Detection of 23–27 kDa GTP-binding proteins in platelets and other cells

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Membrane proteins from rabbit and human platelets were separated by SDS/polyacrylamide-gel electrophoresis and the resolved polypeptides blotted on nitrocellulose. A family of GTP-binding proteins, termed G_n proteins, was detected by incubation of these blots with [α - 32 P]GTP in the presence of Mg^{2+} . A major G_n protein with a molecular mass of 27 kDa (G_{n27}) and lesser amounts of 23, 24 and 25 kDa G_n proteins were observed in platelet membranes; much smaller amounts were in the platelet soluble fraction. Binding of [α - 32 P]GTP by platelet G_n proteins was blocked by GDP, GTP or guanosine 5'-[γ -thio]triphosphate, but not by GMP or adenosine 5'-[β -imidio]triphosphate. Rabbit and human red-cell membranes contained only G_{n27} . When rat tissues were analysed for G_n proteins, the largest amounts were found in brain, which contained two membrane-bound forms (G_{n27} and G_{n26}) and a soluble form (G_{n26}).

INTRODUCTION

Signal transduction between plasma-membrane receptors and target enzymes, such as adenylate cyclase or phosphoinositidase C, is mediated by GTP-binding proteins (G-proteins), as is that between rhodopsin and cyclic GMP phosphodiesterase in retinal rods [1–3]. In addition, G-proteins can directly regulate some ion channels (e.g. [4–6]). Whereas the G-proteins responsible for stimulation and inhibition of adenylate cyclase (G_s and G_i) and for the activation of cyclic GMP phosphodiesterase in rods (transducin) have been fully characterized, those modulating phosphoinositidase C and ion channels in intact cells have yet to be as clearly identified. G-proteins without assigned functions that may be involved in these processes include G_o , which has been purified from brain tissue [17], various incompletely characterized proteins related to G_i [8,9] and a protein isolated from human placenta [10]. All the above G-proteins contain α -, β - and γ -subunits and differ primarily in their GTP-binding α -subunits [3]. Recently, interest has also focused on the p21 proteins coded for by cellular *ras* genes, since these proteins bind GTP, show some structural and functional homology with the α -subunits of the classical G-proteins and are clearly involved in the regulation of cell growth [11].

The most widely used technique for documenting the presence and function of G-proteins has been by ADP-ribosylation of their α -subunits with cholera toxin (G_s and transducin) or pertussis toxin (G_i , G_o and transducin) [1,3]. In this connection, pertussis toxin has been found to block activation of phosphoinositidase C in some cells, such as neutrophils, but not in others [2]. This toxin does not penetrate intact platelets, but, in permeabilized platelets, has variously been reported to enhance [12] or to inhibit [13] the activation of phosphoinositidase C by thrombin. Whichever is correct, agonists that stimulate phosphoinositidase C cause a pertussis-toxin-resistant increase in platelet membrane

GTPase activity [14,15]. These considerations suggest that G-proteins other than G_i or related pertussis-toxin-sensitive species have roles in signal transduction in platelets and other cells, possibly in the regulation of polyphosphoinositide hydrolysis. In some instances, the *ras* p21 proteins may participate in the activation of phosphoinositidase C, since polyphosphoinositide-hydrolysis products are increased in cells transformed by *ras* oncogenes [16] and enhanced expression of normal *N-ras* p21 can increase inositol phosphate formation in response to certain growth factors [17]. However, it is by no means clear that all the GTP-binding proteins that participate in signal transduction have yet been identified. We have therefore attempted to detect novel GTP-binding proteins in platelets and other cells. In the present study, we describe a widely distributed family of largely membrane-bound 23–27 kDa proteins that can be specifically labelled with [α - 32 P]GTP on nitrocellulose blots of proteins separated by SDS/polyacrylamide-gel electrophoresis. Some of our results have been published in a preliminary form [18,19].

