G-proteins are distinct from ras p21 and other known low molecular mass GTP-binding proteins in the platelet

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The 27 kDa platelet membrane protein (G,27) that binds [α-32P]GTP on nitrocellulose blots of SDS-polyacrylamide gels ([1987] Biochem. J. 245, 617–620) was compared with other low molecular mass GTP-binding proteins. Platelet membranes also contained 21 kDa proteins that bound anti-ras p21 antibody and 22–23 kDa proteins that could be ADP-ribosylated by botulinum neurotoxin type D. These groups of proteins were resolved electrophoretically from each other and from G,27. A low molecular mass GTP-binding protein from bovine brain ([1987] Biochem. J. 246, 431–439) was also resolved from G,27. At the levels normally present in cell membranes, only G,-proteins bound significant amounts of [32P]GTP after transfer of protein from SDS-polyacrylamide gels to nitrocellulose.

G,-protein; GTP-binding protein; ras p21 protein; Botulinum neurotoxin; Platelet

1. INTRODUCTION

We have identified a family of GTP-binding proteins that can be specifically labelled by [α-32P]-GTP on nitrocellulose blots of human and rabbit platelet membrane proteins that have been separated by SDS-polyacrylamide gel electrophoresis [1]. These proteins, which we have termed G,-proteins [1], are widely distributed and consist of a major 27 kDa species (G,27) and variable amounts of 23–26 kDa species, depending on the cell type or tissue studied. In the rat, G,27 was the most abundant in brain and substantial amounts were also found in lung, spleen, kidney and adrenal gland [1]. More recently, G,-proteins have also been identified in membranes from mouse tissues and NIH3T3 cells [4], neutrophils [5] and plant hypocotyl [6]. The functions of G,-proteins are not known, but it has been suggested that they could play a role in pertussis toxin-insensitive signal transduction [1,7].

Several low molecular mass GTP-binding proteins of uncertain relationship to the G,-proteins have been identified in mammalian cells. In particular, the 21 kDa products of three closely related and highly conserved ras genes (H-, K- and N-ras), which are present in mutant forms in many human cancers [8], have been shown to be present in variable amounts in many normal tissues [9]. Moreover, ras p21 proteins have been reported to bind GTP after transfer to nitrocellulose blots [10]. In addition, 21–24 kDa membrane proteins present in brain, platelets and some other tissues [11,12] are ADP-ribosylated by certain botulinum neurotoxins in a manner that is stimulated by GTP [13,14] or inhibited by GTP[S] [14,15], suggesting that these proteins bind GTP. A further protein with a low molecular mass GTP-binding subunit has been purified from human placenta and platelets (designated Gp) [16] and appears to be identical to a protein purified from bovine brain that contains a 25 kDa GTP-binding component (G,25) [17]. A similar protein has also been isolated from bovine...
brain by Kikuchi et al. [18]. Here, we have compared platelet G\textsubscript{\alpha}-proteins with \textit{ras} p21, botulinum neurotoxin type D substrates and G\textsubscript{\alpha}25.

2. EXPERIMENTAL

2.1. Materials

Rabbit and human platelet membranes were prepared as in [1]. Prestained proteins for use as molecular mass standards on SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Mississauga, Ontario). Anti-\textit{ras} p21 pan-reactive monoclonal antibody and immunoperoxidase reagents were from Cetus Diagnostics (Emeryville, CA). Samples of pure fusion protein from the expression of \textit{v-H-ras} in \textit{E. coli} [19] and membranes from yeast cells (\textit{Saccharomyces cerevisiae}) overexpressing \textit{v-H-ras} but deficient in the endogenous \textit{RAS} genes were kindly provided by Dr J.B. Gibbs and Dr I.S. Sigal, Merck Sharp & Dohme Research Laboratories (West Point, PA). [adenylate-\textsuperscript{32P}]NAD\textsuperscript{+} (42 Ci/mmol) was from New England Nuclear (Lachine, Quebec) and botulinum neurotoxin type D from Wako (Houston, TX). Purified low molecular mass GTP-binding protein from bovine brain [17] was a generous gift from Dr T.K. Harden (University of North Carolina School of Medicine, Chapel Hill, NC). Other materials were from sources listed elsewhere [1].

2.2. SDS-polyacrylamide gel electrophoresis and nitrocellulose blotting

Samples of platelet membrane suspension were precipitated with trichloroacetic acid for both protein determination [20] and SDS-polyacrylamide gel electrophoresis on 20 cm 13% acrylamide gels [1,21]. To minimize use of immunoreagents and SDS, samples were electrophoresed at 200 V for 45 min using a Bio-Rad mini-protean slab gel apparatus. Gels were incubated for 30 min in transfer buffer with or without 0.05% SDS [1] and the resolved polypeptides were blotted onto nitrocellulose [22]. SDS was omitted from the transfer buffer when blots were used for detection of \textit{ras} p21 proteins.

2.3. Detection of \textit{G\alpha}-proteins on nitrocellulose

Blots were incubated with 1 nM [\textgtp{\alpha}]-\textsuperscript{32P}]GTP (1 \textmu Ci/ml) in the presence of 2 \textmu M MgCl\textsubscript{2}, washed, air-dried and subjected to autoradiography, as described [1]. The location of bound protein was then determined by staining with amido black 10B [23].

