The GTP-binding regulatory proteins of neuroblastoma × glioma, NG108–15, and glioma, C6, cells

Immunochemical evidence of a pertussis toxin substrate that is neither N_i nor N_o

Graeme Milligan, Peter Gierschik*, Allen M. Spiegel and Werner A. Klee+

Laboratory of Molecular Biology, National Institute of Mental Health and **Metabolic Diseases Branch, National Institute of Arthritis, Diabetes, Digestive and Kidney Disease, National Institute of Health, Bethesda, MD 20892, USA*

Received 20 November 1985

Amounts of the guanine nucleotide binding regulatory proteins which are also pertussis toxin substrates (such as N_i and N_o) were measured in rat glioma, C6BU-1, cells and in neuroblastoma × glioma, NG108–15, hybrid cells. Measurements were performed both by quantitating pertussis toxin catalyzed ADP-ribosylation and by quantitative immunoblotting with affinity purified antibodies specific for N_i or N_o. The amounts of pertussis toxin substrate in C6 and NG108-15 cells are 7.5 and 0.6 pmol/mg membrane protein, respectively. These levels are minimum values and higher estimates of the total amounts of N proteins in the two cells are obtained by quantitative immunoblot analysis of the β-subunit common to all N proteins. Immunoblots with specific antibodies show that NG108-15 cells contain 3.8 pmol/mg of N_o and detectable but small (<0.1 pmol/mg) amounts of N_i. In contrast, C6 cell membranes contain no detectable N_o and only 0.14 pmol/mg N_i. Thus, C6 cells contain large amounts of a pertussis toxin substrate which is neither N_i nor N_o.

GTP-binding protein *Immunoblotting* *Pertussis toxin* *ADP-ribosylation*

1. INTRODUCTION

A family of guanine nucleotide binding proteins, the N (or G) proteins, carries hormonal signals across cell membranes [1,2]. N_s and N_i couple hormone and neurotransmitter receptors to the stimulation (N_s) or inhibition (N_i) of adenylate cyclase and transducin (TD) couples rhodopsin to a cyclic GMP phosphodiesterase in rod outer segments [1,2]. Recently a novel N protein (N_o) has been identified as the major pertussis toxin substrate in brain [3–5] but its function has not yet been elucidated.

Bacterial toxins which catalyze ADP-ribosylation of the N proteins have been of particular importance to their study as this covalent modification alters their function. N_s and TD are substrates for cholera toxin [6] while TD, N_i and N_o are substrates for pertussis toxin [6]. All of the N proteins are heterotrimers with unique α- and, perhaps, γ-subunits and similar if not identical β-subunits [1,2].

The major pertussis toxin substrate of C6 glioma cells modulates adenylate cyclase activity [7] and has an α-subunit of M_r 40 000. We have previously described the production and use of antibodies with selectivity towards the α-subunits of N_i and N_o to identify these proteins [8,9]. In this report we use these antibodies to characterize the pertussis toxin substrates of NG108-15 and of C6 cell membranes. Our results show that the major N protein of NG108-15 membranes is N_o whereas that of C6

+ To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

cells differs from N_i, N_o and TD and, therefore, represents a distinct pertussis toxin substrate.

2. MATERIALS AND METHODS

Purified pertussis toxin [10] was the kind gift of Dr R. Sekura, National Institute of Child Health and Human Development. C6BU-1 glioma cells were grown in 850 cm^2 roller bottles in Dulbecco's Modified Eagle's Medium containing 4.5 g/l glucose and 20% (w/v) fetal calf serum [11]. Neuroblastoma × glioma NG108-15 cells were grown as described [12]. Cells were harvested at confluency and cell pastes or membranes [13] were stored at −70°C until used.

A highly purified mixture of N_i/N_o was prepared as described in [5]. Protein determination and pertussis toxin labelling were performed as described [5] except that membranes were not diluted into 20 mM Tris/HCl, 0.05% Lubrol, pH 8.0 before use. Incorporation of [^{32}P]ADP-ribose into protein was determined after SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography by cutting out bands from dried gels and counting in a liquid scintillation spectrophotometer. Immunoblots of SDS–PAGE resolved membrane proteins were performed as described [14]. Quantitative immunoblotting was performed as in [14] except that ^{125}I-labelled protein A (300000 cpm/ml, New England Nuclear) replaced the second antibody. After washing, the nitrocellulose papers were dried and mounted for autoradiography. Autoradiograms were used to localize bands which were cut out and counted. N protein concentrations were calculated from standard curves, generated on each gel slab of known amounts of bovine brain N_i/N_o. The validity of the quantitation procedure was confirmed by experiments in which the recovery of purified N_i/N_o was measured after its addition to crude membranes and compared to purified N_i/N_o alone.

