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G-Protein $\beta\gamma$ Forms: Identity of β and Diversity of γ Subunits†

Haya Tamir, Ahmad B. Fawzi,‡ Amichai Tamir,§ Tony Evans,‖ and John K. Northup*,⊥
Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510
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ABSTRACT: Signal-transducing G-proteins are heterotrimers composed of GTP-binding α subunits in association with a tightly bound complex of β and γ subunits. While the α subunits are recognized as a family of diverse structures, β and γ subunits have also been found as heterogeneous isoforms. To investigate the diversity and tissue specificity of the $\beta\gamma$ complexes, we have examined homogeneous oligomeric G-proteins from a variety of sources. The β and γ subunits isolated from the major-abundance G-proteins from bovine brain, bovine retina, rabbit liver, human placenta, and human platelets were purified and subjected to biochemical and immunological analysis. Protease mapping and immune recognition revealed an identical profile for each of the two distinctly migrating β isoforms (β_{36} and β_{35}) regardless of tissue or G-protein origin. Digestion with V8 protease revealed four distinct, clearly resolved terminal fragments for β_{36} and two for β_{35}. Trypsin and chymotrypsin digestion yielded numerous bands, but again each form had a unique profile with no tissue specificity. Tryptic digestion was found to be conformationally specific with the most resistant structure being the native $\beta\gamma$ complex. With increasing trypsin, the complex was digested but in a pattern distinct from that for denatured β. In contrast to the two highly homologous β structures, examination of this set of proteins revealed at least six distinct γ peptides. Two unique γ peptides were found in bovine retinal G_t and three γ peptides in samples of bovine brain derived G_o/G_i. Human placental and platelet G_i samples each contained a unique γ. Finally, rabbit liver G_i preparations contained three electrophoretically resolvable γ peptides. Antisera raised to the retinal $\beta\gamma$ structure recognize both retinal γ forms and no other forms. The larger γ peptide from liver G_i is recognized by antiserum β-8 which specifically recognizes the human placental γ, the most rapidly migrating γ from liver corresponds with the mobility of the human platelet γ, and the third liver γ was found in our previous study to be recognized by a sequence-specific antiserum which identifies one of the two closely migrating G_o/G_i γ's as the product of the γ_2 gene [Gautam, N., Northup, J. K., Tamir, H., & Simon, M. I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7973–7977]. An additional γ peptide found in some, but not all, G_o/G_i preparations was identified as γ_3. These studies show a far greater diversity of the γ-subunit structure than had previously been recognized. Our results suggest that the function of the β subunit in G-proteins is highly conserved and that biochemical differences among the $G\beta\gamma$ forms are likely to be due to the diversity in γ structures.

The family of guanine nucleotide binding proteins (G-proteins)[1] plays a major role in transducing extracellular signals to cellular targets, thus transmitting a message from an active cell-surface receptor to cellular effectors. The regulation of adenylyl cyclase activity, visual excitation, activation of specific ion channels, and phosphoinositide turnover are mediated by G-proteins [for a review, see Gilman (1987), Casey and Gilman (1988), and Lochrie and Simon (1988)]. All identified signal-transducing members of this family, purified from a variety of sources, are heterotrimeric membrane proteins composed of three subunits: α, β, and γ (Northup et al., 1980; Fung et al., 1981; Hanski et al., 1981; Sternweis et al., 1981; Baehr et al., 1982; Bokoch et al., 1983; Codina et al., 1984; Sternweis & Robishaw, 1984). The α subunits all contain a high-affinity guanine nucleotide binding site, but they differ in size, sequence, their ability to serve as substrates

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‡ Present address: Schering-Plough Research, 60 Orange St., Bloomfield, NJ 07003.
§ Present address: Department of Physiology and Pharmacology, Tel-Aviv University, Sackler Medical School, Tel-Aviv, 69978, Israel.
‖ Present address: Genentech, Inc., South San Francisco, CA 94080.
⊥ Established Investigator of the American Heart Association.

[1] Abbreviations: G-protein, member of the family of signal-transducing GTP-binding regulatory proteins; G_t, vertebrate retinal G-protein (transducin); G_s and G_i, stimulatory and inhibitory G-proteins of the adenylyl cyclase system; G_o, 39-kDa G-protein of high abundance in the brain; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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for ADP-ribosylation catalyzed by bacterial toxins, and their functional properties. At least 16 α structures have been identified to data from cDNA clones. They are thought to confer receptor and effector specificity to the G-proteins. Biochemical studies of adenylyl cyclase and retinal cyclic GMP phosphodiesterase have elucidated a common mechanism for G-protein mediation of receptor activation. The activated receptor initiates exchange of tightly bound GDP for GTP and consequent dissociation of $G\alpha$ from $G\beta\gamma$. $G\alpha$-GTP then regulates specific effector molecules; hydrolysis of GTP terminates the activation, and $G\alpha$-GDP reassociates with $G\beta\gamma$. In vitro reconstitution of isolated β-adrenergic receptor (Hekman et al., 1987) and rhodopsin (Fung, 1983; Kanaho et al., 1984) has shown that receptor recognition of $G\alpha$ is dependent on its association with $G\beta\gamma$.

The diversity among the β subunits is limited so far to only two polypeptide forms. Analysis by SDS–PAGE has shown them as two closely migrating proteins of 36- and 35-kDa apparent mass (Sternweis et al., 1981). Both polypeptides have been described and purified from a variety of sources (Sternweis et al., 1981; Northup et al., 1983; Evans et al., 1987); however, their relative abundance depends on the tissue of origin. Molecular cloning of the retinal β (β_{36}, β_1 gene) identified the protein as an acidic 340 amino acid polypeptide of M_r 37 375 (Sugimoto et al., 1985; Fong et al., 1986). A second gene, termed β_2, has been identified and shown to share 90% homology with the β_1 gene product (Gao et al., 1987a,b; Fong et al., 1987; Amatruda et al., 1988). Recently, an additional cDNA termed β_3 has been isolated and shown to be closely related to β_1 (83% sequence homology) and β_2 (81%) (Levine et al., 1990).

