CHARACTERIZATION OF $G_o\alpha$-LIKE PROTEINS IN THE *DROSOPHILA* CENTRAL NERVOUS SYSTEM

by

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A DISSERTATION

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This thesis is dedicated to the memory of my grandmother,

Mrs. Annamma Verghese

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ABSTRACT

A family of guanine nucleotide binding proteins (G proteins) is involved in signal transduction across biological membranes. These proteins are heterotrimers comprised of α, β and γ subunits. G proteins that have been isolated and characterized from various vertebrate and invertebrate tissue sources show a remarkable degree of sequence conservation even among distantly related species. Thus, it is likely that many of the components of the signal transduction pathway have been evolutionarily conserved.

The functioning of the nervous system involves diverse components that interact through complex integrative events. Many of these events are likely to be mediated by G proteins. The principal objective of this thesis work was to establish *Drosophila* as a model to study G protein function in the nervous system. Many proteins implicated in nervous system function are conserved in *Drosophila*, not only at the sequence level, but also functionally. Furthermore, the development of *Drosophila* is well documented. Thus, the role of G proteins in nervous system development and differentiation can potentially be investigated using the *Drosophila* system.

The early studies described in this thesis successfully showed that levels of GTP binding present in *Drosophila* neuronal membranes were comparable to binding observed in bovine brain extracts. Additionally, these neuronal membrane preparations contained a 40 kD protein modified by pertussis toxin (PTX). A series of

experiments resulted in the tentative classification of the *Drosophila* toxin substrate as a homologue of vertebrate $G_o\alpha$, a G protein found in remarkably high concentrations in vertebrate brain membranes.

In order to confirm the identity of the *Drosophila* $G_o\alpha$-like protein, fly head extracts were probed with antibodies that were specific for the various vertebrate $G\alpha$ subunits. Immunoblots of fly head extracts probed with a vertebrate $G_o\alpha$ antibody recognized a 40 kD protein. The antibody also efficiently immunoprecipitated the PTX substrate of fly heads. These results suggested that the PTX substrate was a $G_o\alpha$-like protein.

Based on these results, a *Drosophila* head cDNA library was screened using the rat brain $G_o\alpha$ cDNA as a probe, in order to obtain the molecular tools that would enable a more extensive characterization of the $DG_o\alpha$-like protein. One of the hybridization positive clones obtained, λDGo21 was completely sequenced. Sequence homology of DGo21 to vertebrate $G_o\alpha$ at the deduced amino acid level was 82%. Another cDNA (DGo59) that differed from DGo21 only at its 5' end (5'untranslated region and including nucleotide 1-63 of the coding region) was also characterized. The existence of two cDNA types suggests that the two forms are generated by alternate splicing mechanisms involving the use of different exons.

The expression of the two DGo forms was studied by Northern analysis. In adult flies, both a head-specific and a body-specific transcript was seen. Another transcript was present in both heads and bodies. Analysis of stage-specific expression of transcripts

during development showed that the head-specific transcript appeared at 10-14 hour stage of development coinciding with the formation of a functional nervous system in this organism. Another transcript was specific to the 0-2 hour stage of embryogenesis (when zygotic transcription is inactive). This transcript corresponded in size to the transcript seen in adult bodies. Since mature females introduce maternal transcripts into the developing oocytes this would account for the body-specific transcript in adult flies. The third transcript was present during all stages of development and in heads and bodies of adult flies.

These results suggest that transcripts encoding $DG_o\alpha$-like proteins are regulated in a tissue-specific as well as a stage-specific manner. *In situ* hybridization to various tissue sections of flies confirms that these transcripts are localized primarily within cell bodies of neurons as well as in the ovaries of mature females.

INTRODUCTION

During the last two decades, research directed in the area of cellular signalling mechanisms has successfully established the importance of GTP binding proteins (G proteins) in signal transduction. The family of G proteins occurs ubiquitously in eukaryotes, where they couple a diverse array of receptors to appropriate effector functions. An examination of the role of G proteins, especially in regard to neuronal signal transduction is presented in this chapter to provide a background for the relevance of the work that is described herein.

TRANSMEMBRANE SIGNALLING:

Fundamental to the successful functioning of a multicellular organism is an efficient communication system that can convey information from one cell to another. Cells can respond to a variety of extracellular signals by coordinating an elaborate network of communication, eventually resulting in target effectors eliciting appropriate intracellular effects. For example, the lower eukaryotes typified by slime mold and yeast can respond to extracellular signals such as pheromones and altered environmental conditions by exhibiting chemotaxis and mating behaviours. In higher plants and animals, extracellular signals can recruit responses ranging from development, growth, differentiation and behaviour to the regulation of gene expression and synthesis and secretion of proteins.

The cell membrane plays a pivotal role in signalling mechanisms by selectively transmitting information from the

external to the internal cellular environment. Most signalling molecules such as pheromones, neurotransmitters, growth factors, hormones and peptides cannot traverse the cell membrane easily. Although in some cases information transfer occurs through the uptake of specific ions or nutrients via transporter molecules or channels, in most cases the cell membrane contains specialized protein structures or receptors that have high affinity binding sites for specific signalling molecules. The receptor can bind its cognate signalling molecule and thereby initiate a sequence of events within the cell that comprises the signal transduction cascade.

A fundamental prerequisite for transmembrane signal transmission is physical contiguity between the extracellular domains of the receptor and the intracellular environment. This permits the signalling molecule (the first messenger) to bind to the receptor extracellularly and in turn elicit a physiological effect within the cell, most often through the generation of a second messenger. Cells have evolved various mechanisms to facilitate transmembrane signalling. These can be placed into the following four categories.

The first category comprises the ligand modulated ion channels. In this case the receptors are multi-subunit membrane proteins which function as ion channels. Binding of a ligand to the receptor causes changes in ion flux due to increased membrane permeability and consequently a depolarization or hyperpolarization of the membrane (e.g. nicotinic acetylcholine receptor, glycine and γ-aminobutyric acid receptors) (1,2).

The second category includes ligands which can regulate an enzymatic activity contained in the receptor protein. This class of

receptors includes the epidermal growth factor receptor, platelet derived growth factor receptor and insulin (3). These receptors have an intrinsic enzymatic (i.e.) tyrosine kinase activity in one of their domains. Binding of the ligand induces activity of the catalytic domain of the receptor resulting in the phosphorylation of selected target residues. The membrane bound guanylate cyclase is also a receptor in this category. The receptor isolated from atrial membranes has a dual activity in that the single polypeptide chain contains a ligand recognition site (for atrial natriuretic peptide, ANP) and an enzymic activity upon binding ANP that leads to the generation of elevated levels of cGMP (4).

The third category is the class of lipophilic molecules such as the steroid hormones including androgens, corticoids, estrogens and progestins. These molecules are able to diffuse across the cell membrane due to their hydrophobicity and ultimately bind to receptors that are present in the nucleus. Generally, this results in altered gene expression within target tissues (5).

The fourth class of cellular signalling is the most diverse. Interposed between the receptor and the effector in this case is a membrane associated component. This is the signal mediator called the G protein. In the nervous system, many types of receptors transduce sensory signals by this mechanism into appropriate effects. For example, in the visual system of vertebrates there are specialized photoreceptor cells that can respond to a photon of light by generating an electrical potential. This is accomplished by a G protein called transducin that couples light stimulation to phosphodiesterase activity (6). Most recently characterized is G_{olf},

the G protein that mediates olfactory responses when odorant molecules bind specific receptors (7). G proteins couple various hormones, neurotransmitters, eicosanoids, peptides and biogenic amines to a multitude of effectors including enzymes and ion channels (8-12). An ever increasing number of G proteins and their receptor mediated effects are being elucidated; already, over 100 different receptors are known to convey a plethora of biochemical and physical information via at least 20 different G proteins to a spectrum of intracellular effectors. Undoubtedly, this is a field that is rapidly expanding as the mechanisms that underlie this mode of signal transduction are uncovered.

The receptors that are coupled to G proteins exhibit an overall similarity in topology (13). Shared structural motifs include seven transmembrane domains that are hydrophobic stretches of α helices that weave through the membrane separated by extracellular and intracellular loops comprised of hydrophilic amino acids. It is believed that ligand binds to the receptor within the hydrophobic pocket that is formed by the membrane spanning regions. Among the receptors that have been characterized, the greatest homology occurs within the membrane spanning regions (20-50%). The amino termini of receptors are glycosylated while the carboxyl termini are particularly rich in serine and threonine residues. G protein coupled receptors in vertebrates include rhodopsin ('light' receptor), the family of opsins, adrenergic, dopaminergic, serotonergic, muscarinic, peptidergic, $GABA_B$, Substance K and Substance P receptors. The list is growing as more information is obtained. Even in lower eukaryotes there exists evidence for receptors of this subtype. For

example, the life cycle of *D. Discoideum* is dependent upon sensing cAMP levels in the extracellular environment; and the budding yeast, *S. cereviseae,* has two mating types that are characterized by the presence of unique mating factors (pheromones) in each. The cAMP receptor in the former instance (14) and the mating factor receptor (15) in the latter have both been characterized as belonging to the G protein receptor family.

The G proteins are a subset of a large superfamily of GTP binding proteins which includes tubulin (cytoskeletal protein), soluble proteins that are important in protein synthesis such as bacterial elongation and initiation factors (EF-Tu and IF-Tu), products of the yeast YPT1 and SEC4 genes, and low molecular weight (21 kD) proteins typified by the *ras* oncogene and *ras* related proteins. The hallmark of this family is the avidity and specificity with which they bind guanine nucleotides. Although all of the GTP binding proteins share structural and functional features, there are specific characteristics that distinguish the G protein subset. For example, all G proteins are heterotrimers comprised of α, β and γ subunits. The Gα proteins are relatively uniform in molecular weight; they are also substrates for covalent modification by bacterial toxins. The function of the G protein is regulated by guanine nucleotides and a divalent cation, Mg^{+2}. The availability of sequence information has permitted comparison of Gαs with other GTP binding proteins such as the prototypic GTP-binding protein, bacterial EF-Tu (16). Not surprisingly, the domains that seem to be conserved in all G proteins are those that are functionally significant

in interaction with the guanine nucleotides such as the guanine nucleotide binding domains and the region governing the GTPase activity. Consensus sequences are therefore implicated in the activities of the Gα subunits.

HISTORY OF G PROTEINS:

The paradigm for transmembrane signalling by G protein coupled receptors has been the hormone regulated adenylate cyclase system. The discovery of the G proteins came through assimilation of the findings of four different groups. In 1974, Rodbell et al reported that guanine nucleotides were required for the stimulatory response of adenylate cyclase to the hormone glucagon assayed in liver membranes (17). Furthermore, the activation of the cyclase was also affected by divalent ions. The results of Maguire et al (18) showed that guanine nucleotides decreased the affinity of β-adrenergic agonists for the receptor while having no comparable effect on the affinities of antagonists. Their studies demonstrated that in the absence of guanine nucleotides, the dissociation constant for binding of agonists were ten-fold lower than their activation constant for adenylate cyclase. Addition of the guanine nucleotides restored their affinity for activation of the cyclase. The significance of these findings were not immediately ascertained.

Cassel and Selinger made progress in the field when they independently developed an assay to measure GTPase activity in turkey erythrocyte membranes (19). The assay detected the full extent of only those GTPase activities having a high affinity for GTP and effectively discriminating between adenine and guanine

nucleotides. Catecholamines were found to stimulate the GTPase activity of the membranes through their action on β-adrenergic receptors. They postulated that a GTPase activity was intrinsic to adenylate cyclase activition. Taken *in toto*, these results suggested that a guanine nucleotide binding component was an obligatory factor in receptor mediated cyclase stimulation. Furthermore, this component could influence the interactions of the receptor with its agonists in a negatively heterotropic manner.

Evidence for a regulatory guanine nucleotide component was unequivocally obtained when Pfeuffer was able to separate the G protein from the catalytic component of cyclase of pigeon erythrocyte membranes using a GTP-sepharose affinity column (20). The purified G protein was able to restore cyclase activity to membranes prepared from a mutant cell line, *cyc-*, which lacked the adenylate cyclase activity, thereby providing a convenient assay. Subsequently, Orly and Schramm (21) were able to reconstitute a functional β-adrenergic adenylate cyclase system by fusion of a cell having intact β-adrenergic receptor and an inactive cyclase with a cell containing no β-adrenergic receptor but with a functional cyclase. By virtue of the ability of the regulatory component of the purified G protein to stimulate adenylate cyclase, it was termed Gs_{α}. Gs protein was first purified from rabbit liver membranes by Northup et al (22) and was characterized by its ability to reconsitute cyclase activity in an S49 cyc- lymphoma cell line that was genetically deficient in Gs_{α}.

CLASSIFICATION OF G PROTEINS / SUBUNIT CHARACTERIZATION:

Historically, G proteins have been classified based on the function and specificity of the α subunit's interactions with effectors. A mnemonic subscript assigned to the α subunit identifies it on the basis of its initially recognized function. Thus, $G_s\alpha$ is important in the stimulation of adenylate cyclase (22), while $G_i\alpha$ was initially characterized on the basis of inhibition of adenylate cyclase (23). Transducin, or $G_t\alpha$, couples light stimulated rhodopsin activation to cGMP phosphodiesterase (6). A Gα protein that was purified from vertebrate brain tissue, where it was present in abundance (1% of the total membrane protein) had no function assigned to it and hence was designated as $G_o\alpha$ (where 'o' stands for "other") (24, 25). $G_p\alpha$ is believed to couple receptor mediated phosphoinositide metabolism (26).

A typical G protein is comprised of three different subunits, α, β and γ that make up the oligomer associated with the plasma membrane. Each of the subunits is encoded by a distinct gene. The α subunit has a site for binding guanine nucleotides and is characterized by having an intrinsic GTPase activity. The molecular weights of α subunits that have been purified range from 39-52 kD. Cloning of α subunits from a variety of tissue sources shows that these molecules are extremely conserved at the sequence level. Remarkably, α subunits have been found in almost all eukaryotes where their occurence has been investigated, including slime mold, yeast and many invertebrates. The sequence of over 30 different α subunits is currently available. Table 1 is a compilation of the α

subunits that have been characterized along with a tentative listing of the receptors and effectors they couple.

It is the generally accepted view that the α subunit lends functional distinction to the G protein while the β and γ subunits perform a regulatory role. Some of the findings that have led to this belief can be summarized as follows. First, reconstitution studies were able to elegantly demonstrate that the purified α_s subunit could restore cyclase activity to a mutant cell line (S49 cyc- lymphoma) which had been categorized as lacking $G_s\alpha$ by a number of different criteria. Second, purified α subunits introduced into membranes isolated from atrial cells elicited ion channel activity recordable by electrophysiological means (27). Third, various cloned α subunits have been expressed in bacterial *E. coli* systems. Translated α subunit proteins obtained in this manner could effectively couple receptor-effector components even in the absence of β and γ subunits (28). Finally, there is a growing body of evidence that details various pathological conditions that result from α subunit dysfunction.

The β subunit is a 35 kD or 36 kD protein depending on the source from which it was cloned or purified (29, 30). In most tissues, the β subunit occurs as a doublet of the 35 and 36 kD forms. Two different genes encode the two forms of β subunit which are 90% homologous. The γ subunit has a molecular weight that has been reported to range from 8-11 kD. Although cDNAs encoding γ subunits have been isolated (31-33) they remain poorly characterized. Little is known about the specificity underlying βγ linkages. In cells, β and γ subunits occur tightly bound together and

TABLE 1:

Properties of G protein Subunits

(Taken from Friessmuth, M., Casey, P.J., Gilman, A.G. " G proteins control diverse pathways of transmembrane signalling" FASEB J. (1989) 3, 2125-2131)

Subunit	Mr (kD)	Receptor	Role (effector)
Gsα (4)	45-52	βAR>>α_2AR>Rho	Activates adenylate cyclase Activates Ca^{2+} channels
Gsα, olf	45	Unknown	Activates olfactory adenylate cyclase (?)
Giα (3)	40-41	Musc,α_2AR,Rho>βAR	Inhibits adenylate cyclase Activation of K^+ channels
Goα	39-40	Musc,α_2AR,Rho	Activation of voltage sensitive Ca^{2+} channels
Gtα, r	39	Rho>α_2AR>>βAR	Activates cGMP phosphodiesterase in rods
Gtα, c	40	Cone opsins	Activates cGMP phosphodiesterase in cones
Gpi	?	α_1AR, TRH (?)	Stimulation of phosphoinositide breakdown
Gzα (Gxα)	40	Unknown	Pertussin-toxin insensitive events (?)
β (2)	35, 36	—	βγ anchors G protein to membrane - modulates receptor-α interactions - inactivates Gα - activates phospholipase A_2 - production of arachidonic acid metabolites
γ (3?)	8-10	—	—

can be separated only under denaturing conditions. These subunits are hydrophobic in nature, and thus may serve to anchor the G protein within the cytoplasmic membrane. Since the βγ subunits are highly conserved they may be shared between different α subunits; for example, the βγ subunits isolated from G_i could be reconstituted with purified $G_t\alpha$ to produce a rhodopsin stimulated GTPase activity (34). The βγ subunits may also have a regulatory role as evidenced by the ability of the βγ subunits from G_i to inhibit $G_s\alpha$ stimulated cyclase (35). Additionally, it is believed that the βγ subunits may have a regulatory role in modulating receptor-Gα interactions since tryptic cleavage of the amino terminus of $G_t\alpha$ (the region believed to be important in receptor and βγ interactions) prevented activation of rhodopsin stimulated phosphodiesterase activity (36). Recent evidence suggests that the βγ subunits may be involved as well in the production of prostaglandins via activation of phospholipase A_2. The resulting arachidonic acid metabolites can activate K^+ channels in cardiac cells independently of the α subunit (37, 38).

THE GTP CYCLE:

In G proteins, guanine nucleotides are able to mediate signal transduction by switching the protein between a "signal-on" activating conformation and a "signal-off" dormant configuration depending on whether the α subunit is bound to GTP or GDP respectively. The major guanine nucleotide of the cell is GTP, present at a concentration of 10^{-4} M, approximately in ten-fold excess of the concentration of intracellular GDP. Figure 1 represents a simple

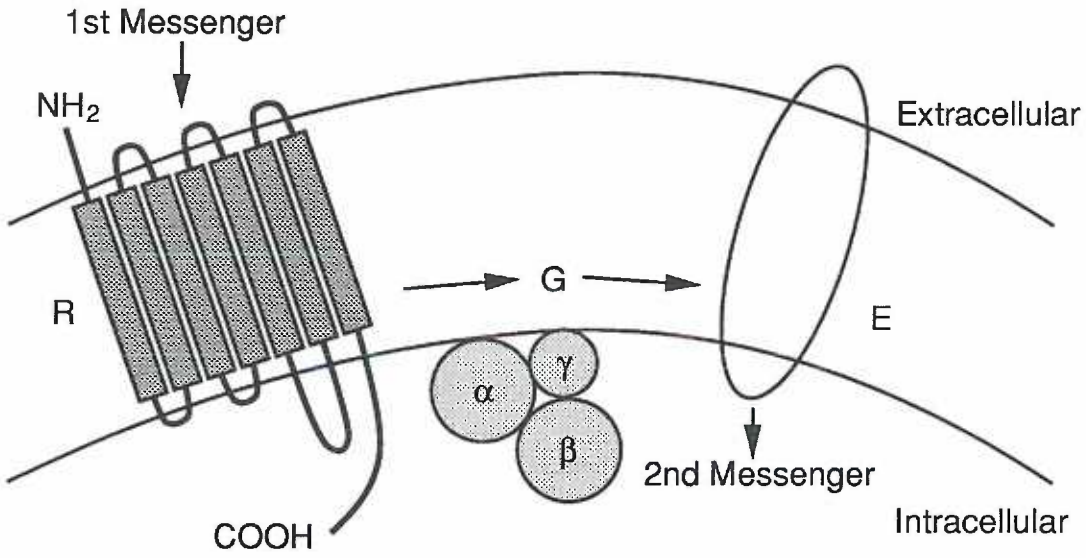
FIGURE 1:

The upper panel depicts a diagram of a G protein-coupled receptor and effector. Extracellular signals (i.e.) the "first messengers" interact with specific receptors (R) that are transmembrane glycoproteins. Typically the receptors have seven membrane-spanning domains, an extracellular amino terminus and an intracellular carboxy terminus. Activated receptor in turn, activates the G protein (G) that is membrane associated. The G protein that has been activated can interact with, and regulate the activity of the effector (E) leading to the generation of an intracellular signal.

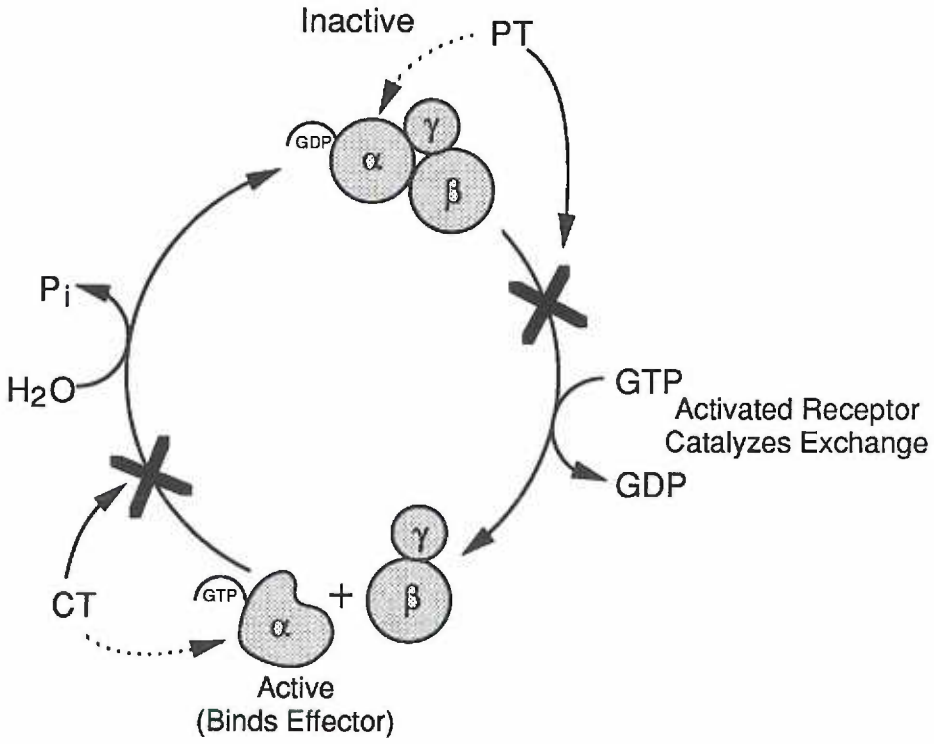
Schematic Diagram of the GTPase Cycle.

The α subunit of the G protein heterotrimer has a GDP bound to it in the basal state of inactivation. Interaction with an activated receptor triggers α-GTP formation and dissociation of the α from β and γ subunits. Termination of activation is signalled by GTP hydrolysis which returns the α subunit to its inactive state (i.e.) α-GDP complexed to $\beta\gamma$. Bacterial toxins transfer (dashed arrows) ADP-ribose from NAD to G protein α subunits. Toxin modification of the α subunits interfere with the normal functioning of the GTP cycle as indicated in the diagram.

(Taken from "Receptor-Effector Coupling By G proteins: Implications for Endocrinology" A.M. Spiegel, 1989)



G-PROTEIN GTPase CYCLE



schematic of the events occurring during the GTP cycle. The proper functioning of the GTP cycle is critical to signal transduction as is evidenced by the pathology resultant upon derangement of any step of the cycle.

Extensive studies have successfully elaborated the kinetics of the cycle (39-42). In the basal state, the G protein exists in a heterotrimeric configuration with GDP bound to the α subunit; in the absence of activation, GDP limits basal GTPase activity. The binding of agonist (i.e.) hormone to the receptor induces a conformational change which causes "opening" of the guanine nucleotide binding site which is rapidly filled by GTP. The rate of binding of GTP to the agonist-receptor-G protein complex approaches diffusion controlled limits, essentially occurring almost instantaneously. (K_m GTP=0.3μM). Mg^{+2} is required for the formation of the active GTP-bound species and at high concentrations (>10mM) stabilizes the latter. The binding of agonist prompts the accelerated dissociation of GDP from the α subunit possibly by lowering the concentration of Mg^{+2} that is otherwise required to facilitate this reaction. For the high affinity Mg^{+2} modulated GTP (GDP) binding site, k_{off} GDP= .03-0.3 min^{-1}. α-Mg-GTP dissociates from the $\beta\gamma$ complex and activates the next component in the cycle, namely the effector.

The lifetime of the active GTP-bound species is finite, controlled by an intrinsic GTPase activity of the α subunit. The reported value for the termination of the α-GTP due hydrolysis (i.e.) k_{cat}, approximates 10 min^{-1} at 30°C, which is in good agreement with the deactivation rate of adenylate cyclase in intact membranes. GDP bound α subunit can then reassociate with the $\beta\gamma$ subunits

resulting in regeneration of the inactive species. The precise mechanism of deactivation has not been detailed.

Salient features of the GTP cycle include the following. The G protein can alter the affinity of the ligand for the receptor in a negative manner. Generally, agonist bound to receptor can activate ten Gα molecules leading to an amplification of the signal. Additionally, as the lifetime of the α-GTP is many seconds, interaction with many effector molecules (approx. one hundred) is possible leading to further amplification of the signal. There exists evidence from the *ras* system that a GTPase activating protein (GAP) can positively influence the rate of GTP hydrolysis significantly (43). Whether or not a homologous factor modulates GTP hydrolysis in G proteins remains to be established. The importance of the GTPase activity of the α subunit is highlighted by the recent finding of a $G_s\alpha$ mutant (GASP), that contained somatic point mutations that destroyed GTPase activity and hence was constitutively active (44). This α subunit showed characteristics of being a putative oncogene. Pituitary tumours containing the mutant $G_s\alpha$ had elevated levels of cAMP and abnormal proliferative capacity.

COVALENT MODIFICATION BY BACTERIAL TOXINS:

A characteristic feature of all α subunits is their susceptibility to bacterial toxins. These toxins have significant deranging effects on the GTPase cycle. The catalytic action of bacterial toxins in modifying the Gα subunits has been used widely in characterizing Gα mediated receptor-effector interactions. In fact, the discovery of the inhibitory G protein (Gi) occurred fortuitously when pertussis toxin

(PTX) was found to abolish receptor mediated cyclase inhibition in rat C6 glioma cells (45). These toxin treated cells contained elevated levels of cAMP. The toxin substrate ($G_i\alpha$) was subsequently purified from these cells using the criterion of toxin binding as an assay (46).

