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COVALENT MODIFICATIONS OF G-PROTEINS

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PERSPECTIVES

G-proteins are a large, ubiquitous group of proteins in which the binding and hydrolysis of GTP play an integral role in the control of their function. This group of proteins comprises the heterotrimeric G-proteins, which have been well characterized and shown to transduce extracellular signals into intracellular responses, and the monomeric (small) G-proteins, whose functions are less well defined but are thought to regulate intracellular signaling pathways. Together, these two superfamilies of G-proteins regulate nearly every cellular activity.

Although each G-protein bears distinctive structural features, which define its role in a specific signal transduction pathway, there is a high degree of homology between members of each superfamily of G-proteins, as well as regions of high homology between the small G-proteins and the heterotrimeric G-proteins. In addition, many members of the G-protein superfamilies are substrates for a large number of covalent modifications. Among these are nearly every covalent modification known (with the exception of glycosylation), including ADP-ribosylation, prenylation, acylation, and phosphorylation. Each of these modifications is critical in controlling the proper interaction of the G-protein with other proteins, as well as with the appropriate membrane compartments. This review will briefly discuss the enzymology and structure of each of these covalent modifications, with an emphasis on their functional significance as they pertain to G-proteins and the potential for pharmacological intervention.

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BACKGROUND

Heterotrimeric G-Proteins

In 1971, Rodbell et al discovered that guanine nucleotides were required for the regulation of receptor-mediated signal transduction (1). This finding ultimately led to the discovery of a family of GTP-binding proteins, now known as G-proteins, which share the function of transducing extracellular stimuli into intracellular responses, usually in the form of a change in concentration of a second messenger molecule. These G-proteins are all heterotrimers consisting of α, β, and γ subunits, of molecular mass 36–52, 35–36, and 6–8 kDa, respectively. To date, molecular cloning techniques have identified in mammalian tissues more than 17 different α subunits, 4 β subunits, and 4 γ subunits, with partial protein sequencing providing evidence for a total of at least 8 different γ polypeptides (2). Homologs of G-protein α, β, and γ subunits have also been identified in yeasts, *Drosophila melanogaster, Caenorhabditis elegans,* and the slime mold *Dictyostelium discoideum* (2). The classification, properties, and function of these different subunits are the subjects of many recent publications (2) and will not be presented in detail here. In summary, the α subunits possess the GTP-binding and hydrolyzing activities and, in most systems, interact with receptor and effector proteins as well. The β and γ subunits form a βγ complex, which is not dissociable under nondenaturing conditions. The major function of the βγ complex appears to be the formation of the holoenzyme, which constitutes the inactivated state of the protein. More recently, however, the βγ complex has also been implicated as a carrier of intracellular signals such as activation of phospholipase A_2 (3–6), adenylate cyclase (6a, 6b), and the mating response in yeast (7). Although the different G-proteins have traditionally been defined by their α subunits, the recent discoveries of greater numbers of β and γ subunits complicate this picture.

Signal transduction processes known to be mediated by G-proteins include the hormone- and neurotransmitter-mediated regulation of adenylate cyclase, phosphoinositide metabolism, and ion channels, as well as the sensory systems of vision, olfaction, and taste. The molecular mechanism of G-protein-mediated signal transduction has been elucidated simultaneously in two systems—the hormone-stimulated increase in adenylate cyclase activity and the light-stimulated phototransduction cascade. A number of excellent reviews on the mechanism of G-protein-mediated signal transduction have been published (8–10); hence, only a brief introduction to their mechanism will be given here. A schematic representation of the general mechanism underlying signal transduction by heterotrimeric G-proteins is shown in Figure 1*A*, with a listing of the well-established signal transduction pathways and the subfamilies involved. In the resting state of the cell, association of the GDP-bound

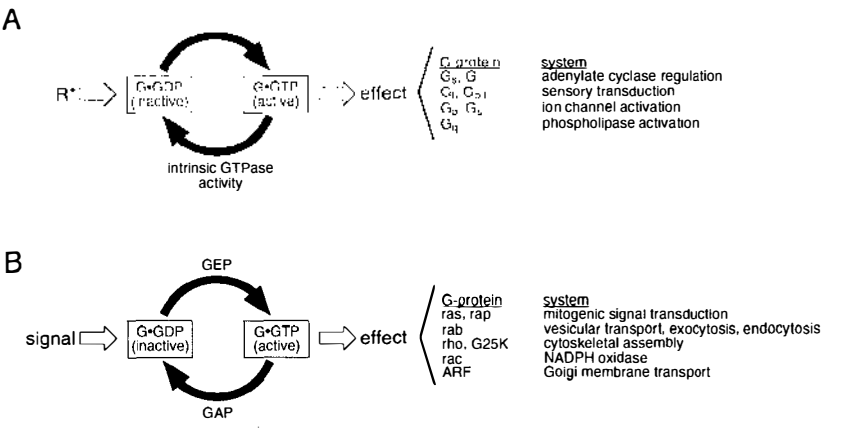

Figure 1 General mechanisms of G-protein-mediated signal transduction. (*A*) Receptor-activated signal transduction of heterotrimeric G-proteins. Activated receptor (R*) catalyzes the exchange of bound GDP for GTP on G-protein, which in turn interacts with an effector protein to produce an intracellular signal. The activated state of G-protein is terminated by an intrinsic GTPase activity. Several well-characterized G-protein-mediated signal transduction systems are listed, with the relevant G-protein. (*B*) Known components of small G-protein-mediated signal transduction. A signal is presumed to drive the small G-protein to its activated, GTP-bound state, a process that may be facilitated by GEPs. After or during completion of the signaling task, the intrinsic GTPase activity of the small G-protein, enhanced by a GAP, returns the protein to its inactive state. Several of the systems in which small G-proteins have been implicated, with the groups involved, are listed.

holoenzyme with the receptor protein results in a high-affinity state of the receptor for its ligand. On binding of a ligand, the receptor activates the G-protein, causing it to exchange its bound GDP for GTP. The binding of GTP changes the conformation of the α subunit, resulting in a release of the $\beta\gamma$ complex. The α subunit then activates or inactivates effector enzymes directly, as in the case of adenylate cyclase, or removes an inhibitory constraint, as in activation of the cyclic GMP (cGMP) phosphodiesterase in the visual phototransduction cascade. The activated α subunit is subsequently turned off by its intrinsic GTPase activity, allowing reassociation with the $\beta\gamma$ complex.

An important hallmark of G-protein-mediated signal transduction is amplification of the primary signal. For most systems, this is achieved in two stages: the first is the activation of 10 to several hundred G-proteins by a single ligand-bound receptor, and the second is the turnover of thousands of second-messenger molecules by each activated effector enzyme. In this way the signal carried by a single ligand molecule may be amplified more than 10^5-fold, as exemplified by the phototransduction cascade of the visual system, capable of detecting a single photon.

Small G-Proteins

For many years, the *ras* oncogene has been known to encode a 21-kDa protein that specifically binds and hydrolyzes GTP (11). The designation of the *ras* gene product as a G-protein, and subsequently as the prototypical small G-protein, was made with the discovery that it possessed regions of homology to the α and γ subunits of the heterotrimeric G-proteins (12). On the basis of their sequence homology with heterotrimeric G-proteins, it was postulated that the ras proteins were also involved in signal transduction processes. This discovery precipitated an intense search for other ras-like proteins. Within the past several years, a large number of proteins similar in size and with various degrees of homology to the ras proteins have been identified (13–15). As with the heterotrimeric G-proteins, many of these genes or gene products have been found in *Dictyostelium discoideum*, yeast, and *Drosophila melanogaster*, as well as in most mammalian tissues. They are separated into groups on the basis of the methods by which they were purified or their cDNAs cloned; the major groups are ras, rab/smg p25, rac, ral, rap/smg p21, rho, G25K/CDC42Hs, and ADP-ribosylation factor (ARF). The multiple names by which some groups are known arise from their independent discoveries in different laboratories. These groups can further be classified into four major families on the basis of amino acid homologies. These are the ras (including rap and ral), rho (including rac and G25K/CDC42Hs), rab, and ARF families (13).

The regions of highest homology within the superfamily of small G-proteins occur in four noncontiguous regions that form the GTP-binding and hydrolysis domains (15). An additional common structural feature is the presence of a cysteine residue at or near the carboxyl terminus of nearly all small G-proteins, which is an important site for posttranslational modification. Members of the ras and rho families possess -Cys-Xaa-Xaa-Xaa carboxyl termini, whereas those of the rab family possess carboxyl termini ending in either a -Cys-Xaa-Cys or -Cys-Cys motif. As will be discussed in detail later in this review, the cysteine residues in all three of these motifs are important sites for prenylation. Thus far, members of the ARF family constitute the only small G-proteins that lack the consensus carboxyl-terminal cysteine. These proteins, which were first discovered by their ability to stimulate ADP-ribosylation of the heterotrimeric G-proteins by cholera toxin (hence the name ADP-ribosylation factor) (16, 17), do not undergo most of the covalent modifications common to the other small G-proteins and will not be discussed in further detail.

