Monoclonal antibodies that distinguish between free and complexed heterotrimeric G protein β subunits

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Abstract Heterotrimeric G proteins were purified from bovine brain by immunoaffinity chromatography on immobilized anti G protein monoclonal antibody 3C2. Release of βγ subunits was effectuated by exposure of immobilized trimeric G proteins to $MgAlF_4$. The resultant βγ subunits were pure and biologically active. Following immunization of mice with purified βγ subunits we obtained monoclonal anti β antibodies showing broad species cross-reactivity. Characterization of the epitope recognized by one such monoclonal antibody, ARC9, indicated involvement of the extreme COOH-terminus, as assessed by its reactivity on β subunits lacking the COOH-terminal 15 residues, obtained by in vitro translation. Although we used native βγ subunits as immunogen, all monoclonal antibodies obtained failed to recognize assembled βγ subunits, and were specific for free β subunits. This property is useful in characterizing the assembly of G proteins from their subunits in living cells.

Key words: Heterotrimeric G protein β subunit; Monoclonal antibody; Immunoaffinity chromatography; Cross-reactivity; Epitope recognition

1. Introduction

Heterotrimeric G proteins (αβγ) transduce signals from a large number of cell surface receptors to a downstream effector system [1–3]. The multiplicity of receptors and the variety of G protein subunits (20 α, 5 β, 8 γ) allow tremendous combinatorial complexity. The specificity of receptor-G protein interactions determines the cellular response, which is in turn dependent on the intracellular machinery employed. Separate from this function in signal transduction is their role as regulators of intracellular trafficking events [4,5].

Upon activation of a receptor by an extracellular signal, which triggers a receptor to interact with the G protein α subunit, the α subunit releases GDP for GTP, resulting in dissociation of the heterotrimer [2]. Both subunits, α and βγ, can then independently regulate downstream effectors [6].

Numerous studies have been performed to characterize the specificity of G protein-receptor interactions. Cell transfections with G protein subunits and antisense oligonucleotide injections have been successful in identifying G protein-receptor combinations capable of assembly and signaling [7,8]. Antibodies against each individual G protein subunit have been invaluable in the studies to dissect the structure and function of these subunits [9–12]. Most of the immunological anti G protein reagents described are polyclonal reagents which have been raised against peptides. Such antibodies are highly specific for one G protein subtype, but they often fail to recognize their epitope under native conditions, as assessed by immunoprecipitation.

Here we describe the generation of monoclonal antibodies (mAb) directed against the β subunit of G proteins. These antibodies were raised against native βγ subunits, as obtained by a single-step immunoaffinity purification from bovine brain membranes, a method that is itself useful for obtaining subunits of good purity in high yield. These monoclonal antibodies show a broad pattern of species and tissue specificity and exhibit unusual properties with respect to the epitope recognized. Although mice were immunized with the native βγ complex, the monoclonal antibodies recognize β only when not associated with γ. We mapped the epitope recognized by mAb ARC9 and localized it to the COOH-terminal 15–20 amino acids of the β subunit. The crystal structure obtained for βγ complexes confirms this region as a putative site of interaction for β and γ [13,14]. This unique pattern of antibody reactivity can be used to score for assembly of heterotrimeric G proteins in living cells.

2. Materials and methods

2.1. Purification of $G_{\beta\gamma}$ subunits

2.1.1. Membrane preparation. Bovine brain membranes from freshly slaughtered cows were prepared as described [15]. Crude membrane preparations in 10 mM Tris-HCl, pH 7.5, 10% (w/v) sucrose were stored at 7.5 mg/ml, at −70°C until used. All subsequent procedures were done at 4°C. G proteins were extracted from 50 ml of brain membranes by addition of 200 ml cold Nonidet P-40/deionized Lubrol lysis buffer (0.5% Nonidet P-40 (NP-40), 0.1% Lubrol (ICN), 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM NaCl, 1 mM PMSF) for 45 min. Lysates were clarified by centrifugation at 11 000 rpm for 45 min in a Sorvall SS-34 rotor. Prior to immunoisolation, the supernatant was mixed with 1/20 volume of a 10% (v/v) solution of formalin fixed *Staphylococcus aureus* for 1 h, followed by a centrifugation at 11 000 rpm (Sorvall SS-34 rotor) for 1 h.

2.1.2. Preparation of immunoaffinity columns. The $G_{o\alpha}$-specific mAb 3C2 (IgG1 isotype) [16] was purified from hybridoma culture supernatant and covalently cross-linked to 20 ml protein A sepharose beads (RepliGen) to a final concentration of 2.4 mg/ml of gel [17]. Purified anti $G_{o\alpha}/G_{i\alpha}$ mAb 3E7 was bound to 5 ml protein A sepharose beads, yielding 1.8 mg antibody per ml resin. The 3E7 antibody retains free α subunits.

2.1.3. Immobilization of G_o and purification of $G_{\beta\gamma}$. 250 ml of lysate was applied to the 3C2 column equilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.015% Lubrol, 5 mM $MgCl_2$ at a flow rate of 1 ml/min, followed by extensive washing (~500 ml) in equilibration buffer. Specific elution of βγ was carried out with 20 column volumes of elution buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 10 mM $MgCl_2$, 50 mM NaCl, 20 mM $AlCl_3$, 10 mM NaF, 25 mM GDP, 1% (w/v) cholate (Sigma)). Prior to elution, the 3E7 column was

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Abbreviations: G proteins, guanine nucleotide binding proteins; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; Ig, immunoglobulin; ARK, adrenergic receptor kinase; BTK, Bruton tyrosine kinase; SDS, sodium dodecyl sulfate; NP-40, Nonidet P-40

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connected in series to the 3C2 column to remove residual free α subunits from the eluted βγ subunits. After application of 2 volumes of elution buffer, elution was stopped for 20–30 min to allow subunit dissociation on the matrix. Elution was continued and fractions of 9–10 ml were collected. Aliquots (50 μl) of each fraction were analyzed as described below. Columns were regenerated with glycine-HCl, pH 2.7, followed by immediate neutralization.

Purified βγ containing fractions were pooled and concentrated by pressure filtration on an Amicon concentrator (YM-10 membrane, cut-off 10 kDa), followed by dialysis against PBS/0.015% Lubrol with three changes of buffer. In some cases 0.3% (w/v) cholate and 1 mM DTT were used in the dialysis buffer, and dialysis preceded concentration. Protein concentrations were determined with BCA protein assay (Pierce).

2.2. Gel electrophoresis

SDS-gel electrophoresis was performed as described [18] on either 12.5% or 15% acrylamide gels. Discontinuous 12.5%-15% gels were prepared by pouring the bottom third of the gel with a 15% acrylamide/bisacrylamide solution, which was then overlaid with a 12.5% solution. Tricine-SDS polyacrylamide gel electrophoresis (PAGE) was done as described [19], either in the variation including 6 M urea or as 16.5% T, 3% C gels. Polypeptides were visualized by silver staining [20]. Radioactively labeled samples were visualized by fluorography using DMSO-PPO and exposure to Kodak XAR-5 films.

