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Immunological Localization of the GTP-Binding Protein G_o in Different Tissues of Vertebrates and Invertebrates

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SUMMARY

Bovine brain contains two GTP-binding proteins, G_i and G_o, which are substrates for ADP ribosylation by pertussis toxin. The G_i protein mediates hormone and GTP inhibition of adenylate cyclase, but the function and the precise tissue distribution of G_o are unknown. To immunologically probe the localization of G_o, we have purified the $G_{o\alpha}$ and $G_{\beta,\gamma}$ subunits of G_o and have raised antibodies against them. The polyclonal anti-$G_{o\alpha}$ antibodies obtained were very selective for $G_{o\alpha}$ compared to $G_{i\alpha}$ or $G_{s\alpha}$. The positive $G_{o\alpha}$ and $G_{\beta,\gamma}$ immunoreactivities were investigated in different tissues of vertebrates and invertebrates on immunoblots after gel electrophoresis of the crude membranes. The anti-$G_{\beta,\gamma}$ antibodies recognized a 35–36-kDa protein in brain of vertebrates such as mammals (rat), avians (pigeon), amphibians (frog), fish (trout), and reptiles (turtle) but not in the invertebrates such as molluscs (snail) and insects (locust). With the anti-$G_{o\alpha}$ antibodies a high level of immunoreactivity was detected at molecular weights of 39,000–40,000 in the brain of invertebrates as well as in the central nervous system of vertebrates. Moreover, ADP ribosylation with pertussis toxin occurred in the nervous system of invertebrates. These results suggest that the GTP-binding proteins of invertebrates either are devoid of $G_{\beta,\gamma}$ subunit or, more probably, possess immunologically different $G_{\beta,\gamma}$ subunits when compared to those of vertebrates. In the vertebrates, $G_{o\alpha}$ immunoreactivity was also present in the peripheral nervous system in areas such as the superior cervical ganglia and sciatic nerve. When examined with the anti-$G_{o\alpha}$ antibodies, the neuro- and adenohypophysis exhibited a similar immunoreactivity which was about 6 times lower than in brain. Our antibodies also recognized a 40-kDa protein in human adipocytes but at a concentration 17 times lower than that recognized in brain. Taken together, these data show that the $G_{o\alpha}$ subunit is well conserved through evolution and, furthermore, confirm that $G_{o\alpha}$ is not strictly limited to the nervous system. This suggests that the protein G_o ensures a function required for neuronal activity but also present in some other non-nervous tissues.

There is increasing evidence demonstrating that extracellular signals leading to intracellular events are in many cases mediated through a family of proteins that bind GTP (for review see Ref. 1). This family of signal-coupling proteins (or G-proteins) has been studied extensively in the hormonal adenylate cyclase and visual phototransduction systems. In these systems, the transduction proteins have been isolated (2–4). For hormone-regulated adenylate cyclase systems there are two proteins: G_s, responsible for hormonal activation of the enzyme, and G_i, which regulates its inhibition. In the visual system another regulatory protein, named transducin (T), mediates the activation of a cGMP phosphodiesterase. All of these proteins are heterotrimers composed of α, β and γ subunits (5).

The α subunit of each of these proteins contains the guanine nucleotide-binding site, a GTPase activity, and can be ADP-ribosylated by the action of bacterial toxins. Cholera and pertussis toxins ADP-ribosylate $G_{s\alpha}$ (M_r = 45,000–52,000) and $G_{i\alpha}$ (M_r = 41,000), respectively, whereas both toxins catalyze the ADP ribosylation of T_α (M_r = 39,000). The β and γ subunits are tightly bound together and common to both G_s and G_i. The γ but probably not the β subunit of G_s and G_i is different from that of transducin (5). In SDS-PAGE analysis, the β subunit often appears as a doublet of 35 and 36 kDa. This observation is believed to be due to the presence of two distinct β subunits rather than the partial proteolysis of the Parger β subunit (6).

Other GTP-binding proteins expected to play a role in the activation of the hydrolysis of the polyphosphoinositides (7, 8) or ion channel opening (9, 10) have not yet been isolated. However, a GTP-binding protein of unknown function, G_o, has been purified from bovine brain (11, 12). The α component of this protein ($G_{o\alpha}$, M_r = 39,000) binds guanine nucleotides and associates with the same $G_{\beta,\gamma}$ subunits as do $G_{s\alpha}$ and $G_{i\alpha}$. The α subunit of G_o can be ADP-ribosylated by pertussis toxin. In bovine brain, G_o is more abundant than G_i and represents up

ABBREVIATIONS: G-protein, any GTP-binding protein that resembles a family of homologous proteins such as G_s and G_i, respectively, stimulatory or inhibitory GTP-binding proteins of adenylate cyclase; $G_{o\alpha}$, the α subunit of GTP-binding protein of unknown function (G_o); $G_{\beta,\gamma}$, the dimer of the β and γ subunits supposedly common to G_s, G_i, and G_o; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

to 1% of membrane proteins (13). The GTPase activity of G_o is more easily detectable than that of G_i (12), but both can reconstitute guanine nucleotide shifts in agonist affinity for brain muscarinic receptor (14, 15).

Immunological studies were carried out with antibodies raised either against purified brain G-protein (16) or against a specific peptide sequence of $G_{o\alpha}$ (17). These studies revealed that brain tissue is the more abundant source of G_o. However, immunoreactivity to various anti-G_o antibodies indicates that a small amount of G_o-like protein can also be detected in peripheral tissues such as liver, heart, and kidney, (16, 17). The predominant localization of G_o in brain and its presence in adrenal medulla (17) indicate that the function of G_o might be related to neuronal activity. In order to gain more insights into the function of G_o, we investigated the precise localization of G_o in central or peripheral nervous system and in non-neuronal tissues both in vertebrates and in invertebrates. Our results demonstrated that $G_{o\alpha}$ is a protein highly conserved and essentially localized in nervous tissues.

