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Isolation and characterization of a cDNA clone for the γ subunit of bovine retinal transducin

(photoreceptor biochemistry/*ras*/signal transduction/molecular evolution)

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ABSTRACT **We have isolated and characterized a cDNA clone that encodes the γ subunit of transducin, the guanine nucleotide binding regulatory protein found in vertebrate photoreceptors. The γ subunit was separated from the α and β subunits of transducin and purified to homogeneity by reversed-phase high performance liquid chromatography. The sequence of the first 45 amino acids at the amino terminus of this polypeptide was then determined by automated Edman degradation. Oligodeoxynucleotide probes corresponding to two nonoverlapping regions of this sequence were synthesized and then used to screen a bovine retinal cDNA library. One probe, $T_\gamma1$, was a mixture of 32 different heptadecamers complementary to all possible mRNA sequences that could encode a portion of the T_γ sequence; the other probe, $T_\gamma2$, was a mixture of 128 different heptadecamers. Thirteen clones that hybridized with $T_\gamma1$ were selected. Only one of these had an insert that also hybridized with $T_\gamma2$. The DNA sequence of this insert encodes a 73-amino acid polypeptide that corresponds to the transducin γ subunit on the basis of amino-terminal sequence, amino acid composition, and carboxyl-terminal sequence. The molecular weight of the mature γ subunit is 8400. It appears to be synthesized as a discrete polypeptide and not as a domain of a larger precursor polyprotein. The transducin γ subunit is very hydrophilic and acidic; it has 19 acidic and 11 basic amino acids as well as three cysteine residues. Furthermore, significant homology was found in comparisons of the nucleic acid sequence corresponding to the carboxyl terminus of the γ transducin transcript with the sequences corresponding to the carboxyl terminus of *ras* oncogene products, suggesting a possible ancestral relationship between these genes.**

Photolysis of rhodopsin triggers an enzymatic cascade in vertebrate photoreceptors that results in the rapid hydrolysis of cyclic GMP (see ref. 1 for a review). This process is mediated through transducin (T), a guanine nucleotide binding protein found specifically in rod outer segments. Photolyzed rhodopsin stimulates hundreds of molecules of transducin to bind GTP, after which each transducin·GTP complex activates a cyclic GMP phosphodiesterase. The resulting hydrolysis of cyclic GMP appears to be involved in visual transduction.

Transducin is a member of a family of membrane-associated guanine nucleotide binding proteins referred to as G proteins (2–4). G proteins all act through a common mechanism; they bind GTP in response to stimulation by specific receptor proteins and then regulate the activity of an enzyme. Transducin is stimulated by photolyzed rhodopsin and activates a phosphodiesterase, whereas other G proteins interact with a variety of hormone or neurotransmitter receptors and either activate or inhibit adenylate cyclase (5). G proteins that stimulate adenylate cyclase are referred to as G_S (6), whereas those that inhibit adenylate cyclase are called G_I (7).

Transducin, like all of the well-characterized G proteins, is made up of three polypeptide subunits (8–10). T_α has an apparent molecular weight (M_r) of 39,000 and contains sites for guanine nucleotide binding (9) and for ADP-ribosylation catalyzed by either cholera toxin (3) or pertussis toxin (11, 12). T_β and T_γ are polypeptides of M_r 36,000 and 6,000–10,000, respectively. G_S, G_I, and transducin each have unique α subunits (ref. 4; unpublished data), whereas the β subunits of all the G proteins that have been examined are nearly identical (4).

In the inactive state, transducin is a complex of T_α, T_β, and T_γ (1). The presence of the β and γ subunits is absolutely required for photolyzed rhodopsin to stimulate T_α to bind GTP (13). When T_α binds GTP, it dissociates from the complex of T_β and T_γ subunits ($T_{\beta,\gamma}$) and activates the phosphodiesterase (9) by relieving the inhibitory constraints imposed on the phosphodiesterase by its own inhibitory subunit (14).

