Volume 237, number 1,2, 168-172 FEB 06299 September 1988

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

Rajinder P. Bhullar* and Richard J. Haslam*+

*Departments of *Biochemistry* and +*Pathology, McMaster University, Hamilton, Ontario L8N 3Z5, Canada*

Received 7 July 1988

The 27 kDa platelet membrane protein (G_n27) that binds [α-^{32}P]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_n27. A low molecular mass GTP-binding protein from bovine brain [(1987) Biochem. J. 246, 431–439] was also resolved from G_n27. At the levels normally present in cell membranes, only G_n-proteins bound significant amounts of [^{32}P]GTP after transfer of protein from SDS-polyacrylamide gels to nitrocellulose.

G_n-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-^{32}P]-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_n-proteins [1], are widely distributed and consist of a major 27 kDa species (G_n27) and variable amounts of 23–26 kDa species, depending on the cell type or tissue studied. In the rat, G_n27 was most abundant in brain and substantial amounts were also found in lung, spleen, kidney and adrenal gland [1]. More recently, G_n-proteins have also been identified in membranes from mouse tissues and NIH3T3 cells [4], neutrophils [5] and plant hypocotyl [6]. The functions of G_n-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_n-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 G_p) [16] and appears to be identical to a protein purified from bovine brain that contains a 25 kDa GTP-binding component ($G_{\alpha 25}$) [17]. A similar protein has also been isolated from bovine

Correspondence address: R.J. Haslam, Department of Pathology, McMaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada

Abbreviations: G_n-proteins, 23–27 kDa proteins detected by binding of [^{32}P]GTP to nitrocellulose blots [1]. A pertussis toxin-sensitive neutrophil G-protein with an α-subunit of 40 kDa has also been designated G_n [2], but is probably identical to G_{i2} [3]

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

brain by Kikuchi et al. [18]. Here, we have compared platelet G_n-proteins with *ras* p21, botulinum neurotoxin type D substrates and $G_{\alpha 25}$.

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-*ras* p21 pan-reactive monoclonal antibody and immunoperoxidase reagents were from Cetus Diagnostics (Emeryville, CA). Samples of pure fusion protein from the expression of v-H-*ras* in *E. coli* [19] and membranes from yeast cells (*Saccharomyces cerevisiae*) overexpressing v-H-*ras* but deficient in the endogenous *RAS* genes were kindly provided by Dr J.B. Gibbs and Dr I.S. Sigal, Merck Sharp & Dohme Research Laboratories (West Point, PA). [*adenylate*-^{32}P]NAD^+ (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 $G_{\alpha 25}$, some 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 *ras* p21 proteins.

2.3. *Detection of G_n-proteins on nitrocellulose*

Blots were incubated with 1 nM [α-^{32}P]GTP (1 μCi/ml) in the presence of 2 μM $MgCl_2$, 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* ras *p21 proteins*

Nitrocellulose blots were incubated overnight at 4°C with a 1:100 dilution of anti-*ras* p21 monoclonal antibody raised against a peptide comprising residues 29–44 of normal *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 μl) contained 100 mM Tris-HCl, pH 8.0, 10 mM thymidine, 5 mM DTT, 1 mM $NADP^+$, 1 mM $MgCl_2$, 10 μM [^{32}P]NAD^+ (5 μCi/assay) and 75–150 μg rabbit platelet membrane protein. The reaction was started by the addition of 10 μg toxin followed by incubation at 30°C for 45 min and was stopped by addition of 100 μl 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 *ras* p21 and G_n-proteins after their transfer to nitrocellulose, using as probes both [^{32}P]GTP (fig.1A) and a monoclonal antibody that recognizes all *ras* proteins (fig.1B). Pure v-H-*ras* fusion protein (0.1 μg) with an apparent molecular mass of 28 kDa [19] bound little [^{32}P]GTP (fig.1A, lane a) but gave a strong immunoperoxidase signal (fig.1B, lane a). In contrast, rabbit and human platelet membrane protein (75 μg) contained G_n-proteins that bound far more [^{32}P]GTP (fig.1A, lanes b,c). Two closely-spaced *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 G_n-proteins (fig.1B, lanes b,c). The amounts of *ras* p21 proteins present in platelet membranes were too small to be detected by [^{32}P]GTP binding. Membranes from yeast cells deficient in the endogenous *RAS* genes but overexpressing v-H-*ras*