2.3. Production of poly- and monoclonal antibodies

The monoclonal antibodies ARC1–ARC10 described in this study were raised as follows. 8 week old female BALB/c and C57BL/6 mice were injected intraperitoneally with 50 μg native βγ in 500 μl PBS/0.015% Lubrol and Freund's adjuvant in 3 weekly intervals with three boosts. Fusions were done according standard procedures [21], hybridoma supernatant was screened in direct and indirect ELISA as described below. Hybridomas were cloned by limiting dilution.

2.3.1. Direct ELISA. βγ was taken from a 20 μg/ml stock in PBS/0.015% Lubrol and diluted 40-fold in 25 mM carbonate buffer (pH 8.9). The complex was allowed to adsorb to 96 well Immunolon 4 plates (Dynatech) overnight and unbound βγ was removed by washing with PBS/0.05% (v/v) Tween. Plates were blocked with PBS/1% (w/v) gelatin at room temperature, washed and 100 μl hybridoma supernatant was added for 2 h. Bound antibody was detected after addition of peroxidase-coupled goat anti mouse antibody (Southern Biotechnology) and tetramethylbenzidine dihydrochloride (Sigma) and H_2O_2 as substrate. Assays were evaluated by visual inspection.

2.3.2. Indirect ELISA. Protein A purified polyclonal rabbit anti βγ was adjusted in coupling buffer to 10 μg/ml, and 100 μl were added per well. Unbound antibody was removed and 100 μl βγ (0.5 μg/ml) in PBS/Tween was added for 2 h at room temperature. The subsequent procedures were identical to those described for direct ELISA.

Antibody isotyping was also done by ELISA, employing isotype specific antibodies (Southern Biotechnology) coupled to plates.

2.3.3. Polyclonal antibodies. A rabbit anti βγ polyclonal antiserum was raised by injecting a NZW rabbit with 250 μg βγ in complete Freund's adjuvant followed by two boosts at 3 weekly intervals containing 250 μg βγ in incomplete adjuvant administered subcutaneously. IgG antibodies were purified on FPLC (Pharmacia) by protein A sepharose chromatography.

A polyclonal rabbit anti γ antiserum was raised against a synthetic peptide (CEAHAKEDLLTPVPASENPF), covering a common γ_2 and γ_3 sequence. This peptide was synthesized on a multiple peptide synthesizer (Advanced Chemtech, model 350), employing conventional Fmoc chemistry. Using the heterobifunctional crosslinker SMPB (Pierce) a bovine serum albumin conjugate was generated and this complex was injected into a rabbit, as described for the βγ antibodies. The polyclonal rabbit anti β_1 antiserum U-49 was raised as described [22] (gift of Dr. S. Mumby, Dallas, TX).

2.4. In vitro transcription and in vitro translation

cDNA constructs (kindly provided by Dr. M. Simon, Pasadena, CA) encoding β_1 and β_2 were transcribed using SP6 (β_1) or T7 (β_2) RNA polymerase (Promega). The optimal amount of RNA needed for translation was determined empirically for each separate chain. In vitro translations in rabbit reticulocyte lysate (Promega) were essentially done as described [23]. Translations were performed for 2 h at 30°C and terminated by addition of Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5% NP-40).

mRNAs encoding COOH-terminal truncations of the β_1 chain were generated by restriction enzyme digestion of the full length cDNA with *Pvu*II, *Dsa*I, *Nsi*I and *Bgl*II respectively. Such linearized plasmids were used for the transcription of mRNA [24].

2.5. Immunoblotting

Polypeptides were resolved on gel systems as indicated in the figure legends and blotted either to nitrocellulose (0.45 μm pore size, Bio-Rad) or to PVDF (Immobilon-P, Millipore). The blots were incubated with the first antibodies (see figure legends), followed by horseradish peroxidase coupled goat anti mouse or horseradish peroxidase coupled goat anti rabbit immunoglobulin (Ig) (Southern Biotechnology) antibody. Bound antibody was visualized by chemoluminescence (ECL detection kit, Kirkegaard and Perry) and exposure to Kodak XAR-5 films.

2.6. Preparation of tissue homogenates

Small scale preparations of crude brain membranes from mouse, rat, and *Xenopus laevis* were performed as described [16]. Adult zebrafish were decapitated, heads were freeze-thawed three times and homogenized in 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA using a Polytron mixer. The homogenate was centrifuged at $500 \times g$ at 4°C for 10 min. Subsequently, the supernatant was centrifuged at $100\,000 \times g$ for 1 h at 4°C. The resulting pellet was resuspended in 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$. *Caenorhabditis elegans* membrane preparation was essentially as described [25], *Drosophila melanogaster* was homogenized identically to the procedure used for zebrafish. Yeast cell homogenate was provided by Drs. F. Solomon and L. Vega (Cambridge, MA). Protein concentration was determined by the method of Bradford.

2.7. GST fusion protein

The expression of the glutathione *S*-transferase (GST)-BTK (PH) fusion protein (222 amino-terminal amino acids) in *E. coli* was essentially as described [26] (construct kindly provided by Drs. F. Alt and W. Khan, Boston, MA). This construct was introduced into *E. coli* and overexpression of the fusion protein was induced with isopropyl-1-thio-β-D-galactopyranoside (Sigma). To purify the fusion protein glutathione-sepharose beads (Pharmacia) were added to the bacterial lysate in NET-N buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40) supplemented with 1 mM PMSF, 50 mM EDTA. Fusion proteins were eluted by competition with 5 mM reduced glutathione (Sigma), and subsequently screened for integrity on SDS-PAGE and silver staining. GST-β ARK 1 fusion protein was provided by Drs. R. Lefkowitz and R. Stoffel (Durham, NC).

Detection of βγ binding to GST-β ARK 1 and GST-BTK (PH). This assay was done as described [27].

