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# Elevated levels of the guanine nucleotide binding protein, $G_o$, are associated with differentiation of neuroblastoma × glioma hybrid cells

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Each of a range of pharmacological agents which function to increase intracellular levels of cAMP caused a morphological 'differentiation' of neuroblastoma × glioma hybrid, NG108-15, cells grown in tissue culture. Associated with this differentiation, increased incorporation of [$^{32}$P]ADP-ribose catalysed by pertussis toxin was noted into a band of some 39–40 kDa in membranes derived from these cells. Immunoblotting using two antipeptide antisera which identify different regions of $G_o\alpha$ demonstrated marked increases in the levels of this polypeptide in membranes of the differentiated cells. However, levels of the $\beta$-subunit did not increase appreciably with differentiation.

Guanine nucleotide-binding protein; cyclic AMP; Differentiation; ADP-ribosylation; Pertussis toxin

## 1. INTRODUCTION

The neuroblastoma × glioma hybrid cell line, NG108-15, is a widely used model system in neurobiology [1]. Reasons for the enduring popularity of these cells include the fact that they are electrically excitable and they express a wide range of receptors for neurotransmitters and hormones which are coupled to a number of distinct second messenger effector systems and ion channels [1]. Dependent upon the nature of the experiments performed upon these cells, they are used in either an 'undifferentiated' [2,3] or 'differentiated' [3,4] state in which the cells extend numerous neurite-like processes. Differentiation is usually achieved by incubation of the cells in tissue culture with various agents which, as a common mode of action, elevate intracellular levels of cAMP.

The function of a number of the receptors on these cells, including both $\delta$-opioid and $\alpha_2$-adrenergic receptors, is attenuated by prior exposure of the cells to pertussis toxin [5,6]. This toxin functions by catalysing an NAD-dependent ADP-ribosylation of certain G-proteins [7]. This modification prevents productive coupling between the G-protein and relevant receptors. As NG108-15 cells express a number of different pertussis toxin-sensitive G-proteins [2,3], attempts have been made to characterize the molecular identity of the G-protein(s) with which specific receptors interact. Using membranes derived from undifferentiated NG108-15 cells, McKenzie et al. [2] concluded that the signal from the $\delta$-opioid receptor was transduced only by $G_i$. In contrast, Hescheler et al. [4], using differentiated NG108-15 cells, concluded that information from the $\delta$-opioid receptor was transduced preferentially via $G_o$. In an attempt to investigate further this apparent dichotomy, we have assessed potential alterations in levels of $G_o$ which might occur with cAMP-induced differentiation in NG108-15 cells. We demonstrate that a range of treatments which increase the effective intracellular concentration of cAMP in these cells and hence produce differentiation also markedly increase levels of the $\alpha$-subunit of $G_o$.

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## 2. MATERIALS AND METHODS

### 2.1. *Tissue culture*

Control, untreated neuroblastoma × glioma hybrid, NG108-15, cells were grown in tissue culture as described [2,3] except that the concentration of foetal calf serum (Gibco, Paisley, Scotland) was 2% (v/v). In the production of differentiated cells, they were maintained as above but with the addition and maintenance of either dibutyryl cAMP (1 mM), 8-bromo cAMP (1 mM), forskolin (10 $\mu$M) or prostaglandin $E_1$ (10 $\mu$M) for 6 days prior to cell harvest. Cell pastes from each of these treatments were maintained at −80°C before membrane preparation [8]. The membranes so produced were stored at −80°C until use.

Pertussis toxin (Porton Products, Porton Down, England) catalysed ADP-ribosylation of membranes of these cells was performed with [$^{32}$P]NAD (New England Nuclear) as in [2]. Protein was estimated by the method of Lowry et al. [9] using bovine serum albumin as standard.

### 2.2. *Immunological analysis*

$G_o\alpha$ was detected immunologically using two distinct antipeptide antisera. Antiserum IM1 was generated in a rabbit against an antigen produced by coupling a synthetic peptide (NLKEDGI SAAKDVK), corresponding to amino acids 22–35 of $G_o\alpha$, to keyhole limpet haemocyanin, with glutaraldehyde, as we have previously described [10]. This antiserum is specific for $G_o\alpha$ [11]. Antiserum OC1 was produced by a similar strategy against a synthetic peptide (ANNLRGCGLY) which corresponds to the C-terminal decapeptide of $G_o\alpha$. It is also specific for $G_o\alpha$ [7]. Antiserum BN1 was produced in a similar fashion against the peptide MSELDQLRQE, which represents the N-terminal decapeptide of the $\beta_1$-subunit of G-proteins. The methodology of the immunoblotting protocols used in these experiments has been described in detail [2,3,10,18].