Although little is known regarding the exact function of most small G-proteins, nearly all appear to be involved in intracellular signal transduction pathways, such as the regulation of cell growth and differentiation, vesicular transport, and cytoskeletal organization (18, 19). Because of this and because

of their resemblance to the heterotrimeric G-proteins, they have been postulated to interact with specific "receptor" and "effector" proteins (18). As is the case with the heterotrimeric G-proteins, these interactions are regulated by the GTP-bound "active" state and GDP-bound "inactive" state of the small G-protein. The known components of the GTP-mediated activation-deactivation cycle common to all small G-proteins are compared with those of the heterotrimeric G-proteins in Figure 1*B*. As shown in this figure, a number of proteins interacting with the small G-proteins have also been described, although their designation as receptor or effector proteins remains elusive. The first of these proteins to be discovered was a GTPase-activating protein (GAP), which stimulates the relatively low GTPase activity of proto-oncogenic ras over 200-fold (20). Because the site of interaction of ras with GAP has been localized to its "effector" binding domain, GAP is presumed to be the downstream effector of ras (21–23). GAPs have subsequently been identified for rab3A/smg p25A (24), rap/smg p21 (25–29), rho (30), and G25K/CDC42Hs (31), ranging in size from ~25 to ~110 kDa. Each GAP appears to be unique and specific for each family of small G-proteins.

In addition to GAPs, the interaction of small G-proteins with other groups of proteins, collectively known as GDP-GTP exchange proteins (GEPs), is well established. These consist of GDP dissociation inhibitors (GDIs) and GDP dissociation stimulators (GDSs), which stoichiometrically interact with the GDP-bound form of the small G-protein and regulate the release of bound GDP and hence the rate of interconversion between their inactive and active states. Thus far, GDIs interacting with rab3/smg p25 (32), rho (33, 34), and an as yet unclassified small G-protein called 24K G (35) have been identified, and GDSs which act on ras (36–39) and rap/smg p21 (40) have also been shown. In contrast to GAPs, GDIs and GDSs are not totally specific and appear to be capable of regulating multiple small G-proteins from different families. Whether this characteristic is physiologically relevant, however, remains to be seen.

Covalent Modifications

It is well known that the proper function of many proteins relies on the covalent addition of a variety of molecular entities to their polypeptide chains. These may be as simple as an additional phosphate group or as large and complex as glycophospholipid moieties. G-proteins, both heterotrimeric and small, appear to be unique in possessing a large number of these modifications, which include ADP-ribosylation, prenylation, acylation, and phosphorylation.

Since this review centers on these modifications as they pertain to members of both classes of G-proteins, with particular regard to their function and potential as sites for pharmacological intervention, a general introduction to their chemistry would be prudent. These covalent modifications are illustrated

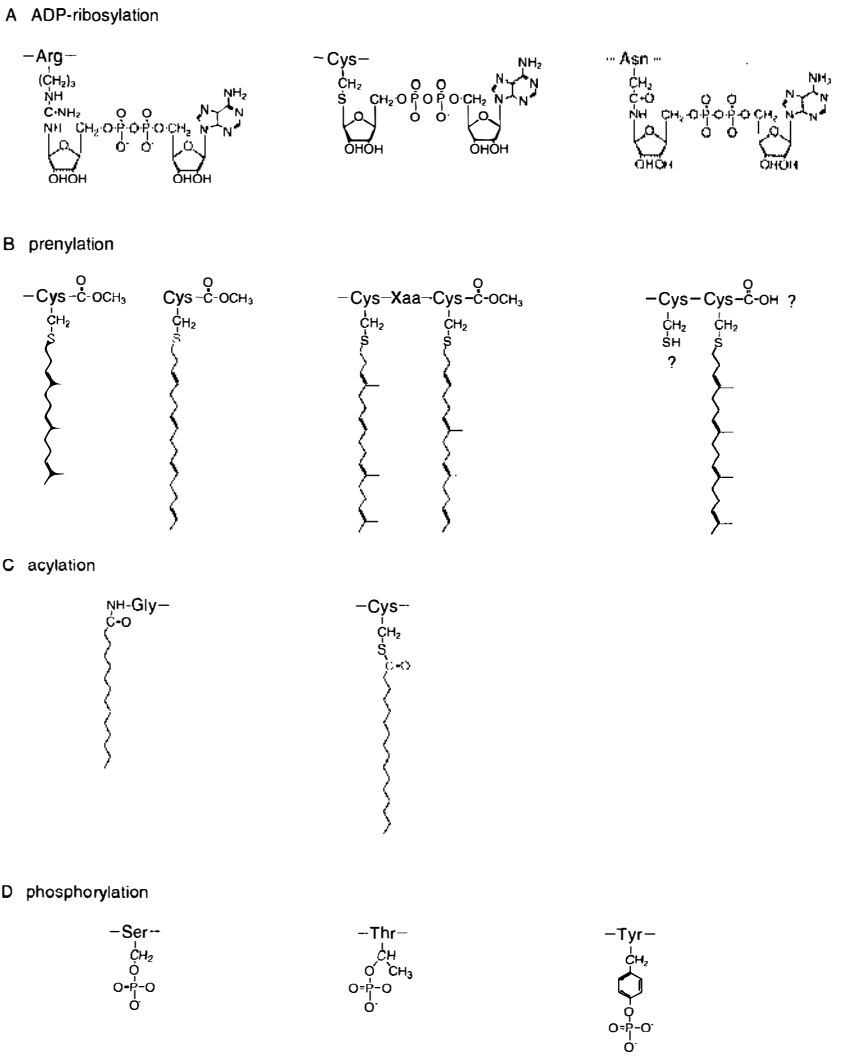

Figure 2 Schematic depiction of covalent modifications known to affect G-proteins. (*A*) ADP-ribosylation of arginine, cysteine, and asparagine by cholera, pertussis, and botulinum toxins, respectively. (*B*) Prenylation and α-carboxyl methylation of proteins with -Cys-Xaa-Xaa-Xaa, -Cys-Xaa-Cys, and -Cys-Cys motifs. Question marks indicate potential sites not yet conclusively shown to be modified. (*C*) Acylation processes relevant to G-proteins, including N-myristoylation and thioester-linked palmitoylation. (*D*) Phosphorylation of serine, threonine, and tyrosine residues, respectively.

generically in Figure 2. ADP-ribosylation involves the transfer of an ADP-ribose moiety from NAD^+ to an acceptor residue, either arginine, cysteine, or asparagine, resulting in formation of the ADP-ribosylated protein and nicotinamide. This mono(ADP-ribosyl)ation is distinct from poly(ADP-ribosyl)ation in structure, substrates, chemistry, and subcellular localization; henceforth, "ADP-ribosylation" will refer only to the former. Prenylation involves the transfer of an isoprenoid moiety from its corresponding pyrophosphate precursor to a cysteine near the carboxyl terminus in a thioether linkage, followed in some cases by proteolysis of three terminal residues to yield a carboxyl-terminal prenyl cysteine and methyl esterification of the newly exposed α-carboxyl group. Acylation is a more general process and includes the attachment of a variety of acyl groups in several possible linkages. The two acylation reactions prominent in the modifications of G-proteins, N-myristoylation and thioester-linked palmitoylation, are outlined in Figure 2. Both processes involve the transfer of the acyl moiety from their coenzyme A (CoA)-activated precursors. In N-myristoylation, a specific signal sequence directs an N-myristoyltransferase to attach a myristate group to an amino-terminal glycine in an amide linkage, a process that appears to be cotranslational and irreversible. In contrast, thioester-linked palmitoylation occurs on candidate cysteine residues and is posttranslational; it is consequently capable of turning over. No palmitoyltransferases have yet been purified or cloned, and palmitoylation appears at least in some cases to be nonenzymatic (41). Phosphorylation is a common regulatory modification, which has been described many times in great detail (42, 43). In the ensuing sections, we will discuss each of these modifications in detail as they pertain to G-proteins and then conclude with a discussion of possible pharmaceutical interventions based on these modifications.

ADP-RIBOSYLATION OF G-PROTEINS

Of the many posttranslational modifications that G-proteins undergo, ADP-ribosylation was the first modification to be identified and is the best characterized thus far. In a strict sense, ADP-ribosylation of G-proteins may not be considered a posttranslational modification, since the available evidence suggests that G-proteins are not ADP-ribosylated under normal physiological conditions. In this modification, the ADP-ribose moiety of NAD is transferred to the GTP-binding α subunit (Figure 2). Although the first and best-known ADP-ribosyltransferases are toxins produced by several strains of bacteria, the corresponding endogenous enzymes have now been described. Each toxin appears to ADP-ribosylate only a specific family of G-proteins. Cholera and pertussis toxins, from the bacteria *Vibrio cholerae* and *Bordetella pertussis*, respectively, catalyze the transfer of ADP-ribose from NAD to the α subunits

of heterotrimeric G-proteins. Most recently, toxins from certain *Clostridium botulinum* strains have been reported to catalyze ADP-ribosylation of certain groups of small G-proteins. Although the ADP-ribosylation of G-proteins by these bacterial toxins is well known to result in serious and potentially fatal clinical manifestations, the physiological relevance of ADP-ribosylation by these endogenous enzymes is not well understood.

Cholera Toxin-Mediated ADP-Ribosylation of Heterotrimeric G-Proteins

Cholera is a highly contagious disease caused by the bacterium *V. cholerae*. The primary manifestation of the disease is massive diarrhea, which can result in death by dehydration if the fluid loss is not treated. This activity was shown at the cellular level to be the result of continuous transport of water and consequent loss of electrolytes from the basal to apical surfaces of the intestinal epithelium. The intracellular activity was subsequently identified as a highly elevated adenylate cyclase activity. These effects were shown to be dependent on the presence of the A subunit of cholera toxin, NAD^+, cellular cytosolic factors, and ATP (44). By analogy to diphtheria toxin, this effect was assumed to involve ADP-ribosylation.