The γ subunits are isolated in tightly bound complexes with the β subunits. While in the past it has been thought that the sole role of the $\beta\gamma$ complex is to deactivate the α subunit, a growing body of evidence suggests their functional involvement in a variety of signaling events. For example, $G\beta\gamma$ causes inhibition of adenylyl cyclase activity (Katada et al., 1984a,b), inhibition of muscarinic receptor stimulation of phospholipase C (Moriaty et al., 1988), activation of cardiac muscarinic K^+ channels (Logothetis et al., 1987), and inhibition of AlF_4^--activated $G_s\alpha$ (Northup et al., 1983) and phospholipase C (Boyer et al., 1989). $G\beta\gamma$ was also found to be required for pertussis toxin catalyzed ADP-ribosylation of $G\alpha$ subunits (Huff & Neer, 1986; Casey et al., 1989). Finally, bovine brain $\beta\gamma$ has been shown to be required for phospholipid vesicle association of G_0 and G_s α subunits (Sternweis, 1986).

The γ subunits have not been fully characterized. The retinal γ protein was sequenced (Ovchinnikov et al., 1985), cloned, and found to be of M_r 8400 (Hurley et al., 1984). Immunological approaches suggested that this γ is distinct (Gierschik et al., 1985; Hildebrandt et al., 1985) and unique to the retina (Roof et al., 1985). Furthermore, it has recently been shown that two other γ subunits are associated with bovine brain G-proteins and one of these γ's is identical with the predicted amino acid sequence of an adrenal γ cDNA clone (Robishaw et al., 1989). Additionally, two new γ cDNA clones have been isolated from bovine brain (γ_3), mouse renal, and mouse retinal libraries (γ_4) (Gautam et al., 1990), thus strongly supporting the idea of the existence of a highly diverse γ-subunit family.

In order to investigate the diversity of G-protein subunits further, we have examined the diversity of β- and γ-subunit structures isolated with the major-abundance G-proteins. Our data show that the G-protein-affiliated β structures are limited to β_1 and β_2 gene products. In contrast, the γ peptides are revealed as considerably more heterogeneous than previously suspected.

Experimental Procedures

Purification of G-Protein. Mixtures of G_0 and G_i were isolated from bovine brain membranes essentially as described (Sternweis & Robishaw, 1984). G_p and G_i were isolated from human placental membranes as described by Evans et al. (1986). Rabbit liver G_i3 was isolated by modifications of previously described methods (Sternweis et al., 1981; Bokoch et al., 1984). The G_i- and G_s-containing fractions from 3 kg of rabbit liver obtained from DEAE and AcA-34 chromatography were pooled from four independent preparations. These were rechromatographed on AcA-34 and subjected to heptylamine–Sepharose and hydroxylapatite chromatography as previously described (Bokoch et al., 1984). Human platelet G_i2 was isolated from 200 units of human platelets, essentially as described for rabbit liver (Bokoch et al., 1984). The G_i fractions were further chromatographed on DEAE-Sephacel in 0.6% Lubrol to separate G_i2 and G_i3 oligomers (identified by specific antipeptide antibodies). All chromatography fractions were assayed for GTPγS binding and pertussis toxin catalyzed ADP-ribosylation as described (Evans et al., 1986). G-protein oligomer fractions used for these experiments displayed $\geq$0.9 mol/mol of GTPγS-binding activity. Transducin was prepared from bovine retinal rod outer segment membranes according to Kuhn's method (Kuhn, 1980) with some modifications (Fawzi & Northup, 1990).

The G-proteins were found to be >90% homogeneous as judged by GTPγS-binding activity and Coomassie blue staining of polyacrylamide gels.

Preparation of $\beta\gamma$ Proteins. The $\beta\gamma$ component of bovine G_t was resolved from $G_t\alpha$ by activation with aluminum fluoride and sequential chromatography on ω-aminooctylagarose (Fung, 1983) and heptylamine–Sepharose in solutions containing magnesium, aluminum, and fluoride.[2] Human placental $\beta_{35}\gamma$ was isolated as previously described (Evans et al., 1987). Fractions of $\beta_{35}\gamma$ were further chromatographed on Ultrogel AcA-54 (LKB) equilibrated and eluted with 20 mM Tris-HCl, pH 8, 100 mM NaCl, and 1% sodium cholate. This chromatography resolved three distinct peaks: β subunits, $\beta\gamma$ subunits, and γ subunits (see Results).

Electrophoresis. SDS–PAGE was performed according to the method of Laemmli (1970). Samples were treated with DTT and *N*-ethylmaleimide in order to increase resolution of the polypeptide bands (Sternweis & Robishaw, 1984; Evans et al., 1987). For protein resolution by preparative electrophoresis, 11% polyacrylamide gels were stained briefly with Coomassie brilliant blue to visualize proteins, and the β bands were excised and stored at −80 °C.

Protease Mapping. Gel slices from preparative electrophoresis were subjected to proteolytic digestion by the method of Cleveland (Cleveland et al., 1977). Proteins were visualized by silver staining (Wray et al., 1981); all gels were run at 6 °C.

Tricine–SDS–PAGE (Schagger & Von Jagow, 1987) was used to visualize the γ subunits; 6 M urea–15.5% acrylamide gels were run at room temperature at a constant voltage of 100 V. Bands were visualized either by Coomassie blue or by silver staining.

Generation of Antisera and Immunoblot Techniques. Rabbit polyclonal antisera were produced as previously described (Evans et al., 1987). Tr-β-11 was raised against pu-

[2] Fawzi, A. B., Fay, D. S., Murphy, E. A., Tamir, H., Erdos, J. J., & Northup, J. K. (1991) *J. Biol. Chem.* (in press).