Materials and Methods

Preparation of purified G_o subunits. Purification of the mixed preparation of G_o and G_i was achieved from bovine brain membranes by successive elution through DEAE-Sephacel (Pharmacia), AcA 34 (LKB), and heptylamine-Sepharose columns as described by Sternweis and Robishaw (11). Pure $G_{o\alpha}$ subunit was obtained from the G_i and G_o mixture by further separation on heptylamine-Sepharose using the same gradient system as in the third step but with the continuous presence of 10 μM $AlCl_3$ and 10 mM NaF. The first fractions, analyzed by silver-stained SDS-PAGE, were judiciously pooled and dialyzed against 20 mM Hepes-NaOH (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 0.1% Lubrol and concentrated by ultrafiltration. The $G_{\beta,\gamma}$ entity was obtained from the mixture of G_i and G_o by chromatography over DEAE-Sephacel in a buffer containing 0.6% Lubrol, $AlCl_3$, and NaF as described by Neer *et al.* (12). The pure $G_{\beta,\gamma}$ sample was dialyzed and concentrated as was done for $G_{o\alpha}$. These purified $G_{o\alpha}$ and $G_{\beta,\gamma}$ fractions were used for the immunization of the rabbits.

Production of antisera. Hybrids from New Zealand White rabbits (HY 278, Elevage Scientifique des Dombes, France) were injected intradermally at multiple sites on the back with 50 μg of purified $G_{\beta,\gamma}$ or $G_{o\alpha}$ prepared as a 1:3 emulsion with complete Freund's adjuvant. Two weeks after the initial injection, the rabbits were injected with 25 μg of identical sample prepared in the same manner with incomplete Freund's adjuvant. Antisera were collected weekly, stored at −80°, and used without further purification. Anti-$G_{o\alpha}$ antibodies were affinity purified onto a column of activated gel (Sepharose CH, Pharmacia) to which the mixture of G_o-G_i had been covalently linked.

Preparation of the membranes from different tissues. The different brains or peripheral tissues were dissected and homogenized in about 20 volumes of 50 mM Tris-HCl (pH 7.5), 3 mM EDTA, 5 μg/ml soybean trypsin inhibitor. 0.1 mM phenylmethylsulfonyl fluoride either with a Polytron (Kinématica) or, for small samples, with a Teflon-glass potter and centrifuged 10 min at 20,000 × *g*. Pellets were taken up in the same buffer for protein determination and stored in liquid nitrogen or at −80°.

SDS-polyacrylamide gel electrophoresis. One hundred μg of protein were pelleted 5 min in an Eppendorf centrifuge. Each particular fraction was solubilized in 10 μl of 50 mM Tris-HCl (pH 6.8), 10% SDS, and 0.5 mM dithiothreitol by incubation for 2 hr at 30°. The samples were cooled mixed with 5 μl of 9 mM *N*-ethylmaleimide (except when stated) and allowed to stand 15 min at room temperature. Then, 15 μl of a solution of 100 mM Tris-HCl, pH 6.8, 10% SDS, 10% β-mercaptoethanol, and 20% glycerol were added and the whole sample was loaded onto the gel. Analysis of the molecular weight of the proteins was performed on 10% acrylamide gels prepared according to the method of Laemmli (18). Proteins were stained with silver or blotted onto nitrocellulose.

Immunoblotting. Proteins were transferred onto nitrocellulose sheets as previously described (19). The nitrocellulose was dried and stained with red Ponceau S in order to assess the quality of the transfer. After two 15-min washes in 10 mM Tris-HCl (pH 7.5), 500 mM NaCl, the nitrocellulose was incubated in the same buffer supplemented with 3% gelatin to block nonspecific protein binding. The blots were then incubated overnight at room temperature in the same buffer containing 1% gelatin and a 1:100 dilution of rabbit antiserum or 0.3% gelatin with the affinity purified antibodies (10 μg/ml). The nitrocellulose sheets were washed six times with 10 mM Tris, 500 mM NaCl, 0.05% Tween 20 over a 2-hr period. The blot was incubated with radioiodinated protein A (70,000–100,000 cpm/ml) in 50 ml of Tris-buffered saline without Tween, containing 1% gelatin and 0.02% NaN_3 for 60 min at room temperature. The blot was then washed extensively in Tris-buffered saline containing 0.05% Tween 20. Dried blots were exposed to Kodak XAR film with Dupont Cronex image-intensifying screens at −80° for 16–48 hr.

Quantitation of $G_{o\alpha}$ and $G_{\beta,\gamma}$ in tissues. For each tissue, three membrane samples containing 10–50 μg of protein and five different amounts of $G_{o\alpha}$ and $G_{\beta,\gamma}$ subunits (0.05–0.5 μg) were loaded on the same gel. The protein A immunoblot was conducted as described above and the autoradiogram was used to identify the relevant bands on nitrocellulose. The bands were carefully cut out for radioactivity determination.

ADP ribosylation of membrane components and purified proteins with pertussis toxin. ADP ribosylation with pertussis toxin was carried out as described by Ribeiro-Neto *et al.* (20) with minor modifications according to the method of Sternweis and Robishaw (11). Briefly, 100 μg of particulate fraction or 1 μg of purified protein were incubated for 60 min at 30° with 1 μM NAD, 0.5–1 μCi of [α-^{32}P]NAD (New England Nuclear), 1 mM ATP, 100 μM GTP, 10 mM thymidine, 1 mM EDTA, 0.1 mM $MgCl_2$, 1 mg/ml L-myristyl phosphatidylcholine, 70 mM Tris-HCl, pH 8, and 100 ng of pertussis toxin (List Biological Laboratories) in a 60-μl assay volume. The reaction was stopped by the addition of 2% SDS with 100 μg/ml bovine serum albumin, and proteins were precipitated overnight at 4° with 10% trichloroacetic acid. After centrifugation for 10 min at 10,000 × *g*, the pellets were washed twice with ethyl ether, dried, and analyzed by SDS-PAGE.

