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Molecular cloning and characterization of cDNA encoding the GTP-binding protein α_i and identification of a related protein, α_h

(guanine nucleotide-binding protein/gene superfamily/signal transduction/adenylate cyclase)

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ABSTRACT **We have cloned and characterized cDNA encoding α_i, the GTP-binding subunit of G_i, a protein that mediates hormonal inhibition of adenylate cyclase and hormonal regulation of other membrane functions. We have also identified cDNA encoding a putative protein, which we have named α_h, that is highly homologous to α_i but different from other known GTP-binding proteins. Both cDNAs were isolated from a bovine pituitary library. The cDNA encoding α_i was identified by finding that the amino acid sequence determined for two tryptic peptides from α_i agreed exactly with amino acid sequences deduced from the cDNA. We also determined the amino acid sequence of peptides derived from α_o, a related 39-kDa protein purified from bovine brain. These sequences are ≈75% identical to the sequence determined for α_i. Southern blot analysis of bovine genomic DNA, using as probes radiolabeled cDNAs for α_i, α_h, and the α subunit of a related protein, transducin, showed that each probe recognized different genomic DNA fragments. Our results suggest a further level of complexity in the organization of the G-protein gene family, with multiple G proteins of very similar structural properties likely to be identified as products of distinct genes.**

The signal-transducing GTP-binding protein G_i is a member of a family of related guanine nucleotide-binding regulatory proteins termed G proteins (reviewed in refs. 1 and 2). G proteins involved in phototransduction [transducin or G_T (3, 4)] and in adenylate cyclase stimulation [G_s (5)] and other G proteins of unknown function [G_o (6, 7)] share structural and mechanistic features with G_i, which itself is implicated in hormonal inhibition of adenylate cyclase, as well as a variety of other hormone responses (8–13). Each G protein consists of α, β, and γ subunits and is most clearly distinguished from other G proteins by the biological and biochemical attributes of its GTP-binding α subunit. The β subunits of the different G proteins are extremely similar to one another and appear to be functionally interchangeable, but the γ subunit of transducin differs from those of the other G proteins (15, 16).

The 41-kDa α subunit of G_i, α_i, can be covalently modified by pertussis toxin, an ADP-ribosyltransferase exotoxin isolated from the bacterium *Bordetella pertussis* (8–10). The 39-kDa α subunit of G_o, α_o, also is ADP-ribosylated by pertussis toxin (6). In addition to α_o and α_i, a less abundant and less well characterized 40-kDa pertussis toxin substrate is found in bovine brain (6). The α_s subunit of G_s is ADP-ribosylated by a different bacterial toxin, cholera toxin (17); transducin is a substrate for ADP-ribosylation by both toxins (18, 19). Toxin-catalyzed ADP-ribosylation of these proteins affects their ability to transduce GTP-dependent hormonal signals.

ADP-ribosylation of α_i by pertussis toxin is associated with a loss of hormone receptor-mediated adenylate cyclase inhibition (8, 11). In some cells, ADP-ribosylation of a 41-kDa GTP-binding membrane protein by pertussis toxin blocks hormonal effects that are *not* mediated by changes in cyclic AMP levels or by adenylate cyclase inhibition. Instead, these hormone actions are correlated with GTP-dependent changes in Ca^{2+} flux or phosphatidylinositol metabolism (11–13).

To understand the mechanism by which G proteins modulate these different kinds of hormonal actions, it is necessary first to define the number of G proteins that exist. It is not known whether each hormone receptor is coupled to its own G protein, whether each effector system is regulated by a particular G protein, or whether G proteins are multifunctional in the cell. Genetic analysis is a powerful way to begin to define the number of G proteins and to gain insight into the extent of G-protein diversity.