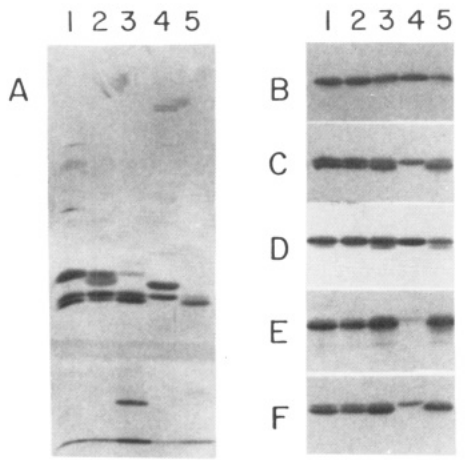

FIGURE 1: SDS–PAGE and immunoblot recognition of G-proteins. Heterotrimeric G-proteins and β subunits were prepared as described under Experimental Procedures, alkylated with *N*-ethylmaleimide, and loaded on an 11% acrylamide gel at approximately 250 ng of β chain/lane. Lane 1, 1 μg of rabbit liver G_i; lane 2, 1 μg of bovine brain G_o/G_i; lane 3, 1 μg of human placental G_p; lane 4, 500 ng of bovine retinal G_t; lane 5, 250 ng of human placental β_{35}. Panel A: silver staining of all proteins. Panels B–F are replicate gels of the samples of panel A transferred to nitrocellulose for Western blots as described under Experimental Procedures. Panel B, antiserum Tr-β-11 that was generated for $G_t\beta\gamma$; panel C, antiserum β-8 that was raised against human placental $\beta_{35}\gamma$. Panels D–F are data for sequence-directed antipeptide antisera (20); panel D; βN1; panel E, βP1–16; panel F; β2N.

rified bovine $G_t\beta\gamma$; antisera β-7 and β-8 were raised to human placental $\beta_{35}\gamma$. Rabbit antipeptide antisera were generated as described (Amatruda et al., 1988). β2N was raised to the peptide CGDSTLTQITAGLDP (amino acids 25–39 of the β_2-predicted protein sequence), βP1–16 was raised to TVGFAGHSGDVMSLS (amino acids 177–191 of β_2), and βN1 was raised to MSELDQLRQEAEQL (initial 14 amino acids of β_1). G-proteins were separated either on SDS–PAGE or on Tricine–SDS–PAGE, electrophoretically transferred to 0.45-μm nitrocellulose membranes, and incubated with specific rabbit polyclonal antisera as previously described (Evans et al., 1987). Visualization of the G-protein bands was performed by using either alkaline phosphatase conjugated goat anti-rabbit IgG $F(ab')_2$ (Sigma) or ^{125}I-labeled goat anti-rabbit IgG $F(ab')_2$ (New England Nuclear) and autoradiography with Kodak XAR film.

Materials. V8 protease (Endoproteinase Glu-C) from *Staphylococcus aureus* and trypsin from bovine pancreas were both obtained from Boehringer Mannheim. α-Chymotrypsin was from Sigma. Tricine, *N*-[tris(hydroxymethyl)methyl]-glycine, was obtained from Boehringer Mannheim. [^{35}S]-GTPγS (1200 Ci/mmol) and [α-^{32}P]NAD^+ (30 Ci/mmol) were obtained from New England Nuclear. Purified pertussis toxin was the generous gift of Dr. Jack Munoz, Rocky Mountain Laboratories. All other materials and reagents were of the highest grade commercially available.

RESULTS

Analysis of β Subunits Visualized by Protein Staining and Immunoblotting. Purified G-proteins were used to study the different β-subunit forms. As seen in Figure 1, panel A (with the exception of lane 5), all of the G-proteins examined are heterotrimers.[3] Each sample represents the most abundant

[3] Human placental G_p used in these experiments may not be a heterotrimeric G-protein. The GTP-binding polypeptide copurifies with a βγ complex indistinguishable from that of isolated human placental G_i.

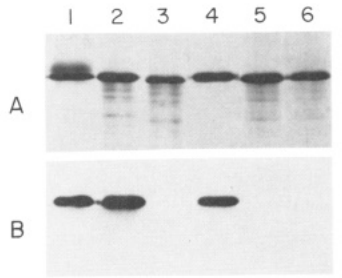

FIGURE 2: Characterization of the homogeneity of β chains. β chains were electrophoretically purified as described under Experimental Procedures, and approximately 1.5 μg of each of the isolated bands was analyzed for purity on 11% acrylamide separating gels. Panel A is a silver stain of the samples. Lane 1, bovine retinal β_{36}; lanes 2 and 3, bovine brain β_{36} and β_{35}, respectively; lanes 4 and 5, human placental G_p β_{36} and β_{35}; lane 6, human placental β_{35}. Panel B presents a replicate gel of the samples from panel A probed for reactivity with antiserum Tr-β-11 as described under Experimental Procedures.

GTP-binding proteins from the following tissues: bovine brain (G_o/G_i that was estimated to be 90% G_o), bovine retina (G_t), rabbit liver, and human placenta (G_i). The β forms associated with these diverse G-proteins (in lanes 1–3) appear as resolved doublets of two closely migrating proteins of similar abundance. Transducin (as shown in lane 4) contains only β_{36} while a human placental β_{35} preparation (lane 5) reveals a low abundance of β_{36}. The latter can been seen in panel B as recognized by antiserum Tr-β-11 that reacts specifically with β_{36}. The immunological characterization of the β subunits of these G-proteins is presented in panels B–F which are Western transblot replicates of panel A. The upper band of the doublet from each source is recognized by the Tr-β-11 serum raised against bovine $G_t\beta\gamma$ as well as the β_1 sequence-directed antiserum βN1 (panel D). Serum β-8 raised against human placental β_{35} recognizes both β bands with equal intensity. Panels D–F reveal the different characteristics of the antipeptide antibodies that were designed to distinguish among the β forms (Amatruda et al., 1988). The β_2 sequence-specific antiserum βP1–16 (panel E) is almost uniquely reactive with the lower band of each doublet; antiserum β2N (panel F) also recognizes the upper bands but much less intensely. In no case did we detect preferential recognition of one of the G-protein β subunits. βP1–16 (panel E) is the most selective antipeptide antisera; however, some recognition of β_{36} is still apparent.