Cassel & Selinger (45) were the first to identify the regulatory component of adenylate cyclase, later known as the stimulatory G-protein, G_S, as the substrate for ADP-ribosylation. They showed that the activation of adenylate cyclase in turkey erythrocyte membranes by cholera toxin appeared to mimic the increases observed in the presence of the nonhydrolyzable GTP analog, Gpp(NH)p, and correctly deduced that the action of cholera toxin was inhibition of the intrinsic GTPase activity of G_S. Subsequently, labeling with radiolabeled NAD^+ confirmed that the α subunit of G_S is the substrate for cholera toxin-mediated ADP-ribosylation (46–48). Maximal toxin activity is observed when the toxin is pretreated with dithiothreitol and/or sodium dodecyl sulfate and cytosol is present. The former effect is due to release of the active A subunit of the toxin, and the latter is due to the presence of ADP-ribosylation factor (16). Interestingly, ADP-ribosylation appears to be reversible in the presence of the toxin and high concentrations of nicotinamide (46). The reversibility of ADP-ribosylation will be discussed below.

Parallel studies were also performed on the retinal G-protein transducin (49). The ADP-ribosylation of the α subunit of transducin was found to occur only in the presence of photolyzed rhodopsin and was stimulated by the presence of Gpp(NH)p and a stoichiometric amount of $\beta\gamma$ complex (50). The GTPase activity of transducin was found to decrease at a rate and to an extent that closely paralled its ADP-ribosylation. The site of ADP-ribose incorporation on transducin catalyzed by cholera toxin has been determined to be Arg174, which corresponds to Arg187/188 of G_S (51).

The physiological significance of ADP-ribosylation reactions is supported by the identification of enzymes that are capable of the cyclical addition and removal of an ADP-ribose moiety, as well as ADP-ribosylation factors that enhance the rate of the transfer reaction. Arginine-specific ADP-ribosyltransferase activity has been detected in a number of eukaryotic tissues (52). In contrast to the bacterial toxins, these enzymes appear to consist of a single subunit and exist in both cytosolic and integral membrane forms. Enzymes that specifically catalyze the hydrolytic removal of ADP-ribose from ADP-ribosylated arginine residues have been identified in many animal species and tissues (53). Together with reports of the identification of NAD:arginine ADP-ribosyltransferase activities in eukaryotic cells, these results suggest that ADP-ribosylation may be a reversible, physiological event (54). Thus far, however, the identification of endogenously ADP-ribosylated G-proteins has not been reported.

Pertussis Toxin-Mediated ADP-Ribosylation of Heterotrimeric G-Proteins

ADP-ribosylation by pertussis toxin differs from that by cholera toxin in both substrate and effect. As with cholera toxin–mediated ADP-ribosylation, pertussis toxin–mediated ADP-ribosylation affects a distinct subset of G-protein α subunits. Whereas G_S is the substrate for cholera toxin, the cellular target for pertussis toxin is the inhibitory G-protein, G_i.

Pertussis toxin actually consists of a mixture of toxins secreted by the bacterium *B. pertussis*. The clinical manifestation of infection by this bacterium is whooping cough (55). At the cellular level, one effect of injections of pertussis toxin into animals is a potentiated insulin secretion in response to elevated levels of glucose in the blood, also seen as an attenuation of epinephrine-induced hyperglycemia (56). The factor responsible for this biological effect was purified and identified as islet-activating protein (IAP) owing to its effect of preventing a reduction of insulin secretion by the pancreatic islet cells despite hypoglycemia (57). The investigation of IAP effects on a number of cell lines suggested that IAP blocked the hormone- or neurotransmitter-mediated inhibition of adenylate cyclase activity (58). Like cholera toxin, the effect of IAP was shown to be dependent on the presence of ATP and NAD. The use of radiolabeled NAD^+ identified the α subunit of G_i as the substrate for ADP-ribosylation by pertussis toxin (58, 59). Later studies also showed that the 39-kDa α subunit of the G-protein G_o, as well as the α subunit of transducin, is a substrate for pertussis toxin-catalyzed ADP-ribosylation (60–64).

In contrast to the ADP-ribosylation of G_S α by cholera toxin, the ADP-ribosylation of G-proteins by pertussis toxin is most efficient in the $\alpha\beta\gamma$ heterotrimeric form (52). Subsequent studies demonstrated that ADP-ribosyla-

tion blocks the interaction of the α subunits of the inhibitory G-proteins with their receptors, thereby preventing the receptor-mediated exchange of GDP for GTP (65, 66). As a result, the inhibition of adenylate cyclase is released, leading to an elevation in the cellular concentration of cAMP. In the case of transducin, the site of pertussis toxin ADP-ribosylation has been shown to be a cysteine residue at the fourth position from the carboxyl terminus (67). Interestingly, in the γ subunit of the heterotrimeric G-proteins and in a number of other proteins, a cysteine residue in this position is modified by prenylation (68–71). Mutation of the α subunit of G_s to mimic the C terminus of the α subunit of G_i, by introduction of a candidate pertussis toxin ADP-ribosylation site, did not result in modification (72, 73). This suggests that the recognition site for pertussis toxin resides outside the region altered or that the cooperative interaction of the α subunit of G_i with other specific protein components is required for ADP-ribosylation.

Similarly to the discovery of NAD:arginine ADP-ribosyltransferases in eukaryotic tissues, an NAD:cysteine ADP-ribosyltransferase activity has been detected and purified from human erythrocytes (74, 75). Incubation of the purified ADP-ribosyltransferase with inside-out erythrocyte membranes and [^{32}P]NAD labeled a 41-kDa polypeptide that migrated at the same position as pertussis toxin-modified α subunit of G_i (76). This result suggested that this subunit is the physiological ADP-ribosylation substrate for this enzyme, although it is not known whether this modification occurs in intact cells.

Since the pertussis toxin-catalyzed ADP-ribosylation of heterotrimeric G-proteins effectively inactivates the modified protein, this process has played a significant role in identifying the involvement of G_i- and G_o-like proteins in various cellular processes. As mentioned previously, the initial discovery that a 41-kDa polypeptide was ADP-ribosylated by pertussis toxin led to the first identification of G_i. Since then, treatment of intact cells or membranes with pertussis toxin followed by assays for specific cellular events has been a test for the involvement of G-protein-mediated pathways (52).

Botulinum Toxin ADP-Ribosylation of Small G-Proteins

Botulinum toxins are potent neurotoxins that act at presynaptic nerve terminals by inhibiting release of neurotransmitters (77, 78). This inhibition of neurotransmitter release was shown not to be due to alteration of Ca^{2+} influx and was surmised to be the result of an ADP-ribosylation reaction based on the similarity of the botulinum toxins to other ADP-ribosylating bacterial toxins (79, 80). The first molecular studies of botulinum toxin involved the addition of purified type C1 or D toxin to various cultured cells and tissue homogenates, resulting in the ADP-ribosylation of 21–26-kDa proteins (81–84). The observed ADP-ribosylation was stimulated by guanine nucleotides, suggesting that the predominant substrates were small G-proteins. At

the same time, other studies reported the ADP-ribosylation of 21–24-kDa proteins by C3 botulinum toxin (85). ADP-ribosylation by C3 toxin was also stimulated by guanine nucleotides and Mg^{2+}, suggesting that the substrates were GTP-binding proteins. Although the ADP-ribosylation of proteins putatively involved in secretion and the inhibition of exocytosis appear to be related events, studies have demonstrated that the two toxin activities are distinct (86–88). Botulinum toxin D appears to inhibit secretion but possesses no ADP-ribosyltransferase activity, whereas botulinum toxin C3 appears to have the opposite effects (86). It is now clear that the toxin responsible for ADP-ribosylation of members of the class of small G-proteins is the *C. botulinum* exoenzyme C3 and not type C1 or D (89). The ADP-ribosyltransferase activities previously associated with the C1 and D toxins were most probably the result of contamination by C3 toxin. The gene for the *C. botulinum* C3 ADP-ribosyltransferase has recently been cloned and sequenced and has been verified to be the active ribosylating component of the various toxin preparations (90). Evidence exists, however, for the presence of a similar but distinct ADP-ribosyltransferase activity in C1 toxin (88). More recently, epidermal cell differentiation inhibitor, an exoenzyme produced by *Staphylococcus aureus* with 35% amino acid sequence homology to C3 toxin, has also been demonstrated to exhibit ADP-ribosyltransferase activity on small G-protein substrates (90a).

Concurrent with the discoveries of the 21–26-kDa botulinum toxin substrates, the identification of a number of low-molecular-mass GTP-binding proteins in the range of 20–30 kDa were reported. In particular, two proteins of ~22 and ~25 kDa from various tissues were observed to be substrates for C3 toxin-mediated ADP-ribosylation and were subsequently identified as products of the *rho* and *rac* gene families (91–95). Other small G-proteins, including c-H ras and ARF, do not appear to be ADP-ribosylated (92). The *rho* gene product was subsequently shown to be ADP-ribosylated on an asparagine residue, corresponding to Asn41 of the *Aplysia rho* sequence (96). The stability of the modification to neutral hydroxylamine treatment suggested an N-glycosidic bond. Comparison with the structure of the ras protein indicates that this residue is in the GAP interaction domain. Although the botulinum toxin–mediated ADP-ribosylation of small G-proteins has been shown to be stimulated by binding of guanine nucleotides, ADP-ribosylation does not appear to have an effect on intrinsic GTPase activity or on GTPγS binding (92). However, it remains to be determined whether ADP-ribosylation has an effect on GAP interaction.

