Site-Specific Antibodies Directed against G Protein β and γ Subunits: Effects on α and $\beta\gamma$ Subunit Interaction

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ABSTRACT: Little is known about the specific domains of G protein β and γ subunits which interact with each other and with the α subunit. We used site-specific anti-peptide antibodies directed against β and γ subunits to investigate domains on β and γ subunits involved in α subunit interaction. Antibodies included four against the transducin (G_t) β subunit (residues 1–10 = MS, 127–136 = KT, 256–265 = RA, and 330–340 = SW) and two against the γ subunit (residues 2–12 = PV and 58–68 = PE). All antisera, when affinity-purified on peptide columns, yielded antibodies capable of recognizing the denatured cognate subunit on immunoblots, but only RA, SW, PV, and PE recognized native $\beta\gamma_t$ subunits. Affinity purification of MS and KT antisera on columns of immobilized native G_t yielded antibodies capable of recognizing native $\beta\gamma_t$ subunits. The functional effects of each antibody preparation on α_t–$\beta\gamma_t$ interaction were assessed by assaying the ability of the preparations to immunoprecipitate $\beta\gamma_t$ subunits in the presence of excess α subunits and by testing the inhibition of $\beta\gamma_t$-dependent ADP-ribosylation of α_t-subunits catalyzed by pertussis toxin. On the basis of the results, we conclude that the domains on $\beta\gamma_t$ which may be directly involved in α_t–$\beta\gamma_t$ interaction include the extreme amino terminus, residues 127–136 and 256–265 of β_t, and the carboxyl terminus of γ_t. The carboxyl-terminal region of β_t and the amino terminus of γ_t are less likely to be directly involved in α_t–$\beta\gamma_t$ interaction.

The guanine nucleotide binding proteins (G proteins)[1] that couple hormone and neurotransmitter receptors to a variety of intracellular effector enzymes and ion channels are heterotrimeric proteins composed of α, β, and γ subunits (Birnbaumer, 1990b; Bourne et al., 1990, 1991; Simon et al., 1991). The β and γ subunits cannot be separated from each other except under denaturing conditions, and they form a functional heterodimeric complex required for efficient receptor-catalyzed G protein activation (Fung & Nash, 1983; Fung, 1983). The α, β, and γ subunits of transducin, the G protein (G_t) of rod photoreceptor cells, have molecular masses of approximately 39, 36, and 8 kDa, respectively. While amino acid sequences of the three subunits are known (Simon et al., 1991), very little is known concerning the three-dimensional organization of the functional domains involved in intersubunit interaction or the changes in their structures accompanying the signaling process. The amino-terminal 2-kDa fragment of the α_t subunit is essential for the α subunit to interact with the $\beta\gamma_t$ subunit (Navon & Fung, 1987). It is not known whether this part of the molecule makes contact directly with the $\beta\gamma_t$ subunit or whether its presence is essential to keep the remainder of the molecule in a conformation capable of interacting with $\beta\gamma_t$. Recent evidence suggests that the γ subunit may be important for α_t–$\beta\gamma_t$ interaction (Fukada et al., 1990; Halpern & Moss, 1990; Vaillancourt et al., 1990).

Site-specific anti-peptide antibodies against G protein α subunits have proven to be powerful tools for investigating interactions among receptors, G proteins, and effectors (Cerione et al., 1988; Simonds et al., 1989a,b; Shenker et al., 1991). To extend this approach to the $\beta\gamma$ subunits, we prepared antibodies against synthetic peptides corresponding to four regions of β_t and two regions of γ_t. We characterized the specificity of these antibodies and then studied their effects on interactions of α_t and $\beta\gamma_t$ in assays involving immunoprecipitation and pertussis toxin-catalyzed ADP-ribosylation.

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Experimental Procedures

Materials. PVDF paper was from Applied Biosystems. Alkaline phosphatase conjugated goat anti-rabbit IgG, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, *p*-nitro blue tetrazolium chloride, and Affi-Gel-10 and -15 were from Bio-Rad. Protein A–agarose was from Pierce. GTP and GTPγS were from Boehringer-Mannheim. [^{32}P]NAD was from Amersham. Round nitrocellulose membranes (25-mm diameter) were from Schleicher & Schuell. Nonidet P-40 was from Sigma. ^{125}I-labeled recombinant protein A (850 μCi/μg) was from Du Pont–NEN. Ready Protein$^+$ scintillation cocktail was from Beckman.

Purification of α_t and $\beta\gamma_t$. HoloG_t was extracted from bovine retinal rod outer segment membranes using GTP (Kühn, 1980; Gierschik et al., 1984). The pure α_t and $\beta\gamma_t$ were prepared from holoG_t by Blue Sepharose chromatography (Shinozawa et al., 1980). The pure α_t subunit, which is eluted from the Blue Sepharose column in high salt, contains bound GDP which results from the intrinsic GTPase activity of this subunit. Thus, α_t in this paper refers to the α_tGDP complex. The pure α_t and $\beta\gamma_t$ were dialyzed against 10 mM MOPS, pH 7.5, and 25% (v/v) glycerol and stored at concentrations of ~500 μg/mL at 4 °C.

[1] Abbreviations: G protein, guanine nucleotide binding protein; α_t and $\beta\gamma_t$, α and $\beta\gamma$ subunits of G_t, the G protein in retinal rod photoreceptor cells; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; PVDF, poly(vinylidine difluoride); HTD-MS and HTD-KT, antibodies against β_t affinity-purified through a holoG_t-conjugated column; Nonidet P-40, (octylphenoxy)poly(ethoxy)ethanol; BSA, bovine serum albumin; PT, pertussis toxin; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); TPCK–trypsin, *N*-tosyl-L-phenylalanine chloromethyl ketone–trypsin.