EXPERIMENTAL

Materials

[α - 32 P]GTP (3700 Ci/mmol) was from ICN Radiochemicals (Irvine, CA, U.S.A.). Nitrocellulose membrane (0.2 μ m pore) was obtained from Bio-Rad Laboratories (Canada) (Mississauga, Ont., Canada). ATP (disodium salt, prepared by phosphorylation of adenosine), ADP, AMP, p[NH]ppA, GTP, GDP, GMP, protein standard solution, Amido Black 10B, PMSF and low- M_r protein standards for SDS/polyacrylamide-gel electrophoresis were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). GTP[S] (tetralithium salt) was obtained from Boehringer Mannheim Canada (Dorval, Que., Canada). A sample of bovine brain G_o (containing some G_i) was provided by Dr. L. Birnbaumer of Baylor College of Medicine

Abbreviations used: G-proteins, guanine nucleotide-binding proteins involved in signal transduction, including G_s , G_i and G_o ; G_n proteins, GTP-binding proteins detected on nitrocellulose blots; p[NH]ppA, adenosine 5'-[β -imidio]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; PMSF, phenylmethanesulphonyl fluoride.

(Houston, TX, U.S.A.), and a sample of bovine brain ADP-ribosylation factor [20] by Dr. R. A. Kahn of the National Cancer Institute (Bethesda, MD, U.S.A.) Pure fusion protein from the expression of v-Ha-ras in *Escherichia coli* [21] was given by Dr. J. B. Gibbs and Dr. I. S. Sigal of Merck Sharp and Dohme Research Laboratories (West Point, PA, U.S.A.).

Preparation of platelet and red-cell membranes and of tissue fractions

Rabbit, human and rat platelets were washed three times in a medium containing 13 mM-trisodium citrate, 5 mM-dextrose and 135 mM-NaCl (pH 6.5) [22] and were finally resuspended at 3×10^9 /ml in buffer at 0 °C containing 100 mM-Tris/HCl, pH 7.5, 5 mM-EDTA and 0.1 mM-PMSF (Buffer A). These suspensions were frozen and thawed twice (with solid CO₂/methanol). Platelet membranes were then usually isolated by centrifugation at 37 500 *g*_{av.} for 40 min at 4 °C and washed once by resuspension and centrifugation in Buffer A. Membranes were finally resuspended in Buffer A and frozen at -50 °C until used. When both particulate and soluble fractions were studied, rabbit platelets were lysed by freezing and thawing as described above, or by sonication [23], and the lysates were centrifuged at 100 000 *g*_{av.} for 60 min at 4 °C. Rabbit and human red-cell membranes were prepared as in [24]. To prepare rat tissue homogenates, diethyl-ether-anaesthetized rats were perfused with Locke's solution containing 20 nM-prostacyclin and 10 units of heparin/ml to clear blood and platelets from the tissues. Individual organs were immediately frozen in liquid N₂ and stored at -50 °C. When required, 0.5 g of each was homogenized in Buffer A for two 10 s periods, with a Polytron homogenizer. For preparation of particulate and supernatant fractions from rat brain, homogenate was centrifuged at 100 000 *g*_{av.} for 60 min at 4 °C.

SDS/polyacrylamide-gel electrophoresis and blotting of proteins

Samples of membrane suspension or tissue homogenate were precipitated with 10% (w/v) trichloroacetic acid for both determination of protein [25] and SDS/polyacrylamide-gel electrophoresis. In the latter case, protein pellets were solubilized in 50 µl of electrophoresis sample buffer [26]. After neutralization of residual acid, the samples were heated at 100 °C for 3 min and analysed by SDS/polyacrylamide-slab-gel electrophoresis, with 13% (w/v) acrylamide in the separating gel [26,27]. Gels were incubated for 30 min in a buffer, pH 8.3, containing 25 mM-Tris, 192 mM-glycine, 20% (v/v) methanol and 0, 0.025 or 0.05% SDS, and the resolved polypeptides were then transferred to nitrocellulose by electroblotting for 16 h at 30 V in the same buffer [28].