Both PTX and cholera toxin (CTX) can transfer the ADP ribose moiety from NAD (nicotinamide adenine dinucleotide) to the α subunits of G proteins (47-51). PTX modifies a cysteine residue that is conserved at the carboxyl terminus of $G_i\alpha$, $G_o\alpha$ and $G_t\alpha$ (24, 46-48). When this modification occurs, the α subunit is no longer able to dissociate from the $\beta\gamma$ subunits. Thus, the toxin uncouples the G protein from the receptor. This toxin causes whooping cough whose symptoms include lymphocytosis, histamine sensitization and hypoglycemia. PTX is also the causative agent in a certain form of encephalopathy that results in major disturbances in learning and memory processes (52).

CTX modifies an arginine residue that is conserved between $G_s\alpha$ and $G_t\alpha$ (53-56). The effect of this modification is to cause persistent activation of the effector, since the α subunit is now unable to hydrolyze the bound GTP and thus return to its inactive GDP-bound state. CTX is responsible for the pathogenesis of the disease, cholera. In patients suffering from this disease, CTX catalyzes ADP ribosylation of $G_s\alpha$ which results in increased concentration of cAMP in the intestinal cells of the mucosa and a lethal secretion of salt and water into the gut (57).

The disruptive action of these toxins has been utilized as an experimental criterion in identifying particular receptor mediated effects as being transduced via G proteins. Now that antibody and

DNA probes are available, G proteins can be identified without the use of toxins. This has led to the discovery of novel G proteins that appear to be intractable to covalent modification by toxins (58, 59). Their function and significance have yet to be studied in detail.

STRUCTURAL MODEL FOR Gα PROTEINS:

The isolation and characterization of cDNA sequences for a variety of α subunits has allowed a general model of protein structure for the α subunit to be predicted (60-64). There is approximately 52% amino acid identity among the α chains of different G proteins. The proposed model is based largely upon the solution of the crystal structure of EF-Tu and the p21 *ras* protein (16, 65-68). Basically, the α subunit is separated into three domains which contain flexible regions (hinges) that allow conformational changes that occur in any one part of the molecule to be transmitted across the molecule.

Four different stretches (called A, C, E, and G) have been assigned in accordance with the model proposed by Halliday (69) as the regions that show the greatest homology with p21 *ras* and the EF-Tu molecule, (i.e.) the GTP binding region. A, C, E and G regions each occur between a β strand and an α helix and are arranged around a central guanine nucleotide binding core. These regions (A, C, E and G) also display sequence homology among the different α chains.

The domains of the α subunit have been specified based on their function. Domain 1 contains the guanine nucleotide binding region, domain 2 is thought to modulate and interact with the

effector while domain 3 comprises both the amino and carboxyl termini regions which are thought to regulate both receptor and $\beta\gamma$ interactions. There is some evidence for the validity of these domain assignments. For example, tryptic cleavage of the amino terminus (of the α subunit) destroys effective receptor and $\beta\gamma$ interactions (36, 70); ADP ribosylation of the carboxyl terminus by PTX uncouples the $G\alpha$ from the receptor (71). The assignment of the effector binding region is based on this region showing the greatest sequence variability in p21 *ras*. (72). Quarternary structure predictions align the carboxyl terminus in spatial proximity to the amino terminus; this facilitates the interactions between $G\alpha$, $G\beta\gamma$ and the receptor.

Diversity among the various α subunits can arise at different levels. As expected, characterization of the α subunits revealed that although they contained unifying features (such as the ability to bind and hydrolyze GTP), necessarily they diverged from each other in regions that potentially governed receptor and effector interactions. For example, the carboxyl termini of the different α subunits (receptor modulating region) of Gi, Gs, Go and Gt are distinct. Additionally, distinct post-translational modifications may occur in the different α subunits. It is known that only the amino termini of $G_i\alpha$ and $G_o\alpha$ are myristoylated (73). The significance of the modification is not completely understood.

Diversity can also occur through regulation at the level of the gene that specifies the α subunit. For instance, it is known that alternative splicing of a single gene of $G_s\alpha$ produces four different gene products (74). Each of the resulting splice variants were able to

activate calcium channels and adenylate cyclase (75). The mechanism underlying the specificity of effector function in this case is not known. Three different genes encode three different forms of $G_i\alpha$ ($G_i\alpha_{1-3}$) (76, 77). Although the $G_i\alpha$s are closely related they do contain divergent sequences. $G_i\alpha$ subunits are important in modulating the inhibitory activity of adenylate cyclase (23, 78), the activation of K^+ channels in cardiac tissue (79) and furthermore, are implicated in the regulation and coupling of phospholipase C activity (80). The distribution of the various α subunits varies in different tissues. Thus, $G_s\alpha$ and $G_{i2}\alpha$ occur ubiquitously whereas $G_{i1}\alpha$ and $G_{i3}\alpha$ appear to have a more restricted distribution. The construction of $G_s\alpha$-$G_i\alpha$ chimeras is currently underway and should provide further insights into the specificity of receptor and effector interactions. Two forms of transducin or $G_t\alpha$ have been isolated to date. These variants differ in their primary site of expression: one is expressed in the rod cells and the other in the cones. Genes encoding both rod-specific and cone-specific transducins have been characterized (81).

Since G proteins have a critical role in signalling processes, it is expected that aberrations in these proteins would result in pleiotropic cellular effects. The following examples will serve to emphasize the clinical relevance of these macromolecules in cellular function as evidenced by the profound impact that results from alterations in their normal levels. In pseudohypoparathyroidism (type 1a variant) there is resistance at the cellular level to multiple agents that act by stimulating cAMP production. The condition reportedly is due to a genetically acquired reduction of upto 50% in $G_s\alpha$ levels (82). In animal models where type 1 diabetes (diabetes

mellitus) has been experimentally induced, there is a loss of expression of $G_i\alpha$ in liver cells (83). In certain rat pituitary tumours, the failure to respond to dopamine may be due to a $G_o\alpha$ deficiency (84). Most recently, in certain human growth hormone secreting pituitary adenomas there is evidence that somatic point mutations in $G_s\alpha$ result in constitutive activation of the α subunit (44). The condition is characterized by elevated concentrations of cAMP leading to uncontrolled proliferation. The existence of these diseases demonstrate the importance of the G proteins to cellular function. In order to understand the mechanisms that are involved in these pathways as well as the complex interactions that permit efficient signal transduction to occur, many questions need to be investigated at a fundamental level.

$G_o\alpha$, A G PROTEIN OF THE NERVOUS SYSTEM:

Within the nervous system, sensory transduction processes are of singular importance. Synaptogenesis, neurotransmission and ion channel activity are functions that putatively require integrative signal transduction. The nervous system in multicellular organisms is laid out with precision and furthermore, functions efficiently through the coordinate regulation of a myriad of complex interactive events. Indeed, the development, differentiation and continued maintenance of the nervous system is dependent upon temporally and spatially regulated transduction events occurring through receptor systems linked to second messenger pathways.

$G_o\alpha$ was first purified from bovine brain by Sternweis and Robishaw (24) and Neer et al (25). During attempts to purify the

brain $G_i\alpha$, they noticed a GTP binding activity that was 10-fold higher than the expected value. This binding was later characterized as being attributable to the activity of $G_o\alpha$, a protein present in brain at levels of approx. 1% of total brain membrane protein. Although experiments using antibodies specific for $G_o\alpha$ have shown that this subunit occurs predominantly in brain tissues; positive immunoreactivity is also seen in liver, heart, kidney and adipocyte membranes (85). Additionally, Go type immunoreactivity was seen in invertebrate neuronal tissues including those of snail, sea slug, locust and tobacco hornworm (86). Peripheral tissues of these invertebrates did not contain any Go-like antigenicity. On the basis of its tissue localization, Go is almost certain to play a major role in neuronal signal transduction.

The function of Go has remained elusive since its purification from brain membranes. Currently, the most likely role for this $G\alpha$ is the mediation of voltage sensitive calcium channel activity (87). Most recently, the studies of McFadzean et al show that in neuroblastoma (NG108-15) cells, noradrenaline (NA) induced Ca^{+2} current (I_{Ca}) inhibition is effectively blocked by a selective antibody against $G_o\alpha$ (88). Although these cells contain $G_i\alpha$ as well, an antibody against $G_i\alpha$ did not inhibit the I_{Ca} response. Earlier, it had been shown that PTX could effectively block NA induced decrease in I_{Ca} in these NG-108 cells (89). However, the Ca channel inhibition could be restored by the addition of purified $G_o\alpha$ subunit to the cells. $G_i\alpha$ was also able to reconstitute the attenuated response albeit with ten-fold less efficiency than the $G_o\alpha$. The authors postulated the

likelihood of $G_o\alpha$ being implicated in this catecholamine induced inhibition of Ca^{+2} current.

Several other neurotransmitters have also been shown to inhibit I_{Ca} in sensory neurons studied in vitro. Neuropeptide Y (NPY) potently blocks calcium currents in neurons of cultured dorsal root ganglion (DRG) cells. This effect is abolished by pretreating these cells with PTX. Introduction of purified $G_o\alpha$ subunit can rescue the inhibition of I_{Ca} (90-92). Enkephalins such as the opioid peptide, D-ala D-leu enkephalin, also mediate inhibition of I_{Ca}, studied in NG108 cells (93). PTX interferes with this inhibition; and this interference can be overcome by the addition of purified $G_o\alpha$ to the cells.

Both $GABA_B$ and α adrenoceptors are known to negatively couple to adenylate cyclase. Furthermore, they inhibit the secretion of Substance P from cultured DRG cells (94). The antinociceptive function of $\alpha 2$ and $GABA_B$ receptors can be blocked by PTX, suggesting Go involvement. The finding of high concentrations of $G_o\alpha$ immunoreactivity in the substantia gelatinosa area of the dorsal horn of the spinal cord (which contains Substance P releasing neurons) is provocative, implicating $G_o\alpha$ in the pain response.

In order to clarify the role of $G_o\alpha$ in neuronal function, immunohistochemical studies using specific antibodies have mapped the localization of this protein within different areas of the rat brain (95, 96). The results show that the distribution of the protein is not uniform within the brain but is restricted; frontal cortex, pyramidal cells of the hippocampus, Purkinje cells, the claustrum and the habenula being areas of concentration. At the cellular level both neuronal and glial cells contained $G_o\alpha$ (97). However, synaptic zones

appeared to be selectively enriched for $G_o\alpha$. Interestingly, the localization studies revealed that the pattern of distribution of the $G_o\alpha$ in rat brain was paralleled by the distribution of protein kinase C sites (mapped on the basis of autoradiography of labelled phorbol ester binding)(98).

A G protein is coupled to phosphoinositide (PI) metabolism, but its identity has remained an open question. Evidence of PTX impairing pathways that are coupled via PI metabolites includes the following. In HL-60 cells, a chemotactic peptide (f-Met-Leu-Phe) couples receptors to activate phospholipase C. This effect is PTX sensitive. Introduction of purified $G_o\alpha$ or $G_i\alpha$ to toxin pretreated cells can restore the expected effector activity (99).

The studies of VanDongen et al (100) show that purified $G_o\alpha$ could directly activate four classes of K^+ channels in hippocampal pyramidal cells of the neonatal rat brain. The ability of $G_o\alpha$ to stimulate these channels was tested in cell-free membrane patches from these neurons. A purified preparation of bovine brain $G_o\alpha$ (activated by GTPγS) could elicit single channel currents at low concentrations (1-10 pM). To confirm that the G protein involved was in fact $G_o\alpha$ and not a contaminant such as $G_i\alpha$ (which is also known to activate K^+ channels), $G_o\alpha$ was expressed as a fusion protein in *E.coli*. The fusion protein $rG_o\alpha$, was able to activate these channels; other recombinant fusion proteins including $rG_s\alpha$ and $rG_i\alpha$ were unable to produce this effect.

A PTX sensitive protein ($G_o\alpha$?) is involved in long term potentiation (LTP) studied in hippocampal slices of rat brain (101). Hippocampal LTP has been described as a stable facilitation of

synaptic responses resulting from very brief trains of high frequency stimulation. The high frequency neural activity is of sufficient duration to postulate that the resulting LTP is the substrate for learning and memory (102). The action of glutamate acting via the NMDA receptor produces increased concentrations of calcium in the post-synaptic neuron. Ca^{+2} has an inductive effect on LTP (103). Protein Kinase C has been shown to have a role in the maintenance of LTP (104).

Previous experiments have shown that both serotonin and $GABA_B$ receptors are present in hippocampal neurons where they couple to K^+ channels (105). Evidence for the involvement of a G protein came from data showing PTX induced uncoupling of hippocampal neuronal responses to baclofen ($GABA_B$ agonist) and also affected LTP. It is tempting to postulate that G proteins may play an important part in learning and memory acquisition, especially in light of the fact that the distribution of Go parallels that of Protein kinase C in the rat brain. The G protein may act at a site upstream of the NMDA induced Ca^{+2} increase.

A role for $G_o\alpha$ in atrial cells has been proposed on the basis of its capacity to reconstitute K^+ channel activity to muscarinic responses in embryonic chick heart cells that have been pretreated with PTX (106). However, the identity of the G protein in this case is more tentative. LHRH and somatostatin influence I_{Ca} by means of a PTX sensitive protein which may be Gi or Go (84, 108). Additionally, in two pituitary tumours, an anomaly that is reportedly in the receptor complex is attributed to a $G_o\alpha$ deficiency. These cells have abnormal responses to dopamine mediated events. On the basis of

experiments using Go specific antisera, it is believed that this G protein may be responsible for cell transformation and the acquisition of neoplastic characteristics in these tumours (89).

Lastly, a role for $G_o\alpha$ has been suggested in developmental and differentiation processes. The evidence includes a marked increase (approximately four-fold) in $G_o\alpha$ subunit concentration upon the differentiation of fibroblasts into adipocytes, studied in 3T3-L1 cells (109). Also, in embryonic chick heart cells there is a marked increase in $G_o\alpha$ on Day 2 of development followed by a transient decrease before steady state levels are acquired (106). The increase in $G_o\alpha$ is paralleled by the appearance of responsiveness to muscarinic acetylcholine agonists in these tissues.

G PROTEIN MEDIATED SIGNAL TRANSDUCTION IN INVERTEBRATES:

As mentioned earlier, G proteins occur ubiquitously in both vertebrate and invertebrate systems. In fact, the availability of molecular probes corresponding to the different G protein subunits has facilitated the isolation of G protein homologs in various species. The following section will summarize the evidence for G protein mediated signal transduction in various invertebrate systems.

Numerous biochemical studies indicate that signal transduction pathways in the slime mold, *D. Discoideum* involve guanine nucleotide binding proteins. In addition, the cAMP receptor of the slime mold displays seven transmembrane domains in keeping with other G protein linked receptors. The transduction processes in the slime mold are critical to its developmental program. cAMP concentration in the environment governs when the cell will initiate

chemotaxis and concomitantly begin aggregating to form a multicellular intermediate. Two different α subunits (expressed at different times of development) are thought to mediate this differentiation program through the generation of distinct second messengers including inositol trisphosphate and diacylglycerol. The cDNA sequences of these α subunits have been characterized and display 45% overall amino acid homology to the mammalian Gαs (110).

G proteins with α, β and γ subunits analogous to mammalian G proteins have also been identified in the budding yeast, *Saccharomyces cereviseae* (111, 112). The mating pheromone response pathway of this organism has components that have structural similarities to mammalian signal transduction. Pheromone receptors are encoded by genes that are homologous to other G protein coupled receptors. Using molecular manipulations to delete the yeast α-like, β-like, and γ-like genes, it was found that deletion of either the β or γ gene abolishes a cell's ability to respond to pheromone whereas deletion of the α gene produces a cell with the pheromone response programme constitutively activated (33). These results suggest that in contrast to most signal transmission pathways where the α subunit is the transducer, in the yeast mating response, β and γ are the elements that carry the signal between receptor and effector.

A number of invertebrates were examined for the presence of PTX substrates by the criterion of toxin modification. In the nervous systems of mollusks (snail), reptiles (turtle) and insects (*Drosophila, Manduca* and *Locusta*) PTX modified a 40 kD protein (86).

Additionally, an antibody against vertebrate $G_o\alpha$ also cross reacted with a 40 kD protein in neuronal tissues of these organisms.

The conservation of the $G_o\alpha$ subunit in neuronal tissues of invertebrates serves to emphasize its central role in neuronal signal transduction. Harris-Warrick et al studied *Helix aspersa* neuronal activity where dopamine mediates a decrease in calcium currents (113). As in the DRG neurons, this effect in snails could be blocked by PTX and furthermore, it was restored by the intracellular injection of mammalian $G_o\alpha$. Examination of immunoreactivity showed that snail extracts prepared from nervous tissue contained a 40 kD band that was recognized by a vertebrate $G_o\alpha$ specific antibody. Taken together with the results of Homburger et al who demonstrated $G_o\alpha$ immunoreactivity in various invertebrate neuronal tissues, this experiment points to the conservation of a functional $G_o\alpha$ protein in the nervous system of these lower eukaryotes.

Biochemical studies that have characterized GTP binding proteins in invertebrates have focussed on insect and cephalopod visual systems. These organisms have well developed visual systems. Many of the component proteins show remarkable homology to their vertebrate counterparts, such as the insect photoreceptor opsins and vertebrate rhodopsin (114, 115). The phototransduction cascade of vertebrates is also surprisingly paralleled in insects. A major difference, however is that in invertebrates the transduction of light results in a depolarizing potential across photoreceptor membranes; vertebrates respond to the light signal with a hyperpolarizing potential due to the efflux of

cations from the photoreceptor cells. cGMP in both systems is believed to be the second messenger although there is evidence for second messengers generated by phosphoinositide hydrolysis playing a major role in insect and cephalopod visual transduction (116).

The first incontrovertible evidence for G proteins in invertebrates came from light induced GTPase activity in octopus, squid, *Limulus* and fly photoreceptors, suggesting that a transducin-like protein participates in visual transduction (117-122). The nucleotide requirements of the reaction were examined as a criterion of the homology to vertebrate transducin. The preferred substrate for the activated rhodopsin was the GDP-bound form of the G protein. Light also increased intracellular levels of inositol trisphosphate in *Limulus*, squid and *Musca*. This suggested that second messengers generated by phospholipase C participated in visual transduction of invertebrates. Recently, the *norpA* gene of *Drosophila* was cloned and shown to encode phospholipase C (123). Mutants in *norpA* fail to exhibit a receptor potential to light stimulation (norp = no receptor potential). Hence, PI activity has an important signalling role in invertebrate visual transduction.

Other related experiments include the following. Visual excitation and adaptation in *Musca* eye membranes were blocked by the addition of GDPβS (124). GDPβS blocks the function of the G protein α subunit by binding irreversibly, thereby preventing the α subunit from exchanging GTP and becoming activated. It is also unable to dissociate from the βγ subunits. Direct injection of inositol trisphosphate into *Musca* eye membranes was able to restore the excitatory response indicating that the phospholipase C in these

membranes is under stringent regulatory control of a G protein. Invertebrates also contain phosphorylating deactivation proteins that terminate the visual transduction cascade. Activated rhodopsin of vertebrates is phosphorylated (by rhodopsin kinase) and subsequently a 48 kD protein (arrestin) binds to the phosphorylated form to signal the end of activation. In blowfly membranes, phosphorylation of a 48 kD component during visual excitation has been demonstrated (125).

The summary of the findings described above highlight the importance of the G proteins in vertebrate and invertebrate neuronal signal transduction. Obviously, these proteins have been under tremendous selective pressure during the course of evolution resulting in their functional conservation in species that are only distantly related.

DROSOPHILA AS AN INSECT MODEL TO STUDY G PROTEINS:

In the last decade, research efforts that have focussed on elucidating the mechanisms of action of the G-proteins, have amassed a wealth of information. Using purified receptor, G-protein and the effector components, it has been possible to reproduce the signal transduction pathway in an *in vitro* environment using phospholipid vesicles and an assay for effector function. Furthermore, molecular cloning of the various G protein subunits has permitted the application of various techniques such as expression of the α subunit cDNAs in heterologous cell lines and site directed mutagenesis of targeted residues within the α subunit to identify domains involved in critical functions such as receptor and effector interactions and

postulated guanyl nucleotide binding sites. Furthermore, analysis of the crystal structure of EF-Tu and the *ras* protein has permitted models of α subunit protein structure to be constructed. Of significance is the fact that almost all the information that has accumulated to date has been obtained from *in vitro* manipulations of vertebrate G protein systems.

There are many gaps in our understanding of G proteins such as the rules that govern the specificity of receptor-effector interactions, the implications of the diversity of the α subunits and their functional significance to the organism; how the fidelity of signal transduction is maintained in an *in vivo* situation where there are multiple receptors, effectors and G proteins, to enumerate only a few pertinent questions. These and indeed many other questions can only be addressed meaningfully in the context of an *in vivo* environment (i.e.) an intact animal.

A well characterized invertebrate system in which to begin to investigate these questions is *Drosophila melanogaster*. Since G proteins are the central elements that orchestrate the signal transduction processes; it is likely that they are highly conserved molecules. In fact this is amply borne out in sequence data available from the molecular cloning of the various α subunits from different vertebrate tissue sources. Furthermore, several genes have been cloned in *Drosophila* that show remarkable homology to their vertebrate counterparts (126). These include the genes encoding structural elements of the cytoskeleton (i.e.) actin, myosin, tropomyosin, spectrin and tubulin; genes involved in neuronal function such as the acetylcholine receptor, acetyl cholinesterase,

sodium channel protein; growth factor receptors like insulin, TGF-β and the EGF receptors; proteins that play a role in second messenger systems like calmodulin and protein kinases. These examples serve to emphasize that although evolutionary divergence of vertebrates and invertebrates occurred over 500 million years ago, basic mechanisms that are involved in the functioning of a multicellular organism have been conserved. In fact it is believed that "complexity in vertebrates arises from the reiteration and adaptation of common, evolutionarily ancient processes" (G. Rubin, 1988)(126).

There are many advantages to using an invertebrate (metazoan) such as *Drosophila* in the dissection of the components that constitute a particular pathway. Sophisticated molecular genetics, to a large extent, was pioneered and perfected as an experimental approach, in this organism. When molecular genetics is used in combination with the current applications of biochemical, molecular biological, electrophysiological, immunochemical and cytological techniques that are available, it is likely that an accurate assessment of the role of G proteins in *Drosophila* will be possible which can then be meaningfully extended to vertebrate systems.

There exists a body of evidence that demonstrates the importance of cAMP mediated processes to integrative neuronal processes in flies such as learning and memory. For example, *rutabaga* and *dunce*, two *Drosophila* mutants that affect learning are defective in adenylate cyclase and cAMP phosphodiesterase activity, respectively (127, 128). Both of these enzymes are central to cAMP metabolism. It is evident that several mechanisms such as phosphoinositide turnover and phosphorylation cascades must

operate in concert to produce complex neuronal behaviours. The common denominator underlying all these neuronal receptor mediated events is likely to be the G protein.

Compelling evidence for guanyl nucleotide coupled receptors came from a series of experiments examining the presence of bioaminergic receptors in *Drosophila* homogenates. Octopamine, for example, has been suggested to function as a hormone, neurotransmitter and neuromodulator in various insect systems (129-132). The binding of octopamine results in the activation of adenylate cyclase; the cascade is suggested to be the parallel of α adrenergic mediated effects in the sympathetic nervous system. The binding of octopamine was decreased substantially in *Drosophila* extracts in which Gpp(NH)p had been added. Gpp(NH)p is a non-hydrolyzable analog of GTP and binds irreversibly to the α subunit of the G protein causing it to dissociate from the $\beta\gamma$ subunits. Since the $\beta\gamma$ subunits are required for the activation of the G protein by the receptor; inefficient ligand-receptor induced signal transmission occurs. Thus, a G protein is implicated in these receptor mediated effects. This evidence lends credence to the prediction that the G proteins will be functionally conserved in the *Drosophila* system.

Once the genes that specify Gα subunit(s) of *Drosophila* have been isolated, the tools will be available to identify the individual genetic components of the complex processes underlying signalling. For instance, the map position of the cloned genes can be determined by *in situ* hybridization to the polytene chromosomes of the salivary glands. The potential of known mutations mapping to this region can then be examined. Furthermore, knowledge of the map position can

be used to construct strains of flies which have deletions or mutations in specific genes of interest. The effect of such mutations can then be assessed using a variety of behavioural paradigms or biochemical assays of functionality.

Knowing regions of the α subunit protein that are critical to vertebrate Gα function, alterations that would have predictable consequences at the molecular level can be introduced into the fly homologs of the Gα genes. The striking advantage provided by studying *Drosophila* is that the effects of these alterations can be studied at the organismal level. Since the developmental stages of the fly have been well documented in terms of the differentiation events that carry the organism from the embryonic stages to the adult form; it is possible that the effects of Gαs in *Drosophila* on development and differentiation can be investigated. These are but a few of the well established manipulations that are possible using *Drosophila* as an insect model to study function.

Using *Drosophila*, my goal was to identify and characterize the G proteins that played an important role in nervous sytem function. In this organism, where embryological and biochemical approaches nicely complement classical genetic methods, investigation of nervous system function is a burgeoning field. The data described herein establishes predictably that G proteins (specifically the *Drosophila* $G_o\alpha$ homolog) are remarkably conserved in *Drosophila* and furthermore on the basis of the various experimental criteria that were applied, indicate that they subserve functions that are critical to the integrity of the nervous system. The work described is a necessary foundation, providing a framework upon which to base

future experiments that will extend the characterization of G proteins and their role in signal transduction in *Drosophila melanogaster*.

Already, structural genes encoding receptors and ion channels have been mapped; and considerable information is available on the mechanisms underlying neuronal pathfinding, biological rhythms and behaviour (such as learning and memory). The characterization studies detailed in this thesis work, could eventually extend our understanding of the role of G proteins in nervous system development and function into the realm of vertebrate systems as well.