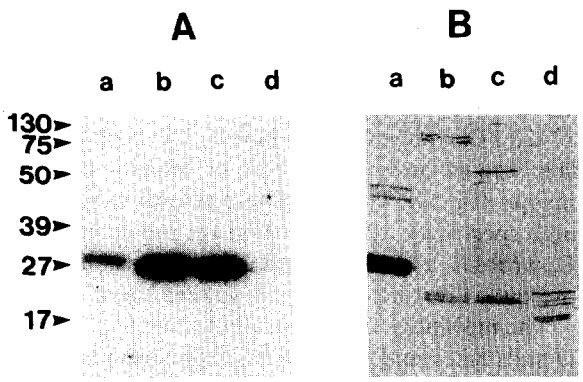

Fig.1. Relationship between the electrophoretic mobilities of the proteins detected on nitrocellulose blots by [^{32}P]GTP-binding and by a monoclonal antibody to *ras* p21 proteins. Pure v-H-*ras* fusion protein (0.1 μg, lane a) and membrane protein from rabbit platelets (75 μg, lane b), human platelets (75 μg, lane c) and yeast cells overexpressing v-H-*ras* p21 (50 μg, lane d) were electrophoresed and blotted onto nitrocellulose. (A) Autoradiograph of a blot probed with [^{32}P]GTP, (B) immunoperoxidase-stained blot probed with anti-p21 monoclonal antibody. The positions of prestained molecular mass marker proteins are shown on the left.

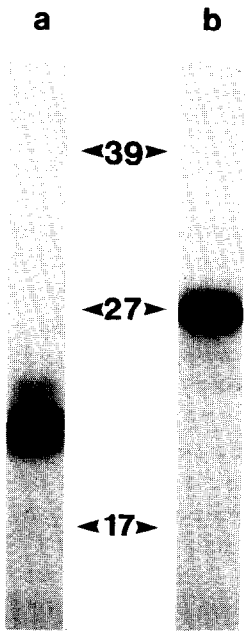

Fig.2. Comparison of the electrophoretic mobilities of the botulinum neurotoxin D substrates and G_n-proteins found in rabbit platelet membranes. Protein (75 μg) from platelet membranes [^{32}P]ADP-ribosylated by botulinum toxin D and from untreated control membranes was electrophoresed and blotted onto nitrocellulose. To detect G_n-proteins, the half of the blot containing control membrane protein was incubated with [^{32}P]-GTP. The autoradiograph above shows: (a) [^{32}P]ADP-ribosylated proteins and (b) G_n-proteins. The positions of prestained molecular mass marker proteins are indicated.

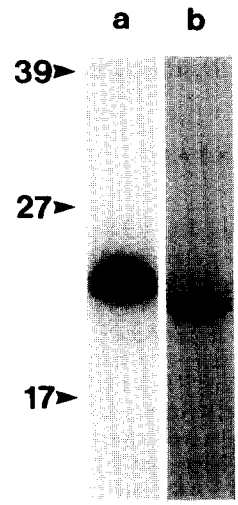

Fig.3. Comparison of the electrophoretic mobilities of the botulinum neurotoxin D substrates and *ras* p21 proteins found in rabbit platelet membranes. [^{32}P]ADP-ribosylated membrane protein (150 μg) was electrophoresed and blotted onto nitrocellulose. Before developing an autoradiograph, the blot was incubated with anti-p21 monoclonal antibody and immunoperoxidase reagents. (a) Autoradiograph showing [^{32}P]ADP-ribosylated proteins, (b) proteins detected using anti-p21 monoclonal antibody. The positions of prestained molecular mass marker proteins are shown.

also showed several immunoreactive bands in the 17–22 kDa range (fig.1B, lane d) but no [^{32}P]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 G_n-proteins and show that G_n and *ras* p21 are both present as distinct species in platelet membranes.

