### **REVIEW ARTICLE**

## Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins

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#### Introduction

All receptors that interact with effector systems to modulate the intracellular levels of a second messenger appear to do so via the intermediacy of members of a family of guanine nucleotide binding proteins (Gproteins). Rodbell and coworkers, whilst studying the ability of the peptide hormone glucagon to stimulate adenylate cyclase activity in hepatocytes, were the first to demonstrate definitively a specific requirement for guanine nucleotides in hormonal function (Rodbell *et al.*, 1971*a*). Since these pioneering studies, it has become increasingly clear that a considerable number of unique but highly homologous G-proteins are the sites of action for guanine nucleotides in these processes. The purpose of this review will be to discuss the techniques that are currently in widespread use to identify and assess the functions of individual members of a subfamily of these signal-transducing G-proteins which are substrates for ADP-ribosylation catalysed by pertussis toxin.

# Guanine nucleotide binding proteins: structure and function

All of the well-characterized G-proteins appear to be heterotrimeric in structure, comprising the following subunits.

(1) A unique  $\alpha$  subunit which binds guanine nucleotides and which possesses an intrinsic GTPase activity. In many, but not all, cases the  $\alpha$  subunits of individual Gproteins may be substrates for ADP-ribosylation catalysed by certain bacterial toxins. These  $\alpha$  subunits range

Go	NGCTLISABERAALERSKAIEKNEKEDGISAAKDUKLLLLGAGESGKSTIVKONKIINEDGFUGEDVKUTKPUVYSNTIQSLAAIVRANDTLGVEYGDKERKADSKMVCDVVSRMEDTEPFS
Gil	NOCTLEARDRAAVERSINTDRHLREDGEKAAREVKLLLLGAGESGKETTVKQHETTHEAGYSEEBCKQYKAVYYENTIQSITATTRANGELKIDFGDAARADDARQL#VLAGAARE GFHT
Gi2	NGCTV3AEDKAAAERSENIDKNLREDGEKAAREVELLLLGAGERGESTIVEQHEIINEDGYSEEECROTRAVYSETIQSIMAIVKANGNLQIDPADPQEADGARQLFALSCAABEQGHLP
Gi3	NGCTLEARDRAAVERSKNIDENEREDGEKAAKEVKLILLGAGESUKSTIVKONKIIHEDGYSEDECKOVKVVVYSKTIDSIIAIIHANGELKIDPGEAREADDAROLFVLAGSANE GVHT
T1 T2	NGAGASABER HSRELEKKEREDABROARTVRILLEGAGBEGRETIVRQMETINODGYSLBBCLEFIATIYGHTLQBILATVRAMTTINIQYGDSARODDABREMHHADTIRE GTMP NGSGABARDRELAKRERELEKKEQBDADREARTVRELELGAGESGRETIVRQMETINODGYSPERCLEYRATIYGNVLQSTLATTRAMPTIGTDYAEVSCVDNGRQENNLADSIBE GTMP
Go	ARLISAMMRLWCDSGIQECENRERREYQEHDEAKYYEDSLDRIGAADYQPTEQDIERTHYKTTGIVETNFTFKNENFREFDVGGREERKKWIHOFEDVTAIIFCVALGCYDOVEHDDETTN
Gil	A KLAGVIKELNK DSCVQACENESEKTQLNDSAATYLNDLDRIAQPHYIPTQQDVLETEVETTGIVETHFYRDLHFANFDVGQQESEKEKWINCFHGVTAIIFCVALEDIDLVLAEDHDN
Gi2	edesgyirremadhgygacfchsrtyglhdsaayylnderriagsdylftgygylrtryrttgivrthftfkdenfrhfdygggrsbrriwihcfrgytalifcyalsatdevlardern
Gi3	s Blagvir Blined gov QACPS RBBEY QLINDEAS YTINDLDRIS QTINI I PTQQDVLRTBVETTQI VETHPIPERLY PENED VGOQRBEBEEVING PBOVTAI I PCVALED TDLVLAED PENN
Τ1	KEMSDIIQELWKDSGIQACEDRASETQLNDSACTYLSDLERLVTPCTVPTEQDVLRSEVETTGIIETQFSFEDLNFRWFDVGQQBSEREKEWIHCFEGVTCIIFIALLSATDHYLVEDDEVN
Т2	PELVEVIRKLWKDGGVQACFDRAAEYQLHDSASYYLNQLDRITAPDYLENEQDYLESRVKTTGIIETKFSVKDLNFRHFDVGGQESERKKNIHCFEGVTCIIFCAALSAYDHVLVEDDEVN
Go	RMHESLMLFDSICNNKFFIDTSILFINKKDLFCEKIKKSPLTICFPETPGSNTTEDRAATIQTGFES KNESPNKEITCHNTGATDTNNIQVFDATTBIIIANNLRCCGLY
Gi1	RMHEENKLPDSICHNKWFTDTSIILFLHKKDLFEEKIKKEPLTICYPEYAGSHTYEEAAAYIQCOFEDLHKERDTKEITTHFTCATDTENVQFVFDAVTDVIIKHNLKDCGLF
Gi2	NHHESHKLFDEICHNKWFTDTIIILFLHKKDLFEBKITQEFLTICFFEYTGANKYDBAASYTQSKFEDLHKBKDTKETYTHFTCATDTKHVQFVFDAVTDVIIKHNLKDCCLF
Gi3	RMHESNKLFDSICNNKWFIDTSIILFLNKKDLPEEKIKRSPLTICYPEYTGSNTYEEAAAYIQCQFEDLNR&EDTKEVYTHPTCATDTKNYQFYFDAYTDVIIKMALECOLY
т1	RMHESLHLENSICHHRYEATTSIVLEINKK#VESEKIXKAHLSICFPDINGPMIXEDAGNYIKVQFLEINMERDVKEIISMTCAIDTQHVKEVFDAVIDIIIKEMLKDCCLF
T2	nhieslilfnstcnikffaatsivlfinkkdlfebrikkvilsicfpeydgnnsybdagnyiksqfldinnkkdvkeiyshitgatotqhvkfyfdavtdiiikenlkdcolf
Prim	ary sequences of the a subunits of the currently identified pertussis toxin-sensitive G-proteins as expressed in rat

Areas of identity are shaded. In cases in which identity at particular residues does not extend across all the polypeptides, then the closest homology to the ' $G_1$ -like' subfamily is indicated. Where each of  $G_1$ ,  $G_1$ ,  $G_2$  and  $G_3$  is represented by a different amino acid at one position then no further homology to  $G_0$ , T1 or T2 is noted. The sequences for  $G_0$ ,  $G_1$ ,  $G_1$ ,  $G_2$  and  $G_3$  are taken from Jones & Reed (1987), that for T1 from Tanabe *et al.* (1985) and that for T2 from Lochrie *et al.* (1985).

Abbreviations used: Gpp[NH]p, guanosine 5'-[ $\beta\gamma$ -imido]triphosphate; GT $\gamma$ S, guanosine 5'-[ $\gamma$ -thio]triphosphate; GDP $\gamma$ S, guanosine 5'-[ $\beta$ -thio]diphosphate.

in apparent molecular mass, under denaturing gel electrophoresis, between 39 and 52 kDa. However, at the level of primary amino acid sequence they are very highly conserved (Fig. 1). As will be discussed below, it is this conservation of sequence and of overall tertiary structure which is the cause of most of the difficulties in attempts to identify these G-proteins unambiguously. It is, however, the individuality of the different  $\alpha$  subunits which both define the separate G-proteins and which can most appropriately and usefully be analysed.

