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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 (G-proteins). 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 α subunit which binds guanine nucleotides and which possesses an intrinsic GTPase activity. In many, but not all, cases the α subunits of individual G-proteins may be substrates for ADP-ribosylation catalysed by certain bacterial toxins. These α subunits range

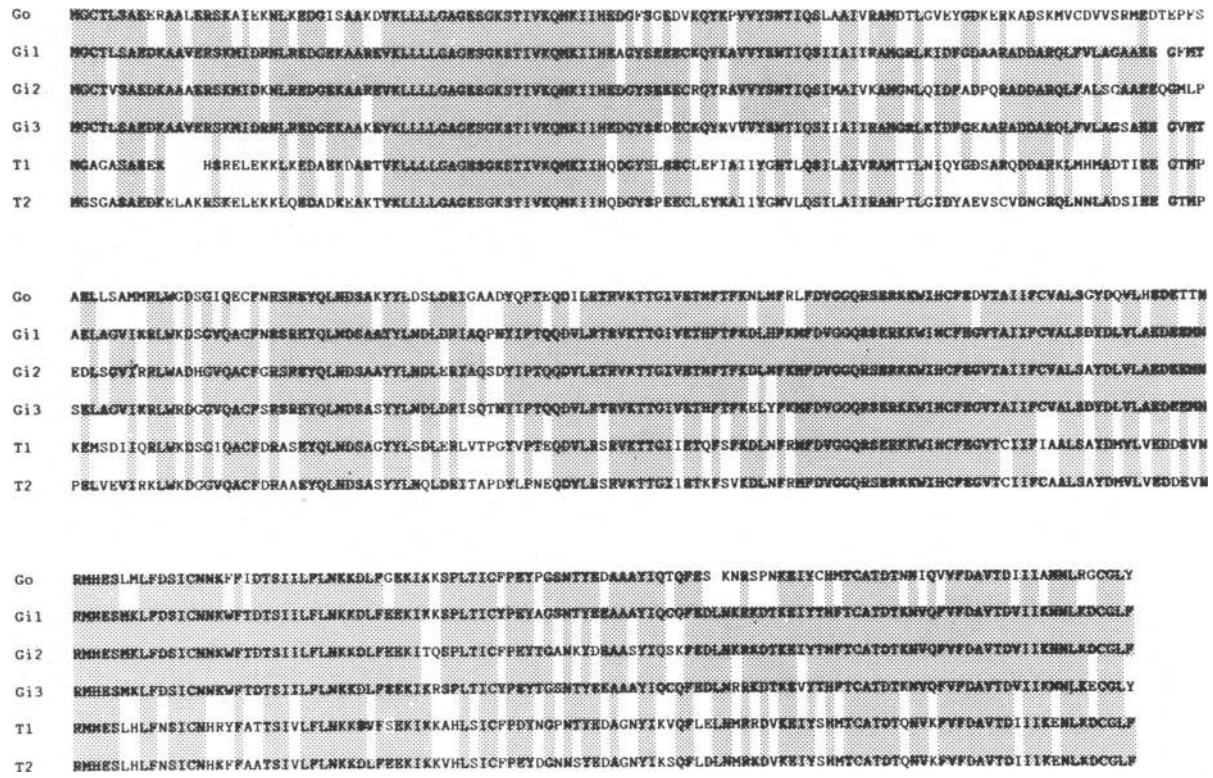

Fig. 1. Primary sequences of the α subunits of the currently identified pertussis toxin-sensitive G-proteins as expressed in rat

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_i-like' subfamily is indicated. Where each of G_i1, G_i2 and G_i3 is represented by a different amino acid at one position then no further homology to G_o, T1 or T2 is noted. The sequences for G_o, G_i1, G_i2 and G_i3 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; GTγS, guanosine 5'-[γ-thio]triphosphate; GDPγS, guanosine 5'-[β-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 α subunits which both define the separate G-proteins and which can most appropriately and usefully be analysed.

(2) A β subunit of some 35–36 kDa on denaturing gels. Until recently it had been generally accepted that a common pool of a single β subunit was shared between different α 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 β 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 β subunit has confirmed the presence of at least two genes encoding β 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 β subunit is able to interact exclusively with a particular subset of α subunits.

(3) A γ subunit. Relatively little attention has been paid to the nature and potential diversity of the γ 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 γ subunit remains tightly associated with the β subunit.