The mobilities of the botulinum neurotoxin type D substrates and G_n-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 G_n-protein (G_n27) (lane b). 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 ($G_{\alpha 25}$) that could be photoaffinity-labelled by 8-azido-[γ-^{32}P]GTP [17], was compared with the platelet G_n-proteins. After SDS-polyacrylamide gel electrophoresis and nitrocellulose blotting, $G_{\alpha 25}$ no longer bound [^{32}P]GTP. However, a trace protein present in the preparation that migrated with the same mobility as G_n27 was highly labelled (fig.4). This again confirms the specificity of the technique for detection of G_n-proteins and shows that G_n27 is distinct from $G_{\alpha 25}$. We also found that $G_{\alpha 25}$ was not ADP-ribosylated by botulinum toxin type D (not shown).

4. DISCUSSION

We have sought to clarify the relationship between G_n-proteins and other low molecular mass GTP-binding proteins in the platelet. The results

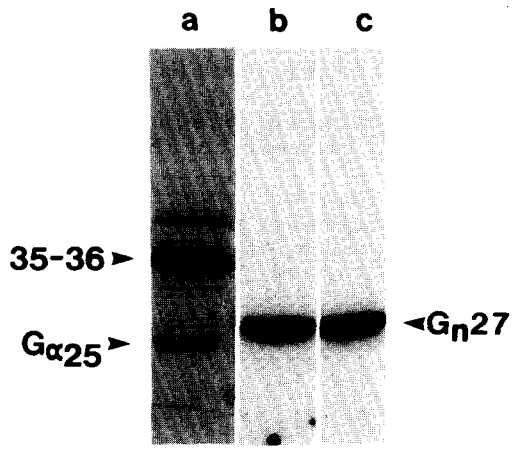

Fig.4. Comparison of the electrophoretic mobilities of bovine brain $G_{\alpha 25}$ and rabbit platelet G_n-proteins. Purified $G_{\alpha 25}$ (5 μg) and rabbit platelet membrane protein (75 μg) were electrophoresed and blotted onto nitrocellulose. The blot was then incubated with [^{32}P]GTP, subjected to autoradiography and stained with amido black. The stained $G_{\alpha 25}$ preparation (a), the corresponding autoradiograph (b) and an autoradiograph of platelet membrane protein (c), are shown.

provide the first evidence that platelets contain *ras* p21 proteins but show that these are widely separated from the G_n-proteins by SDS-polyacrylamide gel electrophoresis. Some evidence was obtained for the presence of two immunoreactive *ras* p21 species in the platelet. Since the antibody used was directed against *ras* p21 amino acid residues 29–44, which are identical in the products of the H-*ras*, K-*ras* and N-*ras* genes [8], we cannot specify which *ras* p21 proteins are expressed in platelets. We have also found that both the G_n- and *ras* p21 proteins are distinct from the platelet membrane proteins ADP-ribosylated on addition of botulinum neurotoxin type D. The latter, which have been detected in platelets previously [12,14, 15], appear to be GTP-binding proteins [15]. However, it should be noted that contaminating C3 toxin may actually be responsible for the ADP-ribosylating activity of type D toxin preparations [14]. Finally, the separation of platelet G_n27 from bovine brain $G_{\alpha 25}$ provides indirect evidence that G_n27 is distinct from the GTP-binding protein (G_p) isolated from human placenta and platelets by Evans et al. [16]. Thus, $G_{\alpha 25}$ co-electrophoresed with the placental protein and both of these were recognized by an antibody to platelet G_p [17]. Moreover, G_p 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 G_n-proteins, *ras* gene products, botulinum toxin substrates and $G_p/G_{\alpha 25}$. 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.

