The Regulatory Components of Adenylate Cyclase and Transducin

A FAMILY OF STRUCTURALLY HOMOLOGOUS GUANINE NUCLEOTIDE-BINDING PROTEINS*

(Received for publication, December 21, 1982)

David R. Manning, and Alfred G. Gilman
From the Department of Pharmacology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

G/F and transducin are guanine nucleotide-binding regulatory proteins that mediate activation of adenylate cyclase and of a rod outer segment cyclic GMP-specific phosphodiesterase, respectively. The substrate for islet-activating protein is a third guanine nucleotide-binding protein that is postulated to mediate inhibition of adenylate cyclase. The extent of structural homology among subunits of all three proteins was examined by analyses of amino acid compositions and electrophoretic patterns of proteolytic peptides. The lower molecular weight subunits (β subunits; Mr = 35,000) of these proteins have identical amino acid compositions and yield similar peptides upon proteolysis with Staphylococcus aureus V8 protease and elastase. The higher molecular weight subunits (α subunits; Mr = 39,000, 41,000, and 45,000) are also similar to each other in these respects. Similarity between the subunits of transducin and the islet-activating protein (IAP) substrate is especially evident. Substantial differences do, however, exist between the lower and higher molecular weight subunits within each protein. In addition, evidence was obtained that the 41,000-Da subunit of the IAP substrate is not derived from the 45,000-Da subunit of G/F. It is concluded that transducin, the IAP substrate, and G/F represent a family of structurally homologous guanine nucleotide-binding regulatory proteins.

GTP is an essential cofactor for hormonal stimulation or inhibition of adenylate cyclase and for light-induced activation of a retinal cyclic GMP-selective phosphodiesterase (reviewed in Refs. 1–3). These actions of GTP require interaction of the nucleotide with specific guanine nucleotide-binding proteins, which then serve as regulators of the appropriate catalytic entity (4–8). The regulatory proteins thus function as transducers of information between receptors for hormones or light and the ultimate effectors.

The stimulatory regulator of adenylate cyclase is the site of action of both guanine nucleotides and fluoride and is, in addition, a substrate for ADP-ribosylation catalyzed by cholera toxin (5, 9–11). A number of hormone–receptor complexes can control the interactions of G/F with GTP and thereby regulate the stimulatory effect of G/F·GTP on the catalytic component of adenylate cyclase. Similarly, transducin mediates activation of the rod outer segment cyclic GMP phosphodiesterase by photolyzed rhodopsin in a guanine nucleotide-dependent manner (7, 12, 13). Both G/F and transducin have been purified to homogeneity (14–17) and characterized. More recently, a third guanine nucleotide-binding protein has also been identified and purified (18). This protein serves as a substrate for ADP-riboisolation by IAP, a toxin produced by Bordetella pertussis. Since ADP-riboisolation of this membrane-bound protein appears to correlate with the effect of the toxin to block inhibition of adenylate cyclase by appropriate neurohormones (19–21), it has been hypothesized that the substrate for IAP is the guanine nucleotide-binding inhibitory regulator of adenylate cyclase (18, 21).

Purified rabbit liver G/F is thought to exist as a dimer consisting of one 35,000-Da subunit and either one 45,000- or one 52,000-Da subunit; the 45,000–35,000-Da dimer is the predominant species (14). Both the 45,000- and 52,000-Da subunits of G/F are ADP-riboisylated during incubation with NAD and cholera toxin (11, 23). The 45,000-Da subunit binds guanine nucleotides (5) and, in this state, can activate the catalytic component of adenylate cyclase in the absence of the 35,000-Da polypeptide. The 35,000-Da subunit is postulated to inhibit activation of adenylate cyclase as a result of its reversible association with the higher molecular weight subunits (5, 24).

Transducin consists of three polypeptides with molecular weights of <10,000, 36,000, and 39,000 (16, 17, 25). The larger subunits, at least, appear to exist in equimolar amounts. The 39,000-Da subunit is ADP-riboisylated upon incubation of rod outer segment disc membranes with NAD and chlora toxin (26) and is also a substrate for ADP-riboisolation by IAP (5). This subunit also binds guanine nucleotides and activates cyclic GMP phosphodiesterase when dissociated (and resolved) from the other subunits (8).