Protein determination. Protein was determined by the method of Lowry *et al.* (21) or by the method of Schultz *et al.* (22) using tritiated dinitrofluorobenzene.

Results

Purification of the subunits of G_o and characterization of the antibodies against $G_{o\alpha}$ and $G_{\beta,\gamma}$. The preparations of mixed G_i and G_o were purified from calf cerebral cortex according to the method of Sternweis and Robishaw (11). The subunits were dissociated by treatment with sodium fluoride and aluminium chloride. Elution through heptylamine-Sepharose or DEAE in the continuous presence of fluoride and aluminium allowed the separation of $G_{o\alpha}$ and $G_{\beta,\gamma}$, respectively (12). Fig. 1 shows an SDS-PAGE silver staining of the various purification steps, along with the purified $G_{o\alpha}$, $G_{\beta,\gamma}$ and G_i proteins. These fractions of pure $G_{o\alpha}$ and $G_{\beta,\gamma}$ were used to raise polyclonal antibodies. The specificity of $G_{o\alpha}$ antibodies was evaluated (Fig. 2, A and B) by the comparison of SDS-PAGE silver stain (Fig. 2A) and immunoblot (Fig. 2B) of different purified fractions. Note that the autoradiogram of the immunoblot was overexposed in order to detect any eventual cross-reactivity with proteins other than the antigen. The anti-$G_{o\alpha}$ antibodies recognized a band of protein at 39–40 kDa in the mixture of G_o plus G_i (Fig. 2B, *lane a*) and in the pure $G_{o\alpha}$ fraction (Fig. 2B, *lane b*). When the amount of $G_{o\alpha}$ was progressively decreased

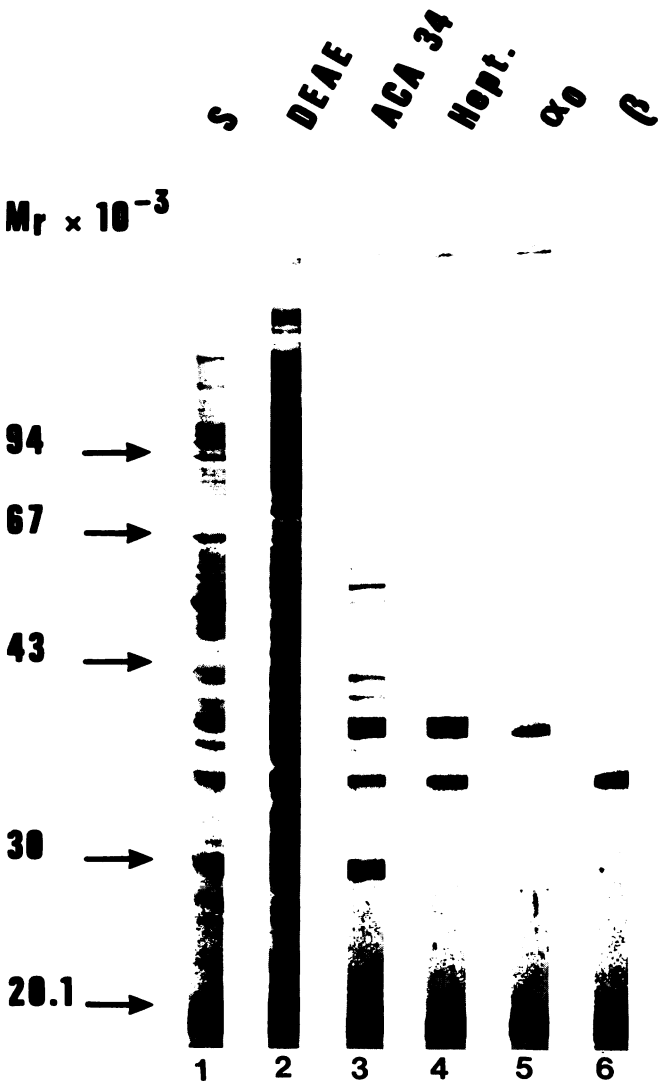

Fig. 1. SDS-polyacrylamide gel analysis of the purification steps of $G_{o\alpha}$, $G_{\beta,\gamma}$ and G_i proteins. The purification of G_o and G_i was achieved as reported (11), and pure $G_{o\alpha}$ and $G_{\beta,\gamma}$ subunits were obtained as described in Materials and Methods. Samples not treated by *N*-ethylmaleimide were: *lane 1*, soluble cholate extract (*S*); *lanes 2–4*, pooled fractions after DEAE, AcA 34, and heptylamine-Sepharose (*Hept.*) columns, respectively; *lane 5*, $G_{o\alpha}$ (α_o); *lane 6*, $G_{\beta,\gamma}$. The gel was stained with silver.

