

# Mapping of $\beta$ -adrenoceptor coupling domains to $G_s$ -protein by site-specific synthetic peptides

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Peptides corresponding to the known sequence of turkey erythrocyte  $\beta_1$ -adrenergic receptor were synthesized and the effects on receptor-mediated cyclase activation were measured. Peptides corresponding to the first and second intracellular loops (T61–71 and T138–159) inhibited at micromolar concentrations the hormone-dependent cyclase activation in turkey erythrocyte membranes. In contrast, the peptide corresponding to the C-terminal part of the third intracellular loop (T284–295) increased the cyclase activity in a hormone-independent manner. Peptides T338–353 and T2–10 and a number of synthetic peptides unrelated to the  $\beta$ -adrenoceptor had no effect.

Adrenoceptor,  $\beta$ ;  $G_s$ -protein; Coupling domain; Synthetic peptide

## 1. INTRODUCTION

$\beta$ -Adrenergic activation of the adenylate cyclase complex involves sequential interaction of the  $\beta$ -adrenoceptor with the stimulatory GTP-binding protein ( $G_s$ ) and the target enzyme. The agonist-occupied receptor promotes exchange of GTP for GDP at the  $\alpha$ -subunit ( $\alpha_s$ ) of  $G_s$ . The GTP- $\alpha_s$  complex subsequently stimulates adenylate cyclase. The intrinsic GTPase activity of  $\alpha_s$  terminates the activation cycle. All components of the signal transduction chain have been purified from several sources, and functionally reconstituted in phospholipid vesicles [1,2].

The  $\beta$ -adrenoceptors from turkey erythrocyte, hamster lung and human placenta membranes have been cloned and sequenced [3–5]. They have a high degree of homology and are similar to some other

G-protein-linked receptors [6,7]. This has led to the conclusion that they all belong to one receptor class. Based on the electron diffraction data of Henderson and Unwin [8] on bacteriorhodopsin and the hydrophathy profile for rhodopsin [9], a structural model of this receptor family was designed which includes seven hydrophobic trans-membrane-spanning regions connected by extracellular and intracellular hydrophilic loops. The turkey erythrocyte  $\beta_1$ -receptor consists of a 50 kDa and a 40 kDa form which are both equally functional. The 40 kDa receptor is formed by limited proteolysis and lacks the N-terminal part including the N-linked carbohydrate chains [10]. The cDNA-deduced sequences of the GTP-binding  $\alpha_s$ -subunits from bovine brain [11], bovine adrenal gland [12] and human liver [13] display greater homologies among each other than the amino acid sequence of  $\beta$ -receptors. The  $\alpha$ -subunits of  $G_s$  consist of two different forms ranging from 44.5 to 46 kDa. Turkey  $\alpha_s$ , which has not yet been cloned, consists of one subunit with an apparent molecular mass of 45 kDa [14]. The assignment of functional domains involved in receptor- $G_s$  interactions was based on site-directed mutagenesis [15–17] or on construction of  $\alpha_2/\beta_2$ -chimeric receptors [18]. In

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*Abbreviations:*  $G_s$ -protein, guanine nucleotide-binding protein mediating stimulation of adenylate cyclase; GTP $\gamma$ S, guanosine 5'-O-(3-thiotriphosphate); DTT, 1,4-dithiothreitol; NMP, *N*-methylpyrrolidone; TFA, trifluoroacetic acid

order to explore further the receptor- $G_s$  coupling domains, we prepared synthetic peptides corresponding to the highly conserved regions of the intracellular part of the  $\beta$ -receptor and have tested them in competition with  $\beta$ -receptor for interaction with  $\alpha_s$ . Unlike methods based on mutagenesis [15–18] where artefacts in structure and function cannot be excluded, the use of synthetic peptides allows one to perform experiments with unmodified components [19]. Our results presented here made it possible to identify regions involved in receptor- $G_s$  coupling.

## 2. MATERIALS AND METHODS

*t*-Boc-amino acids were from Novabiochem, TFA was from Kali-Chemie. NMP was a generous gift from BASF. Other reagents for peptide synthesis were from Applied Biosystems. [ $^{35}$ S]GTP $\gamma$ S (800–1500 Ci/mmol), [ $\alpha$ - $^{32}$ P]ATP (600 Ci/mmol) were from New England Nuclear. All other chemicals and biochemicals were of the highest grade commercially available and were obtained from the same sources as in [2,23].