(2) A  $\beta$  subunit of some 35–36 kDa on denaturing gels. Until recently it had been generally accepted that a common pool of a single  $\beta$  subunit was shared between different  $\alpha$  subunits which were expressed within a single cell type or tissue (Manning & Gilman, 1983). It had been noted, however, that purification of G-proteins from a number of sources led to the resolution of two  $\beta$ subunits (Sternweis et al., 1981; Sternweis & Robishaw, 1984) that were immunologically distinct (Roof et al., 1985; Evans et al., 1987) and the recent identification of clones for two individual forms of the  $\beta$  subunit has confirmed the presence of at least two genes encoding  $\beta$ subunits (Sugimoto et al., 1985; Fong et al., 1987; Gao et al., 1987). No information is currently available as to whether each subtype of the  $\beta$  subunit is able to interact exclusively with a particular subset of  $\alpha$  subunits.

(3) A  $\gamma$  subunit. Relatively little attention has been paid to the nature and potential diversity of the  $\gamma$ 



Fig. 2. The role of GRP binding and hydrolysis in the activation and deactivation of a typical G-protein

subunits. Immunological evidence, however, suggests the expression of a number of forms (Hildebrandt *et al.*, 1985; Gierschik *et al.*, 1985; Evans *et al.*, 1987). In each case these are small polypeptides of some 8 kDa. In all physiological situations the relevant  $\gamma$  subunit remains tightly associated with the  $\beta$  subunit.

The diversities in structure noted at the levels of the  $\beta$ and  $\gamma$  subunits at least raise the possibility of the existence of numerous different holomeric isotypes of the individual G-proteins. However, no evidence to suggest that this can indeed occur has been presented.

The function of the G-proteins is to couple agonistactivated receptors to the effector systems that alter intracellular concentrations of second messengers. As this process must be of limited duration, then the Gprotein is required to undergo a cyclical pattern of activation followed by a subsequent deactivation (Fig. 2). In the resting state the G-protein exists in the holomeric form with GDP bound to the nucleotide binding site of the  $\alpha$  subunit. Upon receptor activation of the G-protein, then the rate of release of GDP, which appears to be the rate limiting step in G-protein activation/deactivation, is enhanced and the released GDP is replaced by GTP (see Gilman, 1987, for review). With GTP in the nucleotide binding site and in the presence of Mg<sup>2+</sup> then the holomeric G-protein can dissociate into an active  $\alpha$  subunit with GTP bound and free  $\beta/\gamma$  subunits. This active  $\alpha$  subunit is then able to interact with the catalytic moiety of a particular secondmessenger-generation system to alter the rate of synthesis of the second messenger. Hydrolysis of the terminal phosphate of the bound GTP by the intrinsic GTPase activity deactivates the  $\alpha$  subunit and in this GDP-bound form it is then able to reassociate with  $\beta/\gamma$  subunits to restore the G-protein to the deactivated state.

#### Identification of G-protein-linked receptors

Alterations in receptor-agonist binding interactions in the presence of analogues of GTP. Early experiments on the binding of ligands to receptors that produce stimulation of adenylate cyclase noted that GTP interferred with the binding of glucagon to its receptors (Rodbell et al., 1971b). It was subsequently noted that guanine nucleotide effects on  $\beta$ -adrenergic receptor binding were limited to agonists and not antagonists (Maguire et al., 1976). Detailed analysis of ligand binding, in a range of systems, indicated that dose-response curves of agonist displacement of [<sup>3</sup>H]antagonist binding were non-ideal and were shown to have Hill coefficients significantly less than 1.0. In contrast, antagonist displacement of [3H]antagonist binding could be adequately fitted to simple models based on the Law of Mass Action and were characterized by Hill coefficients close to 1.0. Full agonists characteristically produced Hill coefficients further removed from the ideal than did partial agonists. The conclusions drawn from these studies were that agonists were able to recognize two states or conformations of the receptor with different affinity, whilst antagonist affinity for these two forms must either be identical, or at least extremely similar (see Birdsall et al., 1980, for example). Furthermore, addition of poorly hydrolysed analogues of GTP, such as Gpp[NH]p or  $GTP\gamma S$ , to the binding incubations reduced the ability of agonists, but not antagonists, to compete for [<sup>3</sup>H]antagonist binding sites and under these conditions the shape of the agonist displacement curves were now close to ideal. These data were consistent with a model whereby agonists interacted with receptors which were in intimate association with a GDP-liganded, and hence unstimulated, G-protein with higher affinity than they did with receptors which were not in such contact with the G-protein. Similar experiments prompted the conclusion that receptors that mediated inhibition of adenylate cyclase must also interact with a G-protein (see Rodbell, 1980) and arguments of this nature were then later extended to provide evidence for the interaction of the Ca<sup>2+</sup> mobilizing receptors with G-proteins. Historically, therefore, guanine nucleotide sensitivity of agonist-binding affinity has often provided the initial suggestion that a particular receptor interacts with a G-protein.

The limitation of this type of approach is that it can offer no information as to the molecular nature of the Gprotein involved. Binding studies performed on membranes derived from tissues pretreated with a toxin isolated from supernatant cultures of Bordetella pertussis have been used to subdivide further the nature of receptor-linked G-proteins (Kurose et al., 1983; Hsia et al, 1984). In these experiments agonist affinity for the displacement of [3H]antagonist binding was reduced in membranes of pertussis toxin-treated cells in comparison to that in membranes from untreated cells. Furthermore, addition of poorly hydrolysed analogues of GTP was not able to reduce agonist affinity for the receptor further, indicating that pertussis toxin pretreatment had modified the relevant G-protein in such a manner that it now appeared to be functionally uncoupled from the receptor. As discussed below, however, these studies are of restricted usefulness due to the limited specificity of this toxin. They were, however, of use in demonstrating that receptors that mediate inhibition of adenylase cyclase do so by interacting with a pertussis toxin-sensitive Gprotein(s) and also that Ca<sup>2+</sup> mobilizing receptors in a number of (Nakamura & Ui, 1985; Ohta et al., 1985), but not all, tissues (Helper & Harden, 1986; Martin et al., 1986), also interact with a pertussis toxin-sensitive Gprotein. Unfortunately, the prospectively erroneous, if most simple, conclusion, i.e. that the G-protein involved in inhibition of adenylate cyclase and stimulation of inositol phospholipid turnover was one and the same, was derived from this approach.

GTPase studies. The interaction of agonist with receptor promotes the release of GDP from a relevant Gprotein and hence allows the binding of GTP. This is followed by the subsequent hydrolysis of the nucleotide by the GTPase activity of the G-protein. Measurement of this enhanced rate of GTPase activity of a membrane in response to agonist thus provides a simple, convenient and direct assessment of the interaction of a receptor with a G-protein(s). This approach was first employed by Cassel & Selinger (1976, 1977) in an avian erythrocyte system to demonstrate interaction of the  $\beta$ -adrenergic receptor with a G-protein. Similar experiments have been performed on a wide range of systems to produce similar conclusions. Despite the simplicity of the experimental protocol, in many systems it has not been possible to demonstrate receptor-stimulation of GTPase activity even though other evidence exists to indicate that a particular receptor interacts with the G-protein signalling system. It is thus worth commenting upon why this may be so. The basal 'GTPase' activity of any membrane system may be considered as a composite of the hydrolysis of GTP by all of the G-proteins within that membrane as well as by other enzymic reactions. Gprotein-related GTP hydrolysis is such that the  $K_{\rm m}$  of these enzymes for GTP is low. Thus it is useful to subtract from the total GTPase activity a blank in which hydrolysis in the presence of a high concentration of GTP (50–100  $\mu$ M) is assessed. Also, the contribution of any particular G-protein to the basal low- $K_m$  GTPase activity will be dependent upon: (1) the relative proportions of the various G-proteins present in the membrane, and (2) their relative rates of GTP hydrolysis. Thus, the observations that receptor-G<sub>s</sub> interactions cannot be measured using this type of assay in a number of systems and have only been reproducibly noted in a few systems, for example in avian erythrocytes (Cassel & Selinger, 1976) and in platelets (Houslay et al., 1986) are a reflection both that levels of G<sub>s</sub> tend to be lower than those of many other G-proteins and that the rate of GTP hydrolysis by purified G<sub>s</sub> is extremely low. As such, receptor-mediated stimulation of this rate may still allow the situation in which the signal of increased hydrolysis of GTP by activated G<sub>s</sub> remains lost within the 'noise' due to the basal rates of hydrolysis by the other Gproteins present.