Study of Structural Characteristics of the β Forms by Means of Peptide Mapping. β subunits from bovine brain G_o/G_i and human placental G_p were separated electrophoretically as described under Experimental Procedures, and the homogeneity of the products was determined by the use of specific antisera as shown in Figure 1. This method was highly reproducible, and there was no evidence of cross-contamination by either of the forms as seen in Figure 2. Peptide maps were designed to study the differences in primary structure between the β forms. Figure 3 presents the results of Cleveland mapping of the proteins shown in Figure 2, after V8 protease digestion. Panel A is the silver stain of the digestion profile for these β forms. The terminal fragments in this reaction are labeled. Analysis of the profile reveals three distinct β_{36} fragments (2a, 3, and 4) and one distinct β_{35} fragment (2b), as well as a fragment of similar mobility present in both β_{35} and β_{36} (1). Replicates of this digestion were transblotted and probed with antisera Tr-β-11, β-7, and β-8. This analysis reveals that the fragment of identical mobility common to the isoforms (1) contains unique epitopes for recognition. Serum Tr-β-11 only recognizes this fragment 1 as derived from $G_t\beta$ and all other β_{36} subunits of the doublets. Serum β-8 also recognizes the β_{35}-specific fragment 2a. As shown for the

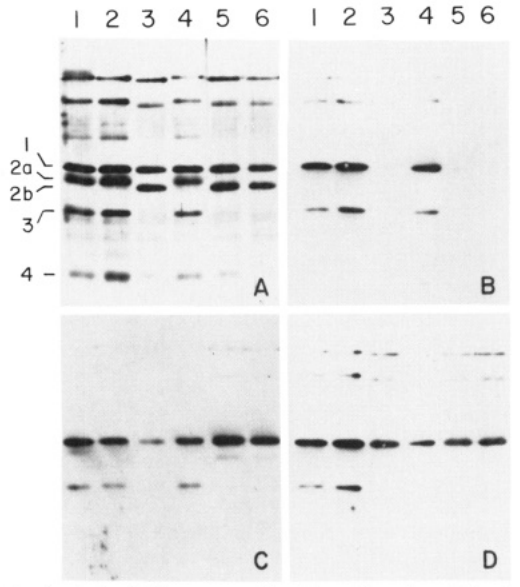

FIGURE 3: Analysis of V8 protease digestion. Digestion of the β chains shown in Figure 2 was performed by the Cleveland method using a 15% separating gel as described under Experimental Procedures. Approximately 1.5 μg of β chain was added per lane with 500 ng of V8 protease. Panel A is a silver stain of the samples. Lane 1, bovine retinal β_{36}; lanes 2 and 3, bovine brain β_{36} and β_{35}, respectively; lanes 4 and 5, human placental G_p β_{36} and β_{35}; lane 6, human placental β_{35}. Panels B–D present immunoblot analysis of replicate gels of this digest. Panel B, Tr-β-11; panel C, β-8; panel D, β-7.

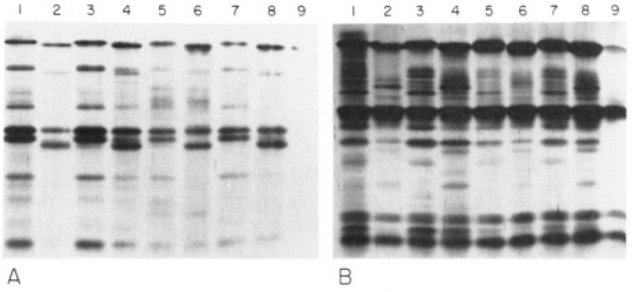

FIGURE 4: Cleveland mapping of β chains. Cleveland mapping of gel-purified β chains was accomplished as described under Experimental Procedures. Panel A presents the silver-staining profile for 50 ng of V8 protease resolved with a 15% separating gel. Panel B is the silver-staining profile for digestion with 500 ng of trypsin separated on a 17% gel. Approximately 1.5 μg of β chain was added per lane. Lane 1, retinal β_{36}; lane 2, human placental β_{35}; lanes 3 and 4, bovine brain G_o/G_i β_{36} and β_{35}; lanes 5 and 6, rabbit liver G_i3 β_{36} and β_{35}; lanes 7 and 8, human placental G_i β_{36} and β_{35}. Lane 9 panel A, 50 ng of V8 protease; lane 9 panel B, 500 ng of trypsin.

full-length proteins, there was no tissue specificity of the protease fragment or reactive epitopes for these antisera.

To test for similarity among the β forms, additional G-proteins were examined. Figure 4 presents further proteolytic characterization of the β proteins shown in Figure 2 with the addition of human placental and rabbit liver $G_i\beta$ polypeptides. Panel A represents V8 digestion while in panel B the tryptic digests of these proteins are shown. As with V8 protease digestion, trypsin digestion reveals numerous fragmentation differences between β_{36} and β_{35} proteins. However, no distinction among the various 36-kDa forms nor among the various 35-kDa forms can be visualized. Chymotryptic digestion of these same proteins also revealed consistent differences between the isoforms with no distinction among the species from these disparate sources. The differences found by means of protease digestion were identical with those found when using homogeneous $\beta\gamma$ preparations that were not subjected to electrophoresis. Figure 5 presents a comparison of

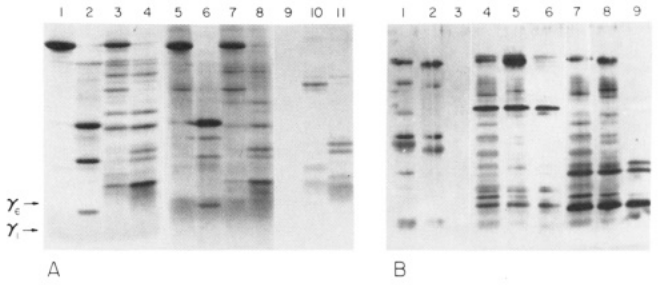

FIGURE 5: Comparison of solution digestion with Cleveland mapping. Panel A, 4 μg of retinal $\beta_{36}\gamma$ or human placental $\beta_{35}\gamma$ was boiled in Laemmli sample buffer and digested in solution with 500 ng of the indicated proteases as described under Experimental Procedures. The digests were resolved on 15% gels and stained with silver. Lanes 1 and 5, no protease added to retinal $\beta_{36}\gamma$ and placental $\beta_{35}\gamma$, respectively. Lanes 2 and 6, β_{36} and β_{35} digested by V8; lanes 3 and 7, β_{36} and β_{35} digested by trypsin; lanes 4 and 8, β_{36} and β_{35} digested by chymotrypsin; lane 9, V8 protease alone; lane 10, trypsin alone; lane 11, chymotrypsin alone. Panel B presents the Cleveland mapping profile for 1.5 μg each of electrophoretically isolated β chains from the human placental G_p performed as described under Experimental Procedures. Lanes 1 and 2, β_{36} and β_{35}, respectively, digested with 100 ng of V8; lanes 4 and 5, β_{36} and β_{35} digestion with 500 ng of trypsin; lanes 7 and 8, β_{36} and β_{35} digestion with 500 ng of chymotrypsin. The proteolytic enzymes are shown in lane 3 (100 ng of V8), lane 6 (500 ng of trypsin), and lane 9 (500 ng of chymotrypsin). The migrations of undigested retinal γ and placental γ are shown with the arrows labeled γ_1 (retinal) and γ_6 (placental) (see footnote 4).