2.8. Tryptic cleavage

Purified βγ complexes (4–6 μg) were treated with 5 μg of L-1-tosyl-

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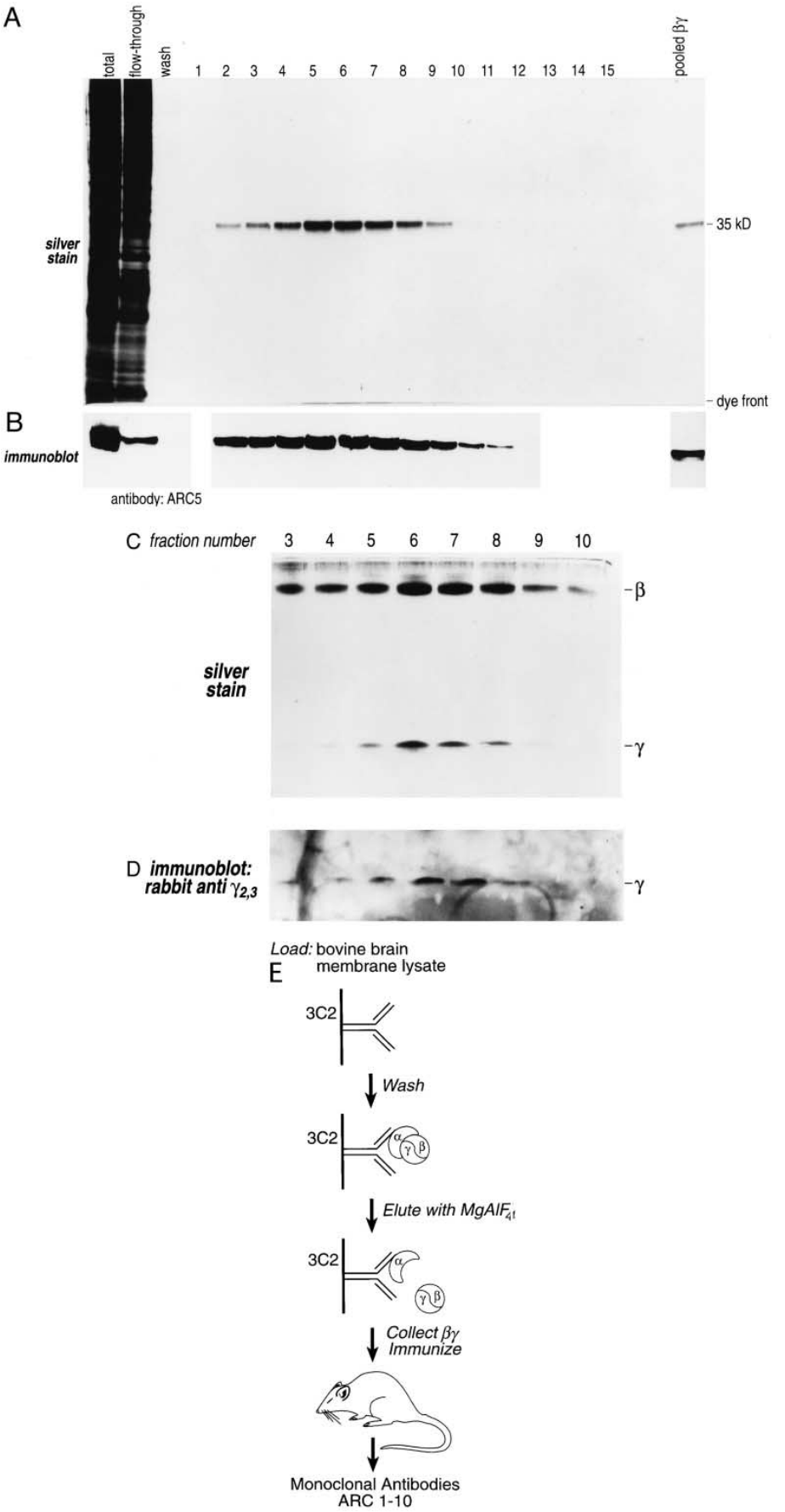
Fig. 1. Immunoaffinity purification of $G_{\beta\gamma}$. Bovine brain membrane lysates were loaded on a column coupled with mAb 3C2. After extensive washing a smaller column coupled with mAb 3E7 was connected in series to remove further α subunits upon elution. The βγ subunits were eluted with a cholate buffer containing $MgAlF_4$ and GDP, and analyzed by SDS-PAGE. Polypeptide chains were visualized by silver staining or detected by immunoblot. A: Eluted samples (1/200 of each fraction) were loaded on a 12.5% SDS-PAGE and stained with silver. Lane 1, NP-40/Lubrol extract from bovine membranes; lane 2, flow through fraction; lane 3, washing fraction; control lane with purified βγ pooled from peak fractions 2–12. B: Immunoblot of purified β subunits. 50 μl aliquots of purified βγ complex containing fractions were subjected to electrophoresis on a 12.5% SDS-PAGE and transferred to nitrocellulose. The blotting membrane was incubated with an anti G_β mAb (ARC5). Lanes designated as in A. C: 30 μl aliquots of eluate fractions subjected to 16.5% T, 6% C, 6 M urea Tris-tricine gel electrophoresis, polypeptides were stained with silver. D: Aliquots of purified βγ complexes were resolved on Tris-tricine gel electrophoresis, transferred to PVDF, and detected with an anti γ_2, γ_3 polyclonal antibody. E: Schedule for the purification of βγ subunits used for the generation of mAbs.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3C2
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Monoclonal Antibodies
ARC 1-10

amido-2-phenylchloromethyl ketone treated trypsin (Sigma) at 30°C for 30 min in 100 µl 50 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.075% (w/v) Lubrol. The reaction was stopped by adding 4×Laemmli sample buffer and boiling for 5 min.

For immunoprecipitation tryptic digestion was stopped by addition of 10×excess (w/w) trypsin inhibitor (Sigma).

2.9. Cell culture, metabolic labeling and immunoprecipitations

All cell lines were routinely grown in DME medium, supplemented with 10% FCS, L-glutamine (2 mM), penicillin (1:1000 dilution U/ml) and streptomycin (100 µg/ml). Species and tissue origin of the cell lines employed are as follows:

U-937 (histiocytic lymphoma, human), IMR-32 (neuroblastoma, human), Glc-2 (small lung cell carcinoma, human), COS-1 (kidney, SV-40 transformed, African green monkey), BC3H1 (brain tumor, smooth muscle-like, mouse), SW 1088 (astrocytoma, human), Jurkat (acute T cell leukemia, human), Neuro-2A (neuroblastoma, mouse).

Adherently growing cells were grown to subconfluence, incubated for 45 min in methionine and cysteine free DME medium, and then labeled with [^{35}S]methionine/cysteine (Express protein labeling mix, NEN) as indicated in the figure legends. For overnight labeling, 50–75 µCi were added to a 10 cm diameter Petri dish of adherently growing cells or to 10^7 cells in suspension. Cells were lysed in 1.5 ml NP-40/Lubrol (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5% NP-40, 0.1% Lubrol, 1 mM EDTA, 1 mM PMSF) lysis buffer. After 45 min at 4°C lysates were depleted of nuclei and cell debris by centrifugation for 10 min at 14000 rpm in an Eppendorf centrifuge, and then precleared twice with normal rabbit serum and *S. aureus*. Immunoprecipitations for recovery of β subunits were done with 4–5 µl ascites of ARC5 or ARC9 mAb, SDS was added as indicated in the figure legends. Immune complexes were precipitated by adsorption to *S. aureus*, followed by 4–5 washes in NET buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40).