In general, much greater success has been achieved using GTPase studies for receptors linked to pertussis toxin-sensitive G-proteins (Koski & Klee, 1981; Aktories & Jakobs, 1981). This is presumably due to the higher abundance of these proteins and their greater enzymic capacity. As with binding studies, however, it has not generally been possible to further subdivide receptor interactions with particular pertussis toxin-sensitive Gproteins due to the lack of specificity of this toxin. Assessments of the specificity of receptor-G-protein interactions within the native membrane have however been addressed by performing GTPase activity experiments (see Table 1). The rationale for these is that activation of the entire population of receptors in a membrane preparation with a saturating concentration of a full agonist will prospectively lead to the activation

#### Table 1. Additivity of receptor-mediated GTPase in membranes of neuroblastoma × glioma NG108-15 cell membranes

The maximum increase in high-affinity GTPase activity caused by receptor-saturating concentrations of either an opioid peptide (DALAMID) or grown factor (foetal calf serum) was assessed in the absence or presence of the other ligand. The data, which demonstrate complete additivity of the individual ligands, is adapted from McKenzie *et al.* (1988*a*). Data of this form are consistent with the hypothesis that the receptors for each of these ligands interact with separate and distinct G-proteins.

	Ligand stimulation above basal level of high-affinity GTPase activity (pmol/min per mg of protein)	
Foetal calf serum	DALAMID	
(20%, v/v)	(10 µм)	
<u> </u>	$6.3 \pm 0.4$	
+	$6.9\pm0.5$	
DALAMID	Foetal calf serum	
(10 µм)	(20%, v/v)	
<u> </u>	12.0+0.3	
+	$12.5\pm0.4$	

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of the full complement G-protein(s) with which that receptor is able to interact. Thus, addivity of receptorstimulated GTPase activity following addition of two agonists which interact with independent receptors would indicate the activation of separate pools of Gprotein, and by extension, different G-proteins. This approach has been elegantly employed by, for example, Houslay et al. (1986a,b) to examine a range of receptor-G-protein interactions in human platelets. A further example of the usefulness of this technique has been the demonstration (McKenzie et al., 1988a) that in membranes of neuroblastoma × glioma hybrid cells, individual receptors for opioid peptides and for a growth factor interact with separate, distinct pertussis toxin-sensitive G-proteins. Of course, if agonist interaction with a particular receptor is able to activate only a small proportion of the population of a particular G-protein, then GTPase additivity would be observed to a second receptor able to activate the same G-protein. This is likely to be the case in some tissues, e.g. brain, where the pertussis toxin-sensitive G-proteins may represent some 1-2% of the total membrane protein (Sternweis & Robishaw, 1984; Neer et al., 1984; Milligan & Klee 1985).

**Bacterial toxins.** Exotoxins isolated from supernatant cultures of *Vibrio cholerae* and of *Bordetella pertussis* have proved to be invaluable tools in the characterization of, in particular, the G-proteins associated with the adenylate cyclase signalling system.

Bordetella pertussis, the causative agent of whooping cough, produces a number of toxins. One of these, named islet activating protein or simply pertussis toxin, was originally shown by Ui and collaborators to produce sustained alterations in receptor-mediated control of cyclic AMP production (Katada & Ui, 1979, 1981). Pertussis toxin consists of six subunits, five of which are dissimilar. Of these, the S1 subunit (28 kDa) is as effective as the holomeric toxin when it is added to membrane preparations, but is totally inactive on whole cells. The other subunits, by contrast, are important in the attachment of the toxin to cells and the entry of toxin into the cell (see Foster & Kinney, 1984, for review). Activation of the toxin in vitro is achieved by treatment with a reducing agent such as dithiothreitol. The S1 subunit is an ADP-ribosyltransferase and catalyses the transfer of the ADP-ribose moiety of NAD<sup>+</sup> to the  $\alpha$ subunit of relevant G-protein substrates. Some of the initial experiments with this toxin demonstrated an enhancement of GTP activation of adenylate cyclase in rat glioma C6 cells. This was concomitant with the transfer of ADP-ribose from NAD<sup>+</sup> to a 41 kDa membrane-associated polypeptide (Katada & Ui, 1982). The release of tonic inhibition of adenylate cyclase activity paralleled by the modification of the 41 kDa protein thus identified this polypeptide as the putatively proposed (Rodbell, 1980), but previously unidentified, inhibitory G-protein of the adenylate cyclase cascade (G<sub>i</sub>). Because an apparently similar protein could be identified in all tissues examined, and because in each tissue apparently only a single polypeptide was modified, it was therefore concluded that any receptor-mediated response which was attenuated by exposure to pertussis toxin must function via G<sub>i</sub> (Fig. 3). This cyclical mode of reasoning was only shown to be flawed following the purification of multiple pertussis toxin substrates (G, and



Fig. 3. Pertussis toxin-catalysed ADP-ribosylation

Membranes of neuroblastoma × glioma hybrid cells were incubated with activated pertussis toxin and [<sup>32</sup>P]NAD<sup>+</sup> as in Milligan (1987). An apparently single band of some 40 kDa was labelled in the presence (a) but not absence (b) of the toxin. However, as noted in the text, this cell line expresses at least two individual pertussis toxin-sensitive G-proteins (G<sub>1</sub>2 and G<sub>o</sub>) of this approximate  $M_r$ . This Figure thus demonstrates the potential difficulty in the unambiguous identification of the molecular identity of a pertussis toxin-sensitive G-protein when assessment is based on pertussis toxin-catalysed ADP-ribosylation alone.

 $G_o$ ) from brain (Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Milligan & Klee, 1985) and then by the recognition that further pertussis toxin-sensitive G-proteins were expressed which were immunologically distinct from brain  $G_i$  and  $G_o$  (Milligan *et al.*, 1986; Gierschik *et al.*, 1986c).

Initial demonstrations that more than a single pertussis toxin-sensitive G-protein could be expressed came from attempts to purify 'G<sub>i</sub>' from bovine brain. Three groups of workers noted that purified preparations of pertussis toxin substrates from brain contained either two (Sternweis & Robishaw, 1984; Milligan & Klee, 1985) or three (Neer *et al.*, 1984) polypeptides in the 39–41 kDa range. These appeared to be at least immunologically if not functionally distinct (see later sections on immunological characterization and on reconstitution studies). Earlier studies on the purification of the pertussis toxin-sensitive G-protein had used tissues such as rabbit liver and had identified but a single pertussis toxin substrate (see, for example, Bokoch *et al.*, 1984).