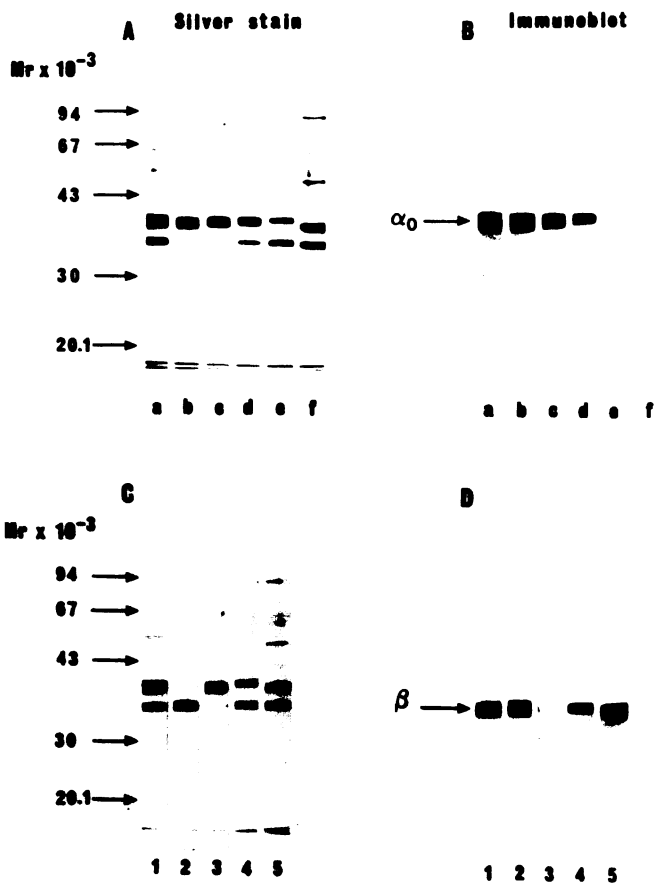

Fig. 2. Analysis of the specific reaction of antisera against $G_{o\alpha}$ and $G_{\beta,\gamma}$ subunits with G-protein on immunoblot. G-protein subunits and transducin not treated with *N*-ethylmaleimide were subjected to electrophoresis through 0% polyacrylamide and either stained with silver (A and C) or blotted onto nitrocellulose and analyzed with the anti-$G_{o\alpha}$ (B) or anti-$G_{\beta,\gamma}$ (D) antisera as described under Materials and Methods. *Lanes a* and *1*, G_o + G_i; *lanes b* and *3*, pure $G_{o\alpha}$; *lane c*, $G_{o\alpha}$ + $G_{i\alpha}$; *lane d*, $G_{o\alpha}$ + g_i; *lanes e* and *4*, G_i; *lanes f* and *5*, transducin, *lanes 2*, pure $G_{\beta,\gamma}$. Each lane was loaded with about 1 μg of protein except *lanes a*, *f*, *1*, and *5* (1.5 μg).

and that of $G_{i\alpha}$ increased, (Fig. 2, A and B, *lanes c–e*), the immunoreactivity diminished and almost vanished with the purified G_i fraction (Fig. 2B, *lane e*). This indicated that the anti-$G_{o\alpha}$ antibodies under our experimental conditions had a negligible cross-reactivity with the 41-kDa $G_{i\alpha}$ subunit. The high specificity of the anti-$G_{o\alpha}$ antibodies is further confirmed by the results obtained with ADP-ribosylated G-proteins (Fig. 3). This figure compares the autoradiogram of the radioactive ADP-ribosylated $G_{o\alpha}$ + $G_{\beta,\gamma}$ and G_i with the immunostaining of the similar nonradioactive ADP-ribosylated fractions. It is clear that while the anti-$G_{\beta,\gamma}$ antibodies cross-reacted with the β subunit of both fractions, the $G_{o\alpha}$ antibodies did not recognize the α subunit of the G_i fraction.

The specificity of the antibodies raised against $G_{\beta,\gamma}$ subunit was evaluated (Fig. 2, C and D) by comparison of SDS-PAGE silver stain (Fig. 2C) and immunoblot (Fig. 2D) of different purified proteins. The anti-$G_{\beta,\gamma}$ antibodies show no cross-reactivity with the α subunit of either G_o (Fig. 2D, *lanes 1* and *3*), G_i (Fig. 2D, *lane 4*), or transducin (Fig. 2D, *lane 5*). On the contrary, all β subunits from brain G-proteins (Fig. 2, C and D, *lanes 1*, *2*, and *4*) and transducin (Fig. 2, C and D, *lane 5*) cross-reacted with the anti-$G_{\beta,\gamma}$ antibodies.

Cross-reactivity of the antibodies with brain membranes from different species. The cross-reactivity of the anti-$G_{o\alpha}$ and anti-$G_{\beta,\gamma}$ antibodies with proteins of the brain membranes of different vertebrate and invertebrate species was investigated. The complete autoradiograms of the immunoblots are shown in Fig. 4. Fig. 4A clearly establishes the existence of a positive immunoreactivity of our anti-$G_{o\alpha}$ antibodies with a protein of M_r = 39,000–40,000 in the nervous system for all of the species tested. Examination of the blot with anti-$G_{\beta,\gamma}$ antibodies (Fig. 4B) revealed that a β-like subunit at 35–36 kDa was detectable only in vertebrate species. This result is in agreement with our previous study in which antibodies against the β subunit of transducin (T_β) exhibited positive immunoreactivity toward the 35–36-kDa protein in vertebrate but not invertebrate tissues (19). Note that the anti-$G_{o\alpha}$ antibody very selectively recognized a protein at 39–40 kDa, whereas few other proteins were stained immunochemically. In contrast, the

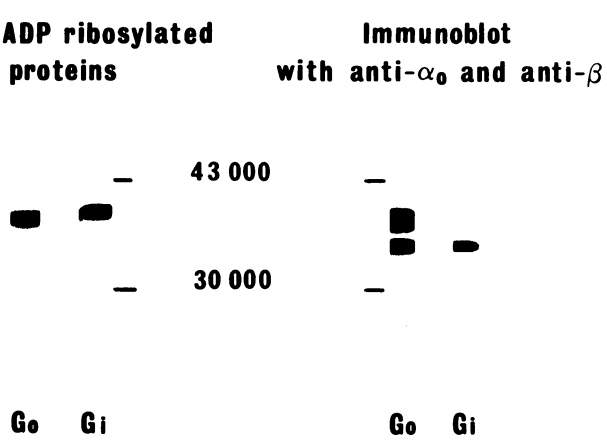

Fig. 3. Specificity of the antibodies against ADP-ribosylated G_o and G_i. Purified $G_{o\alpha}$ plus $G_{\beta,\gamma}$ subunits (*Go*) and G_i, from the fractions shown in Fig. 2, were ADP-ribosylated with pertussis toxin with nonradioactive NAD(10 μM) or with [^{32}P]NAD. $G_{o\alpha}$ + $G_{\beta,\gamma}$ (2.5 μg) and 1.0 μg of G_i of the nonradioactive ADP-ribosylated material were loaded with (*left*) or without (*right*) 2000 cpm of the corresponding ^{32}P-labeled material onto the same gel (see Materials and Methods). After electrophoresis, the gel was blotted onto nitrocellulose. The nitrocellulose was cut and the nonradioactive blot was incubated with the anti-$G_{o\alpha}$ and anti-$G_{\beta,\gamma}$ antibodies and then with radioiodinated protein A. The two blots were exposed for 66 hr. The figure shows only the relevant portion of the autoradiograms.