3. Results

3.1. Immunoaffinity purification of $G_{\beta\gamma}$ subunits

To produce monoclonal antibodies against native βγ subunits a rapid purification procedure using immunoaffinity chromatography was developed. The anti $G_{o\alpha}$ specific mAbs 3C2 and MONO [16] can interact with heterotrimeric G proteins under native conditions and either of these antibodies could be used for the capture of milligram quantities of bovine brain G proteins. We prepared an affinity column coupled with mAb 3C2, resulting in a theoretical binding capacity of 0.32 µmol heterotrimeric G proteins. Elution of βγ subunits from this affinity column could be accomplished by several methods, amongst which were the exposure to GTP-γ

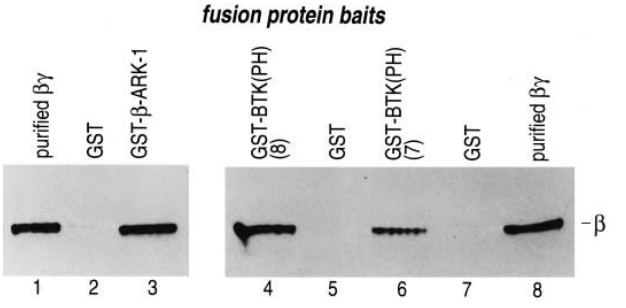

Fig. 2. Immunoaffinity purified βγ subunits interact with GST-β ARK 1 and GST-BTK(PH) fusion proteins. 5 µg immunoaffinity purified βγ in PBS/0.015% Lubrol was incubated with either an equal quantity of GST-β ARK 1 or with GST-BTK(PH) of two different preparations (7,8). Complexes were washed and loaded on a 12.5% SDS-PAGE, transferred to nitrocellulose and immunodetection of bound β subunits was accomplished using mAb ARC5. Purified βγ was used as a positive control, and GST alone served as a negative control.

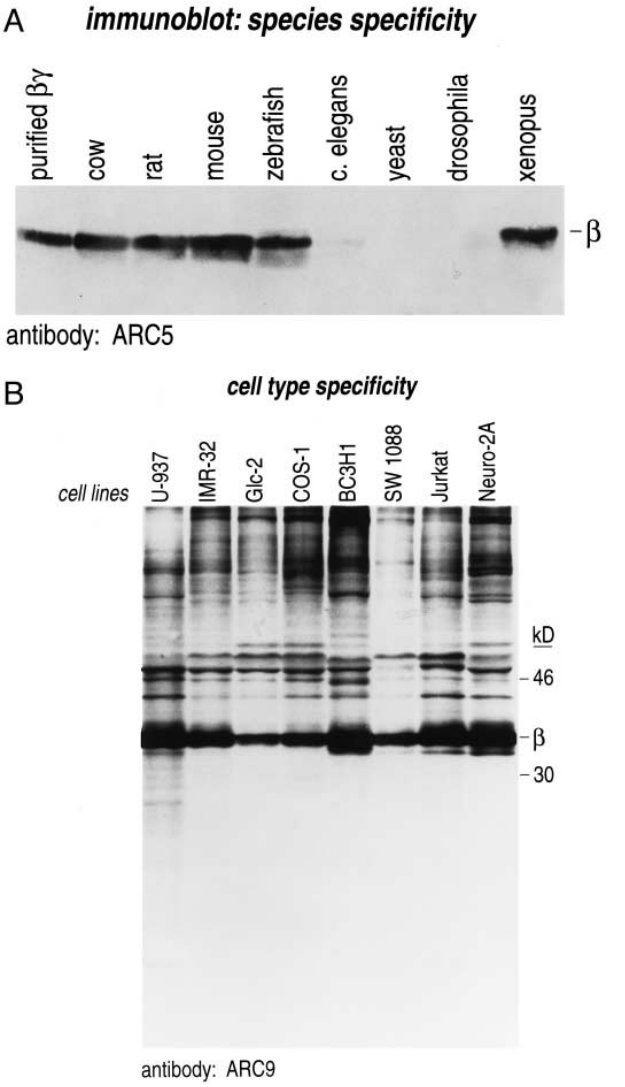

Fig. 3. Specificity of the anti βγ monoclonal antibodies. A: Membranes were prepared from tissue homogenates of the species indicated as described in Section 2. 100 µg of membrane proteins was analyzed by 12.5% SDS-PAGE, blotted to nitrocellulose and incubated with ARC5 (ascites 1:1000). 1 µg purified bovine brain G protein was used as control. The reactivity with *C. elegans* remains marginal even when tested at higher protein concentrations. All anti G_β antibodies (ARC2, 5, 9) showed a comparable pattern of reactivity. B: Cell lines were labeled overnight with ^{35}S]methionine and lysed in NP-40/Lubrol. SDS was then added at a concentration of 0.1%, and β subunits were immunoprecipitated using mAb ARC9.

S or $MgAlF_4$ in the presence of GDP. Prior to elution of the 3C2 resin, a second immunoaffinity column, prepared with the anti $G_{o\alpha}/G_{i\alpha}$ mAb 3E7 was connected in series with the 3C2 matrix. The 3E7 antibody induces dissociation of the brain G_o protein by interacting with α_o and displacing βγ, and also binds free α subunits with high affinity [12]. By connecting the 3E7 column in series, any residual α subunit or trimers present in the 3C2 eluate were removed.

The resulting fractions contained electrophoretically pure β and γ subunits, as visualized by silver staining (Fig. 1A,C) and immunoblotting using a β specific mAb (ARC5, to be described below, Fig. 1B) and a γ_2, γ_3 specific polyclonal antibody (Fig. 1D). Gγ subunits could be further separated on an alternative Tris-tricine gel system, so that the heterogeneity of

different γ subunits was revealed (A. Rehm and H.L. Ploegh, unpublished observation). We conclude that the βγ preparation obtained was heterogeneous with respect to γ composition, in agreement with published data [28,29]. The purification yielded 1.4 mg of βγ complexes starting from 375 mg of membrane protein, which represents approximately 10% of the theoretical binding capacity of the column, and a yield of approximately 70% based on the assumption that 1% of membrane protein consists of G proteins. Prior to concentration, the pooled peak fractions contain βγ in a concentration of up to 18 μg/ml (concentrations in individual peak-fractions will be considerably higher). Concentrated and dialyzed preparations of βγ thus obtained were used as immunogen for the production of both poly- and monoclonal antibodies (Fig. 1E).

3.2. *Production of anti βγ antibodies*

Immunopurified βγ subunits were examined for their biological activity by taking advantage of the known ability of βγ subunits to interact with β-adrenergic receptor kinase (β ARK1) and BTK, two kinases that possess pleckstrin homology domains (PH domains) implicated in the interaction with βγ [27,30,31]. The GST-fusion protein construct for BTK contains the N-terminal unique region (222 amino acids) and covers the PH domain, which is composed of the first 137 amino acids. The βγ subunits prepared by immunoaffinity purification were capable of interacting with both β ARK1 and BTK (Fig. 2), confirming their biological activity.