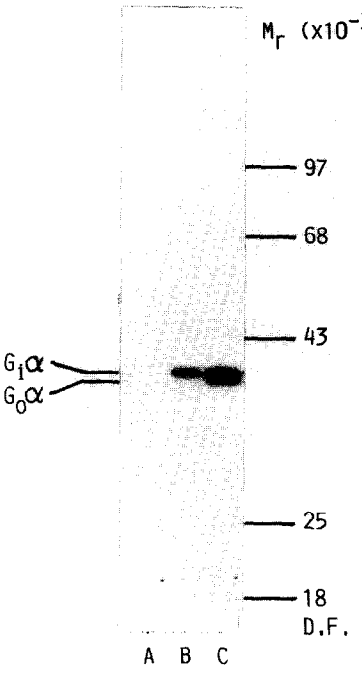


Fig.1. Pertussis toxin-catalysed ADP-ribosylation of membranes of untreated and dibutyryl cAMP differentiated NG108-15 cells. Membranes (25 $\mu$g) of untreated (A,B) or dibutyryl cAMP (1 mM, 6 days) (C) NG108-15 cells were ADP-ribosylated with [$^{32}$P]NAD in the absence (A) or presence (B,C) of thiol-activated pertussis toxin, as described in section 2 and in [8].

## 3. RESULTS

Membranes derived from either untreated or dibutyryl cyclic AMP differentiated neuroblastoma × glioma NG108-15 cells were treated with thiol-preactivated pertussis toxin and [$^{32}$P]NAD and resolved on an SDS-polyacrylamide gel (12.5%, v/v, acrylamide). In each case, apparently two polypeptides of estimated $M_r$ 39000 and 40000 (fig.1) were labelled in the presence of pertussis toxin. These are likely to represent the $\alpha$-subunits of $G_o$ and $G_i$, respectively. Whilst relatively little difference was noted in the incorporation of radioactivity into the 40 kDa polypeptide in either the untreated or differentiated membranes, a marked increase of incorporation was noted in the 39 kDa polypeptide in membranes of the dibutyryl cAMP differentiated cells compared to membranes of untreated cells (fig.1). In the undifferentiated cells only some 5% of radioactivity from the pertussis toxin-catalysed ADP-ribosylation was incorporated into the 39 kDa polypeptide ($G_o$). However, in the differentiated membranes, in a series of experiments, 25–50% of the radioactivity was found in $G_o$.

To ascertain whether the observed increase in pertussis toxin-catalysed ADP-ribosylation was indeed in response to differentiation mediated by cAMP and not to the presence of butyrate, we treated NG108-15 cells with a range of pharmacological agents which increase intracellular levels of cAMP. Growth of NG108-15 cells in the presence of each of prostaglandin $E_1$ (10 $\mu$m), forskolin (10 $\mu$M) and 8-bromo cAMP (1 mM) produced a similar morphological differentiation of the cells to that with dibutyryl cAMP (1 mM) (not shown). Pertussis toxin-catalysed ADP-ribosylation of membranes of each cell preparation demonstrated markedly elevated incorporation of radioactivity into the 39–40 kDa region. Estima-

tion of the radioactivity incorporated into the combination of these polypeptides, by excision of the relevant area of the gel and scintillation counting, demonstrated that differentiation of the cells produced an approx. 2–3-fold increase (table 1).