We have recently begun to isolate cDNA clones for the subunits of bovine transducin in order to determine their primary structures and the genetic relationship between transducin and other members of the G protein family. The approach we have used is to first purify the individual transducin subunits and determine part of their amino acid sequence. Oligodeoxynucleotide probes based on these sequences have been synthesized and used to select phage clones from a bovine retinal cDNA library. The DNA sequences of the isolated clones have then been determined and compared to the respective amino acid sequences.

This report describes the isolation and characterization of a cDNA clone for the γ subunit of transducin. The isolation of cDNA clones for the γ subunit has been more straightforward than for the other subunits because the amino acid sequence of γ can be determined directly by Edman degradation, whereas the α and β subunits have modified amino termini and cannot be sequenced directly.* We have recently determined partial amino acid sequences from proteolytic fragments of T_α and T_β (unpublished data), and this information can be used to isolate cDNA clones corresponding to these subunits.

MATERIALS AND METHODS

Materials. Bovine retinas were obtained from Hormel (Austin, MN). Guanosine 5'-[β,γ]triphosphate (p[NH]ppG) was purchased from Sigma. Restriction enzymes were ob-

Abbreviations: G proteins, a family of guanine nucleotide binding proteins; G_I, inhibitory G protein; G_S, stimulatory G protein; p[NH]ppG, guanosine 5'-[β,γ]imidotriphosphate; kb, kilobases; T, transducin.

*We have attempted to determine amino acid sequence from all three purified subunits by using a gas/liquid solid-phase protein sequenator. T_γ was the only subunit to yield to Edman degradation.

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tained from Boehringer Mannheim and Bethesda Research Laboratories and [γ-^{32}P]ATP (>5000 Ci/mmol; 1 Ci = 37 GBq), from ICN. T4 polynucleotide kinase was purchased from New England Nuclear. The Vydac C4 reversed-phase HPLC column was purchased from Western Analytic; trifluoroacetic acid, carboxypeptidase Y, and 6 M constant-boiling HCl was from Pierce; and acetonitrile was Burdick and Jackson (Muskegon, MI) HPLC grade.

Purification of T_γ. Transducin was extracted from purified bovine rod outer segment membranes as described elsewhere (9) except that 10 μM p[NH]ppG, rather than GTP, was used to elute the transducin from the membranes. The transducin extract was concentrated to 2 mg/ml by ultrafiltration, dialyzed into 20 mM 4-morpholinepropanesulfonic acid (Mops), pH 7.2/1 mM $MgCl_2$/1 mM dithiothreitol and stored at -70°C.

T_γ was purified from this extract on a reversed phase 300-Å pore size Vydac C4 HPLC column, using two Altex 110A pumps and an AXXIOM model 711 controller. The concentrated extract was injected directly onto the column and the protein was eluted with a gradient of increasing acetonitrile concentration in 0.1% trifluoroacetic acid (15). Elution of protein was monitored by absorbance at 214 nm with a Beckman model 160 detector. The gradient conditions are described in the legend of Fig. 1. To obtain enough material for amino acid sequence determination, approximately 2 mg of transducin extract was loaded onto the column, and about 50 μg of purified T_γ was recovered. T_γ was also purified by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by electroelution from the gel by the method of Hunkapiller *et al.* (16). From 600 μg of transducin, about 5 μg of purified T_γ was obtained by this method.

Protein Sequence Analysis. T_γ purified by reversed-phase HPLC or by electroelution was dried, redissolved in approximately 50 μl of water, and loaded directly onto a gas/liquid solid-phase protein sequenator. Phenylthiohydantoin derivatives were analyzed by reversed-phase HPLC as described by Hunkapiller and Hood (17). Residue assignments were made by manual inspection of the HPLC chromatographs.