2.4. Detection of \textit{ras} p21 proteins

Nitrocellulose blots were incubated overnight at 4°C with a 1:100 dilution of anti-\textit{ras} p21 monoclonal antibody raised against a peptide comprising residues 29-44 of normal \textit{ras} p21 proteins. After washing the nitrocellulose, bound antibody was detected using goat anti-mouse IgG-horseradish peroxidase conjugate and tetramethylbenzidine [24].

2.5. ADP-ribosylation of platelet membrane proteins by botulinum neurotoxin D

A modification of the procedure of Ohashi et al. [13] was used. The reaction mixture (200 \mu l) contained 100 mM Tris-HCl, pH 8.0, 10 mM thymidine, 5 mM DTT, 1 mM NAD\textsuperscript{+}, 1 mM MgCl\textsubscript{2}, 10 \mu M [\textsuperscript{32P}]NAD\textsuperscript{+} (5 \textmu Ci/assay) and 75-150 \mu g rabbit platelet membrane protein. The reaction was started by the addition of 10 \mu g toxin followed by incubation at 30°C for 45 min and was stopped by addition of 100 \mu l of 30% (w/v) trichloroacetic acid. Precipitated protein was analyzed by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose.

3. RESULTS

We have compared the electrophoretic mobilities of \textit{ras} p21 and \textit{G\alpha}-proteins after their transfer to nitrocellulose, using as probes both [\textsuperscript{32P}]GTP (fig.1A) and a monoclonal antibody that recognizes all \textit{ras} proteins (fig.1B). Pure \textit{v-H-ras} fusion protein (0.1 \mu g) with an apparent molecular mass of 28 kDa [19] bound little [\textsuperscript{32P}]GTP (fig.1A, lane a) but gave a strong immunoperoxidase signal (fig.1B, lane a). In contrast, rabbit and human platelet membrane protein (75 \mu g) contained \textit{G\alpha}-proteins that bound far more [\textsuperscript{32P}]GTP (fig.1A, lanes b,c). Two closely-spaced \textit{ras}-immunoreactive proteins were also detected in these platelet membranes but had apparent molecular masses of about 21 kDa, significantly less than those of the \textit{G\alpha}-proteins (fig.1B, lanes b,c). The amounts of \textit{ras} p21 proteins present in platelet membranes were too small to be detected by [\textsuperscript{32P}]GTP binding. Membranes from yeast cells deficient in the endogenous \textit{RAS} genes but overexpressing \textit{v-H-ras
Fig. 2. Comparison of the electrophoretic mobilities of the botulinum neurotoxin D substrates and Gα-proteins found in rabbit platelet membranes. Protein (75 μg) from platelet membranes [32P]ADP-ribosylated by botulinum toxin D and from untreated control membranes was electrophoresed and blotted onto nitrocellulose. To detect Gα-proteins, the half of the blot containing control membrane protein was incubated with [γ-32P]GTP. The autoradiograph above shows: (a) [32P]ADP-ribosylated proteins and (b) Gα-proteins. The positions of prestained molecular mass marker proteins are indicated.

also showed several immunoreactive bands in the 17–22 kDa range (fig. 1B, lane d) but no [32P]GTP labelling of proteins of these or higher molecular masses (fig. 1A, lane d). These results indicate the high specificity of this method for the detection of Gα-proteins and show that Gα and ras p21 are both present as distinct species in platelet membranes.

The mobilities of the botulinum neurotoxin type D substrates and Gα-proteins from rabbit platelet membranes were also compared by SDS-polyacrylamide gel electrophoresis. As shown in fig. 2, the toxin ADP-ribosylated major 22–23 kDa and minor 25 kDa platelet proteins (lane a), all of which were well-separated from the major Gα-protein (Gα27) (lane h). Moreover, these botulinum toxin type D substrates migrated more slowly on SDS-polyacrylamide gels than did the ras p21 proteins present in the same membrane preparation (fig. 3, lanes a,b). ADP-ribosylation of the toxin substrates did not affect the migration of the ras-immunoreactive proteins. Purified v-H-ras fusion protein also was not a substrate for the type D toxin, as previously noted by Adam-Vizi et al. [25].

Purified bovine brain protein containing a 25 kDa component (Gα25) that could be photoaffinity-labelled by 8-azido-[γ-32P]GTP [17], was compared with the platelet Gα-proteins. After SDS-polyacrylamide gel electrophoresis and nitrocellulose blotting, Gα25 no longer bound [32P]GTP. However, a trace protein present in the preparation that migrated with the same mobility as Gα27 was highly labelled (fig. 4). This again confirms the specificity of the technique for detection of Gα25 and shows that Gα27 is distinct from Gα25.

We also found that Gα25 was not ADP-ribosylated by botulinum toxin type D (not shown).

4. DISCUSSION

We have sought to clarify the relationship between Gα-proteins and other low molecular mass GTP-binding proteins in the platelet. The results
Moreover, Gp does not appear to be a ras p21 protein [16]. We are thus led to conclude that platelets contain at least four separate families of low molecular mass GTP-binding proteins, namely Gα-proteins, ras gene products, botulinum toxin substrates and Gp/Gα25. Several other ras-related genes have also been identified in mammalian cells [26–29] but it remains to be determined whether their products are found in platelets or are identical to any of the proteins discussed above.

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REFERENCES