MATERIALS AND METHODS

Amino Acid Sequence of Peptides from α_o and α_i. The α_i and α_o proteins were purified from bovine brain (6). The proteins were digested with trypsin or chymotrypsin to generate peptides, which were then purified by reversed-phase HPLC on a Vydac phenyl column (0.45 × 25 cm, Separations Group, Hesperia, CA) using 0.1% trifluoroacetic acid and a linear gradient of 0–60% acetonitrile (120 min) (20). Purified peptides were subjected to automated Edman degradation using an Applied Biosystems (Foster City, CA) 470A sequencer interfaced with an Applied Biosystems 120A PTH (phenylthiohydantoin) analyzer. From α_i, an unambiguous 34 amino acid sequence was obtained from two overlapping tryptic peptides: a 34-residue amino-terminal peptide (beginning Ser-Arg-Glu-), of which the amino-terminal 31 residues were identified, and a 15 amino acid peptide (beginning Ile-Ala-Gln-), of which all 15 residues were identified (shown in Figs. 1 and 2). One tryptic and two chymotryptic peptides were sequenced from α_o, the former yielding 24 residues and the latter two yielding 21 residues and 7 residues. Amino acid assignments could be made for all but one of the 52 total residues sequenced from α_o. These peptides showed 71% sequence identity with the deduced amino acid sequence reported for the α subunit of transducin (α_T) (21–24).

Cloning and Sequencing of cDNA for G Proteins. Based upon the sequence homologies between α_i, α_o, and α_T, we hypothesized that we could identify cDNA clones of α_i and/or α_o by screening a pituitary cDNA library, at low hybridization stringency, with the cloned α_T cDNA. Pituitary

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Abbreviations: G protein, guanine nucleotide-binding protein; α_i, α_s, α_o, and α_T, α subunits of the G proteins G_i, G_s, G_o, and transducin; α_h, putative protein homologous to α_i.

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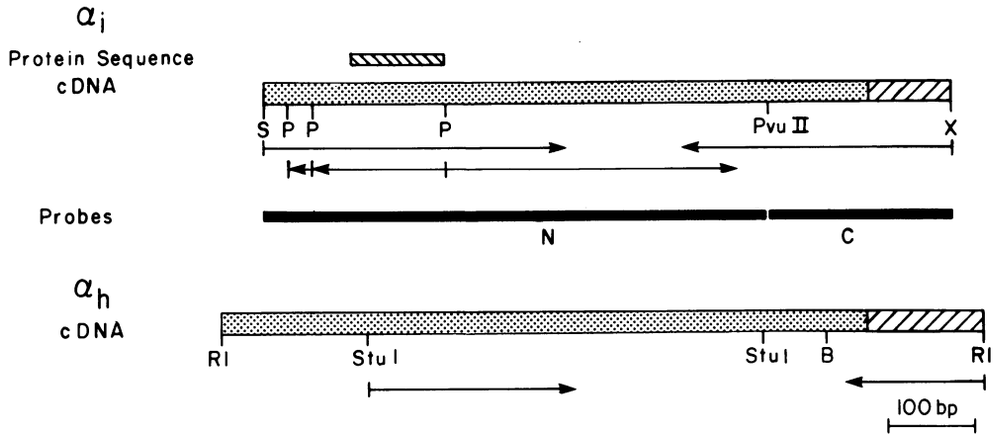
tissue contains G_o, G_i, and G_s (ref. 25; E.J.N., unpublished observations) and probably does not contain the retinal protein transducin. The bovine α_T cDNA clone, $\lambda T\alpha_1$, provided by M. Lochrie and M. Simon (21), was used to screen a bovine pituitary λgt11 library obtained from F. Rottman (26). The $\lambda T\alpha_1$ insert was excised with *Hin*dIII and subcloned into the plasmid pSP64 (Promega Biotec, Madison, WI). This α_T cDNA insert was isolated from the plasmid and labeled by nick-translation for use in plaque hybridization (27). The cDNA library (made from 1.5×10^4 independent bacteriophage plaques) was plated at a density of 3000 plaques per 85-mm plate, transferred to nitrocellulose, and hybridized as described (27), except that the hybridization was carried out at 37°C overnight and the final wash of the filters was at room temperature; a total of 1.5×10^5 plaques were screened. Positive plaques underwent secondary and tertiary screening with the α_T probe before further analysis. Appropriate DNA restriction fragments were subcloned into the bacteriophage M13 sequencing vectors mp18 and/or mp19 for sequencing using the dideoxy chain-termination method (28, 29).