The existence of multiple forms of pertussis toxinsensitive G-proteins has been confirmed and considerably extended by analysis of cDNA clones. At the present time a minimum of some six individual gene products have been identified by this strategy. These include two forms of transducin which are located differentially in either rods or cones (Lochrie et al., 1985; Yatsunami et al., 1985), three genes coding for 'G<sub>i</sub>-like' proteins, named for the chronology of their identification  $G_{1}$ ,  $G_{2}$ and G<sub>3</sub> (Reed & Jones, 1987; Suki et al., 1987; Beals et al., 1987), and G<sub>o</sub> (Itoh et al., 1986; Jones & Reed, 1987) (Fig. 1). In addition, two separate mRNAs which hybridize to a G<sub>o</sub> specific probe have been identified (Jones & Reed, 1987). It is thus possible that yet further forms may be demonstrated. There is thus no justification, in the absence of rigorous biochemical experimentation, to assigning a pertussis toxin-sensitive response to any particular member of this family of proteins. Herein lies the crux of the problem. Despite this, pertussis toxin remains a valuable tool in studies of hormone and neurotransmitter function, provided that the results obtained with the toxin are not overinterpreted.

In contrast to pertussis toxin, cholera toxin has a molecular mass of 84415 and is composed of two protomers, A and B, which interact with one another in a non-covalent manner. The A protomer represents the enzymic moiety of the toxin whilst the B protomer, which consists of five identical polypeptide chains, interacts with a cell surface receptor, ganglioside  $G_{M1}$ and hence in some manner promotes the entry of the A protomer into the cell (see Foster & Kinney, 1984). Like pertussis toxin, the activated A protomer is an ADPribosyltransferase which in the presence of a second protein called ADP-ribosylation factor (ARF) (Kahn & Gilman, 1984) is able to catalyse the transfer of ADPribose from NAD<sup>+</sup> to an arginine residue in the  $\alpha$  subunit of G<sub>a</sub>. This covalent modification stabilizes the GTPbound form of the  $\alpha$  subunit of G<sub>s</sub> and as the GTPase activity of the  $\alpha$  subunit is inhibited then G<sub>a</sub> is maintained in a permanently activated state (Cassel & Selinger, 1977). Adenylate cyclase is thus maximally activated and is no longer sensitive to hormonal activation. It is necessary to preactivate the toxin in vitro by prior treatment with a reducing agent such as dithiothreitol to separate the A and B protomers before the toxin will function enzymically.

The use of  $[^{32}P]NAD^+$  as substrate allows the visualization of polypeptides which are substrates for either cholera or pertussis toxin following gel electrophoresis under denaturing conditions and subsequent autoradiography. In the presence of guanine nucleotides, cholera toxin is able to promote the specific incorporation of radioactivity only into the  $\alpha$  subunit(s) of G<sub>s</sub>. The apparent molecular mass of this polypeptide(s) is normally estimated to be either 45 or 52 kDa and these two forms appear to represent differentially spliced products derived from a single gene (Bray *et al.*, 1986; Robishaw *et al.*, 1986).

Depending upon the tissue studied, pertussis toxin is able to catalyse ADP-ribosylation of at least three polypeptides. Whilst these all migrate in denaturing gel electrophoresis with apparent molecular masses between 39 and 41 kDa, they can be resolved sufficiently to be noted as unique (Neer *et al.*, 1984; Toutant *et al.*, 1987).

In the case of cholera toxin it has been noted that, when the ADP-ribosylation reaction is performed in the absence of guanine nucleotides, then besides incorporation of radioactivity into the  $G_s \alpha$  polypeptide(s), labelling of a polypeptide of some 40 kDa can be observed (Fig. 4). Whilst this observation has been noted for at least a macrophage-like cell line (Aksamit *et al.*, 1985), polymorphonuclear leukocytes (Verghese *et al.*, 1986), a rat glioma cell line (Milligan, 1987), adipocytes (Owens *et al.*, 1985), and a neuroblastoma  $\times$  glioma hybrid cell line (Milligan & McKenzie 1988), this does not appear to be a universal phenomenon (my published work). This polypeptide appears also to be a substrate for pertussis toxin, as prior treatment *in vivo* of either the rat glioma cell line or the neuroblastoma  $\times$  glioma cell line with pertussis toxin prevents subsequent cholera toxin-catalysed ADP-ribosylation of 40 kDa polypeptide. Further, it has been noted that cholera toxin-catalysed ADPribosylation of a 40 kDa polypeptide in adipocytes has functional consequences equivalent to treatment with pertussis toxin (Owens *et al.*, 1985).

The site of pertussis toxin-catalysed ADP-ribosylation in G-proteins that are substrates for this toxin is a conserved cysteine residue located four amino acids from the C-terminus. In contrast, however, the site of action of cholera toxin in G<sub>s</sub> is an arginine residue, which in the postulated tertiary structures of the G-protein (Masters et al., 1986), is located close to the guanine nucleotide binding domain. Interestingly, the primary structure in the region around this arginine is highly conserved in all the G-proteins which have so far been identified and this arginine is invariant in the equivalent position of the other G-proteins. It must then be asked why all Gproteins are not substrates for cholera toxin under all conditions? This may reflect the close proximity of the guanine nucleotide binding domain to the relevant arginine residue and suggests that occupancy of this site by a guanine nucleotide might hinder access of cholera toxin. Evidence in favour of this proposal is provided by the observations that the pertussis toxin substrates only become substrates for cholera toxin in the absence of added guanine nucleotides. Also, addition of agonists for receptors that interact with the pertussis toxin-sensitive G-protein produces an enhancement of cholera toxin catalysis of ADP-ribosylation of this G-protein (Gierschik & Jakobs, 1987; Milligan, 1988; Milligan & McKenzie, 1988) (see Fig. 5).



Fig. 4. Cholera toxin-catalysed ADP-ribosylation: the effect of GRP

Membranes of rat glioma C6 BU1 cells were treated with activated cholera toxin and [<sup>32</sup>P]NAD in the presence (*a*) or absence (*b*) of exogenously added GTP (100  $\mu$ M) as described by Milligan (1987). No incorporation of radio-activity into any of the three polypeptides was noted in the absence of cholera toxin. The 40 kDa band labelled, only in the absence of GTP, in this system, represents the  $\alpha$  subunit of G,2 (Milligan, 1988).



Fig. 5. Agonist enhancement of cholera toxin-catalysed ADPribosylation of a form of G<sub>i</sub> in membranes of NG108-15 cells

Membranes of neuroblastoma × glioma hybrid NG108-15 cells were treated with [<sup>32</sup>P]NAD<sup>+</sup> and either pertussis toxin (lane *a*) or cholera toxin (lanes *b*–*e*) for 2 h at 37 °C. In addition, lane (*b*) contained naloxone ( $10^{-3}$  M), lane (*c*) contained naloxone ( $10^{-3}$  M) + DALAMID ( $10^{-7}$  M), lane (*d*) contained DALAMID ( $10^{-7}$  M) and lane (*e*) contained DALAMID ( $10^{-5}$  M). Radioactivity incorporated into a 40 kDa polypeptide was assessed by densitometric analysis of an autoradiogram following resolution of the samples on an SDS/polyacrylamide gel. Data are adapted from Milligan & McKenzie (1988).

As agonist activation of a receptor-linked G-protein increases the rate of dissociation of GDP from the guanine nucleotide binding site then in the absence of added guanine nucleotides it might be predicted that agonist should produce a G-protein which has been essentially stripped of guanine nucleotide. In this state the pertussis toxin substrate may now become accessible to cholera toxin. Similar strategies involving agonist and GMP had originally been used to strip G-proteins of nucleotide in studies of conformational change and proteolytic sensitivity (see, for example, Hudson et al., 1981). Whilst this represents a novel approach to the identification of receptors linked to G-proteins that are usually considered to be substrates for pertussis toxin, it still does not provide a ready assessment of which of these G-proteins the receptor interacts with. It does however demonstrate a potential use of cholera toxin in the identification of 'pertussis toxin-sensitive G-proteins'. A prospective approach to the identification of specific pertussis toxin-sensitive G-proteins that become activated by particular receptors is likely to involve twodimensional electrophoretic analysis of samples that have been ADP-ribosylated by cholera toxin in the absence of guanine nucleotides in the presence and absence of an agonist for the receptor under investigation. It would then be predicted that only the pertussis toxin sensitive G-protein(s) that could interact with this receptor would show enhanced levels of incorporation of radioactivity catalysed by cholera toxin.

