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Immunochemical Studies of the 36-kDa Common β Subunit of Guanine Nucleotide-Binding Proteins: Identification of a Major Epitope

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SUMMARY

Twenty-four of 24 rabbits immunized with the β subunit common to guanine nucleotide binding proteins developed antibodies reactive on immunoblots with the 15-kDa (amino-terminal) tryptic fragment of β. Only 2 of 24 developed antibodies reactive with the 26-kDa (carboxy-terminal) tryptic fragment. The 15-kDa fragment-reactive antibodies were also detected in several nonimmune sera. Antibodies reactive with the 15-kDa fragment could be affinity-purified from all β-immune sera by adsorption to a fusion protein encoded by a cDNA clone identified by expression vector screening. The 15-kDa fragment antibodies in nonimmune sera did not bind to the fusion protein. Limited amino acid sequence homology between the 36-kDa β subunit and the protein encoded by the cDNA clone suggested that the amino-terminal decapeptide of β contains a major epitope. A synthetic decapeptide, corresponding to the amino terminus of the 36-kDa β subunit, effectively and specifically blocked binding of antibodies in β-immune sera (but not in β-reactive nonimmune sera) to nitrocellulose-bound 15-kDa fragment. The 15-kDa fragment-reactive antibodies could be affinity-purified from β-immune sera on a matrix containing bound decapeptide; affinity-purified antibodies reacted equally well with the 36- and 35-kDa forms of the β subunit. Native transducin β/γ complexes readily blocked binding of 15-kDa fragment-reactive antibodies in immune but not nonimmune sera from binding to the nitrocellulose-bound fragment. The results show that nonimmune sera may contain antibodies directed against an epitope of the 15-kDa fragment that is buried in the native β/γ complex. In contrast, the amino terminal decapeptide of the β subunit is exposed on the surface of the native protein and contains a major antigenic site in both the 35- and 36-kDa forms.

A family of guanine nucleotide-binding proteins (G-proteins) transduces signals across cell membranes by coupling signal detectors to effectors (1, 2). Known G-proteins include G_s and G_i, the G-proteins associated with stimulation and inhibition, respectively, of adenylate cyclase; TD, the G-protein of retinal photoreceptor cells, and G_o, a G-protein of unknown function abundant in brain. G-proteins consist of three distinct polypeptide chains; α subunits bind GTP and are unique for each G-protein. β and γ subunits are tightly associated under nondenaturing conditions; the β/γ complex dissociates from α subunits upon G-protein activation. TD-γ differs immunochemically from other G-γ subunits (3).

All G-proteins purified to date contain a 36-kDa β subunit. The 36-kDa β subunits of G_s, G_i, and TD show similar peptide maps after V8 protease digestion (4). Polyclonal antisera raised against TD-β cross-react with the β subunits of G_s, G_i, and G_o (3, 5–8), and antisera raised against purified brain G-proteins (G_i and G_o) cross-react with β subunits of other G-proteins including TD (5–9). These data suggest that G-protein β 36-kDa subunits are similar if not identical. Recent evidence, based on the nucleotide sequence of cDNA clones encoding G-β and TD-β, suggests that the amino acid sequence of G-β 36-kDa subunits is in fact identical in all G-proteins (10–12).

G-proteins other than TD may also contain a 35-kDa form of the β subunit (13). The latter may differ immunochemically from the 36-kDa form, but its structure has not been definitively determined (5–8). Similarities and differences between the 36- and 35-kDa forms are apparent upon proteolytic peptide mapping (6, 8). Recently, two groups (14, 15) have reported the

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ABBREVIATIONS: G-proteins, guanine nucleotide-binding proteins; G_s and G_i, respectively, stimulatory and inhibitory G-proteins associated with adenylate cyclase; G_o, G-protein of unknown function isolated from brain; TD, transducin, the G-protein of retinal photoreceptor cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone; TBS, Tris-buffered saline; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

sequence of a cDNA that encodes a protein differing by about 34 of 340 residues from the protein encoded by the 36-kDa β subunit cDNA. The identity of the protein encoded by the novel β subunit cDNA is not clear but could be related to the 35-kDa form (14, 15).

Antibodies directed against G-protein β subunits have proven useful as probes of β subunit primary structure, in quantitating the β subunit in crude membrane preparations (3, 5–9), and in phylogenetic studies (9, 16). For β subunit antibodies to be useful in other studies, e.g., as probes of tertiary structure and of function, further information is needed on the location of the epitopes with which β subunit antibodies react. We report here the results of epitope mapping of β subunit antibodies. Using a cDNA clone obtained by screening an expression vector library with β subunit antibodies, we identify an amino-terminal peptide as a major epitope of the β subunit. During the course of these studies, we also found that several sera from rabbits not immunized with β subunit show strong reactivity on immunoblots with the β subunit. The epitope responsible for this reactivity differs from the major epitope recognized by antibodies from animals immunized with β subunit. Antibodies of defined specificity proved useful in determining the extent of surface exposure of different regions of the β subunit in the native molecule.

