Purification of Heterotrimeric GTP-Binding Proteins from Brain: Identification of a Novel Form of G_o

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ABSTRACT: Using high-resolution Mono-Q anion-exchange chromatography, we purified four distinct GTP-binding proteins from bovine brain. Each consists of α and associated β/γ subunits, and each is a substrate for pertussis toxin catalyzed ADP-ribosylation. We defined the relationship between the α subunits of the purified proteins and cloned cDNAs encoding putative α subunits (1) by performing immunoblots with peptide antisera with defined specificity and (2) by comparing the migration on two-dimensional gel electrophoresis of the purified proteins, and of the in vitro translated products of cDNAs encoding α subunits. Purified G proteins with α subunits of 39, 41, and 40 kDa (G_{39}, G_{41}, and G_{40} in order of abundance) correspond to the products of G_o, G_{i1}, and G_{i2} cDNAs. We purified a novel G protein with an α subunit slightly above 39 kDa (G_{39}*). G_{39}* is less abundant than G_{39}, elutes earlier than G_{39} on Mono-Q chromatography, and has a more basic p*I* (6.0 vs 5.6) than G_{39}. G_{39} and G_{39}*, however, are indistinguishable on immunoblots with a large number of specific antisera. The data suggest that G_{39}* may represent a novel form of G_o, differing in posttranslational modification rather than primary sequence.

Heterotrimeric guanine nucleotide binding proteins (G proteins)[1] couple cell surface receptors to effectors (Spiegel, 1987; Gilman, 1987). α subunits, unique for each G protein, may confer specificity in receptor and effector interactions; α subunits also bind guanine nucleotide and are substrates for ADP-ribosylation catalyzed by bacterial toxins such as pertussis toxin. Immunochemical studies (Goldsmith et al., 1987) provide evidence for multiple, distinct pertussis toxin substrates. cDNA cloning also provides evidence for multiple homologous, but distinct, pertussis toxin substrates. Thus, cDNAs encoding G-protein α subunits designated G_{i1}, G_{i2}, G_{i3}, and G_o, each a putative pertussis toxin substrate, have been cloned (Nukada et al., 1986; Itoh et al., 1986; Bray et al., 1987; Jones & Reed, 1987; Van Meurs et al., 1987; Didsbury & Snyderman, 1987; Suki et al., 1987).

Using two-dimensional gel electrophoresis, we were able to resolve at least four pertussis toxin substrates in a partially purified preparation of brain G proteins (Backlund et al., 1988). With specific antibodies, we were able to identify two of these as α subunits of G_o and G_{i1} and suggested that a third might represent G_{i2}. We have now succeeded in purifying four distinct G-protein, pertussis toxin substrates from bovine brain. We characterized these using specific peptide antisera, and also by comparing their migration on 2-D gels to that of the in vitro translation products of G-α cDNA-derived mRNAs. These studies allow definitive identification of three of the four purified proteins as G_{i1}, G_{i2}, and G_o and suggest that the fourth represents a heretofore unrecognized form of G_o.

Experimental Procedures

G-Protein Purification. Bovine brains from freshly slaughtered cows were transported on ice to the laboratory. Membrane preparation and extraction into TED/1% cholate buffer were performed as previously described (Sternweis & Robishaw, 1984; Gierschik et al., 1986). The brain cholate extract was subjected to anion-exchange chromatography using a 3-L DEAE-Sephacel (Pharmacia) bed volume and a linear 7-L gradient from 0 to 0.3 M NaCl in TED/1% cholate buffer. The major peak of guanine nucleotide binding activity, identified by [^{35}S]GTPγS binding, was pooled and concentrated to 50 mL by ultrafiltration using an Amicon YM30 membrane and subjected to gel permeation chromatography using AcA 34 Ultrogel (LKB), 5 × 92 cm column, and TED/1% cholate buffer containing 0.1 M NaCl. The major peak of [^{35}S]-GTPγS binding was again identified, pooled, diluted with 3 volumes of TED/0.6% Lubrol PX buffer, applied to a 3.2 × 30 cm column of DEAE-Toyopearl 650(S), and eluted with an 800-mL gradient of 0–0.25 M NaCl in TED/0.6% Lubrol PX buffer. G-Protein-containing fractions were identified by inspection of Coomassie blue stained gels and immunoblotting (see Results). Mono-Q (Pharmacia) column chromatography was performed as described by Katada et al. (1987).