Two particular difficulties exist in the use, *in vitro*, of bacterial toxin-catalysed ADP-ribosylation as a means of identification and quantification of pertussis toxinsensitive G-proteins. The first is that, depending upon the tissue under investigation, less than the theoretical

maximal degree of ADP-ribosylation may occur (i.e. 1 mol of ADP-ribose/mol of pertussis toxin-sensitive Gprotein). A particular problem which has been noted, but which is only infrequently assessed, is that the [<sup>32</sup>P]NAD<sup>+</sup> can be degraded by NAD-glycohydrolases present and hence is not available as a substrate for the toxin. Rat brain contains high levels of both ' $G_i$ ' (now identified as G<sub>i</sub>l) and G<sub>o</sub>. As assessed by Western blotting techniques, levels of both of these G-proteins increase in neonatal tissue to reach adult levels by 20-30 days (Milligan et al., 1987a). However, results obtained from pertussis toxin-catalysed ADP-ribosylation indicated that levels of these proteins fell sharply with age (Milligan et al., 1987d). Examination of this paradox demonstrated the appearance of an NAD-glycohydrolase with age, such that in older, but not younger, animals, essentially the entire pool of [<sup>32</sup>P]NAD<sup>+</sup> was destroyed in a short time span (Milligan et al., 1987d).

Secondly, the nature of the guanine nucleotide bound to a G-protein can substantially alter the rate of both pertussis and cholera toxin-catalysed ADP-ribosylations. It was noted above that cholera toxin-catalysed ADPribosylation of G-proteins that are traditionally considered to be substrates for pertussis toxin does not appear to occur to any great extent if a guanine nucleotide is bound to the G-protein. However, the nature of the guanine nucleotide bound also affects the rate of pertussis toxin-catalysed ADP-ribosylation. In the presence of the GDP analogue GDP $\beta$ S, the rate of pertussis toxincatalysed ADP-ribosylation of a 40 kDa polypeptide in rat glioma C6 cell membranes was some four times greater than when the same experiment was performed in the presence of the GTP analogue  $GTP\gamma S$  (Milligan, 1987). Experiments such as these are traditionally interpreted to imply that pertussis toxin is better able to interact with the holomeric, unactivated forms of its substrates, which have GDP bound, than with the free, activated  $\alpha$  subunits. Such an interpretation is well supported by observations that the separated  $\alpha$  subunit of the pertussis toxin-sensitive G-protein  $G_0$  is a very weak substrate for ADP-ribosylation but that upon addition of  $\beta/\gamma$  subunits the  $\alpha$  subunit becomes a much improved substrate (Neer et al., 1984). Whilst the above is certainly true, recent observations that pertussis toxin itself has a nucleotide requirement for function (Mattera et al., 1987) hinder interpretation.

A third potential problem with the use of bacterial toxins to identify G-proteins appears at this stage to be largely confined to the use of cholera toxin. A number of recent reports have suggested that prior treatment with cholera toxin can reduce subsequent receptor-mediated hydrolysis of inositol phospholipids (Imboden et al., 1986; Lo & Hughes, 1987). However, at least in the case of vasopressin-stimulation of inositol phosphate generation in rat glomerulosa cells, where pretreatment with cholera toxin reduced the agonist response by some 60%, this does not appear to be due to the toxin modifying a G-protein. Rather, the toxin appeared to either block access of vasopressin to the receptor or to downregulate the vasopressin receptor (Guillon et al., 1988). It is thus a useful cautionary point to note that the toxins may do more than simply catalyse ADP-ribosylation of their available G-protein substrates. As such it is important to demonstrate that toxins used in this type of assay have indeed catalysed ADP-ribosylation of all the available G-protein substrate by further challenging membranes derived from toxin-treated cells with fresh toxin and [<sup>32</sup>P]NAD<sup>+</sup>. Further, the rate of toxincatalysed ADP-ribosylation should reflect the rate of inactivation of receptor-mediated responsiveness. There are also recent reports that subunits of the toxins other than those possessing ADP-ribosyltransferase activity may be responsible for some of the biological effects of the toxins (Strnad & Carchman, 1987).

#### Identification of individual pertussis toxin-sensitive Gproteins

Resolution of the different pertussis toxin-sensitive Gproteins in one-dimensional SDS/polyacrylamide gels is often poor. Whilst this can be improved by prior alkylation of the samples with N-ethylmaleimide (Sternweis & Robishaw, 1984) and a rank order of mobility through such gels noted such that the mobility of  $G_0 >$  $G_{12} > G_{11}$ , greater resolution is generally required. With this in mind, a number of laboratories have adopted the use of two-dimensional electrophoresis. With this approach, resolution of a number of pertussis toxinsensitive G-proteins has been achieved. Initial experiments in this area involved the prior ADP-ribosylation of membrane preparations with pertussis toxin and [<sup>32</sup>P]NAD<sup>+</sup> to function as the detection system. However, by so doing, the electrophoretic mobility of the proteins would be altered such that the polypeptides would be more acidic than the native forms (see for example Deery et al., 1987). More recent attempts have probed nitrocellulose blots of the resolved proteins with specific antibodies so that true isoelectric points can be estimated. In these experiments  $G_i$  migrates with a more basic isoelectric point (6.1) than  $G_{i2}$  (5.65) and the major form of  $G_0$  (5.6). In brain a second form of  $G_0$  can be observed with an isoelectric point near 6.0 (Backlund et al., 1988). In support of these observations, in purification protocols, G<sub>i</sub>1 and a form of G<sub>o</sub> elute in close proximity from anion exchange resins and can be relatively easily resolved from  $G_{12}$  and the major form of  $G_{0}$ , which elute in later fractions (Katada *et al.*, 1987; P. Goldsmith, P.S. Backlund, Jr., G. Milligan, C. G. Unson & A. Spiegel, unpublished work).

A limitation on the use of two-dimensional electrophoresis for the analysis of pertussis toxin-sensitive Gproteins is that in general the  $\alpha$  subunits appear only poorly to penetrate the isoelectric focusing phase of the gel (Heydorn et al., 1986). This leads to a lowering of sensitivity of the system and to marked streaking of the gel if it is overloaded. The resistance to penetration of the first dimension appears to be a property of the Nterminal region of these proteins and may relate to the covalent attachment of myristic acid (Schultz et al., 1987; Buss et al., 1987) to the glycine residue which is present as the N-terminal residue of all the pertussis toxin-sensitive G-proteins which have been characterized. Despite possessing an equivalent N-terminal sequence to the other pertussis toxin-sensitive G-proteins, the individual forms of transducin appear to lack attached myristic acid (Buss et al., 1987). The lack of N-terminal myristic acid may explain why the forms of transducin appear to be peripheral membrane proteins, whereas detergent treatment is generally required to remove the other pertussis toxin-sensitive G-proteins from the plasma membrane. Tryptic removal of an approx. 2 kDa peptide from the N-terminus when the G-protein is liganded with either GTP $\gamma$ S or Gpp[NH]p is sufficient to minimize this difficulty. The remaining 37 kDa polypeptide, which is stable to further tryptic digestion when a GTP, but not GDP, analogue is bound (Katada & Ui, 1982; Eide *et al.*, 1987; McKenzie *et al.*, 1988b), is then able to enter the gel with greater freedom. Such guanine nucleotide control of the tryptic sensitivity of G-proteins had originally been noted for  $G_s$  (Hudson *et al.*, 1981).