Genomic Southern Blots of α_i and α_h. High molecular weight bovine cerebral cortex DNA was prepared (30), digested with appropriate restriction endonucleases, subjected to electrophoresis in 1% agarose gels (12 μg of DNA per lane), and transferred to nitrocellulose filters according to the method of Southern (31). The filters were hybridized with 4×10^7 cpm of ^{32}P-labeled probe at 42°C overnight as described (27), except that the final wash of the filters was at 55°C before exposure for 48 hr on Kodak XRP film at −70°C with intensifying screens.

RESULTS

Four independent bacteriophage plaques were isolated from the bovine pituitary cDNA library (out of 1.5×10^4 independent plaques). Restriction mapping of the cDNA inserts in the bacteriophage revealed that three of the clones had inserts about 1 kilobase (kb) long and portions of these inserts shared identical restriction enzyme sites. A single clone had a ≈3-kb insert; restriction mapping showed it to be distinctly different from the other clones. We determined the nucleotide se-

a



b

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    GlnLeuPheValLeuAlaGlySerAlaGluGluGlyPheMetThrAlaGluLeuAlaGlyValIleLysArgLeuTrpLysAspSerGlyValGlnAlaC
1   CAACTCTTTGTGCTTGCTGGCTCTGCAGAGGAAGGTTTTATGACTGCAGAACTTGCTGGAGTTATAAAGAGACTTTGGAAAGACAGTGGTGTACAAGCCT

    ysPheAsnArgSerArgGluTyrGlnLeuAsnAspSerAlaAlaTyrTyrLeuAsnAspLeuAspArgIleAlaGlnProAsnTyrIleProThrGlnGl
101 GCTTCAACAGATCCCGAGAGTACCAGCTTAATGATTCTGCAGCATACTATTTGAATGATTTGGACAGAATTGCACAACCAAATTATATTCCAACTCAACA

    nAspValLeuArgThrArgValLysThrThrGlyIleValGluThrHisPheThrPheLysAspLeuHisPheLysMetPheAspValGlyGlyGlnArg
201 AGATGTTCTCAGAACTCGAGTGAAAACCACAGGAATTGTTGAGACCCATTTTACTTTCAAAGATCTTCATTTTAAAATGTTTGATGTGGGAGGACAGAGA

    SerGluArgLysLysTrpIleHisCysPheGluGlyValThrAlaIleIlePheCysValAlaLeuSerAspTyrAspLeuValLeuAlaGluAspGluG
301 TCTGAGCGGAAGAAATGGATTCATTGCTTCGAAGGAGTGACCGCCATCATCTTCTGTGTGGCGCTGAGTGACTATGACCTGGTTCTAGCTGAAGATGAAG

    luMetAsnArgMetHisGluSerMetLysLeuPheAspSerIleCysAsnAsnLysTrpPheThrAspThrSerIleIleLeuPheLeuAsnLysLysAs
401 AAATGAACCGAATGCATGAAAGCATGAAGTTATTTGACAGCATATGTAACAACAAATGGTTTACAGATACATCTATTATACTTTTTCTTAACAAGAAGGA

    pLeuPheGluGluLysIleLysLysSerProLeuThrIleCysTyrProGluTyrAlaGlySerAsnThrTyrGluGluAlaAlaAlaTyrIleGlnCys
501 TCTCTTTGAAGAAAAAATCAAGAAGAGCCCTCTAACTATATGCTATCCAGAATATGCAGGCTCAAACACATATGAAGAGGCAGCTGCGTACATTCAGTGT

    GlnPheGluAspLeuAsnLysArgLysAspThrLysGluIleTyrThrHisPheThrCysAlaThrAspThrAsnAsnValGlnPheValPheGluAlaV
601 CAGTTTGAAGACCTCAATAAGAGAAAGGACACAAAGGAAATATACACCCACTTCACTTGCGCCACGGACACCAACAACGTGCAGTTCGTCTTTGAAGCCG

    alThrAspValIleIleLysAsnAsnLeuLysAspCysGlyLeuPheEnd
701 TAACAGACGTCATCATAAAAAATAACCTAAAAGACTGTGGTCTCTTCTGAGTGTTGGCGGCCAAATGGTAAAATGCATTTTCAAACCAAAGTAGTACTTA