Experimental Procedures

Materials. Frozen bovine retinas were obtained from Hormel (Austin, MN). Fresh cow brains were obtained from a local slaughterhouse. New Zealand White female rabbits (Hazleton Co., Denver, PA) were used for all immunizations. TPCK-trypsin was from Sigma, guanosine 5′,3-*O*-triphosphate was from Boehringer, and Blue Sepharose was from Pharmacia. The sources of other materials used in protein determination, SDS-PAGE, and immunoblotting have been described (3). The synthetic peptide corresponding to the amino-terminal decapeptide of the 36-kDa subunit of β, MSELDQLRQE-amide, was obtained from Peptide Technologies Co. (Washington, D. C.). The decapeptide, RLKIDFGESA, unrelated to the β subunit, was kindly provided by Cecilia Unson (Rockefeller University). Peptide purity was assessed by amino acid analysis and high pressure liquid chromatography.

Protein purification. HoloTD was eluted from bovine rod outer segments with either GTP or guanosine 5′(3-*O*-thio)-triphosphate as described (3). TD-α and TD-β/γ were resolved by Blue Sepharose chromatography as described (17). Brain G-proteins were purified by DEAE-Sephacel and Ultrogel AcA-34 chromatography as described (13). Purified β/γ complexes containing the 36- and 35-kDa forms of β were then obtained from early-eluting fractions of a DEAE-Toyopearl column.

Trypsin cleavage of TD-β/γ. TPCK-trypsin (0.93 mg/ml in 0.2 M MOPS buffer, pH 8.1) was incubated with purified TD-β/γ (0.033 μg of trypsin/μg of TD-β/γ) for 5 min at 37°. The reaction was terminated by adding SDS sample buffer and boiling.

Affinity-purification of β antibodies with cDNA clone A. An embryonic chick retina cDNA library was constructed in the expression vector, λ GT11, and screened by standard techniques (18) with an antiserum, AS/1 (3), raised against bovine holoTD. A clone strongly reactive with β-specific antibodies was identified, plaque-purified, and sequenced by the dideoxy method (19). The cDNA clone, designated clone A, was used to infect *Escherichia coli* Y 1090. In brief, an overnight culture of *E. coli* Y 1090, grown in NCZYM medium containing 0.2% maltose, was spun down, resuspended in 10 mM $MgSO_4$, and incubated for 15 min at 37° with sufficient clone A stock to give 10,000–100,000 plaques/150-mm plate. The incubation mixture was combined with 7 ml of NCZYM medium containing 0.75% agarose and pipetted onto 150-mm plates containing 1.5% agar in NCZYM medium. The plates were incubated at 38–39° until covered with visible plaques (2.5–3.5 hr). Nitrocellulose filter discs previously soaked in isopropyl-β-D-thiogalactopyranoside were placed on the plates which were then incubated overnight to allow adsorption of expressed protein onto the filters. The filters were then washed twice in TBS (10 mM Tris-HCl, pH 7.5/500 mM NaCl), and nonspecific protein-binding sites were blocked by incubation for 2 hr in TBS containing 20% goat serum and 0.05% thimerosal. Antisera to be affinity-purified on clone A filters were diluted 1/10 in 10% goat serum, 0.05% thimerosal in TBS and incubated with filters overnight at room temperature on a shaking platform. "Depleted" sera were removed, the filters were washed three times with TBS, and adsorbed antibodies were eluted with two 5-min washes with 10 ml of 0.2 M glycine, 0.5 M NaCl buffer, pH 2.5. The eluate was immediately neutralized to pH 7.2–7.6 with 2 N NaOH.

Other methods. Protein determination, SDS-PAGE, and immunoblotting were performed as described (3). In brief, proteins separated by SDS-PAGE were transferred to nitrocellulose paper (150 mamp constant current overnight). Efficiency of transfer was monitored by Coomassie Blue staining of the gel after transfer, and by Amido Black staining of the nitrocellulose paper. Under the conditions used, essentially quantitative transfer of β/γ subunits and their tryptic fragments was achieved. Nitrocellulose papers were incubated with 3% gelatin in TBS to block nonspecific protein binding, and then in first antibody solutions (at dilutions indicated in figure legends) in 1% gelatin in TBS, overnight at room temperature. The blots were washed twice for 30 min in 0.05% Tween 20 in TBS, and then incubated with second antibody (goat anti-rabbit Ig conjugated to peroxidase, 1/300 dilution) in 1% gelatin in TBS for 2 hr at room temperature. After washing as before, specific bands were developed using 4-chloro-1-naphthol as substrate for antibody-linked peroxidase. For many experiments (see figure legends), multiple immunoblot strips containing separated intact β and γ subunits, as well as the tryptic fragments of the β subunit, were generated as follows. Fifty μg of intact purified TD-β/γ was added to 280 μg of trypsin-digested TD-β/γ in SDS sample buffer. After heating, the entire sample (about 0.8 ml) was placed in a continuous well on top of a 3% polyacrylamide stacking gel, and the components were separated on a 15% polyacrylamide running gel. After transfer to nitrocellulose and blocking with 3% gelatin in TBS, as described (3), the blot was cut vertically into 3-mm strips for incubation with different sera. For immunoblot competition studies, 3-mm strips were incubated in sera diluted (generally 1/100, but see figure legends) in 1% gelatin TBS (final volume, 5 ml) with the specified concentrations of TD-β/γ or synthetic peptide. The amino-terminal β synthetic peptide was coupled to agarose beads, and the derivatized matrix was used to affinity-purify antibodies as described.