The diversities in structure noted at the levels of the β and γ 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 agonist-activated receptors to the effector systems that alter intracellular concentrations of second messengers. As this process must be of limited duration, then the G-protein 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 α 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^{2+} then the holomeric G-protein can dissociate into an active α subunit with GTP bound and free β/γ subunits. This active α subunit is then able to interact with the catalytic moiety of a particular second-messenger-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 α subunit and in this GDP-bound form it is then able to reassociate with β/γ subunits to restore the G-protein to the deactivated state.

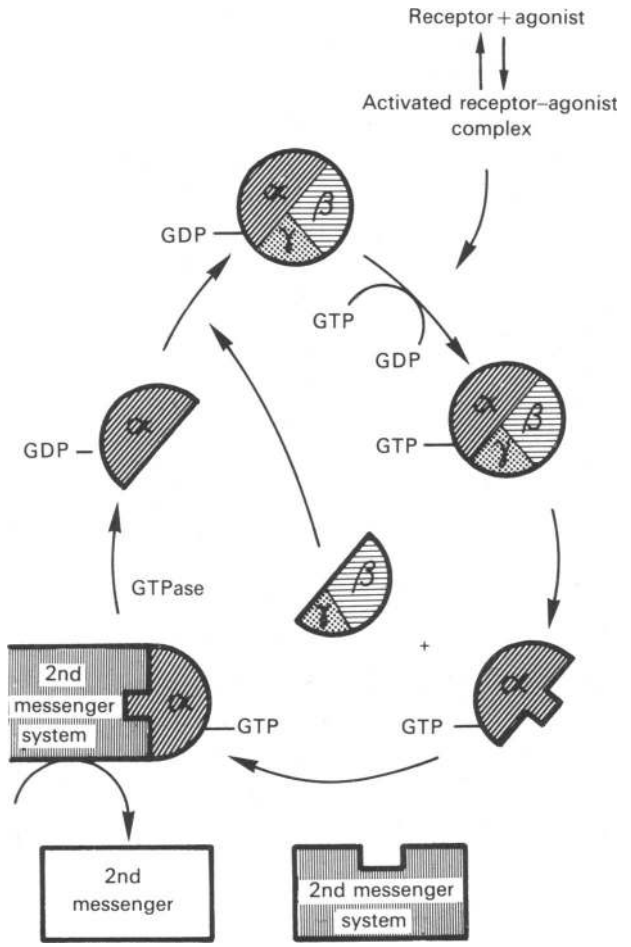

Fig. 2. The role of GRP binding and hydrolysis in the activation and deactivation of a typical G-protein

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.*, 1971*b*). It was subsequently noted that guanine nucleotide effects on β-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 [^{3}H]antagonist binding were non-ideal and were shown to have Hill coefficients significantly less than 1.0. In contrast, antagonist displacement of [^{3}H]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 GTPγS, to the binding incubations reduced the ability of agonists, but not antagonists, to compete for [^{3}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^{2+} 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 G-protein 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 [^{3}H]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 G-protein(s) and also that Ca^{2+} 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 G-protein. 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 G-protein 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 β-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. G-protein-related GTP hydrolysis is such that the K_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 μM) 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_s 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_s tend to be lower than those of many other G-proteins and that the rate of GTP hydrolysis by purified G_s 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_s remains lost within the 'noise' due to the basal rates of hydrolysis by the other G-proteins 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 G-proteins 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 (20%, v/v)	DALAMID (10 μM)
—	6.3±0.4
+	6.9±0.5
DALAMID (10 μM)	Foetal calf serum (20%, v/v)
—	12.0±0.3
+	12.5±0.4

of the full complement G-protein(s) with which that receptor is able to interact. Thus, addivity of receptor-stimulated GTPase activity following addition of two agonists which interact with independent receptors would indicate the activation of separate pools of G-protein, and by extension, different G-proteins. This approach has been elegantly employed by, for example, Houslay *et al.* (1986*a*,*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.*, 1988*a*) 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^+ to the α 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^+ 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_i). 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_i (Fig. 3). This cyclical mode of reasoning was only shown to be flawed following the purification of multiple pertussis toxin substrates (G_i and