Preparation of the Site-Specific Antibodies. Peptides which correspond to amino and carboxyl termini of β_t and γ_t and two internal regions of β_t (Table I) were synthesized, and antisera were raised against these peptides (Goldsmith et al., 1987). The antibodies were affinity-purified by application to columns of either immobilized peptides or holoG_t prepared in the presence of GTPγS to dissociate α_t and $\beta\gamma_t$. The affinity columns were prepared with Affi-Gel-10 or -15 according to the procedure outlined by Bio-Rad. After the column was washed with 100 mM HEPES, pH 7.9, 500 mM NaCl, 0.01% sodium azide, bound antibodies were eluted with a mixture of 100 mM glycine and 2% acetic acid, pH 2.5 (Harlow & Lane, 1988). A single large peak appeared after application of elution buffer, and a second smaller peak appeared after the buffer was changed to the equilibration buffer. Each peak was collected separately and neutralized immediately after elution with 1 M Tris-HCl, pH 7.5. These peaks were dialyzed separately and concentrated with 30 000-MW-cutoff Centriprep (Amicon) at 3000*g* in 100 mM HEPES, pH 7.9, 500 mM NaCl, 0.01% sodium azide at concentrations of 0.5–8.0 mg/mL. The immunoreactivity of the eluates was determined by immunoblotting on a PVDF paper. Both peaks contained protein (Schaffner et al., 1973), but only the first peak showed immunoreactivity against blotted $\beta\gamma_t$. Therefore, we used the first peak for our experiments.

Immunoprecipitation of $\beta\gamma_t$ with Affinity-Purified Antibodies. Immunoprecipitation experiments with β_t- or γ_t-specific antibodies were performed by one of two methods. In the first method, $\beta\gamma_t$ was incubated with various concentrations of antibodies (see figure legends) in 500 μL of 10 mM MOPS, pH 7.5, 200 mM NaCl, 4 mM EDTA, 0.3% Lubrol PX (w/v), 0.05% SDS for 1 h at room temperature. Then, a constant amount of α_t equimolar to $\beta\gamma_t$ was added and the mixture was incubated for an additional 1 h at room temperature, followed by addition of 125 μL of 6% (w/v) protein A–agarose and further incubation for 1 h at room temperature. Preliminary time course studies at room temperature and 4 °C showed maximal effect of antibodies by 1 h (data not shown). The antigen–antibody–protein A–agarose complex was pelleted with a microcentrifuge and washed 3 times in 1 mL of 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 4 mM EDTA, 0.5% Nonidet P-40, 0.05% SDS. Then, the complex was washed once in 1 mL of 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 4 mM EDTA. The immunoprecipitated proteins were eluted from protein A–agarose by suspending the pellet in 80 μL of electrophoresis sample buffer (Laemmli, 1970) and heating at 95 °C for 5 min. The sample was then centrifuged, and 20 μL of supernatant was analyzed by 10% SDS–PAGE in a Tris–tricine buffer system (Schagger & von Jagow, 1987; Simonds et al., 1991) and transferred onto a PVDF paper followed by detection of β_t or α_t with 0.5 μg/mL KT (Table I) or 2 μg/mL AS/7 (Goldsmith et al., 1987), respectively, as the first antibody. Alkaline phosphatase conjugated goat anti-rabbit IgG (Figures 1–4) or ^{125}I-protein A (Figures 7 and 8) was used for detection. This immunoprecipitation protocol was designed to detect inhibition of α_t–$\beta\gamma_t$ interaction by antibody binding on $\beta\gamma_t$.

In the second method, $\beta\gamma_t$ was incubated with equimolar or a 7- or 14-fold molar excess of α_t or a corresponding amount of BSA relative to $\beta\gamma_t$ in the presence or absence of 20 μM $AlCl_3$, 6 mM $MgCl_2$, 10 mM NaF for more than 2 h at 4 °C. Then, antibodies equimolar to $\beta\gamma_t$ were added, and the mixture was incubated for 1 h at room temperature. The $\beta\gamma_t$–antibody–protein A–agarose complex was pelleted, washed, eluted, and analyzed on a PVDF paper as described previously. A loss of β_t immunoreactivity in the pellet was taken as an index of α_t–$\beta\gamma_t$ binding and displacement of antibody from $\beta\gamma_t$. The second protocol allowed more sensitive detection of antibody binding to α_t–$\beta\gamma_t$ contact sites.

Table I: Definition of Synthetic Peptide Antisera Used in This Study

antiserum	peptide antigen[a]	corresponding protein sequence from cDNA (residues)
β_t		
MS	MSELDQLRQE	1–10
KT	KTREGNVRVS[b]	127–136
RA	RADQELMTYS	256–265
SW	GSWDSFLKIWN	330–340
γ_t		
PV	PVINIEDLTEK	2–12
PE	PEDKNPFKELK	58–68

[a] Single-letter amino acid code. [b] Tryptic cleavage site of the native protein is at Arg-129.