Antisera. The characteristics of the antisera used in this study are summarized in Table 1. Antisera PG/4 and BE/1 were from rabbits immunized with ADP-ribose conjugated to bovine serum albumin and keyhole limpet hemocyanin, respectively (20).

Results

Trypsin cleaves both TD-β/γ (21) and brain G-β/γ (22, 23) into stable β fragments of about 26 and 15 kDa; the γ subunit is not cleaved under these conditions. Direct sequence analysis

TABLE 1
Antisera used in this study

Code	Antigen	Ref.
AS/1, AS/4, AS/6	native holoTD	3
CW/1, CW/3, CW/4	denatured holoTD	3
SP/3	denatured TD-β/γ	17
RV/3, RV/6	denatured G_i/G_o	9
U-49	peptide 130–145, β36	5
MP/6	none	
BE/1, PG/4	none for preimmune; ADP-ribose conjugate	20

indicates that the 15-kDa fragment corresponds to amino acids 1–129 of the β subunit (with a blocked amino terminus) and that the 26-kDa fragment represents the remainder of the protein (amino acids 130–340) (10–12).

We have immunized a total of 24 rabbits with G-protein preparations containing β subunit. All 24 developed antibodies reactive with the β subunit on immunoblots. β subunit-reactive antibodies were not detected in preimmune sera from any of these animals (3, 9). As predicted by our previous study (3), only antisera from animals immunized with TD-β/γ recognized TD-γ on immunoblots. All β subunit antisera reacted with the 15-kDa tryptic fragment. Only 2 of 24 β subunit antisera (AS/1 and RV/6) reacted strongly (at 1/100 dilution) with the 26-kDa tryptic fragment of β on immunoblots (not shown).

In testing other antisera from rabbits immunized with proteins other than G-β, we found several that reacted strongly (at 1/100 dilution) on immunoblots with the β subunit. These included animals immunized with preparations (e.g., ADP-ribose conjugated to bovine serum albumin) that could not have been contaminated with β subunit. Moreover, the preimmune serum from such animals was as reactive with β as were post-immunization bleeds. In all such sera tested (MP/6, PG/4, and BE/1, not shown; but see later figures), marked reactivity against the 15-kDa tryptic fragment was evident with little if any reactivity against the 26-kDa fragment.

A cDNA clone obtained by screening an embryonic (14 day) chick retinal expression vector (λ GT11) library with a holoTD antiserum (AS/1) proved useful in defining a major epitope of the β subunit. This clone, designated clone A, was strongly and specifically reactive with antiserum AS/1. After plaque purification of clone A, we used an epitope selection method to define the subpopulation of antibodies within the polyclonal antiserum that were reactive with the antigen expressed by clone A. Preliminary experiments (not shown) indicated that clone A was recognized by β rather than TD-α or TD-γ antibodies. We therefore incubated β subunit-reactive antisera with filters onto which clone A-expressed protein had been adsorbed (see Experimental Procedures). The antisera after incubation with clone A filters and the antibodies specifically bound to clone A filters and eluted with pH 2.5 glycine buffer were tested for β subunit reactivity on immunoblots containing trypsin-cleaved TD-β/γ (Fig. 1).

All 10 sera tested contain 15-kDa fragment-reactive antibodies, 5 of 10 contain TD-γ antibodies, and 2 of 10 contain 26-kDa fragment-reactive antibodies (Fig. 1). In 9 of 10 sera, 15-kDa fragment-reactive antibodies are specifically bound to the clone A-expressed protein (Fig. 1A). The two antisera with 26-kDa fragment-reactive antibodies (RV/6 and AS/1; Fig. 1A, lanes *9* and *10*) provide evidence for the specificity of the clone A epitope selection. In neither case are 26-kDa antibodies adsorbed by clone A filters. Instead, these antibodies remain in the serum after incubation with the filter, as seen in immunoblots performed with "depleted" sera (Fig. 1B, lanes *9* and *10*). Note also that TD-γ-reactive antibodies, where present, are also not adsorbed by clone A filters (Fig. 1B, lanes *4–7* and *10*). Faint γ reactivity detected in lane *5* (Fig. 1A) is presumably due to incomplete washing of the filter incubated with antiserum SP/3. Only the nonimmune serum, MP/6, fails to yield clone A affinity-purified 15-kDa antibodies, despite the fact that β subunit reactivity in this antiserum is all directed against the 15-kDa fragment (Fig. 1B, lane *2*). The 15-kDa fragment-reactive antibodies in two other nonimmune sera tested (BE/1 and PG/4, not shown) also do not bind to the clone A-expressed protein. Thus, 15-kDa fragment-reactive antibodies present in nonimmune sera can be differentiated from those in β-immune sera by lack of reactivity with the protein encoded by clone A.