As in the case of the cholera and pertussis toxin–mediated ADP-ribosylation of the large G-proteins, the modification of rho and rac proteins by botulinum toxin opens the possibility of defining the functions of these proteins. Addition of C3 toxin to the media of cultured cells, however, appears to have mixed

effects. Chardin et al (94) reported the collapse of the actin microfilament network in Vero cells on addition of purified C3 toxin, which suggested that the rho protein plays a role in cytoskeletal assembly. Nishiki et al (97), however, reported that addition of C3 toxin to PC12 cells results in the termination of cell growth and induces neurite outgrowth. More recently, treatment of Vero cells with the *S. aureus* epidermal cell differentiation inhibitor resulted in a redistribution of Golgi markers and morphological changes consistent with the involvement of its small G-protein substrate in vesicular trafficking (97a). In all of these experiments, however, the specific ADP-ribosylation substrates were not defined, and the contrasting effects may occur because of different small G-proteins are predominantly modified in each of these cell lines.

As in the case of cholera toxin–mediated ADP-ribosylation of G_S, a factor has been isolated from bovine brain cytosol which is a specific activator of the botulinum toxin-mediated ADP-ribosylation of bovine brain cytosolic proteins (98). This activator appears to be specific for botulinum toxin ADP-ribosylation and does not enhance the cholera toxin–mediated ADP-ribosylation of G_S. Also of interest is the identification of a mammalian enzyme with properties similar to those of the bacterial C3 toxin, as reported for the ADP-ribosylation of heterotrimeric G-proteins (99). The existence of specific activators and enzymes again suggests a physiological role for ADP-ribosylation of small G-proteins. However, both membrane-bound and cytosolic substrates were found to be stoichiometrically modified by the C3 toxin, suggesting that these proteins are not normally ADP-ribosylated.

PRENYLATION OF G-PROTEINS

Protein prenylation is a very recently discovered posttranslational modification. The covalent addition of a prenyl moiety to a polypeptide was first described for the yeast mating factors (100–103). These short polypeptides, which mediate the mating response between opposite cell types in yeast, were found to contain a unique farnesylcysteine residue at their carboxyl termini. Schmidt et al (104) presented the first evidence that proteins in mammalian cells are also modified by derivatives of mevalonic acid. On the basis of the different fates of mevalonic acid radiolabeled at various positions, the modifying group was deduced to be an isoprenoid. Since then, several methods have been developed for the identification of prenyl groups on modified proteins. One of the most rigorous methods involves chemical cleavage of the modifying prenyl group with Raney nickel, which releases thioether-linked lipids, followed by gas chromatography-coupled mass spectrometry (GC-MS). The advantage of GC-MS analysis is that definitive identification of the prenyl group, as well as its sterochemical configuration, can be determined.

Farnsworth et al (105) and Rilling et al (106) used GC-MS to analyze the total prenylated proteins in HeLa and CHO cells and demonstrated that the predominant prenyl modification in mammalian cells is not the farnesyl group but the 20-carbon geranylgeranyl group. The stereochemistry of both types of prenyl groups was determined to be all-*trans*. A similar method that has been used to analyze prenylated proteins employs methyl iodide cleavage of the prenyl group followed by high-performance liquid chromatography (HPLC) for identification of the released isoprenoid (107). This method, however, does not allow the rigorous assignment of the stereochemistry of the modifying prenyl group. Although both GC-MS and HPLC analyses provide definitive identification of the structure of the modifying isoprenoid, these methods do not reveal the amino acid to which the prenyl group is attached nor its location within the polypeptide. To alleviate these shortcomings, GC-MS identification has been coupled with proteolytic digestion of the prenylated polypeptide to its constituent amino acids followed by reversed-phase HPLC separation of the digest and comparison with authentic standards (108). Such a method can be used to confirm the modification of a cysteine residue and, under carefully controlled conditions, the presence of an α-carboxyl methyl ester.

Prenylation differs from ADP-ribosylation in affecting a very diverse groups of proteins. Among these are yeast mating factor peptides, nuclear lamins, and γ subunits of heterotrimeric G-proteins, nearly all small G-proteins, and certain G-protein-related enzymes such as the retinal cGMP phosphodiesterase and rhodopsin kinase (109). Despite the apparent diversity in the structures and functions of these proteins, all share the common characteristic of possessing a cysteine residue at their carboxyl termini in a -Cys-Xaa-Xaa-Xaa, -Cys-Cys, or -Cys-Xaa-Cys motif. However, it is important to note that not all proteins bearing this distinctive carboxyl-terminal sequence are modified by prenylation. Most notably, the α subunits of the heterotrimeric G-proteins G_i, G_o, and transducin terminate in this motif but are not prenylated (110). Significantly, this cysteine residue is the site of ADP-ribosylation in these proteins (67).

In the next section, the prenylation of large and small G-proteins possessing a -Cys-Xaa-Xaa-Xaa motif will be discussed jointly because of the similarity of the respective mechanisms and putative functions, as well as the coincidence of their discoveries. A subsequent section will deal exclusively with the prenylation of -Cys-Cys and -Cys-Xaa-Cys motifs unique to members of the superfamily of small G-proteins.

Prenylation of G-proteins with the -Cys-Xaa-Xaa-Xaa Motif

With respect to proteins terminating in a -Cys-Xaa-Xaa-Xaa motif, the term "prenylation" is generally intended to encompass a closely coupled series of

posttranslational modifications, involving attachment of an isoprenoid moiety to the sulfhydryl group of a carboxyl terminal cysteine via a thioether linkage, proteolytic removal of three residues downstream of the prenylated cysteine, and methyl esterification of the newly exposed C-terminal cysteine residue (109).

The first indication that G-proteins were modified similarly to the yeast mating factors was the identification of that the RAM1 gene of *Saccharomyces cerevisiae* was essential to the posttranslational modification of both the yeast mating **a** factor and RAS, the yeast homolog of the mammalian ras protein (111). Examination of both sequences revealed that the only similarity was a -Cys-Xaa-Xaa-Xaa sequence at their carboxyl termini, suggesting that this motif signaled the modification of both proteins by the RAM1 gene product. This proposition was strengthened by the discovery that mammalian ras proteins are substrates for an α-carboxyl methyl esterification not previously demonstrated in mammalian proteins (112). These results led to the proposal that a -Cys-Xaa-Xaa-Xaa carboxyl-terminal sequence serves as a general signal for lipidation of the cysteine sulfhydryl group, proteolysis of the three carboxyl-terminal amino acids, and methyl esterification of the resulting carboxyl-terminal cysteine. Following these reports, Gutierrez et al (113) demonstrated that between the newly synthesized and mature ras polypeptides existed another species, of intermediate hydrophobicity and apparent molecular size, which could be selectively partitioned into a detergent phase. Hancock et al (114) subsequently identified this form as being modified by polyisoprenylation on Cys186 and further showed that in H- and N-ras, palmitoylation of an adjacent cysteine residue followed prenylation of Cys186. These results demonstrated that the previous assignment of Cys186 as the site of palmitoyl modification was incorrect (115, 116) and that the carboxyl terminus of ras was indeed processed identically to that of the yeast mating factors. The isoprenoid moiety on ras was subsequently identified as the 15-carbon farnesyl group (107). In yeast, the RAS2 protein is also modified by farnesylation (117).

The next major discovery occurred when Yamane et al (68) and Mumby et al (69) showed that the γ subunits of G-proteins purified from bovine brain and the neural cell line PC12, respectively, are modified by geranylgeranylation and carboxyl methylation on a C-terminal cysteine. These studies represented the first identification of proteins modified by the 20-carbon geranylgeranyl group. Geranylgeranylation of G-protein γ subunits was also demonstrated by [^{3}H]mevalonic acid labeling following expression in a rabbit reticulocyte lysate system (118). Interestingly, the γ subunit of transducin was found to be modified by a farnesyl moiety (70, 71). Subsequently, analysis of purified G25K (119) and rap1B (120), and of metabolically labeled rac, ral, rap1A, and rho during expression in rabbit reticulocyte lysates or

transfected cells (121, 122), showed that all of these small G-proteins are geranylgeranylated. The mechanisms and properties of each step in the prenylation process will now be considered.

PRENYLATION Prenyl modification of proteins with -Cys-Xaa-Xaa-Xaa motifs is highly specific. For polypeptides with a given -Cys-Xaa-Xaa-Xaa sequence, only one type of prenyl group has been found. The information dictating this specificity has been shown unequivocally to reside in the -Cys-Xaa-Xaa-Xaa sequence. Hancock et al (114) first demonstrated that the -Cys-Xaa-Xaa-Xaa motif is sufficient for signaling prenylation by adding this motif to a heterologous protein, protein A, and showing that a product of mevalonic acid was incorporated. More conclusive evidence came from the work of Reiss et al (123), who showed that tetrapeptides modeled after the carboxyl terminus of ras were capable of inhibiting the in vitro farnesylation of recombinant ras protein and could also serve as substrates for the protein farnesyltransferase. The results of these studies indicated that nonpolar aliphatic or aromatic residues in the first and second positions following the cysteine were most effective in inhibiting the farnesylation of ras. The amino acid at the carboxyl terminus was found to have the most influence on the efficacy of the tetrapeptide. Methionine, phenylalanine, and serine were most effective, whereas aliphatic residues at this position were least effective. During this time, it was also becoming apparent that almost all geranylgeranylated proteins possess primary sequences terminating in a leucine residue. Studies with recombinant ras (124) and G25K/CDC42Hs proteins (H. K. Yamane, R. A. Cerione, & B. K.-K. Fung, unpublished results) with various amino acid substitutions at the carboxyl terminus confirmed the importance of the carboxyl-terminal residue in dictating the specificity of the two prenyl modifications. In summary, proteins terminating in a -Cys-Xaa-Xaa-Xaa motif are farnesylated when a moderately polar residue, such as serine, methionine, or glutamine, is present at the carboxyl terminus and are geranylgeranylated when a large hydrophobic residue, such as leucine, valine, isoleucine, or phenylalanine, is the carboxyl-terminal residue. It should be noted, however, that nearly all proteins shown to be geranylgeranylated thus far terminate in leucine.

