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Identification of a G_s-protein coupling domain to the β-aderenoceptor using site-specific synthetic peptides

Carboxyl terminus of $G_{s\alpha}$ is involved in coupling to β-adrenoceptors

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Competition between G_s-protein and the synthetic peptide, GSA 379–394, derived from the carboxyl-terminal region of the α_s-subunit, led to complete inhibition of receptor-mediated adenylate cyclase activation in turkey erythrocyte membranes. Related peptides corresponding to the homologous carboxyl-terminal region of α_t-, α_{i1}- or α_o-subunits did not interfere with β-receptor-G_s coupling. The direct coupling between G_s and adenylate cyclase was not influenced by any of these peptides. These results emphasize the important role of the carboxyl-terminus of G-protein α-subunits for the specific recognition of their corresponding receptors and for signal transduction.

Adrenoceptor, β-; G_s-protein; Coupling domain; Synthetic peptide; Anti-peptide antibody

1. INTRODUCTION

G-proteins transfer information from cell surface receptors to effector enzymes located on the cytoplasmic face of plasma membranes. The stimulatory G-protein (G_s) mediates the transfer of the β-adrenergic signal to the target enzyme adenylate cyclase (C). A better comprehension of the mechanisms of signal transduction may be obtained by identification of the mutual contact sites of each of the components of the adenylate cyclase complex. Using the site-specific peptide competition strategy, we have successfully characterized several receptor contact domains interacting with G_s [1]. In this contribution, we describe the extension of our studies to localize the region of the G_s-protein α-subunit involved in coupling to the β-adrenoceptor.

The signal transducing G-proteins are composed of 3 distinct subunits (α, β and γ). While the α-subunit is capable of binding and hydrolyzing guanine nucleotides and activates the effector enzyme directly, the role of the $\beta\gamma$ complex is still a matter of speculation. By means of cDNA cloning techniques, a large number of GTP-binding α-chains have been identified to date [2]. Most tissues contain two major forms of α_s-subunits ranging from 44.5 to 46 kDa. The human α_s is composed of 4 splice variants which arise from alternative splicing of a single precursor mRNA [3]. Turkey erythrocyte α_s, which has not yet been cloned, consists of only one α-subunit form, shown by SDS-PAGE to have an apparent M_r of 45 kDa [4]. While the extent of sequence identity between the different mammalian α-subunits can be as low as 37%, the homology among different mammalian α_s is nearly 100% [5]. The regions of diverse G-protein α-subunits involved in the binding of nucleotides and in coupling to receptor or effector protein are currently under investigation. Besides a common GTP-binding domain, there are some regions which have been proposed to be essential for an interaction of α-subunits with other components of signal transduction chains [6]. There are several lines of evidence that the carboxyl terminus of α_s represents at least one part of the contact domains interacting with the β-receptor. The most informative evidence came from studies with the UNC-mutant of S49 mouse lymphoma cells which is characterized by a lack of receptor-mediated stimulation of adenylate cyclase. The uncoupling was demonstrated to result from a single arginine-to-proline substitution in the position −6 from the C-terminus [7,8]. Supporting evidence was obtained from studies with the rhodopsin-transducin system where C-terminal peptides of α_t and corresponding antibodies were able to attenuate the receptor-G-protein coupling efficiency [9,10]. Recent structural and functional studies on G_s-interaction to receptor and effector have been performed exclusively by means

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Abbreviations: G_s-protein, guanine nucleotide-binding protein mediating stimulation of adenylate cyclase; α_t, α_o and α_{i1}, α-subunits of corresponding G-proteins; C, adenylate cyclase; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); TFA, trifluoroacetic acid

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of antibodies raised against the carboxyl-terminal decapeptide [18]. In this case, the interpretation of the functional results was ambiguous. Although there was clear evidence for the participation of α_s C-terminus in receptor coupling, an involvement of the same region in effector coupling could not be excluded. Binding of antibodies to the target regions might cause unspecific effects like steric hindrance, or change the conformational transition states required for effective coupling. In the present study, we provide evidence that direct competition using synthetic peptides derived from the C-terminal part of different G-protein α-subunits, when compared to the antibody approach, was more selective in differentiation between α-subunit coupling to receptor and to effector protein. Our results demonstrate that the extreme C-terminal part of the G_s-protein α-subunit is involved preferentially in coupling to the β-receptor.