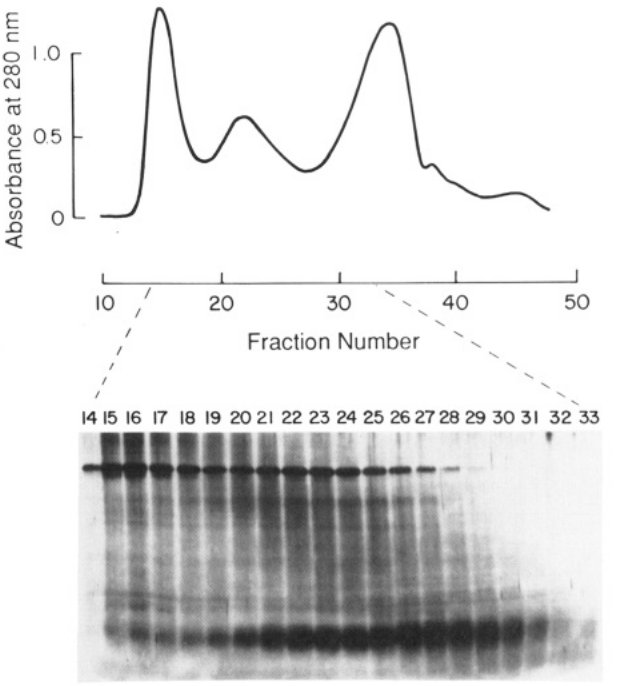

FIGURE 6: Resolution of native $\beta\gamma$ complex from β and γ chains. 6.4 mg of human placental $\beta_{35}\gamma$ was chromatographed on AcA-54 as described under Experimental Procedures. The top panel is the UV absorbance profile at 280 nm for this chromatography. The bottom panel presents the SDS–PAGE analysis of these fractions. Aliquots of the indicated fractions were diluted 1:20 in Laemmli sample buffer, and 20 μL of each was loaded on a 15% SDS gel run and stained with silver as described under Experimental Procedures. Pools from this chromatography were as follows: "β" fractions 15–18; "$\beta\gamma$" fractions 21–25, and "γ" 26–30.

V8, trypsin, and chymotrypsin digestions of $G_i\beta\gamma$ and placental $\beta_{35}\gamma$ with electrophoretically isolated β_{36} and β_{35}. These results indicate that the presence of the γ subunit did not alter the digestion of the denatured β chain.

Purification and Characterization of Native Human Placental $\beta\gamma$. Throughout the course of this work, we have noted variability in the activity of $\beta\gamma$ preparations.[2] This appears

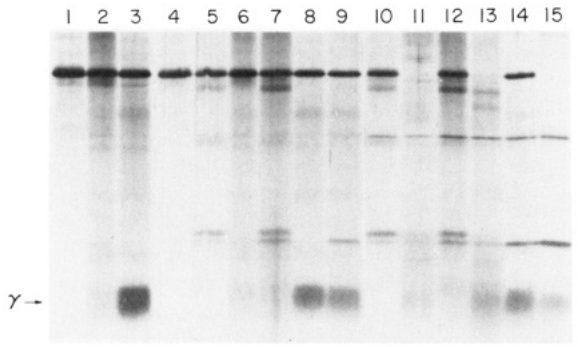

FIGURE 7: Tryptic digestion of native and denatured β. Native $\beta_{35}\gamma$ was isolated from the chromatography shown in Figure 6. An additional sample of human placental $\beta_{35}\gamma$ was first denatured by boiling 2% SDS followed by chromatography on AcA-54 as described under Experimental Procedures. In this chromatography, no $\beta_{35}\gamma$ peak was obtained, with β_{35} and γ peaks corresponding to the "β" and "γ" pools obtained in the experiment of Figure 6. Samples were digested in solution with 100 ng (lanes 4–9) or 500 ng of trypsin (lanes 10–15) for 1 min (lanes 4, 6, 8, 10, 12, and 14) or 30 min (lanes 5, 7, 9, 11, 13, and 15). β chains used were (lane 1) 10 μg of β_{35} peak from denaturing chromatography and (lanes 2 and 3) 10 μg of β_{35} peak and 5 μg of $\beta_{35}\gamma$, respectively, from the experiment of Figure 6. Lanes 4, 5, 10, and 11 are digestions of denatured β_{35}; lanes 6, 7, 12, and 13 are digestions of resolved β_{35}; and lanes 8, 9, 14, and 15 are digestions of $\beta_{35}\gamma$. Digestions were subjected to SDS–PAGE on a 15% separating gel and stained with silver as described under Experimental Procedures.

to be due in part to denaturation of the β chains. To address this, we have devised a chromatographic resolution of native $\beta\gamma$ from denatured β and γ chains. Figure 6 presents the results of chromatography of 6.4 mg of purified human placental $\beta_{35}\gamma$ over a 50-mL Ultrogel AcA-54 column, resulting in a $\beta\gamma$ complex that was found to be functionally very active.[2] Previously we had shown that the activity of native $\beta\gamma$ subunits was resistant to trypsin during a 10-min incubation with a mass ratio of 1:7 trypsin (Northup et al., 1983); however, digest profiles in Figures 4B and 5 show that the β chains are being proteolyzed at somewhat lower mass ratios (1:20 trypsin). Since the β chains in these experiments were denatured in SDS prior to proteolysis, we tested the tryptic digestion of the native $\beta\gamma$ complex as compared with denatured β under the same conditions. Figure 7 presents resolved β and $\beta\gamma$ from the experiment shown in Figure 6 with the addition of a β_{35} that was run through a similar chromatographic procedure, except that the sample was boiled prior to being loaded on the column. While both samples of resolved β_{35} seem to be digested in the same pattern, the $\beta\gamma$ complex is resistant to cleavage at lower enzyme concentrations. In addition, a major digestion fragment is missing from the $\beta\gamma$ peptide map in comparison with denatured β chain. It should be noted that the γ subunit was not digested appreciably and even at high enzyme concentrations the diffuse γ band is still obvious.