Prenylation of -Cys-Xaa-Xaa-Xaa-encoded proteins is now known to be catalyzed by at least two different protein prenyltransferases, a protein farnesyltransferase (123, 125) and a protein geranylgeranyltransferase (124, 126–129a). The prenyl donors are farnesyl pyrophosphate and geranylgeranyl pyrophosphate, respectively. The former is a direct intermediate in the cholesterol biosynthetic pathway, and the latter appears to be a specific substrate for protein prenylation, although it may also serve as a precursor for the higher-molecular-weight dolichols. The specificity of the pre-

nyltransferases appears to be absolute; rat brain farnesyltransferase does not transfer geranylgeranyl groups to -Cys-Xaa-Xaa-Leu-terminating peptides, and vice versa. The enzymes have a specific requirement for the divalent cations Mg^{2+} and Zn^{2+} (130). The former is required in millimolar concentrations and the latter in micromolar concentrations. In an elegant series of experiments, Zn^{2+} was shown to be required for binding of the protein or peptide substrate to the β subunit, while Mg^{2+} is required for transfer of the farnesyl moiety (130).

Several protein prenyltransferases have now been purified from various sources and demonstrated to have similar properties. The rat brain farnesyltransferase is composed of a 49-kDa α subunit and a 46-kDa β subunit. The β subunit has been shown to contain the protein-binding site by chemical cross-linking studies, whereas the α subunit is presumed to contain the prenyl pyrophosphate–binding site (131). Transfection studies indicate that the formation of the αβ heterodimer is an absolute requirement for stability and activity (132). Although the geranylgeranyltransferase has not yet been purified to homogeneity, available evidence suggests that it is also an αβ heterodimer with subunit functions very similar to those of the farnesyltransferase (129a). Moreover, both protein prenyltransferases appear to share identical α subunits, as determined by cross-reactivity with monoclonal and polyclonal antibodies against the purified farnesyltransferase α subunit (133). The mechanism by which the two prenyltransferases share common α subunits that bind different prenyl pyrophosphate precursors has yet to be determined. It is possible that there is cooperativity between the binding sites, as in the case of the myristoyl-CoA:protein N-myristoyltransferase; binding of the "wrong"-chain-length acyl-CoA markedly reduces its affinity for peptide substrates (134).

Genetic evidence has also pointed to the existence of both a farnesyltransferase and a geranylgeranyltransferase in the yeast *S. cerevisiae*. The deduced amino acid sequence of the *RAM1/DPR1* and *RAM2* gene products, previously shown to be essential genes responsible for the maturation of both **a** factor and RAS, are homologous to the β and α subunits of the rat brain farnesyltransferase, respectively (111, 124, 135–137). Protein geranylgeranyltransferase activity in *S. cerevisiae* is dependent on the *CDC43/CAL1* gene, suggesting that it encodes the β subunit of the geranylgeranyltransferase (124, 138). Furthermore, geranylgeranyltransferase activity is reduced in *ram2* mutants, suggesting that *RAM2* encodes a common subunit of both yeast protein prenyltransferases (124, 138).

PROTEOLYSIS Of the three steps in the prenylation of substrate proteins, the least well characterized thus far is the proteolytic removal of the three carboxyl-terminal residues which occurs prior to α-carboxyl methyl esterifi-

cation. Recently, several groups have reported the identification of prenylcysteine proteolytic activity in yeast and mammalian system (139, 140). Canine pancreatic microsomes have been known to catalyze the α-methyl esterification of prenylated proteins expressed in rabbit reticulocyte lysates, implying the presence of a protease activity (141, 142). The specificity of this proteolytic activity, however, is not known. The characterization of protease activity specific to prenylated proteins was described in the yeast *S. cerevisiae* (139). Distinct membrane and cytosolic protease activities were identified and shown to be distinct from carboxypeptidase Y. The cytosolic protease was partially purified and shown to have a molecular mass of ~110-kDa; it cleaved the carboxyl-terminal residues of a farnesylated synthetic yeast ras C-terminal peptide in rapid succession. Protease activity apparently specific to prenylated peptides has also been identified in bovine liver microsomes (140). This activity was found to cleave a farnesylated model tetrapeptide on the carboxyl side of the farnesylated cysteine, releasing a tripeptide. It was not inhibited by addition of up to a fivefold excess of nonfarnesylated tetrapeptide, suggesting that it is specific for prenylated peptides.

α-CARBOXYL METHYLATION The α-carboxyl methylation of mammalian proteins was first demonstrated on ras proteins overexpressed in a fibroblast cell line, which incorporated methyl groups with a stability consistent with α-carboxyl methyl esters (112). The α-carboxyl methylation of 21–26-kDa membrane proteins in rod outer segment membranes and in a macrophage cell line were subsequently reported, suggesting that other small G-proteins were substrates for this modification (143, 144). The small G-protein G25K, purified from bovine brain membranes, was shown to be carboxyl methylated on reconstitution with a methyltransferase activity in detergent-stripped bovine brain membranes (145). By using the same reconstitution assay, the first heterotrimeric G-protein shown to be carboxyl methylated was the γ subunit of brain G-proteins (146).

A methyltransferase activity which catalyzed the α-carboxyl methylation of farnesylated or geranylgeranylated synthetic peptide substrates was identified in endoplasmic reticulum membranes of rat liver homogenates (147, 148). The corresponding S-acylated and geranylated peptides were poor substrates for the methyltransferase, suggesting that both the nature and length of the prenyl group are important for recognition by the enzyme. Furthermore, *N*-acetyl-farnesylcysteine has been shown to be a substrate for α-carboxyl methyltransferases from various tissues and yeast, indicating that upstream residues are not involved (149–152). Study of analogs of *N*-acetyl-farnesylcysteine showed that *S*-farnesyl-3-thioproprionic acid is a good stubstrate for

the methyltransferase in rod outer segment membranes, defining this structure as the minimal recognition unit for the methyltransferase (151).

In *S. cerevisiae*, mutations of the *STE14* gene have been shown to abolish farnesylcysteine methyltransferase activity (152–154). The assignment of the *STE14* gene product as the methyltransferase was verified by its expression in *Escherichia coli* and by the demonstration that the resulting protein catalyzed α-carboxyl methylation of farnesylcysteine substrates in vitro (152). The *STE14* gene was required for in vivo carboxyl methylation of **a** factor and RAS proteins, indicating that a common enzyme may be responsible for the methylation of all prenylated proteins (152). As in the case of the mammalian enzymes, the *STE14* gene product is found exclusively in the membrane fractions.

In contrast to the prenylation and proteolysis steps, methyl esterification of the prenylated cysteine may be reversible. The methyl ester is susceptible to hydrolysis at higher pH and by nonspecific esterase activity in various proteases (108). The presence of a methylesterase activity in rod outer segments has been suggested (150). Thus far, however, no methylesterase activity specific for prenyl cysteine methyl esters has been identified. In conjunction with the reversibility of the methylation reaction is the possibility that methylation plays a role in the function of -Cys-Xaa-Xaa-Xaa-encoded proteins, as methylation does in bacterial chemotaxis. Although evidence for this form of regulation in mammalian cells is limited, a recent study has shown the presence of an unmethylated form of G25K/CDC42Hs in rabbit brain cytosol, which translocates to membranes upon being carboxyl methylated (152a). It is noteworthy that in yeasts, the absence of an α-carboxyl methyl ester on mating factors results in the nearly complete loss of activity (102, 103, 152).

Prenylation of Small G-proteins with -Cys-Cys and -Cys-Xaa-Cys Motifs

A number of small G-proteins possess a carboxyl-terminal cysteine in a -Cys-Xaa-Cys or -Cys-Cys motif. In contrast to the -Cys-Xaa-Xaa-Xaa motif, the -Cys-Xaa-Cys and -Cys-Cys carboxyl-terminal motifs appear to be unique to members of the rab family of small G-proteins. Farnsworth et al (105) were the first to determine that small G-proteins possessing this motif were modified by prenylation. They showed that both cysteine residues of the -Cys-Ala-Cys sequence of rab3A are modified by geranylgeranylation. Studies of the -Cys-Cys-terminating proteins rab1B, rab2, ypt1, and ypt3 from the yeast *Schizosaccharomyces pombe*, and the -Cys-Xaa-Cys-terminating proteins rab4 and rab6, have also shown that these proteins incorporate geranylgeranyl groups when expressed in rabbit reticulocyte lysates or COS cells (155–158). The stoichiometries of these modifications have not been unequivocally

demonstrated, but the available evidence suggests that only one of the two cysteine residues is geranylgeranylated (159, 159a). The apparently exclusive geranylgeranylation of -Cys-Xaa-Cys- and -Cys-Cys-encoded proteins is consistent with the greater abundance of the geranylgeranyl moiety from analysis of whole-cell extracts (105, 106).