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[1] Abbreviations: G protein, guanine nucleotide binding protein; G_s, G protein associated with stimulation of adenylyl cyclase; G_i, G protein originally identified in terms of inhibition of adenylyl cyclase; G_o, G protein of undefined function abundant in brain; G_{i1}, G_{i2}, and G_{i3}, three homologous but distinct G proteins arbitrarily named in order of their cDNA cloning; G_{41}, G_{40}, G_{39}, and G_{39}*, G proteins with α subunits of 41, 40, 39, and 39.5 kDa, respectively, on SDS–PAGE; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; TED, 20 mM Tris-HCl (pH 7.5), 1 mM NaEDTA, and 1 mM DTT; 2-D gel, two-dimensional gel; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Guanine Nucleotide Binding Assay. [^{35}S]GTPγS (New England Nuclear, specific activity 1335 Ci/mmol) binding of fractions from DEAE-Sephacel and AcA 34 Ultrogel columns was performed as described (Sternweis & Robishaw, 1984); samples were incubated for 60 min at 30 °C.

Pertussis Toxin Labeling. Pertussis toxin (Islet-activating protein, List Biochemicals) was activated for 15 min at 30 °C in 10 mM DTT at a final concentration of 100 μg/mL. Mono-Q column fractions (in TED/0.7% CHAPS buffer) were diluted into buffer to give final concentrations of 0.1% Lubrol PX, 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT. Toxin labeling was performed in a final volume of 0.1 mL containing 10 μL of column fraction as above, 10 μL of activated toxin (1 μg), and 80 μL of buffer consisting of 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM ATP, and 3.5 μM [^{32}P]NAD (New England Nuclear, specific activity 28 Ci/mmol). The reaction, 1 h at 30 °C, was stopped by adding an equal volume of SDS–PAGE sample buffer and boiling for 3 min. Aliquots were then subjected to SDS–PAGE (10% gels); the gels were fixed, and autoradiography was performed with an image-intensifying screen (Cronex) at −70 °C.

SDS–PAGE, Immunoblots, and 2-D Gel Electrophoresis. SDS–PAGE and immunoblotting were performed as previously described (Gierschik et al., 1986) except that samples were routinely treated with *N*-ethylmaleimide (Sternweis & Robishaw, 1984). 2-D gels were performed as previously described (Backlund et al., 1988).

Antisera. Polyclonal rabbit sera used for immunoblots are listed in Table I. Production and characterization of RV/3 (Gierschik et al., 1986), AS/6 and -7, and LE/2 and -3 (Goldsmith et al., 1987) and of LD, SQ, GA, and MS antisera (Goldsmith et al., 1988) were previously described. Peptides used as antigens for production of the remaining antisera were synthesized as previously described (Goldsmith et al., 1987), except that the peptide used to immunize IM/1 was synthesized with an amino-terminal cysteine as a potential site for coupling with a bifunctional reagent, and the β-peptide was synthesized by Peptide Technologies (Washington, DC) as the carboxy-terminal amide. All peptides were conjugated to keyhole limpet hemocyanin (Sigma) using glutaraldehyde, and immunization and affinity purification of antisera were performed as described (Gierschik et al., 1986; Goldsmith et al., 1987).

In Vitro Transcription and Translation. Rat cDNAs encoding G_{i1}, G_{i2}, G_{i3}, and G_o were from Jones and Reed (1987). The pGEM-2 plasmid vectors (Promega Biotec) containing cDNA inserts were linearized with either *Hin*dIII (G_{i1}, G_{i2}, G_{i3}) or *Xmn*I (G_o) restriction endonucleases (New England Biolabs). In vitro RNA transcription was done with 2 μg of linearized plasmid DNA and 10 units of either SP6 (G_{i1}, G_{i2}, G_{i3}) or T7 (G_o) RNA polymerase under conditions specified by the supplier (Promega Biotec). Methylguanylate cap (Pharmacia) was added at the beginning of the 2-h incubation at 37 °C.

In vitro translation of the RNA (Pelham & Jackson, 1976) was performed with the rabbit reticulocyte lysate system (Bethesda Research Laboratories). L-[^{35}S]Methionine (Amersham) was used at a final concentration of 1 mCi/mL, and the incubation was for 1 h at 30 °C.