2. MATERIALS AND METHODS

t-Boc-amino acids were from Novabiochem, TFA was from Kali-Chemie. Other reagents for peptide synthesis were from Applied Biosystems. [α-^{32}P]ATP (600 Ci/mmol) was from New England Nuclear. MBS and EDCI were from Sigma. AuroProbe BL plus GAR immunogold reagent and IntenSE BL silver enhancement system were from Janssen (Belgium). All other chemicals and biochemicals were of the highest grade commercially available and were obtained from the same sources as in [1].

2.1. *Peptide synthesis*

Peptides were synthesized by the solid-phase Merrifield method on an Applied Biosystems 430A synthesizer. Amino acids were coupled in DMF as symmetrical anhydrides with the exception of Gln and Asn which were coupled as HOBt-esters. Deprotection, cleavage and purification were carried out as described earlier [1].

2.2. *Antipeptide antibodies*

Peptide-specific antibodies were produced by immunization with Cys-containing peptides coupled to keyhole limpet hemocyanin (KLH) [11]. 10 mg of KLH in 0.5 ml 10 mM phosphate buffer, pH 7.4, were reacted by slow addition of 2 mg *m*-maleimidobenzoyl *N*-succinimide ester (MBS) in 0.1 ml dimethylformamide for 1 h. The activated carrier was passed through a 1 × 5 cm Sephadex G-25 column in 50 mM phosphate buffer, pH 7, and added to 10 mg of the reduced peptide. After 3 h, 10 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) were added, the mixture was readjusted to pH 7 and kept for 1 h before dialysis against phosphate-buffered saline at pH 7.2. Alternatively, peptides containing no Cys residues were activated first by reacting with 1 mg EDCI per mg peptide in a minimal volume of water (pH ≈3) prior to conjugation to KLH at pH 9 [12].

New Zealand White rabbits were immunized by multisited intradermal or subcutaneous injections of a total of 300–500 μg peptide-KLH conjugate in complete Freund's adjuvant. Boost injections were applied after 3 and 5 weeks (and every 4 weeks thereafter) with incomplete Freund's adjuvant. Blood was obtained before immunization and 10 days after the boost injections. Serum was stored frozen at −20°C.

For ELISA, microtiter plates were coated with 100 ng of peptide in 50 μl 10 mM phosphate buffer per well and baked at 40°C overnight [13]. Washing and blocking followed standard procedures. Serial dilutions of peptide antisera were applied. The titer was calculated from the 50% point of the dilution curve.

2.3. *Immunoblotting*

Samples (50–100 ng G-protein or 100 μg membrane protein) were subjected to SDS-PAGE on 11% gels. Electrophoretic transfer of proteins to nitrocellulose membranes was carried out in 25 mM Tris, 192 mM glycine, pH 8.3, at constant current (0.15 A) for 15 h at 4°C. Nitrocellulose sheets were blocked with 5% dried skimmed-milk in 10 mM Tris, 150 mM NaCl, pH 7.3, and incubated for 2 h with 1:400 dilutions of antisera, followed by 1:100 dilution of AuroProbe BL plus goat anti-rabbit IgG for 2 h, washed with 0.1% bovine serum albumin in phosphate-buffered saline, pH 7.4, and further silver-enhanced [14]. To test the ability of peptides to block the antisera reactivity, the antisera (1:400 dilution) were preincubated with increasing amounts of peptides for 1 h at 0°C.

2.4. *Membrane preparations and purification of G_s/G_i-proteins*

Purified turkey erythrocyte membranes were prepared according to Puchwein et al. [15]. G_s- and G_i-proteins from turkey erythrocytes were purified according to Hanski et al. [4]. Deactivation of G_s was carried out as described by Feder et al. [16].

2.5. *Adenylate cyclase assay*

Adenylate cyclase activity in intact membranes was measured at 32°C for 20 min in a final volume of 150 μl containing 20 mM Hepes, pH 7.8, 45 mM NaCl, 3 mM $MgCl_2$, 5 mM theophylline, 10 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.5 mM ATP and 1–3 μCi [α-^{32}P]ATP per assay. [^{32}P]cAMP formed was isolated and determined according to Salomon et al. [17].