anti-$G_{\beta,\gamma}$ antibodies cross-reacted with proteins at molecular weights higher than 36,000. This occurred especially with the mammal brain of bovine and rat.

Cross-reactivity of the antibodies with peripheral nervous and non-nervous tissues of vertebrates. Different peripheral tissues mainly from rat, bovine, and human were examined to assess the immunoreactivity toward the anti-$G_{o\alpha}$ and anti-$G_{\beta,\gamma}$ antibodies. Fig. 5 shows positive immunoreactivity in three neuronal tissues other than the central nervous system: the posterior pituitary, the superior cervical ganglia, and the sciatic nerve. There are also reports concerning an immunoreactivity in non-neuronal tissues (16, 17). Similarly, we demonstrated a G_o-like material in two tissues not yet examined: first, in the anterior pituitary in which the immunostaining was quite equivalent to what was found in innervated posterior pituitary (Fig. 5) and, second, in human adipocyte (Fig. 5), in which there was also a positive immunoreactivity. However, in the adipocyte purified membrane preparation, the immunoreactive protein had a slightly lower mobility (40 kDa) in SDS-PAGE than those of rat brain central cortex or anterior pituitary (39 kDa). Indeed, a positive immunoreactivity was also obtained in both tissues with the affinity purified anti-$G_{o\alpha}$ antibodies (data not shown).

In order to estimate the relative amount of the $G_{o\alpha}$ and $G_{\beta,\gamma}$ immunoreactivities in the central and peripheral tissues, we quantified the radioactivity of the ^{125}I-iodinated protein A bound to the corresponding immunoblotted protein (see Materials and Methods). The relationships between the amount of protein A and the quantity of either the pure $G_{o\alpha}$ or $G_{\beta,\gamma}$ subunits were established (data not shown). From these linear standard curves, the quantities of $G_{o\alpha}$ and $G_{\beta,\gamma}$ subunits present in the same tissues were calculated and are reported in Table 1. Our results confirm that $G_{o\alpha}$ represents about 1% of the rat brain membranes and also indicate that the concentrations of $G_{o\alpha}$-like protein in anterior pituitary and adipocyte membranes

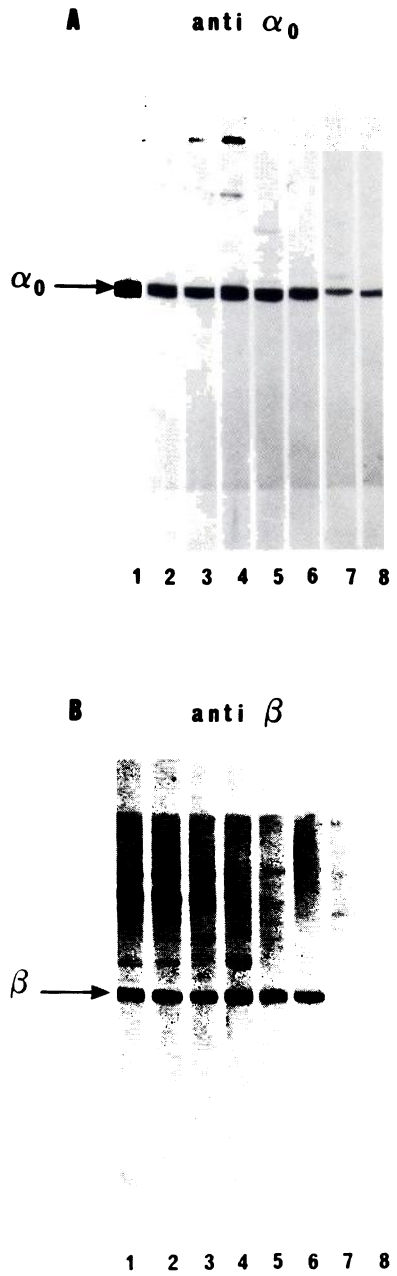

Fig. 4. Immunoblot analysis of brain particulate fraction from various species with anti-$G_{o\alpha}$ and anti-$G_{\beta,\gamma}$ antisera. One hundred μg of membrane proteins were subjected to electrophoresis through 10% polyacrylamide gels, blotted onto nitrocellulose, and analyzed with anti-$G_{o\alpha}$ (A) and anti-$G_{\beta,\gamma}$ (B) antisera as described under Materials and Methods. Proteins loaded were from: *lane 1*, calf; *lane 2*, rat; *lane 3*, turtle; *lane 4*, pigeon; *lane 5*, frog; *lane 6*, trout; *lane 7*, snail; *lane 8*, locust.

were 6 and 17 times lower than in brain cortex, respectively. In these experiments the quantity of $G_{o\alpha}$-like material was always slightly higher than that of $G_{\beta,\gamma}$ suggesting the presence of free $G_{o\alpha}$ in the tissues.