RESULTS

Using specific, high-affinity guanine nucleotide binding as an assay (Sternweis & Robishaw, 1984), we purified G proteins from a cholate extract of bovine brain membranes. Successive chromatography on columns of DEAE-Sephacel, AcA 34 Ultrogel, and DEAE-Toyopearl 650(S) (TSK-Toyopearl) was performed as described under Experimental Procedures. Fractions from TSK-Toyopearl chromatography were analyzed by SDS–PAGE and Coomassie blue staining (Figure 1A). The predominant proteins in fractions 28–31 are 36- and 35-kDa proteins, identified by immunoblotting as the two forms of β subunit common to G proteins (data not shown). Since neither fluoride nor other G-protein activators were used in our purification scheme, the appearance of free β subunits may reflect spontaneous dissociation of β/γ from α subunits, particularly G_o-α (Sternweis & Robishaw, 1984; Neer et al., 1984), during the course of purification.

TSK-Toyopearl fractions 32–45 contain, in addition to β subunits, a heterogeneous group of proteins in the range of 39–43 kDa. Early fractions, 32–36, contain an apparent doublet of 39 and 41 kDa, whereas later-eluting fractions 39–44 contain as many as three or four bands. To obtain further purification of G proteins, we subjected TSK-Toyopearl fractions to chromatography on a high-resolution anion-exchange Mono-Q column (Katada et al., 1987). Figure 1, parts B, C, and D, shows the results of fractionation of TSK-Toyopearl fractions 33, 38, and 40, respectively, by anion-exchange Mono-Q chromatography. TSK fraction 33 is resolved into two distinct peaks of protein (Figure 1B, left), an early eluting peak consisting of α_{39}* with associated β/γ and a later eluting peak of α_{41} with associated β/γ (Figure 1B, right). TSK fraction 38 is resolved into four distinct peaks (Figure 1C, left). The small, earliest eluting peak consists exclusively of α_{39}*; although all of the subsequent fractions contain α_{39} protein, the second and fourth peaks consist primarily of α_{41} and α_{40} proteins, respectively. The third and largest peak of protein contains predominantly α_{39} (Figure 1C, right). TSK fraction 40 is resolved by Mono-Q chromatography into two major peaks consisting of α_{39} and α_{40}, respectively, preceded by a minor peak (fraction 22) containing α_{41} (Figure 1D).

Mono-Q fractions representing each of the four, distinct peaks were subjected to SDS-PAGE, Coomassie blue and silver staining, and pertussis toxin catalyzed ADP-ribosylation. Coomassie blue staining revealed slight contamination of the G_{40} peak with α_{39}, and of the G_{39} peak with α_{41} (Figure 2). α_{39}* migrates subtly, but reproducibly, more slowly than α_{39} (Figure 2). Each peak contains both 36- and 35-kDa forms (36 > 35 kDa) of β subunit; there was no indication of preferential association of either form of β subunit with any particular α subunit (Figure 2). Likewise, there was no apparent difference in the γ subunits (visualized by silver staining a 15% gel) associated with the four forms of G protein (not shown). α subunits in each peak serve as pertussis toxin substrates; α_{41} and α_{40} showed higher incorporation of ADP-ribose than α_{39} and α_{39}* (not shown).

To identify the G proteins contained in the four peaks, we performed immunoblot analysis with specific peptide antisera (Table I). An antiserum, GA/1, raised against a highly conserved sequence common to G-α subunits, and similar to one described by Mumby et al. (1986), recognized each of the α subunits and gave an immunoblot pattern (Figure 3, left) comparable to that seen with Coomassie blue staining. Antiserum AS/7, shown previously (Goldsmith et al., 1987) to recognize G_i but not G_o, reacts with α_{41} and α_{40}, but not with either α_{39} or α_{39}*. AS/7 does reveal the α_{41} contaminating the G_{39} peak. Since AS/7 does not differentiate between G_{i1} and G_{i2} which share identical carboxy-terminal decapeptide sequences, we also used antisera raised against a decapeptide sequence that is distinct for each form of G_i (Table I). Antiserum LD/1, raised against a G_{i1}-specific sequence, reacts

