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Receptor and effector interactions of G_s

Functional studies with antibodies to the α_s carboxyl-terminal decapeptide

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Antibodies generated to a synthetic decapeptide, RMHLRQYELL, representing the carboxyl-terminus of G_s-α have been characterized in immunoblots and functional studies. This antibody, designated RM, reacts exclusively with a doublet of proteins of 52 and 45 kDa in immunoblots of bovine brain and wild-type S49 murine lymphoma cell membranes. No such reactivity is seen in membranes from *cyc*$^-$ S49 cells, which lack G_s. RM blocks receptor-mediated activation of G_s and adenylyl cyclase in membranes from wild-type S49 cells. RM could also immunoprecipitate adenylyl cyclase activity in detergent extracts from GTP[γ]S- or fluoride-preactivated bovine brain membranes; thus binding of α_s to effector and carboxyl-terminal antibody was mutually compatible. Such experiments provide an approach for the elucidation of functionally relevant interactions of G-proteins with receptors and effectors in the membrane.

GTP-binding protein; Adrenergic receptor, β-; Adenylyl cyclase; Immunoprecipitation

1. INTRODUCTION

The central role in many transmembrane signalling pathways is occupied by one of a family of guanine nucleotide-binding proteins (G-proteins) [1–3]. This family comprises a group of structurally related heterotrimers of $\alpha\beta\gamma$ subunit composition positioned at the cytoplasmic face of the membrane which transduce signals from activated receptors to specific effectors within the cell. The α-subunits bind guanine nucleotides, possess GTPase activity and are targets for mono-ADP-ribosylation catalyzed by certain bacterial toxins. By virtue of their relatively divergent primary sequence, the α-subunits are believed to confer functional specificity to the oligomer. G-protein activation by specific receptors is characterized by binding of GTP in exchange for GDP and the concomitant dissociation of the α-subunit from a tightly coupled $\beta\gamma$ complex; dissociation allows one or both subunits to modulate target effectors. G-proteins mediate the effects of receptor activation on enzyme function and ionic flux in many systems [1–3].

Our burgeoning knowledge of G-α protein sequence predicted from cDNA and genomic cloning studies [1–3] overshadows our understanding of the structural requirements which govern the specificity of interaction with effector and receptor. The carboxyl-terminal region of G-α was postulated to be involved in receptor coupling by Masters et al. [4] based on several lines of evidence. Homology of the α-subunit of the retinal G-protein, transducin, with arrestin, the '48 kDa' protein of retina which competes with transducin for binding to photo-rhodopsin, is confined to the carboxyl-terminal region of α-transducin [4,5]. Furthermore G-α subunits which are functionally uncoupled from receptors by pertussis toxin undergo a mono-ADP-ribosylation at a cysteine

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residue four amino acids from the carboxyl-terminus [1–3]. More recently, a point mutation was identified in the genome of the *unc* variant of S49 mouse lymphoma cells [6,7] which results in a Pro for Arg substitution six amino acids from the carboxyl-terminus of α_s, and accounts for the uncoupling of receptor from G_s characteristic of this phenotype [8].

Less well defined are the domains of G-α which interact with and discriminate among effector molecules. A variable domain bounded by two highly conserved regions in the amino-terminal half of G-α was postulated by Masters et al. [4] to be involved in effector interaction. A comparable region has been implicated in effector signalling in the GTP-binding proteins EF-Tu [9] and $p21^{ras}$ [10]. An elegant test of this model demonstrated, however, that a chimeric α-chain expressed from cDNA encoding the amino-terminal 60% of α_i and the carboxyl-terminal 40% of α_s retained the effector specificity expected of α_s, stimulating adenylyl cyclase [11].