$G_{o\alpha}$ immunoreactivity and ADP ribosylation in neuronal and non-neuronal invertebrate tissues. In order to further characterize the $G_{o\alpha}$-immunoreactive material found in invertebrates, we carried out an immunological comparison of the brain and the peripheral tissues of *Locusta migratoria*. It appeared from this study that no positive immunoreactivity could be detected at 39–40 kDa in peripheral tissues like sali-

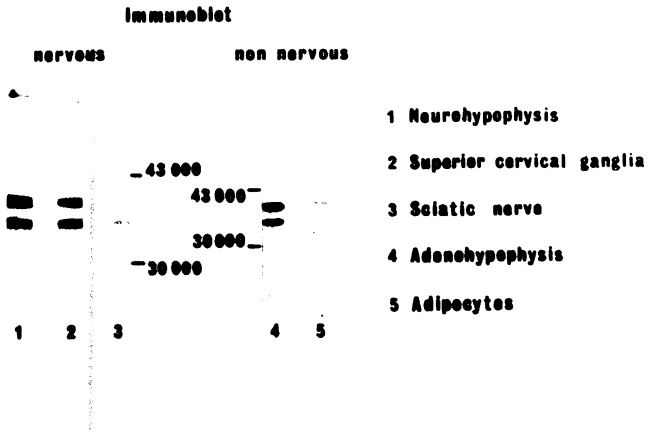

Fig. 5. Autoradiogram of the immunoblot analysis of the same peripheral tissues with anti-$G_{o\alpha}$ and anti-$G_{\beta,\gamma}$ antisera. About 100 μg of the particulate fraction of each tissue were analyzed as described in the legend of Fig. 4. The "nervous" tissues analyzed were neurohypophysis (*lane 1*), superior cervical ganglia (*lane 2*), sciatic nerve (*lane 3*), and the "non-nervous" tissues were adenohypophysis (*lane 4*) and human adipocyte (*lane 5*). Except for the latter, all tissues were from rat.

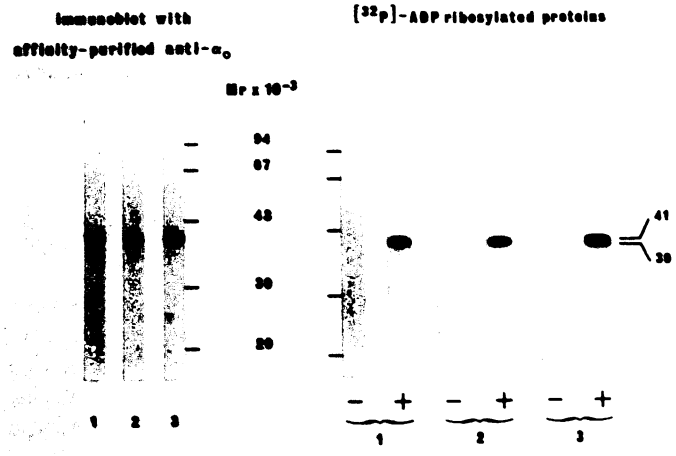

Fig. 6. $G_{o\alpha}$ and $G_{\beta,\gamma}$ immunoreactivities and ADP ribosylation in neuronal invertebrate tissues. Autoradiograms of the immunoblot analysis (*left*) and of [^{32}P]ADP-ribosylated protein (*right*) of 100 μg of nervous tissues of snail (*lane 1*), locust (*lane 2*), and 1 μg of G_o + G_i (*lane 3*) are shown. [^{32}P]ADP ribosylation was carried out as described in Materials and Methods without (−) or with (+) pertussis toxin.

TABLE 1

Qualitative and quantitative presence of the anti-$G_{o\alpha}$ and $G_{\beta,\gamma}$ immunoreactivities in various tissues of different species

The presence of immunoreactivity was assessed on autoradiograms after electrotransfer of the proteins from SDS-PAGE onto nitrocellulose and successive incubations with the antibodies and radioiodinated protein. A. Quantitation of immunoreactivity was determined by counting the relevant band of nitrocellulose as described in Materials and Methods.

Tissue	Percentage of membrane protein	
	$G_{o\alpha}$	G_β
Rat		
Cerebral cortex	1.05 ± 0.12	0.83 ± 0.15
Anterior hypophysis	0.17 ± 0.05	0.13 ± 0.03
Human		
Adipocyte	0.06 ± 0.02	0.05 ± 0.01
Platelet	ND	0.17 ± 0.03

* ND, subunit not detectable in the tissue.

vary glands, male accessory glands, and flight muscles (data not shown). By contrast, the affinity purified antibodies against $G_{o\alpha}$ cross-react with a protein at 39–40 kDa (Fig. 6) in the brains of snail (Fig. 6, *lane 1*) and of locust (Fig. 6, *lane 2*), confirming the similarity between vertebrate $G_{o\alpha}$ and an invertebrate G-protein. In agreement with this finding is the fact that ADP ribosylation with pertussis toxin (Fig. 6, *right*) occurred in neuronal tissues of both snail and locust. This latter result corroborates the finding of Kopf *et al.* (23) who showed a pertussis substrate at 39 kDa in the sperm of sea urchins.

Discussion

We have determined the distribution of the GTP-binding protein G_o immunoreactivity in brain and peripheral nervous and non-nervous tissues of various species. Only a few studies have been devoted to the localization of this protein of unknown function first identified in bovine brain. The antibodies used previously to probe G_o were raised against either purified $G_{o\alpha}$ (16, 17) or a peptide sequence specific of $G_{o\alpha}$ (17) and, finally, against preparation of G_o and G_i (13). In the latter case, one rabbit antiserum was selective for the G_o subunits. Our anti-$G_{o\alpha}$ antibodies compare favorably with the others since it hardly detected, at a 1:100 dilution, the α subunit of G_i, whereas the band at 39–40 kDa was heavily labeled (Figs. 2 and 3).