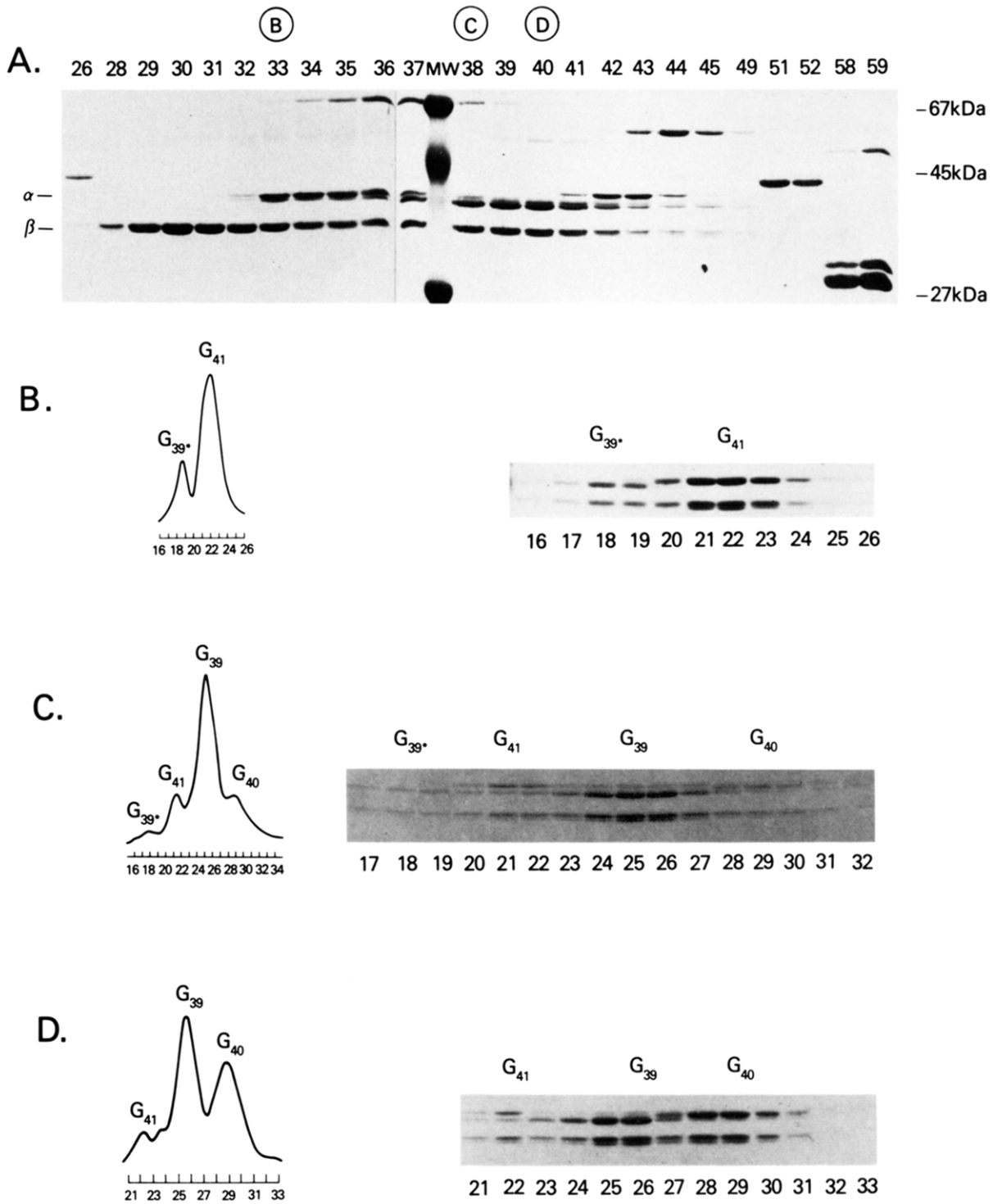

FIGURE 1: Analysis by SDS–PAGE and Coomassie blue staining of fractions from (A) TSK-Toyopearl chromatography and (B–D) Mono-Q chromatography. Forty microliters of each fraction was treated with *N*-ethylmaleimide and SDS–PAGE sample buffer and separated on a 10% gel. Fractions shown in (A) were run on two separate gels (division between fractions 36 and 37). The molecular weight standards shown (MW) are bovine serum albumin (67 kDa), ovalbumin (45 kDa), and α-chymotrypsinogen (27 kDa). Portions of the gel above and below these standards did not contain stained proteins with the exception of the dye front containing presumptive γ subunits (not shown). The positions of G-protein α and β subunits are indicated. The TSK-Toyopearl fractions (25 mL each) are numbered at the top, and those fractions subjected to Mono-Q chromatography (33, 38, and 40) are labeled. The Mono-Q chromatographic profiles of these fractions are shown in panels B–D of the figure. The left side of panels B–D shows the tracing of OD_{280} absorption for the G-protein-containing fractions, and the right side shows the Coomassie blue stained gel of the corresponding fractions. Fractions (1 mL each) are numbered at the bottom, and the G protein in each peak is labeled. Only the α and β subunit-containing portion of the gel is shown.