Carboxypeptidase Y Digestion and Amino Acid Analysis. Approximately 55 μg (7 nmol) of purified T_γ in 0.5% $NaDodSO_4$/0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.3, were digested with 0.43 μg (7 pmol) of carboxypeptidase Y at 23°C. Norleucine (4 nmol) was included as an internal standard. Aliquots (1.8 nmol) were taken at 5, 20, 60, and 100 min, treated as described by Martin *et al.* (18) to remove $NaDodSO_4$, and analyzed on a Durrum D-500 amino acid analyzer. Amino acid analysis was performed by hydrolyzing 1-nmol aliquots of HPLC-purified T_γ in constant-boiling 6 M HCl for 12, 24, and 48 hr. An additional aliquot was first treated with performic acid to convert cysteine to cysteic acid and then hydrolyzed. The amino acid compositions of these hydrolysates were then determined. Values for serine and threonine were determined by extrapolation to zero time to correct for their destruction during hydrolysis.

Gel Electrophoresis of Proteins and DNA. $NaDodSO_4$/polyacrylamide gel electrophoresis of proteins was performed as described by Laemmli (19), using a 16% gel. The molecular weight markers used were ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lysozyme, 14,000; and bovine pancreatic trypsin inhibitor, 6000.

DNA samples were digested with restriction enzymes, electrophoresed in agarose, and blotted to nitrocellulose according to standard procedures (20).

DNA Sequence Analysis and Preparation of Oligodeoxynucleotides. DNA sequencing was performed by the dideoxy method of Sanger *et al.* (21).

Oligodeoxynucleotides were synthesized on an automated DNA synthesizer using phosphoramidite chemistry (22).

Screening of the Bovine Retinal cDNA Library. A bovine retinal cDNA library in the λgt10 phage vector was constructed and generously supplied to us by Jeremy Nathans (Stanford University School of Medicine) (23). Replicate nitrocellulose filter blots (24) of the cDNA library were hybridized to mixed oligodeoxynucleotide probes that were labeled at their 5′ ends by using [γ-^{32}P]ATP. Hybridizations were performed in 6× SET (0.9 M NaCl/150 mM Tris·HCl, pH 8.0/6 mM EDTA), 5× Denhardt's solution (0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone), and 0.1% $NaDodSO_4$ at 42°C for 2–4 hr. Filters were washed three times for 5 min in 0.9 M NaCl/90 mM sodium citrate, pH 7.2/0.2% $NaDodSO_4$ at room temperature and autoradiographed for 1–4 days at -70°C, using Kodak XAR-5 film and an intensifying screen (Kodak X-Omatic regular).

RESULTS

Purification of Transducin and Its γ Subunit. To isolate T_γ cDNA clones, it was first necessary to determine a portion of the amino acid sequence of the γ subunit. Transducin was extracted from bleached bovine rod outer segment membranes by using a standard protocol (9). The extract contained the three polypeptide subunits of transducin as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis (results not shown).

T_γ was then purified from the extract by reversed-phase HPLC on a Vydac C4 column using a gradient of increasing acetonitrile concentration in 0.1% trifluoroacetic acid. An example of a purification of T_γ by this method is shown in Fig. 1 along with a $NaDodSO_4$/polyacrylamide electrophoresis gel of the purified T_γ. Approximately 6 nmol of T_γ was prepared by this method for amino acid sequence determination and amino acid analysis. Approximately 0.6 nmol of T_γ was also prepared from the transducin extract by electroelution from a $NaDodSO_4$/polyacrylamide electrophoresis gel of the transducin extract.

Amino Acid Sequence of T_γ. Fig. 2 shows the 45 amino acid sequence that was determined by automated Edman degradation of approximately 3 nmol of HPLC-purified T_γ. The sequence of T_γ purified by electroelution was also determined and it was identical. The sequence in Fig. 2 does not, however, represent the complete amino acid sequence of T_γ.