801 CATGTGGATCTCTCTAGAGTCT  822
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FIG. 1. Cloning and sequencing of the cDNA for α_i. (*A*) cDNA inserts for $p\alpha_i$ and for $p\alpha_h$, with relevant restriction sites for *Sma* I (S), *Pst* I (P), *Pvu* II, *Xba* I (X), *Eco*RI (RI), *Stu* I, and *Bam*HI (B). The stippled area in each cDNA bar represents coding sequence; the hatched cDNA is 3′ untranslated region. The hatched bar above the $p\alpha_i$ cDNA corresponds to the region of sequence identity with the α_i protein. Arrows below the cDNA clones show the direction and extent of sequencing. Probes labeled N and C (for "NH_2" and "COOH") were purified after cutting the $p\alpha_i$ cDNA with *Pvu* II to generate 5′ and 3′ nonoverlapping fragments for use in probing Southern blots. bp, Base pairs. (*B*) Nucleotide sequence and deduced amino acid sequence for the $p\alpha_i$ cDNA. The deduced amino acid sequence for $p\alpha_h$ is shown in Fig. 2.

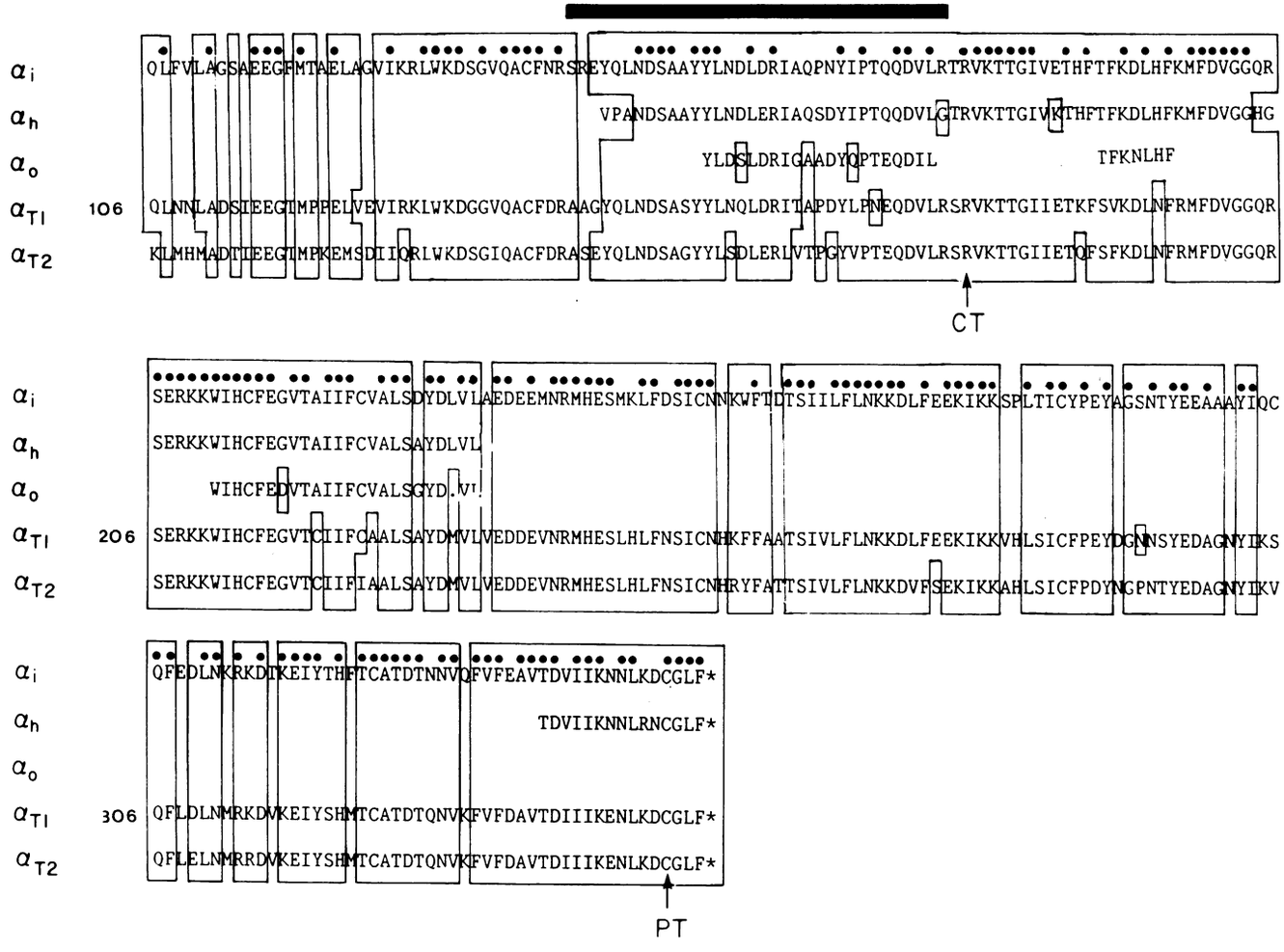

FIG. 2. Sequence homologies among the G proteins. The amino acid sequence of α_i is shown aligned with the corresponding amino acid sequence from α_h, both deduced from the cDNA clones described in the text. These are aligned with the two reported sequences of transducin from ref. 21 (α_{T1}) and refs. 22–24 (α_{T2}); the numbering system is based on residue assignments from α_{T1} (the pα_i and pα_h cDNA clones are not full length and thus the numbering of their amino acid residues cannot be meaningfully assigned). Also shown is the sequence of three peptides from α_o, purified and sequenced as described in *Materials and Methods* (the single residue that could not be unambiguously identified is denoted by a small dot). The solid bar above the α_i sequence shows the region in which peptide sequence was obtained from the purified protein. The residues in transducin that are ADP-ribosylated by cholera toxin and by pertussis toxin are labeled CT and PT, respectively. The large boxes enclose amino acids that are either identical to α_i or are conserved according to the Dayhoff classification (33). The large dots above the sequences mark residues that are identical in all available G-protein sequences. The single-letter amino acid abbreviations are A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