We describe here the development and characterization of antibodies to the carboxyl-terminal decapeptide of α_s as a probe of G_s function. These antibodies potently inhibit receptor-dependent activation of G_s in membranes. Antibody binding to this carboxyl-terminal domain is compatible with α_s binding to and activation of effector, as demonstrated by the GTP[γ]S-dependent immunoprecipitation of adenylyl cyclase activity. This approach promises to provide a means of identifying functionally relevant interaction of receptors and effectors with specific G-proteins in situ.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis, conjugation and immunization*

The decapeptide RMHLRQYELL was synthesized by the solid-phase method described by Barany and Merrifield [12] using an Applied Biosystems 430A automated peptide synthesizer according to [13]. Conjugation of peptide to KLH according to Reichlin [14] and immunization of New Zealand White rabbits were performed as in [13,15].

2.2. *Affinity purification of antibodies*

Affinity purification of antibodies to the decapeptide from crude antisera on a column of peptide covalently linked to agarose (Affigel 15, BioRad) was performed as described [13,15]. Prior to use, antibodies were exchanged into 150 mM NaCl/50 mM Tris-HCl (pH 7.5) by gel filtration in Sephadex G-25 M (PD-10 prepacked columns, Pharmacia). Concentration of affinity-purified antibodies and normal rabbit immunoglobulin (Pel-Freez) was determined spectrophotometrically using the relation $A_{278}(1\%) = 13.8$ [16], assuming a molecular mass of 150000 g/mol for immunoglobulin.

2.3. *Membrane preparations*

Membranes of bovine brain were prepared according to Sternweis and Robishaw [17]. Membranes from wild-type and *cyc*$^-$ S49 murine lymphoma cells were prepared as described [18]. Membranes were stored under liquid N_2 until use. After thawing all subsequent steps were performed at 0–4°C unless otherwise indicated.

2.4. *Antibody treatment of membranes*

Membrane aliquots (3–6.5 mg/ml) were thawed and diluted with 4 vols ice-cold 150 mM NaCl/10 mM $MgCl_2$/20 mM Tris-HCl (pH 7.5) (NMT) buffer. After centrifugation at 48000 × *g* for 30 min, the supernatant was discarded and the membrane pellet resuspended in 1 vol. NMT. Normal rabbit immunoglobulin or affinity-purified RM antibodies were added in 1/20–1/10 vol. to a final concentration of 1–100 μg/ml. When the concentration dependence of RM antibody effects was being examined, serial dilutions were made into a solution of normal rabbit immunoglobulin to keep total immunoglobulin concentration constant. After incubation for 2 h at 4°C, 30-μl aliquots were assayed for adenylyl cyclase activity as described below.

2.5. *Immunoprecipitation*

Membranes (3–6.5 mg/ml) were thawed and diluted with 4 vols ice-cold NMT. After centrifugation at 48000 × *g* for 30 min, the membrane pellet was resuspended in 1 vol. NMT. Membranes were incubated for 20 min at 30°C, with or without 100 μM GTP[γ]S or 10 mM NaF as indicated in the figure legends. They were then chilled on ice, and recentrifuged as before. The supernatant fractions were discarded, and the membranes resuspended in 1 vol. NMT. An equal volume of 2% (w/v) $C_{12}E_9$ Lubrol (Sigma P-9641) in NMT was added, and after brief mixing, the sample was incubated on ice for 30–60 min. The insoluble material was removed by centrifugation, and the supernatant representing the detergent extract (2–3 mg/ml in protein) was recovered. Control or affinity-purified RM antibody was then added in 1/20 or 1/10 vol. to a final concentration of 20–100 μg/ml, and the samples incubated for 4 h at 4°C. Immunoprecipitation was effected by addition of *Staphylococcus aureus* cells [Pansorbin, Calbiochem; cells previously exchanged into 1% (w/v) $C_{12}E_9$ Lubrol in NMT by centrifugation; 1 μl of 10% (w/v) suspension added per μg IgG], incubation for 30 min at 4°C and centrifugation in a Beckman microfuge. The supernatant fraction was carefully removed, and the pellet washed once, then resuspended to the original volume with 1% (w/v) $C_{12}E_9$ Lubrol in NMT containing 3–5 mg/ml bovine serum albumin as carrier. Aliquots (30 μl) of the unfractionated detergent extract containing antibody (total) and the resuspended immunoprecipitate (pellet) were assayed for adenylyl cyclase activity, as described below, or analyzed for α_s content by SDS-PAGE and immunoblotting with RM antibodies (see below).