Recently, the identification and purification of a protein geranylgeranyltransferase activity specific for proteins with the -Cys-Xaa-Cys motif have been reported (124, 159a, 160, 160a). In contrast to the farnesyltransferase and geranylgeranyltransferase described in the previous section, synthetic peptides with the -Cys-Xaa-Cys motif are not substrates for this enzyne and do not competitively inhibit geranylgeranylation of the substrate protein. Furthermore, mutant H-ras proteins terminating in -Cys-Cys are not prenylated (156). These results strongly suggest that other regions of the substrate proteins are required for recognition by this enzyme. The apparent requirement for a secondary recognition site is plausible since the -Cys-Xaa-Cys and -Cys-Cys motifs appear to be restricted to a highly homologous group of small G-proteins, whereas a large number of structurally diverse proteins possess the -Cys-Xaa-Xaa-Xaa motif.

In *S. cerevisiae* the *BET2* gene encodes a polypeptide responsible for the membrane localization of ypt1 and sec4 (161). The BET2 protein has been postulated to be a component of the -Cys-Cys geranylgeranyltransferase on the basis of the homology of its predicted amino acid sequence (34% identity) to that of *RAM1/DPR1* and the fact that ypt1 and sec4 terminate in -Cys-Cys sequences (124, 161). The processing of RAS and **a** factor is unaffected by mutations to *BET2*, indicating that it does not encode a prenyltransferase subunit common to the yeast farnesyltransferase.

Prenylated proteins terminating in -Cys-Xaa-Cys motifs have been shown to undergo methylation of the α-carboxyl group (158, 162). In contrast to the processing of -Cys-Xaa-Xaa-Xaa-encoded proteins, proteolysis of amino acid residues downstream of the prenylated cysteine is not required. Prenylated proteins ending in the -Cys-Cys motif have not been observed to be methyl esterified (158). The significance and mechanism of this specificity of methylation is not known.

Function of Prenylation

Although the exact functional role of prenylation remains to be elucidated, a large body of evidence, both cytochemical and functional, points to a role in membrane localization. Direct evidence for the involvement of prenyl groups in membrane binding has been provided by limited proteolysis which cleaves a small (~1-kDa) carboxyl-terminal fragment from several small G-proteins (145, 163). In all cases, the smaller prenylated carboxyl-terminal fragment has been shown to bind to membranes while the remainder of the protein is

found in the supernatant fraction. The GTP-binding and hydrolyzing functions of the amino-terminal fragment remain unaffected. In vivo, mutational analyses have shown that the absence of prenylation results in translocation of the mutant G-protein from the membranes to the cytosol (114, 164–166). These results clearly demonstrate a general role of prenylation in membrane localization.

As discussed previously, proteins can be modified by either a farnesyl or geranylgeranyl group. What, if any, are the functional differences between the two prenyl modifications? A priori, the geranylgeranyl group, with its additional five-carbon isoprene unit, is likely to be more hydrophobic and therefore expected to confer tighter binding of the prenylated protein to membranes. Perhaps the clearest example of a difference between these prenyl groups resides with the prenylated γ subunits of transducin and brain G-proteins. Consistent with these differences in prenylation and the membrane avidity of geranylgeranyl groups over farnesyl groups, transducin heterotrimers can be eluted from rod outer segment membranes with low-ionic-strength buffers, while brain G-proteins require detergents for solubilization (167, 168).

In addition to the prenyl moiety, other regions of the protein may be involved in facilitating membrane binding. Studies of the ras proteins have demonstrated that although farnesylation of the carboxyl terminal cysteine increases hydrophobicity, it is by itself insufficient to direct membrane localization (114). In H-, N-, and K-ras(A) proteins, the membrane localization necessary for transforming activity is conferred by palmitoylation of an upstream cysteine (Cys181 or Cys184). This acyl modification is discussed in more detail in a subsequent section. In K-ras(B) and most other small G-proteins, which lack an adjacent upstream cysteine, membrane localization is conferred by a polybasic domain immediately preceding the prenylated cysteine (169). Mutants in which the positively charged lysine residues constituting this domain were changed to neutral glutamine residues illustrated a direct correlation between membrane localization and the number of positively charged residues in this domain. All mutants were prenylated normally. The presence of the polybasic domain is believed to facilitate membrane binding by charge-charge interactions with the negatively charged phospholipids or proteins on the membrane surface. In support of this hypothesis, mutation of the contiguous lysine residues to arginine residues preserved the membrane-localizing function of this domain (169). As an additional example of differences between the two prenyl modifications, a geranylgeranylated mutant K-ras(B) protein associated with a membrane fraction even in the absence of a polybasic domain (169).

Although the importance of prenylation in membrane localization has clearly been demonstrated for the ras proteins, studies of other small G-proteins suggest that the prenyl group may also have other functions.

Disruption of the -Cys-Xaa-Xaa-Xaa motif has been shown to abolish both membrane binding and biological activity (114, 164). Removal of the carboxyl-terminal prenylation signal and replacement with an amino-terminal myristoylation site restores transforming activity (170), suggesting that the sole function of prenylation is membrane binding and not interaction of the prenyl group with a membrane-bound receptor. On the other hand, interaction of the small G-protein smg p21B/rap1B with smg p21 GDI is dependent on its posttranslational modification by prenylation (163). Proteolytic cleavage of smg p21B demonstrated that the small carboxyl-terminal fragment is required for interaction with its GDI. Studies with synthetic peptides have shown that a geranylgeranyl group is required for inhibition of this interaction (171). The use of smg p25A/rab3A with various degrees of carboxyl-terminal processing further verified that only forms that were geranylgeranylated formed a complex with smg p25A GDI (172). The interaction of smg p25A GDI with the geranylgeranyl moiety of smg 25A is consistent with a possible role of the GDI in translocating this small G-protein from the membrane to the cytosol. Along similar lines, farnesylation of the γ subunit of transducin has been shown to be required for interaction with the α subunit (70, 173).

ACYLATION OF G-PROTEINS

Many forms of protein acylation, differing in both the acyl group and the method of attachment, have been described (174, 175). Of particular interest to the study of G-proteins is N-myristoylation, in which a myristate moiety is attached to an N-terminal glycine residue via an amide bond. The consensus sequence for N-myristoylation has been established to be an amino-terminal glycine residue (following cleavage of the initiator methionine) with a hydroxyamino residue commonly found four amino acids downstream. Using an extensive panel of peptides, Towler et al (176, 177) have made a comprehensive probe of the substrate specificity of the *N*-myristoyltransferase from *S. cerevisiae*. Their results indicate that in addition to an N-terminal glycine the second most desirable feature is the presence of a small uncharged amino acid, such as alanine, or a hydroxyamino acid four residues downstream from the glycine. The remaining residues near the amino terminal appear to be more tolerant with respect to the range of amino acids allowed. Although a hexamer can be sufficient as a substrate, the effect of the addition of further residues depends on their identity. It is clear that in certain proteins, additional regions of the polypeptide may play a role in determining whether it is a substrate for the *N*-myristoyltransferase (178).

Deichaite et al (179) have shown that *N*-myristoyltransferase activity is present in nearly every eukaryotic cell type. They further demonstrated in an elegant experiment that myristoylation is a cotranslational rather than post-

translational event. Using a rabbit reticulocyte lysate expression system synchronized for the start of translation, they showed that the attachment of a myristate group to the $p60^{v\text{-}src}$ protein occurs before the nascent polypeptide is less than 100 residues long. Furthermore, myristoylation did not occur after synthesis of the $p60^{v\text{-}src}$ protein was completed.

In addition to N-myristoylation, a number of small G-proteins are posttranslationally modified by palmitoylation, in which a palmitate group is attached to a cysteine residue in a process closely linked to prenylation. The myristoylation of heterotrimeric G-protein α subunits and palmitoylation of ras proteins will be considered separately.

Myristoylation of Heterotrimeric G-Proteins

The possibility that G-protein α subunits are acylated stemmed from reports that the activated α subunits, following release from the associated βγ complexes, remain localized to plasma membranes despite the lack of any obvious hydrophobic domains (168). This membrane localization was thought to be required for promoting the subsequent reassociation of α subunits with βγ complexes following deactivation by the inherent GTPase activity. Additionally, the deduced amino acid sequences of some G-protein α subunits were known to possess an N-terminal sequence characteristic of the modification site for N-myristoylation.

The myristoylation of endogenous G-protein α subunits was first found in isolated proteins from bovine brain (174) and human astrocytoma cells (180). Schultz et al (174) demonstrated that the α subunit of G_o purified from bovine brain possessed a stoichiometric amount of myristate which was released by acid hydrolysis. Buss et al (180) metabolically labeled human astrocytoma cells with either [^{35}S]methionine, [^{3}H]palmitate, or [^{3}H]myristate and immunoprecipitated G-protein α subunits by using specific antipeptide antibodies. Using this technique, the 40- and 41-kDa α subunits of G_i, as well as the α subunit of G_o, were found to be myristoylated. The stability of radiolabeled myristate to hydroxylamine treatment was consistent with attachment through an amide linkage. The α subunit of G_s was not myristoylated, consistent with the fact that the deduced amino acid sequences of both the 45- and 52-kDa forms of this α subunit do not show the presence of an N-terminal glycine (181).

Subsequently, the myristoylation of specific G-protein α subunits was studied in depth by the transient expression and labeling in COS cells containing the appropriate cDNA constructs (182, 183). These studies verified that the α subunits of G_{i1}, G_{i2}, G_{i3}, G_o, and G_z were substrates for myristoylation and, furthermore, that these α subunits, as well as α subunit of G_s overexpressed in COS cells, were localized to membrane fractions.