Resolution of Different Forms of the γ Subunit from a Variety of Sources. Even SDS gels with high-percentage acrylamide (up to 17%) did not resolve γ peptides well. Further, γ proteins tended to appear as very diffuse bands (Figure 5, panel A, and Figure 7). In order to overcome this obstacle, we have used a rather new system (Schagger & Von Jagow, 1987) that takes advantage of the properties of Tricine and urea. Tricine at the usual pH used (6.8–8.8) as the trailing ion migrates faster than glycine, so stacking and destacking of small peptides can be achieved, and since urea reduces the effective pore size in the gels, the end results are elimination of the need to use a higher acrylamide concentration and a better resolution of small molecular weight proteins. We find this system less complicated than others published (Hilde-

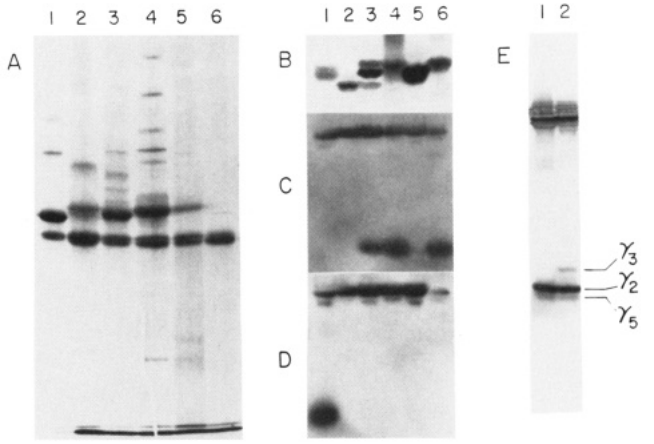

FIGURE 8: PAGE and immunoblot analysis of γ forms. G-protein oligomers were isolated as described under Experimental Procedures. Panel A presents an 11% SDS gel analysis of the composition of these proteins visualized with silver: lane 1, 2.7 μg of $G_t\alpha\beta\gamma$; lane 2, 4.2 μg of bovine brain G_o/G_i $\alpha\beta\gamma$; lane 3, 3.5 μg of human platelet G_i $\alpha\beta\gamma$; lane 4, 3.6 μg of rabbit liver $G_i\alpha\beta\gamma$; lane 5, 4.2 μg of human placental $G_i\alpha\beta\gamma$; lane 6, 1.2 μg of human placental $\beta_{35}\gamma$. Panels B–D are replicate 15.5% acrylamide–6 M urea–tricine gels run as described under Experimental Procedures. Samples were (lane 1) 5.4 μg of $G_t\alpha\beta\gamma$, (lane 2) 3.9 μg of human platelet $G_i\alpha\beta\gamma$, (lane 3) 5.2 μg of rabbit liver $G_i\alpha\beta\gamma$, (lane 4) 5.6 μg of human placental $G_i\alpha\beta\gamma$, (lane 5) 5.6 μg of bovine brain G_o/G_i $\alpha\beta\gamma$, and (lane 6) 1.7 μg of human placental $\beta_{35}\gamma$. Panel B presents the silver staining of the region of γ migration. Replicate gels were transferred to nitrocellulose and probed for recognition with polyclonal antisera as described under Experimental Procedures, and the regions of β and γ migration are shown in panels C and D. Panel C, antiserum β-8; panel D, antiserum Tr-β-11. Examination of two bovine brain G_o/G_i preparations is shown in panel E: lane 1, 2.5 μg of the G_o/G_i shown in panels A–D; lane 2, 2.5 μg of an independent G_o/G_i preparation.

brandt et al., 1984, 1985), and we now show that γ peptides can be clearly resolved by using this Tricine–urea system. Figure 8 presents an 11% Laemmli gel side by side with high-resolution gels for a set of homogeneous G-proteins which includes bovine retinal G_t, bovine brain G_o/G_i, human platelet G_i, human placental G_i, and rabbit liver G_i, as well as human placental $\beta_{35}\gamma$. The proteins seen in the silver-stained 11% gel (panel A) were all >90% pure as judged by Coomassie staining and GTPγS-binding activities (data not shown). Panel B shows the region of migration of the γ peptides on a 15.5% polyacrylamide–6 M urea gel. As previously reported, retinal $G_t\gamma$ migrates distinctly from γ peptides of other tissues (Hildebrandt et al., 1985). Surprisingly, the γ components from liver and brain G-proteins are distinctly heterogeneous, with three different R_f's in the liver and three additional R_f's in the brain. The faster migrating γ of liver G_i appears to correspond to the γ subunit affiliated with platelet G_i2, while the most slowly migrating γ in liver corresponds to the γ protein found in placental G_i and $\beta_{35}\gamma$. Western transblot replicates of this gel, that are shown in panels C and D, identify the upper γ in liver G_i as immunologically similar to placental γ while all the other γ peptides are unrecognized. Similarly, antisera raised against bovine $G_t\beta\gamma$, represented by Tr-β-11 in panel D, react exclusively with retinal γ subunit. A separate high-resolution gel analysis of bovine brain G_o/G_i preparations is shown in panel E. The protein load in this experiment was reduced to examine the γ composition of the closely migrating γ bands, previously refered to as "γ_6" and "γ_5" (Robishaw et al., 1989). This gel shows that both G_o/G_i preparations contain these two γ bands, while one of them also contains a more slowly migrating γ that has been identified as γ_3 by reactivity with peptide-directed antisera (Gautam et al., 1990). In other experiments where the protein loads were reduced

as well, bovine G_t was found to consist of two distinct bands which were both recognized by antiserum Tr-β-11 (data not shown).

