Identification of the GTP-binding Protein Encoded by Gi3 Complementary DNA*

(Received for publication, February 12, 1988)

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Three closely related, but distinct, GTP-binding proteins (G-proteins) are encoded by cDNAs arbitrarily designated G₁₁, G₁₂, and G₁₃. The in vitro translated products of mRNAs prepared from G₁₁, G₁₂, and G₁₃ cDNAs migrate as 41-, 40-, and 41-kDa proteins, respectively, on sodium dodecyl sulfate-polyacrylamide gels. Antisera were raised against synthetic decapetides corresponding to a divergent sequence (residues 159-168 for G₁₁, and 203-212 for G₁₃) of the three cDNAs and tested on immunoblots for reactivity with three purified G-proteins, G₁₁ and G₁₀ from brain and G₁₃ from HL-60 cells. LD antisera (G₁₃ peptide) react only with brain G₁₃, LE antisera (G₁₉ peptide) react only with brain G₁₀, and SQ antisera (G₁₀ peptide) react exclusively with HL-60 G₁₃. The results indicate that the 41-kDa G-protein purified from HL-60 cells differs from the purified brain 41-kDa protein and suggest that the HL-60 cell protein corresponds to that encoded by G₁₃ cDNA.

Heterotrimeric GTP-binding proteins (G-proteins) couple cell-surface receptors to effectors (1, 2). Distinct α-subunits bind guanine nucleotides and are thought to confer specificity in G-protein-receptor and -effector interactions. cDNA cloning has identified three distinct, but highly (>85%) homologous, G-protein α-subunits, arbitrarily designated G₁₁, G₁₂, and G₁₃ in order of cloning (2, 3). The predicted amino acid sequence of G₁₁ cDNA corresponds to that determined for a 41-kDa protein purified from bovine (4) and rat (5) brain. The amino acid sequences predicted by G₁₂ and G₁₃ cDNAs do not correspond to sequences derived for purified G-proteins. Using antisera (LE/2 and LE/3) raised against a synthetic decapetide with a sequence uniquely predicted by G₁₂ cDNA, we were able to identify tentatively a 40-kDa G-protein, the major pertussis toxin substrate purified from neutrophils, as the protein encoded by G₁₂ cDNA (6). In order to identify the putative product of G₁₃ cDNA, and in an effort to develop reagents capable of distinguishing closely related G-proteins, we raised antisera against unique, synthetic peptides corresponding to G₁₀ and G₁₃, respectively. Using these antisera, we report here the tentative identification of a 41-kDa G-protein purified from HL-60 cells as the protein encoded by G₁₃ cDNA.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—α-subunit peptides (Table I) were prepared exactly as described previously (6). The β-subunit peptide (Table I) was obtained from Peptide Technologies (Washington, D.C.) and was synthesized as the carboxyl-terminus amide.

Antisera—Peptide conjugation and immunization schedules were as previously described (6). The antisera used in this study are defined in Table I.

Protein Purification—41- and 40-kDa G-proteins were purified from bovine brain essentially as described by Katseda et al. (7). The 41-kDa G-protein purified from HL-60 cells (8) was kindly provided by R. Uhing, P. Polakis, and R. Snyderman (Duke University and Genentech).

In Vitro Transcription and Translation—Rat cDNAs encoding G₁₁, G₁₀, G₁₉, and G₁₃ (3) were the kind gift of D. Jones and R. Reed (Johns Hopkins University). The cDNAs were contained in the pGEM-2 vector (Promega Biotec). The sense strands of G₁₀, G₁₃, and G₁₉ cDNAs were oriented 3' to the SP6 RNA polymerase promoter. The plasmids containing these cDNAs were linearized with HindIII restriction endonuclease (New England Biolabs). The G₁₀ cDNA sense strand was oriented 3' to the T7 RNA polymerase promoter; this plasmid was linearized with XmnI restriction endonuclease (New England Biolabs). In vitro RNA transcription was performed using 2 µg of linearized plasmid DNA and 10 units of either SP6 or T7 RNA polymerase (Promega Biotec). The reaction included (final concentrations): Tris/HCl (pH 7.4), 40 mM; MgCl₂, 6 mM; spermidine, 2 mM; NaCl, 10 mM; dithiothreitol, 10 mM; and ATP, GTP, UTP, 0.5 mM each. Methylnuamide cap structure (Pharmacia LKB Biotechnology Inc.) 0.25 mM, was added with 0.1 mM GTP at the beginning of the 2-h incubation at 37 °C. After 26 min, GTP, 0.15 mM, was added to give a final concentration of GTP of 0.25 mM. After 2 h, reaction products were extracted with phenol/chloroform, precipitated with 10% (v/w) original volume 3 M sodium acetate (pH 6.0) and 2.5 times original volume ethanol, vacuum dried, resuspended in water treated with diethyl pyrocarbonate (Sigma), and stored at -20 °C.