quence of the transducin-homologous regions of these clones (Fig. 1). We concluded that one cDNA clone encoded α_i because 34 out of the 34 amino acid residues we sequenced from the α_i protein were encoded precisely by this cDNA (designated pα_1, Fig. 1).** This region of identity with α_i subtends a sequence in which homology among the other G proteins is 60% (Fig. 2). The single open reading frame in this cDNA easily aligns with both reported transducin cDNA sequences as well as with the peptide sequence from α_o. The pα_i cDNA fragment extends beyond a termination codon into what is presumed to be the 3′ untranslated region, but the poly(A)-addition consensus sequence AATAAA was not found in the sequence of 100 bp in the 3′ direction; the additional DNA in this insert (1 kb) extending 3′ to this region has not yet been sequenced. The cDNA encoding α_i is not full length and extends in the 5′ direction to a sequence corresponding to transducin amino acid residue 106 before encountering a poly(T) track and losing the open reading frame. This probably reflects an artifact created during construction of the cDNA library; thus, the α_i amino-terminal sequence is not represented in this clone.

Partial sequencing of the other transducin-homologous cDNA clone reveals an open reading frame, which, upon translation, shows marked similarity to all other G-protein sequences. This clone clearly represents a distinct sequence (Figs. 1 and 2; refs. 21–24) and was termed pα_h. Fig. 2 documents the extensive homology among the G-protein cDNA clones pα_i and pα_h, the two previously reported transducin sequences (21–24), and the α_o protein sequence. The sequence of α_i is 90% identical to the sequence of α_{T1} and α_{T2}. However, it is only 51% identical to the sequence of α_s (34, 35). The sequence of α_h is more similar to α_i (87% identity over the 107 residues sequenced) than to either of the transducins (71% and 69% identity) or to α_s (46% identity). Over the 52 residues of α_o protein sequence, there is 77% identity with the deduced sequence of α_h and α_i. When conservative amino acid substitutions are allowed (33), the percentage (identity plus homology) increases by 5–15% in every instance.