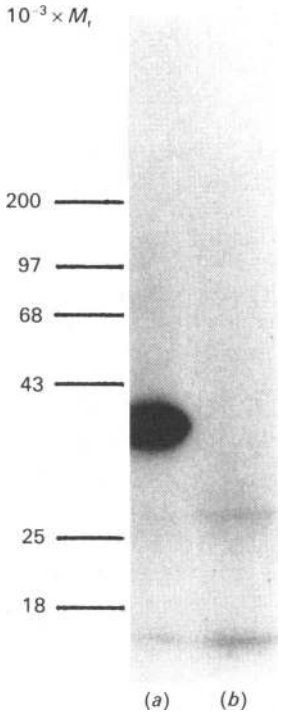

Fig. 3. Pertussis toxin-catalysed ADP-ribosylation

Membranes of neuroblastoma × glioma hybrid cells were incubated with activated pertussis toxin and [^{32}P]NAD^+ 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_i2 and G_o) 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.*, 1986*c*).

Initial demonstrations that more than a single pertussis toxin-sensitive G-protein could be expressed came from attempts to purify 'G_i' 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 toxin-sensitive 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_i-like' proteins, named for the chronology of their identification G_i1, G_i2 and G_i3 (Reed & Jones, 1987; Suki *et al.*, 1987; Beals *et al.*, 1987), and G_o (Itoh *et al.*, 1986; Jones & Reed, 1987) (Fig. 1). In addition, two separate mRNAs which hybridize to a G_o 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 ADP-ribosyltransferase which in the presence of a second protein called ADP-ribosylation factor (ARF) (Kahn & Gilman, 1984) is able to catalyse the transfer of ADP-ribose from NAD^+ to an arginine residue in the α subunit of G_s. This covalent modification stabilizes the GTP-bound form of the α subunit of G_s and as the GTPase activity of the α subunit is inhibited then G_s 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 α subunit(s) of G_s. 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 α 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 × 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 × 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 ADP-ribosylation 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_s 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 G-proteins 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).

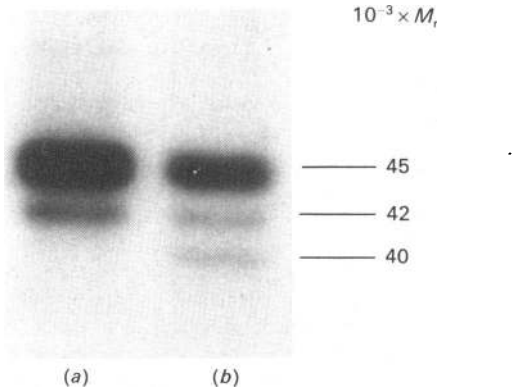

Fig. 4. Cholera toxin-catalysed ADP-ribosylation: the effect of GRP

Membranes of rat glioma C6 BU1 cells were treated with activated cholera toxin and [^{32}P]NAD in the presence (*a*) or absence (*b*) of exogenously added GTP (100 μM) as described by Milligan (1987). No incorporation of radioactivity 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 α subunit of G_i2 (Milligan, 1988).

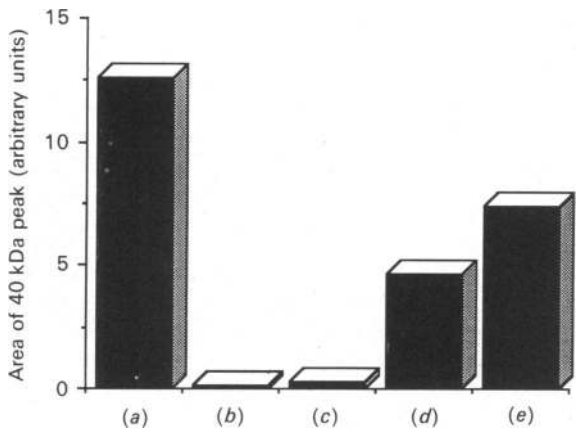

Fig. 5. Agonist enhancement of cholera toxin-catalysed ADP-ribosylation of a form of G_i in membranes of NG108-15 cells

Membranes of neuroblastoma × glioma hybrid NG108-15 cells were treated with [^{32}P]NAD^+ 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 two-dimensional 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 toxin-sensitive 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 G-protein). A particular problem which has been noted, but which is only infrequently assessed, is that the [^{32}P]NAD^+ 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_i1) and G_o. 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.*, 1987*a*). However, results obtained from pertussis toxin-catalysed ADP-ribosylation indicated that levels of these proteins fell sharply with age (Milligan *et al.*, 1987*d*). 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 [^{32}P]NAD^+ was destroyed in a short time span (Milligan *et al.*, 1987*d*).