MATERIALS AND METHODS

Preparation of *Drosophila* Head Homogenates:

Adult flies were frozen, mechanically agitated to separate heads from bodies, and heads isolated by sieving. Typically, 2g of heads or bodies was homogenized in 20ml of ice-cold homogenization buffer (20mM TrisHCl, pH 8.0, 1mM EDTA) containing protease inhibitors (1mM phenylmethyl-sulfonyl fluoride, 1mM benzamidine, 1mM aprotinin, 0.1μg/ml pepstatin) in a Polytron for six 15-sec high speed bursts. The homogenate was centrifuged (2,000 x g, 10min at 4°C), the pellet resuspended in homogenization buffer, and recentrifuged. The supernatant obtained from both the spins were combined and filtered through two layers of cheesecloth. The filtrate was then subjected to ultracentrifugation (100,000 x g for 60min at 4°C). The final pellet was resuspended in minimal volume of homogenization buffer to form the crude membrane preparation.

Solubilization of *Drosophila* Crude Head Homogenates:

Drosophila membranes prepared as described above were incubated with an equal volume of dilution buffer containing 20mM Tris.HCl, pH 8.0, 1mM EDTA, 40mM $MgCl_2$, 2mM dithiothreitol and 2% cholate. The mixture was resuspended using a Dounce homogenizer and incubated with shaking at 4°C for 1hr. The membranes were then subjected to ultracentrifugation (100,000 x g for 60min at 4°C). The supernatant obtained after this step served as the detergent solubilized membrane extract.

Determination of Protein Concentration:

Protein concentrations were determined by staining with Amido Black (133) using bovine serum albumin as the standard. Briefly, 0.5-30mg of protein (in 200µl dH_2O) was incubated with 30µl of a solution containing 1.0M Tris.HCl (pH 7.5) and 2% SDS. 50µl 90% TCA was added and the mixture vortexed briefly. Samples were then filtered through a Millipore HA filter. The sample tube was rinsed with 200µl 6% TCA and the wash solution was filtered. The area of the filter was then gently washed with 1-2ml of 6% TCA. The filter was placed for 30 min in a solution of 0.1% Amido Black10B in methanol: acetic acid: water (45:10:45), after which the filter was briefly rinsed with dH_2O and then rinsed in a solution of methanol: acetic acid:water (90:2:8). After a final rinse with dH_2O, the filter was air dried. The stained spots were punched out and placed in tubes containing 1ml 25mM NaOH and 0.05mM EDTA in 50% ethanol for 20min with occasional vortexing. To determine the protein concentration of the sample, the absorbence of the solution was read at 630nm.

Pertussis Toxin Labelling:

Pertussis toxin was obtained from List Biologicals. In a typical 50µl assay, crude membranes (5.0µg of total protein) were incubated with pertussis toxin (10µg/ml) which had been preactivated at 37°C for 15min in 5mM dithiothreitol. Reactions were performed in a buffer consisting of 100mM Tris, pH 8.0, 2.5 mM $MgCl_2$, 1mM EDTA, 10mM dithiothreitol, 10mM thymidine, 1mM ATP, and ^{32}P-labelled NAD at 10mM and 2.5µCi per assay. The reaction was incubated at

30°C for 1hr and terminated by the addition of an equal volume of 4 x Laemmli sample buffer. Samples were boiled for 5min and run on 11% SDS-PAGE gels. Gels were then fixed and dried for autoradiography.

Cholera Toxin Labelling:

Cholera toxin was pre-activated by incubation at 37°C for 10 min in the presence of 0.5% SDS and 2.5mM dithiothreitol in 25mM Tris.HCl (pH 7.5). For the toxin labelling reaction, crude membranes were incubated with cholera toxin using reaction conditions as described for the pertussis toxin labelling. However, the incubation mix included 100-200μg of helper protein extract, the preparation of which is described below.

Preparation of Helper Protein Extract:

Heparinized chicken blood was collected and centrifuged at 10,000 x g for 5min. Packed chicken erythrocytes that were obtained in the pellet were mixed with an equal volume of 0.13M NaCl solution containing 0.01% sodium azide, 2 kallikrein inhibitory units per ml of aprotinin, and 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.3); the erythrocytes were lysed by repeated freezing and thawing, and the lysate was centrifuged at 10,000 x g for 5 min to obtain the cytosol. The separated cytosol was made free of endogenous nucleotides by incubation with 10% Norit A for 30 min at 0°C. The final supernatant was used as a protein factor to promote ADP ribosylation catalyzed by cholera toxin (134).

GTPγS Binding Assay:

G proteins were quantitated by their ability to bind GTPγS. In a standard assay, protein samples were diluted into 10mM NaHepes, pH 8.0, 1mM EDTA, 1mM dithiothreitol, 0.1% w/v Lubrol. 20μl of diluted protein sample was mixed with 20μl of 50mM NaHepes, pH 8.0, 40mM $MgCl_2$, 1mM EDTA, 1mM dithiothreitol, 200mM NaCl, 2mM GTPγS, and (^{35}S)GTP$_\gamma$S (10^5 cpm). The samples were incubated at 30°C for 40-60 min. GTPγS bound to protein was determined by dilution of the samples with ice-cold filtration buffer (20mM Tris-Cl, pH 8.0, 100mM NaCl, 25mM $MgCl_2$) followed by rapid filtration of the samples through BA85 nitrocellulose filters (Schleicher and Schuell). The filters were then washed 4 times with 2ml of the filtration buffer. Filters were dried and dissolved in 10ml of liquid scintillant for analysis of retained radioactivity (24).

Immunoblots:

Peptide antibodies or polyclonal antisera were used to visualize crossreacting proteins. Peptides were synthesized, rabbits immunized, and antibodies affinity purified as described by Goldsmith et al (135). The polyclonal antisera were prepared using the $G_o\alpha$ purified from bovine brain as an antigen to immunize rabbits (136). Purified $G_o\alpha$ subunit was a gift of Dr. J. K. Northup. Western blots were performed as described by Otter et al (137). Cross-reacting proteins were visualized using alkaline phosphatase-conjugated second antibodies (Promega) or ^{125}-I Protein A (138) followed by autoradiography.

Immunoprecipitation:

Samples labelled by pertussis toxin were boiled for 5 min following the addition of aprotinin to 0.2mg/ml and SDS to 0.1%. Samples were then incubated for 10 min on ice with an equal volume of fixed Staphylococcus A cells which had been resuspended in TBST (10mM Tris.Cl, pH 8.0, 150mM NaCl, 0.05% Tween 20) to a concentration of 10mg of protein/ml. The reaction mix was subsequently spun briefly (15sec) in a Microfuge and affinity purified antibody added to the supernatant at the concentrations indicated in the figure legends. Antibody binding was competed by preincubation of the antibody with a 10-fold excess (by weight) of the indicated peptides (or purified protein) in TBST containing 100mg/ml bovine serum albumin at room temperature for 1hr. Samples were incubated with affinity purified antibody or antibody plus peptide for 2-4hr at 4°C followed by the addition of an equal volume of resuspended Staphylococcus A cells and further incubation for 45 min at 4°C. Finally, Staphylococcus A cells were washed twice with TBST by centrifugation, resuspended in 1 x Laemlli sample buffer, boiled for 5 min and spun briefly. The supernatant was then loaded on an 11% SDS-PAGE gel. The gel was subsequently fixed and dried for autoradiography.

Two-Dimensional Gel Electrophoresis:

Pertussis toxin labelled membranes were electrophoresed on two-dimensional (NEPHGE/SDS) gels and visualized by autoradiography. All procedures were essentially as detailed by O'Farrell (139).

Radiolabelling of Nucleic Acid Probes:

(i) Nick Translation of Isolated DNA Fragments

DNA fragments were isolated from agarose gels as per instructions of the Gene Clean Kit (Bio101, Cat.# 3105). In a standard labelling reaction, 500ng of DNA was nick translated using the BRL Nick Translation Reagent Kit (Cat. #8160SB); according to the instructions provided therein. In order to remove unincorporated nucleotides from the probe, the nick translated sample was applied to a G-50 Sephadex column equilibrated with a solution of 10 mM Tris.Cl, pH 7.6, 100mM NaCl, 1mM EDTA, 0.5% SDS. The labelled fragments were obtained in the void volume. Typical incorporations from these reactions yielded radiolabelled DNA of >1 x 10^8 dpm/μg.

(ii) Oligolabelling DNA:

This method allows DNA fragments to be labelled with specific activities of approximately 10^9 dpm/μg or higher. In a typical oligolabelling reaction, between 8-64ng of DNA maybe efficiently labelled directly in low melt agarose. The procedure for oligolabelling was essentially that described by Feinberg, A.P. and Vogelstein, B. (140). The labelled fragment is applied to a G-50 Sephadex column as has been described, to remove any unincorporated nucleotides.

Screening of a cDNA library:

An adult *Drosophila* head cDNA library in λgt11 (provided by P. Salvaterra) was titered using *E.coli* Y1088. Approximately 50,000 plaques were plated onto NZCYM plates for screening using

nitrocellulose filters (Schleicher and Schuell). The filters were denatured in alkali, neutralized, air-dried and baked for two hours at 80°C under vacuum. Filters were probed under low stringency hybridization conditions using a 920-bp EcoR1-Hpa11 fragment corresponding to the entire coding region of a rat $G_o\alpha$ cDNA (provided by H.Itoh) to identify *Drosophila* cDNAs homologous to vertebrate $G_o\alpha$. Hybridizations were carried out in 5 x SSC, 30% formamide, 50mM NaPi, 1% non-fat dry milk at 37°C and filters washed in 1 x SSC, 0.1% SDS at 50°C. Approximately 5 x 10^5 recombinant phage were screened. Positive phage were picked into SM and stored at 4°C over chloroform. Phage were plaque purified (until a single, well isolated plaque could be picked) by repeated screening at reduced densities.

Plate Lysate Preparation:

Approximately 10^5 pfu of a bacterial phage suspension were plated with 50-100µl of *E. coli* Y1088 bacteria. The bacterial suspension was prepared by resuspending the cells obtained from an overnight bacterial culture in 0.4 vols 10mM $MgSO_4$. The plate was incubated at 37°C for 9-14hrs to allow the plaques to reach confluency. 5ml of SM was added directly to the plate and incubated with constant, gentle shaking for 3-5 hrs to elute the phage. The SM was aspirated off the plates, centrifuged with chloroform to remove any bacterial debris and stored at 4°C. The preparation was subsequently titered to quantitate pfu/ml. Typically, values of 10^9-10^{10} pfu/ml were obtained for lysate stocks prepared in this manner.

Preparation of Lambda DNA:

High titer stocks (10^9 pfu/ml or higher) of positive lambda clones produced from plate lysates were used to prepare λDNA. A bacterial culture of *E.coli* Y1088 cells that had been grown to an O.D. of 1.5 was centrifuged to obtain the cells which were then resuspended in 0.4 x vols 10mM $MgSO_4$. 50-100µl of this bacterial suspension was infected with approx. 1 x 10^6 pfu from a plate lysate stock. The infection was allowed to proceed for 15 min at 37°C and then innoculated into 80ml of FRM broth. The infections were allowed to proceed for 6-8 hrs at 37°C or until bacterial lysis was evident. The culture was then centrifuged at 10,000 x g for 15 min at 4°C. RNase and DNase were added to the supernatant at a concentration of 1mg/ml. After 30 min at 37°C, 4gm of polyethyleneglycol (PEG 8000) and 1M NaCl (solid) were added and the mixture was then incubated at 4°C for 3 hr. The PEG precipitated phage particles were collected by centrifugation (10,000 x g for 30 min). The pellet was resuspended in 0.5ml SM, centrifuged (2,000 x g for 5 min); and the supernatant extracted with an equal volume of chloroform. The aqueous phase was then incubated with 5µl 10% SDS and 5 µl 0.5M EDTA (pH 8.0) at 68°C for15 min. The solution was phenol-chloroform extracted (3X) and ethanol precipitated to recover the phage DNA.

Southern Blot Analysis:

λDNA isolated as described in the previous section, was restriction digested with various enzymes. Genomic DNA was

prepared from whole flies as per the procedure of Kidd et al (1983)(141) and restriction digested. Digested DNA (λDNA/genomic DNA) was electrophoresed on 0.8%-1.0% agarose gels and the DNA transferred by capillary action to Zetabind membranes (Cuno Laboratory Products). For hybridization under low stringency conditions, the membranes were hybridized at 37°C in buffer containing 5 X Denhardt's solution, 25% formamide, 1% SDS, 50mM NaPi (pH 7.2) and 5 X SSC. Washes were done at 1 X SSC; 0.5% SDS at 50°C. High stringency conditions included hybridization in 5 X Denhardt's, 50% formamide, 1% SDS, 50mM NaPi (pH 7.2) and 3 X SSC at 37°C. Washes were done at 0.1X SSC; 0.5% SDS at 65°C.

Preparation of Total RNA:

Total RNA was prepared from isolated heads, bodies and whole adult flies as well as from the various developmental stages (embryos, larvae, pupae) as described by Cathala et al (142). Briefly, 1gm of frozen flies (or heads, bodies, embryos, larvae, pupae) was homogenized in 7ml of buffer (5M guanidine isothiocyanate, 10mM EDTA, 50mM Tris.Cl pH 7.5 and 8% β-mercaptoethanol) with a Polytron at low speeds for 2-5 min until the sample was solubilized. 5-7 volumes of 4M LiCl were added to the homogenate and precipitated overnight on ice. The homogenate was then centrifuged at 10,000 r.p.m. in a SS-34 (Beckman) rotor at 0°C for 30 min to obtain a pellet which was resuspended in approximately 3ml of 50mM Tris.Cl, pH 7.5, 5mM EDTA, 0.5% SDS. The resulting suspension was digested at 43°C for 3 hrs (with occasional vortexing) using Proteinase K at a concentration of 150 mg/ml. 90μl of 5M NaCl was

added and the solution then extracted 3X with phenol:chloroform: isoamyl alcohol, 50:50:1. The final aqueous phase was precipitated with 2.5 volumes of ethanol overnight. The RNA was collected by centrifuging for 30 min at 0°C. Poly(A)+ RNA was selected on oligo(dT)-cellulose (Collaborative Research) as described by Maniatis (143).

Northern Blot Analysis:

Total RNA or Poly(A)+ RNA was separated on 1.2% agarose, 6% formaldehyde gels and blotted to Nytran (Schleicher and Schuell). Hybridizations were done in 50% formamide, 5% SDS, 0.4M sodium phosphate, pH 7.2, 1mM EDTA at 37°C. Washes were done at 0.1 X SSC, 0.5% SDS at 65°C.

In Situ Hybridizations to Polytene Chromosomes:

Mouth parts of climbing third instar larvae of *Drosophila* were dissected in *Drosophila* Ringer's solution (0.128M NaCl, 0.15mM $CaCl_2$, 4.68 mM KCl) to obtain intact salivary glands which were then transferred to a silanized coverslip. The glands were fixed for 5 min in lactic acid: dH_2O: acetic acid (3:2:1) and inverted onto slides. The salivary glands were prepared for hybridization as follows. Glands were dispersed using mechanical pressure such as a weighted pencil or the blunt edge of a forceps to flatten and spread the chromosomes. The chromosomes were periodically examined under a dissecting microscope to ensure that the morphology was preserved and that chromosomes were non-refractile. Slides were incubated overnight at 4°C. Coverslips were rapidly removed from the slides by freezing

the slides (face down) on a slab of dry ice. Slides were rapidly plunged into 95% ethanol for 10 min and air dried.

The DNA probe used for hybridization and the hybridization conditions were essentially as described in the Genius Kit (Boehringer Mannheim) which results in a digoxygenin-11-dUTP labelled derivative of the DNA being formed. Slides were washed after hybridization and processed to develop the colour reaction. After a brief rinse in Giemsa stain, slides were dehydrated and mounted using permanent mounting medium.

In Situ Hybridizations to Tissue Sections:

Hybridization of tissue sections was done with RNA probes generated in vitro by using either T3 or T7 promoters after the subcloning of the appropriate fragments into pBS(M13-). RNA transcripts were labelled using (α-(^{35}S)thio)UTP. RNA was prepared and hybridized to 8μM tissue sections that had been fixed with 4% formaldehyde, then treated with 0.2M HCl for 20min, 2 X SSC for 30 min at 60°C, Proteinase K (0.2μg/ml) at 37°C for 15min, and 4% formaldehyde for 20min, and then acetylated and dried (144). After hybridization overnight at 50°C, slides were rinsed briefly in 4 X SSC and then incubated for 15 min at 60°C in 50% formamide, 0.3M NaCl, 30mM Tris, pH 7.5, 1mM EDTA, 10mM dithiothreitol. This procedure greatly improved the signal to noise ratio. Slides were then washed in 4 1 of 2 X SSC (room temperature, 30 min), 0.1 X SSC (55°C, 10min) and 0.1 X SSC (room temperature, 10 min). Slides were dried and coated with photographic emulsion (Kodak NTB2). As described in Quan et al, (145).

Miscellaneous Procedures:

Nucleic acid manipulations were essentially performed as desribed in Maniatis (143). These included plasmid purifications (CsCl method); rapid isolation of plasmid DNA (alkaline denaturation method); preparation of competent *E.coli* cells and transformation of bacteria (into *E.coli* JM101 or MC1061).

Restriction digestions were performed as per the enzyme manufacturers' directions.

Nucleotide sequencing of restriction fragments that were subcloned into pBS(M13-)(Stratagene) or M13 was by the standard Sanger dideoxy nucleotide chain termination method (146). Sequencing reactions were performed using (^{35}S)dATP and Sequenase (US Biochemicals).

CHAPTER 1

Introduction:

The characteristic feature of all G proteins is their ability to bind guanine nucleotides with high affinity. The binding of nucleotides (GTP) is the pivotal event in the signal transduction cascade which activates the G protein and allows it to transduce the signal from the receptor to the effector component. As shown in Fig.1, the α subunit of the G protein has GDP bound to it in its inactive state. Although cells contain high concentrations of GTP, in the inactive configuration the α subunit does not bind GTP. When ligand binds to its receptor, the GDP moiety of the α subunit is rapidly exchanged, being replaced by GTP.

The active GTP bound form of the α subunit is then able to dissociate from the β and γ subunits with which it is normally associated. The signal cascade continues with the α-GTP triggering activation of the effector component. The latter produces the physiological effect within the cell, most often through generation of second messengers. Activation does not continue indefinitely. First, the α-GTP modulates receptor-agonist affinities in a negative manner. Second, a GTPase activity intrinsic to the α subunit hydrolyzes the GTP, thus returning the α subunit to its basal state of inactivation, i.e., α-GDP. In this state, the α subunit can reassociate with the β and γ subunits and is ready for the next round of activation. Amplification of the signal occurs at two levels. First, the receptor-agonist complex activates many G proteins and secondly, due to the relatively poor hydrolytic activity of α-GTP, many effector

molecules are activated. A two-fold amplification in signal transmission is thus achieved. Mg^{+2} is implicated at many stages of the GTP cycle. The details of the kinetics of the cycle are described on page 14.

The ability of the Gα subunits to bind guanine nucleotides has been exploited in a well defined assay. The rationale for the assay is based on the principle that by using non-hydrolyzable analogs of GTP, it is possible to quantitate the GTP binding activity of Gα subunits in a membrane preparation. Typically, a radiolabelled (S^{-35}), non-hydrolyzable derivative of GTP (guanosine 5'-3- *O*-(thio)triphosphate) is used in the GTPγS assay. This assay has been routinely used, particularly to characterize Gα subunits during their purification from various membrane sources (24).

This chapter describes the results of GTP binding studies that were carried out on *Drosophila* membrane preparations. The GTP binding assay was used as a preliminary quantitative measure to facilitate comparison of binding values in fly neuronal extracts and vertebrate tissue extracts. Thus, a detailed analysis of the kinetics of GTP binding in *Drosophila* membranes was not undertaken. Moreover, the prerequisite for such an analysis would be purified α subunit from fly heads. The results that follow do however establish that *Drosophila* neuronal membranes contain high levels of GTP binding and remarkably, values for this binding compared favourably with those obtained in vertebrate brain extracts.

Results and Discussion:

Crude homogenates of *Drosophila* heads were prepared and extracted in the presence of 1% cholate essentially as described in Methods. A non-linear relationship exists between the GTPγS bound and increasing concentrations of crude membranes. Hence, GTP binding has only been extensively characterized in detergent solubilized membranes. Furthermore, assay conditions were selected and optimized so as to parallel conditions that are routinely used in assaying for GTP binding in vertebrate membranes.

Fig.2 shows the results obtained from GTP binding in *Drosophila* neuronal membranes. In the range of protein concentrations used (0-200μg/ml), a linear relationship with amount of GTPγS bound is observed. Low concentrations of GTPγS used in the assay (2-10μM) allowed for the detection of proteins that bind GTP with relatively high affinity, k_d in the submicromolar range. Within this range of GTP concentration, low affinity GTP binding proteins such as the cytoskeletal protein, tubulin, are excluded. Furthermore, the assay conditions included incubating the membranes with elevated concentrations of ATP or ATPγS (1mM) in order to block all non-specific nucleotide binding sites. In the absence of ATP or ATPγS, the non-specific binding was observed to be non-saturable. Fig.3 shows that at 10μm GTPγS, all GTP binding sites are saturated. The specific binding was computed as the difference in the values obtained when membranes were assayed in the presence of ATPγS alone versus ATPγS and GTPγS.

Drosophila neuronal membranes extracted with 1% cholate results in a soluble protein preparation with a specific activity of

GTPγS binding of approximately 900 pmoles/mg of protein. This level of binding is approximately that observed in cholate extracts of vertebrate brain membranes. Table 2 lists the specific activity of binding seen in various vertebrate membranes. The values listed have been standardized to 10mg protein/ml to facilitate the comparison.

These preliminary experiments demonstrated that *Drosophila* heads contained measurable GTP binding activity. Furthermore, these binding studies indicate that this activity was present in fly neuronal extracts at levels comparable to vertebrate neuronal tissues.

FIGURE 2:

Increasing concentrations of cholate extracted *Drosophila* membranes (at1.17mg/ml) were used to determine the linear range of the GTP binding assay.

GTP binding assays on *Drosophila* neuronal membranes were performed as described in Methods. Binding assayed in the absence of added nucleotide is represented by circles; binding in the presence of 1mM ATPγS is represented by the squares and binding in the presence of 1mM ATPγS and 1mM GTPγS is represented by the triangles. The specific activity of binding per mg of protein is computed as the difference between the values obtained from the ATPγS curve and the GTPγS curve.

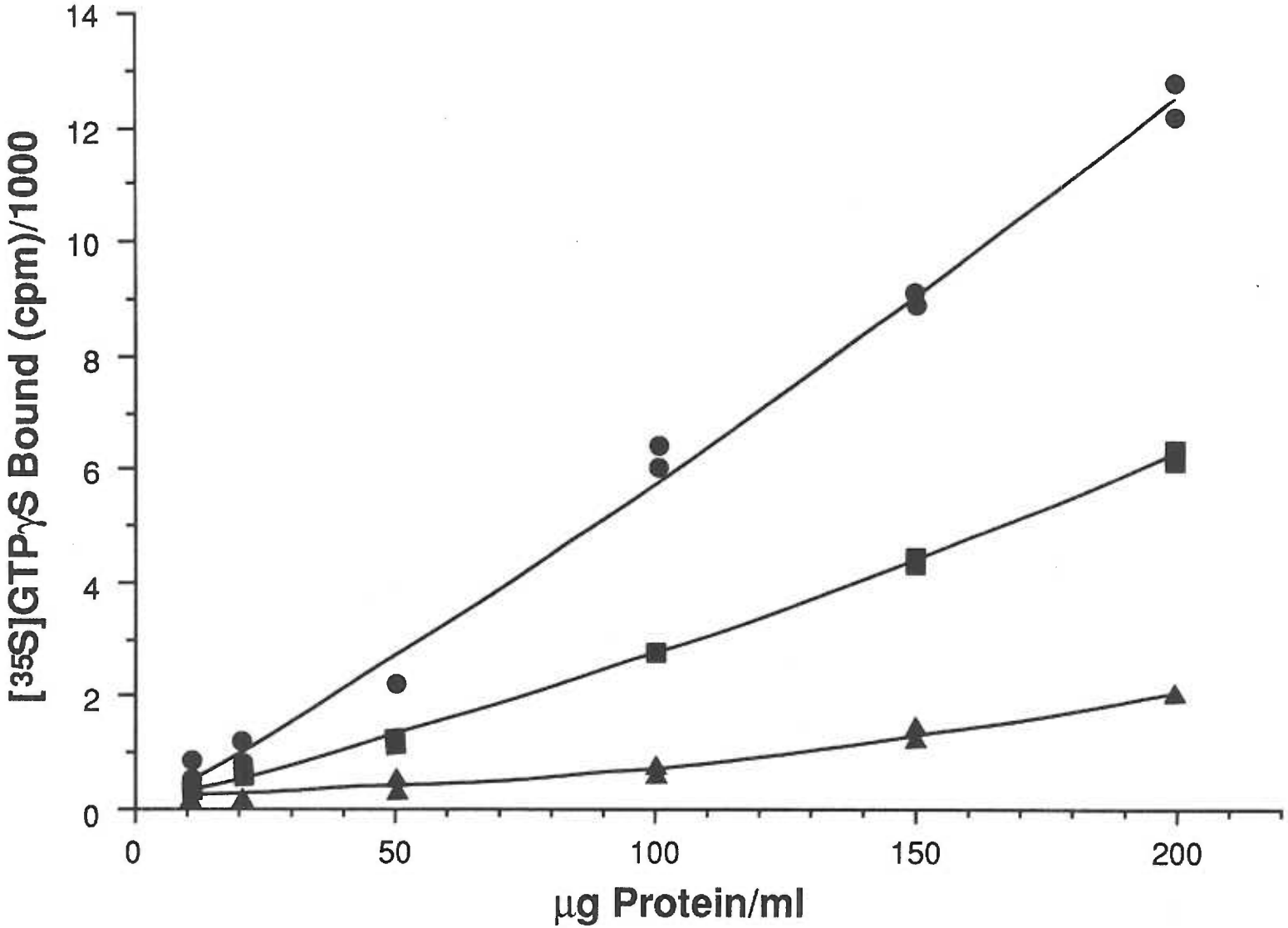

[35S]GTPγS Bound (cpm)/1000
14
12
10
8
6
4
2
0
0
50
100
150
200
µg Protein/ml

FIGURE 3:

Increasing concentrations of (^{35}-S) GTPγS were used to determine the saturating concentration of GTPγS in the assay.

Protein concentration of the solubilized extract used to assay GTP binding was 1.17 mg/ml. Assays were performed as described in Methods. The figure shows that at 10μM GTPγS concentration all of the GTP binding sites are saturated.