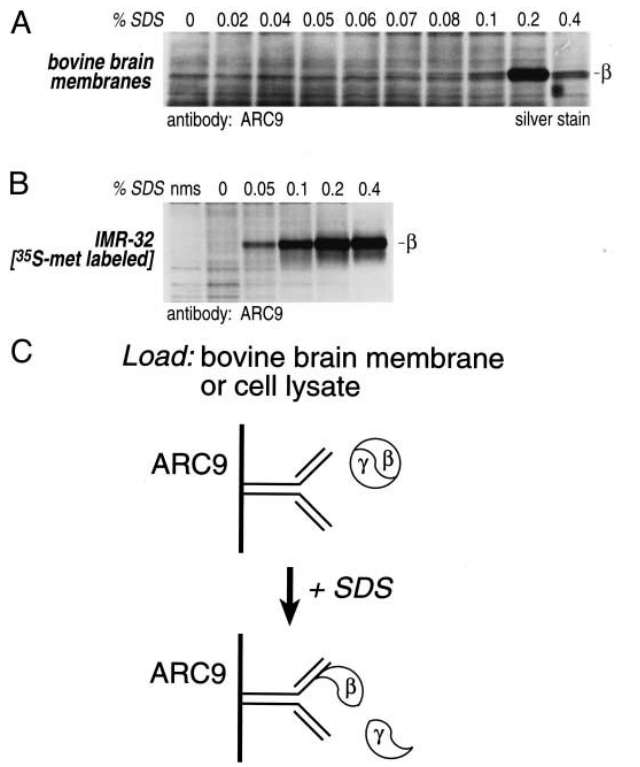

Fig. 4. The recovery of G protein β subunits in immunoprecipitations requires SDS. A: Bovine brain membranes (7.5 mg/ml) were lysed as in Fig. 1. Aliquots of lysate were then exposed to different concentrations of SDS, ranging from 0 to 0.4% (w/v) as indicated. MAb ARC9 was used to immunoprecipitate β subunits. The position of β is indicated on the right. Samples were analyzed on a 12.5% SDS-PAGE (A, B). B: IMR-32 neuroblastoma cells were metabolically labeled overnight with [^{35}S]methionine. Lysates were first prepared in NP-40/Lubrol buffer, and SDS was then added to the concentrations indicated. G_β subunits were recovered using mAb ARC9, normal mouse serum (nms) served as a negative control. C: ARC9 recognizes the β subunit after dissociation of the γ subunit induced by SDS.

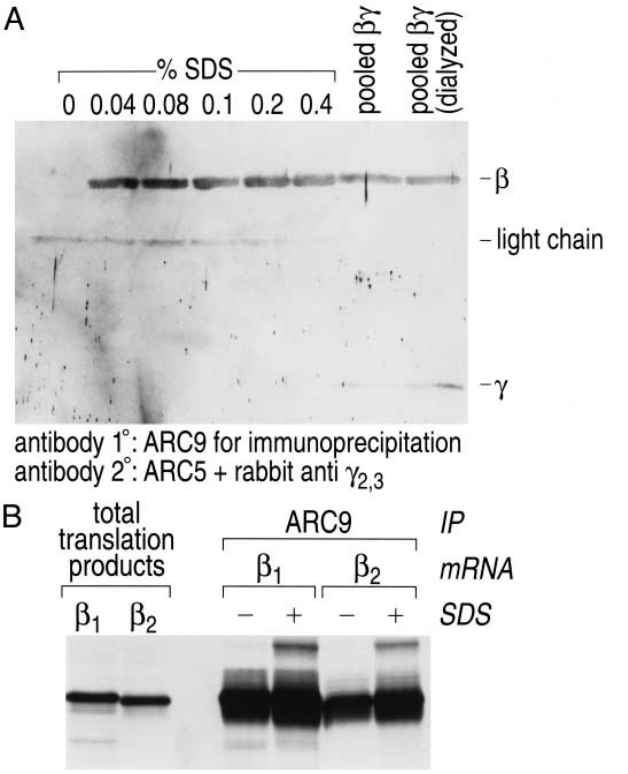

Fig. 5. Recognition of β by mAb ARC9 and the presence of γ subunits are mutually exclusive. A: Bovine brain membranes were lysed in NP-40/Lubrol. Immunoprecipitations with mAb ARC 9 were done in the presence of variable concentrations of SDS, followed by separation on a discontinuous 12.5%-15% SDS-PAGE. Polypeptides were transferred to PVDF and detected with a mixture of an anti G_β mAb (ARC5) and a polyclonal anti G γ_2, γ_3 antibody. The positions of β chain, γ chain and antibody light chain are indicated on the right. Control lanes contain 1 μg purified βγ subunits from pooled fractions (aliquots taken before and after dialysis, see Section 2). B: G protein β_1 and β_2 subunits were translated in vitro and analyzed for reactivity with mAb ARC9 in the presence (+) and absence (−) of 0.2% SDS. Total translation products and immunoprecipitates were loaded on a 12.5% SDS-PAGE.

Using βγ subunits purified by immunoaffinity chromatography, polyclonal antibodies were raised in rabbits (Fig. 1E). The purified IgG fraction of a polyclonal antiserum was then used for indirect ELISA as capture reagent for purified βγ and for immunoblotting.

Following immunization of mice, 10 stable antibody-producing hybridomas (ARC1–10) were obtained from two fusions, eight of which turned out to be of the IgM isotype. Because of the limited usefulness of the IgM antibodies for biochemical analysis much of the further immunochemical characterization was carried out with the IgG isotype reagents ARC5 (IgG1) and ARC9 (IgG3) and with the polyclonal rabbit anti βγ serum.

3.3. *Specificity of the anti βγ monoclonal antibodies*

The specificity of three mAbs was examined using immunoblots. First, the antibodies ARC2 (IgM), ARC5 (IgG1), and