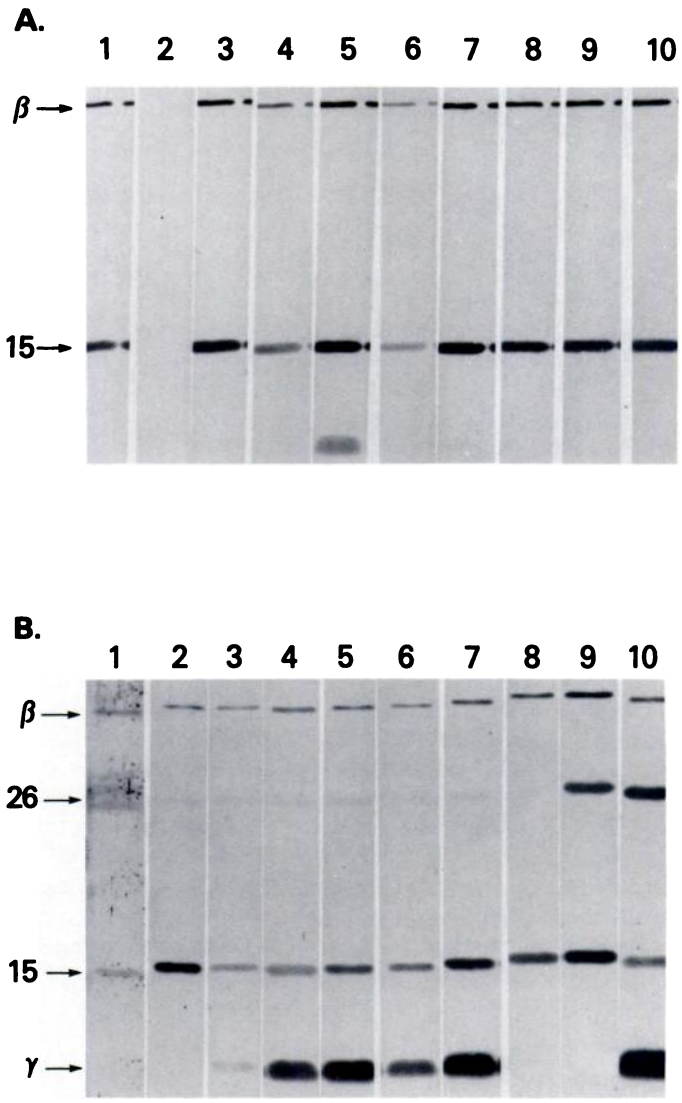

Fig. 1. Affinity purification of antibodies against the 15-kDa tryptic fragment of the β subunit with cDNA clone A. Filters containing the clone A fusion protein were prepared as described under Experimental Procedures. Ten different β subunit-reactive sera were adsorbed onto individual filters. The sera were saved after incubation with filters; the latter were extensively rinsed with buffer, and specifically bound antibodies were eluted as described under Experimental Procedures. TD-β/γ was cleaved partially with trypsin, separated on a 15% polyacrylamide gel, and transferred to nitrocellulose paper. Individual strips were used in immunoblots with (A) affinity-purified antibodies eluted from clone A filters or (B) sera after adsorption to clone A filters. →, the positions of β and γ subunits, and of the tryptic fragments, 26 and 15 kDa, of the β subunit. The sera used were: *1*, AS/6; *2*, MP/6; *3*, CW/1; *4*, AS/4; *5*, SP/3; *6*, CW/4; *7*, CW/3; *8*, RV/3; *9*, RV/6; and *10*, AS/1.

To define the basis for clone A reactivity with most β subunit antisera, we excised the cDNA insert from λ GT11 with EcoRI, subcloned it in M13, and obtained the nucleotide sequence (19, 24). The insert was only 173 base pairs long and encoded a single open reading frame.[3] A search of the National Biomedical

[3] Sequence available from the authors upon request.

TABLE 2

Comparison of predicted amino acid sequences of homologous domains of proteins encoded by clone A and β subunit cDNAs (single letter amino acid code)

Clone A	(residues 17–26)	K S E L D E L Q E E
β (36-kDa form; Refs. 10–12)	(residues 1–10)	M S E L D Q L R Q E
β (Ref. 15)	(residues 1–10)	M S E L E Q L R Q E

Research Foundation database failed to reveal any proteins with high homology to the insert sequence. Comparison of the clone A predicted amino acid sequence with that of bovine TD-β (10), however, showed a small region of homology involving amino acids (arbitrarily numbered) 17–26 of clone A and the amino terminus, amino acids 1–10, of the 36-kDa form of TD-β (Table 2). Six of 10 amino acid identities and two substitutions of glutamic acid for glutamine are present in this peptide. These results suggest that clone A cDNA encodes a protein other than TD-β (see Discussion), but that a sequence within clone A is sufficiently homologous to TD-β to be recognized by β subunit antibodies reactive with this epitope.