Previous studies have shown that a positive G_o immunoreactivity is present in the central nervous system of several vertebrate species like rats, humans, chickens, and frogs (13). Here we extended (Fig. 4) these approaches to the nervous system of reptiles (turtle) and invertebrates such as molluscs (snail) and insects (locust). The positive immunoreactivity found in their nervous systems seems to infer that a relatively large amount of a G_o-like protein is present in the brains of all animals. The fact that the immunoreactivity is directed against a 39–40-kDa subunit in all of the species tested suggests that this protein is highly conserved during evolution. The constant presence of the 39–40-kDa protein contrasts with that of the $G_{\beta,\gamma}$ subunits which could not be detected in invertebrate tissues with antibodies against $G_{\beta,\gamma}$ subunits of bovine brain. Indeed, we have shown previously that antibodies against $T_{\beta,\gamma}$ recognizes a 35–36-kDa subunit only in vertebrates (19). These concordant data raise the question of whether or not invertebrate G-proteins are associated with a $G_{\beta,\gamma}$-like subunit. If not, one might expect that the appearance of the coupling between the $G_{o\alpha}$-like protein and a $G_{\beta,\gamma}$ protein corresponds to a modification of the $G_{o\alpha}$ function. Alternatively, in invertebrates, the 39–40-kDa protein might be coupled to a peculiar subunit which does not cross-react immunochemically with our mammal anti-$G_{\beta,\gamma}$ antibodies. The fact that invertebrate tissues can also be ADP-ribosylated argues in favor of the existence of an invertebrate $G_{\beta,\gamma}$ subunit, since in vertebrates the ADP ribosylation by pertussis toxin requires the presence of the $G_{\beta,\gamma}$ subunits. Along with this result, it was recently reported in photoreceptor membranes of cephalopods that there exists immunoreactivity toward $T_{\beta,\gamma}$ antibodies (24). Together, these results indicated the existence, at least in some invertebrate tissues, of a protein similar to the vertebrate G_β subunit.

The relatively large amount of the G_o-like protein in brain, including that of invertebrates, reinforced the idea that its unknown function is essential for neuronal activity. One can think of synaptogenesis, liberation of neurotransmitters, ionic

channel activity, etc. It is also possible that its function is expressed in many cell types but more abundantly in nervous tissues, e.g., Ca^{2+} movement. In order to delineate the function of G_o, we addressed the following questions in term of the localization of G_o. Is the G_o protein limited to the central nervous system or is it also present in peripheral nervous tissues? Could G_o protein be localized in non-neuronal tissues?

Our data clearly show that, in nervous tissues like posterior pituitary, superior cervical ganglia, and also sciatic nerve, a G_o-like protein does exist (Fig. 5). One might thus wonder if the positive and weak immunoreactivity found in previous studies in heart, kidney, tracheal muscle, and rod outer segment are due to the innervation of those tissues. If this explanation cannot be ruled out for some tissues, it is interesting to note that the cardiac-sarcolemma preparation, probably devoid of any nerve tissue, also exhibited a 39–40-kDa immunoreactivity (17). We report here for the first time the existence of an immunoreactivity in the rat adenohypophysis and human adipocytes. These two latter tissues which cannot be suspected of having nerve tissue contamination clearly establish the fact that non-neuronal tissues can also exhibit a positive immunoreactivity toward the antibodies against $G_{o\alpha}$.

The question which now arises from this observation is the following. Is the $G_{o\alpha}$-like subunit present in the peripheral non-nervous tissues identical or only homologous to the brain $G_{o\alpha}$ subunit? For the adipocyte membranes, two reports indicated that ADP ribosylation leads to incorporation of ^{32}P in two proteins (26, 27). The first one, having a molecular weight of 41,000 on SDS-PAGE, is presumably $G_{i\alpha}$. The second one was described at 39 or 40 kDa and it was supposed to be the α subunit of G_o (27). However, the mobility in SDS-PAGE of the immunoreactive material of human adipocyte seems higher than that from bovine brain $G_{o\alpha}$ (Fig. 5). This slight discrepancy in protein mobility cannot be attributed to a species difference since rat and dog adipocytes have an identical immunological pattern (data not shown). Our data would suggest the existence of a G-like protein in adipocytes which might be different from the brain G_o.

The other peripheral tissue which has a substantial amount (although 5 times lower than in brain) of $G_{o\alpha}$ immunoreactivity is the anterior pituitary. The almost equal immunoreactive material in anterior and posterior pituitary rules out a possible contamination of the anterior pituitary by the posterior pituitary during the dissection. Here there was no difference in the mobility on SDS-PAGE between the $G_{o\alpha}$ proteins from rat or bovine brain and the $G_{o\alpha}$-like protein in anterior pituitary. Thus, the anterior pituitary might have the same $G_{o\alpha}$ subunit as the one in the brain.

One should be careful, however, before drawing any definitive conclusions concerning the identification of $G_{o\alpha}$ immuno-like material. Several laboratories have shown by cloning techniques that all of the α subunits of the G-proteins have considerable homologies. Furthermore, by screening cDNA libraries, several unknown G-proteins have been identified (28). Thus, one might detect, using antibodies against a member of the G-protein family, another G-protein with different function. Nevertheless, at the present time, our data suggest that the G_o protein, although highly expressed in brain, is not exclusive for neuronal activity. The function of G_o should be involved in a common mechanism present in the nervous system and the anterior hypophysis. One possibility is that the G_o function may be related to a phenomenon such as exocytosis. In this regard, immunofluorescent techniques will permit us to advance our understanding of the function of G_o by enabling us to study its precise cellular and subcellular localization.