**Nakado *et al.* (32) have also published the sequence of cDNA for α_i from bovine cerebral cortex. Except for two amino acids, the deduced sequences of α_i from pituitary and from cerebral cortex are identical.

The sequence differences between $p\alpha_h$ and $p\alpha_i$ suggest that they represent the products of different genes. This idea is supported by analysis of genomic DNA from bovine brain (Fig. 3*a*). Southern blots of bovine cerebral cortex genomic DNA probed with ^{32}P-labeled $p\alpha_i$ show several bands. In contrast, genomic Southern blots probed with ^{32}P-labeled $p\alpha_h$ cDNA identify a single band (different from the bands detected with $p\alpha_i$), suggesting that α_h is encoded by a single gene, as has also been found for α_T and α_s (21–24, 36).

To ascertain whether the multiple bands labeled by $p\alpha_i$ represent different genomic coding blocks or whether they represent multiple genes, nonoverlapping $p\alpha_i$ probes were prepared (Fig. 1). The $p\alpha_i$ cDNA clone was cut with *Pvu* II and, as predicted from the nucleotide sequence, gave rise to two fragments, which were purified, ^{32}P-labeled, and used as probes in genomic Southern blots (Fig. 3*b*). The 5′ fragment (N) identified multiple bands with each of three restriction enzyme digestions of bovine genomic DNA, as did the 3′ (C) fragment. For the *Bam*HI and *Hin*dIII digestions, there were two bands labeled in common by the N and C probes, and a single band was labeled in common in the *Eco*RI digestion.

DISCUSSION

These studies have characterized cDNA encoding α_i and have identified the existence of cDNA encoding a related protein, which we have named α_h. These proteins are highly homologous to one another and to other G proteins. However, the sequences of α_i, α_h, and the two tranducin α subunits are more similar to each other than to α_s. Analysis of bovine genomic DNA (Fig. 3) shows that different genomic fragments are identified by the cDNAs for α_i, α_h, and transducin, confirming that these G proteins are products of distinct genes. Southern blots probed with radiolabeled α_i gave a complex pattern of bands, suggesting the existence of more than one gene for α_i. The presence of more than one gene for α_i may simply reflect different alleles in a heterozygotic cow; alternatively, multiple α_i genes may be differentially regulated in different cell types. Preliminary experiments suggest that multiple α_i genes may be present in other species, including mouse and human (data not shown).

The protein encoded by $p\alpha_h$ cDNA has not yet been identified but is likely to be a member of the G-protein family

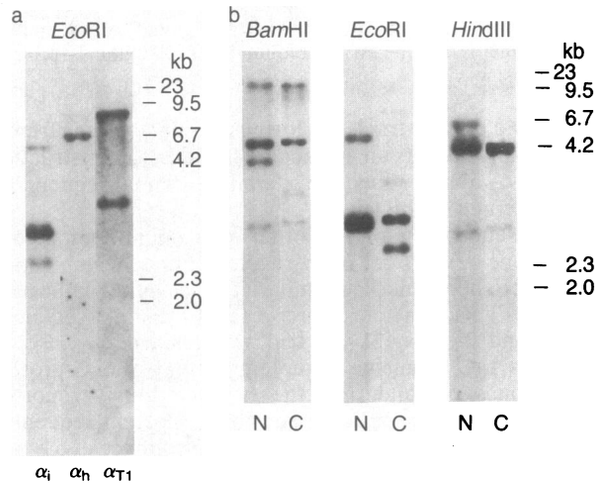

FIG. 3. Genomic Southern blots. (*a*) Blots were probed with the $p\alpha_i$, $p\alpha_h$, or transducin (α_{T1}) cDNAs (see *Materials and Methods*); data are shown for bovine genomic DNA digested with *Eco*RI. (*b*) Blots were probed with either the N-terminal or C-terminal probe from the α_i cDNA (see Fig. 1), as shown pairwise for genomic DNA digested with *Eco*RI, *Bam*HI, or *Hin*dIII. Markers at right in *A* and *B* show migration of fragments from *Hin*dIII digestion of bacteriophage λ DNA.