In vitro translation of the RNA (9) was performed with the rabbit reticulocyte lysate system (Bethesda Research Laboratories). K⁺ and Mg²⁺ concentrations in the reaction were optimized (translation of 2 µg of each RNA, L-[³⁵S]methionine (Amersham Corp.) was used at a final concentration of 1 µCi/ml. Incubation for 1 h at 30 °C was terminated by heating the reaction mixture at 100 °C in SDS-PAGE sample buffer for 5 min.

Other Methods—Cholate extracts of bovine brain membranes were prepared as described (6). SDS-PAGE and immunoblotting were performed as previously described (6), except that 10% polyacrylamide gels contained half of the usual concentration of Bis-acrylamide (final 0.13 g/100 ml rather than 0.27). This was found to give better resolution of α-subunits (10).

RESULTS

To define the molecular weights of the proteins encoded by G-protein α-subunit cDNAs, we prepared mRNAs from the corresponding cDNAs and translated these in vitro with the reticulocyte lysate system. [³⁵S]Methionine-labeled protein products were subjected to SDS-PAGE and visualized by fluorography. A labeled protein of approximately 47 kDa (migrating just above the ovalbumin marker protein) was present in the reticulocyte lysate without addition of exoge-

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The abbreviations used are: G-protein, guanine nucleotide-binding protein; G₁₁, a G-protein originally identified in terms of inhibition of adenylcyclase; G₁₂, a G-protein with α-subunit of 41 kDa; G₁₉, a G-protein with α-subunit of 40 kDa; G₁₃, a G-protein of unclear function, abundant in brain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
TABLE I

Definition of synthetic peptide antisera used in this study

| Antiserum | Peptide antigen* | Corresponding cDNA sequence |
|-----------|------------------|----------------------------|
| LD/1, LD/2 | LDR1AQP KNPIY | G₁₂ (residues 159–169) |
| LE/2, LE/3 | LERIAQ SDYI | G₁₂ (residues 160–169) |
| SQ/2, SQ/3 | LDR1S SQNYI | G₆K (residues 159–168) |
| AS/6, AS/7 | KENLKD CGLF | Transducin (C terminus) (G₄₁ and G₄₃ C terminus = KNL K DCGLF; G₆K C terminus = K NL K E C GL Y) |
| GA/1, GA/3 | GAGESKSTIVK | All G₄ (residues 40–51 in G₁₁) |
| MS/1, MS/2 | MSEL DQLRQE | β, subunit (N terminus) |

* Single letter amino acid code.
* Sequence of human cDNA (14–16); rat cDNA (3) substitutes threonine for second serine residue.

decapetide of transducin-α. These antisera recognize not only transducin but also the 41-kDa G-protein (G₄₁) purified from brain as well as the 40-kDa G-protein (G₆K) purified from neutrophils (6). The latter two proteins correspond to G₄₃ and G₆₀, respectively; these cDNAs have an identical carboxyl-terminal sequence, differing by a single residue from transducin-α (Table I). To distinguish G₆₀ from G₄₁, we generated antisera, LE/2 and LE/3, against a peptide corresponding to residues 160–169 predicted by G₆₀ cDNA. This decapeptide differs in sequence for all three forms of G₆₀ cDNA (Table I). LE antisera reacted specifically with the neutrophil G₆₀ and not the brain G₄₁. This allowed tentative identification of the former as the product of G₆₀ cDNA (6).

In an effort to develop G₁₁- and G₆₀-specific reagents, we immunized rabbits with the synthetic peptides corresponding to residues 159–168 predicted by G₆₀ and G₆₃ cDNAs (Table I). The resultant antisera (LD and SQ for G₆₀ and G₆₃, respectively), as well as several others (Table I), were tested for specific reactivity with three purified G-holoproteins: G-proteins with α-subunits of 41 and 40 kDa (G₄₁ and G₆₀, respectively) purified from brain and a G-protein with 41-kDa α-subunit (G₄₃) purified from HL-60 cells (8).