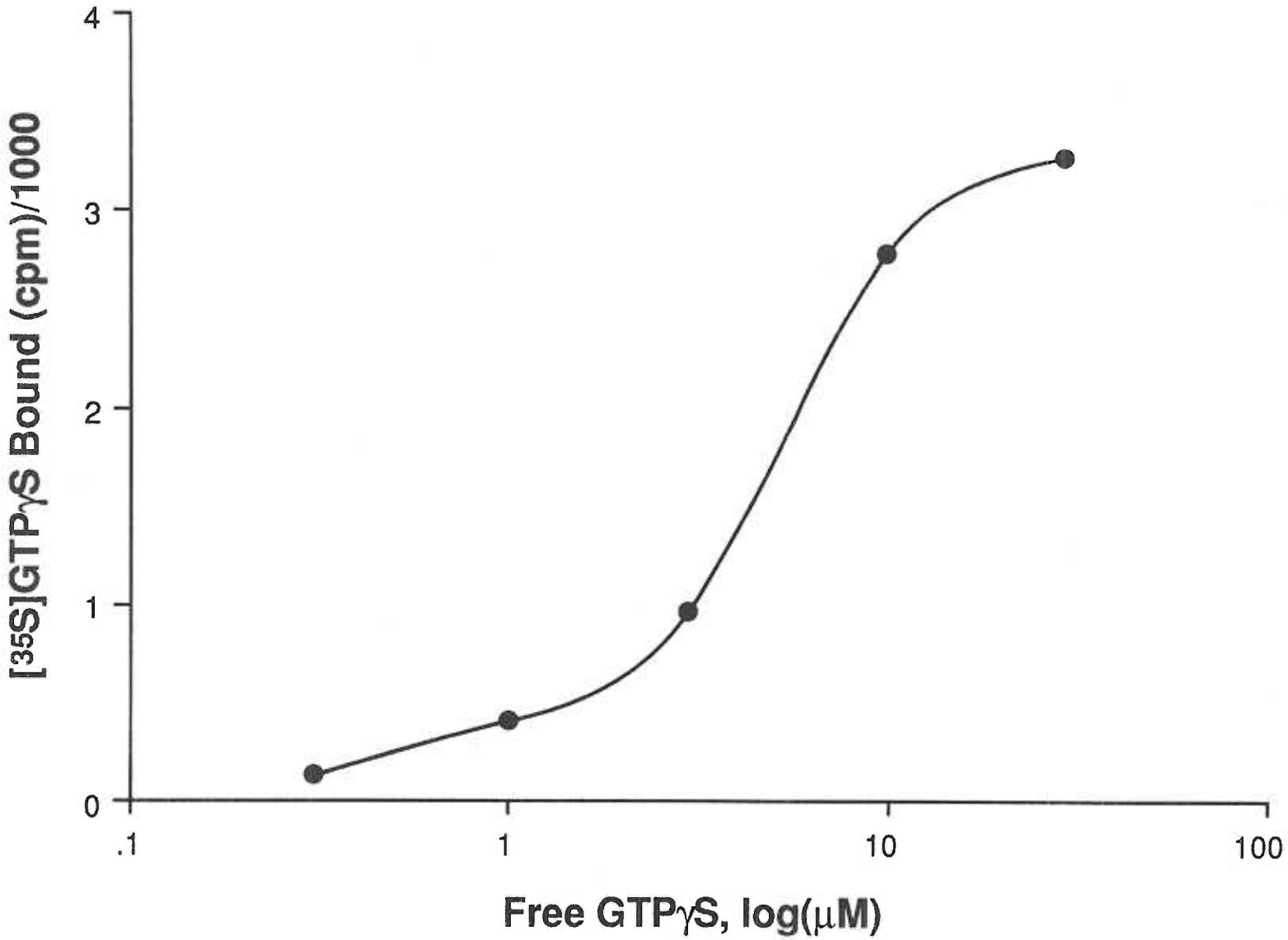

4
3
2
1
0
[35S]GTPγS Bound (cpm)/1000
.1
1
10
100
Free GTPγS, log(μM)

TABLE 2:

Binding of a non-hydrolyzable analog of GTP (i.e.) ^{35}-S GTPγS is measured as described in Methods. Binding activity was assayed for a variety of cholate extracts from different membrane sources. The values obtained have been standardized to 10mg/ml protein.
#1-6 taken from Sternweis, P.C. and Robishaw, J.D. (1984) J. of Biol. Chem. Vol. 259, 13806-13813

Source of Extract	GTPγS Binding nmol ml-1
1. Bovine Heart	0.71
2. Wt-S49 Lymphoma	0.92
3. Rat Liver	0.31
4. Rabbit Liver	0.39
5. Rat Brain	2.44
6. Bovine Brain	2.26
7. Drosophila Heads	1.90

CHAPTER 2

Introduction:

Almost all known biochemically characterized G proteins are substrates for covalent modification by bacterial toxins. Both CTX and PTX catalyze mono-ADP-ribosylation of the various Gαs. These toxins are multi-subunit proteins. The A subunit contains the activity of the toxin while the other subunits form an oligomer that is responsible for attachment to the cell membrane. The oligomer binds to the cell membrane and thus facilitates the entry of the A subunit into the cytoplasm of the cells. Toxin modification has been used as a basis to classify the different G protein α-subunits. Hence, $G_s\alpha$ and $G_t\alpha$ are substrates for CTX induced modification at an Arg residue that is conserved between the two proteins; while $G_i\alpha$, $G_o\alpha$ and $G_t\alpha$ serve as substrates for PTX modification. PTX will modify these α subunits only when they are complexed to the βγ subunits; the modification occurs at a Cys that is present 4 residues from the carboxyl terminus of each protein.

In order to further the characterization of the G proteins that were present in the *Drosophila* central nervous system, crude homogenates of fly heads and bodies (see Methods) were assayed for their susceptibility to PTX and CTX. The following section describes the identification of a major 40 kD PTX substrate present in *Drosophila* heads.

Results and Discussion:

The results of the previous chapter demonstrated that fly heads contained significant levels of GTP binding activity. Since the α subunits of the G proteins are substrates for CTX or PTX, the next criterion to identify *Drosophila* G proteins centered on the toxin modification assay. Like the GTP binding assay, the conditions required for toxin labelling have been extensively detailed. In fact, the assay was used as a means to purify $G_i\alpha$, the cyclase inhibiting G protein from rat C6 glioma cells (23). As described in Methods, if ^{32}P-labelled NAD is used in the ADP ribosylation reaction, covalently modified proteins are radiolabelled and can then be identified by separation on an SDS-PAGE system followed by autoradiography.

(i) CTX Labelling in Drosophila Membranes:

In vertebrate membranes, CTX labels $G_s\alpha$ and/or $G_t\alpha$. However, the activation of the toxin requires the presence of an ADP ribosylation factor (ARF) also known as a helper protein (134). Addition of this helper protein (isolated from chicken erythrocyte membranes) to the toxin labelling reaction, facilitates the dissociation of the active protomer of the toxin from the other subunits.

Labelling of *Drosophila* heads with CTX produced results that were difficult to interpret (data not shown). This was due to the following. First, the incubation conditions that were required for CTX labelling of *Drosophila* membranes apparently activated an endogenous ADP ribosyl transferase activity. This resulted in membranes being labelled even in the absence of added toxin. Endogenous ADP-ribosyl transferases are reportedly present in a

variety of mammalian tissues (147-149). However, in most mammalian tissues these enzymes do not recognize substrates with the degree of specificity as seen for the *Drosophila* heads.

Secondly, proteolysis of the CTX labelled membranes resulted in several low molecular weight proteins being separated on the SDS-PAGE system. These results have been corroborated by Hopkins et al (150). They noted that CTX labelling in *Drosophila* membranes appeared to modify 50 kD and 37 kD proteins. However, these proteins were modified (i.e.) labelled even when assay conditions omitted the toxin. They suggest that due to the high proteolytic activity present in fly heads, degradation of the α subunits results in sites that are not normally accessible to the action of endogenous ribosyltransferases becoming substrates for ADP ribosylation.

In light of the above results, the CTX labelling in *Drosophila* heads was not characterized further. PTX labelling of fly heads was pursued and is described in the following section.

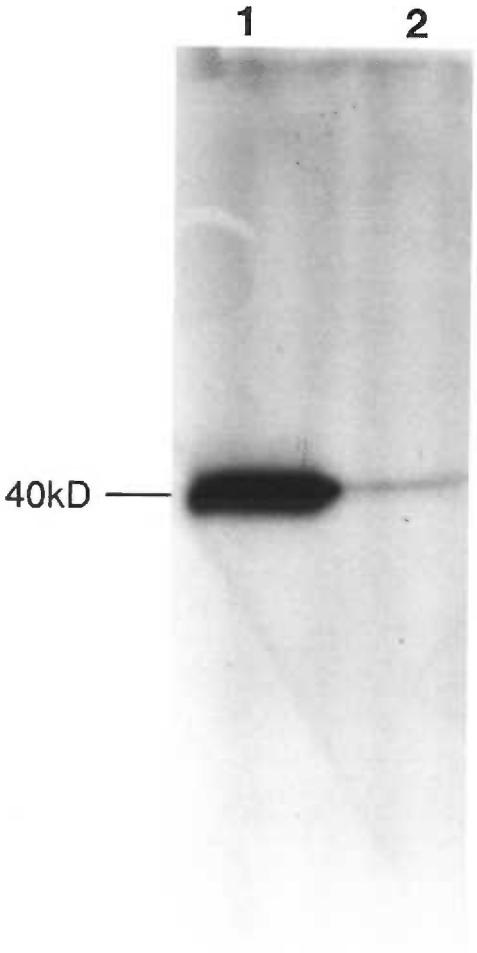
(ii) PTX Labelling in Drosophila Membranes:

Crude membrane preparations of *Drosophila* head homogenates were ADP ribosylated by PTX as described in Methods. The labelled protein migrated on SDS-PAGE gels with an apparent molecular mass of 40 kD. As shown in Fig.4, this protein can be completely solubilized from crude membranes by cholate extraction. This result correlates the GTP binding activity that was measured in the solubilized head extracts with the PTX modified substrate. Additionally, no labelling was observed in membranes that were prepared from *Drosophila* body homogenates.

FIGURE 4:

Modification of cholate extracted *Drosophila* membranes with Pertussis Toxin

Lane 1, 5.0µg of cholate extracted *Drosophila* neuronal membranes (see Methods) were labelled by PTX; *lane 2*, 5.0µg of the residual pellet (obtained after detergent extraction) was labelled similarly with PTX. Intense labelling was observed at 40 kD. The autoradiogram was overexposed to determine if other minor bands were labelled.


1
2
40kD

Modification of vertebrate α subunits by PTX occurs only if the α subunit is complexed with the βγ subunits (151). Thus, treatment of membranes with agents such as GTPγS and Mg^{+2} which promote the dissociation of the heterotrimeric complex inhibit the modification of α subunits by PTX. As shown in Fig.5, the addition of GTPγS reduced modification of the 40 kD protein by PTX in *Drosophila* head membranes. Labelling is almost completely eliminated in the presence of both GTPγS and Mg^{+2}. These results are consistent with the presence in the *Drosophila* nervous system of a G protein whose α subunit is modified by PTX. In addition, the ability of this protein to serve as a toxin substrate is modulated in a manner similar to that observed in vertebrates (152-154).

There are a number of PTX substrates in vertebrate tissues that migrate on SDS-PAGE gels with apparent molecular mass of 39-40 kD (i.e.) $G_i\alpha$, $G_o\alpha$ and $G_t\alpha$. In order to determine the complexity of the PTX labelled moiety in fly heads, PTX labelled membranes were separated by two-dimensional gel electrophoresis. However, as the labelled substrate migrated as a long streak in the isoelectric focussing dimension as shown in Fig.6, any resolution of complexity was not possible by this method. This phenomenon has been previously reported for vertebrate G proteins, suggesting that it may be a general property of Gα subunits (150).

(iii) Characterization of the PTX Substrate:

In vertebrates, PTX catalyzes ADP ribosylation of the α subunits of Gi, Go and Gt. On SDS-PAGE gels, these toxin substrates migrate with an apparent molecular mass of 39-41 kD. By analogy to

FIGURE 5:

Modification of *Drosophila* membranes by Pertussis Toxin

Homogenates of Adult *Drosophila* heads or bodies were prepared and labelled with PTX as described in Methods. *Lanes 1 and 4*, head homogenates incubated with PTX and ^{32}-P labelled NAD. *Lane 2*, head homogenates incubated with ^{32}-P labelled NAD in the absence of PTX. *Lane 3*, body homogenates incubated with ^{32}-P labelled NAD and PTX. *Lane 5*, incubation as in lanes 1 and 4 following the addition of GTPγS to 1mM. *Lane 6*, incubation as in lanes 1 and 4 following the addition of GTPγS to 1mM and $MgCl_2$ to 50mM.

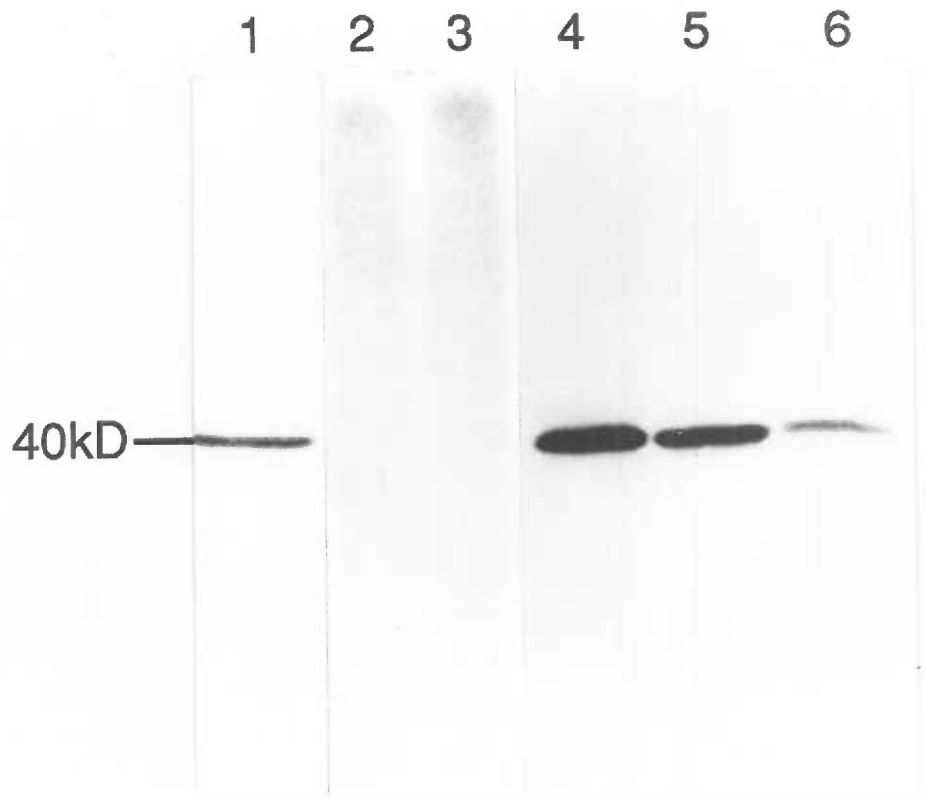
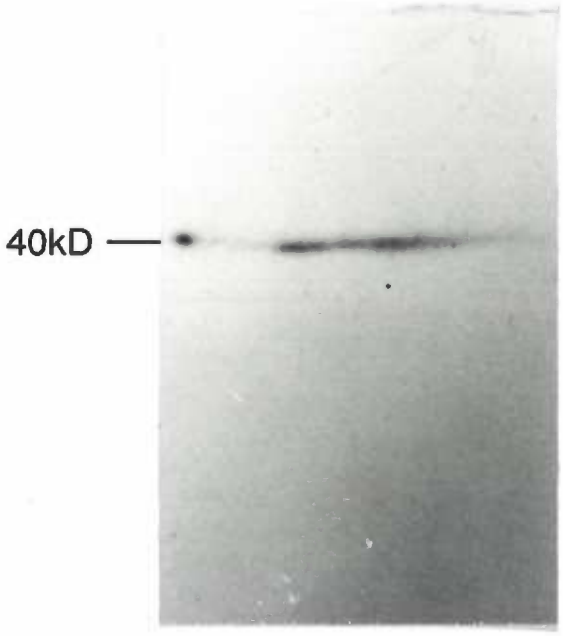

1
2
3
4
5
6
40kD

FIGURE 6:

Analysis of Pertussis Toxin labelled membranes by Two-dimensional gel electrophoresis

Drosophila neuronal membranes were PTX labelled as described in Methods and then subjected to two-dimensional gel electrophoresis. Only the region of the gel that appeared labelled is shown in the figure.


40kD

the vertebrate system, the PTX substrate present in *Drosophila* nervous tissue could correspond to the *Drosophila* homolog of Gi, Go and/or Gt. If the PTX substrate represents the α subunit of a G protein used in *Drosophila* visual transduction, (i.e. the *Drosophila* $G_t\alpha$ homolog), it should be absent or reduced in head membranes prepared from mutants homozygous for the eyes absent mutation (*eya/eya*) This recessive mutation is completely penetrant and eliminates all eye structures with the exception of ocelli.

As shown in Fig.7, the 40 kD PTX substrate is present in both wild type and *eya/eya* membranes. The level in *eya/eya* membranes is only slightly reduced. This result suggests that in fly heads a substantial amount of a PTX substrate is present in tissues other than the eye. Thus, it is unlikely that the PTX substrate is a Gα which participates in visual transduction.

The characterization of the PTX substrate in fly heads was pursued as follows. Wild type heads were dissected to remove eyes and equivalent amounts of protein homogenates from eyes and heads (free of eyes) were then compared to examine PTX labelling. As seen in Fig.8, PTX labelling was observed only in the head homogenates. Eye homogenates were not labelled at observable levels. These results are consistent with those shown in Fig.7. This data then confirms the presence of a 40 kD PTX substrate that appears to localize to *Drosophila* neuronal membranes. Furthermore, the labelling of the substrate is influenced by nucleotides and divalent cations in a way similar to vertebrate Gαs. These studies were insufficient in determining the identity of the *Drosophila* PTX substrate based on analogy to vertebrate Gαs that are recognized PTX

FIGURE 7:

Pertussis toxin modified proteins in wild type and *eyes absent* head homogenates

PTX-modified proteins in adult head homogenates from wild type and eyes absent flies. *Lanes 1, 2 and 3*, 5, 10 and 25µg of protein from wild type heads labelled with PTX. *Lanes 4, 5 and 6*, 5, 10 and 25µg of protein from *eya/eya* heads labelled with PTX.

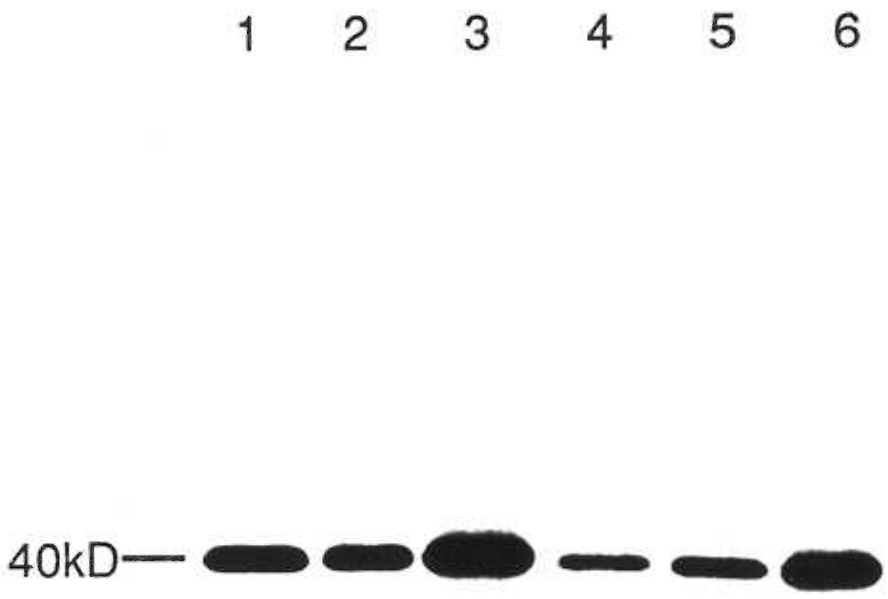
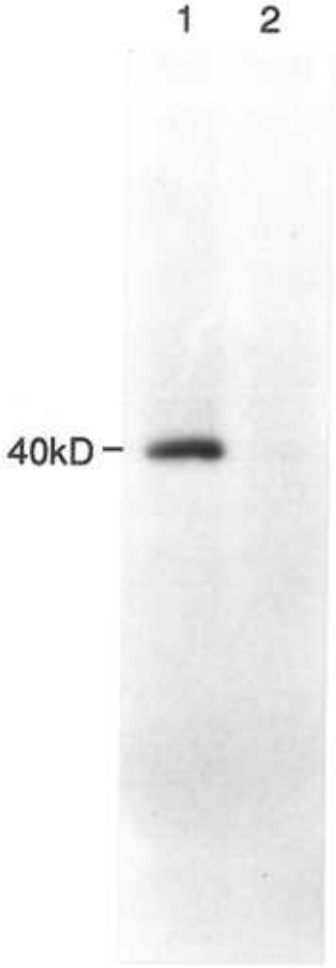

1 2 3 4 5 6
40kD—

FIGURE 8:

Pertussis Toxin labelling of *Drosophila* heads and eyes

Drosophila heads were dissected to remove the eyes and the heads and eyes were homogenized separately as described in Methods. Equivalent amounts of protein from crude head membranes and eye membranes were then PTX labelled. *Lane 1*, 5.0μg head membranes; *lane 2*, 5.0μg eye membranes


1
2
40kD

substrates. Previous studies in invertebrates have documented evidence for a 40 kD PTX substrate present in the nervous system of locusts, snail, sea urchin, tobacco horn worm and octopus (86). Furthermore, in neurons of *H.aspersa*, dopamine modulates calcium current activity which could be blocked by PTX. Injection of mammalian $G_o\alpha$ into these neurons could restore the dopamine induced decrease of calcium currents (113). Taken together, the evidence for $G\alpha$ subunits being conserved in the invertebrate nervous system is compelling and merits continued characterization.

CHAPTER 3

Introduction:

The study of G proteins has gained a significant impetus with the availability of antibodies that are able to specifically discriminate between the various vertebrate Gαs, although these are closely related in sequence. The antibodies that are available include polyclonal antisera that were raised against the Gαs as well as peptide-specific antibodies that were generated to sequences that were found only in each of the Gαs. For example, peptide specific antibodies can differentiate each of the three forms of $G_i\alpha$ (155, 156). Previous studies have convincingly demonstrated that these antibodies do not crossreact with any but the Gαs that they were specifically raised against.

The results described in the previous chapters define the presence of a PTX substrate in fly heads that also correlates well with a high GTP binding activity similar to that reported in rat and bovine brain membranes. In these latter membranes the GTP binding activity is chiefly contributed by $G_o\alpha$. In order to extend the characterization of G proteins that were present in *Drosophila* at the immunochemical level, vertebrate Gα antibodies were used in a series of experiments that are detailed in this section.

Results and Discussion:

The initial immunochemical characterization of *Drosophila* head homogenates was pursued using polyclonal antisera specific for bovine brain $G_o\alpha$ (provided by Dr.John Northup). These antisera had

been preadsorbed with purified $G_i\alpha$. Since the $G_o\alpha$ protein is most related to the $G_i\alpha$ family of proteins, preadsorption ensured that the antiserum did not contain any $G_i\alpha$ cross-reactivity. The specificity of the antisera for vertebrate $G_o\alpha$ has been unequivocally demonstrated (136). *Drosophila* head homogenates probed with these antisera ($G_o\alpha$#1 and $G_o\alpha$#2) shows immunoreactive material migrating on SDS-PAGE at 40 kD as shown in Fig. 9.

Fig.10 demonstrates that a 40 kD protein found in *Drosophila* head membranes is also recognized on Western blots by two antibodies generated to peptides found at the amino (GC) and carboxyl (GO) termini of vertebrate $G_o\alpha$. The binding of the GO and GC antibodies can be competed by preincubation with the cognate peptide and, in the case of GO reactivity, can be competed by preincubation of the antibody with purified vertebrate $G_o\alpha$ protein and not by preincubation with peptides which represent sequences found at the carboxyl terminus of vertebrate $G_s\alpha$ or $G_t\alpha$. No proteins were found in *Drosophila* head membranes which bound a variety of peptide antibodies specific for the various vertebrate $G\alpha$ proteins on Western blots (Figs. 11A and 11B). In addition, no GO cross-reactive material appears to be present in body membranes.

Immunoprecipitation of the PTX Substrate:

In order to confirm that the GO and GC antibodies were indeed recognizing the 40 kD PTX substrate in *Drosophila* neuronal membranes, these antibodies were used in immunoprecipitation experiments as described in Methods. Fig.12 shows the results of

FIGURE 9:

Polyclonal antisera to vertebrate $G_o\alpha$ recognize a 40 kD protein in *Drosophila* heads

Crude membranes (50μg) prepared from wildtype and *eyes absent* flies (as described in Methods) were separated on 11% polyacrylamide gels and transferred to nitrocellulose for immunoblotting. *Lane 1*, wild type and *lane 2*, *eyes absent* heads probed with a 1:1000 dilution of $G_o\alpha$ (#1) antiserum. *Lane 3*, wild type and *lane 4*, *eyes absent* heads probed with a 1:1000 dilution of $G_o\alpha$ (#2). The blots were incubated with ^{125}I labelled goat anti-rabbit IgG to visualize cross-reacting proteins.