Currently, the most convenient approach to the identification of individual species of pertussis toxinsensitive G-proteins involves the use of specific antisera (Fig. 6). With the purification of ' $G_i$ ' from a number of tissues, considerable efforts have been made to generate polyclonal antisera selective for individual pertussis toxin-sensitive G-proteins. These attempts have not been universally successful. Often, the use of a mixture of holomeric pertussis toxin-sensitive G-proteins isolated from brain as antigen has led to the generation of antisera containing antibodies directed against the  $\alpha$ subunit of  $G_{0}$  and against the  $\beta$  subunit but within these antisera no antibodies were generated which selectively recognize forms of the  $\alpha$  subunit of G<sub>i</sub> (Gierschik et al., 1986a; Huff et al., 1985; Roof et al., 1985). Why G<sub>o</sub> appears to be immunodominant in comparison to G<sub>i</sub> is not obvious given the marked homology between these proteins at the primary sequence level. At least within our own attempts to produce antisera by this route we have only succeeded in generating anti-G<sub>o</sub> antisera (Gierschik et al., 1986a) and at least anecdotal evidence, and the lack of reports to contradict this, suggest that this has generally been the experience of other workers. Recently however, Katada et al. (1987) appear to have generated a polyclonal anti-G, antiserum using purified brain G-protein as antigen. In a number of cases anti- $G_{o}$  antiseral generated in this direct fashion show some degree of cross-reactivity with other pertussis toxinsensitive G-proteins (see Huff et al., 1985, for example) and this may alter from different bleeds of the same antiserum. In many respects, given the very high levels of homology between members of this sub-family of Gproteins, it should not be surprising if a degree of crossreactivity were noted with polyclonal antisera. However, based on the recognition that domains which must interact selectively with particular classes of receptors





Membranes of cells of the neuroblastoma × glioma hybrid cell line NG108-15 were resolved on SDS/polyacrylamidegel electrophoresis (10 %, v/v) and immunoblotted with (a) an antipeptide antiserum against the C-terminal decapeptide of G<sub>o</sub>, (c) an equivalent antiserum against the C-terminal decapeptide of 'G<sub>i</sub>' and (b) a mixture of these two antisera. The two individual antisera each recognize a single polypeptide whilst the mixture recognizes a doublet. The polypeptide identified by the G<sub>o</sub>-specific antiserum migrates further in the gel than that identified by the 'G<sub>i</sub>' antiserum. and with different second messenger-effector systems must be exposed at the surface of the protein and hence potentially available as antigenic epitopes, then as these are likely to represent the most divergent sections of the G-protein it may be appreciated how fairly selective antisera might be generated. Anti-G<sub>o</sub> antisera of this class were first used to demonstrate that  $G_0$  and  $G_1$  were distinct entities (Gierschik et al., 1986a) and that G<sub>o</sub> did not simply represent a proteolytic fragment of  $G_i$  (Huff et al., 1985; Milligan & Klee, 1985). However, in the absence of an antiserum generated against purified  $G_{i}$ , it was the cross-reactivity with brain  $G_i$  of a polyclonal antiserum raised against holomeric bovine rod transducin (Pines et al., 1985) that first permitted the mapping of tissue and regional location of brain  $G_i$  (now called  $G_i$ 1). These antisera were also applied to studies of the developmental regulation of  $G_i$  and of  $G_o$  and were used to demonstrate that observed alterations in the levels of the pertussis toxin-labelling of membranes of NIH 3T3-L1 pre-adipocytes during differentiation could mask the independent regulation of amounts of two separate pertussis toxin substrates (Gierschik et al., 1986b). Rather different results on the relative expression of G<sub>i</sub> and G<sub>o</sub> in this same experimental system have been detailed more recently by Watkins et al. (1987). These same antisera have been used to assess the relative concentrations of  $G_i$  and  $G_o$  in a number of tissues (Milligan et al., 1987a,c; Luetje et al., 1987). Anti-Gprotein antisera have also been productively utilized in immunocytochemical assays to determine the distributions of specific G-proteins (Worley et al., 1986; Lad et al., 1987; Terishima et al., 1987).

The inability of these polyclonal anti-G<sub>o</sub> and anti-G<sub>i</sub>1 selective antisera to stain a 40 kDa pertussis toxin substrate that was present in high levels in both human neutrophils (Gierschik et al., 1986c) and in rat glioma C6 cells (Milligan et al., 1986) further demonstrated that pertussis toxin-sensitive G-proteins other than these two were expressed in various tissues. Whilst the nature of the major pertussis toxin substrate(s) in these cells was not apparent at that stage, this has subsequently been identified as G<sub>i</sub>2 by the use of selective antipeptide antisera (see below). However, as recently noted by Jones and Reed (1987), based on their identification of four separate pertussis toxin-sensitive G-proteins from a rat olfactory neuroepithelial cDNA library, "the specificity of polyclonal or monoclonal antisera should be well characterized for definitive immunoanalysis".

Based on considerations of this nature a number of laboratories have produced anti-peptide antisera directed against short synthetic peptides which, from either conventional protein sequencing or analysis of cDNA clones, can be predicted to be present in particular pertussis toxin-sensitive G-proteins. A particularly elegant example of the potential of this approach has been provided by the generation of anti-peptide antisera directed against sequences unique to two individual forms of transducin which, as deduced from separate cDNA clones, were some 78% identical at the amino acid level (Lerea et al., 1986). One of these antisera specifically stained rods and the other cones, demonstrating that the two G-proteins represented cell-type specific forms of transducin. Mumby et al. (1986) were the first to generate a series of antipeptide antisera against synthetic peptides corresponding to sequences of individual G-protein  $\alpha$  subunits and were able to

demonstrate that these antisera displayed greater specificity for particular G-proteins on Western blots that did antisera generated against purified G-protein  $\alpha$  subunits. However, no antipeptide antisera against G, were produced in these studies. Because it had previously been noted that a polyclonal anti-transducin antiserum, CW6, which could be demonstrated to recognize an epitope close to the C-terminus of the  $\alpha$  subunit of this protein, was able to cross-react with brain G<sub>1</sub> (Pines et al., 1985; Milligan & Klee, 1985), then Spiegel, Unson and Milligan (see for example Falloon et al., 1986; Milligan et al., 1987b; Goldsmith et al., 1987) produced antipeptide antisera directed against the C-terminal decapeptide of transducin with the hope that these antisera would also cross-react with G<sub>i</sub>. This peptide sequence could be predicted from cDNA clones of transducin whilst the equivalent sequence of G<sub>i</sub> was not available at that time. These antisera did in fact recognize brain G, as well as transducin on western blots, indicating that the C-terminal sequence of G, must be similar to that of transducin. In retrospect this was not an unexpected result, as cDNA studies later demonstrated that brain G<sub>i</sub> contained but a single conservative substitution within this region when compared to transducin. Somewhat more surprisingly, these antisera detected high levels of a 40 kDa pertussis toxin substrate in human neutrophils (Falloon et al., 1986; Goldsmith et al., 1987) and in glioma C6 cells (Goldsmith et al., 1987; Milligan et al., 1988), whereas it had previously been noted that antisera against  $G_0$  or the predominant  $G_1$ -like protein of brain  $(G_1)$  were unable to recognize these forms. Further cDNA cloning studies of libraries constructed from glioma C6 cells (Itoh et al., 1986) and leukocytic tissues (Didsbury et al., 1987) demonstrated the potential expression of a second G<sub>i</sub>-like protein (G<sub>i</sub>2) which had an identical C-terminal decapeptide to that of G<sub>i</sub>1 and which was some 88% similar overall at the primary sequence level. An antipeptide antiserum directed against a 10-amino-acid sequence corresponding to amino acids 160-169 of this protein equally recognized the 40 kDa pertussis toxin-sensitive polypeptide of neutrophils and glioma C6 cells, confirming the identity of this protein as G<sub>1</sub>2 (Goldsmith et al., 1987; Milligan et al., 1988). This antiserum, which did not recognize G<sub>i</sub>1, also identified low levels of G<sub>1</sub>2 in brain tissue (Backlund et al., 1988), which migrated between G<sub>i</sub>1 and G<sub>o</sub> on denaturing gel electrophoresis. These results were consistent with the idea that the three pertussis toxin-sensitive polypeptides first identified in brain by Neer et al. (1984) represented  $G_{i}1$ ,  $G_{i}2$  and  $G_{o}$ . As noted above, the recent cloning of a third ' $G_{i}$ -like' protein from both rat (Jones & Reed, 1987) and human tissues (Suki et al., 1987) further clouds attempts to demonstrate unequivocally the absolute molecular identity of pertussis toxin-sensitive Gproteins.