The homology between a portion of clone A and the amino terminus of the β subunit, and the specific binding of 15-kDa fragment-reactive antibodies in all β-immune sera tested to clone A-expressed protein suggested that the amino terminus of the β subunit may represent a major epitope of the protein. To obtain more rigorous evidence for this hypothesis, we tested the effect of a synthetic peptide corresponding to the amino-terminal decapeptide of the β subunit, MSELDQLRQE-amide, on binding of antibodies to intact β and its 15-kDa tryptic fragment. The effects of the β-specific peptide were compared to a control decapeptide, RLKIDFGESA, with a sequence unrelated to that of the β subunit. Fig. 2A shows that antibodies affinity-purified from AS/1 by adsorption to clone A-expressed protein react on immunoblots, as expected, with intact β subunit and the 15-kDa fragment. Binding to both of these proteins is blocked in a concentration-dependent manner by the β-specific decapeptide (Fig. 2A, lanes *a–d*) but not by the control peptide (Fig. 2A, lanes *e–h*). In Fig. 2B, we show the results of a similar experiment in which 100 μg of β-specific peptide were compared to 100 μg of the control peptide in blocking the binding of several crude antisera to trypsin-cleaved and uncleaved β/γ. For each of the four immune sera (first four sets of *lanes* in Fig. 2B), the β peptide effectively blocked antibody binding to intact β and, in particular, to the 15-kDa fragment. Note that binding of antibody to the γ subunit or to the 26-kDa fragment (third set of *lanes* in Fig. 2B—AS/1) of β was not blocked by the β peptide. This, along with the inability of the control peptide to block antibody binding, indicates the specificity of the β peptide effect. Both preimmune sera tested (PG/4 and MP/6—last two sets of *lanes* in Fig. 2B), in contrast, were not blocked from binding to β subunit and the 15-kDa fragment by either the β or the control peptide.

The ability of the synthetic decapeptide corresponding to the amino terminus of 36-kDa β to compete effectively for binding to 15-kDa fragment-reactive antibodies in β-immune sera suggested that a matrix containing covalently bound synthetic peptide should be useful in affinity-purification of such antibodies from β-immune sera. Indeed, we found that passage of antisera over a column of agarose-bound peptide allowed purification from crude sera of specific β and 15-kDa fragment-reactive antibodies. Fig. 3 shows that crude antiserum AS/1 reacts with both the 36- and the less abundant 35-kDa forms of the β subunit purified from bovine brain (Fig. 3B). Antibodies affinity-purified from AS/1 by passage over the peptide column

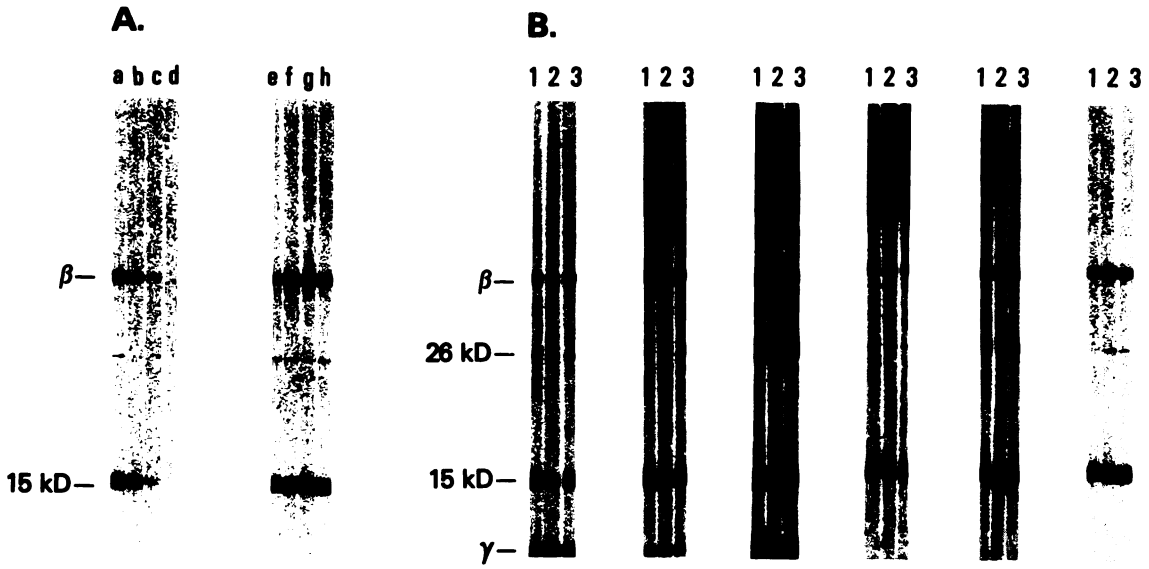

Fig. 2. Competition by synthetic peptides for binding to β subunit antibodies. Trypsin-cleaved and uncleaved TD-β/γ were separated on 15% polyacrylamide gels and transferred to nitrocellulose. Individual strips were cut and incubated with sera and peptides (final volume of first antibody solution = 5 ml) as follows. A. Antibodies were affinity-purified from AS/1 by adsorption to filters containing clone A fusion protein (dilution approximately 1/400); lanes *a*, *e*: no peptide; *b*, *f*: 1 μg; *c*, *g*: 10 μg; *d*, *h*: 100 μg peptide; lanes *b*, *c*, *d*: peptide MSELDQLRQE-amide; lanes *f*, *g*, *h*: peptide RLKDIFGESA. B. Antisera (each at 1/100 dilution; *left* to *right*) were: SP/3, CW/1, AS/1, AS/6, PG/4, and MP/6. Lane *1*, no peptide; lane *2*, 100 μg of MSELDQLRQE-amide; lane *3*, 100 μg of RLKIDFGESA. The positions of the intact β and γ subunits and of the 26- and 15-kDa fragments of β are indicated. The faintly stained, thin band seen in several lanes near the position of the 26-kDa tryptic fragment is not identical with the latter and may represent a minor tryptic fragment that includes the amino terminus.