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References

1. Stryer, L., and H. R. Bourne. G-proteins: a family of signal transducers. *Annu. Rev. Cell Biol.* **2**:1–44 (1986).
2. Northup, J. K., P. C. Sternweis, M. D. Smigel, L. S. Schleifer, E. M. Ross, and A. G. Gilman. Purification of the regulatory component of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **77**:6516–6520 (1980).
3. Bokoch, G. M., T. Katada, J. K. Northup, E. L. Hewlett, and A. G. Gilman. Identification of the predominant substrate for ADP-ribosylation by islet activating protein. *J. Biol. Chem.* **258**:2072–2075 (1983).
4. Kühn, H. Light- and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes. *Nature (Lond.)* **283**:587–589 (1980).
5. Hildebrandt, J. D., J. Codina, W. Rosenthal, L. Birnbaumer, E. J. Neer, A. Yamazaki, and M. W. Bitensky. Characterization by two-dimensional peptide mapping of the gamma subunits of Ns and Ni, the regulatory proteins of adenylate cyclase, and of transducin, the guanine nucleotide-binding protein of rod outer segments of the eye. *J. Biol. Chem.* **260**:14867–14872 (1985).
6. Roof, D. J., M. L. Applebury, and P. C. Sternweis. Relationships within the family of GTP-binding proteins isolated from bovine central nervous system. *J. Biol. Chem.* **260**:16242–16249 (1985).
7. Haslam, R. J., and M. L. M. Davidson. Guanine nucleotides decrease the free (Ca^{++}) required for secretion of serotonin from permeabilized platelets. Evidence of a role for a GTP-binding protein in platelet activation. *FEBS Lett* **174**:90–95 (1984).
8. Nakamura, T., and M. Ui. Simultaneous inhibitions of inositol phospholipid breakdown, arachidonic acid release, and histamine secretion in mast cells by islet-activating protein, pertussis toxin. *J. Biol. Chem.* **260**: 3584–3593 (1985).
9. Pfaffinger, P. J., J. M. Martin, D. D. Hunter, N. M. Nathanson, and B. Hille. GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature (Lond.)* **317**:536–538 (1985).
10. Holz, G. G., IV, S. G. Rane, and K. Dunlap. GTP-binding proteins mediate transmitter inhibition of voltage dependent calcium channels. *Nature (Lond.)* **319**:670–672 (1986).
11. Sternweis, P. C., and J. D. Robishaw. Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem.* **259**:13806–13813 (1984).
12. Neer, E. J., J. M. Lok, and L. G. Wolf. Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. *J. Biol. Chem.* **259**:14222–14229 (1984).
13. Gierschik, P., G. Milligan, M. Pines, P. Goldsmith, J. Codina, W. Klee, and A. Spiegel. Use of specific antibodies to quantitate the guanine nucleotide-binding protein Go in brain. *Proc. Natl. Acad. Sci. USA* **83**:2258–2262 (1986).
14. Florio, V. A., and P. C Sternweis. Reconstitution of resolved muscarinic cholinergic receptors with purified GTP-binding proteins. *J. Biol. Chem.* **260**:3477–3483 (1985).
15. Kurose, H., T. Katada, T. Haga, K. Haga, A. Ichiyama, and M. Ui. Functional interaction of purified muscarinic receptors with purified inhibitory guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *J. Biol. Chem.* **261**:6423–6428 (1986).
16. Huff, R. M., J. M. Axton, and E. J. Neer. Physical and immunological characterization of a guanine nucleotide-binding protein purified from bovine cerebral cortex. *J. Biol. Chem.* **260**:10864–10871 (1985).
17. Mumby, S. M., R. A. Kahn, D. R. Manning, and A. G. Gilman. Antisera of designed specificity for subunits of guanine nucleotide-binding regulatory proteins. *Proc. Natl. Acad. Sci. USA* **83**:265–269 (1986).
18. Laemmli, U. K. Cleavage of structural protein during assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
19. Audigier, Y., C. Pantaloni, J. Bigay, P. Deterre, J. Bockaert, and V. Homburger. Tissue expression and phylogenetic appearance of the beta and gamma subunits of GTP binding proteins. *FEBS Lett.* **189**:1–7 (1985).
20. Ribeiro-Neto, F. A. P., R. Mattera, J. D. Hildebrandt, J. Codina, J. B. Field, L. Birnbaumer, and R. D. Sekura. ADP-ribosylation of membrane components by pertussis and cholera toxin. *Methods Enzymol.* **109**:566–572 (1985).
21. Lowry O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
22. Schultz, R. M., J. D. Bleil, and P. M. Wassarman. Quantitation of nanogram amounts of protein using (^3H)-dinitrofluorobenzene. *Anal. Biochem.* **91**:354–356 (1978).

23. Kopf, G. S., M. J. Woolkalis, and G. L. Gerton. Evidence for a guanine nucleotide-binding regulatory protein in invertebrate and mammalian sperm. *J. Biol. Chem.* **261**:7327–7331 (1986).
24. Tsuda, M., T. Tsuda, Y. Terayama, Y. Fukada, I. Akino, G. Yamanaka, L. Stryer, T. Katada, M. Ui, and T. Ebrey. Kinship of cephalopod photoreceptor G-protein with vertebrate transducin. *FEBS Lett.* **198**:5–10 (1986).
25. Deleted in proof.
26. Malbon, C. C., P. J. Rapiejko, and J. A. Garcia-Sainz. Pertussis toxin catalyzes the ADP-ribosylation of two distinct peptides, 40 and 41 kDa, in rat fat cell membranes. *FEBS Lett.* **176**:301–306 (1984).
27. Owens, J. R., L. T. Frame, M. Ui, and D. M. F. Cooper. Cholera toxin ADP-ribosylates the islet-activating protein substrate in adipocyte membranes and alters its function. *J. Biol. Chem.* **260**:15946–15952 (1985).
28. Itoh, H., T. Kozasa, S. Nagata, S. Nakamura, T. Katada, M. Ui, S. Iwai, E. Ohtsuka, H. Kawasaki, K. Suzuki, and Y. Kaziro. Molecular cloning and sequence determination of cDNAs for alpha subunits of the guanine nucleotide-binding proteins Gs, Gi and Go from rat brain. *Proc. Natl. Acad. Sci. USA.* **83**:3776–3780 (1986).

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