The protein tentatively termed the IAP substrate has been purified from rabbit liver and consists of a 35,000- and a 41,000-Da subunit (18). The 41,000-Da subunit binds guanine nucleotides and is ADP-riboisylated by IAP. The latter modification presumably results in attenuation of hormonal inhibition and enhancement of hormonal activation of adenylate cyclase (19–21). The purified substrate for IAP is not ADP-riboisylated by chlora toxin. As with G/F and transducin, the subunits of the IAP substrate dissociate in conjunction with

* This work was supported by American Cancer Society Grant BC240 and by United States Public Health Service Grant NS18153. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by National Research Service Award GM08401.

The abbreviations used are: G/F, guanine nucleotide-dependent stimulatory regulator of adenylate cyclase; IAP, islet-activating protein.
binding of appropriate guanine nucleotides to the larger subunit (18).

Despite the overt similarities between G/F, transducin, and the substrate for IAP, the precise extent to which they are related is unknown. Moreover, the relationship between the individual subunits of these proteins has not been assessed. Toward this end, we have examined the amino acid compositions of the subunits of these three proteins and the electrophoretic patterns of peptides generated therefrom by the action of two proteases. The data provide strong evidence for structural homology among all three proteins. However, certain differences do exist among these proteins, as well as between their constituent subunits.

**EXPERIMENTAL PROCEDURES**

G/F and the IAP substrate were purified from rabbit liver membranes by methods described by Sternweis et al. (14) and Bokoch et al. (18), respectively. Fractions of the IAP substrate that were completely resolved from G/F were utilized. Transducin was purified from bovine retinas essentially by the procedures of Papamost and Dreyer (27). Individual subunits of the three proteins were purified electrophoretically by a procedure similar to that of Stephens (28) in preparation for analysis of amino acid composition. Specifically, 300–500 μg of transducin, the IAP substrate, or G/F were collected by precipitation with trichloroacetic acid, solubilized in sample buffer containing 1% sodium dodecyl sulfate (29), and electrophoresed on a discontinuous sodium dodecyl sulfate-polyacrylamide slab gel (11% acrylamide) as described by Laemmli (29). Electrode buffers contained 0.1 mM mercaptoacetic acid to minimize oxidative destruction of amino acids. Following electrophoresis, protein bands were visualized with 4 M sodium acetate (30). Portions of gels containing individual protein subunits were excised, rinsed with H2O, minced, and placed over a sodium dodecyl sulfate-polyacrylamide tube gel (3 cm x 1 cm; 5% acrylamide). Dialysis tubing, prepared by extensive boiling in 600 mM NaHCO3 and 5 mM EDTA and then in H2O, was knotted and attached to the bottom (anodic end) of the tube. Protein was then eluted electrophoretically into the dialysis tubing with an electrical field (140 V) maintained for 14 h. The eluted protein was subsequently dialyzed against 0.1% sodium dodecyl sulfate. Extensive precautions were taken to minimize contamination of these preparations with other proteins or amino acids, e.g. solutions were made with freshly distilled H2O immediately prior to use and glassware was washed with ChemSolv (American Scientific Products, McGaw Park, IL), followed by rinsing with glass-distilled H2O.

Amino acid analyses were performed with an Aminco amino acid analyzer using ophthalaldehyde as the detection reagent (31). Hydrolysis of proteins was effected in HCl (constant boiling grade) containing 0.02% phenol at 110 °C for 20 h in vacuo. Recovery of amino acids was determined by addition of noreleucine to samples prior to hydrolysis. Side chain amides are converted to corresponding acids by these hydrolytic procedures. Values for serine and threonine were not corrected for limited destruction occurring during hydrolysis (32). Values for proline, cysteine, and tryptophan were not determined. Quantities of amino acids not specifically attributable to the protein being analyzed (33) were assessed with samples derived from polyacrylamide gels to which no protein had been added. With the exception of glycine, this contamination represented 5–20% of total amino acids present in samples of protein. Contamination by glycine represented 25–75% of the total glycine present; the latter value was due in part to the low content of glycine of several of the polypeptides studied. Amino acid compositions are expressed as moles of amino acid/mol of total amino acids analyzed X 100. Values for each protein are the average of at least four separate determinations using subunits derived from at least two separate electrophoretic preparations and at least two separate preparations of purified holoprotein.