Pertussis Toxin-Catalyzed ADP-Ribosylation of Transducin. $\beta\gamma_t$ (4 pmol) was incubated with various amounts of antibodies for 1 h at 4 °C. Then, α_t (55 pmol), preactivated pertussis toxin (10 μg), and 75 μL of ADP-ribosylation buffer containing 1 mM ATP, 1 mM DTT, 1 mM EDTA, 20 μg/mL of pyruvate kinase, 3 mM potassium phosphoenolpyruvate, 10 mM thymidine, 10 mM Tris-HCl, pH 8.0, 100 μM GTP, and 2.5 μM [^{32}P]NAD (specific activity 800 Ci/mmol) were added and the mixture was incubated for 30 min at 30 °C. The reaction was stopped with 500 μL of 2% SDS and 0.1 mg/mL of BSA. After precipitation with 500 μL of 30% TCA, the sample was filtered onto nitrocellulose membrane and washed several times with 6% TCA. The radioactivity was counted with liquid scintillation after the filters were dissolved with scintillant (Ready Protein$^+$, Beckman). Proteins were determined by staining with amido-black (Schaffner & Weissman, 1973).

All of the experiments were done within a week after the preparation of G_t subunits because we noticed a significant decrease in incorporation of [^{32}P]NAD catalyzed by pertussis toxin during long-term storage of G_t. Different antibodies were compared in the same experiment to minimize interassay variation.

Results

Specificity of Affinity-Purified Antibodies Defined by Immunoblots of $\beta\gamma$ Cleaved with Trypsin. To investigate domains on $\beta\gamma_t$ for interaction with α_t, we made four synthetic peptides of β_t and two synthetic peptides of γ_t on the basis of G_t cDNA sequences (Table I). Peptides synthesized corresponded to the amino and carboxyl termini of both subunits and to two internal sequences of β_t chosen on the basis of their predicted high hydrophilicity and surface exposure. Rabbits were immunized, and antisera were purified with columns of immobilized peptides. For the examination of immunoreactivities and specificities of affinity-purified antibodies, native $\beta\gamma$ was trypsinized and subjected to SDS–PAGE and immunoblotted onto the PVDF paper (Figure 1). As the native β subunit has only one site accessible for tryptic cleavage at Arg-129, trypsin treatment gives rise to two stable fragments of 26 kDa (carboxyl terminus) and 14 kDa (amino terminus) (Fung & Nash, 1983; Fong et al., 1987). As expected, MS recognized uncleaved β_t and the 14-kDa amino-terminal fragment. SW, RA, and KT antibodies recognized uncleaved β_t and the 26-kDa carboxyl-terminal fragment. The sequence of the KT synthetic peptide is KTREGNVRVS[2] and contains

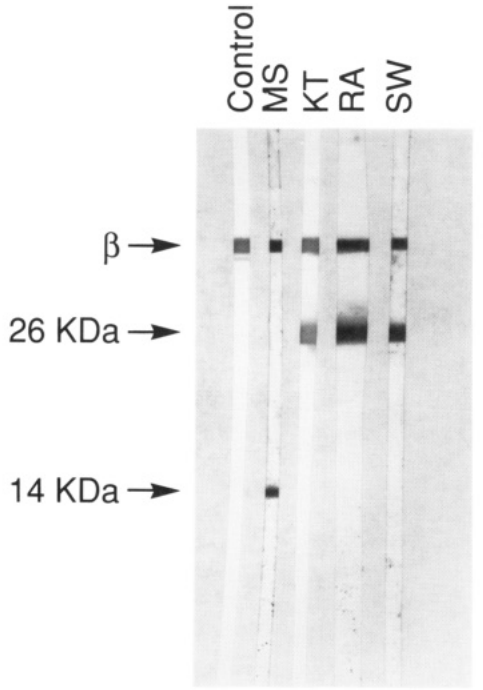

FIGURE 1: Recognition of trypsin-cleaved βγ with affinity-purified β antibodies. Purified bovine brain βγ subunits (Goldsmith et al., 1988) were incubated with or without TPCK-treated trypsin (1:30 w/w) at 37 °C for 5 min at a protein concentration of 0.24 mg/mL in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.6% Lubrol PX containing 100 mM NaCl. The reaction was stopped by the addition of three volumes of SDS-containing buffer and boiling for 3 min. A portion of each sample containing 12 μg of βγ was applied to a 15% acrylamide gel in a preparative well 5.5 cm wide. Prior to sample addition, 6 μg of undigested βγ was added as an internal reference. After SDS–PAGE, immunoblotting was performed on strips of PVDF paper as described in Experimental Procedures. Trypsinized fragments were detected with 0.5 μg/mL of MS, KT, RA, and SW antibodies (Table I). The lane marked control is uncleaved βγ detected with 0.5 μg/mL of RA antibody. The positions of uncleaved β, the carboxyl-terminal 26-kDa fragment, and the amino-terminal 14-kDa fragment are indicated with arrows.

the Arg-129 corresponding to the tryptic cleavage site of native β_t. Residues 130–136 retain antigenicity even after tryptic cleavage of three amino acids (KTR) of the amino-terminal portion of the KT peptide. Therefore, residues 130–136 must encompass at least one antigenic determinant seen by the KT antibody. PV and PE antibodies do not recognize brain γ but specifically recognize γ_t (Figure 2). Immunoreactivity of the PE antibody to denatured γ_t is stronger than that of the PV antibody.