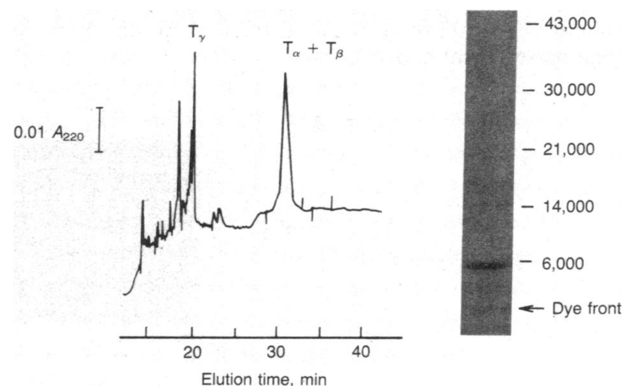

FIG. 1. Separation of the transducin subunits by reversed-phase HPLC. An analytical separation of the transducin subunits is shown. The transducin (50 μg) was injected onto a Vydac C4 column (4 × 25 mm) equilibrated with 0.1% trifluoroacetic acid in water at a flow rate of 1.0 ml/min. An increasing gradient of acetonitrile was used to elute the polypeptides. Five minutes after the injection, the acetonitrile concentration was raised to 40% over 5 min, then to 50% over 5 min, to 60% over 10 min, and finally to 70% over 15 min. Protein elution was monitored by absorbance at 220 nm. In the preparative separation 2 mg of transducin was applied. The purified T_γ is shown on a $NaDodSO_4$/16% polyacrylamide electrophoresis gel on the right along with molecular weight markers.

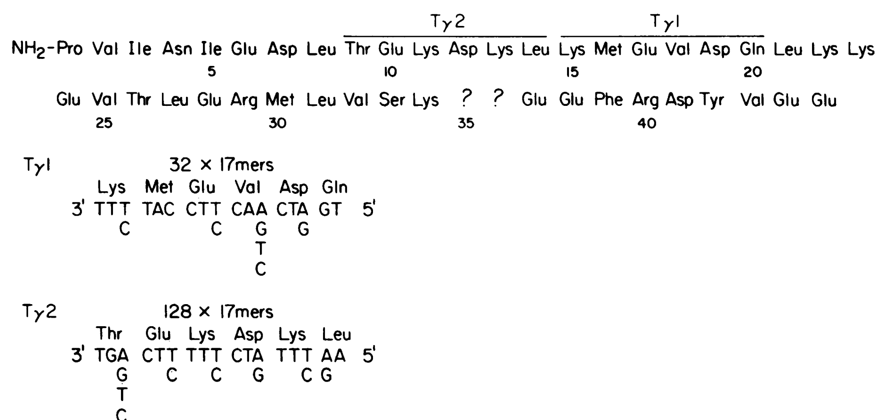

FIG. 2. Partial amino acid sequence of the bovine retinal transducin γ subunit. The oligodeoxynucleotide probes $T_\gamma 1$ and $T_\gamma 2$ that were used to screen the bovine retinal cDNA library are shown in the lower portion of the figure.

HPLC-purified T_γ was digested with carboxypeptidase Y and was found to have a carboxyl-terminal serine residue, not a glutamic acid. Furthermore, the amino acid composition of purified T_γ determined by amino acid analysis did not correspond to the composition of the sequence shown in Fig. 2, suggesting that the sequence was incomplete.

Isolation and Characterization of a T_γ cDNA Clone. Two nonoverlapping T_γ-specific oligodeoxynucleotide probes were synthesized (22) and are shown in Fig. 2. Probe $T_\gamma 1$ is a mixture of 32 different heptadecamers that is complementary to all possible mRNA sequences that could encode that region. Similarly, $T_\gamma 2$ is a mixture of 128 different heptadecamers. Probe $T_\gamma 1$ had the lowest level of redundancy, so it was first 5'-end-labeled by using T4 polynucleotide kinase and then used to probe nitrocellulose filter replicas of a λgt10 bovine retinal cDNA library. This library has been used for isolation of rhodopsin cDNA clones (23). From a screening of approximately 30,000 plaques, 13 were selected that hybridized with the $T_\gamma 1$ probe. DNA was isolated from each of these clones, digested with *Bam*HI and *Bgl* II, and analyzed after transfer to nitrocellulose by hybridization with the 5'-end-^{32}P-labeled $T_\gamma 1$ and $T_\gamma 2$ oligodeoxynucleotides (Fig. 3). All of the selected clones had inserts that hybridized with $T_\gamma 1$ (Fig. 3*A*); however, only one of these also hybridized with probe $T_\gamma 2$ (Fig. 3*B*). The size of the insert in this phage is about 1.2 kilobases (kb), as estimated by its restriction digest pattern (not shown).