2.6. *Adenylyl cyclase assays*

Adenylyl cyclase activity in 30–40 μl membrane suspension and/or detergent extract, prepared as above, was determined in 100 μl final assay volume which contained in addition 0.1 mM [α-^{32}P]ATP (2–5 × 10^6 cpm), 1 mM dithiothreitol, 0.2 mg/ml

creatinine phosphokinase, 1.8 mg/ml creatine phosphate, 20 μM cAMP, 10 mM $MgCl_2$ and 30 mM Tris-HCl (pH 7.5). Samples from bovine brain included 1 mM isobutylxanthine (Fluka). Other components when present included NaF, GTP, GTP[γ]S, forskolin, and (−)-isoproterenol at final concentrations of 10 mM, 100 μM, 100 μM, 100 μM and 500 μM, respectively. Reactions were carried out at 30°C for 20 min, and terminated by addition of carrier ATP and [^{3}H]cAMP. Radiolabeled cAMP was isolated by the method of Salomon et al. [19] as described in [20].

2.7. *SDS-PAGE and immunoblotting*

These procedures were performed as in [15,21]. Immunoblots of detergent extracts containing antibody or subfractions generated by immunoprecipitation used [^{125}I]iodo-recombinant protein A (New England Nuclear, NEX 146-L; 0.2–0.5 μCi/ml) in place of secondary antibody, followed by autoradiography for 1–6 days in Kodak X-Omatic cassettes with regular intensifying screens at −70°C.

3. RESULTS

Antisera were generated in two rabbits against the synthetic decapeptide, RMHLRQYELL, corresponding to the carboxyl-terminal of α_s predicted from cDNA sequences in several species [22–27]. Attention was directed to this region because of its possible role in coupling to receptors (see above) and because of the success of previous antisera developed against the carboxyl-terminal decapeptides of transducin-α [13,28] and G_o-α [29]. Immune sera from both rabbits, designated RM/1 and RM/2, but not preimmune sera, identified a doublet of 45 and 52 kDa on immunoblots of proteins in a cholate extract of bovine brain membranes. Affinity purification of antibodies on a column of immobilized peptide-agarose generated a reagent (RM antibody) which identified only the 45 and 52 kDa doublet in bovine brain membranes and membranes from wild-type S49 murine lymphoma cells (fig.1). This reactivity could be blocked by coincubation with low concentrations of free peptide (not shown). In contrast, no reactivity was seen in membranes from *cyc*$^-$ S49 cells [30], which lack mRNA corresponding to G_s-α [31] (fig.1, lane 3). These findings demonstrate the specific reactivity of the anti-peptide antibodies with both of the major size classes of α_s identified by protein purification [32] and cDNA cloning [22–27].

Because the carboxyl-terminus of several Gα subunits is accessible in membranes to pertussis toxin [1–3], we reasoned that RM might recognize G_s in native membranes as well, with possible functional effects. S49 murine lymphoma cells which possess β-adrenergic receptors coupled to adenylyl cyclase via G_s (see [33]) were used to examine the functional consequences of RM treatment. Isoproterenol-dependent activation of G_s mediated by the β-adrenergic receptor was inhibited by RM in a concentration-dependent fashion in membranes of wild-type S49 murine lymphoma cells with an IC_{50} value of approx. 25 nM (fig.2). Receptor-independent activation of G_s by fluoride [34] was also inhibited by RM. Such inhibition was much weaker, however, as antibody concentrations up to 0.25 μM (38 μg/ml) produced only a 30% inhibition of fluoride stimulation in S49 cell membranes (fig.2). All of the inhibitory effects of RM on G_s function in S49 cells could be prevented by coin-