Preparation and characterization of antisera CW6 and RV3 has been documented elsewhere [8,9]. Antibodies directed against N_i and N_o were affinity purified from these antisera by adding the sera diluted 1:3 in 50 mM glycine/HCl, pH 7.5, 500 mM NaCl (buffer A) to a column of sepharose to which purified N_i/N_o had been covalently coupled (09.5 mg N_i, 0.5 mg N_o/ml resin). 10 vols of diluted antiserum were used per volume of N protein resin which had been extensively pre-equilibrated with buffer A. After mixing overnight, the diluted antiserum was removed and the resin was washed with 25 vols of buffer A. Specifically bound antibodies were eluted from the resin with 100 mM glycine/HCl, pH 2.5, and 500 mM NaCl, immediately neutralized with 0.1 M NaOH and used as the first antibody at dilutions equivalent to a 1/100 dilution of original antiserum.

3. RESULTS

Membranes prepared from C6 cells were treated with [α-^{32}P]NAD in the presence or absence of activated pertussis toxin for various times. Toxin dependent incorporation of [^{32}P]ADP-ribose into protein was limited to a single band of M_r 40000 (fig.1a). Incorporation of radioactivity reached a maximal level of 7.5 pmol/mg protein after 4 h incubation at 37°C (fig.1b). This value is likely to represent a minimum estimate of the levels of pertussis toxin substrate of C6 membranes because the [α-^{32}P]NAD substrate is hydrolysed in the presence of C6 membranes at a rate of 15%/h. In parallel experiments with membranes from the neuroblastoma × glioma cell line NG108-15, where degradation of [α-^{32}P]NAD did not occur, ADP-ribosylation of pertussis toxin substrate had not reached a plateau by 4 h (fig.1b).

We attempted to identify the 40 kDa pertussis toxin substrate of C6 cells with specific antibodies against N_i or N_o. Antiserum RV3 contains antibodies directed against the α-subunit of N_o and the β-subunit common to all N-proteins. As little as 0.25 pmol N_o can be reproducibly detected with affinity purified antibodies from this antiserum. No α-subunit of N_o was detected in C6 membranes whereas a similar amount of membranes from NG08-15 cells produced a good signal corresponding to 3.8 pmol/mg protein (fig.2). The β-subunit in both C6 and NG108-15 membranes was easily detected.

We examined the possibility that quantitation of the α-subunit of N_o might be interfered with by other proteins in the C6 membranes. Recovery of the α-subunit of N_o was at least as good when purified N_o was added to C6 membranes before electrophoretic separation as when the purified proteins were run alone (fig.3).