A restriction map and partial DNA sequence of the cloned insert are shown in Fig. 4. Translation in one reading frame produces a protein sequence that agrees precisely with the amino-terminal 45 amino acid sequence of T_γ and also clarifies the assignment of two cysteines at positions 35 and 36. In addition, the open reading frame continues until an in-phase termination signal (TAA) is reached at nucleotide 223. The sequence encodes a polypeptide that is 73 amino acids long, beginning with the proline at position 1. The amino acid composition of the sequence agrees very closely with the composition of T_γ determined by amino acid analysis of HPLC-purified T_γ (Table 1). The termination codon is followed by a 134-base-pair 3' untranslated region that is about 76% A+T and contains the mRNA 3' consensus polyadenylation signal (24) A-A-T-A-A-A starting at position 349. However, no poly(A) tract was found, which suggests that this cDNA clone may be incomplete at its 3' terminus.

DISCUSSION

We have isolated a cDNA clone coding for the γ subunit of bovine retinal transducin and deduced from it the complete amino acid sequence of this subunit. T_γ appears to be synthesized as a discrete polypeptide rather than as a domain of a polyprotein because many of the features of a eukaryotic translation initiation site are found. (*i*) An initiator codon (ATG) immediately precedes the amino-terminal proline. (*ii*)

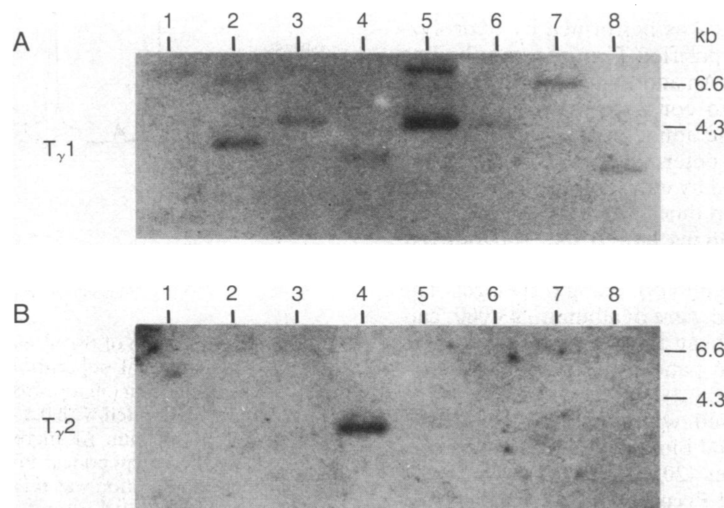

FIG. 3. Southern blot analysis of cDNA clones that hybridized to oligodeoxynucleotide probe $T_\gamma 1$. DNA from each of these clones was digested with *Bam*HI and *Bgl* II, separated on a 1.0% agarose gel, transferred to nitrocellulose, and probed with the designated ^{32}P-labeled oligodeoxynucleotide probes $T_\gamma 1$ (*A*) and $T_\gamma 2$ (*B*). The fragments corresponding to the inserts of 8 of the 13 selected phages are shown.

There is an adenosine three nucleotides upstream from the presumed initiator ATG codon, in common with about 80% of the eukaryotic mRNAs that have been examined (25). (*iii*) There is an inverted repeat about 10 base pairs upstream from the ATG. Stem and loop structures have been found to precede the initiator codon in several other mRNAs (26). Although the DNA sequence 5′ to the coding region did not include any nonsense codons, this region does not show any sequence corresponding to the T_α or T_β carboxyl-terminal sequences (12) or to any of the α or β subunit sequences that we have determined (unpublished data). These sequences might be present if T_γ were synthesized along with other transducin subunits as part of a precursor "protransducin" molecule (27). Finally, a termination codon, TAA, is found after the serine at position 73.