Incubation of blots with [α -³²P]GTP

The procedure was a modification of a method used for labelling *ras* p21 proteins [29]. Nitrocellulose blots were usually incubated for 30 min at 20 °C in buffer containing 50 mM-Tris/HCl, pH 7.5, 0.3% Tween 20, 2 µM-MgCl₂ and 1.0 µCi of [α -³²P]GTP/ml (final concn. 1.0 nM). Exceptions to this procedure are indicated in the Figure legends. After washing twice in the same buffer without [α -³²P]GTP, blots were air-dried and bound ³²P was detected by autoradiography (12–72 h at -50 °C, by

using a Cronex intensifying screen from Picker International, Brampton, Ont., Canada). In some experiments, bound ³²P was determined by counting Čerenkov radiation [27]. For molecular-mass determinations, protein standards were electrophoresed in parallel with the experimental samples. After autoradiography, blots were stained with Amido Black 10B [30].

RESULTS AND DISCUSSION

In an attempt to detect GTP-binding proteins in cell membranes, nitrocellulose blots of membrane proteins resolved by SDS/polyacrylamide-gel electrophoresis were incubated with [α -³²P]GTP. Under the conditions described in the Experimental section, a family of 23–27 kDa proteins was specifically labelled (Fig. 1). We have named these GTP-binding proteins 'G_n proteins', to reflect the fact that, even after electrophoresis and blotting on to nitrocellulose, they were able to bind guanine nucleotides with high affinity.

Rabbit and human platelet membranes contained a major 27 kDa labelled species (G_n27) and lesser amounts of a 24 kDa species (G_n24) (Fig. 1, lanes *a* and *b*). Traces of 25 kDa and 23 kDa G_n proteins were also observed. Rabbit and human red-cell membranes contained only G_n27 (Fig. 1, lanes *c* and *d*). Preparation of platelet

Fig. 1. Detection of GTP-binding proteins on nitrocellulose blots and determination of their molecular masses

Membrane protein (75 µg) was electrophoresed and blotted on to nitrocellulose using transfer buffer containing 0.025% SDS. The blot was then incubated with 0.3 nM- [α -³²P]GTP and 0.1 µM-MgCl₂, as described in the Experimental section. The lanes on the autoradiograph show labelled membrane proteins from: (*a*) rabbit platelets; (*b*) human platelets; (*c*) rabbit red cells; (*d*) human red cells. The molecular masses of labelled proteins were calculated from standards running with the mobilities shown on the right.

Fig. 2. Effects of the blotting and incubation conditions on the labelling of G_n proteins with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$

Samples of rabbit platelet membrane protein (70 μg) were electrophoresed and blotted on to nitrocellulose using transfer buffer containing no SDS (lanes *a*–*c*), 0.025% SDS (lanes *d*–*f*) or 0.05% SDS (lanes *g*–*i*). The blots were then incubated with 1.0 nM- $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ and 2 μM - MgCl_2 (lanes *a*, *d* and *g*), 5 mM- MgCl_2 (lanes *b*, *e* and *h*) or 5 mM-EDTA (lanes *c*, *f* and *i*). An autoradiograph is shown.