The most recent addition to this family of proteins  $(G_13)$  is extremely similar to  $G_11$  and in rat tissues differs in only some 22 of 354 amino acids (6%). These modifications are scattered throughout the amino acid sequence and as such it is difficult to identify potential areas of amino acid sequence which might allow selective antipeptide antisera to be produced. The *C*-terminal of rat  $G_13$  does in fact differ in two positions from that of  $G_11$  and  $G_12$ , but both (Asp and Phe in  $G_11$  and  $G_12$  to Glu and Tyr in  $G_13$ ) are but minor alterations. Preliminary and somewhat circumstantial evidence,



Fig. 7. The C-terminal decapeptides of the pertussis toxinsensitive G-proteins

The marked homology of sequence of the individual proteins in this region would suggest that, with the exception of  $G_o$ , each of the other polypeptides would be unlikely to display marked selectivity in interactions with receptors if this domain was to represent the sole area of contact between receptors and G-proteins.

which relies upon the detection of mRNAs coding for both  $G_i^2$  and  $G_i^3$  but not  $G_i^1$  in the human cell line HL60, has been presented in conjunction with Western blot analyses, which could be interpreted to imply that antisera against the *C*-terminal region of tranducin do recognize  $G_i^3$  as well as the other ' $G_i$ -like' G-proteins (Murphy *et al.*, 1987).

As transducin is limited in distribution to photoreceptor-containing tissues, then C-terminal antisera have been used to detect the presence and levels of 'G<sub>i</sub>like' proteins in a range of tissues which do not express transducin (Gawler et al., 1987; Milligan et al., 1987b). Of particular note, they have been used to demonstrate that the lack of functional G<sub>i</sub> activity in hepatocyte membranes derived from alloxan- or streptozotocindiabetic rats corrected with reduction of the amounts of the  $\alpha$  subunit of a form of G<sub>1</sub> to some 10% of that of untreated rats (Gawler et al., 1987). They have also been used to show that chemical treatment of rats to produce a hypothyroid state leads to a 2-fold elevation of levels of G<sub>i</sub> in membranes of adipocytes of these animals which correlated with reduced functioning of agonists which stimulate adenylate cyclase in these cells (Milligan, 1987b). These antibodies have been particularly useful as they do not cross-react with  $G_0$ , in which the C-terminal region differs in five of the ten amino acids of the antigen (Fig. 7). These antisera can also be used to detect ADPribosylated forms of 'G<sub>1</sub>-like' proteins and of transducin although the cysteine residue, which is the ADP-ribose acceptor site, is within the antigenic epitope. The covalently modified  $\alpha$  subunits are detected as more slowly migrating forms than the unmodified polypeptides in one-dimensional denaturing gel electrophoresis (Goldsmith et al., 1987).

#### **Reconstitution studies**

A fundamental aim of many biochemical investigations is to purify parts of a multicomponent system and to then attempt to reconstitute the function of that system to assess the specific roles of the different polypeptides. Signal transduction systems are admirably suited to this task, as in the native membrane separate receptor, Gprotein and effector entities can be identified.

The isolation of a variant form of the S49 lymphoma cell line which did not generate cyclic AMP in response to  $\beta$ -adrenergic agonists, despite the expression of  $\beta$ adrenergic binding sites (Insel *et al.*, 1976), played a major part in the definition of the role of G<sub>s</sub>. Named cyc<sup>-</sup> (Bourne *et al.*, 1975), analysis of this mutant demonstrated that a normal adenylate cyclase was present in membranes of this cell but that it lacked a substrate for cholera toxin (Johson *et al.*, 1978). More recently, further experimentation has shown it to lack both the structural polypeptide of G<sub>s</sub> $\alpha$ , as assessed in immunoblotting studies, and relevant mRNA (Harris *et al.*, 1985). This cell line thus facilitated the purification of G<sub>s</sub> by functioning as a highly sensitive acceptor system in reconstitution studies.

Similar mutants of pertussis toxin-sensitive G-proteins are not available. However, membranes of cells which have been pretreated with pertussis toxin can in many regards be considered to be functionally lacking these proteins. Reconstitution of membranes of these pertussis toxin-substrate<sup>-</sup> cells with various purified G-proteins (Katada et al., 1984a; Milligan & Klee, 1985; Milligan et al., 1985) thus should allow an assessment of the ability of receptors to interact with the exogenously provided G-proteins. A G-protein which can interact with a particular receptor should then be both able and sufficient: (1) to restore high-affinity agonist binding to the receptor, (2) to produce agonist-stimulated highaffinity GTPase activity and (3) to restore receptormediated alterations in second messenger generation. In short, a relevant G-protein should reverse the attenuation of receptor functioning which was produced by pertussis toxin. A considerable number of studies of this nature have been performed, usually with the aim of attempting to assess whether ' $G_i$ ' and  $G_o$  mediate the effects of different receptors. The overall pattern to emerge from the majority of these studies is that little selectivity is shown by receptors for the different pertussis toxinsensitive G-proteins (see Asano et al., 1985; Kikuchi et al., 1986, as examples). However, with hindsight, it is easy to argue that the validity of these studies needs to be closely re-examined because the number of pertussis toxin-sensitive G-proteins which have recently been identified, and their similarity, indicate that these reconstitution experiments may not have been performed with homogeneous populations of individual G-proteins. Equally, the role of presumably similar or identical  $\beta/\gamma$ subunits, associated with the individual  $\alpha$  subunits, in these experiments are difficult to assess. Inhibition of adenylate cyclase activity in reconstitution systems containing resolved adenylate cyclase catalytic subunit and  $\alpha$ or  $\beta/\gamma$  subunits of either G<sub>i</sub> or transducin have however indicated that the major inhibitory role is played by the  $\beta/\gamma$  subunits, presumably by combining with, and inactivating, the  $\alpha$  subunit of G<sub>s</sub> (Cerione *et al.*, 1986).

The exact roles of the different subunits, however, still remains a contentious issue. In the S49 cyc<sup>-</sup> model (Katada *et al.*, 1984*b*), somatostatin is able to produce a receptor-mediated inhibition of adenylate cyclase activity (Jakobs *et al.*, 1983), suggesting a direct role for the  $\alpha$ subunit of G<sub>i</sub>. More interestingly, the use of resolved  $\alpha$ subunits of G<sub>i</sub>1, G<sub>i</sub>2 and G<sub>o</sub> in reconstitution studies has suggested that only a 41 kDa polypeptide (G<sub>i</sub>1  $\alpha$  ?) is able to produce inhibition of adenylate cyclase (Katada *et al.*, 1987). Similar debates remain as to the roles of  $\alpha$ and  $\beta/\gamma$  subunits of pertussis toxin-sensitive G-protein (named G<sub>k</sub>) in the control of muscarinic receptor-linked K<sup>+</sup> channels in heart (Codina *et al.*, 1987; Logothetis *et al.*, 1987) (see Neer, 1988, for a detailed discussion).