with α_{41} in the G_{41} peak and also with contaminating α_{41} in the G_{39} peak. In contrast, G_{i2}-specific antiserum, LE/3, reacts only with α_{40} in the G_{40} peak (Figure 3). Antiserum SQ/2, raised against a G_{i3}-specific peptide, and specifically reactive (Goldsmith et al., 1988) with an α_{41} purified from HL-60 cells (Uhing et al., 1987), showed no detectable reactivity in any of the G-protein peaks (not shown). Antiserum GO/1, raised against the carboxy-terminal decapeptide of G_o-α, reacts equivalently with α_{39} and α_{39}*, but with neither α_{41} nor α_{40}. GO/1 does reveal the α_{39} contaminating the G_{40} peak (Figure 3). Three additional G_o-specific antisera, RV/3, IM/1, and GC/1 (Table I), like GO/1, reacted equivalently and spe-

Table I: Antisera Used in This Study

antiserum	antigen[a]	specificity
RV/3	purified G_i/G_o	G_o-α, β
MS/1	β_1 cDNA (1–10)	β
GA/1	G_{i1} cDNA (40–51)	common G-α
AS/6, AS/7	transducin-α (341–350)	transducin-α and G_i-α
GC/1	G_o cDNA (2–17)	G_o-α
GO/1	G_o cDNA (345–354)	G_o-α
IM/1	G_o cDNA (22–36)	G_o-α
LD/1, LD/2	G_{i1} cDNA (159–168)	G_{i1}-α
LE/2, LE/3	G_{i2} cDNA (160–169)	G_{i2}-α
SQ/2, SQ/3	G_{i3} cDNA (159–168)	G_{i3}-α

[a] For all but RV/3, synthetic peptides were used as antigens; the numbers in parentheses refer to the residues of the cDNA-predicted sequence of peptide antigens.

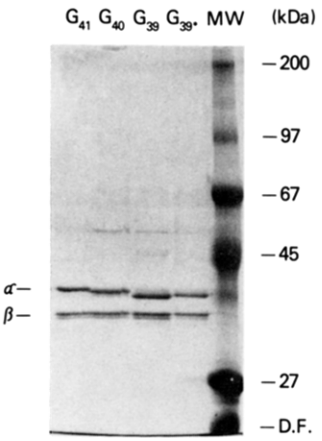

FIGURE 2: SDS–PAGE and Coomassie blue staining of purified G proteins. Forty microliters each of Mono-Q fractions containing G_{41}, G_{40}, G_{39}, and G_{39}* was separated on a 10% gel and stained with Coomassie blue. The positions of α subunits and the β doublet are indicated. Presumptive γ subunits run with the dye front (D.F.). The approximate molecular weight (MW) of marker proteins (Bethesda Research Labs prestained "high" standards) run in parallel is also indicated.

cifically with α_{39} and α_{39}* (not shown). GC/1 and GO/1 antisera were raised against synthetic peptides corresponding to the amino (2–17) and carboxyl (345–354) termini, respectively, of G_o-α (Table I). Their equivalent reactivity with α_{39} and α_{39}* helps exclude the possibility that one of the two proteins is a proteolytic product of the other.

In order to compare the purified brain G proteins by an additional, independent method, we analyzed the peak Mono-Q fractions by 2-D gel electrophoresis and silver staining (Figure 4). A mixture of purified G proteins (Figure 4, G-MIXTURE) showed four major spots, labeled a–d, corresponding in mobility to the four major pertussis toxin substrates we described previously (Backlund et al., 1988). Analysis of the individual purified G proteins (Figure 4) revealed a major spot for each corresponding to a (α_{39}), b (α_{40}), c (α_{39}*), or d (α_{41}), as well as smaller acidic satellite spots (labeled a′, b′, c′, or d′). α_{41} has the most basic p*I* (approximately 6.1), whereas α_{40} and α_{39} are the most acidic (p*I*'s about 5.65 and 5.6, respectively). Importantly, the position of α_{39}* (p*I* 6.0) is completely distinct from that of α_{39}.