Discussion

In contrast with the α subunits, for which at least 16 cDNA clones have now been isolated, β and γ subunits have been thought to be highly conserved. It has been argued that $\beta\gamma$ subunits perform an identical function common to all G-proteins while divergent, receptor- and effector-specific functions are performed by the α subunits. The identification of three cDNA structures encoding β and two encoding γ subunits does not appear to support this argument. Previous studies have only revealed two protein structures each for β and γ subunits. Therefore, we have examined the β and γ structures affiliated with a diverse set of G-proteins to reveal any tissue or protein specificity in these subunits. Our results confirm the conservation of β-subunit structure but reveal an unexpected diversity of γ structures.

In all G-proteins we have examined, only two separable β polypeptides were observed. Both protease and immunological analyses of the two β chains showed identity of the β_{36} and β_{35} forms from the disparate G-proteins. These results extend our previous examination of the homology between the β forms (Evans et al., 1987) and complement studies using two-dimensional peptide mapping (Woolkalis & Manning, 1987) which concluded that there is homology, but distinction, between β_{36} and β_{35} proteins. The protein sequence for the two β forms at present is limited to the β_1 gene product. However, recognition of the β proteins by sequence-directed antisera designed to be specific for β_1 or β_2 gene products (Gao et al., 1987a; Amatruda et al., 1988) has confirmed the presence of β_1 epitopes in all the β_{36} bands and β_2 epitopes in all the β_{35} bands. Further, the identical fragmentation profiles obtained with V8 protease, trypsin, and chymotrypsin for all β_{36} and β_{35} proteins suggest that these have identical primary structures among all the examined G-proteins. These data also suggest that if the β_3 gene product is present in these bands it is in identical proportion with β_1 and/or β_2 gene products in all examined G-proteins.

The complete protein sequence of β_{35} is still unknown; our attempts to determine it failed, mainly due to the hydrophobic nature of the polypeptide and a blocked amino terminus. Therefore, our comparison has been based on deduced sequences, and the two regions of nonhomology between the β_1 and β_2 gene products. The digestion profiles with V8 protease presented in this paper show slight discrepancies with the somewhat more complex profiles we have previously reported (Evans et al., 1987). This probably is due to an increased resolution of the β chains by the techniques we report herein and our ability to confirm the homogeneity of the products using specific antisera. The V8 profiles reveal four distinct terminal digestion products resolved from β_{36} and two unique fragments from the β_{35} form. Examination of the deduced structures reveals a Glu at residue 215 in β_1 which is substituted with Asp in β_2. This could account for one additional fragment from β_{36} assuming that all the other Glu sites are equally exposed to the enzyme after the β chain has been denatured. It seems more likely that not all sites are equally exposed to proteolysis in SDS solution since β_{35} digestion yields only two distinct products while the primary structure of $\beta 2$ gene product predicts five fragments of greater than 3 kDa that should have been resolved by our gel system. Therefore, we conclude that the protease profiles also reflect differential exposure of sites between the two protein structures in SDS solution.

While we have been unsuccessful in obtaining sequence data for the β_{35} protein, we can identify the fragments containing the amino terminus based upon the reactivity of the antiserum βPN1–16. The terminal fragment we have designated V8 – 1 (for which no sequence could be determined from >1 nmol of peptide) was recognized by βPN1–16. The homologous fragment from β_{36} proteins contained the major epitope(s) for all β_{36}-reactive antisera and the amino-terminal peptide-directed serum βN1. Others have also noted that the dominant, if not unique, antigenic site(s) for β subunits existed within the amino-terminal 15-kDa portion of the molecule based upon a tryptic cleavage (Zaremba et al., 1988).

Our results also reveal a conformational dependence of tryptic proteolysis of the β_{35} protein. The native $\beta_{35}\gamma$ structure was not only more resistant to proteolysis, but also yielded a distinct fragmentation profile; the proteolytic intermediates present in digests of denatured β_{35} never appeared in the digestion of native $\beta_{35}\gamma$. This conformational specificity of proteolysis may be a useful probe for defining native β chains in preparative purification fractions of $\beta\gamma$. We have resolved native $\beta_{35}\gamma$ from denatured β_{35} and γ chains by sizing chromatography, and we have shown that identically migrating β_{35} and γ chains are resolved from preparations which were not purposefully denatured as well as from boiled $\beta\gamma$ samples. The tryptic proteolysis of the β_{35} chain resolved from an untreated $\beta_{35}\gamma$ sample was found to be identical with that for denatured β_{35} and distinct from that for the native $\beta_{35}\gamma$ complex. Resolved β_{35} chain was found to be inactive in reconstitution of rhodopsin-catalyzed GTPγS binding to $G_t\alpha$ while the $\beta_{35}\gamma$ peak was enriched in activity (data not shown). Intriguingly, the resolved β_{35} chain migrates as an approximately 70-kDa protein, suggesting a dimeric structure in detergent solution. This β_{35} dimer is probably not analogous to the suggested $\beta\gamma$ dimer found for the retinal G-protein (Baehr et al., 1982) since the latter protein was presumably active. Alternatively, the apparent β_{35} dimer we have resolved from active $\beta_{35}\gamma$ complexes may represent an anomalous migration of the unfolded β chain in this chromatography.

