., Spiegel, A. M. & Milligan, G. (1988) *Biochem. J.* **249**, 653–659
7. Palm, D., Münch, G., Malek, D., Dees, C. & Hekman, M. (1990) *FEBS Lett.* **261**, 294–298
8. Simonds, W. F., Goldsmith, P. K., Woodard, C. J., Unson, C. G. & Spiegel, A. M. (1989) *FEBS Lett.* **249**, 189–194
9. Masters, S. B., Stroud, R. M. & Bourne, H. R. (1986) *Protein Eng.* **1**, 47–54
10. Nilsson, L. & Nygard, O. (1985) *Eur. J. Biochem.* **148**, 299–304
11. Ryananov, A. G., Shestakova, E. A. & Natapov, P. G. (1988) *Nature (London)* **334**, 170–173
12. Traub, L. M., Evans, W. H. & Sagi-Eisenberg, R. (1990) *Biochem. J.* **272**, 453–458
13. Udrisar, D. & Rodbell, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6321–6325
- 13a. Traub, L. M. & Sagi-Eisenberg, R. (1992) *J. Biol. Chem.*, in the press
14. Pierce, B. M. F. & Robinson, M. S. (1990) *Annu. Rev. Cell Biol.* **6**, 151–171
15. Morris, S. A., Ahle, S. & Ungewickell, E. (1989) *Curr. Opin. Cell Biol.* **1**, 684–690

16. Keen, J. H. (1990) *Annu. Rev. Biochem.* **59**, 415–438
17. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Graham, J. (1984) in *Centrifugation: A Practical Approach* (Rickwood, D., ed.), pp. 168–171, IRL Press, Oxford
19. Kreibich, G., Ulrich, B. L. & Sabatini, D. D. (1978) *J. Cell Biol.* **77**, 464–487
20. Hortsch, M. & Meyer, D. I. (1985) *Eur. J. Biochem.* **150**, 559–564
21. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
22. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
23. Gershoni, J. M. (1988) *Methods Biochem. Anal.* **33**, 1–58
24. Lambert, N. (1989) in *Protein Purification Methods: A Practical Approach* (Harris, E. L. V. & Angal, S., eds.), pp. 101–108, IRL Press, Oxford
25. Savits, A. J. & Meyer, D. I. (1990) *Nature (London)* **346**, 540–544
26. Fujiki, Y., Hubbard, A. L., Fowler, S. & Lazarow, P. B. (1982) *J. Cell Biol.* **93**, 97–102
27. Beck, K. A. & Keen, J. H. (1991) *J. Biol. Chem.* **266**, 4442–4447
28. Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E. & Klausner, R. D. (1990) *J. Cell Biol.* **111**, 2295–2306
29. Donaldson, J. G., Lippincott-Schwartz, J. & Klausner, R. D. (1991) *J. Cell Biol.* **112**, 579–588
30. Allan, V. J. & Kreis, T. E. (1986) *J. Cell Biol.* **103**, 2229–2239
31. Duden, R., Griffiths, G., Frank, R., Argos, P. & Kreis, T. E. (1991) *Cell* **64**, 649–665
32. Waters, M. G., Serafini, T. & Rothman, J. E. (1991) *Nature (London)* **349**, 248–251
33. Linderman, J. J. & Lauffenburger, D. A. (1988) *J. Theor. Biol.* **132**, 202–245
34. Shepherd, V. L. (1989) *Trends. Pharmacol. Sci.* **10**, 458–462
35. Wistow, G. J., Katial, A., Craft, C. & Shinohara, T. (1986) *FEBS Lett.* **196**, 23–28
36. Wilden, U., Hall, S. W. & Kühn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1174–1178
37. Aridor, M., Traub, L. M. & Sagi-Eisenberg, R. (1990) *J. Cell Biol.* **111**, 909–917
38. Aridor, M. & Sagi-Eisenberg, R. (1990) *J. Cell Biol.* **111**, 2885–2891
39. Higashijima, T., Burnier, J. & Ross, E. M. (1990) *J. Biol. Chem.* **265**, 14176–14186
40. Cheung, A. H., Sigal, I. S., Dixon, R. A. F. & Strader, C. D. (1989) *Mol. Pharmacol.* **34**, 132–138
41. Valiquette, M., Bonin, H., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. & Bouvier, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5089–5093

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