Effect of Affinity Purification through Different Columns on Recognition of Native $\beta\gamma_t$ by Antibodies. As described above, we confirmed that each antibody can recognize denatured β_t or γ_t on immunoblots. Next, we examined if each antibody can recognize native proteins. Immunoprecipitation of $\beta\gamma_t$ in the absence of any detergent or in the presence of only 0.3% Lubrol PX produced nonspecific precipitation of $\beta\gamma_t$ and α_t with protein A–agarose only or nonspecific IgG–protein A–agarose complex (data not shown). Therefore, we included both 0.3% Lubrol PX and 0.05% SDS in the incubation buffer for immunoprecipitation (Mazzoni & Hamm, 1989). Immunoprecipitation of $\beta\gamma_t$ with PE, PV, SW, or RA antibodies affinity-purified through columns of immobilized peptides indicates that these antibodies can recognize native $\beta\gamma_t$ (Figure 3). Thus, these specific regions are presumably exposed to the surface of native $\beta\gamma_t$, and they exist in a conformation recognized by at least a subset of the polyclonal antibodies affinity-purified with the peptide column.

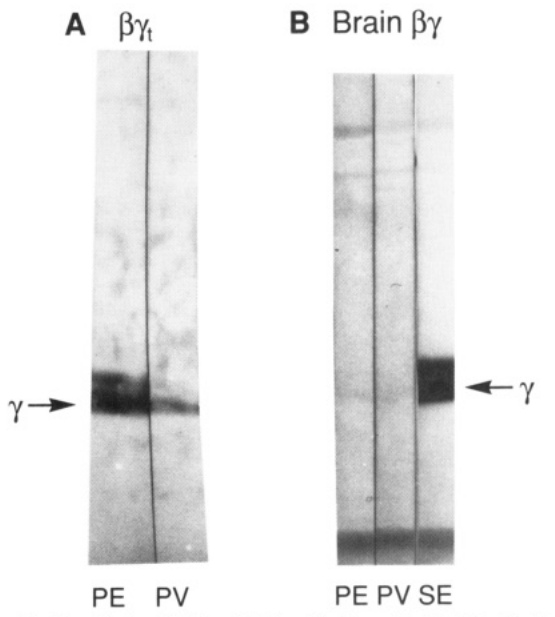

FIGURE 2: Specificity of PV and PE antibodies. Purified bovine brain βγ and $\beta\gamma_t$ were immunoblotted with 10 μg/mL of PV and 10 μg/mL of PE antibodies. Panels: A, $\beta\gamma_t$; B, bovine brain βγ. SE antibody is raised against the carboxyl terminus of brain γ (γ2) (residues 55–64, PASENPEREK) and detects at least two forms of brain γ subunit (Simonds et al., 1991). With PE and PV only faint cross-reactivity with brain γ is seen. A doublet was variably seen for γ_t with PE; the significance of this is unclear.

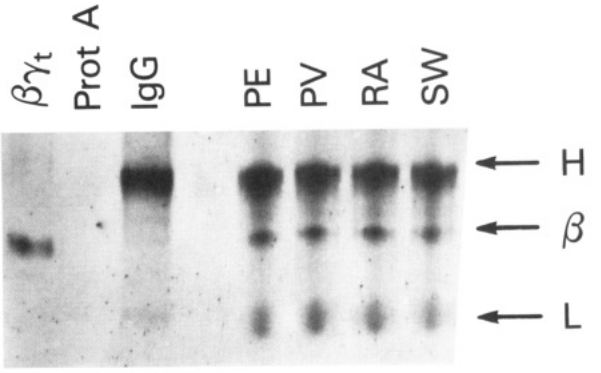

FIGURE 3: Immunoprecipitation of $\beta\gamma_t$ with RA, SW, PV, and PE antibodies. A total of 0.3 nmol of purified $\beta\gamma_t$ was incubated with 0.7 nmol of each antibody or unrelated IgG in the presence of 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 4 mM EDTA, 0.3% Lubrol PX, and 0.05% SDS for 1 h at room temperature. The antibody–$\beta\gamma_t$ complex was immunoprecipitated with 25 μL of 6% protein A–agarose, washed, and analyzed with KT antibody as in Experimental Procedures. In this figure and in subsequent figures (Figures 4, 7, and 8) of immunoprecipitation experiments, the actual data shown represent immunoblots of immunoprecipitates. Lanes: $\beta\gamma_t$, $\beta\gamma_t$ precipitated with 10% SDS and 30% TCA; prot A, $\beta\gamma_t$ precipitated without any IgG; IgG, precipitation with unrelated IgG; PE, PV, RA, and SW, precipitation with specific antibodies as labeled. H and L denote positions of IgG heavy and light chains, respectively, detected on the immunoblot of immunoprecipitate.

In contrast, KT and MS antibodies affinity-purified through columns of immobilized peptides were far less effective in immunoprecipitating native $\beta\gamma_t$ despite their strong reactivity on immunoblots with denatured $\beta\gamma_t$. There are two possibilities to explain the inability of MS and KT antibodies to immunoprecipitate β_t. One is that the two antibodies cannot recognize these regions because they are sterically hindered in the β_t–γ_t contact domains of native subunits. Another possibility is that MS and KT antibodies selected by binding to the cognate peptide cannot recognize or have only weak

[2] Single-letter amino acid code.

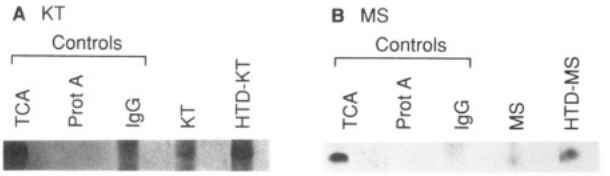

FIGURE 4: Immunoprecipitation of $\beta\gamma_t$ with KT and MS antibodies. Antisera against KT and MS peptides were purified through columns of immobilized peptide (KT and MS) or GTPγS-treated holoG_t (HTD-KT and HTD-MS). The affinity-purified antibodies were then used for immunoprecipitation, and the pellet was analyzed by SDS–PAGE and immunoblotting with KT antibody. Panels: A, KT; B, MS antibodies. Lanes: TCA, Bγ_t precipitated with SDS and TCA; prot A, precipitation without any IgG; IgG, unrelated IgG; KT or MS, antibody purified through a column of immobilized KT (panel A) or MS (panel B) peptide; HTD-KT or HTD-MS, antibody purified through a column of GTPγS-treated holoG_t. The amount of volume applied to lane 1 in panel B was $^1/_{10}$ of that applied to lane 1 in panel A.