Pertussis toxin-catalysed ADP-ribosylation of G-proteins can be modulated by a range of factors other than the absolute levels of the $\alpha$-subunits of the G-proteins present in the membranes. We thus performed Western blot analysis of membranes derived from untreated and cyclic AMP differentiated NG108-15 cells with an antipeptide antiserum (OC1) which identifies the extreme C-terminus of the $\alpha$-subunit of $G_o$. In membranes derived from either untreated cells or cells grown in the presence of any of the agents employed, antiserum OC1 identified an apparently single polypeptide of 39 kDa (fig.2). No matter which pharmacological agent was used for the cellular differentiation process, markedly increased levels of

Table 1

Pertussis toxin-catalysed ADP-ribosylation of membranes of untreated and cAMP-differentiated NG108-15 cells

| Treatment | [$^{32}$P]ADP-ribose incorporated into 39 + 40 kDa band (cpm) | % of cpm in untreated membranes |
|---|---|---|
| Expt 1 | | |
| Untreated | 912 ± 22 | 100 |
| Dibutyryl cAMP (1 mM) | 2215 ± 236 | 243 |
| Forskolin (10 $\mu$M) | 1804 ± 160 | 198 |
| Expt 2 | | |
| Untreated | 504 ± 22 | 100 |
| Prostaglandin $E_1$ (10 $\mu$M) | 1212 ± 30 | 276 |
| 8-Bromo cAMP (1 mM) | 1393 ± 26 | 240 |

NG108-15 cells were maintained for 6 days in the absence or presence of agents which elevate intracellular cAMP levels, as detailed in section 2. Membranes (25 $\mu$g) derived from these cells were prepared and treated with thiol-activated pertussis toxin and [$^{32}$P]NAD as in section 2. The samples were then alkylated with *N*-ethyl maleimide [18] resolved by SDS-PAGE (10%, v/v, acrylamide) and autoradiographed. The autoradiograph so generated was used as a template to locate the radioactivity at approx. 40 kDa, which was excised from the dried gel and determined by liquid scintillation counting. No radioactivity was incorporated into polypeptides of this approximate size in the absence of pertussis toxin. Data for the cpm are means ± SE ($n = 4$). Data represent incorporation of radioactivity into a combination of $G_i$ (40 kDa) and $G_o$ (39 kDa) as it is not possible to separate these polypeptides sufficiently well to allow the excision of bands comprising solely one or the other

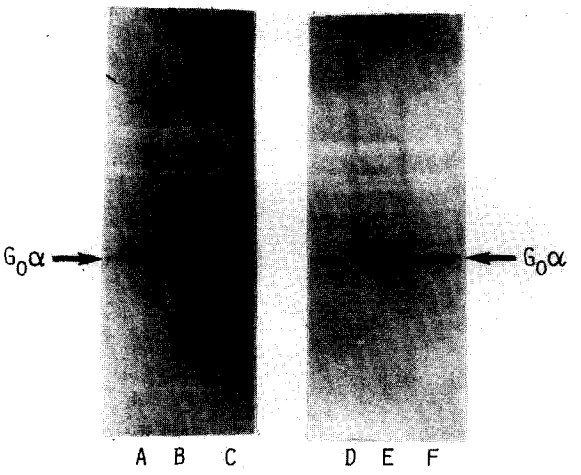


Fig.2. Immunoblotting of membranes of untreated and cAMP-differentiated NG108-15 cells to detect $G_o\alpha$ with antiserum OC1. Membranes (100 $\mu$g) of untreated (A,D) and cAMP-differentiated NG108-15 (B,C,E,F) cells were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted using antiserum OC1 (1:5000 dilution) as the primary agent. Lanes: (B) cells differentiated with dibutyryl cAMP (1 mM); (C) with forskolin (10 $\mu$M); (E) with 8-bromo cAMP (1 mM); (F) with prostaglandin $E_1$ (10 $\mu$M). Similar results were seen in 3 further experiments.

$G_o\alpha$ were identified in the cell membranes by this antiserum. As it was possible that cellular differentiation was associated with a modification close to the C-terminus of $G_o\alpha$ that increased the interaction of the polypeptide with the anti-$G_o$ antibodies in antiserum OC1, we repeated the experiments with a second antiserum (IM1) which identifies a peptide sequence present close to the N-terminus of $G_o\alpha$. Identical results were obtained with the second antiserum (fig.3) thus confirming the presence of markedly elevated levels of $G_o\alpha$ associated with cAMP-mediated differentiation of NG108-15 cells.