Antiserum CW6, recognizes the α-subunits of N_i

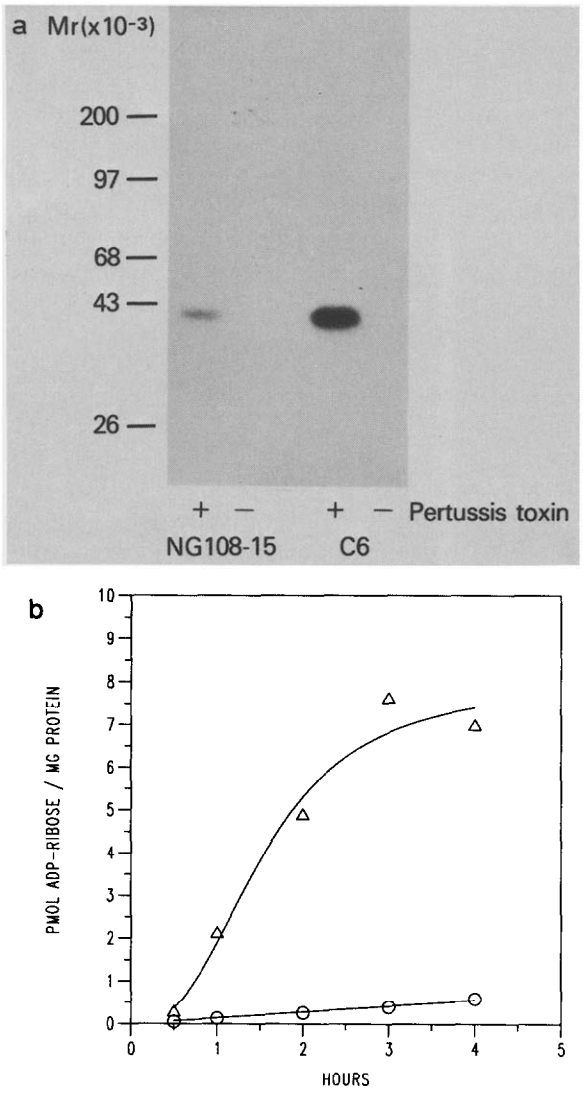


Fig.1. Pertussis toxin catalysed ADP-ribosylation of C6 and NG108-15 membranes (a) 5.75 μg C6 or NG108-15 membrane proteins were ADP-ribosylated in the presence or absence of preactivated pertussis toxin as described in section 2 for 1 h at 37°C. At the termination of incubation, protein was precipitated with deoxycholate/trichloroacetic acid [23]. After neutralization and addition of denaturing solution, samples were applied to a 10% SDS–PAGE and separated overnight at 50 V. The gel was silver stained [24] and dried and then subjected to autoradiography for 24 h. (b) C6 (5.75 μg protein (Δ) or NG108-15 (28.75 μg protein (○)) membranes were ADP-ribosylated in the presence of preactivated pertussis toxin for the times shown. After electrophoresis as above, the gel was stained with Coomassie blue, dried and autoradiographed. The autoradiograph so produced was used to localize the ADP-ribosylated proteins.

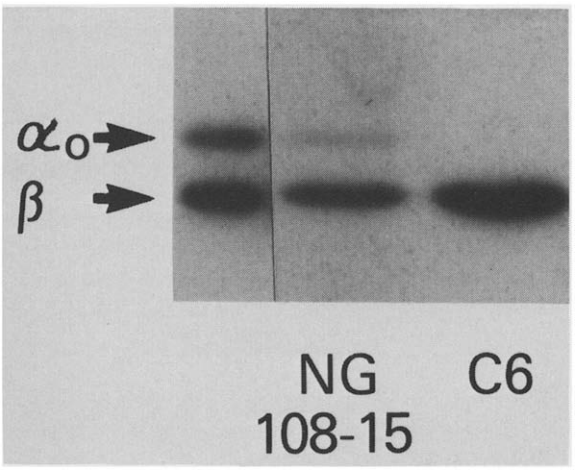


Fig.2. Detection of the α-subunit of N_o and β-subunits in membranes of C6 and NG108-15 cells. C6 and NG108-15 membranes (200 μg) were subjected to 10% SDS–PAGE and then immunoblotted with affinity purified antibodies against the α-subunit of N_o and β (derived from antiserum RV3). ^{125}I-labelled protein A was used in place of second antibody and detected by autoradiography. A standard lane containing 2.7 pmol N_o α-subunit and 7.1 pmol β-subunit is shown for reference.