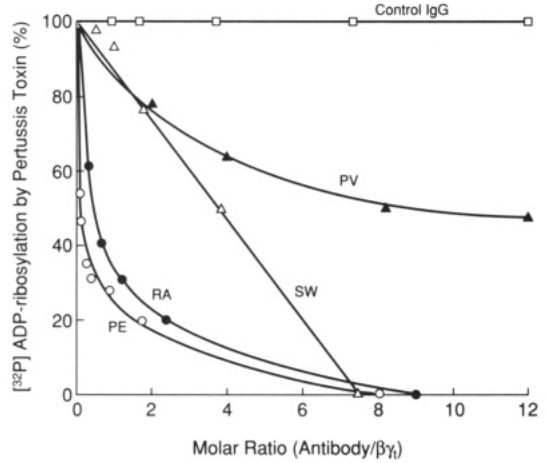

FIGURE 5: Dose-dependent inhibition of PT-catalyzed ADP-ribosylation with antibodies against $\beta\gamma_t$. A total of 4 pmol of $\beta\gamma_t$ was preincubated with various concentrations of antibodies for 1 h at room temperature. Then, 55 pmol of α_t, 10 μg/mL of preactivated pertussis toxin, and 80 μL of reaction mixture containing [^{32}P]NAD were added and the mixture was incubated at 30 °C for 30 min. The reaction was stopped, filtered, and measured as in Experimental Procedures. Antibody symbols: PE (○), RA (●), SW (△), and PV (▲) and unrelated IgG (□) as a control. The data are expressed as percentages of the control value without IgG.

affinity for native β_t. Therefore, we affinity-purified KT and MS antisera through a column containing immobilized native $\beta\gamma_t$ in an effort to isolate from the polyclonal antiserum a population of antibodies capable of recognizing the native subunit. These antibodies, termed HTD-KT and HTD-MS, could not only recognize denatured β_t but could also immunoprecipitate native $\beta\gamma_t$ (Figure 4).

Inhibition of Pertussis Toxin-Catalyzed α Subunit ADP-Ribosylation with Antibodies against $\beta\gamma_t$. PT catalyzes ADP-ribosylation on cysteine in the fourth position from the carboxyl terminus of α_t (West et al., 1985), and the presence of $\beta\gamma$ subunit is essential for this reaction (Tsai et al., 1984; Neer et al., 1984). $\beta\gamma_t$ was preincubated with an antibody, then the PT-catalyzed ADP-ribosylation reaction was started by addition of α_t, PT, and the reaction mixture as described in Experimental Procedures. SW, RA, and PE antibodies inhibited PT-catalyzed ADP-ribosylation in a concentration-dependent manner and completely inhibited at a molar ratio of $\beta\gamma_t$ to antibody less than 1:10 (Figure 5). PV antibody inhibited in a concentration-dependent manner, but the potency was much weaker than that of SW, RA, and PE, and inhibition was incomplete even in the presence of a 10-fold molar excess of PV antibody to $\beta\gamma_t$.

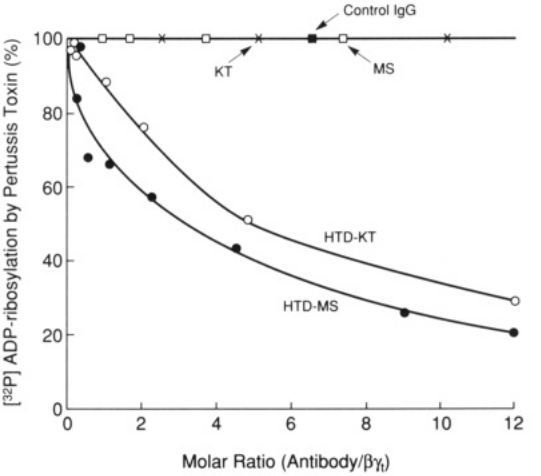

FIGURE 6: Inhibition of pertussis toxin-catalyzed ADP-ribosylation with antibodies affinity-purified through holoG_t-conjugated column. Experiments were performed as in the legend to Figure 5. HTD-KT (○) and HTD-MS (●) were affinity-purified through a holoG_t-conjugated column, and KT (×) and MS (□) were affinity-purified through a peptide-conjugated column. Data are expressed as in Figure 5.

Whereas MS and KT antibodies affinity-purified through columns of immobilized peptides could not inhibit ADP-ribosylation, HTD-MS and HTD-KT antibodies could inhibit PT-catalyzed ADP-ribosylation in a concentration-dependent fashion (Figure 6). This result is consistent with the result of the immunoprecipitation experiment. Both results suggest that the native conformation of the amino terminus (residues 1–10) and the KT site (residues 127–136) of β_t are significantly different from the denatured conformation. Apparently, only a subset of antipeptide antibodies from each antiserum is capable of recognizing the epitope in its native context. This subset of antibodies is selected for by affinity purification using native transducin.