Acknowledgements: R.P.B. is the recipient of a Canadian Heart Foundation Fellowship. This work was supported by grants from the Heart and Stroke Foundation of Ontario (T.443) and the Medical Research Council of Canada (MT-5626).

REFERENCES

[1] Bhullar, R.P. and Haslam, R.J. (1987) Biochem. J. 245, 617–620.
[2] Gierschik, P., Sidiropoulos, D., Spiegel, A. and Jakobs, K.H. (1987) Eur. J. Biochem. 165, 185–194.
[3] Murphy, P.M., Eide, B., Goldsmith, P., Brann, M., Gierschik, P., Spiegel, A. and Malech, H.L. (1987) FEBS Lett. 221, 81–86.
[4] Nagahara, H., Nishimura, S., Sugimura, T. and Obata, H. (1987) Biochem. Biophys. Res. Commun. 149, 686–692.
[5] Bokoch, G.M. and Parkos, C.A. (1988) FEBS Lett. 227, 67–70.
[6] Drobak, B.K., Allan, E.F., Comerford, J.G., Roberts, K. and Dawson, A.P. (1988) Biochem. Biophys. Res. Commun. 150, 899–903.
[7] Lapetina, E.G. and Reep, B.R. (1987) Proc. Natl. Acad. Sci. USA 84, 2261–2265.
[8] Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827.
[9] Tanaka, T., Ida, N., Shimoda, H., Waki, C., Slamon, D.J. and Cline, M.J. (1986) Mol. Cell. Biochem. 70, 97–104.
[10] McGrath, J.P., Capon, D.J., Goeddel, D.V. and Levinson, A.D. (1984) Nature 310, 644–649.
[11] Ohashi, Y. and Narumiya, S. (1987) J. Biol. Chem. 262, 1430–1433.
[12] Aktories, K., Weller, U. and Chhatwal, G.S. (1987) FEBS Lett. 212, 109–113.
[13] Ohashi, Y., Kamiya, T., Fujiwara, M. and Narumiya, S. (1987) Biochem. Biophys. Res. Commun. 142, 1032–1038.
[14] Rošener, S., Chhatwal, G.S. and Aktories, K. (1987) FEBS Lett. 224, 38–42.
[15] Aktories, K. and Frevert, J. (1987) Biochem. J. 247, 363–368.
[16] Evans, T., Brown, M.L., Fraser, E.D. and Northup, J.K. (1986) J. Biol. Chem. 261, 7052–7059.
[17] Waldo, G.L., Evans, T., Fraser, E.D., Northup, J.K., Martin, M.W. and Harden, T.K. (1987) Biochem. J. 246, 431–439.

[18] Kikuchi, A., Yamashita, T., Kawata, M., Yamamoto, K., Ikeda, K., Tanimoto, T. and Takai, Y. (1988) J. Biol. Chem. 263, 2897-2904.
[19] Stein, R.B., Robinson, P.S. and Scolnick, E.M. (1984) J. Virol. 50, 343-351.
[20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
[21] Laemmli, U.K. (1970) Nature 227, 680-685.
[22] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
[23] Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502-514.
[24] Bos, E.S., Van der Doelen, A.A., Van Rooy, N. and Schuurs, A.H.W.M. (1981) J. Immunoassay 2, 187-204.
[25] Adam-Vizi, V., Knight, D. and Hall, A. (1987) Nature 328, 581.
[26] Madaule, P. and Axel, R. (1985) Cell 41, 31-40.
[27] Chardin, P. and Tavitian, A. (1986) EMBO J. 5, 2203-2208.
[28] Lowe, D.G., Capon, D.J., Delwart, E., Sakaguchi, A.Y., Naylor, S.L. and Goeddel, D.V. (1987) Cell 48, 137-146.
[29] Touchot, N., Chardin, P. and Tavitian, A. (1987) Proc. Natl. Acad. Sci. USA 84, 8210-8214.