Interestingly, the α subunit of transducin transiently expressed in COS cells was found to be myristoylated (183), although the protein purified from retinal rods was found to contain only minor amounts of myristate groups (180). More recent studies have found that the purified α subunit of transducin is indeed fatty acylated on an N-terminal glycine by a heterogeneous mixture of lauroyl, myristoyl, (*cis*-Δ^5)-tetradecaenoyl, and (*cis,cis*-Δ^5,Δ^8)-tetradecadienoyl groups (183a).

The function of N-myristoylation appears to vary. In the case of $p60^{v\text{-}src}$, myristoylation appears to be essential for interaction with a membrane-bound receptor (184). Although interaction of N-terminal peptides of $p60^{v\text{-}src}$ with its receptor was clearly shown to require myristoylation, N-terminal peptides from other myristoylated proteins did not interact with the receptor, indicating that the presence of the myristate group is essential but not sufficient for recognition by the receptor. In the case of myristoylated G-protein α subunits, mutagenesis of the N-terminal glycine to alanine in the α subunits of G_{i1} and G_o followed by expression of the mutant cDNAs in COS cells resulted in α subunits that were not myristoylated and whose localization shifted from membrane to cytosol (182, 183). Recently, a more in-depth study of the function of myristoylation in G-proteins, involving coexpression of the α subunit of G_o with the N-myristoyltransferase in *E. coli*, was undertaken (185). The resulting α subunit of G_o was found to consist of a mixture of recombinant nonmyristoylated and myristoylated forms, which could be purified to homogeneous preparations and used to investigate the functional role of the myristate group by comparing the properties of the two preparations. The most striking result was the inability of the nonmyristoylated α subunit to interact effectively with βγ complexes as determined by several criteria. In contrast, the myristoylated form behaved identically to the corresponding purified brain protein. These results are consistent with reports that the amino terminus of the α subunit of transducin is required for its interaction with the βγ complex (73, 186, 187). The failure of nonmyristoylated α subunits to form a stable complex with βγ, known to bind strongly to membranes (188), may explain its release from the membranes to the cytosol. More recently, the role of myristoylation in G-protein function was investigated through the study of gip2, an oncogenic α subunit of G_i with transforming activity (188a, 188b). Removal of the site of myristoylation by site-directed mutagenesis resulted in nonmyristoylated gip2 which lacked transforming activity (188c). While these studies indicate a role for myristoylation in protein-protein interaction, the exact nature of this interaction remains to be elucidated.

The relative importance of myristoylation in βγ interaction and membrane localization may vary among the various α subunits. The best evidence for this variability is the fact that the α subunit of G_s is not myrisytoylated, yet

requires detergent for solubilization. Additionally, recombinant α subunits of some G-proteins (G_{i1}, G_{i2}, G_{i3}, and G_0) modified by the less hydrophobic myristate analog 10-(propoxy)decanoate (11-oxamyristate) remained in the supernatant, while the distribution of others (G_t and G_z) remained unchanged (183). These results suggested that myristoylation is critical to the localization of some, but not all, α subunits. Since these oxatetradecanoic acid analogs can enter cells and their incorporation appears to be variable with different protein substrates, Duronio et al (178) have suggested that they may be of therapeutic significance.

Palmitoylation of Small G-Proteins

Thus far, only members of the ras family of small G-proteins have been shown to be modified by palmitoylation (114–116). Within this group, the H-, N-, and K-ras(A) proteins are palmitoylated on a cysteine residue immediately upstream of the farnesylated cysteine in a thioester linkage. The palmitoyl moiety has been shown to be essential for high-avidity membrane binding, which in turn is required for ras transforming activity (114). The only exception is K-ras(B), which lacks the upstream cysteine residue (11). K-ras(B), as well as most other small G-proteins, instead possesses a polybasic domain upstream of the prenylated cysteine that facilitates membrane localization (169). This mechanism has been discussed in a previous section.

Although the palmitoylation of ras is being discussed under the topic of acylation, the palmitoylation is intrinsically linked to the prenylation of the ras proteins. Evidence for this close link is the fact that for a number of years, the ras proteins were thought to be palmitoylated on Cys189 (115, 116), the cysteine residue subsequently shown to be farnesylated (114). The reason for this misinterpretation is that farnesylation of Cys189 is required for the palmitoylation of an upstream cysteine residue, Cys186 (114). Consequently, site-directed mutagenesis to remove Cys189 abolishes palmitoylation, but does so indirectly by preventing the farnesylation of this cysteine.

Magee et al (189) demonstrated that, in contrast to prenylation, the palmitoylation of ras is dynamic with a half-life of approximately 20 min, compared with about 1 day for the ras protein. The higher turnover of the palmitate group reflects the lability of the thioester linkage and the observation that palmitoylation may occur nonenzymatically (41). These authors further showed that the membrane-bound form, but not the cytosolic form, of ras is palmitoylated. This is consistent with observations that although farnesylation increases its hydrophobicity, palmitoylation is required for strong membrane binding (169). Although evidence is lacking, it is possible that the palmitoylation of G-proteins is a regulated, reversible process designed for cyclical translocation of the ras protein between the membrane and cytosol.

PHOSPHORYLATION OF G-PROTEINS

Many proteins, including G-proteins, are substrates for phosphorylation. However, in contrast to the modifications described to this point, the phosphorylation of G-proteins is the least well defined, particularly with regard to the heterotrimeric G-proteins.

Phosphorylation of Heterotrimeric G-Proteins

The first evidence that G-proteins may be regulated by phosphorylation came with the observation that treatment of platelet membranes with partially purified protein kinase C resulted in phosphorylation of a 41-kDa protein to a level approximated at 1 mol of P_i incorporated per mol of substrate (190). The observed phosphorylation was dependent on Ca^{2+} and phosphatidylserine and was inhibited by the addition of $\beta\gamma$ complexes. More recent results with recombinant G-proteins have shown that the α subunit of G_z is stoichiometrically phosphorylated by partially purified protein kinase C on a serine residue near the amino terminus, whereas the recombinant α subunits of G_{i1}, G_{i2}, and G_{i3} are not labeled under the same conditions (191). Phosphorylation of the α subunits of several G-proteins has also been reported to occur on both serine and tyrosine residues in a reconstituted system containing purified insulin receptor protein kinase (192), protein kinase C (193), and $pp60^{c\text{-}src}$ (194). Although these results clearly demonstrate that the α subunits of G-proteins can be phosphorylated in vitro, a link with the physiological effects has yet to be conclusively demonstrated. The possibility that G-proteins are substrates for phosphorylation by protein kinase C and receptor tyrosine kinases is intriguing, however, in that it may represent the potential for crosstalk between the adenylate cyclase, phosphatidylinositol, and growth factor receptor tyrosine kinase second-messenger systems.

A more convincing phosphorylation of heterotrimeric G-proteins occurs in vivo on the β-subunit homolog (STE4) of the yeast *S. cerevisiae*, in response to binding of mating factors from cells of the opposite mating type to a cell surface receptor. Cole & Reed (195) demonstrated that the *STE4* gene product was phosphorylated in *MAT***a** haploid cells in response to treatment with α factor, as well as in *MAT*α cells in response to **a** factor. The phosphorylated product was not observed in an isogenic strain in which the *STE4* gene was deleted. Analysis of the phosphorylated STE4 protein showed the presence of phosphoserine. Deletion of regions of the protein believed to contain the sites of phosphorylation resulted in the absence of mating factor–induced phosphorylation. Yeast expressing the mutant STE4 protein displayed partial characteristics of the mating response during vegetative growth and displayed greater sensitivity and responsiveness in mating factor-induced events than

wild-type cells. These results suggested that phosphorylation may be a negative regulatory signal, with deletion of the phosphorylation sites producing a constitutively activated protein.

Phosphorylation of Small G-Proteins

Shortly after their discovery, the viral ras proteins were shown to be modified by autophosphorylation (196). The phosphorylated residue was determined to be Thr59, which is the site of one of two point mutations responsible for oncogenic activation. This autophosphorylation is most probably the artifactual consequence of this mutation, which introduces a phosphorylation site adjacent to the binding pocket for the guanine nucleotide phosphate groups. This autophosphorylation reaction may consequently not have any significance in the normal function of cellular ras proteins.

Ras proteins are also phosphorylated in vitro by protein kinase A and protein kinase C (197) and in vivo on stimulation of cells expressing high levels of ras by activators of these protein kinases (198, 199). The site of phosphorylation of H-ras was identified by site-directed mutagenesis as Ser177 (198), while that of K-ras was deduced to be Ser181 (199). Although the physiological significance of these modifications has not been determined, their localization to the carboxyl-terminal hypervariable domain resembles the phosphorylation of smg p21B/rap1B, whose effects have been better characterized and will be discussed below (200). With regard to the possible physiological significance of phosphorylation of the mammalian ras proteins, it has been clearly demonstrated that ras GAP is phosphorylated by mitogen receptor protein kinases (201–204). It is possible that the phosphorylations of ras and GAP are coordinately regulated under physiological conditions.

The RAS2 protein of *S. cerevisiae*, which is responsible for the regulation of adenylate cyclase, has also been shown to be an excellent substrate for protein kinase A in vitro (205). In this case, phosphorylated RAS2 exhibited a greatly decreased ability to activate adenylate cyclase in an in vitro assay. Furthermore, phosphorylation did not inhibit the ability of RAS2 to bind or hydrolyze GTP, suggesting that the observed decrease was mediated by an effect on interaction with another protein, possibly adenylate cyclase.