### 2.1. Peptide synthesis

Peptides were synthesized by the solid-phase Merrifield method on an Applied Biosystems 430A synthesizer. The following amino acids with protected side chains were used: Cys (pMeBzl); Asp (OBzl), Glu (OBzl), Ser (Bzl), Thr (Bzl), Tyr (2-Br-Z); His (Z); Lys (Cl-Z); Met = O and Arg (MTS). Amino acids were coupled as HOBt esters in NMP. The coupling yield exceeded 99% in all steps. Deprotection and cleavage from the resin was done with a trifluoromethanesulfonic acid/thioanisole/ethanedithiol/*m*-cresol/TFA (1:1:0.5:0.5:10, v/v) mixture [20]. The reaction was performed at 0°C for 2 h and at 20°C for 1 h. Reduction of methionine sulfoxide was achieved by adding 30% (v/v) dimethyl sulfide to the reaction mixture. The reaction was then allowed to proceed for another 1 h at 20°C. The crude peptides were precipitated into ether and purified to >95% purity by gel filtration over Sephadex G-10 and preparative RP-HPLC. Trifluoroacetate was exchanged against Cl<sup>-</sup> by chromatography on Amberlite IRA-400. Purity was checked by analytical HPLC and amino acid analysis. Peptide concentrations in the biological assays were calculated gravimetrically by adding one Cl<sup>-</sup> per positive charge to the molecular mass of the peptide.

### 2.2. Membrane preparations

Purified turkey erythrocyte membranes were prepared according to Puchwein et al. [21]. Rabbit heart membranes and solubilized adenylate cyclase preparation were prepared as in [22].

### 2.3. $G_s$ preparation

$G_s$  from turkey erythrocytes was purified according to Hanski et al. [14]. Deactivation of  $G_s$  was carried out as described by Feder et al. [2]. All operations were carried out at 4°C.

### 2.4. Reconstitution of purified $G_s$ in phospholipid vesicles

Reconstitution was carried out as described by Hekman et al.

[23]. 15  $\mu$ l of a phosphatidylethanolamine/phosphatidylserine/cholesterol hemisuccinate (12:8:5) lipid mixture (5 mg/ml) were added to 215  $\mu$ l reconstitution buffer (20 mM Hepes, pH 7.6, 20 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT and 0.2% Lubrol PX) which contained 3–6 pmol purified  $G_s$ . The reconstitution mixture was kept at room temperature for 5 min. Detergent was removed by gel filtration on a 7  $\times$  0.4 cm Sephadex G-25 (fine) column. The amount of  $G_s$  incorporated into phospholipid vesicles was determined by binding of 1  $\mu$ M [ $^{35}$ S]GTP $\gamma$ S [23].

### 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  $\mu$ l containing 20 mM Hepes, pH 7.8, 45 mM NaCl, 3 mM MgCl<sub>2</sub>, 5 mM theophylline, 10 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.5 mM ATP and 1–3  $\mu$ Ci [ $\alpha$ - $^{32}$ P]ATP per assay. [ $^{32}$ P]-cAMP formed was isolated and determined according to Salomon et al. [24].

### 2.6. $G_s$ activation

$G_s$  was activated with or without synthetic peptides as indicated. Hormone-induced activation of  $G_s$  in membranes was achieved with  $10^{-5}$  M l-isoproterenol,  $10^{-7}$  M GTP $\gamma$ S (or  $10^{-5}$  M GTP) and 0.5 mM MgCl<sub>2</sub> at 37°C for the times indicated. The peptides were preincubated with the membranes for 30 min at 4°C and subsequently incubated for 10 min at 30°C. The hormone-independent activation of purified  $G_s$  in phospholipid vesicles was carried out at 32°C in the presence of 0.5 mM MgCl<sub>2</sub> and  $10^{-7}$  M GTP $\gamma$ S.

## 3. RESULTS AND DISCUSSION

Four peptides (see table 1 and fig.1) corresponding to the conserved regions of the cytoplasmic loops and the N-terminal segment of the C-terminus of the turkey  $\beta_1$ -receptor were synthesized. Each sequence was chosen in a way that the peptide contained 3–5 hydrophobic amino acids for anchorage in the cell membrane. The remainder of the peptide chain consisted mainly of hydrophilic amino acids (table 1). The ability of the peptides to interfere with the hormone-mediated signal transduction pathway was tested with intact turkey erythrocyte membranes. Peptide T2–10 corresponding to the extracellular N-terminal segment (fig.1) and unrelated peptides of similar length and charge served as controls. Receptor-mediated  $G_s$  activation was followed in the presence of either GTP or GTP $\gamma$ S.