The results of the immunoblots comparing LD, LE, and SQ reactivity are shown in Fig. 2. A β-subunit-specific antiserum, MS/1, was included in all first antiserum incubations to visualize the β-subunits of the purified G-proteins. MS/1 antiserum alone revealed only the β-subunits (Fig. 2, upper left). A “G-α common” antiserum, GA/1, similar to that used by Muncy et al. (11) was used to visualize all three α-subunits (Fig. 2, upper left). Antiserum AS/7 reacts equally well with brain G₄₁ and G₆₀ but reacts significantly less well with HL-60 G₄₃ (compare Fig. 2, upper middle versus upper left). These results suggest that brain G₆₀, like neutrophil G₆₀, corresponds to G₄₃, since AS antisera react equivalently with the identical carboxyl-terminal decapetide of G₄₁ and G₆₂. G₄₃ differs from G₄₁ and G₆₂ by two additional conservative substitutions in the carboxyl-terminal decapetide (Table I). The lower reactivity of AS/7 with HL-60 G₄₃ is consistent with tentative identification of this protein as G₄₃-α. AS/7 does not react at all with G₄₀-α which differs from the peptide antigen by 5 of 10 residues in the carboxyl terminus (6). LD antisera (Fig. 2, bottom left) reacts only with brain G₄₁, consistent with its identification as G₄₁. LE antisera (Fig. 2, bottom middle) reacts exclusively with brain G₄₀, presumptive evidence that this protein is G₆₂. Finally, SQ antisera (Fig. 2, bottom right) reacts only with the HL-60 G₄₃. This strongly suggests that this protein represents the product encoded by G₆₀ cDNA.

To provide further evidence for the specific reactivity of the peptide antisera, we assessed the ability of the synthetic peptides used as antigens (residues 159–168 for G₄₁ and G₆₀, 160–169 for G₆₀, Table I) to compete for antibody binding. Fig. 3 shows the results of one such experiment, in which LD,
unit-specific antiserum, was included at 1:250 dilution in all incubations. GA/1 and AS/7 were used at 1:250 dilution, and LD/1, LE/3, and SQ/2 at 1:100 dilution. Arrows indicate the positions of α- and β-subunits.

FIG. 2. Reactivity of antisera with purified GTP-binding proteins. G-Holoproteins (brain G$_{41}$ and G$_{40}$ = G-proteins containing 41- and 40-kDa α-subunits, respectively, purified from brain; HL-60 G$_{41}$ = G-protein containing 41-kDa α-subunit purified from HL-60 cells) were separated by SDS-PAGE on a 10% gel (approximately 1 µg/lane of brain G$_{41}$ and G$_{40}$ and approximately 0.5 µg/lane of HL-60 G$_{41}$), and immunoblotted with the indicated antisera. MS/1, β-subunit-specific antisera, was included at 1:250 dilution in all incubations. GA/1 and AS/7 were used at 1:250 dilution, and LD/1, LE/3, and SQ/2 at 1:100 dilution. Arrows indicate the positions of α- and β-subunits.

FIG. 3. Specificity of LD, LE, and SQ antisera assessed by competition with synthetic peptides. Purified brain G$_{41}$ and G$_{40}$ and HL-60 G$_{41}$ were separated by SDS-PAGE (10% gel) and immunoblotted. Individual nitrocellulose strips (approximately 1 µg/lane for brain G$_{41}$, 0.5 µg/lane for brain G$_{40}$, and 0.25 µg/lane for HL-60 G$_{41}$) were incubated with the indicated antisera at 1:100 dilution (5 ml of total volume of 1% gelatin/Tris-buffered saline). Synthetic peptides (10 µg/ml) were added to the antisera as follows: lane 1, no peptide; lane 2, G$_{159-168}$ peptide; lane 3, G$_{160-169}$ peptide; and lane 4, G$_{159-168}$ peptide. SQ/2 antisera shows unexplained reactivity with the β-subunit (fainter band beneath α-subunit) which is unaffected by specific peptide antigen (lane 4).

LE, and SQ antisera were tested on immunoblots with the purified proteins, brain G$_{41}$, brain G$_{40}$, and HL-60 G$_{41}$, respectively, to which they specifically react. For each antisera, reactivity on immunoblot was effectively blocked by 10 µg/ml of the corresponding synthetic decapeptide antigen. An equivalent concentration of either of the other two peptides did not affect antibody binding.

A similar experiment assessed the specific reactivity of LD and LE antisera with plasma membrane proteins from bovine cerebral cortex. Two different LD antisera detect the same 41-kDa protein, and two different LE antisera reveal a 40-kDa protein (Fig. 4). Reactivity with the specific protein band is blocked by as little as 1 µg/ml of the corresponding peptide antigen (Fig. 4, lanes 2 and 3), but the G$_{15}$ peptide does not affect LE reactivity nor does G$_{12}$ peptide affect LD reactivity (not shown). This is entirely consistent with the differential reactivity of LD and LE antisera with purified brain G$_{41}$ and G$_{40}$, respectively (Fig. 2). Concentrations of G$_{15}$ peptide as high as 100 µg/ml, moreover, fail to block specific reactivity of LD or LE antisera with brain membranes (Fig. 4, lanes 4–6). As yet, we have not detected specific reactivity of SQ antisera with protein(s) in brain membranes.