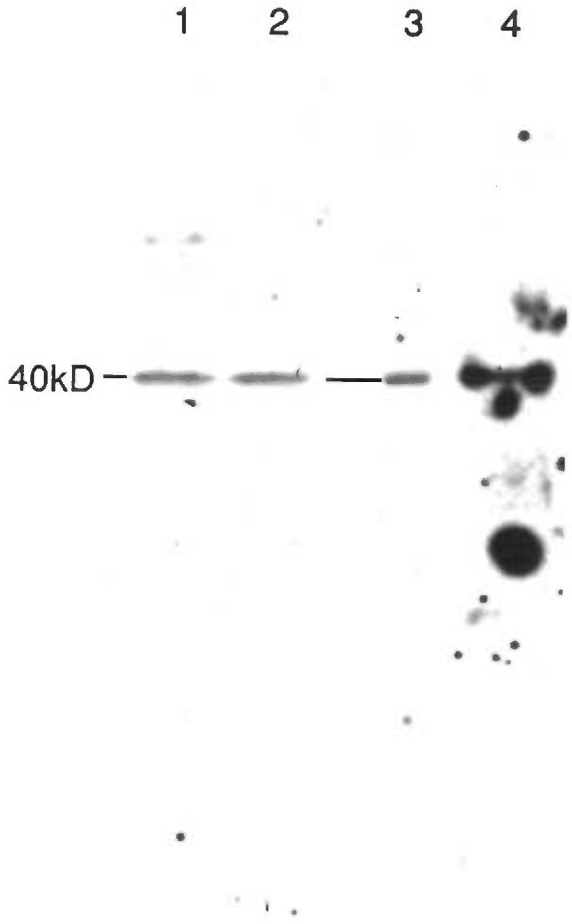

1
2
3
4
40kD

FIGURE 10:

Antibodies to peptide sequences found in vertebrate $G_o\alpha$ recognize a 40 kD protein in *Drosophila* head homogenates

In each lane, the protein from two heads from either wild-type (*lanes 1, 3, 4 and 6*) or *eyes absent* (*lanes 2and 5*) flies have been separated on 11% SDS PAGE, transferred to nitrocellulose, and probed with GO antibody (*lanes 1 and 2*) or GC antibody (*lanes 4 and 5)* at a concentration of 1μg/ml. The immunoreactivity is specifically competed by pre-incubation of each antibody with 10-fold excess of the cognate peptide (GO, *lane 3*; GC, *lane 6*)

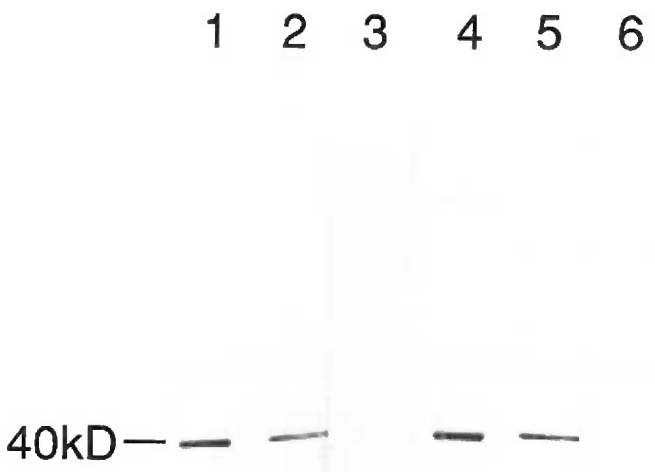

1 2 3 4 5 6
40kD

FIGURE 11 A:

Competition of $G_o\alpha$ immunoreactivity

Lanes 1, 2 and 3, 50μg of head membranes from wild-type flies separated and transferred as in Fig.10, and then probed with GO antibody (0.77μg/ml, *lane 1*), GO antibody pre-incubated with 10-fold excess, by weight, GO peptide (*lane 2*). GO antibody pre-incubated with purified Go protein (16μg, *lane 3*), *lane 4,* 50μg of crude membranes from bodies of wild-type flies separated, transferred and probed with GO antibody (0.77μg/ml).

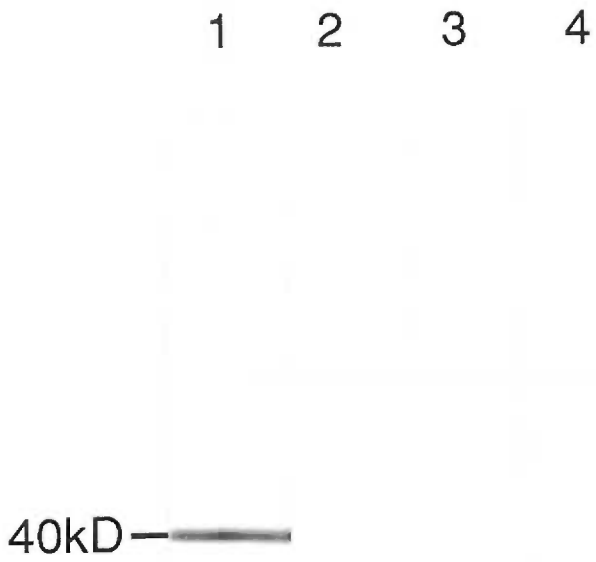

1
2
3
4
40kD

FIGURE 11 B:

Competition of $G_o\alpha$ immunoreactivity with peptide sequences found at the C-terminus of $G_s\alpha$, $G_i\alpha$ and $G_t\alpha$.

In each lane, 50μg of head homogenate from wildtype flies have been separated and transferred to nitrocellulose. The immunoblots are then probed with antibody or antibody plus peptide. *Lanes 1 and 2*, same as in Figure 11A. *Lane 3*, GO antibody preincubated with 10-fold excess (by weight) RM peptide; *lane 4*, GO antibody preincubated with 10-fold excess (by weight) of AS peptide. RM and AS represent the C-terminal 10 residues of vertebrate $G_s\alpha$ and $G_t\alpha$, respectively. AS will also recognize vertebrate $G_i\alpha1$ and $G_i\alpha2$

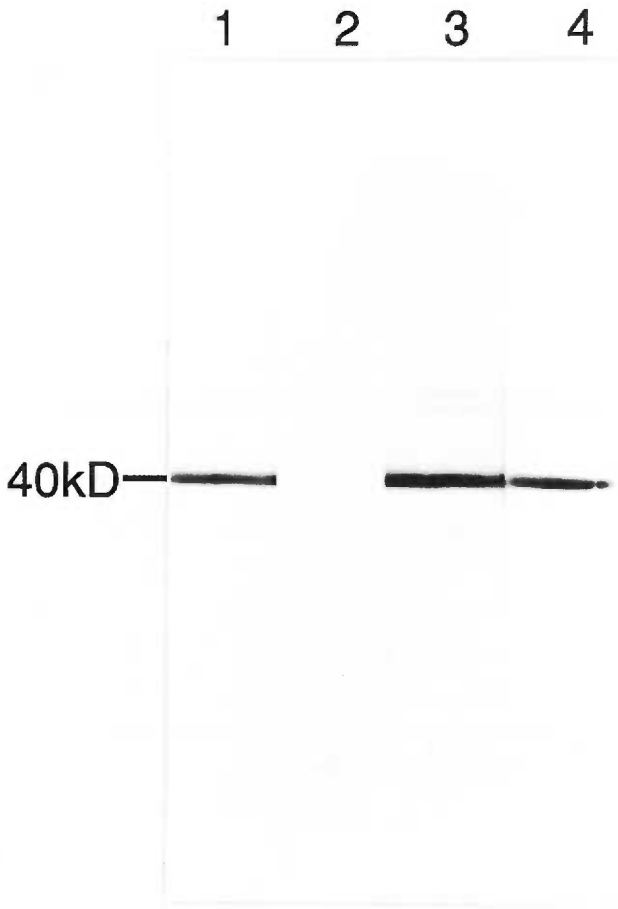
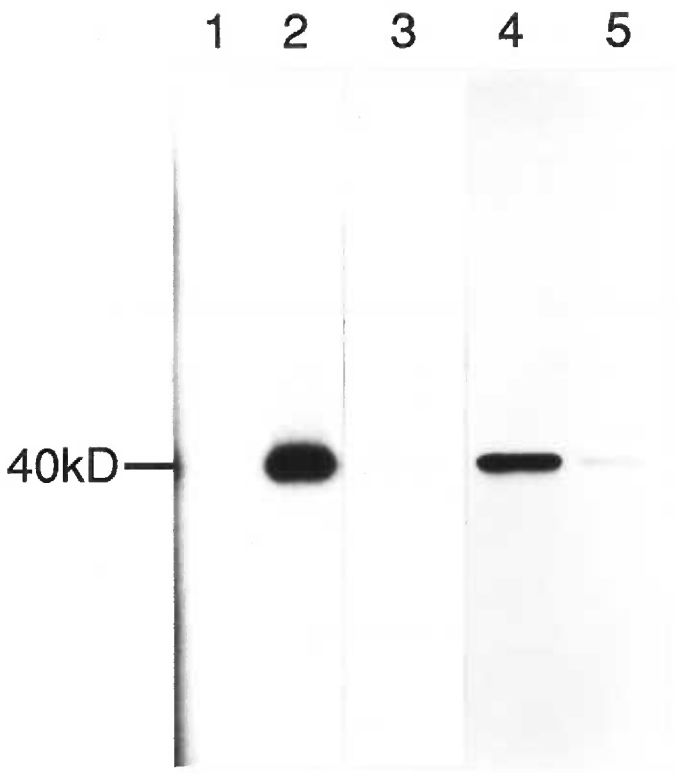

1
2
3
4
40kD

FIGURE 12:

Immunoprecipitation of the Pertussis Toxin substrates in head homogenates with the GO and GC antibodies
Samples were labelled with PTX and proteins immunoprecipitated with affinity purified GO or GC antibodies (1 μg each/reaction) as described in Methods. *Lane 1*, proteins precipitated by Staph. A cells in the absence of antibody, *lane 2*, immunoprecipitation with the GO antibody, *lane 3*, immunoprecipitation with GO antibody pre-incubated with a 10-fold excess of GO peptide, *lane 4*, immunoprecipitation with the GC antibody, *lane 5*, immunoprecipitation with the GC antibody pre-incubated with 10-fold excess of GC peptide


1
2
3
4
5
40kD

these experiments. Both the GC and GO antibodies were able to immunoprecipitate the PTX substrate of fly heads. In each case, the immunoprecipitation is largely, if not completely competed by pre-incubation of the antibody with the cognate peptide. From the relative intensity of the bands present on the autoradiogram, it appears that the GO antibody immunoprecipitates the protein more effectively. Fig.13 shows that when increasing amounts of the GO antibody were added to PTX labelled membranes, increasing amounts of the 40 kD, PTX labelled protein was immunoprecipitated with a corresponding decrease in the amount of label remaining soluble. Therefore, it is likely that the concentration of the antibody is the only limiting factor in precipitating all of the PTX labelled material in *Drosophila* head membranes.

Fig.14 shows the immunoprecipitation of the PTX substrate using the polyclonal antibody that was generated against bovine brain $G_o\alpha$ (provided by Dr. John Northup). The data obtained was completely consistent with the results when antibodies generated to $G\alpha$ peptide sequences were used.

The data described in this section establish that the PTX substrate is present primarily in *Drosophila* head tissues other than the eye. This protein is recognized specifically by antibodies against vertebrate $G_o\alpha$. These observations strongly suggest that the 40kD PTX substrate is a *Drosophila* $G_o\alpha$-like protein. The absence of immunoreactivity and a PTX substrate in body membranes, suggests that the level of this protein is reduced in body membranes relative to head membranes. The difference in PTX labelling then, most

FIGURE 13:

Pertussis toxin labelled proteins immunoprecipitated with increasing amounts of GO antibody

PTX labelled head homogenates (5.0μg) were prepared for immunoprecipitation as described in the Methods and incubated with 1μg (*lanes1, 2*), 2.5μg (*lanes 3, 4*), 7.5μg (*lanes 5, 6*) and 10μg (*lanes 7, 8*) of GO antibody. Following incubation, antibody-bound protein (*lanes 1, 3, 5, 7*) was precipitated by the addition of Staph. A cells. Unbound protein (*lanes 2, 4, 6, 8*) remained in the supernatant.

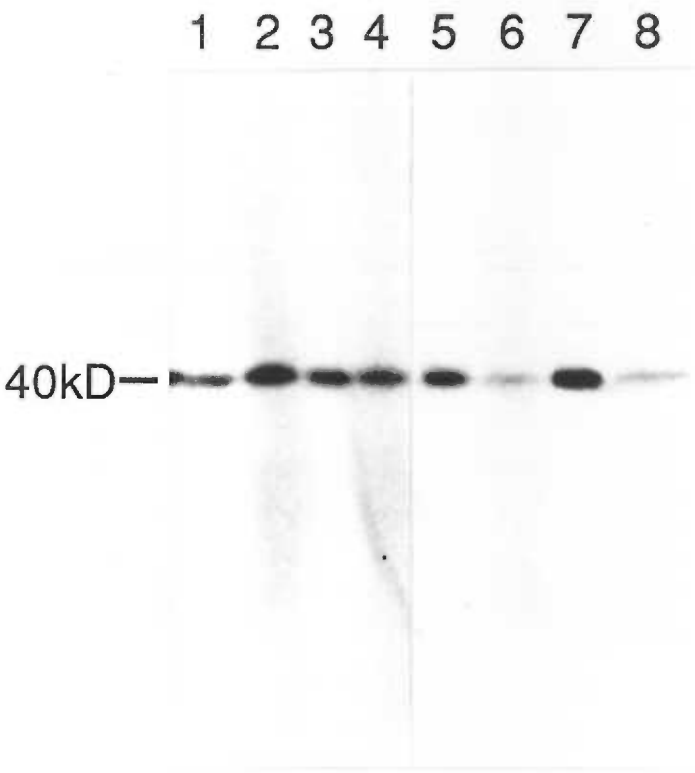
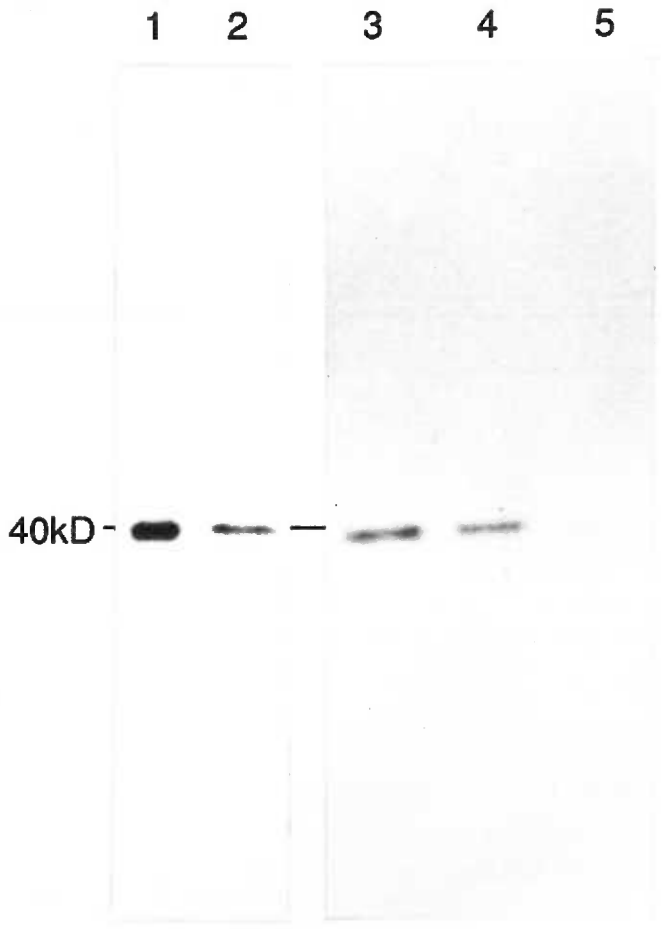

1 2 3 4 5 6 7 8
40kD—

FIGURE 14:

Immunoprecipitation of the Pertussis toxin substrate of *Drosophila* membranes with polyclonal antisera to vertebrate $G_o\alpha$

PTX labelled membranes from wild type and *eyes absent* flies were prepared for immunoprecipitation as described in Methods. *Lane 1*, PTX labelled head membranes from wild type flies, *lanes 2 and 3*, PTX labelled head membranes (wild type) immunoprecipitated with 5.0µl $G_o\alpha$#1 antiserum, *lane 4*, PTX labelled head membranes (*eyes absent*) immunoprecipitated with 5.0µl $G_o\alpha$#1 antiserum, *lane 5*, wild type membranes immunoprecipitated using 5.0µl $G_o\alpha$#1 preimmune antiserum.


1
2
3
4
5
40kD

probably reflects a reduced level of protein rather than a difference in the efficiency of the labelling enzyme in the two membrane preparations.

CHAPTER 4

Introduction:

The studies described in the previous chapters demonstrate the existence of a 40 kD protein in *Drosophila* head membranes which is modified by PTX in a manner similar to that observed for vertebrate G-protein α subunits. This PTX modified protein appears to be in head tissues other than the eye. Remarkably, this protein is also recognized by antibodies generated to peptides found in vertebrate $G_o\alpha$, suggesting that the PTX labelled protein is, in fact, the *Drosophila* analog of $G_o\alpha$.

This chapter details the analysis of *Drosophila* $G_o\alpha$-like proteins pursued at the level of the gene. Molecular characterization was initiated by screening a *Drosophila* head cDNA library for putative $G_o\alpha$-like clones. Positive clones were completely sequenced to facilitate comparison of the vertebrate and fly $G_o\alpha$s at both the nucleotide and amino acid levels. Genomic Southern blot analysis was performed to determine the number of copies of the $G_o\alpha$-like ($DG_o\alpha$) gene that were present in the *Drosophila* genome; the cytogenetic map position corresponding to the $DG_o\alpha$ gene was determined. Finally, the expression of the mRNAs that encoded *Drosophila* $G_o\alpha$-like proteins was examined by Northern blot analysis as well as by *in situ* hybridization to tissue sections.

Results and Discussion:

(i) Isolation of Drosophila cDNAs encoding $G_o\alpha$-like Proteins:

Recombinants from a *Drosophila* head cDNA library in λgt11 were isolated using the entire coding region of the rat brain $G_o\alpha$ cDNA (corresponding to a BamH1-Nco1, 920 bp fragment) as a probe under reduced stringency conditions. Upon initial screening of 500,000 recombinants, 15 hybridization positive clones were identified. These cDNA clones were plaque purified and λDNA was prepared, as described in Methods. DNA from these lambda clones was digested with Bam H1 and EcoR1 and subjected to Southern blot analysis. The probe used for the hybridization was the coding region of the rat brain $G_o\alpha$ cDNA (this probe had been used earlier to identify $DG_o\alpha$-like cDNAs). Based on this analysis, as shown in Fig.15, twelve of the recombinants produced similar restriction patterns. Of these, λDGo21 (#10 in Fig.15) had the longest insert (approximately 2.2 kb), and hence was selected for nucleotide sequencing.

The EcoR1 insert of λDGo21 was isolated and the DNA subcloned into pBS(M13-) (Stratagene) for DNA sequencing. The strategy used for sequencing this clone as well as the restriction map for λDGo21 is shown in Fig.16.

(ii) Comparison Analysis Between λDGo21 and Gα Sequences:

Fig.17 shows the complete nucleotide sequence and the derived amino acid sequence of λDGo21. The insert contains one long open reading frame coding for a protein of 354 residues. (Calculated molecular weight=40,491). The initiator methionine was assigned on

FIGURE 15:

Southern Blot Analysis of putative $G_o\alpha$-like clones obtained from screening a *Drosophila* head cDNA library

Lambda phage DNA was prepared from each of the 15 $G_o\alpha$ hybridization positive clones as described in Methods. Approx. 5μg of phage DNA from these clones was restriction digested, gel electrophoresed and transferred to nitrocellulose for Southern blot analysis. A radioactively labelled probe corresponding to the coding region of the rat brain $G_o\alpha$ cDNA was used for the hybridization (see Methods). The numbers at the top of the figure depict the respective clone numbers; for example, 4= λ clone #4. The λ DNA was digested with EcoR1 (lanes denoted A) and BamH1 (lanes denoted B).

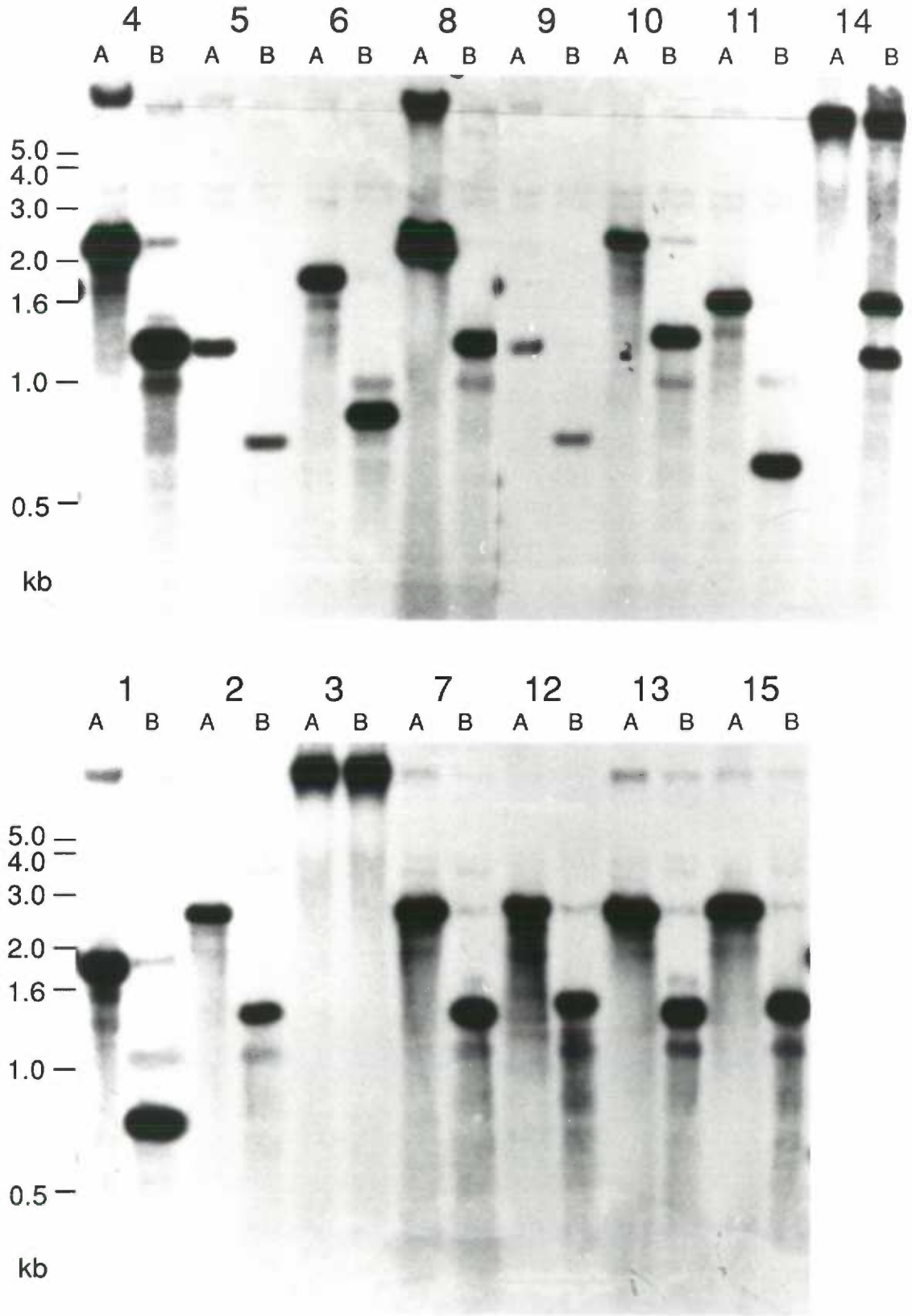

4 5 6 8 9 10 11 14
A B A B A B A B A B A B A B A B
5.0
4.0
3.0
2.0
1.6
1.0
0.5
kb
1 2 3 7 12 13 15
A B A B A B A B A B A B A B
5.0
4.0
3.0
2.0
1.6
1.0
0.5
kb

FIGURE 16:

Restriction Map and Nucleotide Sequencing Strategy for the *Drosophila* $G_o\alpha$-like cDNA

A partial restriction map for the *Drosophila* $G_o\alpha$-like ($DG_o\alpha$) cDNA is diagrammed. Horizontal arrows depict the size of the fragments that were subcloned for sequencing and also indicate the direction of the nucleotide sequence determination. The scale of the diagram is indicated to the lower right of the figure.