2.6. *Activation of G_s in turkey erythrocyte membranes*

The competition experiments in the presence or absence of synthetic peptides or specific antibodies were carried out exclusively with intact turkey erythrocyte membranes. The final peptide concentrations or antisera dilutions were as indicated in the legends to the figures. Hormone-induced activation of G_s was achieved with 10^{-5} M L-isoproterenol, 5×10^{-7} M GTPγS and 0.5 mM $MgCl_2$ at 37°C for 5 min. To measure the fluoride-mediated cyclase activity, the membranes were preincubated with 30 μM $AlCl_3$, 6 mM $MgCl_2$ and 10 mM NaF for 40 min at 30°C. The synthetic peptides were preincubated with membranes for 60 min at 4°C, while the incubation with antibodies was carried out overnight at 4°C with gentle shaking.

3. RESULTS AND DISCUSSION

In a previous paper [1], we reported the mapping of some interaction sites between β-adrenoceptor and G_s in turkey erythrocyte membranes using the technique of site-specific peptide competition. In the present study we have focussed on the carboxyl-terminal region of α_s and describe the ability of α_s peptide GSA 379–394 to attenuate β-adrenergic signal transduction. In addition we have tested C-terminal peptides from transducin α, α_o- and α_{i1}-subunit for possible interference in the same test system. Amino acid compositions of C-terminal peptides and their sequence alignment are shown in table 1. As shown in fig.1, the addition of the peptide

Table 1

Comparison of amino acid sequences of C-terminal parts of different G-protein α-subunits

α_s (379–394)	C R D I I Q R M H L R Q Y E L L
α_{i1} (339–354)	V T D V I I K N N L K D C G L F
$\alpha_{t(r)}$ (335–350)	V T D I I I K E N L K D C G L F
α_o (339–354)	V T D I I I A N N L R G C G L Y

The sequences were taken from Lochrie and Simon [19]

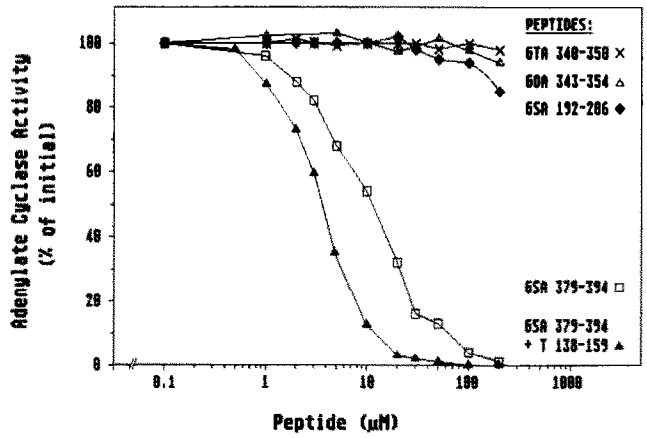

Fig.1. Effects of synthetic peptides on β-receptor-mediated cyclase activation in turkey erythrocyte membranes. Before activation, the membranes were incubated in the presence of peptides for 60 min at 4°C. Subsequently, the hormone-induced G_s-activation was carried out for 5 min at 37°C with 5×10^{-7} M GTPγS and 10^{-5} M L-isoproterenol or 10^{-5} M L-propranolol. The antagonist-induced activation, which was about 5% of total activity, has been subtracted. The peptide GTA 340–350 (×) represents the C-terminus of transducin α, GOA 343–354 (△) represents the C-terminus of α_o, GSA 192–206 (◆) and GSA 379–394 (□) are derived from α_s, and T 138–159 represents the second intracellular loop of the β_1-adrenoceptor.

GSA 379–394, corresponding to the extreme carboxyl-terminal region, to the intact turkey erythrocyte membranes caused strong concentration-dependent inhibition of hormone-induced cyclase activation with IC_{50} values of 12 μM. Complete uncoupling was observed above 100 μM. The carboxyl-terminal part of transducin α-subunit (α_t) has been shown to be involved directly in the coupling of light receptor rhodopsin to the GTP-binding protein transducin as reported by Hamm et al. [9]. With respect to the partial sequence homology and comparable functional properties of the C-terminal regions of α_s and α_t, we investigated whether transducin-C-terminal peptide GTA 340–350 causes comparable effects in β-adrenergic signal transduction. As depicted in fig.1, the peptide GTA 340–350 was not able to compete for β-receptor-G_s coupling. Furthermore, two other undecapeptides derived from the C-terminal region of pertussis toxin substrates α_{i1}- and α_o-subunits showed no inhibitory effects (fig.1). These results taken together with the data of Hamm et al. [9] emphasize the importance of the carboxyl-terminal regions of G-protein α-subunits for the specific recognition of the appropriate target receptor. As additional control, the peptide GSA 192–206, which corresponds to the middle part of α_s, did not exhibit any effects at comparable concentrations (fig.1).