DISCUSSION

The sequences of human (12–16), rat (3, 5), and bovine (4) cDNAs encoding three distinct, but closely related, forms of G$_{i}$ have recently been reported. To define the functions and distribution of the putative proteins encoded by these cDNAs, it would be helpful to identify their protein products. In vitro translation of mRNA transcribed from G$_{15}$, G$_{12}$, and G$_{3}$ cDNAs indicates that the respective products migrate on SDS-PAGE as 41-, 40-, and 41-kDa proteins. The G$_{i}$ translation product migrates as a 39-kDa protein. The differences in migration between the translation products are subtle but reproducible. The differences are not directly related to size as G$_{15}$, G$_{12}$, and G$_{3}$ cDNAs encode 354 amino acid proteins, whereas G$_{12}$ cDNA encodes a 355-amino acid protein.

Antisera we raised against synthetic peptides proved capable of distinguishing among various purified G-proteins.
Antisera AS/6 and AS/7, raised against the carboxyl-terminal decapeptide of transducin-α (6), reacted differentially with Gα4 proteins purified from brain and HL-60 cells, respectively. Since G4 shows lower homology in carboxyl-terminal sequence to the antigenic peptide than Gα4 and Gα2, the weaker reactivity of AS/7 with HL-60 Gα4 suggested the latter could represent Gα3. Stronger evidence comes from immunoblots with three antisera, LD, LE, and SQ, raised against a decapeptide showing differences in sequence between Gα1, Gα2, and Gα3. For Gα1 and Gα2, no species differences in the sequence of the relevant decapeptide (residues 159-168) and 160-169, respectively) have been reported (3-5, 12, 13). For the Gα4 peptide (residues 159-168), there is a single amino acid difference between the human (14-16) and rat (3) sequences. We chose the human Gα3 sequence for generation of SQ antisera, even though it shows one difference from Gα3. This makes it more likely that differences in antibody reactivity would reflect differences in G-protein type rather than species differences.

Each of the three antisera reacted exclusively with a different purified G-protein, LD with brain Gα4, LE with brain Gα2, and SQ with HL-60 cell Gα4. The specificity of antibody reactivity is further emphasized by competition for antibody binding with the synthetic peptides used as antigens. Even though the amino acid sequences of the peptide antigens show relatively minor differences, only the antigenic peptide proved capable of blocking the reactivity of the corresponding antisera.

The unique pattern of immunoreactivity of these peptide antisera enable us to identify several purified G-proteins as products of defined cDNAs. Although the peptide antisera we have used (Table I) probe three discrete regions of Gα sequence, our assignments must be considered tentative until the purified proteins are directly sequenced. LD antisera identify brain Gα1 as Gα1, in good agreement with direct protein sequencing (4, 5). LE antisera identify brain Gα2 as Gα2. In one of the original reports on purification of pertussis toxin substrates from brain, a 40-kDa protein in addition to Gα1 and Gα2 was observed (17). Recently, this protein was purified and shown to differ from brain Gα1 and Gα2 (7). Our results strongly suggest that brain Gα1 is the same protein, namely Gα1, as Gα1 purified from neutrophils (18) and HL-60 cells (8, 19). Gα1 purified from HL-60 cells was shown by peptide mapping to differ from Gα1 (8). The unique reactivity of HL-60 Gα1 with AS and SQ antisera suggests that this is the protein encoded by Gα3 cDNA. This is consistent with cloning of Gα3 cDNA from an HL-60 cell library (14) and our previous identification of Gα3 mRNA in HL-60 cells (20). Whereas Gα3 mRNA appears widely distributed in brain and peripheral tissues, Gα1 mRNA is relatively brain-specific (21). In contrast Gα3 mRNA is widely distributed in peripheral tissues but undetectable with the methods we have used in brain. This is consistent with the apparent absence, or very low abundance, of Gα3 immunoreactivity in brain. Our results suggest that Gα1 in brain is principally, if not exclusively, Gα1 in peripheral tissues likely consists, at least in part, of Gα3.

All three forms of Gα are pertussis toxin substrates (6-8, 17-19). Several effector functions, including adenyl cyclase inhibition, stimulation of a K+ channel, and stimulation of phospholipase C in cells such as neutrophils are regulated by pertussis toxin-sensitive G-proteins (1, 2). As yet, it is not clear which, if any, of the forms of Gα regulates these effectors.

Results of reconstitution experiments employing “purified” G-protein preparations must be interpreted cautiously given the possible heterogeneity of such preparations. The immunologic tools described in this report offer the possibility of specifically identifying closely related G-proteins and should prove useful in elucidating the functions of this important class of signal transducers.

Acknowledgments—We are grateful to R. Reed and D. Jones for providing cDNA clones and sequences prior to publication, and to R. Uhing, P. Polakis, and R. Snyderman for providing purified HL-60 cell G-proteins.

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2 M. Brann and A. Spiegel, manuscript in preparation.