Proteolytic peptides of the protein subunits were generated and resolved by the procedures of Cleveland et al. (34), as modified by Lam and Kasper (35). Transducin, the IAP substrate, or G/F was initially subjected to sodium dodecyl sulfate-polyacrylamide tube gel electrophoresis (8% acrylamide; 20 μg of protein/tube), and the gel was then equilibrated in 50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 20% glycerol for 30 min. The equilibrated gel was placed horizontally above a discontinuous sodium dodecyl sulfate-polyacrylamide slab gel consisting of a 3.5-cm stacking gel and a 7-cm separating gel (15% acrylamide). Equilibration buffer containing 0.05% bromphenol blue and either 30 μg of Staphylococcus aureus V8 protease or 16 μg of porcine elastase was layered above the tube gel. Electrophoresis was initiated with 10–15 mA at a temperature of 15 °C. As the dye front neared the separating gel, current was discontinued for 30 min to allow proteolysis; electrophoresis was then reinitiated at 30 mA. Gels were finally stained with Coomassie brilliant blue R-250 (0.25%).

**RESULTS**

Relative mobilities of the electrophoretically purified subunits of transducin, the IAP substrate, and G/F upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis are illustrated in Fig. 1. Mobilities of the smaller (β) subunits are identical, corresponding approximately to previously reported structural homology among all three proteins. However, certain differences do exist among these proteins, as well as between their constituent subunits.

**IAP SUBSTRATE**

| Subunit | G/F |
|---------|-----|
| Transducin | 35 39 35 41 35 45 |
| PLASE | 35 |
| BSA | 39 |
| OVAL | 35 |
| CA | 41 |
| TI | 45 |

**FIG. 1. Electrophoretic profile of purified subunits of transducin, the IAP substrate, and G/F.** Electrophoretically purified subunits of the three proteins were subjected to discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (29) and were visualized by staining with Coomassie blue. Approximately 2 μg of each subunit were employed. Phosphorylase b (PLASE), bovine serum albumin (BSA), ovalbumin (OVAL), carbonic anhydrase (CA), and soybean trypsin inhibitor (TI) were the standards (Mr = 92,500, 66,200, 45,000, 31,000, and 21,500, respectively).

**FIG. 2. Amino acid compositions of the subunits of transducin.** Electrophoretically purified subunits of transducin were hydrolyzed and resultant amino acid residues were analyzed as described under “Experimental Procedures.” Vertical brackets represent ±1 S.E. (n = 6).
molecular weights of 35,000. Those of the larger (α) subunits correspond approximately to reported values of 39,000, 41,000, and 45,000 Da, respectively. The 52,000-Da subunit of G/F was not available in quantities sufficient for electrophoretic purification.

Amino acid compositions of electrophoretically purified subunits are depicted in Figs. 2-5. Compositions of the three 35,000-Da subunits are indistinguishable and are notable only for lower contents of lysine and higher contents of arginine than averages reported for proteins in general (37). Compositions of the 39,000-, 41,000-, and 45,000-Da subunits are also quite similar to each other and are characterized by low contents of glycine and high contents of arginine. There are, however, significant differences among the α subunits in the content of methionine. These differences were corroborated by analyses of peptides derived by cleavage with cyanogen bromide (results not shown). Distinct differences also existed between amino acid compositions of the α and β subunits within each protein. Especially notable were differences in threonine, serine, glutamic acid (i.e. glutamic acid plus glutamine), glycine, and lysine.

Analysis of proteolytic fragments derived from electrophoretically resolved subunits provided a more sensitive means of assessing primary structural characteristics. Electrophoretic patterns of peptides generated upon concurrent electrophoresis of the subunits of transducin, the IAP substrate, or G/F with S. aureus V8 protease are illustrated in Fig. 6. Degradation of intact subunits was complete and yielded several peptides discernable in the 9,000–22,000 Da region. There is striking similarity among patterns of peptides derived from the 35,000-Da subunit of each protein. Those of the IAP substrate and G/F are identical and only differ from that of transducin in the relative intensity of staining. In each case, five bands were resolved, corresponding to molecular weights of approximately 10,000, 12,000, 15,000, 18,000, and 21,500.