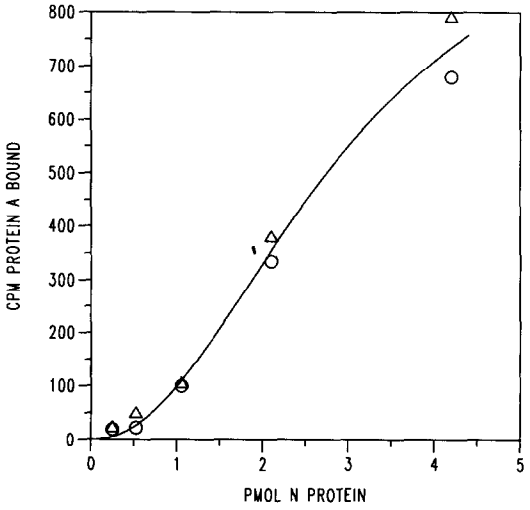
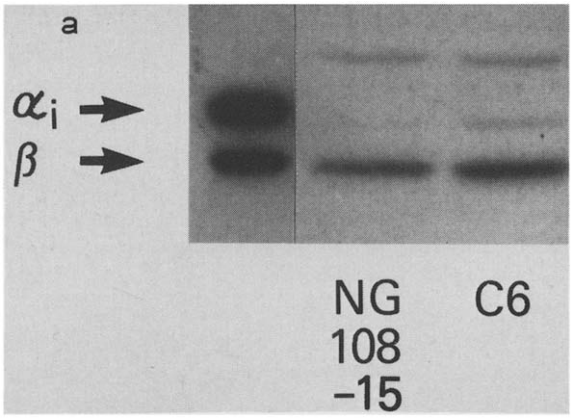


Fig.3. Quantitation of N proteins in the presence and absence of membrane proteins. N_o (0.25–4.2 pmol) was added to a 10% SDS–PAGE either alone (Δ) or in the presence of 200 μg C6 membrane protein (○). After resolution overnight at 50 V, the proteins were immunoblotted with affinity purified antibodies against N_o, followed by treatment with ^{125}I-protein A and then autoradiographed. Appropriate bands on the nitrocellulose were excised and counted.



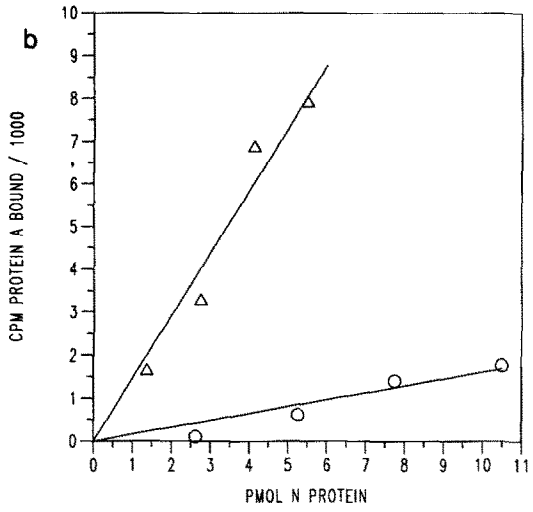


Fig.4. Detection of the α-subunit of N_i and β-subunits in membranes of C6 and NG108-15 cells. (a) C6 and NG108-15 membranes (200 μg protein) were subjected to 10% SDS-PAGE and then immunoblotted with affinity purified antibodies which recognize the α-subunit of N_i and β. ^{125}I-protein A was used in place of second antibody and detected by autoradiography. A standard lane containing 1.4 pmol N_i α-subunit and 7.1 pmol β-subunit is shown for reference. (b) After electrophoresis, N_i (1.4–5.5 pmol (△) and N_o (2.6–10.5 pmol (○)) were immunoblotted with affinity purified antibodies against the α-subunits of N_i or N_o and stained with ^{125}I-protein A. After autoradiography, appropriate bands were cut and counted. Standard curves similar to these were constructed for each individual experiment. Similar curves for β reactivity were also produced.

and TD strongly and of N_o very weakly (in addition to recognizing the common β-subunit). Antibody, affinity purified from this serum, weakly stains a band at M_r 39000 in C6 membranes (fig.4a). This band cannot represent the α-subunit of N_o because this would have been much more efficiently recognized by antibodies from antiserum RV3; it is unlikely to represent the α-subunit of TD as this protein is believed to be restricted to photoreceptor containing tissues. The band may represent the α-subunit of N_i, but if so, the incorporation of radioactivity (30 cpm) is less than 2% of that expected from the 1.5 pmol pertussis toxin substrate applied to the SDS-PAGE (2000 cpm, see fig.4b). A third band at M_r 45000 is also detected with affinity purified CW6 antibodies in C6 and in NG108-15 membranes (fig.4a); the significance of this staining is unclear. Weak, but positive staining in the α-subunit of N_i region with antibodies affinity purified from antiserum CW6 was observed in NG108-15 membranes (fig.4a); the levels, too low to quantitate accurately, are <0.1 pmol/mg protein.