membranes in the presence of additional proteolytic inhibitors (0.2 mM-leupeptin and 1 mM-benzamidine) did not affect the pattern of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ binding (results not shown), suggesting that, even if the lower- M_r G_n proteins are proteolytic fragments of G_n27 , they are present in intact platelets. Investigation of the experimental conditions affecting the detection of G_n proteins showed that inclusion of 0.025–0.05% SDS in the transfer buffer yielded an optimal recovery of G_n proteins on nitrocellulose (e.g. Fig. 2, lanes *a*, *d* and *g*). Addition of 0.1–2.0 μM - MgCl_2 during incubation of blots with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ gave good labelling of both G_n27 and G_n24 (Figs. 1 and 2), but, in the presence of 5 mM-EDTA, binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to the G_n proteins was abolished (Figs. 2, lanes *c*, *f* and *i*). Addition of 5 mM- MgCl_2 decreased the labelling of G_n24 , though not of G_n27 (Figs. 2, lanes *b*, *e* and *h*). These results suggest that Mg^{2+} ions are required for binding of GTP by the G_n proteins, though high Mg^{2+} concentrations may decrease the affinity of G_n24 for GTP. Incubation of the labelled blots with buffer containing 2% SDS led to removal of bound ^{32}P without major loss of protein, indicating a non-covalent interaction. Binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to rabbit platelet membranes was specific in that it was unaffected by the addition of 10 μM -GMP or 10 μM -p[NH]ppA to the incubation medium and only partly blocked by 10 μM -ATP (Fig. 3). However, 0.1 μM -GDP, -GTP[S] or -GTP diminished binding substantially, and 10 μM -GDP, -GTP[S] or -GTP abolished binding (Fig. 3).

In a survey of whole rat tissues, the largest amounts of G_n protein were found in brain, which contained one

Fig. 3. Specificity of nucleotide binding by rabbit platelet G_n proteins

Samples of rabbit platelet membrane protein (70 μg) were electrophoresed and blotted on to nitrocellulose by using transfer buffer containing 0.05% SDS. Strips containing individual samples were incubated with 1 nM- $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ and 2 μM - MgCl_2 in the absence or presence of other nucleotides, and the autoradiographs shown were developed. Lanes are for the following treatments: (*a*) no other nucleotide; (*b*) 10 μM -GMP; (*c*) 10 μM -p[NH]ppA; (*d*) 10 μM -ATP; (*e*) 0.10 μM -GDP; (*f*) 0.10 μM -GTP[S]; (*g*) 0.10 μM -GTP; (*h*) 10 μM -GDP; (*i*) 10 μM -GTP[S]; (*j*) 10 μM -GTP.

Fig. 4. Detection of G_n proteins in different rat tissues

Samples of protein (100 μg) from rabbit platelet membranes and homogenates of various rat tissues were electrophoresed and blotted on to nitrocellulose by using transfer buffer containing 0.05% SDS. The blot was incubated with 1 nM- $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ and 2 μM - MgCl_2 , and the above autoradiograph was developed. Lanes show protein from (*a*) rabbit platelet membranes and (*b*–*j*) rat tissue homogenates, as follows: (*b*) platelets; (*c*) lung; (*d*) liver; (*e*) brain; (*f*) heart; (*g*) spleen; (*h*) adrenal gland; (*i*) kidney; (*j*) skeletal muscle.

species with the same M_r as platelet G_n27 , and another species, of 26 kDa (G_n26), distinct from those observed in platelets (Fig. 4). Although the amounts present varied widely, G_n proteins were found in all tissues examined (Fig. 4). The specific activity of the total G_n protein in each tissue (i.e. c.p.m. of $^{32}\text{P}]\text{GTP}$ bound to nitrocellulose/100 μg of protein) was expressed as a percentage of that found in brain. Values obtained (means $\pm$ S.E.M., four determinations) were as follows: platelets, $44 \pm 6\%$; adrenal gland, $26 \pm 6\%$; lung, $25 \pm 4\%$; kidney, $11 \pm 4\%$; spleen, $10 \pm 2\%$; liver, $8 \pm 3\%$; heart muscle, $7 \pm 2\%$; skeletal muscle, $5 \pm 1\%$. The specific activity of G_n proteins in rabbit platelet membranes was $74 \pm 9\%$ of that in whole rat brain. The subcellular distribution of G_n proteins was studied in rabbit platelets and rat brain (Fig. 5). The specific activity of G_n protein in the 100000 g_{av} fraction of sonicated platelets was $11 \pm 1\%$ (mean $\pm$ S.E.M., four determinations) of that in the particulate fraction. Prolonged exposure of autoradiographs revealed traces of both G_n27 and G_n24 in the soluble fraction. Larger amounts of soluble G_n protein were detected when EDTA and PMSF were omitted from Buffer A, suggesting that membrane-bound G_n proteins can be released by proteolysis (results not shown). In brain, the specific activity of G_n protein in the soluble fraction was 26% of that in the particulate fraction (mean of two determinations). The soluble fraction contained G_n26 and traces of G_n24 , but no G_n27 (Fig. 5).