As G-proteins are usually considered to exist as heterotrimeric complexes of $\alpha + \beta/\gamma$ subunits we examined whether levels of the $\beta$-subunit were increased in parallel with $G_o\alpha$ upon cAMP-induced differentiation of the cells. Using an antipeptide antiserum (BN1), directed against the extreme N-terminus of the $\beta 1$ polypeptide, we identified a single polypeptide of 36 kDa in membranes of NG108-15 cells. However, we noted only a small increase in levels of this polypeptide caused by any of the differentiation procedures employed (fig.4).

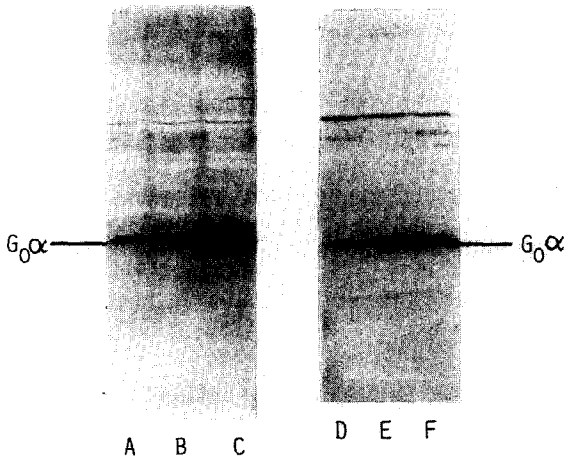


Fig.3. Immunoblotting of membranes of untreated and cAMP-differentiated NG108-15 cells to detect $G_o\alpha$ with antiserum IM1. Details of this experiment are as recorded in the legend to fig.2 except that the primary antiserum was a 1:200 dilution of IM1. Lanes: (A,D) untreated cells; (B) cells treated with 8-bromo cAMP (1 mM); (C) with prostaglandin $E_1$ (10 $\mu$M); (E) with dibutyryl cAMP (1 mM); (F) with forskolin (10 $\mu$M). The same pattern of immunoreactivity was observed in three other experiments.

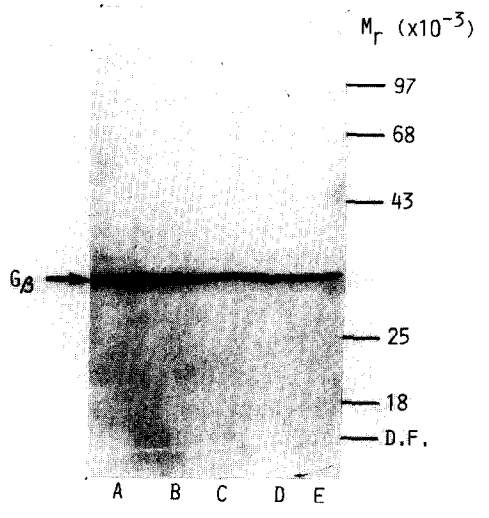


Fig.4. Immunoblotting of membranes of untreated and cAMP-differentiated NG108-15 cells with antiserum BN1 to detect $\beta$-subunit. Details of this experiment are as recorded in the legend to fig.2 except that the primary antiserum was a 1:200 dilution of BN1. A single band of 36 kDa was observed in all samples, with only minor variations in intensity of the staining. Cells differentiated with: (A) dibutyryl cAMP (1 mM), (B) forskolin (10 $\mu$M), (C) undifferentiated cells, (D) 8-bromo cAMP (1 mM), (E) prostaglandin $E_1$ (10 $\mu$M).

## 4. DISCUSSION

A number of G-proteins which can act as substrates for pertussis toxin-catalysed ADP-ribosylation have been identified, both from protein purification schemes [12] and via analysis of cDNA clones [13]. These G-proteins display extreme homology in their primary sequence and as such, questions have been posed as to whether the individual proteins function to transfer information between different receptors and effector systems or whether they are essentially isoforms of one another. Reconstitution of purified fractions of $G_i$ and $G_o$, generally isolated from brain which expresses high levels of both proteins, with a variety of purified or partially purified receptor species in artificial systems, has frequently suggested that $G_i$ and $G_o$ display similar abilities to interact with a particular receptor [14,15]. Whilst these experiments demonstrate that the individual G-proteins do not appear to display a high degree of selectivity in receptor recognition, such investigations do not allude to whether more specific interactions occur in membrane or whole cell systems. The neuroblastoma × glioma hybrid cell line NG108-15 is a suitable model with which to address questions of this nature as it represents a homogeneous cell population and is known to express both $G_o$ and a subtype of $G_i$ [3,16]. Further, it expresses a $\delta$-opioid receptor which interacts with a G-protein(s) which is sensitive to treatment with pertussis toxin [5,6].