In order to define the p*I* of the proteins encoded by cloned G-α cDNAs, we used the latter to transcribe individual mRNAs which were then translated in vitro. We analyzed the [^{35}S]methionine-labeled in vitro translation products both by SDS–PAGE and by 2-D gel electrophoresis. On SDS–PAGE, the translation products of G_{i1}, G_{i2}, G_{i3}, and G_o cDNAs migrate as 41-, 40-, 41-, and 39-kDa proteins, respectively (Goldsmith et al., 1988). To correlate the positions of the translation products with those of purified G proteins, reticulocyte lysate containing translation products and a mixture of purified G proteins were combined before 2-D gel electrophoresis. The proteins were transferred to nitrocellulose paper; the positions of the translation products were revealed by autoradiography (Figure 5, left) and those of the purified G proteins by immunoblotting with specific antibodies (Figure 5, right). An endogenous reticulocyte lysate protein ("R" in Figure 5) serves as a convenient reference marker. For G_{i1} (Figure 5A,B) and G_o (Figure 5G,H), the p*I*'s of the translation products correspond precisely to those of the major immunoreactive forms of α_{41} and α_{39}, respectively. For G_{i2} (Figure 5C,D), the p*I* of the translation product is slightly more acidic than that of the major immunoreactive form of α_{40} and corresponds instead to that of the minor, acidic satellite of α_{40}. The 2-D gel position of the G_{i3} translation product does not correspond to that of any of the major immunoreactive forms of purified brain G proteins. The p*I*, approximately 5.8, corresponds to that of a minor immunoreactive spot revealed by antiserum AS/6 (Figure 5E,F). The position of α_{39}* (the minor, most basic immunoreactive spot in Figure 5H) does not correspond to that of any of the four translation products.

The latter result, together with the equivalent reactivity of α_{39} and α_{39}* with G_o-α-specific antisera, suggested the possibility that α_{39}* differs from α_{39} by some posttranslational modification. To assess the possibility that α_{39}* could somehow be formed artifactually from authentic α_{39} during the course of chromatographic purification of the brain cholate extract, we performed immunoblots on bovine brain membrane proteins, freshly extracted with cholate and separated by 2-D gel electrophoresis. Immunoblots with G_o-α-specific antisera showed a pattern virtually identical with that seen in Figure 5H; i.e., the antibody revealed spots corresponding to purified α_{39} (Figure 4, a and a′) and to purified α_{39}* (Figure 4, c).

Discussion

The first reports on purification of pertussis toxin substrates from brain (Sternweis & Robishaw, 1984; Neer et al., 1984) described two G proteins with α subunits of 41 and 39 kDa. The initial report of Neer et al. (1984) also recognized a 40-kDa protein. Recently, Katada et al. (1987), using Mono-Q chromatography, resolved the 40-kDa protein from 41- and 39-kDa species and showed that it is immunochemically distinct. We have employed similar methods to isolate these three proteins from brain, and in addition have purified a novel form of G protein. The latter, designated G_{39}*, is lower in abundance than G_{39}, elutes earlier than G_{39} on TSK-Toyopearl chromatography, and can be completely resolved from G_{39} by mono-Q chromatography.

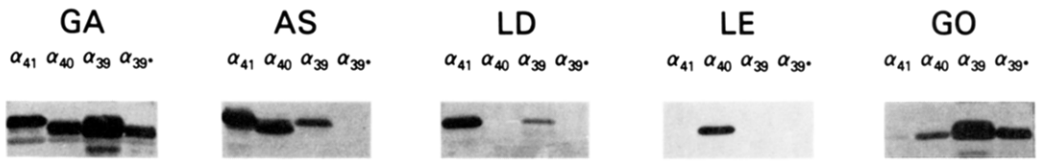

FIGURE 3: Immunoblot analysis of purified G proteins with specific peptide antisera. Twenty microliters each of Mono-Q fractions containing purified G_{41}, G_{40}, G_{39}, and G_{39}* was separated on a 10% gel and immunoblotted with the indicated antisera (GA/1, AS/7, and GO/1 at 1:250 dilution; LD/1 and LE/3 at 1:100 dilution).