Immunoprecipitation of $\beta\gamma_t$ in the Presence of Various Amounts of Antibodies. If binding sites of antibodies are directly or indirectly involved in α_t–$\beta\gamma_t$ interaction, the antibodies might be expected to block α_t–$\beta\gamma_t$ interaction. Each antibody was preincubated with $\beta\gamma_t$ followed by the addition of α_t and immunoprecipitation with protein A–agarose. In the presence of an equimolar ratio of antibodies relative to $\beta\gamma_t$, α_t associated with immunoprecipitated $\beta\gamma_t$ was detected with each of the six antibody preparations (data not shown). In contrast, in the presence of excess antibodies relative to $\beta\gamma_t$, five antibodies (RA, SW, HTD-KT, HTD-MS, and PE) immunoprecipitated $\beta\gamma_t$ without detectable α_t (Figure 7). However, PV antibody immunoprecipitated both $\beta\gamma_t$ and α_t (Figure 7). Therefore, RA, SW, HTD-KT, HTD-MS, and PE antibodies directly or indirectly prevent α_t–$\beta\gamma_t$ interaction, whereas PV antibody is less effective in doing so. This result is consistent with results of inhibition of PT-catalyzed ADP-ribosylation, in which RA, SW, HTD-KT, HTD-MS, and PE antibodies strongly inhibited ADP-ribosylation but PV inhibited ADP-ribosylation only weakly and incompletely (Figures 5 and 6).

Immunoprecipitation of $\beta\gamma_t$ in the Presence of Various Amounts of α_t. To investigate further the interactions between antibodies and $\beta\gamma_t$ vs α_t and $\beta\gamma_t$, we conducted the following experiment. A constant amount of $\beta\gamma_t$ was preincubated with three different concentrations of α_t ($\beta\gamma_t$:α_t = 1:1, 1:7, 1:14) followed by the addition of β_t or γ_t antibodies equimolar to $\beta\gamma_t$ and immunoprecipitation. If antibody binds to a region of $\beta\gamma_t$ involved in α_t interaction, addition of α_t should reduce the amount of $\beta\gamma_t$ immunoprecipitated by antibody. We could

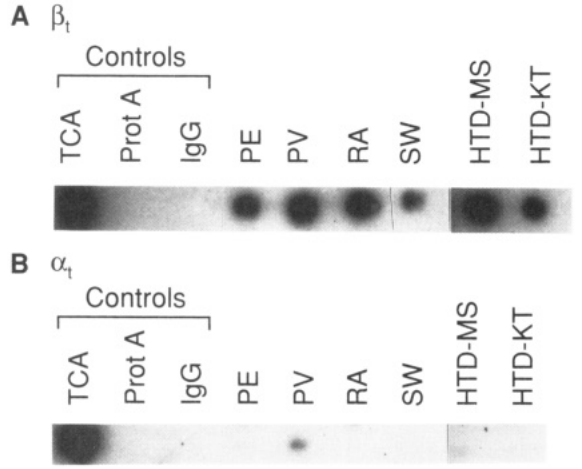

FIGURE 7: Inhibition of immunoprecipitation of α_t with $\beta\gamma_t$ by antibody binding. A total of 220 pmol of $\beta\gamma_t$ was preincubated with 2.2 nmol of antibodies in 0.5 mL as in Experimental Procedures. Then, 220 pmol of α_t was added and the mixture was incubated for 1 h at room temperature followed by the addition of 125 μL of 6% protein A–agarose. The incubation mixture was centrifuged and the pellet was washed as in Experimental Procedures. The pellet was then analyzed by SDS–PAGE and immunoblotting on a PVDF paper with (A) KT (β_t) or (B) AS (α_t) antibody. Lanes: TCA, precipitation with 10% SDS and 30% TCA; prot A, precipitation only with protein A–agarose; IgG, precipitation with unrelated IgG and protein A–agarose; PE, PV, RA, SW, HTD-MS, and HTD-KT, precipitation with specific antibodies as labeled.

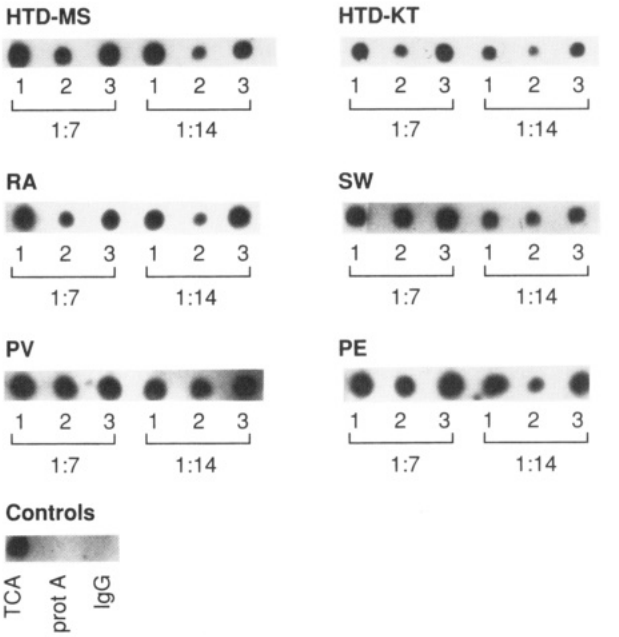

FIGURE 8: Inhibition of immunoprecipitation of $\beta\gamma_t$ with antibodies by two different amounts of α_t. A total of 220 pmol of $\beta\gamma_t$ was incubated with a 7-fold (lanes marked 1:7) or a 14-fold (lanes marked 1:14) molar excess of α_t or a corresponding amount of BSA for 2 h on ice. Then, antibodies (HTD-MS, HTD-KT, RA, SW, PV, and PE) were added and incubated for 1 h on ice followed by immunoprecipitation and immunoblotting with KT antibody. The lanes labeled 1 in all panels represent added BSA; lanes 2 represent added α_t to $\beta\gamma_t$; lanes 3 represent added fluoride-activated α_t. Controls: lane TCA, $\beta\gamma_t$ precipitated with 10% SDS and 30% TCA; prot A, precipitation without any IgG; IgG, immunoprecipitation with unrelated IgG.