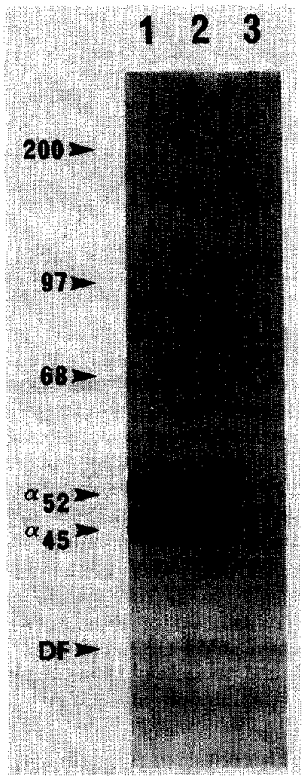

Fig.1. Immunoblotting of G_s-α with RM antibody. Membrane samples of bovine brain (lane 1, 50 μg), wild-type S49 murine lymphoma cells (lane 2, 100 μg) or *cyc*$^-$ S49 cells (lane 3, 100 μg) were separated in 10% polyacrylamide gel by SDS-PAGE, and after transfer to nitrocellulose immunoblotted with affinity-purified RM antibody at 10 μg/ml. Molecular masses (in kDa) of marker proteins and G_s-α subunits, and position of dye front (DF) are indicated to the left. Note minor cross-reacting species at ~75 kDa in lanes 2,3.

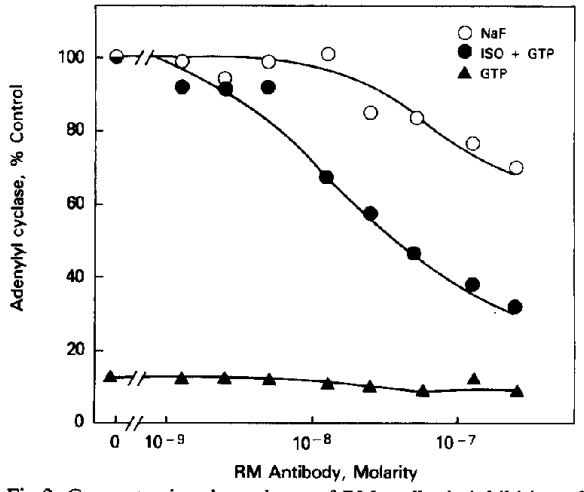

Fig.2. Concentration dependence of RM antibody inhibition of isoproterenol and fluoride activation of G_s. Wild-type S49 cell membranes were incubated with increasing amounts of RM antibody for 2 h at 4°C as shown, after which aliquots were assayed for adenylyl cyclase activity with 10 mM NaF (○), or with 100 μM GTP with (●) or without (▲) 500 μM (−)-isoproterenol. Control values (100%) (in pmol cAMP/min per mg) were 84 (○) and 60 (●, ▲). Values represent means of triplicate determinations.

cubation with the cognate peptide (not shown). In contrast to the results in S49 cell membranes, RM inhibited β-adrenergic and fluoride-activated cyclase activity with similar efficacy in turkey erythrocyte membranes (not shown).

The mutual compatibility of α_s interaction with RM and effector was studied in detergent extracts of bovine brain membranes pretreated with GTP[γ]S. Such pretreatment promotes persistent activation of adenylyl cyclase, whether measured in the membranes or in the subsequent detergent extract, presumably mediated by a stable α_s-GTP[γ]S complex. Incubation of detergent extract from preactivated membranes with RM (19–91 μg/ml) produced a variable inhibition of adenylyl cyclase activity (23–50%) relative to extract incubated with the equivalent amount of normal rabbit immunoglobulin (not shown). Immunoprecipitation of immunoglobulins with protein A (Pansorbin *S. aureus* cells) significantly depleted cyclase activity in extracts incubated with RM (35–81%, n = 9), but not in those with normal IgG. This cyclase activity could be recovered in the RM immunoprecipitates upon resuspension (fig.3). No such activity could be recovered in normal IgG immunoprecipitates or RM immunoprecipitates from extracts lacking GTP[γ]S preactivation (fig.3). Parallel immunoblotting revealed comparable immunoprecipitation of α_s by RM with or without preactivation (fig.3, inset). Preactivation of brain membranes with fluoride in place of GTP[γ]S gave comparable results (not shown).