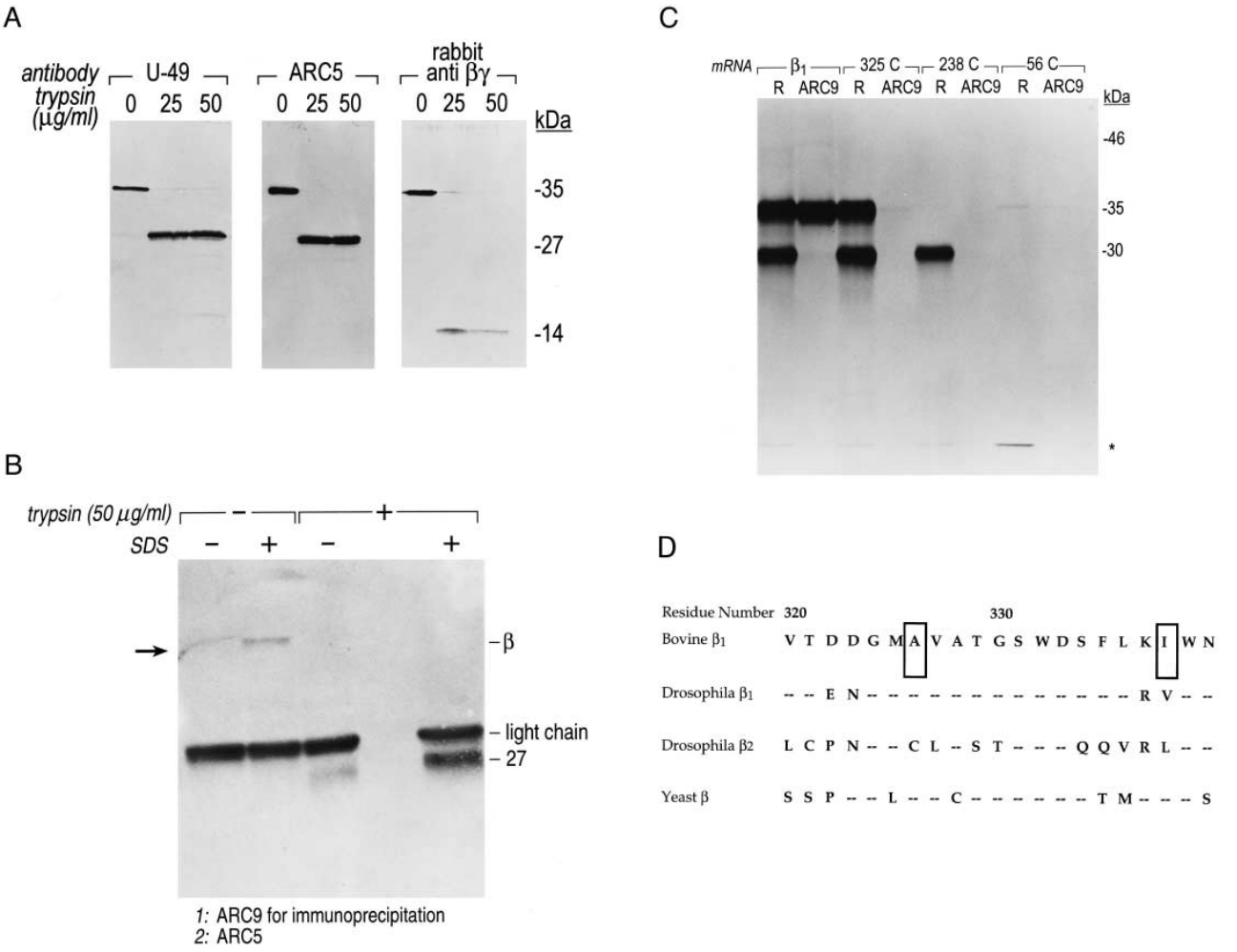

Fig. 6. Epitope recognition of ARC5, ARC9 and rabbit anti βγ serum. A: Purified βγ complexes (4–6 μg) were digested with trypsin as described in Section 2. Samples were loaded on a 12.5% SDS-PAGE, transferred to nitrocellulose and detection of β chains and tryptic fragments was accomplished using the antibodies U-49 (1:10 000), ARC5 (1:1000), and rabbit anti βγ serum (1:20 000). ARC5 and ARC9 both recognize the COOH-terminal 27 kDa fragment (data shown for ARC5). B: Tryptic cleavage of βγ (4–6 μg) complexes removes γ subunits from the 27 kDa fragment and allows epitope recognition in the absence of SDS. After trypic digestion immunoprecipitations were performed in the absence and presence of SDS using ARC9. Immunoprecipitates were resolved on 12.5% SDS-PAGE, transferred to nitrocellulose and detection of full length β chains and tryptic fragments was done with ARC5. (Note that the arrow indicates an artifact, upon inspection of duplicate blots there is no immunoprecipitation product of full length β in the absence of SDS). C: mRNAs encoding the full length β_1 chain (340 amino acids) or COOH-terminally truncated forms (C) were translated in vitro using reticulocyte lysate. Lysates were divided and subjected to immunoprecipitation with mAb ARC9 and polyclonal rabbit anti βγ serum (R). Immunoprecipitates were analyzed on 12.5% SDS-PAGE. The 56 amino acid long truncated β chain does not resolve in this gel system and runs with the dye-front as indicated (*). D: Amino acid sequence alignment of β subunits from different species. Alignment is based on the homology of four β subunits comprising the WD-40 repeat 7 (single letter code). Substitutions in *Drosophila* and yeast compared to bovine β_1 are printed in capital letters, homologies are indicated by dashes. Residues important for specific interactions with γ subunits are boxed.

ARC9 (IgG3) all recognized a polypeptide doublet at 35 and 36 kDa in crude brain membrane extracts of bovine origin (Figs. 1 and 3A, data shown for ARC5), clearly distinguishable from the polypeptide stained by anti G_α antibody 3E7 (data not shown). Second, ARC2, ARC5 and ARC9 reacted with a 35/36 kD polypeptide doublet present in highly purified G protein preparations (Fig. 3A).

When analyzing the pattern of reactivity of the ARC2, ARC5 and ARC9 on crude membrane preparations from a variety of species (shown for cow, *Xenopus*, *C. elegans*, yeast, *Drosophila*, mouse, rat, zebrafish in Fig. 3A), we observed reactivity not only with G proteins from the immunizing species, but also with β subunits from *Xenopus*, zebrafish, mouse, and rat. Marginal reactivity was observed for *C. elegans*, while no reactivity was seen for *Drosophila* and yeast. This survey was not extended beyond the species documented, nor have the conditions for preparation of the appropriate homogenates been optimized.

All mammalian cell lines examined, regardless of cell type or tissue origin, revealed strong immunoreactivity in immunoblot and immunoprecipitation (Fig. 3B).

3.4. *G protein β subunits are recovered from non-ionic detergent-lysates upon exposure to SDS*

Our initial attempts to immunoprecipitate βγ subunits from ^{35}S-labeled cell lysates were unsuccessful, and contrasted with the reactivities of total cell extracts prepared from the same cells in immunoblots (data not shown). Even though the initial immunizations were performed with βγ not previously exposed to denaturants and of proven biological activity,

these results suggested that the mAbs might preferentially recognize free β subunits, as generated following exposure to SDS. To examine whether association of βγ influences the reactivity of the ARC antibodies, we immunoprecipitated βγ subunits from bovine brain membranes, following solubilization in NP-40/ Lubrol and exposure to SDS (Fig. 4A). We observed an increase in the reactivity of ARC9 with β subunits starting at 0.06% of SDS with maximal recovery at 0.2–0.4% SDS. Similar results were obtained with mAb ARC5 (data not shown). Although β and γ subunits are considered a functional and structural complex, we failed to recover γ subunits as co-immunoprecipitates in assays using ARC5 or ARC9 in the presence of these low concentrations of SDS. Similar samples analyzed on 15% SDS-PAGE gels and on Tris-tricine gels confirmed the recovery of β, whereas γ could not be detected.

The ability of ARC5 and ARC9 to recognize β subunits upon exposure to SDS, as well as their suitability for immunoblotting procedures suggest that they recognize epitopes that require unfolding of the β subunit, or that are masked when γ subunits are present.