Evidence for the phosphorylation of other small G-proteins has been scattered, but substantial amounts of information have been obtained for two small G-protein families. The major small G-protein family in which phosphorylation has been well characterized is the rap/smg p21/Krev group. smg p21A and smg p21B purified from human platelet membranes and bovine brain membranes were shown to be phosphorylated stoichiometrically in a cell-free system by protein kinase A in a cAMP-dependent manner (206, 207). Additionally, this small G-protein was phosphorylated in intact human platelets on stimulation by cAMP-elevating agonists prostaglandin E_1 and iloprost and by treatment with dibutyryl cAMP, suggesting that it is a physiological

substrate (207, 208). The site of phosphorylation of smg p21B was shown to be Ser179, very near the prenylated carboxyl terminus (200). Functionally, this phosphorylation has been demonstrated to reduce its binding to membranes and enhance its GDS-stimulated GDP-GTP exchange (200). These results are consistent with a report that smg p21 purified from the cytosol of human platelets was shown to be a substrate for protein kinase A only after removal of endogenous phosphate groups by treatment with alkaline phosphatase (209). The authors further suggest that phosphorylation serves as a signal for translocation of the membrane-bound form of this protein to the cytosol.

Phosphorylation of members of another small G-protein, G25K was described by Hart et al (210). G25K was shown to be substrate for the epidermal growth factor (EGF) receptor/tyrosine kinase in a reconstituted lipid vesicle system. The phosphorylation was observed to be stimulated by EGF, and G25K incorporated maximally ~2 mol of phosphate groups per mol of GTPγS-binding activity. Bacterially expressed recombinant ras proteins were not phosphorylated under these conditions and may be the result of a lack of prenyl modification. This report suggests a possible role for this protein in mitogenic pathways.

POTENTIAL PHARMACOLOGICAL SIGNIFICANCE OF G-PROTEIN MODIFICATIONS

Because of the wide variety of cellular processes that they regulate, G-proteins represent prime targets for pharmacological intervention in many disease states. When the involvement of G-proteins in disease states is discussed, the implication of the ras proteins in many cancers immediately comes to mind. A closely related and rapidly emerging topic concerns the recent discovery that several mammalian oncogenes are homologous to and display GAP, GDI, or GDS activity in vitro (211–213). Less well known is that genetic defects in G-protein function have been implicated in pseudohypoparathyroidism (214), diabetes (215), Albright hereditary osteodystrophy (216), and hereditary obesity in mice (217, 218). A somatic mutation of the α subunit of G_s has been shown to result in McCune-Albright syndrome (218a) or acromegaly (219), depending on whether it is expressed early in embryonic development or in the adult pituitary, respectively. Elucidation of the site of these mutations has uncovered a class of α subunits with oncogenic potential (188a, 188b, 219a). When one considers that many defects in G-protein-linked receptors and effectors may be compensated for by modulating the activity of the relevant G-protein, the pharmacological significance of G-proteins grows substantially. As discussed throughout this review, covalent modifications of G-proteins are essential to their interaction with other proteins and with their target membranes, as well as to their intrinsic activities. Cholera and whooping cough, for instance, are well known to be the direct result of the ADP-ribosylation

of G-proteins by bacterial toxins (55). More recently, the newly discovered rab geranylgeranyltransferase was found to have a striking similarity to the choroideremia gene product, indicating the possibility of a direct link between a defect in prenylation and this form of retinitis pigmentosa (159a). Undoubtedly, other G-protein-related diseases, both from the list above and among pathogenic states not yet identified to be G-protein-related, will be found to result from defects in posttranslational modifications. Some G-protein-related diseases may also potentially be treated by altering their properties through their posttranslational modifications. Consequently, we discuss here potential therapies based on the covalent modifications of G-proteins. As with any discussion of this type, some of the ideas discussed may find potential significance in the near future, while others will be further removed or of research interest only.

The most promising, or certainly the most popularized, pharmaceutical treatment of a G-protein-related disease is the treatment of certain cancers by interfering with the prenylation of oncogenic ras proteins. The loss of transforming activity on blocking of prenylation is well established (164, 220). The most straightforward method for inhibiting prenylation is by limiting the synthesis of 3-hydroxy-3-methylglutaryl (HMG)-CoA and hence prenyl pyrophosphates through the use of HMG-CoA reductase blockers such as compactin (221, 222) and mevinolin (223). Although these drugs are used in the treatment of hypercholesteremia and can be used to block protein prenylation in cultured cells, their lack of specificity may pose a problem with the function of other prenylated proteins. An intriguing result is the observation by Crowell et al (224) that the cyclized isoprenoid limonene, which occurs only in plants, is a specific inhibitor of the prenylation of low-molecular-weight G-proteins. A more specific approach entails the use of protein farnesyltransferase inhibitors, based on the structures of substrate tetrapeptides. Since it is likely that a single protein farnesyltransferase modifies all substrate proteins, other farnesylated proteins, such as nuclear lamin B, will also presumably be affected. The challenge in the future will be to find suitable inhibitors that target ras farnesylation without affecting other prenylated proteins or the intrinsic properties of the enzyme. If the farnesyl modification of ras is required for its interaction with a downstream effector, as is the case for the geranylgeranyl group of rap1A in the interaction of this small G-protein with its GDI, a more specific, and consequently more promising, approach would involve the use of prenylated analogs corresponding to the carboxyl terminus of ras. Furthermore, the same approach could be used to reverse oncogenic activation involving other small G-proteins.

Other steps in the maturation of ras may also serve as targets for the pharmaceutical disruption of oncogenic ras activity. One possibility is palmitoylation, since it is at this step that the ras proteins become tightly membrane bound. Such an intervention would require a better understanding

of the enzymology of palmitoylation. Another point of intervention is the α-carboxyl methylation step. Inhibitors such as *N*-acetylfarnesylcysteine have been shown to be effective in blocking this step (149), and it may be possible to design specific inhibitors for different prenylated proteins.

ADP-ribosylation is a G-protein modification clearly linked to a disease state. ADP-ribosylation of G-proteins by exotoxins secreted by *V. cholerae* and *B. pertussis* results in cholera and whooping cough, respectively (55). Unfortunately, pharmacological intervention in the treatment of these diseases has been limited. As discussed previously, ADP-ribosylation appears to be reversible in the presence of excess nicotinamide. Consistent with this result, the fluid loss associated with cholera has been shown to be reduced by administration of nicotinamide (225). It is possible that, under certain circumstances, the effects of these toxins will be prevented or reduced by the administration of peptides that mimic the ADP-ribosylation sites of the affected G-proteins. Another area in which toxins of this type may be of potential pharmaceutical use is in the design of recombinant toxins, where a chimeric protein composed of a toxin domain and a targeting domain is used to selectively kill undesirable cells (226). Diphtheria toxin, which ADP-ribosylates elongation factor 2 and consequently inhibits protein synthesis, has been used in experimental studies as a selective cytotoxin. It is possible that toxins that ADP-ribosylate G-protein will be used in a similar fashion, perhaps when uncoupling of a specific signal transduction pathway is desired.

A more moderate alternative to the therapeutic ADP-ribosylation of G-proteins is the modulation of G-protein–receptor coupling by alteration of its myristoylation. The oxatetradecanoic acid analogs of myristic acid have been shown to permeate cells and covalently modify substrate G-proteins (178). Furthermore, modification with the 11-oxamyristate analog has been shown to result in the translocation of some G-proteins from the membrane to the cytosol (183). Clearly, a better understanding of the implications of this translocation, as well as the effects of these analogs and their metabolites on other acylated proteins, would have to precede the potential pharmacological use of these analogs. In the meantime, myristic acid analogs represent a valuable tool in delineating the effects of myristoylation in G-protein function.

Phosphorylation is such a widespread covalent modification and regulatory mechanism that its general inhibition would clearly have undesirable effects. The best-characterized G-protein phosphorylation is the protein kinase A–catalyzed phosphorylation of smg p21B/rap1B. This phosphorylation has been shown to enhance its sensitivity to smg p21 GDS, which directly or indirectly results in translocation from the membrane to the cytosol as part of its function. In this case, the use of peptide analogs corresponding to the phosphorylation site may be useful in disturbing this cycle when necessary.

CONCLUDING REMARKS

Knowledge of the many covalent modifications that members of both the heterotrimeric and small G-protein superfamilies undergo has expanded at a remarkable rate. The speed with which these discoveries have been made has been facilitated by the strong similarities in structure and function among these superfamilies, which have allowed discoveries in one group of proteins to be applied to the others. Consequently, possible pharmacological interventions in the treatment of G-protein-related diseases, based on an advanced knowledge of their covalent modifications, are already emerging. Such therapies may herald a new era in the pharmacological treatment of disease, since G-proteins have been implicated in the regulation of a diverse array of important cellular processes. This era is near for the heterotrimeric G-proteins, whose mechanism of action and regulatory pathways are already well elucidated. The future for pharmacological intervention in small G-protein-related diseases is equally bright, because these proteins have been implicated in cell proliferation and differentiation, vesicular transport, and secretion. Defects in these processes may be the underlying cause of many cancers and neurological disorders. Understanding of the covalent modifications of the small G-proteins has far outpaced the knowledge of their functions, which will be critical to the development of successful drug therapies for these diseases. Fortunately, clearer pictures of the functions of the small G-proteins are already beginning to emerge, and the next several years should witness the elucidation of these pathways.

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