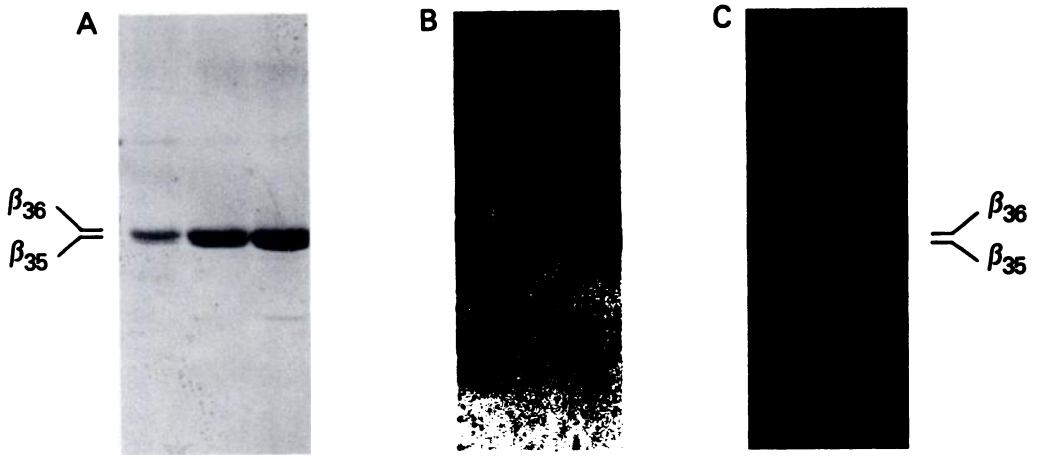

Fig. 3. Immunoblot of purified β 35- and 36-kDa proteins with crude and affinity-purified β antibodies. Fractions from DEAE-Toyopearl chromatography containing purified bovine brain β/γ subunits (approximately 5, 10, and 15 μg/lane from *left* to *right*) were separated by SDS-PAGE (10% gel) and either stained with Coomassie Blue (A) or transferred to nitrocellulose paper for immunoblotting (B and C). In B, crude AS/1 antiserum (1/100 dilution) was used as first antibody. In C, the first antibody solution contained antibodies (1/20 dilution) affinity-purified from AS/1 by passage over a matrix with covalently bound amino-terminal β-decapeptide (see Experimental Procedures). Only the portion of the gel and blots containing the β subunits is shown. The positions of the 36- and 35-kDa forms are indicated.

are also reactive with both forms of the β subunit (Fig. 3C). The amino terminal decapeptide encoded by a novel β subunit cDNA (15) is shown for comparison with the 36-kDa β sequence in Table 2. Note that there is only a single conservative substitution of a glutamate for aspartate residue in position 5 in the novel cDNA. This suggests that the protein encoded by this cDNA should be recognized by antibodies affinity-purified on the synthetic peptide column. The ability of these antibodies to recognize the 35-kDa β subunit suggests, but clearly does not prove, that the protein encoded by the novel β subunit cDNA is the 35-kDa form of β.

To gain insight into the topographic location of epitopes within the native TD-β/γ complex, we performed competitive binding studies. For these experiments we tested various dilutions of antisera on immunoblots and found a concentration range within which 50% reduction in concentration led to a detectable reduction in band intensity on immunoblots. Identical nitrocellulose strips containing both trypsin-cleaved and uncleaved TD-β/γ (see Experimental Procedures) were incubated with appropriate concentrations of various antibodies. Increasing concentrations of soluble, native TD-β/γ were added to test for competition with nitrocellulose-bound protein in binding antibodies.

Added TD-β/γ (Fig. 4) effectively blocked antibody binding to the 15-kDa fragment of β in the case of crude antiserum AS/1 (Fig. 4, lanes *2A–D*) and for antibodies affinity-purified from AS/1 on clone A filters (Fig. 4, lanes *6A–D*). For antiserum RV/6, only the highest concentration of TD-β/γ added led to displacement of the 15-kDa fragment antibodies (Fig. 4, lane *1D*). In contrast to AS/1, 15-kDa fragment antibodies in preimmune sera were not blocked by even the highest concentrations of added TD-β/γ (Fig. 4, lanes *3A–D*, *4A–D*). Note also that added TD-β/γ effectively competes with TD-γ antibodies in AS/1 (Fig. 4, lanes *2A–D*).

Fig. 4 also compares the ability of added TD-β/γ to block binding of 26-kDa fragment antibodies. In the two antisera raised against purified G-proteins, 26-kDa antibodies are not displaced even at the highest TD-β/γ concentration (RV/6, lanes *1A–D*; AS/1, lanes *2A–D*, Fig. 4). In contrast, antibodies in an antiserum raised against a synthetic peptide corresponding to the first 16 amino acids of the 26-kDa fragment (5) are readily displaced by added TD-β/γ (Fig. 4, lanes *5A–D*). As expected, the peptide antiserum shows no reactivity against either the 15-kDa fragment of β or against the γ subunit.

Discussion

The amino acid sequence of the 36-kDa G-protein β subunit is highly conserved among vertebrates. This is reflected in cross-reactivity of β subunits from amphibia, birds, and other mammals with antibodies raised against the bovine protein (9), and in direct sequence comparison of the bovine and human proteins (10, 12). Nonetheless, the β subunit is highly immunogenic; all 24 rabbits immunized with β subunit developed significant antibody titers. Using the same immunization protocol, we have noted a lower incidence of antibody production in rabbits injected with G-protein α and/or γ subunits.