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 ADP-ribosylation 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 GDPβS, the rate of pertussis toxin-catalysed 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 GTPγS (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 α subunits. Such an interpretation is well supported by observations that the separated α subunit of the pertussis toxin-sensitive G-protein G_o is a very weak substrate for ADP-ribosylation but that upon addition of β/γ subunits the α 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 chal-

lenging membranes derived from toxin-treated cells with fresh toxin and [^{32}P]NAD$^+$. Further, the rate of toxin-catalysed 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 G-proteins

Resolution of the different pertussis toxin-sensitive G-proteins 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_o > G_i2 > G_i1, 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 toxin-sensitive G-proteins has been achieved. Initial experiments in this area involved the prior ADP-ribosylation of membrane preparations with pertussis toxin and [^{32}P]NAD$^+$ 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_i1 migrates with a more basic isoelectric point (6.1) than G_i2 (5.65) and the major form of G_o (5.6). In brain a second form of G_o can be observed with an isoelectric point near 6.0 (Backlund *et al.*, 1988). In support of these observations, in purification protocols, G_i1 and a form of G_o elute in close proximity from anion exchange resins and can be relatively easily resolved from G_i2 and the major form of G_o, 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 G-proteins is that in general the α 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 *N*-terminal 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 GTPγS 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.*, 1988*b*), 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 toxin-sensitive 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 α subunit of G_o and against the β subunit but within these antisera no antibodies were generated which selectively recognize forms of the α subunit of G_i (Gierschik *et al.*, 1986*a*; Huff *et al.*, 1985; Roof *et al.*, 1985). Why G_o appears to be immunodominant in comparison to G_i 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_o antisera (Gierschik *et al.*, 1986*a*) 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_i antiserum using purified brain G-protein as antigen. In a number of cases anti-G_o antisera generated in this direct fashion show some degree of cross-reactivity with other pertussis toxin-sensitive 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 G-proteins, it should not be surprising if a degree of cross-reactivity were noted with polyclonal antisera. However, based on the recognition that domains which must interact selectively with particular classes of receptors

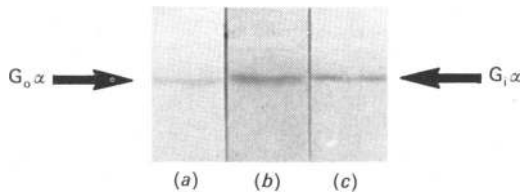

Fig. 6. Antipeptide antisera directed against the *C*-terminal decapeptides of 'G_i' and G_o discriminate between these two forms

Membranes of cells of the neuroblastoma × glioma hybrid cell line NG108-15 were resolved on SDS/polyacrylamide-gel electrophoresis (10%, v/v) and immunoblotted with (*a*) an antipeptide antiserum against the *C*-terminal decapeptide of G_o, (*c*) an equivalent antiserum against the *C*-terminal decapeptide of 'G_i' 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_o-specific antiserum migrates further in the gel than that identified by the 'G_i' 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_o antisera of this class were first used to demonstrate that G_o and G_i were distinct entities (Gierschik *et al.*, 1986*a*) and that G_o 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_i1). 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.*, 1986*b*). Rather different results on the relative expression of G_i and G_o 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.*, 1987*a*,*c*; Luetje *et al.*, 1987). Anti-G-protein 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_o and anti-G_i1 selective antisera to stain a 40 kDa pertussis toxin substrate that was present in high levels in both human neutrophils (Gierschik *et al.*, 1986*c*) 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_i2 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 α 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 α subunits. However, no antipeptide antisera against G_i 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 α subunit of this protein, was able to cross-react with brain G_i (Pines *et al.*, 1985; Milligan & Klee, 1985), then Spiegel, Unson and Milligan (see for example Falloon *et al.*, 1986; Milligan *et al.*, 1987*b*; 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_i. This peptide sequence could be predicted from cDNA clones of transducin whilst the equivalent sequence of G_i was not available at that time. These antisera did in fact recognize brain G_i as well as transducin on western blots, indicating that the *C*-terminal sequence of G_i must be similar to that of transducin. In retrospect this was not an unexpected result, as cDNA studies later demonstrated that brain G_i 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_o or the predominant G_i-like protein of brain (G_i1) 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_i-like protein (G_i2) which had an identical *C*-terminal decapeptide to that of G_i1 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_i2 (Goldsmith *et al.*, 1987; Milligan *et al.*, 1988). This antiserum, which did not recognize G_i1, also identified low levels of G_i2 in brain tissue (Backlund *et al.*, 1988), which migrated between G_i1 and G_o 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_i1, G_i2 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 G-proteins.