by several criteria: the sequence of α_h is highly homologous to α_i and α_o and contains amino acid residues that are potential sites for modification by both cholera toxin and pertussis toxin. Previous work showed that bovine brain contains a minor 40-kDa substrate for ADP-ribosylation by pertussis toxin, in addition to the 41-kDa α_i and 39-kDa α_o proteins (6). It may be that this protein is α_h. Recent immunologic studies suggest that NG 108-15 and C6 glioma cells contain a pertussis toxin substrate that is neither α_i nor α_o (25).

The GTP-binding proteins encoded by the *ras* genes of mammals and yeast, the elongation factors EF-Tu and EF-G, and the transducin α subunit α_T share four regions of sequence homology totaling about 60 residues (2, 21–24, 36, 37, 38). These residues may be involved in the GTP-binding site (37, 38). The partial α_i sequence includes two of these regions (residues 192–205 and 243–253), whereas our α_h and α_o sequences include one of them. In these regions, the *ras* protein, EF-Tu, and EF-G are equally homologous to α_i, α_o, α_h, and α_T.

The cysteine located four residues from the carboxyl terminus is the site at which transducin is modified by pertussis toxin (14). Since α_i is a substrate for ADP-ribosylation by pertussis toxin, we would expect the sequence homology at the carboxyl terminus (Fig. 2); this site is absent from α_s (34, 35). The carboxyl-terminal region of the α_T molecule is an especially important one, since it seems to be involved in the interaction of transducin with rhodopsin (19). The analogous interaction in the hormone-responsive system is the interaction of α_i or α_s with hormone receptors. Indeed, ADP-ribosylation of α_i does uncouple G_i from hormone receptors (39–41). The homology in this region may help explain why rhodopsin can substitute for receptors and modulate G_i activity in reconstitution experiments (42, 43). We would further suggest that hormone receptors and rhodopsin will be found to be homologous in the regions where they interact with the α components of GTP-binding regulatory proteins. Sequence homology between the β-adrenergic receptor and rhodopsin has been reported recently (45).

Cholera toxin ADP-ribosylates transducin at an arginine residue (18) that is also present in α_i and α_h (Fig. 2). A large body of evidence suggests that α_i is not ADP-ribosylated by cholera toxin *in vivo* (2). However, a recent report (44) describes a 41-kDa protein that is ADP-ribosylated *in vitro* by both pertussis and cholera toxins.

The biological functions of α_i, α_h, and α_o are not completely defined. ADP-ribosylation of a 41-kDa membrane G protein in many tissues is associated with the loss of hormone-mediated inhibition of adenylate cyclase, but a direct inhibitory interaction of α_i with the adenylate cyclase catalytic unit has not been demonstrated (E.J.N. and J.W.W., unpublished observations). There are other examples of cells in which pertussis toxin-catalyzed ADP-ribosylation of a 41-kDa membrane G protein results in a blockade of signal transduction by receptors that do not inhibit adenylate cyclase (11–13). These pertussis toxin substrates may be α_i or may be homologous G proteins of similar size. Most cell types in which there is a hormonal inhibition of adenylate cyclase also show an associated hormonal inhibition of a Ca^{2+}-dependent secretory response, as well as hormonal regulation of phosphatidylinositol metabolism and of protein kinase C activity (11–13). It seems likely that signal transduction across biological membranes is mediated by a family of highly homologous but distinct G proteins. Our studies point out the complexity of the family of G proteins. A central question is whether there is a separate G protein for each pharmacological class of receptors or whether the diversity of G proteins reflects the interaction of specific G proteins with the different effector systems in plasma membranes. Further

studies into the structure and genetic organization of these proteins are likely to provide new insights into this ubiquitous gene superfamily.

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