To rule out the possible interference of the peptide GSA 379–394 in the interaction of G_s to adenylate cyclase, receptor-independent cyclase activation was carried out. For that purpose the membranes were first preactivated with AlF_4^-, then allowed to interact with peptides before finally measuring cyclase activation (fig.2). The peptide GSA 379–394 inhibited slightly (20% at 100 μM) the fluoride-mediated G_s activation of adenylate cyclase. These results show clearly that the interruption of signal transduction by peptide GSA 379–394 is caused mainly by inhibition of receptor-G_s coupling.

As previously reported, the β-receptor peptide T 138–159 inhibited strongly the hormone-induced cyclase activation indicating that the second intracellular loop of turkey erythrocyte β-receptor is directly involved in coupling to G_s [1]. The simultaneous addition of the peptides GSA 379–394 and T 138–159 to the membranes decreased the IC_{50} value to 3 μM indicating that the combined action of the peptides might be synergistic rather than additive (fig.1).

Using an approach similar to that described recently by Simonds et al. [18], we studied the possible effects of antibodies raised against the α_s peptides, GSA 379–394 and GSA 192–206, on the transduction of hormone signals in the adenylate cyclase system. Both antisera were characterized for their specificity in immunoblots and were shown to be highly selective for α_s (fig.3). The antibody AB-GSA 192–206 showed weak cross-reactivity with the α_i-subunit. Although the peptide sequences were taken from the known sequences of mammalian α_s-subunits, the anti-peptide antibodies recognized equally well the α_s from turkey erythrocyte membranes. This is in accordance with the fact that both mammalian and avian G_s-proteins are able to activate adenylate cyclase, as has already been shown using purified components of β-adrenergic signal chain reconstituted in phospholipid vesicles [16]. As depicted

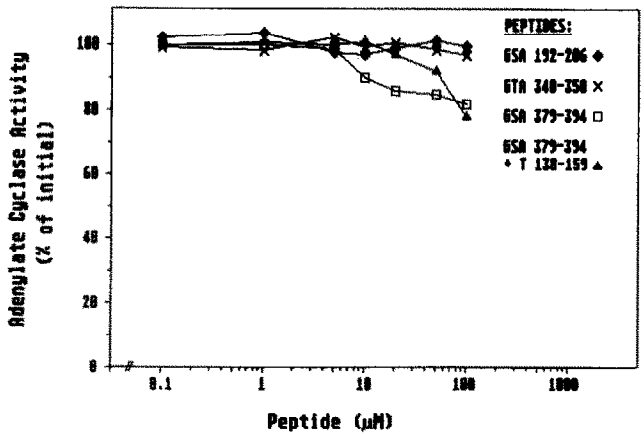

Fig.2. Effects of synthetic peptides on G_s-mediated cyclase activation in turkey erythrocyte membranes. Fluoride-activated membranes were incubated with peptides for 60 min at 4°C and the cyclase activation was carried out for 20 min at 32°C as described in section 2. The peptide GTA 340–350 (×) represents the C-terminus of transducin α, GOA 343–354 (△) represents the C-terminus of α_o, GSA 192–206 (◆) and GSA 379–394 (□) are derived from α_s, and T 138–159 represents the second intracellular loop of the β_1-adrenoceptor.