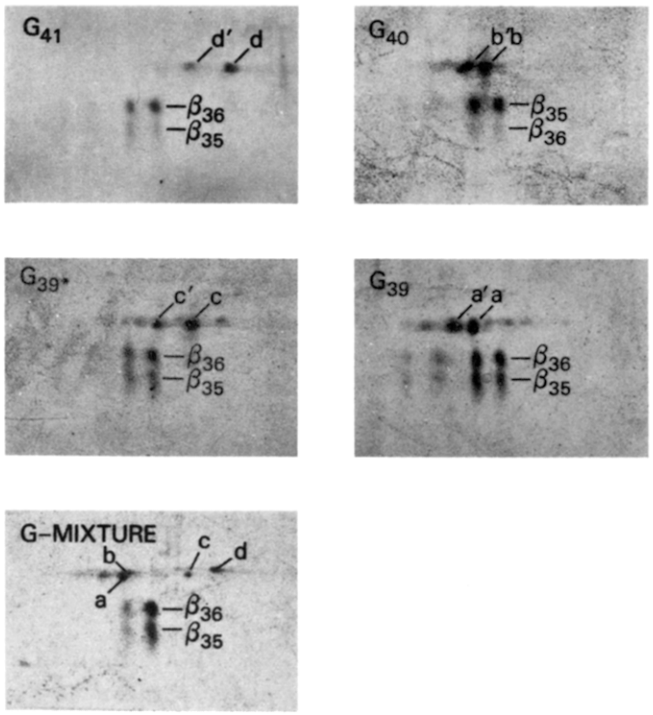

FIGURE 4: Analysis of purified G proteins by two-dimensional gel electrophoresis. A mixture of brain G proteins (G-MIXTURE) and Mono-Q fractions (10 μL each) containing purified G_{41}, G_{40}, G_{39}*, and G_{39} were subjected to two-dimensional gel electrophoresis and silver stained. The positions of the two forms of β subunit (36 and 35 kDa) are indicated. The individual α subunits are labeled as follows: a = α_{39}, b = α_{40}, c = α_{39}*, and d = α_{41}. a′, b′, c′, and d′ indicate more acidic satellites of the major spots. The approximate p*I* range is from 6.5 to 5.1 (right to left). Only the G-protein-containing region of the gels is shown. Only minor silver-stained contaminants were apparent in other portions of the gel.

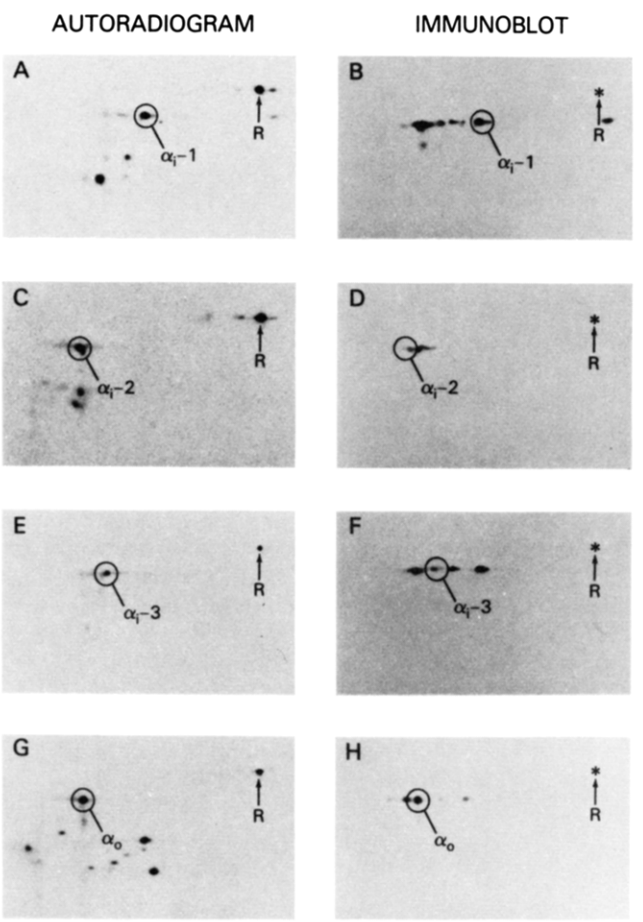

FIGURE 5: Analysis by two-dimensional gel electrophoresis of the products of in vitro translation of G protein α-subunit cDNAs (panels A, C, E, and G), and of the immunoreactive forms of purified brain G protein (panels B, D, F, and H). Aliquots of reticulocyte lysate containing [^{35}S]methionine-labeled translation products (A and B = G_{i1}, C and D = G_{i2}, E and F = G_{i3}, G and H = G_o) were added to a mixture of purified G proteins (see Figure 4, G-MIXTURE) and subjected to two-dimensional gel electrophoresis. The separated proteins were transferred to nitrocellulose paper. Nitrocellulose blots were incubated with rabbit antisera (see Table I) [AS/6 (panels B and F), LE/3 (panel D), and IM/1 (panel H)], and immunoreactive spots were revealed with peroxidase-conjugated goat anti-rabbit Ig. The same blots were also used for autoradiography of [^{35}S]-methionine-labeled proteins (panels A, C, E, and G). An approximately 47-kDa labeled protein present in the reticulocyte lysate mixture without addition of exogenous mRNA is indicated by the arrow labeled "R" in the autoradiograms. This protein serves as a convenient reference for migration on the 2-D gel. Its location on the immunoblots is indicated with the asterisk labeled "R". The spots representing the translation products of each cDNA-derived mRNA are circled and labeled in the autoradiograms (panels A, C, E, and G). Spots of lower molecular weight that are not circled on the autoradiograms represent variable amounts and kinds of truncated product for each mRNA. The immunoreactive spots circled and labeled on the immunoblots (panels B, D, F, and H) correspond to the positions of the labeled translation products in the autoradiograms.