3.5. Detection of metabolically labeled β subunits

When cells were metabolically labeled overnight with [^{35}S]methionine and immunoprecipitates were prepared with ARC5 or ARC9 from non-ionic detergent lysates, only weak signals were obtained for the β subunits. Inclusion of $MgAlF_4$ in the lysate did not improve recovery (data not shown), a result consistent with the suggestion that mAbs ARC5 and ARC9 recognize the unfolded β subunit, or require release of the γ subunit to allow epitope recognition.

Addition of increasing concentrations of SDS to metabolically labeled IMR-32 cell lysates resulted in enhanced recovery of the β subunit (Fig. 4B). Optimal recovery was obtained by addition of SDS to a final concentration between 0.2% and 0.4% SDS, whereas the minimal requirement for SDS was 0.05%. Few if any β subunits were detected in the absence of SDS. The amount of cells used for these titration experiments was kept constant to maintain a fixed protein-detergent ratio. Under these conditions, other multimeric proteins such as MHC class I molecules still retain their quaternary structure [32], as evident from their resistance to extraction or washing in 0.1% SDS. We suggest that this relatively mild treatment with the strong detergent SDS is likely to result in the selective loss of γ subunits (Fig. 4C).

Further evidence for the conclusion that γ exerts a masking effect on the epitopes recognized by ARC5 or ARC9, is provided in Fig. 5A. Bovine brain membranes were first lysed in NP-40/Lubrol, followed by immunoprecipitation with ARC9 in the presence of different concentrations of SDS. Samples were resolved on a discontinuous 12.5%-15% SDS-PAGE gel and blotted onto PVDF. The β subunits were visualized with ARC5, whereas γ subunits were detected with rabbit anti γ_2, γ_3 serum. We observed increased recovery of β starting with 0.04% SDS and maximal recovery at 0.2–0.4% SDS. Although the quantity of β precipitated at 0.2% SDS is comparable to β in both control lanes containing purified βγ, we failed to detect γ in the immunoprecipitates by immunoblotting, yet these γ subunits are easily detected in unfractionated βγ complexes.

3.6. In vitro translation of G_β subunits

The in vitro translation system provides properly conformed polypeptides capable of exerting enzymatic functions, i.e. receptor kinases like BTK and TSK can mediate auto- and transphosphorylation reactions (A. Rehm and H.L. Ploegh, unpublished observation). Accordingly, β and γ subunits translated in vitro are capable of assembly, albeit inefficiently [33,34].

When β_1 and β_2 were translated in vitro using reticulocyte lysate, and immunoprecipitations were performed with ARC9 in buffer containing either NP-40 or supplemented with 0.2% SDS, recovery of β was equal under both conditions (Fig. 5B). Therefore, free β subunits translated in vitro can be recognized after solubilization in non-ionic detergent buffer and do not require SDS for recognition by ARC9.

3.7. Identification of epitopes recognized by ARC5 and ARC9

The epitope recognized by ARC5 and ARC9 was mapped to the COOH-terminal 15–20 amino acids, based on the ability of ARC5 and ARC9 to recognize the COOH-terminal 27 kDa tryptic fragment, but not the NH_2-terminal 14 kDa fragment, and the failure to recognize a truncated β chain, lacking the 15 COOH-terminal residues, obtained by in vitro translation (Fig. 6A,C). The polyclonal rabbit anti βγ antiserum recognized exclusively the 14 kDa NH_2-terminal fragment, consistent with the observation that a truncated 56 amino acid comprising NH_2-terminal fragment, as obtained by in vitro translation, was immunoprecipitated (Fig. 6A,C). The presence of the strongly labeled 30 kDa fragment visible in in vitro translations we attribute to premature termination of translation, because this fragment is reactive with the rabbit anti βγ serum, but not with ARC9. The size difference between the 325 amino acid truncated β and the full length β (340 amino acids) is not evident in this gel, but can be readily visualized by comparing the respective input mRNAs on gel.

The tryptic cleavage of βγ complexes leaves γ intact, which is found in association with the 14 kDa fragment. Accordingly, the 27 kDa fragment could be immunoprecipitated in the absence of SDS (Fig. 6B). At least qualitatively, we may infer that removal by trypsin of the 14 kDa NH_2-terminal fragment exposes the ARC9 epitope in the absence of SDS.

Inspection of β sequences suggest that the ARC5/ARC9 epitope involves the COOH-terminal WD40 repeat (Fig. 6D).

4. Discussion

To generate monoclonal antibodies against native βγ subunits we developed a rapid isolation procedure that provided us with highly purified βγ subunits and that may prove to be generally useful. The elution of βγ by application of $MgAlF_4$ yielded a preparation that contained pure and biologically active βγ complexes, as demonstrated by immunoblot, silver staining and binding to GST fusion proteins in vitro (Figs. 1 and 2). Our antibody based immunoaffinity purification readily tolerates minor modifications with respect to Mg^{2+}, Al^{3+}, F^- and GDP concentrations in the elution buffer. We could induce complete release of $G_{o\alpha}$ from the antibody resin in the presence of glycine-HCl (pH 2.7) for regeneration of the column. Analysis by gel electrophoresis revealed that these α subunits were completely devoid of βγ (data not shown). This is probably due to the relatively low affinity of $G_{o\alpha}$ for βγ subunits [35,36].

Although the immunogen raised to generate both poly- and monoclonal reagents was the biologically active purified βγ

preparation, we were surprised to see that none of the antibodies produced showed any significant reactivity with native βγ subunits, either derived from bovine brain membranes or from metabolically labeled cells, as assessed by immunoprecipitation. The purification protocol used to recover βγ did not employ denaturants or extremes of pH, and neither did the ELISA to screen hybridoma supernatant. This aspect will be discussed further below.

The reactivity of ARC5 and ARC9 with βγ subunits from different species reveals that Drosophila and yeast β, which do not react with the anti β mAbs, exhibit highest divergence in the 40 amino-terminal amino acids and in the carboxy-terminal WD 6/WD 7 domain compared to vertebrate βs [25].

Both regions contribute to a large number of interactions at the βγ interface [13]. Specifically, Phe-64 in the COOH-terminal portion of $G_{t\gamma}$ forms extensive hydrophobic interactions with $G_{t\beta}$. Residues involved in the COOH-terminus of β are in sheet 7, and include Ile-338 and Ala-326.

We used a combination of in vitro translation and tryptic cleavage to demonstrate that the reactivity of the mAb ARC9 mapped to the COOH-terminal 15–20 amino acids of β (Fig. 6A,C). Upon inspection of the published structure we conclude that a domain on β located here is likely to be accessible to antibodies only after dissociation of γ [13]. Moreover, appearance of trypsin-sensitive βγ complexes in the course of biosynthesis correlates with loss of antibody reactivity (A. Rehm and H.L. Ploegh, submitted). The COOH-terminal 6 residues of γ and its attached isoprenoid moiety can potentially form a stable conformation in the presence of membranes [13], and membrane attachment obviously does not interfere with interactions of β and γ polypeptide chains. Further evidence for the suggested location of the epitope is provided by the observation that after tryptic digestion of βγ complexes the resulting 27 kDa fragment can be immunoprecipitated in the absence of SDS, whereas the intact β chain associated with γ requires SDS to be recognized (Fig. 6B). The 27 kDa COOH-terminal derived tryptic fragment is devoid of γ, the latter is found in association with the NH_2-terminal 14 kDa fragment of β [37,38].