Similar caveats must currently exist for other reconstitution studies which have been performed using artificial phospholipid vesicles as the milieu for interactions of purified' G-proteins and purified or partially purified receptors (Florio & Sternweis, 1985; Cerione et al., 1985a; Kurose et al., 1986). However, in some instances a degree of selectivity in receptor-G-protein interactions has been noted in such reconstitution systems (Cerione et al., 1985b). Given the complexity of the expression of the different pertussis toxin-sensitive G-proteins, in which, for example Jones & Reed (1987) noted the transcription of detectable levels of mRNAs for  $G_0$ ,  $G_1$ ,  $G_2$  and  $G_i$ 3 in all tissues which they examined, it will probably require the production of protein expressed from transfection experiments (Nukada et al., 1987; Graziano et al., 1987) before reconstitution studies can be definitively performed. In this regard however, bacterially expressed protein may not be suitable if it lacks various post-translational modifications, such as the attachment of myristic acid, which presumably plays a role in the interaction of the  $\alpha$  subunit with the membrane.

#### Use of antisera to assess receptor-G-protein interactions

Based on the knowledge that the site of pertussis toxincatalysed ADP-ribosylation, in G-proteins which are substrates for this toxin, is a conserved cysteine residue located four amino acids from the C-terminus, and also that this modification attenuates productive interactions between receptor and G-protein, it has been proposed that the C-terminal region of G-proteins is likely to represent a (the) site of receptor-G-protein contact (Masters et al., 1986; Bourne et al., 1987; McKenzie et al., 1988a,b; Hamm et al., 1987). In the case of  $G_s$ , this contention has been elegantly validated by the analysis of sequence of clones of  $G_s$  isolated from both wild type and the unc (uncoupled) mutant of the S49 lymphoma cell line (Sullivan et al., 1987). The unc mutant does not produce cyclic AMP upon  $\beta$ -adrenergic activation (Haga et al., 1977) although the structural polypeptides for each of  $\beta$ -adrenergic receptor, G<sub>s</sub> and adenylate cyclase are expressed. Bourne and coworkers have demonstrated that this mutation consists of a single base change in the gene coding for the  $\alpha$  subunit of  $\hat{G}_s$ . This results in the exchange of an arginine residue six amino acids from the C-terminus in the wild type to a proline residue in the unc mutant (Sullivan et al., 1987). Similar results have been produced by Rall & Harris (1987). Consistent with this is the observation that the  $\alpha$  subunit of G<sub>s</sub> from the unc mutation has an isoelectric point more acidic than that of wild type (Schleifer *et al.*, 1980). This alteration appears to be sufficient to prevent productive interaction between agonist-bound  $\beta$  receptor and the mutant form of G<sub>s</sub>.

As similar mutants of the pertussis toxin-sensitive Gproteins have not been identified, then alternative strategies have had to be employed to address the question of which domain(s) of these G-proteins is (are) important for receptor coupling. One approach which has been successful has been to utilize either anti-peptide or monoclonal antisera directed against epitopes located

either within or close to the putative receptor-recognition domain of pertussis toxin-sensitive G-proteins. Milligan and coworkers used an anti-peptide antiserum directed against the C-terminal decapeptide of the 'G,-like' proteins to demonstrate that a  $\delta$  opioid receptor on the neuroblastoma × glioma hybrid NG 108-15 interacted exclusively with 'G<sub>i</sub>' in this cell line (McKenzie et al., 1988*a*,*b*) despite the fact that both ' $G_i$ ' and  $G_o$  and potentially other uncharacterized pertussis toxin-sensitive G-proteins are expressed by these cells (Milligan et al., 1986). These results are at variance with the conclusions of Hescheler et al. (1987) who concluded that in this cell line the opioid receptor was able to interact preferentially with  $G_0$  to modulate the function of  $Ca^{2+}$ channels, based on reconstitution studies using 'purified'  $G_i$  and  $G_o$  from brain. However, as pointed out above, these fractions were unlikely to represent homogeneous populations of a single G-protein and given the difficulty in resolving  $G_0$  from  $G_1^2$  in purification protocols (Katada *et al.*, 1987; P. Goldsmith, P. S. Backlund Jr., G. Milligan, C. G. Unson & A. Spiegel, unpublished work), then considerable contamination of this ' $G_0$ ' preparation must be considered likely. Thus, despite the conclusive evidence that Ca<sup>2+</sup> channels can be modulated by pertussis toxin-sensitive G-proteins (Hescheler *et al.*, 1987; Scott & Dolphin, 1987) the molecular identity of the Gprotein(s) has not yet been rigorously demonstrated.

Further evidence to support the selectivity of this type of antibody approach was provided in the studies of McKenzie *et al.* (1988*a*) by the observation that a growth factor receptor on this cell line, which interacts with a second pertussis toxin-sensitive G-protein, was not uncoupled from its response by antibodies affinitypurified from the anti-peptide, anti-'G<sub>i</sub>' antiserum. This showed that the G-protein linked to the growth factor receptor must possess a C-terminal sequence distinct from that of  $G_i$ . A similar approach has been utilized by Hamm and coworkers to study the interaction of rhodopsin and transducin by employing monoclonal antisera which were demonstrated to recognize an epitope near to the C-terminal region of transducin (Deretic & Hamm, 1987; Hamm et al., 1987). These monoclonals were able to uncouple transducin from rhodopsin but monoclonals directed against epitopes elsewhere on transducin did not.

Because the C-terminal 30 amino acids of all the ' $G_i$ like' G-proteins are so homologous, then it must be surmised that if the extreme C-terminus of the Gproteins was to represent the only area of receptor contact then there would be little prospect that the individual forms of 'G<sub>i</sub>' could interact with different receptors (Fig. 7). However, the region between 30 and 60 amino acids from the C-terminus of the pertussis toxin-sensitive G-proteins is one of the regions of these proteins which displays a degree of divergence at the level of amino acid sequence, and it may be that this region represents the key area in defining receptor-Gprotein contacts. Further experiments of this type may help to define the specificity or otherwise of interactions between individual receptors and different pertussis toxin substrates.

Antisera directed against sequences within the other major area of sequence divergence between these pertussis toxin-sensitive G-proteins may be useful in probing interactions of G-proteins and second messenger generation systems, as this area is likely to represent the effector-recognition domain. Other defined antisera may be useful in studies on the interactions between the individual subunits of the G-proteins. In this regard Navon & Fung (1987) have recently used monoclonal antisera directed against the *N*-terminus of the  $\alpha$  subunit of transducin to demonstrate that this region is involved in contact between the  $\alpha$  and  $\beta/\gamma$  subunits.

Although the amino acid sequences of the pertussis toxin-sensitive G-proteins may be very strongly conserved, the nucleotide sequences of the genes coding for these are less so, due to the degeneracy of the genetic code. Thus, oligonucleotides derived from cDNAs offer the potential to be very selective probes for the analysis of transcription of mRNAs coding for proteins which are very similar in amino acid sequence (Brann et al., 1987; Murphy et al., 1987; Jones & Reed, 1987). Although probes of this nature cannot be directly used to assess receptor-G-protein interaction, a combination of the use of oligonucleotide probes to confirm or deny the expression of individual G-proteins, and antibody probes, suggests techniques for the further progress in our understanding of the details of signal transduction processes that involve pertussis toxin-sensitive G-proteins.

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