Immunochemical (Huff et al., 1985; Gierschik et al., 1986) and other methods (Sternweis & Robishaw, 1984) indicated that α_{41} and α_{39} are distinct proteins. Amino acid sequencing of α_{41} and α_{39} purified from brain showed that these correspond to the proteins encoded by G_{i1} and G_o cDNAs, respectively (Nukada et al., 1986; Itoh et al., 1986; Van Meurs et al., 1987). After the present work was completed, Itoh et al. (1988) reported that the sequence of α_{40} purified from porcine brain corresponds to that of G_{i2} cDNA. The putative product of G_{i3} cDNA has not been identified by direct amino acid sequencing of purified protein, but using peptide-specific antisera, we have recently shown that an α_{41} purified from HL-60 cells corresponds to the G_{i3} cDNA product (Goldsmith, et al., 1988).

We used two independent methods to compare the four G proteins purified from brain, and to define their relationship to G-α cDNA-encoded proteins. Immunoblots with specific peptide antisera demonstrate that α_{40} and α_{41} correspond to G_{i2} and G_{i1}, respectively. α_{39} and α_{39}* were indistinguishable immunochemically; they each reacted with a G-α common antiserum, and with four different G_o-specific antisera. They each failed to react with several G_i-specific antisera.

2-D gel electrophoresis confirmed that α_{41} and α_{39} correspond to G_{i1} and G_o, respectively. The translation product encoded by G_{i2} cDNA migrated closest to purified α_{40} protein, but coincided with an acidic satellite rather than the major electrophoretic form of the protein. The significance of this observation, and indeed of the multiple species of each purified protein apparent on 2-D gel electrophoresis, is unclear. Certain G-protein α subunits have been shown to undergo at least one posttranslational modification, myristoylation (Buss et al., 1987), but the relationship of this, or other possible modifications, to the charge heterogeneity detected on 2-D gels is unknown at present.

We were unable to detect immunochemically a purified brain protein corresponding to the G_{i3} cDNA. The translation product encoded by G_{i3} cDNA, moreover, does not comigrate with any of the major purified brain G proteins. We conclude that if the protein encoded by G_{i3} cDNA is present at all in brain, it must be at a substantially lower concentration than the G proteins described herein.

On 2-D gel electrophoresis, α_{39} and α_{39}* are entirely, and reproducibly, distinct. Their difference in p*I* is greater than

that observed between the major and minor forms of each individual purified protein. Their migration on 2-D gels and their distinct chromatographic behavior suggest that G_{39} and G_{39}* differ structurally; the failure of four different G_o antisera to distinguish between α_{39} and α_{39}*, however, suggests that the proteins might differ with respect to posttranslational modification rather than primary sequence. α_{39} and α_{39}* are unlikely to arise from each other by proteolysis, since they differ only slightly in size on SDS–PAGE, and react equivalently with antisera specific for epitopes on both the amino and carboxy termini of G_o-α. α_{39}* is also unlikely to be formed artifactually during purification of G proteins from brain cholate extract, since immunoblots of brain membrane proteins freshly extracted with cholate reveal G_o-immunoreactive spots with pI's corresponding to those of both pure α_{39} and pure α_{39}*. Direct amino acid sequencing and other structural analyses will be required to define the relationship of the two proteins.

The purification of a distinct α_{40} from brain, and of an apparently novel form of G_o, raises important questions regarding analysis of G-protein function. G proteins, in addition to regulating adenylyl cyclase, and cGMP phosphodiesterase in retinal photoreceptors, are involved in control of phospholipase C and of various ion channels (Spiegel, 1987; Gilman, 1987). The particular G protein(s) responsible for effector regulation in each case has (have) not been carefully defined. Results of reconstitution studies performed with "purified" G proteins must be interpreted cautiously. G-protein preparations apparently homogeneous by one-dimensional SDS–PAGE may in fact contain several structurally, and possibly functionally, distinct entities. The methods described here for purification and identification of G proteins should permit more rigorous definition of G-protein specificity in receptor and effector coupling.

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