The amino acid composition of HPLC-purified T_γ matches well with the composition predicted from the cDNA sequence (Table 1). The subunit is very hydrophilic and is acidic; it has 19 acidic residues and 11 basic residues. It should also be noted that the subunit has three cysteine residues. Ho and Fung (28) recently reported that under nondenaturing conditions T_γ does not react with N-[^{3}H]ethylmaleimide. This suggests that the three cysteines in T_γ either are in the form of disulfide bonds or are inaccessible to the aqueous environment. We have found that T_γ separates completely from T_β on the C4 reversed-phase column even in the absence of reducing agents. This suggests that there are no disulfide linkages between the β and the γ subunits. The highly ionic nature of the γ subunit implies that the $T_{\beta,\gamma}$ complex is held together by electrostatic forces.

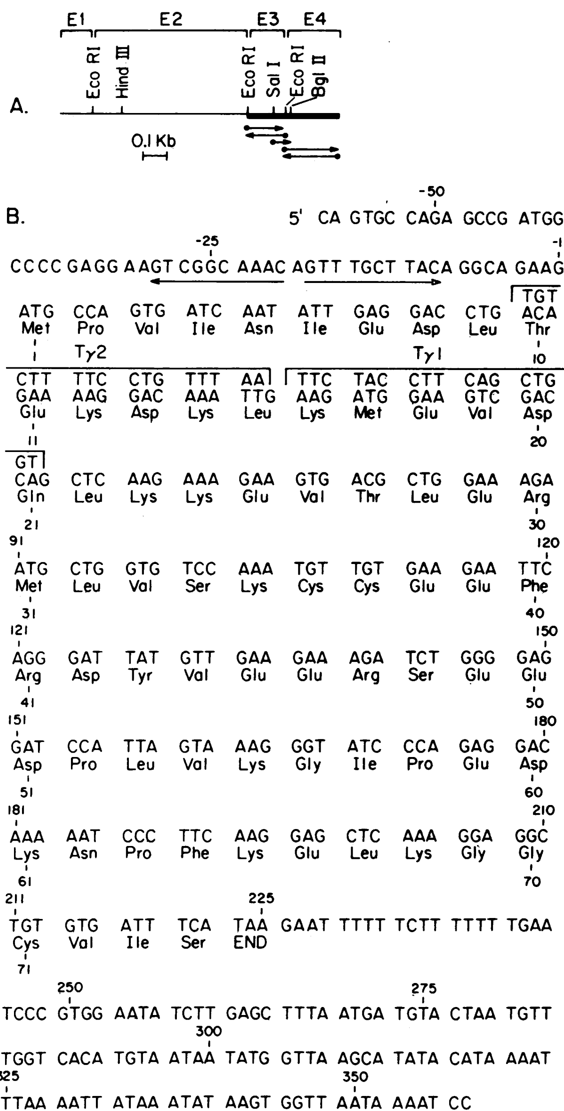

FIG. 4. (*A*) Complete *Eco*RI and partial *Hin*dIII, *Sal* I, and *Bgl* II restriction map. Four *Eco*RI fragments are designated E1–E4. The portion of the DNA insert encoding T_γ is indicated in the bold line and the DNA sequencing strategy is shown by the arrows. (*B*) DNA and protein sequences from the insert of the T_γ cDNA clone. The two arrows that precede the initiator codon underline a nearly perfect 10-base-pair inverted repeat; the underlined sequence starting at position 349 designates a polyadenylylation signal. $T_\gamma1$ and $T_\gamma2$ represent the two nonoverlapping regions of oligodeoxynucleotide probe hybridization.

Table 1. Comparison of the amino acid composition of HPLC-purified T_γ determined by amino acid analysis with the composition of the protein encoded by the cDNA clone

	Composition, mol %	
Residue	From amino acid analysis	Based on cDNA sequence
Asp	11.0	11.0
Thr	3.3	2.7
Ser	5.0	4.1
Glu	18.0	17.7
Pro	5.6	5.5
Gly	6.7	5.5
Ala	1.3	0
Val	8.4	9.6
Met	1.8	2.7
Ile	4.2	5.5
Leu	10.4	9.6
Tyr	1.1	1.4
Phe	2.2	2.7
His	0.5	0
Lys	12.3	13.6
Arg	4.4	4.1
Cysteic acid	3.8	4.1

Results are the average of two separate sets of amino acid analyses.