not find any reduction in the amount of immunoprecipitated $\beta\gamma_t$ in the presence of an equal molar ratio of α_t to $\beta\gamma_t$ (data not shown). In contrast, in the presence of a 7- and 14-fold molar excess of α_t to $\beta\gamma_t$, the quantity of immunoprecipitated $\beta\gamma_t$ markedly decreased when we used PE, RA, HTD-MS, and HTD-KT antibodies for immunoprecipitation of $\beta\gamma_t$; the decrease was α_t concentration-dependent and concentration-specific, as BSA had no effect (Figure 8). α_t activated by incubation with $AlCl_3$, $MgCl_2$, and NaF, moreover, failed to inhibit immunoprecipitation of $\beta\gamma_t$ even when added at a 14-fold molar excess of α_t to $\beta\gamma_t$ (Figure 8). This result would be predicted from a model in which fluoride activation causes dissociation of α_t from $\beta\gamma_t$ (Sternweis & Gilman, 1982). Inhibition by α_t of $\beta\gamma_t$ immunoprecipitation with SW antibody was less clear cut, and it did not occur at all with PV antibodies.

Discussion

The association–dissociation equilibrium of α and $\beta\gamma$ subunits has been fundamental to proposed mechanisms of action of the G proteins (Birnbaumer et al., 1990a). In solution, activation of G proteins generates dissociated α and $\beta\gamma$ subunits. The domains of G protein α subunits which may interact with receptors, effectors, and $\beta\gamma$ subunits have been characterized in some detail (Birnbaumer et al., 1990a). Several different methods have been used to investigate interaction domains of G protein subunits. These include cross-linking (Hingorani et al., 1988), chemical modifications (Ho et al., 1989; Vaillancourt et al., 1990), proteolysis (Winslow et al., 1986), the use of monoclonal antibodies (Mazzoni & Hamm, 1989), and site-directed mutagenesis (Johnson & Dhanasekaran, 1989; Osawa et al., 1990). Most of these studies focused on the α subunit of G proteins. However, little is known regarding domains of $\beta\gamma$ subunits involved in α subunit interaction.

Recently, a diversity of $\beta\gamma$ subunits has been reported and the possibility of functional differences among $\beta\gamma$ subunits has been suggested (Simon et al., 1991). There is debate regarding the notion of whether or not $\beta\gamma$ subunits are common to all heterogeneous α subunits or whether each α subunit associates only with specific $\beta\gamma$ subunits (Marbach et al., 1990). Investigation of the domains of $\beta\gamma$ involved in α–$\beta\gamma$ interaction could provide further understanding about the selectivity and functional importance of $\beta\gamma$ subunits. A monoclonal antibody against γ_t was shown to block α–$\beta\gamma$ subunit interaction, but the epitope for this monoclonal antibody was not defined (Halpern & Moss, 1990). Thus, the domain recognized by this reagent is unclear. A polyclonal antibody against β_t was shown to block partially the regulation of adenylate cyclase by nonhydrolyzable analogues of GTP and to impair the nucleotide exchange occurring on brain membranes (Corrèze et al., 1987). The site of binding for this reagent of β_t was not defined. Peptide antibodies offer an advantage in functional studies in that their epitopes are relatively well-defined (particularly for short peptide immunogens). For this reason, we chose to study the effect of a panel of peptide antibodies raised against $\beta\gamma_t$ on α_t–$\beta\gamma_t$ interaction.

We first defined the specificity and reactivity of each antibody against the denatured and native subunits. For two of the antisera (MS and KT), the antibodies affinity-purified on a peptide column failed to recognize the native subunits but affinity purification on a column of immobilized native protein yielded antibodies reactive with the native subunits. This suggests that the population of crude antipeptide antibodies within an antiserum contains a subpopulation capable of recognizing the native β_t structure; this subpopulation may be lost during affinity purification of the antiserum on immobilized peptide columns. Evidence suggests that synthetic peptides shorter than 30 amino acid residues do not adopt a stable conformation in solution (Scheraga, 1981; Wright et al., 1988) and that they exist in a large number of different

and transient conformational states that are in equilibrium (Jackson et al., 1985). Only a small fraction of certain synthetic peptides conjugated to solid support might share the conformations of the corresponding sequence in native G protein subunits. The amino terminus of β_t (MS peptide) might be such a peptide. Recently, Lupas et al. have suggested that residues 1–31 of β_t comprise a domain with a unique, coiled-coil structure (Lupas et al., 1991). This conformation may be dramatically different from that of the corresponding residues in denatured β_t and the corresponding synthetic peptide.