All animals immunized with β subunit developed antibodies directed against one or more epitopes on the 15-kDa tryptic fragment. Only 2 of 24 developed antibodies directed against 26-kDa fragment epitope(s). Thus, the high immunogenicity of the β subunit is due to one or more sites on the 15-kDa fragment. All β antisera tested in another study (23) reacted exclusively with the 15-kDa fragment. Sera from several rabbits not immunized with β subunit showed reactivity against β on immunoblots. In all cases, reactivity was directed against epitope(s) on the 15-rather than the 26-kDa fragment. Although we cannot give a precise estimate of the prevalence of β subunit antibodies in sera from rabbits not immunized with β, detection of six such sera during screening of less than 36 total sera indicates that this is not a rare phenomenon.

We used a cDNA clone obtained by expression vector screening to define a major epitope of the β subunit on the 15-kDa tryptic fragment. Although the fusion protein expressed by clone A is specifically reactive with β subunit antisera, it is unlikely that the protein encoded by clone A cDNA is the chick form of the 36-kDa β subunit. Since the β subunit sequence is highly conserved between species, one would expect more than the very limited homology noted between clone A cDNA and

Fig. 4. Competition between native, soluble TD-β/γ and nitrocellulose-bound denatured protein for binding to antibodies. Trypsin-cleaved and uncleaved transducin β/γ were separated on 15% polyacrylamide gels and transferred to nitrocellulose; individual strips were cut and used for immunoblots with the following sera: *1*, RV/6; *2*, AS/1, *3*, MP/6; *4*, PG/4; *5*, U-49; and *6*, antibodies affinity-purified from AS/1 by adsorption to filters containing clone A fusion protein. Dilutions were 1/100 for lanes *1–4*, 1/30,000 for lanes *5*, and 1/200 for lanes *6*. TD-β/γ was added with sera during immunoblot first antibody incubation as follows: *A*, 0; *B*, 5; *C*, 10; and *D*, 25 μg. →, the β and γ subunits and the 15- and 26-kDa tryptic fragments of the β subunit.

bovine TD-β. We have not defined the identity of the protein encoded by clone A. Immunoblots of total chick retinal proteins performed with antibodies affinity-purified by clone A-expressed protein reveal only the 36-kDa β subunit.[4]

Irrespective of the identity of the clone A-encoded protein, it is extremely likely that β subunit antibodies recognize the clone A protein on the basis of the homologous peptide in the two proteins (Table 2). This suggests that the homologous region, the amino-terminal decapeptide of β, represents a major epitope of the protein. Without exception, incubation of immune sera with immobilized clone A protein allowed purification of β antibodies specific for the amino-terminal, 15-kDa fragment. In contrast, β antibodies from nonimmune sera, despite being directed against the 15-kDa fragment, did not bind to clone A protein. This implies that 15-kDa antibodies in nonimmune sera are directed at a site distinct from the amino-terminal decapeptide of β.

The effect of a synthetic decapeptide corresponding to the amino terminus of β on antibody binding provided further evidence that this decapeptide contains a major antigenic site of the β subunit. The β peptide specifically blocked binding of antibodies affinity-purified on clone A-expressed protein and of antibodies within several immune sera to the β subunit and its 15-kDa tryptic fragment on immunoblots. β- and 15-kDa fragment-reactive antibodies in nonimmune sera (e.g., PG/4, MP/6) were not blocked by the β peptide.

Experiments involving competition for binding to β antibodies between nitrocellulose-bound, denatured proteins and soluble, native β/γ subunits provided further evidence for differences between 15-kDa antibodies from immunized versus nonimmunized animals. In immunoblots, antibodies react with proteins that have been denatured by SDS and have had disulfides reduced with sulfhydryl reagents. Epitopes exposed by such treatment may not be exposed in the native protein. Thus, competition for antibody binding between denatured and native protein can provide information on the topography of epitopes in the native protein. Native TD-β/γ competes effectively with nitrocellulose-bound protein for binding to 15-kDa fragment-reactive antibodies affinity-purified with the clone A protein. This suggests that the epitope expressed by the clone A protein, presumptively within the amino terminal decapeptide of β, is exposed in the native protein. Native TD-β/γ does not block binding of 15-kDa fragment-reactive antibodies in sera from nonimmune animals. This not only distinguishes the two sets of 15-kDa antibodies but also implies that the epitope on the 15-kDa fragment recognized by antibodies from nonimmunized rabbits is buried in the native β subunit. We can only speculate that such antibodies arise in response to a protein (or proteins) other than the β subunit, but with an epitope homologous to a region of the denatured 15-kDa fragment. In certain antisera, notably RV/6, heterogeneity of 15-kDa antibodies is evident. Although a subset of these antibodies binds to clone A protein, depletion of 15-kDa antibody reactivity is less marked than for other antisera. Displacement of 15-kDa antibodies by native β is also less evident for RV/6.