These patterns differ substantially, however, from those
obtained from the \( \alpha \) subunits. Peptides derived from the 39,000- and 41,000-Da subunits of transducin and the IAP substrate produce electrophoretic patterns quite similar to each other, differing primarily in relative intensity of staining. Peptides with identical mobilities have apparent molecular weights of 10,500, 12,500, and 14,000. Peptide patterns from the 45,000- and 32,000-Da subunits of G/F are identical with each other and are comprised primarily of 12,000-, 13,000-, and 17,000-Da species. These electrophoretic patterns differ somewhat from those derived from the 39,000- and 41,000-Da subunits of transducin and the IAP substrate. These differences were corroborated by examination of discernable peptides derived from the minor 41,000 Da IAP substrate contaminant present within various preparations of G/F that were analyzed (results not shown). However, a minor 17,000-Da peptide is obtained from the 41,000-Da subunit of the IAP substrate and the larger subunits of G/F. In addition, a minor 14,000-Da peptide is also derived from the 45,000-Da subunit of G/F. Peptide mobility and staining patterns obtained for each subunit with \( S. aureus \) V8 protease and elastase (see below) were consistent upon repetition and were observed upon simultaneous proteolysis of various subunits within the same polyacrylamide slab gel (results not shown).

Electrophoretic patterns of peptides generated upon concurrent electrophoresis of the subunits of transducin, the IAP substrate, or G/F with pancreatic elastase are illustrated in Fig. 7. Although degradation of subunits was not necessarily complete, peptides were generated in quantities sufficient for analysis. Those of the 35,000-Da subunits are virtually identical, again differing only slightly in staining intensity. In each case, six proteolytic fragments with molecular weights of 13,000, 16,000, 17,000, 21,000, 24,000, and 32,000 were resolved. These peptides differ substantially from those produced from the \( \alpha \) subunits. The electrophoretic patterns of peptides derived from the 39,000- and 41,000-Da subunits of transducin and the IAP substrate are nearly identical with each other but differ from those of the 45,000- and 52,000-Da subunits of G/F. Those of the latter two subunits are similar but not entirely identical.

**DISCUSSION**

Structural and functional homology among transducin, the substrate for IAP, and G/F has been inferred previously from their electrophoretic and hydrodynamic behavior (8, 14, 18), their capacity to undergo ADP-ribosylation (11, 18, 26), and their ability to bind guanine nucleotides (5, 8, 18). While the precise extent to which functional homology exists is presently debated, the primary structural characteristics of these proteins have now been assessed directly by analyses of amino acid compositions and proteolytic peptides. The data obtained demonstrate extensive structural homology among all three GTP regulatory proteins, but also reveal specific differences. Amino acid compositions and peptide electrophoretic profiles of the 35,000-Da subunits of each protein are virtually identical. These structural characteristics, however, differ appreciably from those of the higher molecular weight subunits. Differences such as the relative contents of glycine and serine in the \( \alpha \) and \( \beta \) subunits are of an extent sufficient to demonstrate that the 35,000-Da subunits are, in fact, distinct gene products. The higher molecular weight subunits exhibit amino acid compositions quite similar to each other, differing significantly only in their content of methionine. Analyses of peptides produced by proteolytic procedures in general demonstrate marked structural similarity between transducin and the substrate for IAP, but differences between these proteins and G/F. These peptide differences, together with the differences in the contents of methionine, suggest that the 41,000-Da subunit of the IAP substrate is not derived from the 45,000-Da subunit of G/F. That it is derived from the 52,000-Da subunit of G/F cannot be precluded, but this possibility seems unlikely. Rather, proteolytic peptides of the 52,000-Da subunit bear striking similarity to those of the 45,000-Da subunit, indicating that the former is either a precursor to the latter or a closely related isoform. Hudson and Johnson (38) reached a similar conclusion about this similarity, based on their analysis of ADP-ribosylated proteolytic fragments of the two polypeptides.

We therefore conclude that transducin, the IAP substrate, and G/F comprise a family of structurally homologous guanine nucleotide-binding regulatory proteins. All contain structurally similar 35,000-Da subunits, and all are probably subject to the regulatory properties ascribed to that subunit (24). All contain structurally similar, though not identical, higher molecular weight subunits capable of binding guanine nucleotides, and all are substrates for ADP-ribosylation catalyzed by specific bacterial toxins. Knowledge of the precise extent of the homology among these proteins now awaits direct determinations of amino acid sequences.

Acknowledgments—We would like to express our sincere gratitude to Dr. Brent Reed for advice and assistance in obtaining amino acid compositions. We also thank Dr. Phuong Lan Tran for initial advice regarding this work. Frank Roganti provided excellent technical assistance, and Wendy Deener provided skillful assistance in preparation of the manuscript.

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The regulatory components of adenylate cyclase and transducin. A family of structurally homologous guanine nucleotide-binding proteins.

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J. Biol. Chem. 1983, 258:7059-7063.

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