4. DISCUSSION

An outstanding problem in our understanding of signal transduction by G-proteins is the struc-

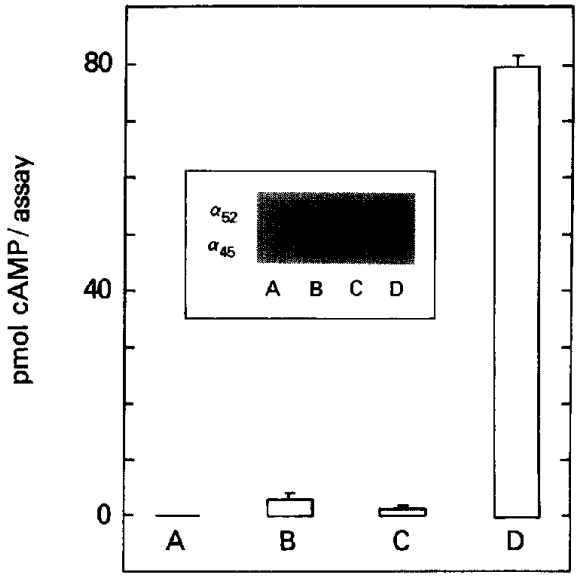

Fig.3. Immunoprecipitation of adenylyl cyclase activity and α_s by RM antibody. Detergent extracts were prepared from bovine brain membranes with or without preactivation by 100 μM GTP[γ]S for 20 min at 30°C, as described in section 2. Extracts (3 mg/ml) were incubated with normal immunoglobulin or RM antibody (50 μg/ml; 0.33 μM) for 4 h at 4°C, following which immunoprecipitation was initiated by addition of protein A *S. aureus* cells (Pansorbin). After centrifugation, the supernatant was removed and the pellet washed then resuspended in detergent buffer. Adenylyl cyclase activity was determined on 20-μl aliquots from each fraction. Assays included 100 μM GTP[γ]S, 1 mM isobutylmethylxanthine, and 1 mg/ml BSA. Activity in the pellets (immunoprecipitates) is shown. (A,B) No preactivation; (C,D) GTP[γ]S preactivation of membranes. (A,C) Normal immunoglobulin; (B,D) RM antibody. Activity recovered in pellet D represented approx. 25% of total starting activity in the extract containing RM antibody. (Inset) Parallel immunoblot analysis of α_s content in pellets generated by immunoprecipitation. (A–D) As indicated above. Recovery of both forms of α_s in pellets B,D was approx. 30% of the α_s present in starting material as estimated by densitometry. Immunoblot developed with RM antibody at 10 μg/ml, followed by [^{125}I]iodo-protein A. Position of 52 and 45 kDa forms of α_s indicated.

tural basis for the specificity required to discriminate among various receptors and effectors in the membrane. While little is known about the repertoire of γ-subunits present in G-protein heterotrimers, only two forms of β-subunit have been identified which are 90% homologous [35,36]. Thus, the more variable α-subunits must in large part provide the requisite structural specificity to ensure fidelity in signal transduction. Indeed, the resolved α-subunits are necessary and sufficient for stimulation of effectors including adenylyl cyclase [37] and cGMP phosphodiesterase [38].

We present here the characterization of a site-specific probe of G_s function, an antibody to the decapeptide comprising the carboxyl-terminus of α_s. This extreme carboxyl-terminal region of the G_α-subunit may play a role in receptor interaction as postulated by Masters et al. [4] based on evidence cited above. Furthermore, Cerione et al. [28] have recently shown that antibodies to the comparable region of transducin$_\alpha$ block the interaction of holotransducin with the photoreceptor rhodopsin in reconstituted phospholipid vesicles. Interest in this region was further heightened by the finding that the domain governing effector specificity may also reside in the carboxyl-terminal 40% of G_α [11]. Highly selective reagents for α_i1 and α_i2 [13] and for α_o [29] have been previously generated exploiting carboxyl-terminal sequence divergence for the development of antibodies to synthetic peptides.