The 26-kDa antibodies from the two reactive sera, RV/6 and AS/1, are poorly blocked by native TD-β/γ. We cannot exclude the possibility that this reflects low affinity of 26-kDa fragment antibodies in RV/6 and AS/1, but favor the interpretation that inability of native TD-β/γ to block binding of RV/6 and AS/1 antibodies to nitrocellulose-bound 26-kDa fragment is due to the lack of exposure of the epitopes recognized by these antibodies in native TD-β/γ. Antibodies raised against a synthetic peptide representing the amino-terminal 16 amino acids of the 26-kDa fragment (5) are readily blocked by native protein. These results, suggesting that the site recognized by the peptide antiserum is exposed in the native protein, are consistent with the accessibility of this same site to tryptic proteolysis in the native, soluble protein (21), and even in the membrane-bound protein (22, 23).

Various parameters, including hydrophilicity (25), flexibility, and surface exposure (26), have been used to predict which

[4] A. Spiegel, P. Bray, and R. Vinitsky, unpublished observations.

sites on a protein are most likely to be antigenic. Fig. 5 shows a plot of relative hydropathicity of bovine TD-β (27). The amino terminus, corresponding to the epitope identified with the clone A protein, shows the greatest degree of hydrophilicity. Thus, the evidence on surface exposure of this region obtained from competitive binding studies and the effect of the synthetic β decapeptide are consistent with the idea that the amino terminus of β contains a major epitope of the protein. Interestingly, the next most hydrophilic site is near amino acids 129–130, the exposed tryptic cleavage site between the 15- and 26-kDa fragments. We have not detected antibodies directed against this site in any of our antisera. The only antisera showing 26-kDa fragment reactivity, RV/6 and AS/1, recognize a different site as shown by competitive binding studies. That antibodies against this site can be generated, and detected, is shown by the studies with the peptide antiserum U-49 (5). Only continuous epitopes (i.e., those defined by a linear sequence of amino acids) are readily measured by immunoblotting. Since this method may not detect discontinuous epitopes present in the native protein, we cannot exclude the existence of important epitopes of this type in the native β subunit.

In addition to the 36-kDa form of β subunit, some tissues, notably placenta (6), contain a 35-kDa form. Immunochemical differences have been noted between the two forms. Thus, peptide antiserum U-49 reportedly recognizes only the 36-kDa form (5). Polyclonal antisera raised against TD-β/γ (36-kDa form of β) have been reported to preferentially recognize the 36-kDa form (6–8). The present studies show that antibodies directed against the amino-terminal decapeptide of 36-kDa β react equally well with the 35-kDa form. This region is likely to be highly homologous in the two forms of the protein. A novel β subunit cDNA (15), in fact, encodes a protein with a single conservative substitution from the β 36-kDa form in this region. Since the amino-terminal decapeptide is a major epitope of the β subunit, we would predict that antisera raised against TD-β/γ should in general contain a population of antibodies against this epitope capable of recognizing both the 36- and the 35-kDa forms. This was the case for AS/1 (Fig. 3) and several other antisera raised against TD-β/γ (not shown). Antibodies against additional epitopes that are unique to the 36-kDa form may be present in such sera and account for preferential reactivity of such sera with the 36- rather than the 35-kDa subunit.

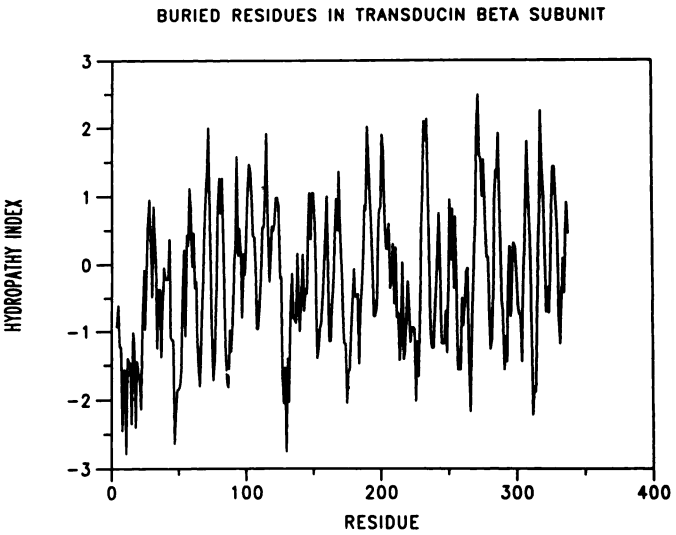

Fig. 5. Hydropathicity profile of the β subunit. The hydropathicity of the β subunit is plotted with upward deflections indicating greater hydropathicity.

The β subunit must have multiple domains for interaction with the γ chain, with α subunits, and perhaps with membrane sites and/or receptors. The efficacy of native TD-β/γ in competing for binding to antibodies directed against the amino-terminal decapeptide and against amino acids 130–145 provides evidence that these domains are not occluded in the native complex by γ subunit. Future studies, using antibodies directed against specific regions of the β subunit, should help in defining the functional domains of this protein. The present studies are significant in this respect, in that they indicate that a subset of antibodies within sera from virtually all rabbits immunized with β subunit recognizes an epitope within the amino-terminal decapeptide. As predicted by the competition studies with the synthetic β peptide, this population of antibodies can be affinity-purified on a matrix to which the β peptide has been covalently bound. This will allow preparative scale purification of region-specific antibodies for functional and other studies.

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