HTD-MS, HTD-KT, RA, SW, and PE antibodies at an excess molar ratio to $\beta\gamma_t$ significantly inhibited PT-catalyzed ADP-ribosylation. In contrast, PV antibody was only a weak inhibitor of PT-catalyzed ADP-ribosylation. The α subunits of pertussis toxin-sensitive G proteins are ADP-ribosylated by the toxin only if they exist as $\alpha\beta\gamma$ trimers (Neer et al., 1984; Tsai et al., 1984). Therefore, inhibition of ADP-ribosylation by antibodies is consistent with prevention of α_t–$\beta\gamma_t$ interaction. There is no evidence for direct binding of pertussis toxin to $\beta\gamma$. Thus, inhibition of pertussis toxin-catalyzed ADP-ribosylation by antibodies is unlikely to be due to disruption of pertussis toxin–$\beta\gamma$ interaction. Nonetheless, this possibility cannot be completely excluded by the present data. Other possibilities such as interference with an interaction site for pertussis toxin or a combined effect on both α_t and pertussis toxin interaction with $\beta\gamma_t$ can also not be formally excluded. SW antibody exhibited a unique, linear inhibition profile (Figure 5). β_t shows a repeated structure that may be involved in protein–protein interaction (Fong et al., 1986). SW antibody is directed against one set of this repeated sequence. This may be relevant to the unique inhibition pattern of PT-catalyzed ADP-ribosylation with SW antibody.

Five β_t or γ_t antibodies, not including PV antibody, could interfere with coimmunoprecipitation of α_t with $\beta\gamma_t$. We conducted several experiments at various concentrations of antibodies and found that demonstration of this effect required a molar excess of antibody relative to $\beta\gamma_t$. In the presence of a limiting amount of antibody relative to $\beta\gamma_t$, α_t was always found in immunoprecipitation in uniform amounts for all antibodies tested (data not shown). In contrast, binding of antibodies, with the exception of PV, to $\beta\gamma_t$ in a molar excess of antibodies relative to $\beta\gamma_t$ completely blocked immunoprecipitation of α_t in association with $\beta\gamma_t$ (Figure 7). $\beta\gamma_t$ shows a concentration-dependent tendency to form oligomers even in the presence of detergent (Baehr et al., 1982; Sternweis, 1986; Mazzoni & Hamm, 1989). Thus, it is likely that in the presence of a substoichiometric ratio of antibodies to $\beta\gamma_t$ the interaction between the oligomers of G_t through $\beta\gamma_t$ produces incomplete blockade of α_t–$\beta\gamma_t$ interaction and results in immunoprecipitation of α_t by antibody-bound $\beta\gamma_t$ oligomers.

A molar excess of antibodies inhibited α_t–$\beta\gamma_t$ interaction as reflected in both PT-catalyzed ADP-ribosylation and immunoprecipitation assays. The presence of an excess of antibodies may be required to block immunoprecipitation of α_t because of heterogeneity in the population of affinity-purified antibodies. Peptides may be able to assume significantly different conformations in immunized rabbits and when immobilized on columns, and the immune system of rabbits can produce heterogeneous antibodies against injected peptides (Lerner, 1984; Jackson et al., 1985; Wright et al., 1988) reflecting in part a diversity of conformations in peptide–carrier protein conjugates and their degradation products. The conformation of agarose-coupled peptides may also vary depending on the amino acid side chains involved in covalent attachment. At any particular time, the proportion of synthetic peptides existing in the conformation most representative of the native proteins may be as little as 10^{-4}–10^{-5} (Jackson et al., 1985). For this reason, an inoculum of synthetic peptide may be thought of as containing many thousands of conformationally and antigenically distinct species. Therefore, to get an adequate concentration of the subset of antibodies which efficiently recognize native $\beta\gamma_t$, the use of an excess of peptide-reactive antibodies may be required.

There are at least two possible explanations for the ability of antibodies in a molar excess relative to $\beta\gamma_t$ to block completely α_t–$\beta\gamma_t$ interaction. One is direct disruption of α_t–$\beta\gamma_t$ interaction by antibody; the other is that antibody binding on $\beta\gamma_t$ indirectly induces conformational changes of $\beta\gamma_t$ reducing its affinity for α_t. Selectivity in antipeptide antibody effects has been demonstrated in experiments in which antibodies directed against the carboxyl-terminal decapeptide of G protein α subunits were shown to disrupt receptor–G protein interactions but not G protein–effector interactions (Simonds et al., 1989b). Nevertheless, there are several reports regarding indirect conformational changes after antibody binding on proteins (Colman, 1988; Friguet et al., 1989). Inhibition of subunit interactions by synthetic peptides (as opposed to peptide antibodies) could provide evidence for direct involvement of the corresponding residues. For example, the carboxyl-terminal decapeptide of α_t has been shown to inhibit receptor interaction of the corresponding subunit (Cerione et al., 1988). We initially tested the peptides used to generate antisera in the present study for ability to inhibit pertussis toxin-catalyzed ADP-ribosylation but found nonspecific inhibition that precluded further studies with this approach.

The present data allow certain tentative conclusions regarding regions of $\beta\gamma_t$ involved in α_t subunit interaction: MS, KT, RA, and PE regions may be directly involved in α_t subunit interaction, since these antibodies showed evidence of interfering with α_t–$\beta\gamma_t$ interaction in all three functional assays tested. There remains, however, the possibility that the inhibition observed with one or more of these antibodies is mediated by indirect conformational changes. The SW region may be only indirectly involved in α_t subunit interaction, since SW inhibited PT-catalyzed ADP-ribosylation, but its immunoprecipitation of $\beta\gamma_t$ was only marginally affected by molar excess of added α_t. The PV region is likely essentially uninvolved in α_t subunit interaction, since this antibody showed only weak interference in each of the functional assays. These tentative conclusions may now be tested further with complementary methods including site-directed mutagenesis of the relevant regions.

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