Carboxypeptidase Y digestion of purified T_γ confirms the carboxyl-terminal serine predicted from the cDNA. The molecular weight of mature T_γ calculated from the sequence shown in Fig. 4 is 8400. The methionine encoded by the initiator codon is not found in the mature subunit; it is presumably removed from the polypeptide during posttranslational processing.

The size of the cloned DNA insert is about 1.2 kb. We have rescreened 40,000 plaques from the bovine retinal cDNA library with probes corresponding to *Eco*RI fragments E1 and E2 shown in Fig. 4*A* and also with fragments E3 and E4. We found that the frequency of phages in the library that hybridize with the combined probes E3 and E4 is 0.05%. The frequency of phages that hybridize with E1 and E2 is 0.01%. Furthermore, none of these phages hybridized with both probes. This indicates that the cDNA sequences in fragments E1 and E2 may not be derived from T_γ mRNA. This cDNA may have been ligated to the T_γ cDNA during construction of the cDNA library. Characterizing other T_γ cDNA clones that were isolated with fragments E3 and E4 has confirmed this.

The function of T_γ is not currently understood. It is tightly bound to the β subunit and the $T_{\beta,\gamma}$ complex appears to be required for the interaction of T_α with photolyzed rhodopsin (13). γ subunits have recently been found to be associated with other G proteins besides transducin. The inhibitory G protein, G_I, from rabbit liver (29) and from human erythrocytes (30), the stimulatory G protein, G_S, from human eryth-

rocytes (30), and the "other" G protein from bovine brain (31), G_O, all have small molecular weight (6,000–10,000) subunits that remain tightly associated with their β subunits throughout several purification steps. Reversed-phase HPLC, polyacrylamide gel electrophoresis, and silver staining of two of these γ subunits show that they are substantially different from one another.† Thus, some of the specificity for a particular class of G protein may reside in the γ subunit sequence.

Gilman (5) has recently suggested that the G proteins are related to the GTP binding *ras* family of proteins on the basis of homology between a short stretch of amino acids at the carboxyl terminus of the α subunit of transducin and a sequence in the middle of the *ras* protein. There is also some homology between the carboxyl-terminal sequences of T_γ and of the *ras* proteins. Powers *et al.* (32) have noted that *ras* proteins from different sources all terminate with the amino acid sequence C-A-A-X, in which A represents an aliphatic amino acid, C represents cysteine, and X is a carboxyl-terminal serine, methionine, or cysteine. The carboxyl-terminal sequence of T_γ fits this pattern. Furthermore, there is 44% homology between the DNA sequences starting at exon 4 of human c-Ha-*ras*1 protooncogene (nucleotides 451–570 in ref. 33) and nucleotides 107–225 of T_γ. To maximize the homology, the sequences were aligned by introducing only one gap, consisting of a single nucleotide between nucleotides 162 and 163 of T_γ. It is possible that these two families of proteins (ras and the G proteins) are derived from common ancestral genes. Domain functions in the ras protein may be embodied in the different subunits of transducin and both proteins may function in transducing signals at the cellular level. Recent evidence suggests that ras interacts directly with cell-surface receptor proteins (34, 35).

The existence of T_γ cDNA clones now makes possible direct sequence comparison of the γ subunits of transducin and other G proteins. Homologous cDNAs can be isolated from cDNA libraries constructed from other tissues to make these comparisons. In addition, this T_γ cDNA clone can be used along with cDNA clones for T_α and T_β to study the location and arrangement of genes for transducin and the other G proteins.

†An unpublished comparison of T_γ with the γ subunit derived from rabbit liver G_I, done in collaboration with G. Bokoch and A. G. Gilman.

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