Dependent upon the nature of the experiments to be performed, these cells are grown in either the absence or presence of an agent which elevates intracellular cAMP. This treatment produces a morphological differentiation of the cells [1]. In membranes from untreated NG108-15 cells, the opioid peptides cause inhibition of adenylate cyclase activity [17]. McKenzie et al. [2,20] have demonstrated that the interaction of the opioid receptor with the G-protein signalling system, as assessed by measuring enkephalin-stimulation of high-affinity GTPase activity, is essentially completely attenuated by antibodies directed against the extreme C-terminus of $G_i\alpha$ [10]. In contrast antibodies which identify the equivalent region of $G_o\alpha$ have no effect. These data argue strongly that in membranes of the untreated cells the entire opioid receptor signal is transduced via $G_i$. How-

ever, as well as causing inhibition of adenylate cyclase, opioid peptides reduce $Ca^{2+}$ currents across membranes of NG108-15 cells [4]. This process can, however, only be observed in cAMP-differentiated cells. Using this approach, Hescheler et al. [4] have observed a reconstitution of opioid peptide-mediated reduction in $Ca^{2+}$ currents in pertussis toxin-pretreated differentiated cells by the addition of $G_o$. This process could be mimicked, but not as effectively, by a purified preparation of $G_i$.

The potential dichotomy between these two observations, although they are not strictly comparable, has led us to examine what happens to the levels of $G_o$ during cAMP-mediated differentiation of NG108-15 cells. By performing pertussis toxin-catalysed ADP-ribosylation of membranes of both untreated and dibutyryl cAMP-differentiated cells and resolving the 39 kDa $G_o$ and 50 kDa $G_i$ (fig.1), we noted that radioactivity incorporated into the 39 kDa polypeptide was markedly increased in the differentiated membranes in comparison to the untreated membranes. By contrast, little alteration in labelling of the 40 kDa polypeptide was noted under the two conditions. Further, the relative labelling of 39 to 40 kDa polypeptides in the membranes from untreated cells was small, presumably indicating that the absolute amount of $G_o$ was very low in comparison to $G_i$.

As a series of factors, including the state of association of $\alpha$ and $\beta/\gamma$ subunits and the presence or absence of various nucleotides, are known to modulate the rate of pertussis toxin-catalysed ADP-ribosylation of relevant G-proteins [18,19], we then endeavoured to confirm that the increased incorporation of radioactivity into the 39 kDa polypeptide in the differentiated membranes was indeed a reflection of elevated amounts of $G_o\alpha$. For these experiments we used either of two anti-peptide antisera, which we have generated [8,11], which display specificity for this polypeptide. One of these (OC1) identifies the extreme C-terminus of $G_o\alpha$, the other (IM1) a region close to the N-terminus. Western blotting with either of these antisera confirmed the presence of markedly elevated levels of $G_o\alpha$ in the differentiated membranes. This was the case, irrespective of the pharmacological manipulation used to induce the differentiation (figs 2,3).

Based on these observations, the suggestion [2,20] that in membranes of undifferentiated NG108-15 essentially all of the $\delta$-opioid receptor-mediated GTPase activity is at the level of $G_i$ may well reflect the fact that very little $G_o$ is present. As such, this low level of $G_o$ would provide very little GTPase activity, even if it were also coupled to the opioid receptor. As it is not possible to measure $Ca^{2+}$ currents successfully in untreated NG108-15 cells, then an assessment of the role of $G_o$ in these cells in controlling this process is not feasible. It may however be that opioid peptide-mediated inhibition of adenylate cyclase is transduced via $G_i$ in NG108-15 cells whilst the effects of the same agonists on $Ca^{2+}$ currents are transduced via $G_o$. Experiments using C-terminal $G_i$ and $G_o$ antisera to attempt to uncouple opioid receptor modulation of $Ca^{2+}$ currents in differentiated NG108-15 cells are likely to be useful in assessing this possibility.

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