As the C-terminal decapeptide sequence of α_s is relatively divergent from other G_αs, it was not unexpected that RM proved highly selective for the two forms of α_s. When several tissues were tested on immunoblots, no evidence of cross-reactivity with more abundant G-proteins, such as G_o, G_i1 and G_i2 in bovine brain and G_i2 and G_i3 in S49 cells, was observed. Recent experiments with a panel of soluble recombinant G_α subunits in *E. coli* lysates showed that RM uniquely immunoprecipitated α_s (unpublished).

RM potently inhibited the G_s-mediated β-adrenergic stimulation of adenylyl cyclase. This finding supports the view that the carboxyl-terminus of G_α is essential for interaction with receptor [4] and parallels observations with antibodies (AS/7) to the C-terminus of transducin-α which blocked its activation by photorhodopsin [28]. The same AS/7 antibody was found to block opioid receptor, but not serum-stimulated GTPase in NG108-15 cell membranes [39]. RM thus uncouples G_s from receptor, producing a state resembling the *unc* S49 mutant phenotype in this regard.

The inhibition seen to direct activation of G_s by fluoride was unexpected, suggesting antibody effects are more generalized than other 'uncoupling' lesions involving the extreme G_α C-terminus, such as the Arg^{389} to Pro mutation in *unc* S49 $G_s\alpha$ [6,7] or ADP-ribosylation by pertussis toxin of α_i and α_o [1–3]. In the case of the latter modifications, AlF_4^- or GTP[γ]S can still activate the G-protein involved as demonstrated by subunit dissociation, effector activation or stabilization to tryptic digestion. If conformational change in this C-terminal domain of G_α plays an essential part in the concerted or sequential exchange of guanine nucleotide and dissociation of $\beta\gamma$, then antibody binding could dampen or sterically hinder such motion, producing a 'global' inhibition. ADP-ribosylation or the *unc* mutation might preclude receptor binding without impairing the overall mobility of this region. The marked difference in sensitivity to RM of receptor-mediated and fluoride activation of G_s in S49 cell membranes is consistent with dual mechanisms of inhibition.

Immunoprecipitation of adenylyl cyclase activity by RM was dependent on preactivation of G_s with GTP[γ]S or fluoride. In contrast, RM immunoprecipitated α_s regardless of the state of activation. The potential stability of the interaction between α_s and the catalytic component of adenylyl cyclase has been noted by others who described comigration of the two during affinity purification of the catalyst [40,41]. The stability to RM immunoprecipitation of an α_s-cyclase complex may prove indicative of the strength of interaction of α_s with other putative effector molecules [42]. The ability of RM to bind to an activated α_s-effector complex suggests that the extreme C-terminal decapeptide region does not figure critically in the putative effector interaction domain of the G_α subunit [11].

The experiments described here offer a means of targeting the functional interaction of specific G-proteins with receptors and effectors using G_α carboxyl-terminal antibodies. This approach is unique in that antibodies of predefined specificity

which recognize domains in native G-proteins in the membrane and which can precipitate active G_α-effector complexes are employed to obtain information on functional interaction. Specific antibodies against synthetic peptides comprising G_α domains may allow unequivocal assignment of function to a particular G-protein, even in complex biological membranes expressing a multiplicity of receptors, G-proteins and effectors. Reconstitution and transfection experiments involving G-proteins, receptors and effectors evaluate the potential for interaction among components at an experimentally defined stoichiometry, but do not address the question of their endogenous roles. With a clear definition of the relevant functional interactions will come an understanding of the structural features governing the discrimination required of signal-transducing G-proteins.

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