Alteration of a conformational epitope upon isoprenylation of the large hepatitis delta antigen has been observed [39]. We cannot formally discriminate between the γ polypeptide or the isoprenoid moiety attached to its COOH-terminus masking the epitope. An in vitro translation system using β cotranslated with isoprenylation resistant γ could not be used to prove modification independent β recognition, since this system does not support quantitative assembly, and levels of unassembled β subunits by far exceed those which are complexed (A. Rehm and H.L. Ploegh, unpublished observation). Transfections of cells with isoprenoid-resistant γ subunits are likewise inconclusive, since the ARC antibodies react with all β subunits tested so far and endogenous and transfected γ subunits can not be readily discriminated.

In conclusion, we have developed a rapid and simple procedure for the purification of native βγ subunits, which enabled us to generate monoclonal antibodies with unexpected properties. Two representative examples, the antibodies ARC5 and ARC9, exhibit an unprecedented broad species and tissue specificity. These antibodies are useful for immunoblotting and immunoprecipitation studies on cells from a variety of species, which make them convenient tools to study G protein function and transport in living cells. ARC5 and ARC9 recognize their epitopes on β only after dissociation of γ. This property should be useful to study assembly events of heterotrimeric G proteins in cells during the course of biogenesis, a quality of importance for understanding how specific signaling cascades are put together and maintained.

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References

[1] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[2] Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) Nature 349, 117–127.
[3] Simon, M.I., Strathmann, M.P. and Gautam, N. (1991) Science 252, 802–808.
[4] Bomsel, M. and Mostov, K. (1992) Mol. Biol. Cell 3, 1317–1328.
[5] Ferro-Novick, S. and Novick, P. (1993) Annu. Rev. Cell Biol. 9, 575–599.
[6] Neer, E.J. (1995) Cell 80, 249–257.
[7] Kleuss, C., Scheruebl, H., Hescheler, J., Schultz, G. and Wittig, B. (1992) Nature 358, 424–426.
[8] Quick, M.W., Simon, M.I., Davidson, N., Lester, H.A. and Aragay, A.M. (1994) J. Biol. Chem. 269, 30164–30172.
[9] Simonds, W.F., Butyrinski, J.E., Gautam, N., Unson, G. and Spiegel, A.M. (1991) J. Biol. Chem. 266, 5363.
[10] Muntz, K.H., Sternweis, P.C., Gilman, A.G. and Mumby, S.M. (1992) Mol. Biol. Cell 3, 49–61.
[11] Pronin, A.N. and Gautam, N. (1994) Methods Enzymol. 237.
[12] Yilla, M., Oleinick, J. and Ploegh, H.L. (1996) FEBS 387, 16–22.
[13] Sondek, J., Bohm, A., Lambright, D.G., Hamm, H.E. and Sigler, P.B. (1996) Nature 379, 369–374.
[14] Wall, M.A., Coleman, D.E., Lee, E., Iniguez-Lluhi, J.A., Posner, B.A., Gilman, A.G. and Sprang, S.R. (1995) Cell 83, 1047–1058.
[15] Sternweis, P.C. and Robishaw, J.D. (1984) J. Biol. Chem. 259, 13806–13813.
[16] Van der Voorn, L., Tol, O., Hengeveld, T.M. and Ploegh, H.L. (1993) J. Biol. Chem. 268, 5131–5138.
[17] Schneider, C., Newman, R.A., Sutherland, D.R., Asser, U. and Greaves, M.F. (1982) J. Biol. Chem. 257, 10766–10769.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Schaegger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[20] Blum, H., Beier, H. and Gross, J.H. (1987) Electrophoresis 8, 93–99.
[21] Köhler, G. and Milstein, C. (1975) Nature 256, 495–497.
[22] Mumby, S.M., Kahn, R.A., Manning, D.R. and Gilman, A.G. (1986) Proc. Natl. Acad. Sci. USA 83, 265–269.
[23] Bijlmakers, M.-J., Benaroch, P. and Ploegh, H.L. (1994) EMBO J. 13, 2699–2707.
[24] Bijlmakers, M.J.E., Benaroch, P. and Ploegh, H.L. (1994) J. Exp. Med. 180, 623–629.
[25] Van der Voorn, L., Gebbink, M., Plasterk, R.H.A. and Ploegh, H.L. (1990) J. Mol. Biol. 213, 17–26.
[26] Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–40.
[27] Koch, W.J., Inglese, J., Stone, W.C. and Lefkowitz, R.J. (1993) J. Biol. Chem. 268, 8256–8260.
[28] Morishita, R., Nakayama, H., Isobe, T., Matsuda, T., Hashimoto, Y., Okano, T., Fukada, Y., Mizuno, K., Ohno, S., Kozawa, O., Kato, K. and Asano, T. (1995) J. Biol. Chem. 270, 29469–29475.
[29] Wilcox, M.D., Schey, K.L., Dingus, J., Mehta, N.D., Tatum, B.S., Halushka, M., Finch, J.W. and Hildebrandt, J.D. (1994) J. Biol. Chem. 269, 12508–12513.
[30] Tsukada, S., Simon, M.I., Witte, O.N. and Katz, A. (1994) Proc. Natl. Acad. Sci. USA 91, 11256–11260.

[31] Pitcher, J.A., Inglese, J., Higgins, J.B., Arriza, J.L., Casey, P.J., Kim, C., Benovic, J.L., Kwatra, M.M., Caron, M.G. and Lefkowitz, R.J. (1992) Science 257, 1264–1267.
[32] Wiertz, E.J.H.J., Jones, T.R., Sun, L., Bogyo, M., Geuze, H.J. and Ploegh, H.L. (1996) Cell 84, 769–779.
[33] Schmidt, C.J., Thomas, T.C., Levine, M.A. and Neer, E.J. (1992) J. Biol. Chem. 267, 13807–13810.
[34] Neer, E.J., Denker, B.M., Thomas, T.C. and Schmidt, C.J. (1994) Methods Enzymol. 237, 226–239.
[35] Pang, I.-H., Smrcka, A.V. and Sternweis, P.C. (1994) Methods Enzymol. 237, 164–174.
[36] Asano, T., Morishita, R. and Kato, K. (1991) J. Biochem. 110, 571–574.
[37] Fung, B.K.-K. and Nash, C.R. (1983) J. Biol. Chem. 258, 10503–10510.
[38] Thomas, C.T., Sladek, T., Yi, F., Smith, T. and Neer, E.J. (1993) Biochemistry 32, 8628–8635.
[39] Hwang, S.B. and Lai, M.M.C. (1994) J. Virol. 68, 2958–2964.