The most recent addition to this family of proteins (G_i3) is extremely similar to G_i1 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_i3 does in fact differ in two positions from that of G_i1 and G_i2, but both (Asp and Phe in G_i1 and G_i2 to Glu and Tyr in G_i3) are but minor alterations. Preliminary and somewhat circumstantial evidence,

G_i1	K N N L K D C G L F
G_i2	K N N L K D C G L F
G_i3	K N N L K E C G L Y
G_o	A N N L R G C G L Y
TD1	K E N L K D C G L F
TD2	K E N L K D C G L F

Fig. 7. The *C*-terminal decapeptides of the pertussis toxin-sensitive 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_i2 and G_i3 but not G_i1 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_i3 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_i-like' proteins in a range of tissues which do not express transducin (Gawler *et al.*, 1987; Milligan *et al.*, 1987*b*). Of particular note, they have been used to demonstrate that the lack of functional G_i activity in hepatocyte membranes derived from alloxan- or streptozotocin-diabetic rats corrected with reduction of the amounts of the α subunit of a form of G_i 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_i in membranes of adipocytes of these animals which correlated with reduced functioning of agonists which stimulate adenylate cyclase in these cells (Milligan, 1987*b*). These antibodies have been particularly useful as they do not cross-react with G_o, 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 ADP-ribosylated forms of 'G_i-like' proteins and of transducin although the cysteine residue, which is the ADP-ribose acceptor site, is within the antigenic epitope. The covalently modified α 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, G-protein 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 β-adrenergic agonists, despite the expression of β-adrenergic binding sites (Insel *et al.*, 1976), played a major part in the definition of the role of G_s. Named cyc$^-$ (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 $G_s\alpha$, as assessed in immunoblotting studies, and relevant mRNA (Harris *et al.*, 1985). This cell line thus facilitated the purification of G_s 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$^-$ cells with various purified G-proteins (Katada *et al.*, 1984*a*; 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 high-affinity GTPase activity and (3) to restore receptor-mediated 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 toxin-sensitive 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 β/γ subunits, associated with the individual α subunits, in these experiments are difficult to assess. Inhibition of adenylate cyclase activity in reconstitution systems containing resolved adenylate cyclase catalytic subunit and α or β/γ subunits of either G_i or transducin have however indicated that the major inhibitory role is played by the β/γ subunits, presumably by combining with, and inactivating, the α subunit of G_s (Cerione *et al.*, 1986).

The exact roles of the different subunits, however, still remains a contentious issue. In the S49 cyc$^-$ 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 α subunit of G_i. More interestingly, the use of resolved α subunits of G_i1, G_i2 and G_o in reconstitution studies

has suggested that only a 41 kDa polypeptide (G_i1 α ?) is able to produce inhibition of adenylate cyclase (Katada *et al.*, 1987). Similar debates remain as to the roles of α and β/γ subunits of pertussis toxin-sensitive G-protein (named G_k) in the control of muscarinic receptor-linked K^+ 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.*, 1985*a*; 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.*, 1985*b*). 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_o, G_i1, G_i2 and G_i3 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 α subunit with the membrane.

Use of antisera to assess receptor–G-protein interactions

Based on the knowledge that the site of pertussis toxin-catalysed 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.*, 1988*a*,*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 β-adrenergic activation (Haga *et al.*, 1977) although the structural polypeptides for each of β-adrenergic receptor, G_s 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 α subunit of 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 α subunit of G_s 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 β receptor and the mutant form of G_s.

As similar mutants of the pertussis toxin-sensitive G-proteins 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_i-like' proteins to demonstrate that a δ opioid receptor on the neuroblastoma × glioma hybrid NG 108-15 interacted exclusively with 'G_i' 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_o 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_o from G_i2 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_o' preparation must be considered likely. Thus, despite the conclusive evidence that Ca^{2+} channels can be modulated by pertussis toxin-sensitive G-proteins (Hescheler *et al.*, 1987; Scott & Dolphin, 1987) the molecular identity of the G-protein(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 affinity-purified from the anti-peptide, anti-'G_i' 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 G-proteins was to represent the only area of receptor contact then there would be little prospect that the individual forms of 'G_i' 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–G-protein 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 α subunit of transducin to demonstrate that this region is involved in contact between the α and β/γ 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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