Since the N proteins are all heterotrimeric and contain a common β-subunit, quantitation of this subunit using affinity purified antibodies should give a reliable estimate of total N protein concentration. Quantitation of binding of antibodies derived from antiserum RV3 indicates a β-subunit concentration of 17 pmol/mg protein in NG108-15 membranes, whereas the corresponding measurement with antibody purified from serum CW6 yields estimates of 14 pmol/mg protein. With membranes derived from C6 cells the same antibodies led to estimates of β-subunit concentration of 43 pmol/mg protein and 29 pmol protein, respectively. The latter values are approx. 4-times

Table 1

Amounts of N protein subunits determined by immunoblotting

	pmol/mg membrane protein	
Subunit	C6	NG108-15
α_i	0.15	<0.1
α_o	ND[a]	3.8
β	37	15

[a]Not detectable

higher than those estimated by pertussis toxin catalyzed ADP-ribosylation. Clearly, however, even if this lower estimate is used, only a small fraction of the pertussis toxin substrate of C6 cells can be accounted for by N_i, N_o, or TD. In table 1 we have summarized the results of our analyses of the N proteins in the 2 cell lines.

4. DISCUSSION

It was originally believed that pertussis toxin caused covalent modification of a single protein, the α-subunit of N_i [15,16]. Recent experiments have shown that a second pertussis toxin substrate, N_o, exists in brain [3–5] and in heart [17]. Antibodies capable of recognizing the α-subunit of one or another of these proteins have been produced and characterized [8,9,14,18]. The results presented here suggest that yet a third pertussis toxin substrate exists which may represent the major such protein in cells such as glioma C6.

In this report we have attempted to identify the pertussis toxin substrates of C6 and NG108-15 cell membranes. At least 7.5 pmol/mg protein of ADP-ribose was incorporated into C6 membranes upon incubation with pertussis toxin. This value is probably an underestimate because membrane-bound NADase significantly reduces the levels of substrate during this assay. With C6 membranes degradation of NAD proceeds at some 15%/h. Membranes of NG108-15 cells do not cause significant hydrolysis of NAD and have not reached maximal levels of ADP-ribose incorporation within 4 h at which time 0.6 pmol/mg protein have been incorporated. It is likely that ribosylation is equally incomplete in C6 membranes. The amount of β-subunit in C6 membranes, which is a measure of total N protein concentration, is 36 pmol/mg protein. Because N_s levels are usually much lower than those of N_i or N_o it is unlikely that many of the large number of β-subunits are associated with N_s. A similar discrepancy in the amounts of N proteins based upon estimates of β-subunit concentration or on pertussis toxin catalyzed ADP-ribosylation has also been observed in human neutrophils (Gierschik, P. et al., submitted), and our results with NG108-15 membranes also suggest that some N protein may be unaccounted for.

Affinity purified antibodies which recognize the α-subunit of N_o did not stain Western blots of C6 membrane protein. These antibodies easily detected the 3.8 pmol/mg N_o present in NG108-15 cells, which show less than 10% of the pertussis toxin catalyzed ADP-ribosylation than C6 membranes. Thus, N_o is either absent or below current levels of detection in C6 membranes. Antibodies which recognize the α-subunits of N_i and TD stain a 40 kDa band in C6 membranes. However, as judged against standards of purified N_i, this could account for less than 2% of the pertussis toxin substrate present. This band migrates in SDS-PAGE with a mobility slightly greater than that of the purified α-subunit of N_i. Possibly this is an artifact due to the disparity in protein concentrations in these preparations, since in mixing experiments, small quantities of the purified α-subunit of N_o added to C6 membranes also migrate slightly faster than the purified α-subunit of N_o alone (not shown). Under similar conditions, the β-subunit of C6 membranes also runs ahead of the β-subunit of purified N proteins.

It is unlikely that the inability of the antibodies to recognize N_o in C6 cell membranes is due to species diversity. Although the antisera were raised against bovine N proteins, and the C6 cell line is derived from rat, these antibodies have been shown to cross react over a wide range of vertebrate species, including rat [9].