The G_n proteins were compared with other known GTP-binding proteins. A sample containing purified G_i and G_o from bovine brain (1 μg) was electrophoresed and blotted on nitrocellulose, but no binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ in the 39–41 kDa range was detected (result not shown). This may reflect irreversible denaturation of these proteins during SDS/polyacrylamide-gel electrophoresis. A sample (1 μg) of bovine brain ADP-ribosylation factor, a 21 kDa protein that binds GTP only in the presence of Mg^{2+} ions [20], was treated similarly and was also not detected. In contrast, v-Ha-*ras* fusion protein (0.5 μg) gave a signal similar in strength to that obtained from the G_n proteins in 70 μg of rabbit platelet membrane protein (results not shown). This fusion protein migrated with an apparent molecular mass of 27 kDa, similar to the value of 28 kDa reported previously [21]. However, as the known cellular *ras* gene products have molecular masses of 21 kDa [11], the G_n proteins are clearly distinct. Furthermore, a monoclonal

Fig. 5. Subcellular distribution of G_n proteins in rabbit platelets and rat brain

This autoradiograph shows the G_n proteins present in membrane fractions (lanes *a*, *c* and *e*) and supernatant fractions (lanes *b*, *d* and *f*) prepared from rabbit platelets lysed either by freezing and thawing (lanes *a* and *b*) or by sonication (lanes *c* and *d*), or prepared from rat brain subjected to Polytron homogenization (lanes *e* and *f*). Samples containing 70 μ g of protein were analysed by SDS/polyacrylamide-gel electrophoresis and blotted on to nitrocellulose by using transfer buffer containing 0.05% SDS. The blot was incubated with 1 nM- $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ and 2 $\mu\text{M-MgCl}_2$.

antibody that recognizes all known *ras* p21 proteins did not cross-react with the G_n proteins (R. P. Bhullar & R. J. Haslam, unpublished work). Our method was not sufficiently sensitive to detect *ras* p21 proteins in tissue samples. It is not clear whether the G_n proteins have previously been detected by others, using different techniques. However, a 25 kDa protein can be photo-affinity-labelled with azido-GTP in rat liver membranes [31]. In addition, Evans *et al.* [10] have purified a G-protein with an α -subunit of 21 kDa from human placental membranes; they indicated that this protein was also present in platelets. Martin *et al.* [32] have briefly reported the isolation from bovine brain of a similar protein with a 25 kDa α -subunit. Thus, although the molecular masses of the G_n and placental proteins appear very different, we cannot exclude the possibility that they are related.

The specific and high-affinity Mg^{2+} -dependent binding of GTP by the G_n proteins and their predominant location in cell membranes suggest that these proteins may function analogously to the α -subunits of the classical G-proteins and thus have a role in pertussis-toxin-insensitive signal transduction. As no major pertussis-toxin substrates outside the 39–41 kDa range have been observed in platelets or other cells [3,13,33], the G_n proteins are candidates for mediation of the toxin-resistant activation of phosphoinositidase C, as well as other less well-understood transduction processes. In the platelet, the latter include the opening of receptor-operated Ca^{2+} channels in the plasma membrane [34], the stimulation of Na^+/H^+ exchange by adrenaline and ADP [35], and possibly the activation of phospholipase A_2 at low cytosolic Ca^{2+} concentrations [35,36]. The ready detection of G_n proteins on nitrocellulose blots provides a specific assay for these proteins comparable with ADP-ribosylation of G-proteins by bacterial toxins or use of specific antibodies and should facilitate their purification and the analysis of their functions.

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