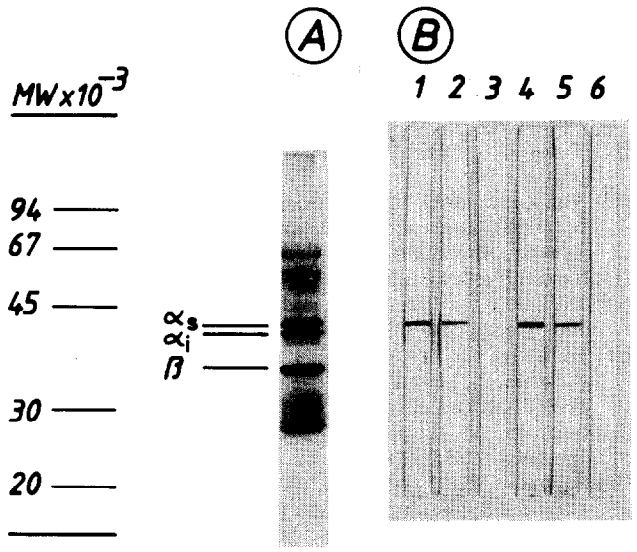

Fig.3. Analysis of specificity of anti-α_s-peptide antisera to the partially purified G_s/G_i preparation by immunoblotting. (A) SDS-PAGE and silver staining of G_s/G_i fraction used for immunoblotting. The amounts of α_s- and α_i-subunits were 50–100 ng. (B) Immunoblotting of G_s/G_i fraction with antiserum AB-GSA 379–394 (lane 1) and antiserum AB-GSA 192–206 (lane 4). The lanes 2 and 3 represent the addition of 10 nM and 1 μM of the peptide GSA 379–394 to the antiserum AB-GSA 379–394 prior to immunostaining. The lanes 5 and 6 show the addition of 10 nM and 1 μM of the peptide GSA 192–206 to the antiserum AB-GSA 192–206.

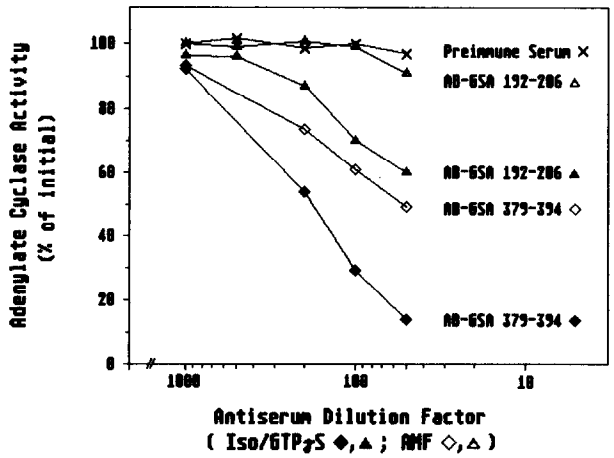

Fig.4. Inhibition of β-adrenergic signal transduction in turkey erythrocyte membranes by antibodies raised against synthetic peptides derived from α_s-subunit. Membranes were preincubated with the antiserum AB-GSA 379–394 (◇, ◆) or AB-GSA 192–206 (△, ▲) overnight at 4°C. The receptor-induced cyclase activation (◆, ▲) was carried out with 10^{-5} M L-isoproterenol and 5×10^{-7} M GTPγS while the fluoride-mediated activation (◇, △) was achieved with 10 mM NaF, 30 μM $AlCl_3$ and 6 mM $MgCl_2$ as described in section 2.

in fig.3, the preincubation of the antibodies AB-GSA 379–394 and AB-GSA 192–206 with their corresponding peptides prevented in concentration-dependent manner the specific recognition of α_s in immunoblots.

When we compared the capability of peptides or their corresponding antibodies to serve as functional probes, significant differences became apparent. In analogy to the peptide results (see fig.1), the antibody AB-GSA 379–394 did indeed strongly inhibit hormone-stimulated cyclase activation, with a half-maximal inhibition at approximately 1:200 dilution (fig.4). However, the antibody AB-GSA 192–206 still caused 40% inhibition of initial activity at 1:50 dilution, which points to a more global contribution of the recognition site to the total reactivity. The receptor-independent cyclase activation (fluoride activation, see fig.4) was also impaired to a considerable extent when the antibody AB-GSA 379–394 was used. The results from peptide competition appear much more selective, since the peptide GSA 379–394 attenuated exclusively the receptor-G_s coupling. Bearing this in mind, the partial inhibition of fluoride-stimulated cyclase activity by AB-GSA 379–394 and the corresponding effects reported by Simonds et al. [18] have to be interpreted cautiously. Our data from peptide competition make it clear that there is no direct involvement of the carboxyl-terminal part in coupling of α_s to adenylate cyclase. There is an additional advantage in studies using site-specific peptide competition. Whereas peptides could be synthesized easily with a defined number of residues, heteroclonal antibodies generally represent an unknown mixture of composite sites.

The methods used here provide the potential tools to search for other regions of G_s interaction with the β-adrenoceptor. Thus, more specific synthetic peptides and the reconstituted system with purified components [16] should provide more information on structure-function relationships. Additionally, we expect to identify more precisely the crucial amino acids of the C-terminal region of different α-subunits which seem to determine the receptor specificity for different G-proteins. The strategy of site-specific peptide competition has proved to be superior to the anti-peptide antibody technique where non-functional effects like steric hindrance, loss of lateral and conformational mobility and precipitation of components can lead to ambiguities in the interpretation of the results. Lacking these disadvantages, the synthetic peptides represent a powerful tool for the identification of functional domains in protein-protein interactions.

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