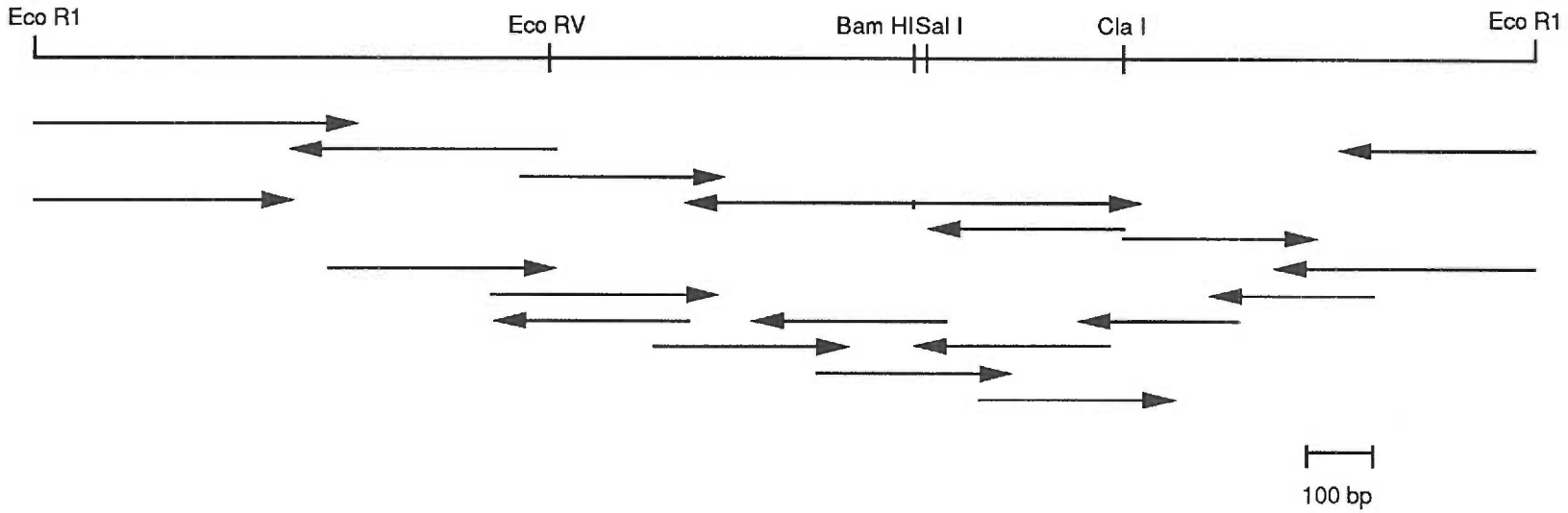
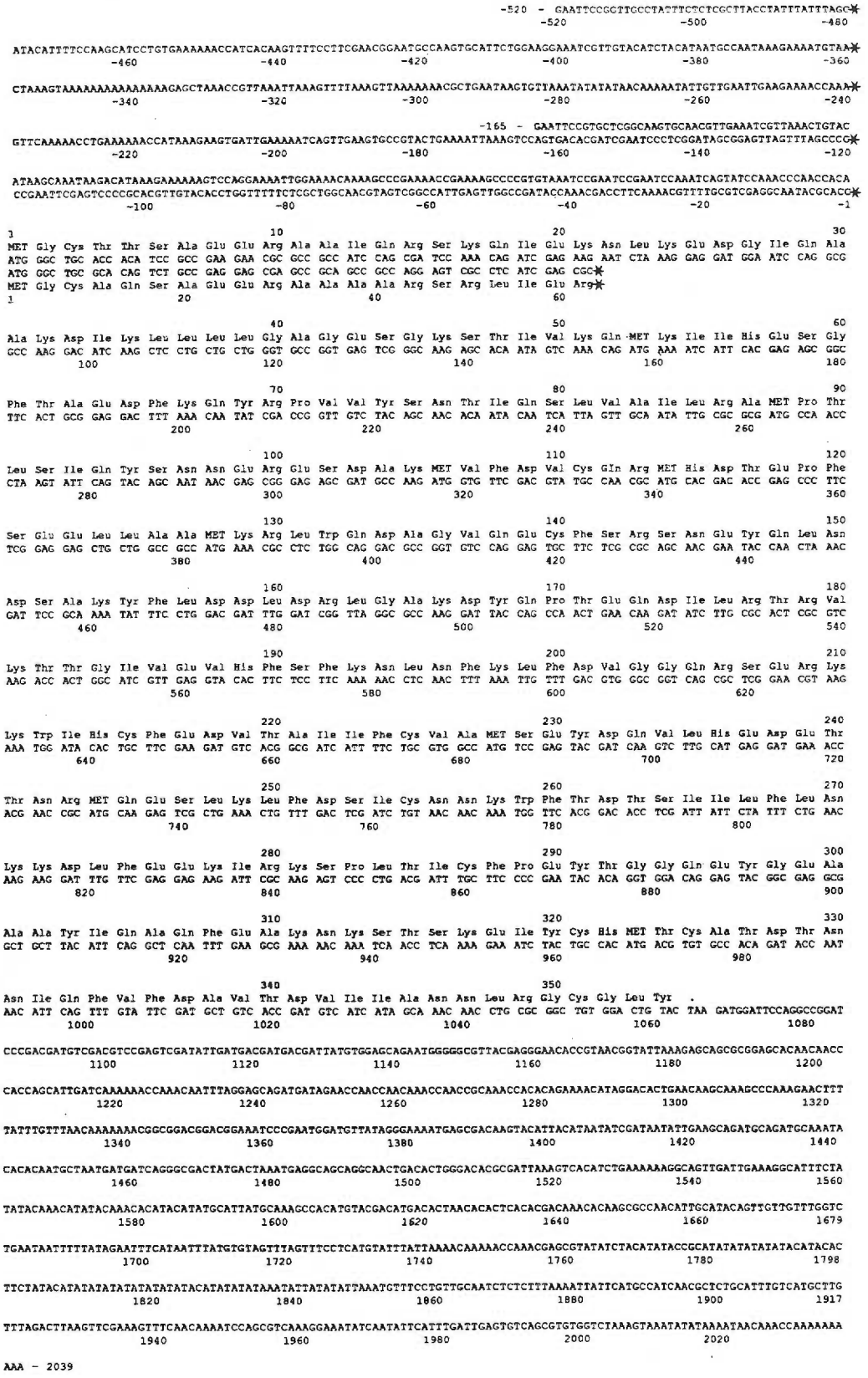

Eco R1
Eco RV
Bam HI
Sal I
Cla I
Eco R1
100 bp

FIGURE 17:

Sequence of λDGo21 and λDGo59

Nucleotide sequence and derived amino acid sequence of λDGo21 and λDGo59. Nucleotide 1 is the first nucleotide of the ATG translation start codon. Only the 5'-untranslated region and region coding for the first 21 residues of λDGo59 are shown. The nucleotide sequence of this cDNA is identical to λDGo21 in the remaining coding region. Only the sequence of λDGo59 (lines marked with asterisks) which differs from λDGo21 is shown.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-520 -500 -480
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-460 -440 -420 -400 -380 -360
CTAAAGTAAAAAAAAAAAAAAAAGAGCTAAACCGTTAAATTAAAGTTTTAAAGTTAAAAAAACGCTGAATAAGTGTTAAATATATATAACAAAAATATTGTTGAATTGAAGAAAACCAAA
-340 -320 -300 -280 -260 -240
-165 - GAATTCCGTGCTCGGCAAGTGCAACGTTGAAATCGTTAAACTGTAC
GTTCAAAAACCTGAAAAAACCATAAAGAAGTGATTGAAAAATCAGTTGAAGTGCCGTACTGAAAATTAAAGTCCAGTGACACGATCGAATCCCTCGGATAGCGGAGTTAGTTTAGCCCC
-220 -200 -180 -160 -140 -120
ATAAGCAAATAAGACATAAAGAAAAAAGTCCAGGAAAATTGGAAAACAAAAGCCCGAAAACCGAAAAGCCCCGTGTAAATCCGAATCCGAATCCAAATCAGTATCCAAACCCAACCACA
CCGAATTCGAGTCCCCGCACGTTGTACACCTGGTTTTTCTCGCTGGCAACGTAGTCGGCCATTGAGTTGGCCGATACCAAACGACCTTCAAAACGTTTTGCGTCGAGGCAATACGCACG
-100 -80 -60 -40 -20 -1
1 10 20 30
MET Gly Cys Thr Thr Ser Ala Glu Glu Arg Ala Ala Ile Gln Arg Ser Lys Gln Ile Glu Lys Asn Leu Lys Glu Asp Gly Ile Gln Ala
ATG GGC TGC ACC ACA TCC GCC GAA GAA CGC GCC GCC ATC CAG CGA TCC AAA CAG ATC GAG AAG AAT CTA AAG GAG GAT GGA ATC CAG GCG
ATG GGC TGC GCA CAG TCT GCC GAG GAG CGA GCC GCA GCC GCC AGG AGT CGC CTC ATC GAG CGC
MET Gly Cys Ala Gln Ser Ala Glu Glu Arg Ala Ala Ala Ala Arg Ser Arg Leu Ile Glu Arg
1 20 40 60
40 50 60
Ala Lys Asp Ile Lys Leu Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr Ile Val Lys Gln MET Lys Ile Ile His Glu Ser Gly
GCC AAG GAC ATC AAG CTC CTG CTG CTG GGT GCC GGT GAG TCG GGC AAG AGC ACA ATA GTC AAA CAG ATG AAA ATC ATT CAC GAG AGC GGC
100 120 140 160 180
70 80 90
Phe Thr Ala Glu Asp Phe Lys Gln Tyr Arg Pro Val Val Tyr Ser Asn Thr Ile Gln Ser Leu Val Ala Ile Leu Arg Ala MET Pro Thr
TTC ACT GCG GAG GAC TTT AAA CAA TAT CGA CCG GTT GTC TAC AGC AAC ACA ATA CAA TCA TTA GTT GCA ATA TTG CGC GCG ATG CCA ACC
200 220 240 260
100 110 120
Leu Ser Ile Gln Tyr Ser Asn Asn Glu Arg Glu Ser Asp Ala Lys MET Val Phe Asp Val Cys Gln Arg MET His Asp Thr Glu Pro Phe
CTA AGT ATT CAG TAC AGC AAT AAC GAG CGG GAG AGC GAT GCC AAG ATG GTG TTC GAC GTA TGC CAA CGC ATG CAC GAC ACC GAG CCC TTC
280 300 320 340 360
130 140 150
Ser Glu Glu Leu Leu Ala Ala MET Lys Arg Leu Trp Gln Asp Ala Gly Val Gln Glu Cys Phe Ser Arg Ser Asn Glu Tyr Gln Leu Asn
TCG GAG GAG CTG CTG GCC GCC ATG AAA CGC CTC TGG CAG GAC GCC GGT GTC CAG GAG TGC TTC TCG CGC AGC AAC GAA TAC CAA CTA AAC
380 400 420 440
160 170 180
Asp Ser Ala Lys Tyr Phe Leu Asp Asp Leu Asp Arg Leu Gly Ala Lys Asp Tyr Gln Pro Thr Glu Gln Asp Ile Leu Arg Thr Arg Val
GAT TCC GCA AAA TAT TTC CTG GAC GAT TTG GAT CGG TTA GGC GCC AAG GAT TAC CAG CCA ACT GAA CAA GAT ATC TTG CGC ACT CGC GTC
460 480 500 520 540
190 200 210
Lys Thr Thr Gly Ile Val Glu Val His Phe Ser Phe Lys Asn Leu Asn Phe Lys Leu Phe Asp Val Gly Gly Gln Arg Ser Glu Arg Lys
AAG ACC ACT GGC ATC GTT GAG GTA CAC TTC TCC TTC AAA AAC CTC AAC TTT AAA TTG TTT GAC GTG GGC GGT CAG CGC TCG GAA CGT AAG
560 580 600 620
220 230 240
Lys Trp Ile His Cys Phe Glu Asp Val Thr Ala Ile Ile Phe Cys Val Ala MET Ser Glu Tyr Asp Gln Val Leu His Glu Asp Glu Thr
AAA TGG ATA CAC TGC TTC GAA GAT GTC ACG GCG ATC ATT TTC TGC GTG GCC ATG TCC GAG TAC GAT CAA GTC TTG CAT GAG GAT GAA ACC
640 660 680 700 720
250 260 270
Thr Asn Arg MET Gln Glu Ser Leu Lys Leu Phe Asp Ser Ile Cys Asn Asn Lys Trp Phe Thr Asp Thr Ser Ile Ile Leu Phe Leu Asn
ACG AAC CGC ATG CAA GAG TCG CTG AAA CTG TTT GAC TCG ATC TGT AAC AAC AAA TGG TTC ACG GAC ACC TCG ATT ATT CTA TTT CTG AAC
740 760 780 800
280 290 300
Lys Lys Asp Leu Phe Glu Glu Lys Ile Arg Lys Ser Pro Leu Thr Ile Cys Phe Pro Glu Tyr Thr Gly Gly Gln Glu Tyr Gly Glu Ala
AAG AAG GAT TTG TTC GAG GAG AAG ATT CGC AAG AGT CCC CTG ACG ATT TGC TTC CCC GAA TAC ACA GGT GGA CAG GAG TAC GGC GAG GCG
820 840 860 880 900
310 320 330
Ala Ala Tyr Ile Gln Ala Gln Phe Glu Ala Lys Asn Lys Ser Thr Ser Lys Glu Ile Tyr Cys His MET Thr Cys Ala Thr Asp Thr Asn
GCT GCT TAC ATT CAG GCT CAA TTT GAA GCG AAA AAC AAA TCA ACC TCA AAA GAA ATC TAC TGC CAC ATG ACG TGT GCC ACA GAT ACC AAT
920 940 960 980
340 350
Asn Ile Gln Phe Val Phe Asp Ala Val Thr Asp Val Ile Ile Ala Asn Asn Leu Arg Gly Cys Gly Leu Tyr .
AAC ATT CAG TTT GTA TTC GAT GCT GTC ACC GAT GTC ATC ATA GCA AAC AAC CTG CGC GGC TGT GGA CTG TAC TAA GATGGATTCCAGGCCGGAT
1000 1020 1040 1060 1080
CCCGACGATGTCGACGTCCGAGTCGATATTGATGACGATGACGATTATGTGGAGCAGAATGGGGGCGTTACGAGGGAACACCGTAACGGTATTAAAGAGCAGCGCGGAGCACAACAACC
1100 1120 1140 1160 1180 1200
CACCAGCATTGATCAAAAAACCAAACAATTTAGGAGCAGATGATAGAACCAACCAACAAACCAACCGCAAACCACACAGAAAACATAGGACACTGAACAAGCAAAGCCCAAAGAACTTT
1220 1240 1260 1280 1300 1320
TATTTGTTTAACAAAAAAACGGCGGACGGACGGAAATCCCGAATGGATGTTATAGGGAAAATGAGCGACAAGTACATTACATAATATCGATAATATTGAAGCAGATGCAGATGCAAATA
1340 1360 1380 1400 1420 1440
CACACAATGCTAATGATGATCAGGGCGACTATGACTAAATGAGGCAGCAGGCAACTGACACTGGGACACGCGATTAAAGTCACATCTGAAAAAAGGCAGTTGATTGAAAGGCATTTCTA
1460 1480 1500 1520 1540 1560
TATACAAACATATACAAACACATACATATGCATTATGCAAAGCCACATGTACGACATGACACTAACACACTCACACGACAAACACAAGCGCCAACATTGCATACAGTTGTTGTTTGGTC
1580 1600 1620 1640 1660 1679
TGAATAATTTTTATAGAATTTCATAATTTATGTGTAGTTTAGTTTCCTCATGTATTTATTAAAACAAAAACCAAACGAGCGTATATCTACATATACCGCATATATATATACATACAC
1700 1720 1740 1760 1780 1798
TTCTATACATATATATATATATATACATATATATAAATATTATATATTAAATGTTTCCTGTTGCAATCTCTCTTTAAAATTATTCATGCCATCAACGCTCTGCATTTGTCATGCTTG
1820 1840 1860 1880 1900 1917
TTTAGACTTAAGTTCGAAAGTTTCAACAAAATCCAGCGTCAAAGGAAATATCAATATTCATTTGATTGAGTGTCAGCGTGTGGTCTAAAGTAAATATATAAAATAACAAACCAAAAAAAA
1940 1960 1980 2000 2020
AAA - 2039

the basis of sequence alignment with other Gα subunits which have a conserved Met-Gly-Cys sequence at the amino terminus. In addition, the nucleotide sequence around the proposed initiator ATG, CCACAATGG, is a good match to both the Kozak consensus sequence (157, 158) for translation initiation (CC(A/G)CCATGG) and the proposed *Drosophila* translation initiation sequence ((C/A)AA(A/C)ATG) (159).

Fig.18A shows the alignment of the protein encoded by λDGo21 with the amino acid sequence of the various vertebrate Gα proteins and the *ras* oncogene. Fig.18B shows a comparison of the amino acid sequence of this protein with the *Drosophila* $G_s\alpha$-like and $G_i\alpha$-like proteins. These comparisons establish that λDGo21 codes for a Gα protein. There is striking homology of this protein to other G protein α subunits including the *ras* protein. The homology is noted in regions that are believed to be important for GTP binding and hydrolysis (A, C, E and G; Figs.18A and18B).

The Halliday regions (see introduction, p.18) are those regions that have an almost invariant sequence and readily identifiable functions. In EF-Tu, these regions are characteristically preceded by short hydrophobic stretches that are presumably β sheets and followed by longer stretches of polar residues that are likely α helices. This motif has been maintained in other nucleotide binding proteins that have been characterized. Halliday regions are found occurring in a specific order of sequence and spacing. Thus, the A, C, E and G regions are found arrayed along the primary protein structure from the amino to the carboxyl terminus. The C, E and G regions are separated from each other by about 20-50 amino acids;

FIGURE 18 A:

Alignment of the amino acid sequence of λDGo21 with vertebrate Gα proteins and the ras oncogene.

The derived amino acid sequence of the λDGo21 insert has been aligned with the amino acid sequence of bovine $G_o\alpha$, $G_s\alpha$, rod $G_t\alpha$, $G_i\alpha_2$ and the ras oncogene. Only residues identical to those of the *Drosophila* protein have been boxed. The postulated guanine nucleotide binding regions (A, C, E, and G are indicated).

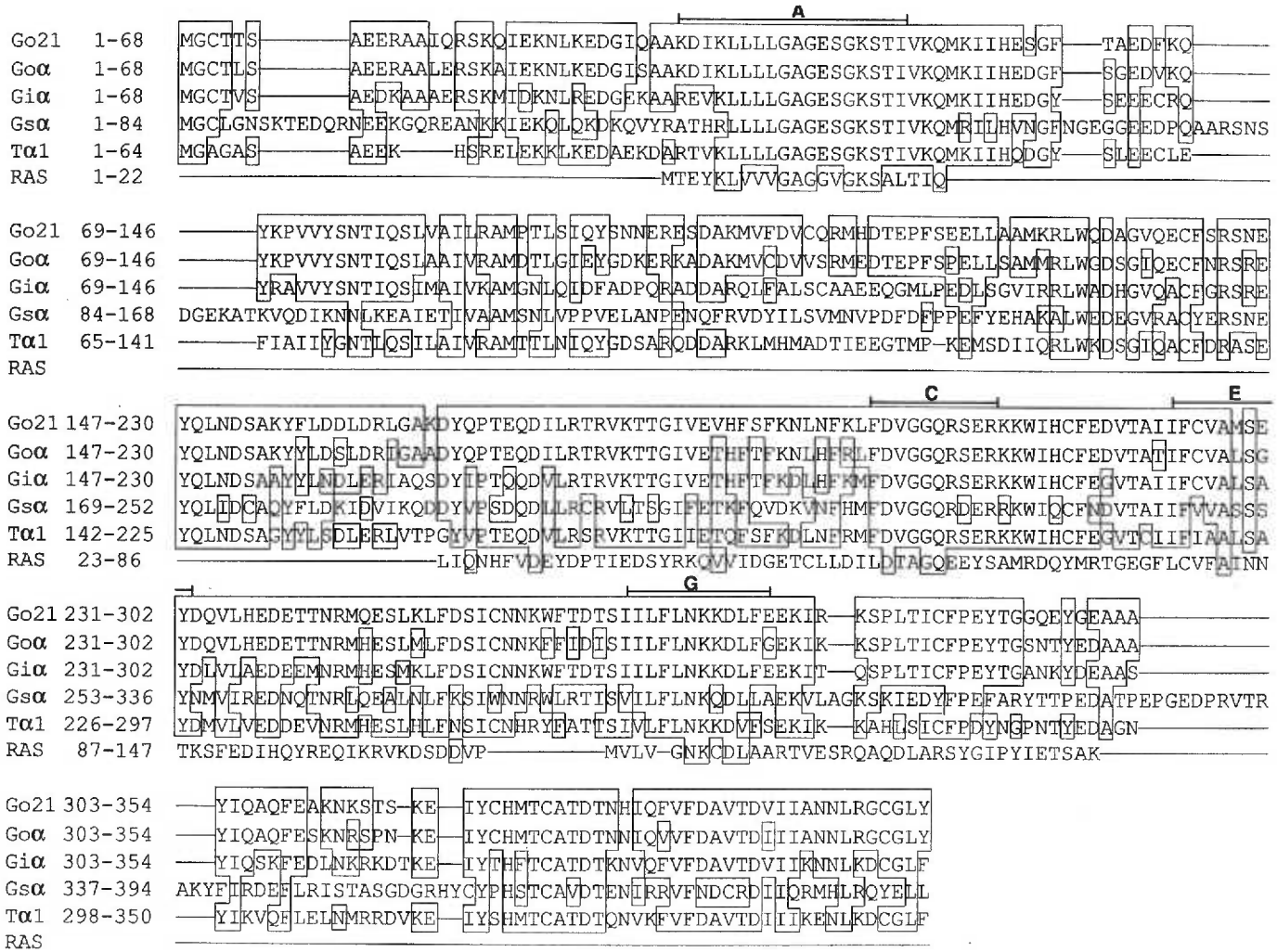
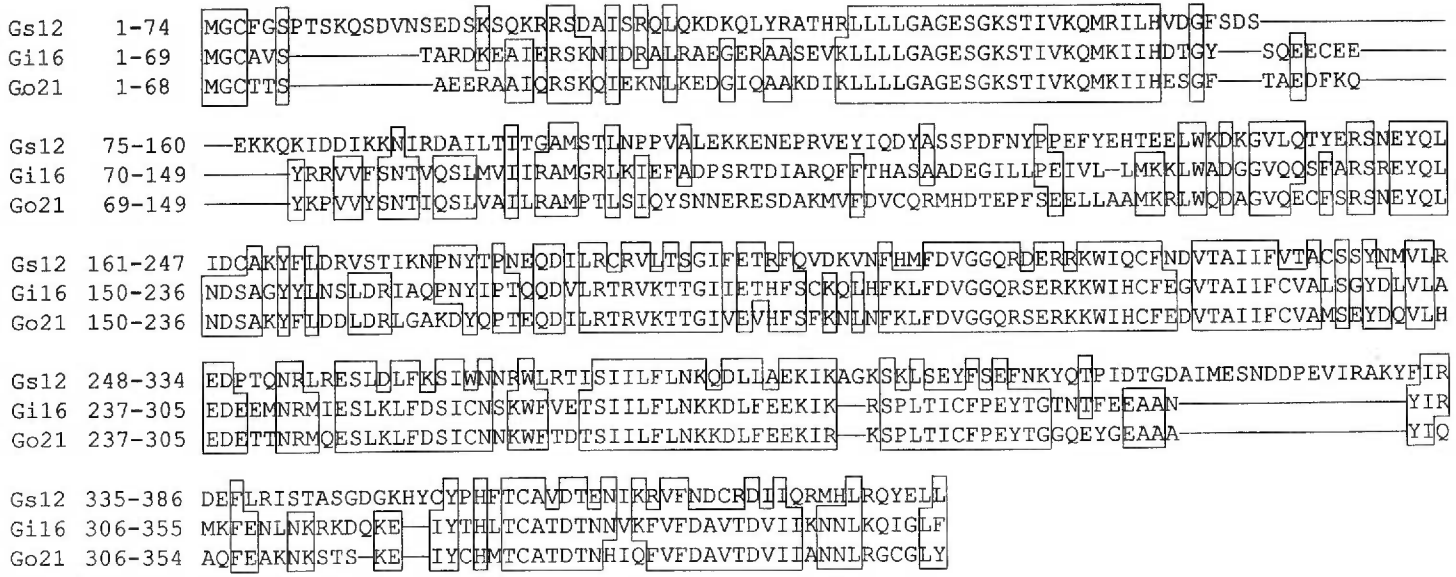

A
C
E
G
Go21 1-68 MGCTTS AEERAAIQRSKQIEKNLKEDGIQAAKDIKLLLLGAGESGKSTIVKQMKIIHESGF TAEDFKQ
Goα 1-68 MGCTLS AEERAALERSKAIEKNLKEDGISAAKDIKLLLLGAGESGKSTIVKQMKIIHEDGF SGEDVKQ
Giα 1-68 MGCTVS AEDKAAAERSKMIDKNLREDGEKAARETKLLLLGAGESGKSTIVKQMKIIHEDGY SEEECRQ
Gsα 1-84 MGCLGNSKTEDQRNEEKGQREANKKIEKQLQKDKQVYRATHRLLLLGAGESGKSTIVKQMRILHVNGFNGEGGEEDPQAARSNS
Tα1 1-64 MGAGAS AEEK HSRELEKKLKEDAEKDARTVKLLLLGAGESGKSTIVKQMKIIHQDGY SLEECLE
RAS 1-22 MTEYKLVVVGAGGVGKSALTIQ
Go21 69-146 YKPVVYSNTIQSLVAILRAMPTLSIQYSNNERESDAKMVFDVCQRMHDTEPFSEELLAAMKRLWQDAGVQECFSRSNE
Goα 69-146 YKPVVYSNTIQSLAAIVRAMDTLGIEYGDKERKADAKMVCDVVSRMEDTEPFSPELLSAMMRLWGDSGIQECFNRSRE
Giα 69-146 YRAVVYSNTIQSIMAIVKAMGNLQIDFADPQRADDARQLFALSCAAEEQGMLPEDLSGVIRRLWADHGVQACFGRSRE
Gsα 84-168 DGEKATKVQDIKNNLKEAIETIVAAMSNLVPPVELANPENQFRVDYILSVMNVPDFDFPPEFYEHAKALWEDEGVRACYERSNE
Tα1 65-141 FIAIIYGNTLQSILAIVRAMTTLNIQYGDSARQDDARKLMHMADTIEEGTMP KEMSDIIQRLWKDSGIQACFDRASE
RAS
Go21 147-230 YQLNDSAKYFLDDLDRLGAKDYQPTEQDILRTRVKTTGIVEVHFSFKNLNFKLFDVGGQRSERKKWIHCFEDVTAIIFCVAMSE
Goα 147-230 YQLNDSAKYYLDSLDRIGAADYQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCVALSG
Giα 147-230 YQLNDSAAYYLNDLERIAQSDYIPTQQDVLRTRVKTTGIVETHFTFKDLHFKMFDVGGQRSERKKWIHCFEGVTAIIFCVALSA
Gsα 169-252 YQLIDCAQYFLDKIDVIKQDDYVPSDQDLLRCRVLTSGIFETKFQVDKVNFHMFDVGGQRDERRKWIQCFNDVTAIIFVVASSS
Tα1 142-225 YQLNDSAGYYLSDLERLVTPGYVPTEQDVLRSRVKTTGIIETQFSFKDLNFRMFDVGGQRSERKKWIHCFEGVTCIIFIAALSA
RAS 23-86 LIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINN
Go21 231-302 YDQVLHEDETTNRMQESLKLFDSICNNKWFTDTSIILFLNKKDLFEEKIR KSPLTICFPEYTGGQEYGEAAA
Goα 231-302 YDQVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIK KSPLTICFPEYTGSNTYEDAAA
Giα 231-302 YDLVLAEDEEMNRMHESMKLFDSICNNKWFTDTSIILFLNKKDLFEEKIT QSPLTICFPEYTGANKYDEAAS
Gsα 253-336 YNMVIREDNQTNRLQEALNLFKSIWNNRWLRTISVILFLNKQDLLAEKVLAGKSKIEDYFPEFARYTTPEDATPEPGEDPRVTR
Tα1 226-297 YDMVLVEDDEVNRMHESLHLFNSICNHRYFATTSIVLFLNKKDVFSEKIK KAHLSICFPDYNGPNTYEDAGN
RAS 87-147 TKSFEDIHQYREQIKRVKDSDDVP MVLV GNKCDLAARTVESRQAQDLARSYGIPYIETSAK
Go21 303-354 YIQAQFEAKNKSTS KE IYCHMTCATDTNHIQFVFDAVTDVIIANNLRGCGLY
Goα 303-354 YIQAQFESKNRSPN KE IYCHMTCATDTNNIQVVFDAVTDIIIANNLRGCGLY
Giα 303-354 YIQSKFEDLNKRKDTKE IYTHFTCATDTKNVQFVFDAVTDVIIKNNLKDCGLF
Gsα 337-394 AKYFIRDEFLRISTASGDGRHYCYPHSTCAVDTENIRRVFNDCRDIIQRMHLRQYELL
Tα1 298-350 YIKVQFLELNMRRDVKE IYSHMTCATDTQNVKFVFDAVTDIIIKENLKDCGLF
RAS

FIGURE 18 B:

Alignment of the amino acid sequence of λDGo21 with *Drosophila* Gα proteins

Comparison of the derived amino acid sequence of λDGo21 with the amino acid sequence of the *Drosophila* $G_s\alpha$-like and $G_i\alpha$-like proteins. Identical residues are boxed. Sequences are taken from Quan et al (1989) for $G_s\alpha$ and Provost et al (1988) for $G_i\alpha$(145, 174).