Unlike the β chains, the γ peptides that are resolved during oligomeric G-protein purifications are heterogeneous and exhibit tissue and G-protein specificity. Bovine retina expresses two resolvable γ subunits based on mobility in 6 M urea–acrylamide gels. These correspond to the two γ forms reported by Fukada and co-workers for bovine G_t (Fukada et al., 1989). Bovine brain expressed three γ proteins, two of which were previously identified in bovine brain G_o/G_i mixtures (Robishaw et al., 1989). Human platelet and placenta G_i proteins each contain a uniquely migrating γ form, while liver G_i is the source of three resolvable γ subunits. Reactivity with antiserum β-8 reveals an immunologically similar γ in the liver G_i with identical mobility with placental γ. As previously reported, the retina expressed a unique γ chain corresponding to the γ_1 gene (Hurley et al., 1984). We also find as reported that all retinal γ-recognizing sera tested reacted with both γ types in G_t (Evans et al., 1987; Fukada et al., 1989; Gautam et al., 1990). Since the carboxyl terminus of retinal γ contains the putative consensus sequence Cys-a-a-X, which was indicated by recent work to be the site for isoprenylation (Yamane et al., 1990; Mumby et al., 1990), it is possible that the distinctly migrating γ peptides represent modified and nonmodified chains. Since the γ peptides are associated with oligomer G_t, it is also possible that they both are mature products of distinct genes like the rod- and cone-specific alleles of $G_t\alpha$ (Yatsunami & Khorana, 1985; Lochrie et al., 1985).

We have recently reported that antisera designed to be specific for sequences deduced for the γ_2 gene product react with a brain γ and liver γ of identical mobility (middle γ of liver, see Figure 8, panel B lane 3), while anti-γ_3 recognized a G_o/G_i-specific γ band (Gautam et al., 1990). Two γ proteins migrating with apparent molecular weights of 6K and 5K [termed γ_6 and γ_5 by Robishaw et al. (1989)] have been partially sequenced, and a clone containing the sequences of the 6-kDa protein was isolated and found to be identical with γ_2. The 5-kDa peptide yielded a single fragment whose sequence does not correspond to sequences found in γ_1, γ_2, γ_3, or a fourth partial length clone termed γ_4 (Gautam et al., 1990). The antiserum β-8 recognizes a placental and a liver γ of identical mobility, distinct from the brain γ peptides. Therefore, it seems reasonable to assume that this is an additional, distinct γ which is not γ_1, γ_2, γ_3, or brain 5-kDa γ. We have provisionally named it "γ_6".[4] We also suggest the existence of another novel γ subunit, based on gel resolution, which is expressed in liver and platelets, and we provisionally name it "γ_7". The identities of these additional γ forms await sequence confirmation.

Our data point to a highly conserved structure for the β subunits with considerable heterogeneity of γ structure. Thus, while the $\beta\gamma$ complex may carry out a conserved function in the G-protein family, the heterogeneity of γ peptides suggests diverse specificities. One possible explanation for this is that β chains contain a common site for α-subunit recognition, interchangeable among G-proteins, while γ chains may confer a G-protein-specific receptor recognition. The apparent interchangeability of $\beta\gamma$ forms has been reported for disparate biochemical assays (Kanaho et al., 1984; Cerione et al., 1987). Only the deactivation of $G_s\alpha$ and coupling of β-adrenergic receptors to $G_s\alpha$ have been reported to distinguish between retinal and other $\beta\gamma$ forms (Cerione et al., 1987; Casey et al., 1989). We have recently proposed that the γ subunit provides receptor specificity, and we have found that while $G_t\alpha$ does not distinguish between placental $\beta_{35}\gamma_6$ and retinal $\beta_{36}\gamma_1$, the rhodopsin-catalyzed activation of $G_t\alpha$ does.[2] The diversity of γ structures which are revealed by our work may thus correspond to the diversity of receptor structures with which G-proteins must specifically interact.

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Properties of the Two Terminal Oxidases of *Escherichia coli*†

Anne Puustinen, Moshe Finel,‡ Tuomas Haltia, Robert B. Gennis,§ and Mårten Wikström*
Helsinki Bioenergetics Group, Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10A, 00170 Helsinki, Finland

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ABSTRACT: Proton translocation coupled to oxidation of ubiquinol by O_2 was studied in spheroplasts of two mutant strains of *Escherichia coli*, one of which expresses cytochrome *d*, but not cytochrome *bo*, and the other expressing only the latter. O_2 pulse experiments revealed that cytochrome *d* catalyzes separation of the protons and electrons of ubiquinol oxidation but is not a proton pump. In contrast, cytochrome *bo* functions as a proton pump in addition to separating the charges of quinol oxidation. *E. coli* membranes and isolated cytochrome *bo* lack the Cu_A center typical of cytochrome *c* oxidase, and the isolated enzyme contains only 1Cu/2Fe. Optical spectra indicate that high-spin heme *o* contributes <10% to the reduced minus oxidized 560-nm band of the enzyme. Pyridine hemochrome spectra suggest that the hemes of cytochrome *bo* are not protohemes. Proteoliposomes with cytochrome *bo* exhibited good respiratory control, but H^+/e^- during quinol oxidation was only 0.3-0.7. This was attributed to an "inside out" orientation of a significant fraction of the enzyme. Possible metabolic benefits of expressing both cytochromes *bo* and *d* in *E. coli* are discussed.

The branched respiratory chain of *Escherichia coli* contains two different terminal oxidases, viz., cytochrome *bo* and cytochrome *d* [see Anraku and Gennis (1987) for a review]. These two ubiquinol oxidases are expressed differently in different growth conditions. Mutants able to express only one of the two nevertheless appear to be capable of normal growth (Au et al., 1985).

Both enzymes are located in the plasma membrane where their activity generates an electrochemical gradient of protons, which can subsequently be used to drive synthesis of ATP and transport of nutrients (Anraku, 1988).

Both oxidases of *E. coli* have been isolated, purified, and characterized (Kita et al., 1984ab; Matsushita et al., 1984; Miller & Gennis, 1983). Cytochrome *bo* contains two cytochrome *b* type heme groups, suggested to be protohemes, and has been reported to contain two copper ions (Kita et al., 1984a). The protein, as deduced from its gene structure in *E. coli*, strongly resembles that of cytochrome aa_3 (Saraste et al., 1988; Chepuri et al., 1990). The carbon monoxide action spectrum of cytochrome *bo* (Castor & Chance, 1955) showed that one of the two hemes (here called *o*) reacts with CO and

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* Author to whom correspondence should be addressed.
‡ Present address: Laboratory of Molecular Biology, Medical Research Council, Hills Road, Cambridge CB2 2QH, England.
§ Permanent address: School of Chemical Sciences, University of Illinois at Urbana—Champaign, 505 South Mathews Ave., Urbana, IL 61801.

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