Our conclusion from these experiments is that the pertussis toxin substrate of C6 membranes is predominantly a protein other than N_i or N_o. The weak reactivity observed with anti-N_i antibody suggests either that C6 also contains a small amount of N_i (0.14 pmol/mg membrane protein) or that the antibody weakly recognizes the novel N protein. Because pertussis toxin treatment of C6 cells leads to stimulation of adenylate cyclase activity, the novel N protein may interact with this effector system. However, all N proteins, which dissociate on activation to release the common β-subunit, would be expected to modulate adenylate cyclase activation by altering the equilibrium interaction between subunits of N_s [19]. The previously recognized pertussis toxin substrates are able to substitute for one another, to some extent, in functional assays [20–22]. Specificity of hormone response may be determined primarily by the receptor and could, therefore, be independent of the nature of the specific N protein present in any given cell.

REFERENCES

[1] Gilman, A.G. (1984) Cell 36, 577–579.

[2] Spiegel, A.M., Gierschik, P., Levine, M.A. and Downs, R.W. jr (1985) N. Engl. J. Med. 312, 26–33.

[3] Sternweis, P.C. and Robishaw, J.D. (1984) J. Biol. Chem. 259, 13806–13813.

[4] Neer, E.J., Lok, J.M. and Wolf, L.G. (1984) J. Biol. Chem. 259, 14222–14229.

[5] Milligan, G. and Klee, W.A. (1985) J. Biol. Chem. 260, 2057–2063.

[6] Milligan, G., Simonds, W.F., Streaty, R.A., Tocque, B. and Klee, W.A. (1985) Biochem. Soc. Trans. 13, 1110–1113.

[7] Katada, T. and Ui, M. (1982) J. Biol. Chem. 257, 7210–7216.

[8] Pines, M., Gierschik, P., Milligan, G., Klee, W.A. and Spiegel, A. (1985) Proc. Natl. Acad. Sci. USA 82, 4095–4099.

[9] Gierschik, P., Milligan, G., Pines, M., Codina, J., Klee, W. and Spiegel, A. (1986) Proc. Natl. Acad. Sci. USA, in press.

[10] Sekura, R.D., Fish, F., Manclark, C.R., Meade, B. and Zhang, Y.L. (1983) J. Biol. Chem. 258, 14647–14651.

[11] Amano, T., Hamprecht, B. and Kemper, W. (1974) Exp. Cell Res. 85, 399–408.

[12] Klee, W.A. and Nirenberg, M. (1974) Proc. Natl. Acad. Sci. USA 71, 3414–3477.

[13] Sharma, S., Nirenberg, M. and Klee, W.A. (1975) Proc. Natl. Acad. Sci. USA 72, 590–597.

[14] Gierschik, P., Codina, J., Simons, C., Birnbaumer, L. and Spiegel, A. (1985) Proc. Natl. Acad. Sci. USA 82, 727–731.

[15] Kurose, A., Katada, T., Amano, T. and Ui, M. (1983) J. Biol. Chem. 258, 4870–4875.

[16] Murayama, T. and Ui, M. (1983) J. Biol. Chem. 258, 3319–3326.

[17] Malbon, C.C., Mangano, T.J. and Watkins, D.C. (1985) Biochem. Biophys. Res. Commun. 128, 809–815.

[18] Huff, R.M., Axton, J.M. and Neer, E.J. (1985) J. Biol. Chem. 260, 10864–10871.

[19] Katada, T., Bokoch, G.M., Northup, J.K., Ui, M. and Gilman, A.G. (1984) J. Biol. Chem. 259, 3568–3577.

[20] Florio, V.A. and Sternweis, P.C. (1985) J. Biol. Chem. 3477–3483.

[21] Cerione, R.A., Codina, J., Kilpatrick, B.F., Staniszewski, C., Gierschik, P., Somers, R.L., Spiegel, A.M., Birnbaumer, L., Caron, M.G. and Lefkowitz, R.J. (1985) Biochemistry 24, 4499–4503.

[22] Cerione, R.A., Staniszewski, C., Benovic, J.L., Lefkowitz, R.J., Caron, M.G., Gierschik, P., Somers, R., Spiegel, A.M., Codina, J. and Birnbaumer, L. (1985) J. Biol. Chem. 260, 1493–1500.

[23] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.

[24] Merril, C.R., Dunau, M.L. and Goldman, D. (1981) Anal. Biochem. 110, 201–207.