Gs12 1-74 MGCFGSPTSKQSDVNSEDSKSQKRRSDAISRQLQKDKQLYRATHRLLLLGAGESGKSTIVKQMRILHVDGFSDS
Gi16 1-69 MGCAVS TARDKEAIERSKNIDRALRAEGERAASEVKLLLLGAGESGKSTIVKQMKIIHDTGY SQEECEE
Go21 1-68 MGCTTS AEERAAIQRSKQIEKNLKEDGIQAAKDIKLLLLGAGESGKSTIVKQMKIIHESGF TAEDFKQ
Gs12 75-160 EKKQKIDDIKKNIRDAILTITGAMSTLNPPVALEKKENEPRVEYIQDYASSPDFNYPPEFYEHTEELWKDKGVLQTYERSNEYQL
Gi16 70-149 YRRVVFSNTVQSLMVIIRAMGRLKIEFADPSRTDIARQFFTHASAAADEGILLPEIVL-LMKKLWADGGVQQSFARSREYQL
Go21 69-149 YKPVVYSNTIQSLVAILRAMPTLSIQYSNNERESDAKMVFDVCQRMHDTEPFSEELLAAMKRLWQDAGVQECFSRSNEYQL
Gs12 161-247 IDCAKYFLDRVSTIKNPNYTPNEQDILRCRVLTSGIFETRFQVDKVNFHMFDVGGQRDERRKWIQCFNDVTAIIFVTACSSYNMVLR
Gi16 150-236 NDSAGYYLNSLDRIAQPNYIPTQQDVLRTRVKTTGIIETHFSCKQLHFKLFDVGGQRSERKKWIHCFEGVTAIIFCVALSGYDLVLA
Go21 150-236 NDSAKYFLDDLDRLGAKDYQPTEQDILRTRVKTTGIVETHFSFKNLNFKLFDVGGQRSERKKWIHCFEDVTAIIFCVAMSEYDQVLH
Gs12 248-334 EDPTQNRLRESLDLFKSIWNNRWLRTISIILFLNKQDLLAEKIKAGKSKLSEYFSEFNKYQTPIDTGDAIMESNDDPEVIRAKYFIR
Gi16 237-305 EDEEMNRMIESLKLFDSICNSKWFVETSIILFLNKKDLFEEKIK RSPLTICFPEYTGTNTFEEAAN YIR
Go21 237-305 EDETTNRMQESLKLFDSICNNKWFTDTSIILFLNKKDLFEEKIR KSPLTICFPEYTGGQEYGEAAA YIQ
Gs12 335-386 DEFLRISTASGDGKHYCYPHFTCAVDTENIKRVFNDCRDIIQRMHLRQYELL
Gi16 306-355 MKFENLNKRKDQKE IYTHLTCATDTNNVKFVFDAVTDVIIKNNLKQIGLF
Go21 306-354 AQFEAKNKSTS KE IYCHMTCATDTNHIQFVFDAVTDVIIANNLRGCGLY

the A region is generally more variable but is likely to be within 20-40 amino acids of the amino terminus of the Gα subunit.

The A region has a consensus sequence of Gly-X-X-X-X-Gly-Lys. In DGo21 it occurs between residues 32-49. Mutations in the analogous region of *ras* reduce GTP binding and GTPase activity (160) This region (in EF-Tu) is near the α-phosphate of GDP. The C region has the consensus sequence, Asp-X-X-Gly. In EF-Tu, the asp can chelate the magnesium ion closest to the β-phosphate of GDP. Mutations in this region of *ras* cause decreased GTPase activity(161). The corresponding region in λDGo21 is between 200-209. The E region, residues 223-231, is characterized as being very hydrophobic. Along with other hydrophobic amino acids of the consensus regions, this forms a hydrophobic pocket. Mutations in this region of *ras* result in drastically reduced affinity for GTP (162). The G region has the consensus sequence, Asn-Lys-X-Asp. The region determines nucleotide binding specificity. Mutations in this region can completely abolish nucleotide binding (163). The analogous region in λDGo21 is found between residues 265-276.

The *Drosophila* protein is most similar to vertebrate $G_o\alpha$ (82.2% identical) and less, but still significantly similar to vertebrate $G_i\alpha$, $G_t\alpha$ (62-67% identical) and $G_s\alpha$ (39% identical). The protein is also similar to *Drosophila* $G_i\alpha$-like and $G_s\alpha$-like proteins (164, 145). The similarity to vertebrate $G_o\alpha$ is particularly striking, especially in regions where $G_o\alpha$ diverges from other Gα proteins. For example, residues 99-131 of λDGo21 are 75% identical to vertebrate $G_o\alpha$ and only 17-25% identical to $G_i\alpha$, $G_s\alpha$ or $G_t\alpha$. This segment is one of three regions which differ significantly between all α subunits and thus

maybe involved in mediating interactions specific for each α subunit (16).

The protein encoded by λDGo21 has a cysteine 4 residues from the carboxyl terminus and therefore is likely to be modified by PTX (54). Since the sequences of the *Drosophila* $G_i\alpha$ and $G_s\alpha$-like proteins lack a cysteine in this position, these results are consistent with those described above indicating that the PTX substrate in *Drosophila* head membranes is a $G_o\alpha$-like protein. In addition, the amino- and carboxyl-terminal regions of the protein coded by λDGo21 contain sequences similar to those used to generate the GC and GO antibodies. The *Drosophila* amino terminal sequence, Gly-Cys-Thr-Thr-Ser-Ala-Glu-Glu-Arg-Ala-Ala-Ile-Gln-Arg-Ser-Lys, differs in three positions (underlined) from the GC peptide, Gly-Cys-Thr-Leu-Ser-Ala-Glu-Glu-Arg-Ala-Ala-Leu-Glu-Arg-Ser-Lys. The carboxyl terminal decapeptide sequence, Ala-Asn-Asn-Leu-Arg-Gly-Cys-Gly-Leu-Tyr is identical to that used to generate the GO antibody. This cDNA then should also code for a protein which is recognized by these antibodies. Thus, it is likely that λDGo21 codes for a 40 kD protein in *Drosophila* head membranes which is modified by PTX and recognized by the GC and GO antibodies.

(iii) Description of another $G_o\alpha$-like cDNA:

Additional cDNAs have been isolated which on the basis of restriction mapping and sequence information are likely to represent alternately spliced transcripts of the *Drosophila* $G_o\alpha$-like gene. One of these cDNAs, λDGo59, has been completely sequenced. As shown in Fig.17, λDGo59 is identical to λDGo21 with the exception of the 5'-

untranslated region and the region coding for the amino-terminal 21 amino acid residues. Over the amino-terminal coding region, the two $G_o\alpha$-like proteins differ in 7 of 21 residues while the corresponding cDNAs differ in 27 of 63 nucleotides. The amino terminus of the protein coded by λDGo59, Gly-Cys-Ala-Gln-Ser-Ala-Glu-Glu-Arg-Ala-Ala-Ala-Ala-Arg-Ser-Arg, differs in six positions from the GC peptide (underlined) and may also crossreact with the GC antibody. The carboxyl-terminus of the protein encoded by λDGo59 and 3'-untranslated regions are identical to that of λDGo21.

This cDNA also encodes a protein which is likely to be a PTX substrate and recognized by the GO antibodies. The protein product of λDGo59 differs minimally in molecular weight (40,430) from that encoded by λDGo21 (40,491). These protein species would not have been resolved by the SDS-PAGE techniques used in Figs.4-10. Thus, two $G_o\alpha$-like proteins are expressed in the *Drosophila* nervous system which probably arise by alternate splicing. Of 14 $G_o\alpha$-like clones that have been characterized, 12 are like λDGo21 and two are like λDGo59. This suggests that λDGo21 mRNAs should be expressed more abundantly than λDGo59 mRNAs. Although in vertebrates, molecular cloning has so far identified only one class of $G_o\alpha$ cDNA, alternate splicing as described here for *Drosophila* to produce $G_o\alpha$ variants may explain the novel forms of $G_o\alpha$ which have been described (58, 59).

The structure of the $G_o\alpha$ gene in *Drosophila* has been partially resolved by Yoon et al and Desousa et al (165, 166). Genomic clone analysis showed that the gene (approx. 40kb) is comprised of eight coding exons that are separated by introns. Exons 4-7 which

comprise the carboxyl half of the protein are found clustered within a 1kb region, separated by introns that are approx. 70bp. Exons 2 and 3 are also clustered but exon 3 is separated from exon 4 by a very large intron that spans 13kb. Interestingly, the results show that there are two first exons which can be differentially spliced to exon 2 to generate two alternate cDNAs. This would account for the two different cDNAs, λDGo21 and λDGo59 that were characterized. The two forms of $DG_o\alpha$ share exons 2-7. The first exons are found at different regions of the genomic DNA. The size of the intron separating exon 1 from exon 2 is 2.7kb for λDGo21 while in λDGo59 it is 9.0kb.

When the structure of the fly $G_o\alpha$ was examined for intron-exon boundaries, the results showed that these boundaries matched those found in the gene structure of the $G_i\alpha$ (76), suggesting the two diverged from a single primordial gene.

(iv) Genomic Southern Analysis:

Fly genomic DNA was restriction digested and blotted onto Zeta-bind membranes for Southern analysis. DNA probes corresponding to the coding region of *Drosophila* $G_o\alpha$ subunit were prepared and hybridized as described in Methods. The resulting hybridization pattern, shown in Fig.19 is consistent with the $G_o\alpha$ gene being present in a single copy in the fly genome.

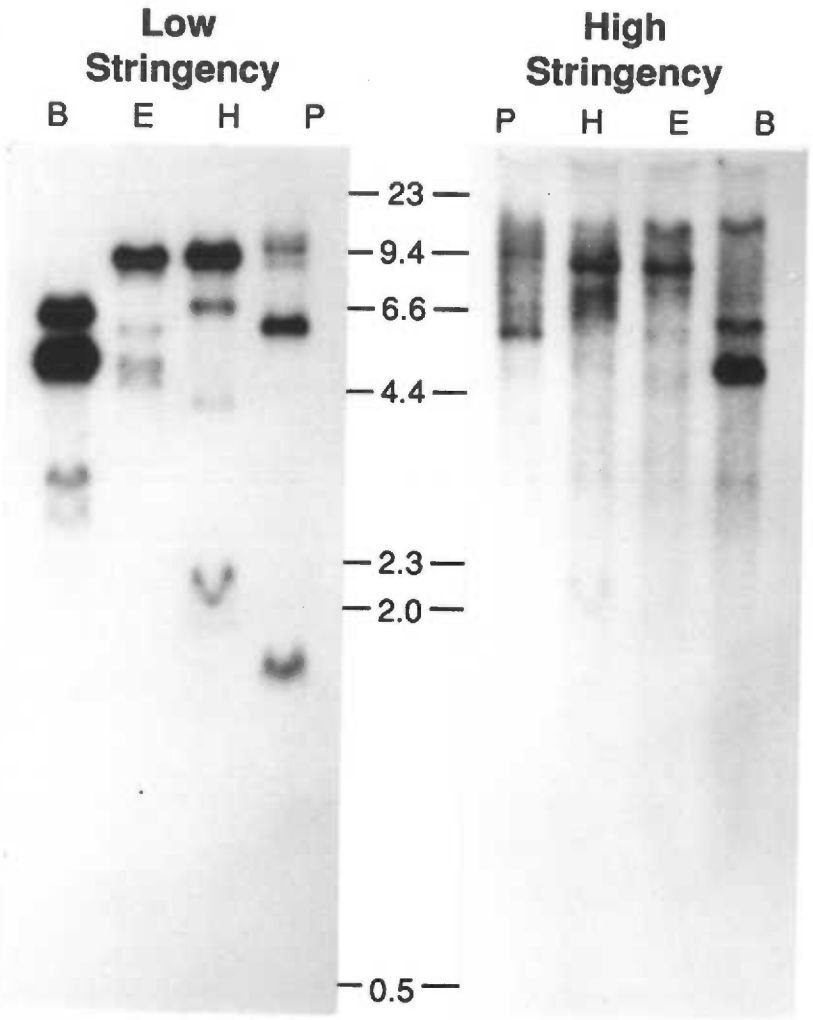
(v) In situ Hybridization to Polytene Chromosomes:

To determine the cytogenetic localization of the DNA, clone (λDGo21) was used as a probe for *in situ* hybridization to polytene

FIGURE 19:

Southern Blot Analysis of *Drosophila* Genomic DNA

Drosophila DNA was cut with BamH1 (B), EcoR1 (E), HindIII (H) or PstI (P) and analyzed by hybridization to the 2.2kb EcoR1 fragment of λDGo21 under high or low stringency conditions as described in Methods. 5.0μg of DNA was run in each lane. HindIII fragments of λDNA were used as size (kb) markers.


Low
Stringency
B
E
H
P
High
Stringency
P
H
E
B
23
9.4
6.6
4.4
2.3
2.0
0.5

chromosomes of larval salivary glands. The probe hybridized to position 47A on the right arm of the second chromosome. No mutations have been reported to map to this position of the genome.

(vi) Expression of Transcripts Coding for $G_o\alpha$–like Proteins in Drosophila:

Northern blots of poly(A+) RNA prepared from whole flies, adult heads and bodies were hybridized with probes corresponding to either the coding sequence or 3'-untranslated region of λDGo21. The results, shown in Fig.20, indicate that at least three different transcripts can be identified with both probes. A 3.5kb mRNA appears enriched in bodies, a 6.0kb appears enriched in heads and a 4.2kb mRNA appears present in both heads and bodies. Since probes to the 3'-untranslated region identify a similar set of transcripts, each class of transcript may contain different 5' ends as a result of alternate splicing.

Fig.21 shows Northern blots of whole fly, head and body poly(A+)RNA hybridized with probes representing sequences specifically found in the 5'-untranslated regions of λDGo21 and λDGo59. A 133-bp EcoR1-Hinf1 fragment (nucleotides -165 to -32) corresponding to the 5'-untranslated region of λDGo21 and a 121-bp BamH1-Nco1 fragment (nucleotides -115 to +4) corresponding to the 5'- untranslated region of λDGo59 were respectively used as the probes specific to λDGo21 and λDGo59. The results indicate that sequences found in this region of λDGo21 are found in RNAs of each of the size classes identified in Fig. 20. The distribution of sequences found in the 5'-untranslated region of λDGo59 is apparently more

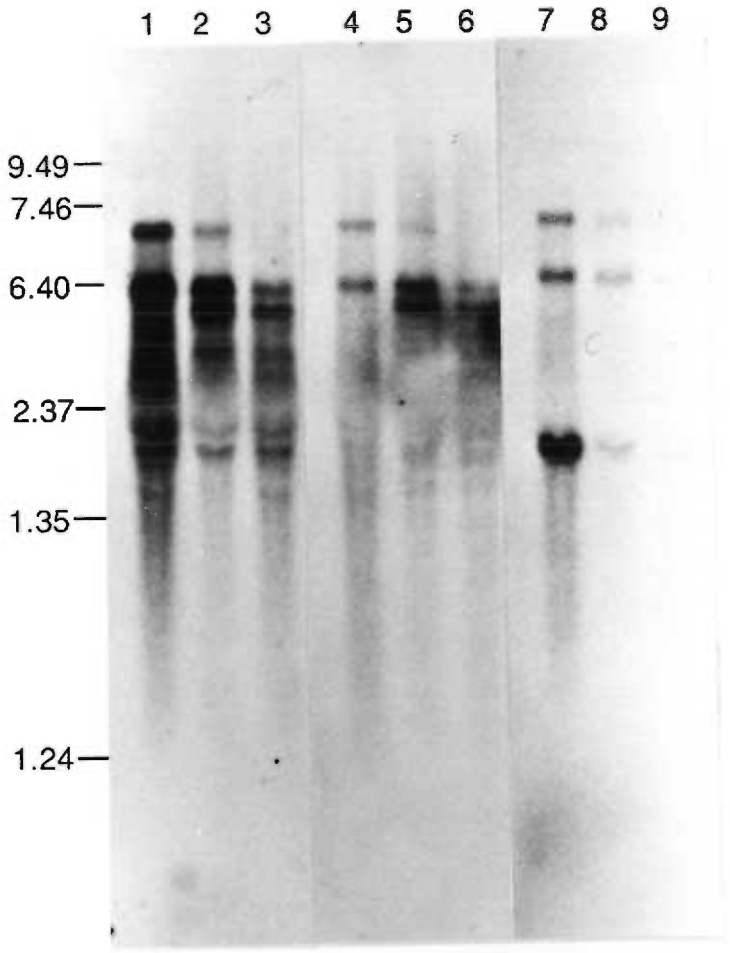
FIGURE 20:

Northern Blot Analysis of λDGo21 Expression

Poly(A+) RNA (15μg each) was isolated from heads (*lanes 1 and 4*), whole flies (*lanes 2 and 5*), and bodies (*lanes 3 and 6*) and probed either with a 1.2kb EcoR1-BamH1 fragment representing the 5'-untranslated and entire coding region of λDGo21 (*lanes 1-3*) or a 0.8kb BamH1-EcoR1 representing the 3'-untranslated regions of this cDNA (*lanes 4-6*).

FIGURE 21:

Northern Blot Analysis of Expression of λDGo21 and λDGo59 Poly(A+)RNA (15μg each) isolated from heads (*lanes 1, 4 and 7*), whole flies (*lanes 2, 5 and 8*) and bodies (*lanes 3, 6 and 9*) and probed as follows. *Lanes 1-3* were probed with an 812-bp BamH1-EcoR1 fragment representing the 3'-untranslated region of λDGo21. *Lanes 4-6* were probed with a 133-bp EcoR1-Hinf1 fragment, corresponding to nucleotides -165 to -32 of the 5'-untranslated region of λDGo21. *Lanes 7-9* were probed with a 121-bp fragment corresponding to nucleotides -115 to +4 found in the 5'-untranslated region of λDGo59. Hybridization conditions are described in Methods.


1 2 3 4 5 6 7 8 9
9.49
7.46
6.40
2.37
1.35
1.24

restricted. These sequences appear to be found in transcripts which are expressed more abundantly in the head and are rarely found in body $G_o\alpha$ transcripts.

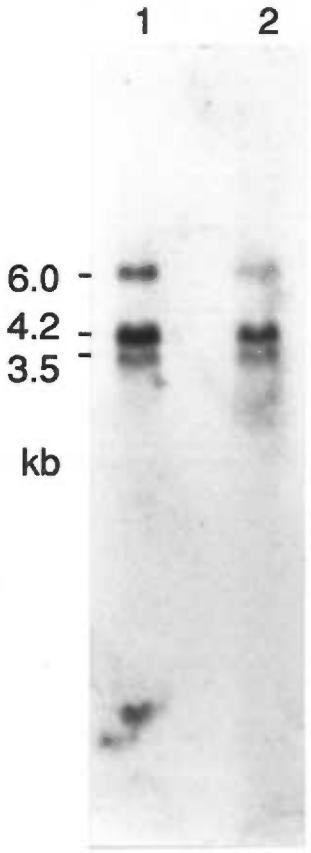
These observations indicate that the formation of the variety of transcripts coding for $G_o\alpha$-like proteins in *Drosophila* are likely to be produced by complex splicing schemes involving the alternate use of a number of exons. In the recent work described by J. Yoon et al (1989) and De Sousa et al (1989) (165, 166), these authors have characterized the genomic structure of the $DG_o\alpha$ gene and suggest that alternate splicing mechanisms result from an alternative splice junction at the amino terminus region of the gene. However, the $DG_o\alpha$ gene structure that they report does not account for all the size transcripts that are seen on Northerns, indicating that the characterization of the $DG_o\alpha$ gene is incomplete.

In order to assess whether *eya* flies contained all the transcripts seen in wild-type flies, Northern analysis was performed on total RNA prepared from wildtype and *eya* flies. The EcoR1 fragment corresponding to λDGo21 was used as the probe for hybridization. Since this probe contains all of the coding region that is common to both λDGo21 and λDGo59, it is expected to recognize all the transcripts that correspond to the two DGo forms. The results of the Northern are shown in Fig.22. All of the transcripts that were recognized in wildtype flies appeared to be present in the *eya* flies. Since the pattern of hybridization that was seen in wildtype and *eya* flies was similar this indicated that the expression of $DG_o\alpha$-like message occurred predominantly in head tissues exclusive of the

FIGURE 22:

Northern Blot Analysis of Expression of λDGo21 in wild type and *eyes absent* flies

Total RNA was prepared from wild type and *eyes absent* flies as described in Methods. *Lanes 1 and 2* represent 10μg of wild type and *eyes absent* RNA, respectively. The blot was probed with the 1.2kb EcoR1-BamH1 fragment that corresponds to the coding region of λDGo21.


1
2
6.0
4.2
3.5
kb

eyes. This result is also consistent with the eyes not containing detectable levels of PTX modified protein. (see Fig.8).

(vii) Stage specific Expression of Transcripts Encoding $DG_o\alpha$-like Proteins:

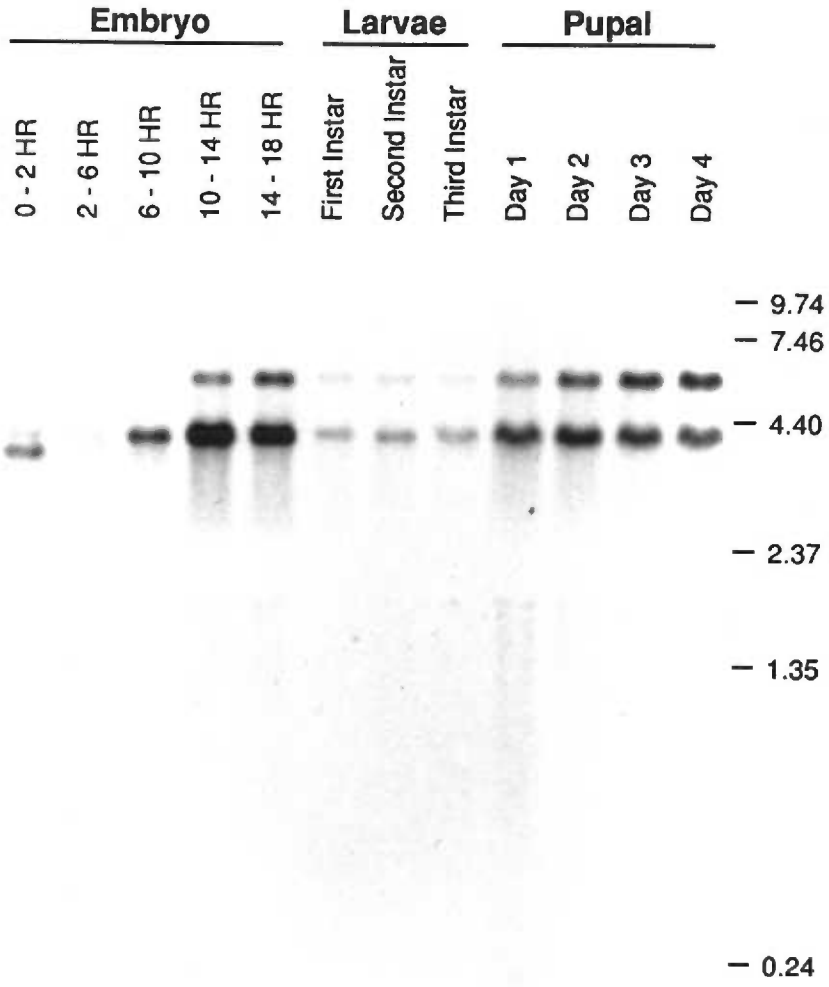
Total RNA was prepared from embryos, larvae and pupae at the various developmental stages as described in Methods. In order to address whether the transcripts that corresponded to $DG_o\alpha$-like messages were regulated in a stage specific manner, Northern blots were analyzed using probes that were specific for the EcoR1 fragment of λDGo21 (which contained coding regions that are common to both λDGo21 and λDGo59) and the 5' untranslated region of λDGo59 (121-bp BamH1-Nco1 fragment).

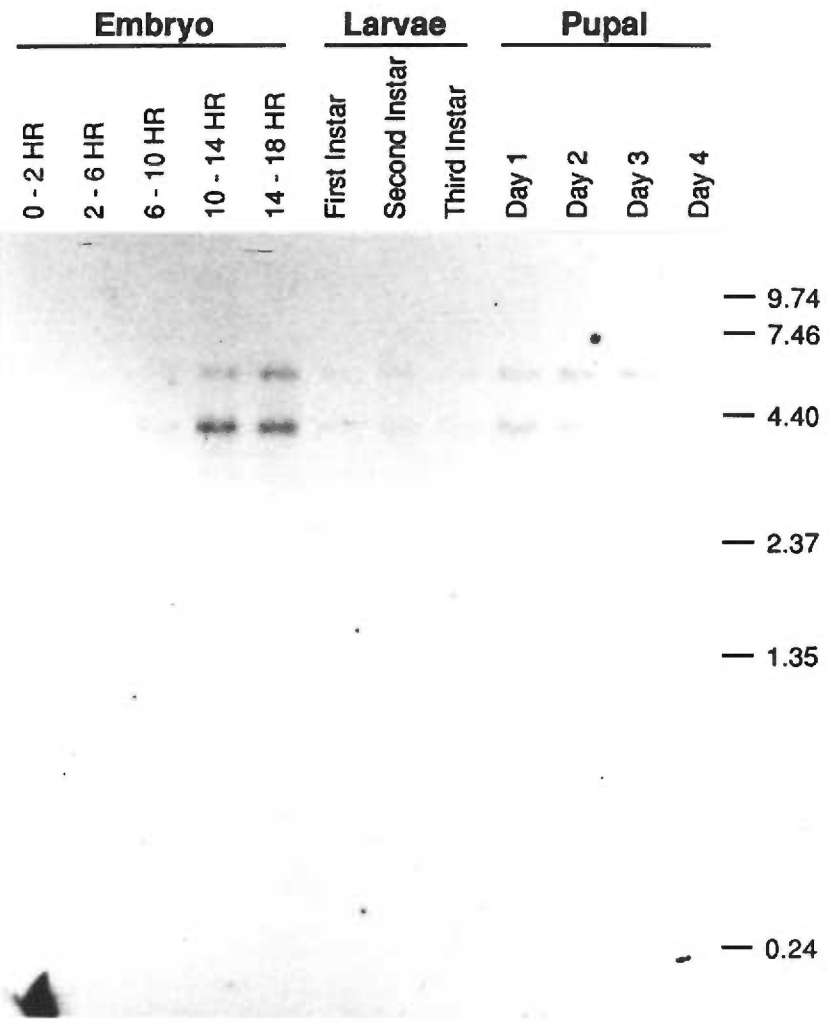
As shown in Fig. 23A, all developmental stages expressed the 4.2kb message. The 3.5kb message, on the other hand, appeared only in the 0-2hr embryo. No transcription occurs during the first two hours of embryo development. Transcripts that are found in these early embryos are transcribed maternally and subsequently introduced into maturing oocytes within the ovaries of female flies. Hence the 3.5kb and the 4.2kb transcripts are likely to be maternal transcripts. The 3.5kb transcript is not present after the 0-2hr stage of development but reappears in total RNA prepared from adult bodies. Developing oocytes in the ovaries which contain the maternally placed transcripts would account for the observation of the 3.5 kb message in the adult bodies (see fig. 25).

FIGURE 23A and 23B:

Stage-specific developmental expression of λDGo21 and λDGo59

Total RNA was prepared from the various embryonic, larval and pupal stages as described in Methods. The developmental stages are labelled. 30μg of total RNA from each stage was loaded in each lane. The blot was then hybridized with a 1.2kb EcoR1-BamH1 fragment that corresponds to the coding region of λDGo21 (Fig. 23A) or a 121-bp fragment corresponding to the 5'-untranslated region of λDGo59 (Fig.23B). Positions of molecular size standards are indicated (0.24-9.5kb RNA"ladder," Bethesda Research Laboratories)


Embryo
Larvae
Pupal
0 - 2 HR
2 - 6 HR
6 - 10 HR
10 - 14 HR
14 - 18 HR
First Instar
Second Instar
Third Instar
Day 1
Day 2
Day 3
Day 4
9.74
7.46
4.40
2.37
1.35
0.24


Embryo
Larvae
Pupal
0 - 2 HR
2 - 6 HR
6 - 10 HR
10 - 14 HR
14 - 18 HR
First Instar
Second Instar
Third Instar
Day 1
Day 2
Day 3
Day 4
9.74
7.46
4.40
2.37
1.35
0.24

Several studies have established the importance of the maternal transcripts to the developing embryo (167-169). Many different maternally specified gene products have been characterized that are critical to specifying the axial polarity of the embryo and as precursors to pattern formation such as segmentation. Pattern formation in *Drosophila* proceeds in a hierarchical manner (i.e.) maternal genes initiate this process by specifying anterior-posterior polarity to the egg. Subsequently this coarse positional information is refined by regulatory interactions between zygotic gene products during later stages of development. It is likely that the $G_o\alpha$ maternal transcripts will have an important role in transducing early development and differentiation processes.

The main events that transpire during *Drosophila* neurogenesis are as follows (170, 171). Between 4-9 hr of embryo development, neuroblasts are seen to be rapidly dividing. These cells are the precursors of the ganglion mother cells that later on will cluster and form the segmental ganglia characteristic of the insect nervous system. At the 10 hr stage of embryo development, the ventral nervous system, which until then had been a loose aggregate of segmentally arranged neuroblasts and ganglion mother cells, now proceeds to form connectives, that is, nerve formation begins. Principal transverse connections between the two brain lobes are also established during this period. Connections between the brain and frontal ganglia are also formed. Thus, this period is marked by a series of neural integrative events.

From Fig. 23A, it is clear that the 6.0 kb message first appears at the 10-14 hr stage of *Drosophila* development. Since this stage is

coincident with the formation of neuronal circuitry, it raises the possibility that $G_o\alpha$ proteins play a role in coupling receptors to early sensory transduction events. Interestingly, the nervous system is functional at 13 hours of embryogenesis as inferred from acetylcholinesterase activity (172, 173).

Fig. 23B shows that the probe specific to the 5'-untranslated region of λDGo59 appears to recognize the 6.0 kb and 4.2 kb transcripts, albeit weakly.
The probe does not recognize the 3.5 kb maternal transcript. These results are consistent with those of Fig.20, showing that the distribution of sequences found in the 5'-untranslated region of λDGo59 appear to be restricted and present at lower levels than those corresponding to λDGo21.

(viii) In situ hybridizations to Drosophila tissue sections:

Using riboprobes corresponding to the 3'-untranslated regions of λDGo21, the distribution of transcripts coding for this $G_o\alpha$-like protein in the CNS of *Drosophila* has been examined. As shown in Fig. 24, in a horizontal section of a *Drosophila* head, hybridization was highest in the cortex of the optic lobes and midbrain containing the neuronal cell bodies. An exception was the cortex of the lamina (first order optic neuropil) which showed only low levels of hybridization. In contrast to the cortex, the central neuropil regions composed of axons, synapses and fiber tracts showed only background levels of hybridization. The eyes, and interestingly, the fat body showed low levels of hybridization. Muscle had no detectable levels of hybridization. Control sections probed with sense strand transcripts

had low, uniform levels of hybridization. *In situ* hybridization with riboprobes specific to the 5'-untranslated region of each of the cDNAs (λDGo21 and λDGo59) showed no apparent difference in the distribution of these two forms in adult *Drosophila* heads.

The distribution of the $G_o\alpha$ transcript is quite similar to that previously reported for $G_s\alpha$ with two exceptions. $G_s\alpha$ transcript was not detected in the fat body and was at low levels in the optic lobe relative to the mid-brain. $G_o\alpha$ transcripts had a more uniform distribution throughout all cortical regions except for the lamina, which for both $G_s\alpha$ and $G_o\alpha$ (as well as $G_i\alpha$) were very low. Although, Fig.24 also shows that the transcripts coding for this $G_o\alpha$-like protein are present in the eyes, this observation is inconsistent with the results of Yoon et al and deSousa et al (165, 166). It is possible then, that the hybridization that is observed in the eyes is a fixation artifact. Also, from the results shown in Figs.7 and 8, the eyes do not appear to be the primary site of expression of the PTX substrate in the *Drosophila* nervous system.

Fig.25 shows the localization of transcripts corresponding to λDGo21 as seen in ovary sections of adult female flies. Hybridization is seen in developing and mature oocytes as well as in the nurse cells that surround the oocytes. These results serve to underline the possibility that the 3.5 kb body transcript (see Figs.19 and 23A) maybe a maternal transcript which is packaged into the unfertilized egg.

FIGURE 24:

In situ hybridization of λDGo21 probes to *Drosophila* head tissue sections

Riboprobes were generated to an 812-bp fragment from λDGo21 representing 3'-untranslated regions. A, dark field illumination; B, bright field illumination. Hybridization is detected in the cortical regions of the brain and optic lobes containing the neuronal cell bodies. Hybridization is absent in the central neuropil containing neuronal processes. Lower, yet significant levels of hybridization are observed in fat bodies and eyes. No hybridization is observed in muscle tissue. E, eye; ON, optic neuropil; BN, brain neuropil; F, fat body; M, muscle; arrows, cortex of the brain and optic lobe.

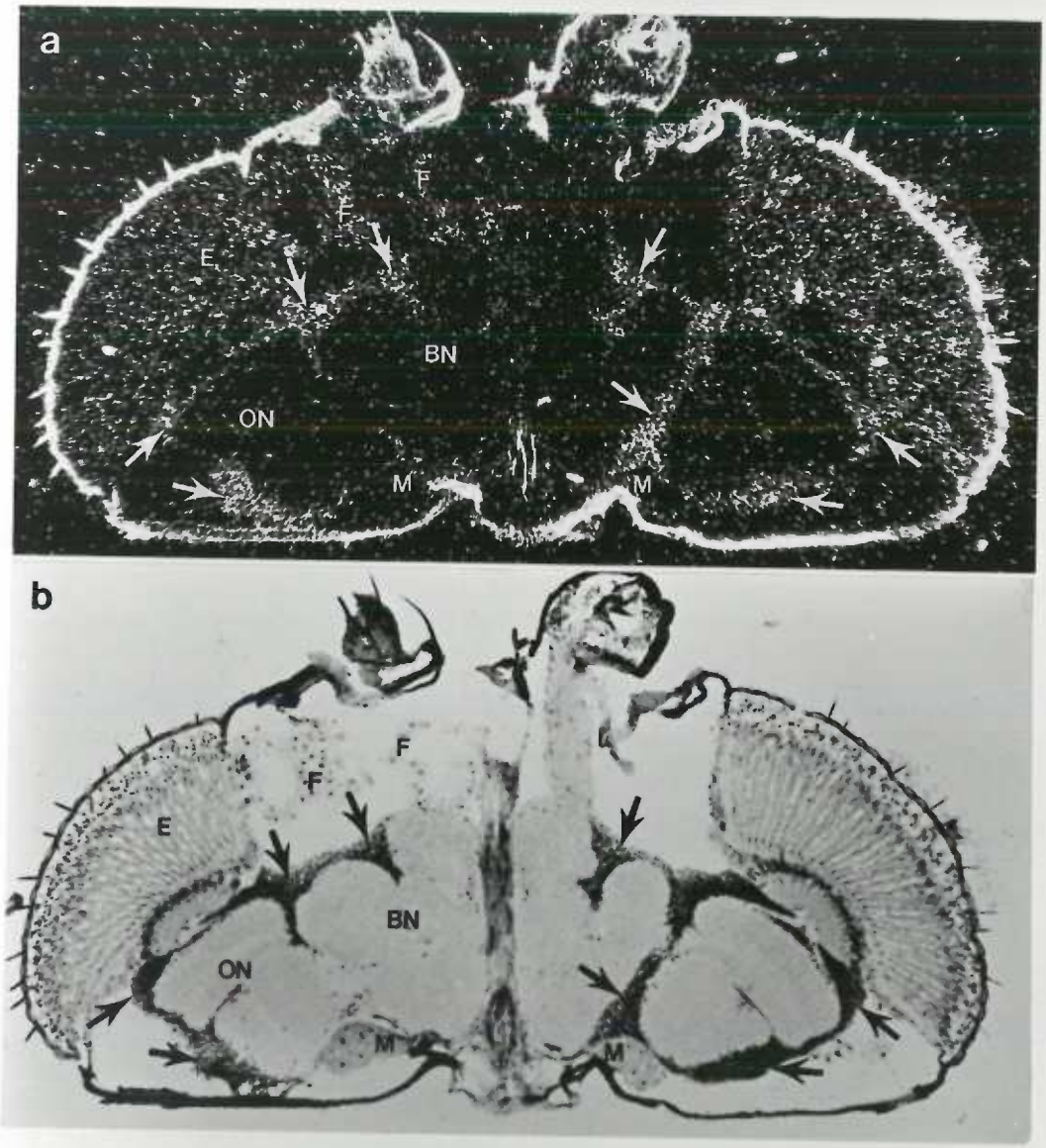
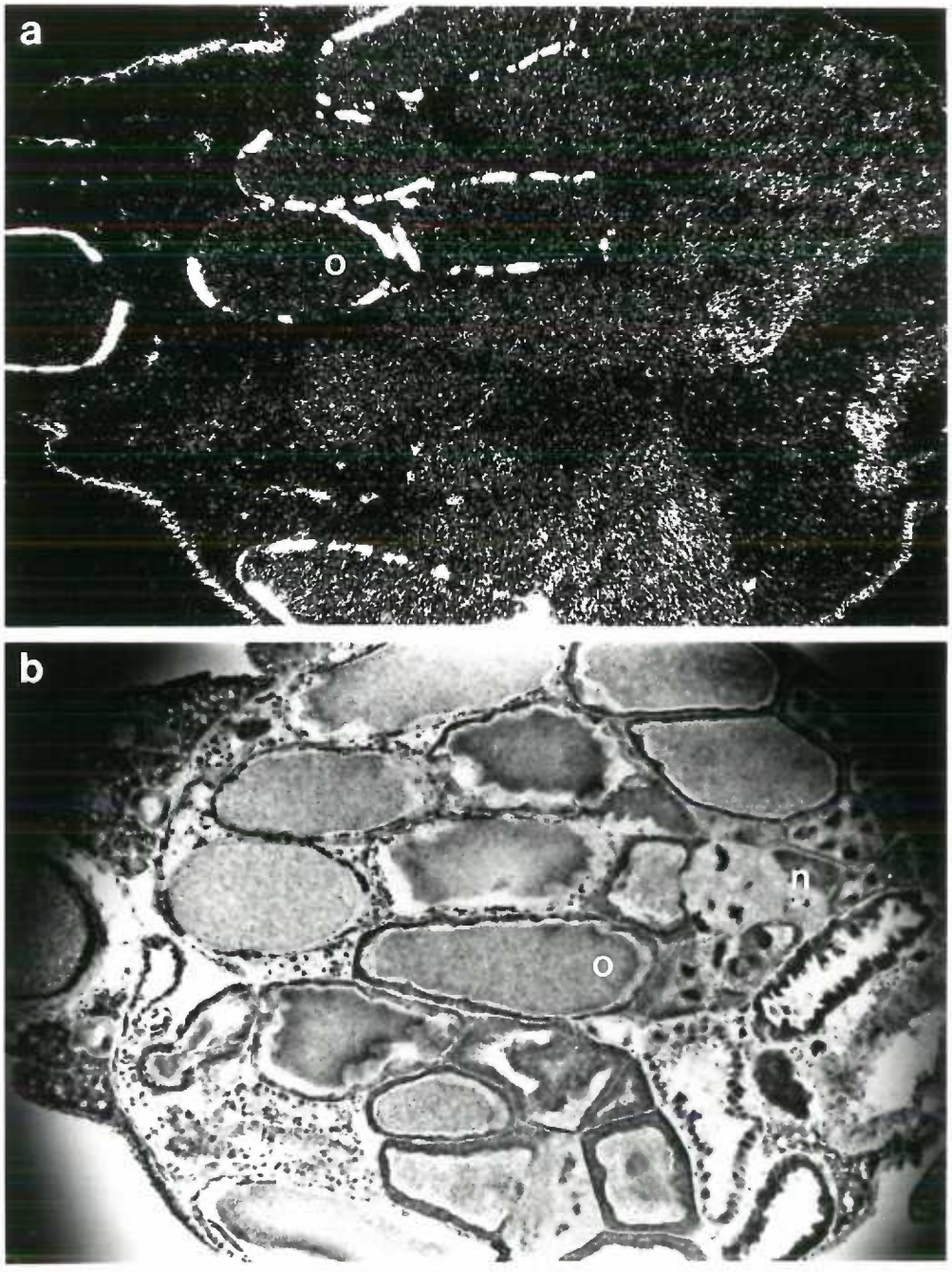

a
F
F
E
BN
ON
M
M
b
F
F
E
BN
ON
M
M

FIGURE 25:

In situ hybridization of λDGo21 probes to *Drosophila* ovary sections Riboprobes were generated to an 812-bp fragment representing the 3'-untranslated regios of λDGo21. a, dark field illumination; b, bright field illumination. Hybridization is seen in developing and mature oocytes (o) as well as in the nurse cells (n) surrounding the oocytes.


a
o
b
n
o

SUMMARY AND CONCLUSIONS

The critical role of G proteins in signal transduction had been conclusively established prior to the initiation of the work described herein. Our goal was to develop *Drosophila* as an invertebrate system in which to characterize the role of G proteins. The development and differentiation of the *Drosophila* nervous system have been extensively studied and furthermore, there exist paradigms such as biochemical and behavioural assays by which integrative nervous system function can be tested in this organism. Hence, the nervous system was the system of choice in which to study G proteins.

The work that is described in this thesis was initiated with experiments employing established criteria to identify G proteins that were present in the nervous system of *Drosophila*. The first assays that were performed on fly neuronal membranes measured the binding of a non-hydrolyzable analog of GTP (i.e.) ^{35}SGTPγS. The results revealed that fly heads contained a GTP binding activity that compared remarkably well with GTP binding levels that were observed in vertebrate brain homogenates. In vertebrates, this activity is largely contributed by $G_o\alpha$, the so-called 'other' Gα subunit.

The second assay that was used to characterize fly neuronal G proteins was modification by bacterial toxins. These toxins covalently modify the α subunit of G proteins in vertebrates. PTX modified a *Drosophila* substrate that migrated on SDS gels with apparent molecular weight of 40 kD. By analogy to vertebrate

systems where $G_o\alpha$ (40 kD) is a PTX substrate found at high concentrations within the nervous system, it was likely that the *Drosophila* neuronal toxin substrate represented a $G_o\alpha$ homologue. Moreover, the *Drosophila* PTX substrate was modulated by nucleotides in a manner similar to vertebrate toxin substrates.

This last observation suggested that the *Drosophila* G protein is likely to be coupled to β and γ subunits. This deduction was based on the fact that GTPγS interfered with PTX labelling. Studies in vertebrates, had shown that GTPγS had the effect of dissociating the G protein heterotrimer. Since the toxin had a requirement for the β and γ subunits to be complexed to the α subunit, heterotrimer dissociating agents prevented toxin modification. As expected, *Drosophila* neuronal membranes pretreated with GTPγS and Mg^{+2} were poor substrates for PTX indicating a need for β and γ subunits. Recent cloning of a β subunit gene of *Drosophila* extends the evidence for the G proteins being conserved as heterotrimers in *Drosophila* (174).

The availability of a spectrum of Gα antibodies that have cognate specificity for the many vertebrate Gαs that have been characterized, provides an invaluable tool with which to ascertain the nature and identity of homologous Gαs in *Drosophila*. The experiments described in Chapter 3 were successful in establishing unambiguously that the *Drosophila* Gα present in nervous tissue crossreacted solely with $G_o\alpha$ specific antibodies. These antibodies were also able to immunoprecipitate the fly neuronal PTX substrate. Based on these results, indications were that the *Drosophila* neuronal G protein that was PTX modified was indeed a $G_o\alpha$ homologue.

In order to confirm these results and establish the homology of the putative *Drosophila* $G_o\alpha$ to its vertebrate counterpart at the molecular level, the next step entailed screening a *Drosophila* head cDNA library using $G_o\alpha$ cDNA (from rat brain) as a probe. Of the putative $G_o\alpha$-like cDNAs that were obtained from this screening, the clone containing the largest insert was chosen for sequencing. Comparison of the sequence data with vertebrate $G\alpha$ sequences showed that the deduced amino acid sequence bore 82% sequence identity with vertebrate $G_o\alpha$. (The similarity of the *Drosophila* $G_o\alpha$ to vertebrate $G_s\alpha$, $G_t\alpha$ and $G_i\alpha$ was between 39-67%).

The data described in Chapter 4 establishes, on the basis of homology, that the *Drosophila* cDNAs encode G proteins. G proteins from several sources have been cloned, including simple eukaryotic organisms such as yeast and slime mold. The homology of these G proteins to vertebrate G proteins is considered to be sufficient to establish their being bona fide $G\alpha$s despite sequence similarity that is only on the order of approx. 40%. Additionally, most of the homology is restricted to regions that are involved in the binding of the guanine nucleotide. In marked contrast, the *Drosophila* $G_o\alpha$ is conserved at 82% (amino acid identity) to the rat brain $G_o\alpha$. The cDNAs diverge in parallel from other $G\alpha$s in the regions of $G_o\alpha$ that confer specificity to it; namely at the carboxyl terminus where the vertebrate $G\alpha$s diverge from each other. One of the functions of $G_o\alpha$ in the vertebrate nervous system is to couple receptors to modulation of ion channel activity. Convincing evidence exists for $G_o\alpha$ linking receptors such as α-adrenergic, serotonergic and dopaminergic as well as neuropeptides like neuropeptide Y to the

inhibition of Ca^{+2} channel activity. $G_o\alpha$ has also been shown to activate K+ channels directly in brain membranes.

With the remarkable degree of conservation of $G_o\alpha$ that is seen in *Drosophila*, it is predictable that a similar function of Ca^{+2} and/or K^+ channel modulation would be subserved by this G protein in this organism. It is highly unlikely that the *Drosophila* $G_o\alpha$-like protein would be mediating a novel and yet uncharacterized function. This lends credence to the idea that the invertebrate and vertebrate $G\alpha s$ diverged from an ancient primordial gene and therefore are expected to conserve many of the functions for which they initially evolved. The molecular characterizations of the *Drosophila* $G_o\alpha$-like cDNA are described completely in Chapter 4.

An interesting finding that emerged from the sequencing of other $G\alpha$ cDNAs was the characterization of an alternate form of $G_o\alpha$ in flies. This cDNA diverged from the initial clone only at its amino terminus, specifically in seven residues of the first 21 (in addition to the 5'-untranslated region). In vertebrates, there is evidence for distinct forms of $G_o\alpha$. Goldsmith et al have recently purified a novel $G_o\alpha$ ($G_o\alpha$*) from bovine brain(155). $G_o\alpha$* is immunologically indistinguishable from $G_o\alpha$. This form has not been characterized at the molecular level. The elucidation of the gene structure of vertebrate $G_o\alpha$ will be important to confirm whether $G_o\alpha$ subtypes exist.

The diversity that was observed in the *Drosophila* cDNAs in all probability arises from alternate splicing mechanisms. The isolation and characterization of genomic clones corresponding to $DG_o\alpha$-like subunits substantiates our finding. The $DG_o\alpha$ gene is reported to be

comprised of eight exons. The two alternate forms show variability in the pattern of usage of the first exon. Moreover, a putative splice site is present at the amino terminus region of the $DG_o\alpha$ gene where the two clones diverge. These findings suggest that this may be one viable mode for *Drosophila* Gαs to generate diversity so as to facilitate interaction with a wider spectrum of effectors (or receptors).

Northern analysis examining the developmental expression of the two forms of cDNAs brought to light an interesting finding. Two transcripts are seen to be expressed early in embryonic development. Since only maternal transcripts are present at the 0-2 hr stage of development of the embryo, the role of Go in development and differentiation may be a significant one. Maternal transcripts are important in early developmental events such as specification of axial polarity and pattern formation. In fact, zygotic transcription does not occur in the embryo until the thirteenth mitotic division of the embryo (at two hours of development). One of the transcripts (body-specific) is specifically present only in the 0-2 hr embryo, while the other persists through development into the adult. A third transcript (head-specific) can be detected in the 10-14 hr embryo. This phase of development corresponds to the formation of complex neuronal circuitry in *Drosophila* and coincides with the condensation of the nervous system. Taken together, these results suggest that Go proteins may play a role in development and differentiation, especially within the nervous system.

The $G_o\alpha$ gene was determined to be present in single copy in the fly genome by Southern blot analysis. The resolution of *in situ*

localization of the gene to polytene chromosomes did not permit correlation with any previously known mutations which mapped to this region. *In situ* hybridization to *Drosophila* tissue sections allowed the localization of transcripts that correspond to the $DG_o\alpha$ gene. By this technique, ovaries of mature female flies were shown to contain significant levels of hybridization. Cell bodies of neuronal cells (of head sections) were also seen to contain $DG_o\alpha$ transcripts. Very little hybridization was seen in the neuropil, the region that contains the processes of the neurons. The data reiterates the likelihood of *Drosophila* $G_o\alpha$ playing a central role in sensory transduction as it pertains to nervous system function.

The initial characterization of $G_o\alpha$ like proteins in the central nervous system of *Drosophila* has been encouraging and leads us to believe that this protein is conserved in this organism at a fundamental level. Furthermore, the early studies described in this thesis culminated in the isolation of a $G_o\alpha$ homologue with remarkable homology to vertebrate $G_o\alpha$. The experiments detailed have provided a framework upon which to base future studies. These might incude the following.

The cloning and isolation of the $DG_o\alpha$ cDNA has provided a molecular tool that can be utilized advantageously to address a number of questions that are pertinent to signal transduction within the nervous system. For instance, Northern analysis of the different developmental stages suggests that there are $DG_o\alpha$ transcripts that are regulated in a stage specific manner. This information can be used in the design of experiments that will assess the significance of these stage specific transcripts.

First, a thorough characterization of the gene structure of *Drosophila* $G_o\alpha$ will be necessary to correlate how the various stage specific and tissue specific transcripts might be generated. The partial gene structure that has been reported for the *Drosophila* $G_o\alpha$ gene does not account for all the different transcripts that are observed on Northern analysis. Complex splicing events that involve the use of different exons in combination with the use of different promoters might account for the variety of transcripts that are present.

Future experiments might include the injection of antisense RNA (for specific transcripts) into the developing embryos, and then observing the resulting effects at further developmental stages or even at the behavioural level. For example, the effect of obliterating a specific transcript can be examined within the developing nervous system. There are distinct anatomical features that identify the progression of normal nervous system development. Since $G_o\alpha$ is predicted to have a major role in development and differentiation, the effects of disrupting the normal $G_o\alpha$ expression might have pleiotropic effects on the organism.

Evidence has suggested that there are at least two forms of $DG_o\alpha$-like cDNAs that are produced by alternate splicing. The sequence of these two forms differs at the amino terminus. Peptide specific antibodies have been used extensively in the detection of subtypes of $G\alpha$s in vertebrates. Antibodies can now be synthesized that have unique specificities for the two forms of the $DG_o\alpha$ proteins that are generated as a result of two $DG_o\alpha$ cDNAs. Using these antibodies, *in situ* localization of these proteins can be determined.

This might provide further insights into regulatory mechanisms that underlie the spatial and temporal expression of these alternate forms.

One of the principal advantages of using *Drosophila* as an insect model, is the ease with which genes (once identified) can be manipulated and then reintroduced into the organism to study the effects of alterations that have been introduced. These are well established procedures that allow the construction of transgenic flies using P-element mediated transformations. Using P-element vectors that carry alterations that will disrupt the $G_o\alpha$ gene, or by using the cytogenetic map position of the $DG_o\alpha$ gene to create a deletion mutant, it is possible that these mutations (or deletions) can be then examined for effects on the organism by any of a number of different criteria.

These experiments serve to demonstrate just some of the many possible approaches that can be taken towards extending current understanding of the role of G proteins in neuronal signal transduction. No doubt the results obtained will have considerable bearing on the significance of G proteins in the nervous system of vertebrates. The conservation of the components of signal transduction in *Drosophila* is yet another comforting example of the underlying uniformity of cellular machinery.

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