As shown in table 1 and fig.2, peptides T61–71 and T138–159 inhibited cyclase activity in membranes in a concentration-dependent manner with IC<sub>50</sub> values of >200 and 15  $\mu$ M, respectively, suggesting that these peptides compete for receptor-binding domains on  $G_s$ . The peptide T338–353 and

Table 1

Effects of synthetic peptides on receptor-mediated cyclase activation

	Amino acid composition	IC <sub>50</sub> (μM)	Adenylate cyclase activity at 100 μM peptide (%)
(A) β-Receptor peptides			
T61-71	LVIAAIGRTQR	>200	80
T138-159	DRYLAITSPPFRYQSL		
	MTRARAK	15	10
T284-295	REHKALKTLGII	200	130
T338-353	FNPIIYCRSPDFRKAF		100
(B) Control peptides			
T138-159 [Met = O <sup>153</sup> ]	DRYLAITSPPFRYQSL		
	(Met = O)TRARAK	20	15
T2-10	GDGWLPPDC		94
H404-418	LDSQGRNCSTNDSPL		95
H404-418 [Nle <sup>411</sup> ]	LDSQGRN(Nle)		
	STNDSPL		98

Peptides indicated with T or H correspond to β<sub>1</sub>-turkey or β<sub>2</sub>-hamster cDNA sequences, respectively. 100% adenylate cyclase activity (640 pmol/mg per min) corresponds to the hormonally stimulated cyclase in turkey erythrocyte membranes in the absence of synthetic peptides

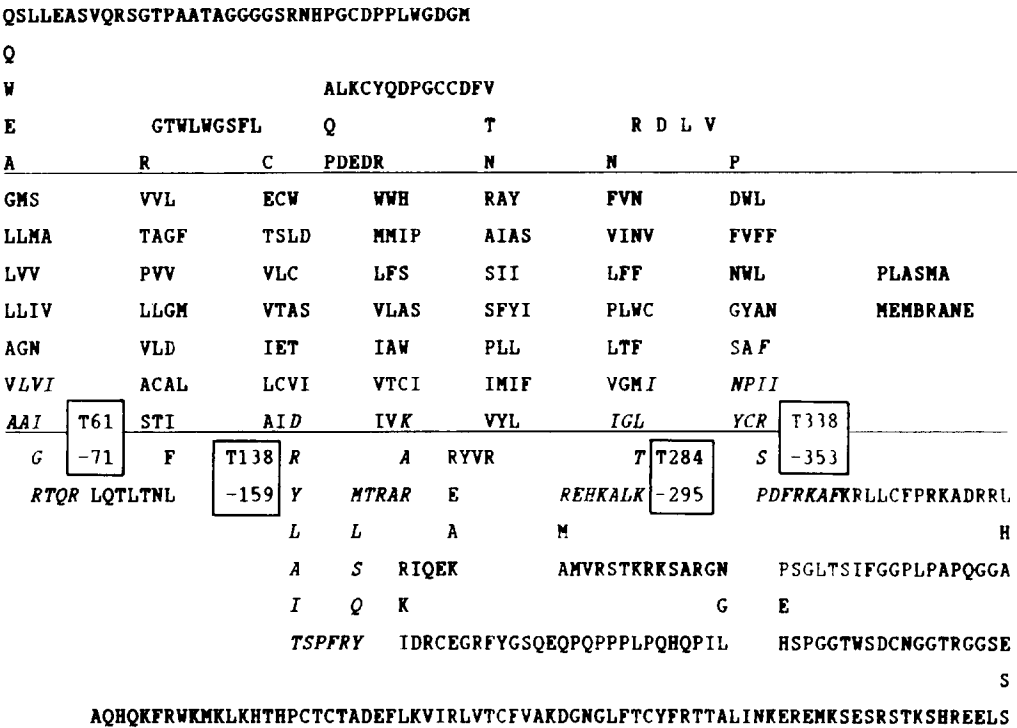


Fig.1. Position of the synthetic peptides in the proposed structure of turkey erythrocyte β<sub>1</sub>-adrenergic receptor (cf. [3]). Letters in italics represent the synthetic peptides.

the unrelated control peptides showed no effect up to 200  $\mu$ M. Peptide T284-295 showed an opposite effect enhancing cyclase activity in a concentration-dependent manner. Peptide T284-295 was able to activate cyclase in turkey erythrocyte membranes, presumably via  $G_s$ -protein, without agonist stimulation and even in the presence of  $\beta$ -adrenergic antagonists (fig.3). Agonist-independent cyclase stimulation by T284-295 in native turkey erythrocyte membranes was confirmed in reconstituted liposomes. Phospholipid vesicles containing purified  $G_s$  prepared from turkey erythrocytes were preincubated with T284-295 for 10 min at 30°C and the time course of GTP $\gamma$ S binding was followed by activation of a crude myocardial cyclase preparation according to Metzger et al. [22]. Since peptide T284-295 increased 2-fold the initial rate of GTP $\gamma$ S binding measured after 2 min, it may be concluded that T284-295 can act as a receptor substitute.

A possible influence of the receptor peptides on the coupling of  $G_s$  to the catalytic subunit (C) in membranes was tested by using  $AlF_4^-$ -activated  $G_s$ . The peptides T61-71, T138-159 and T284-295 were allowed to interact with fluoride-activated membranes for 30 min at 4°C and the extent of cyclase activation was measured (fig.4). Only peptide T138-159 inhibited slightly the  $AlF_4^-$ -induced cyclase activation (22% at 200  $\mu$ M). This effect could be explained assuming that the peptide

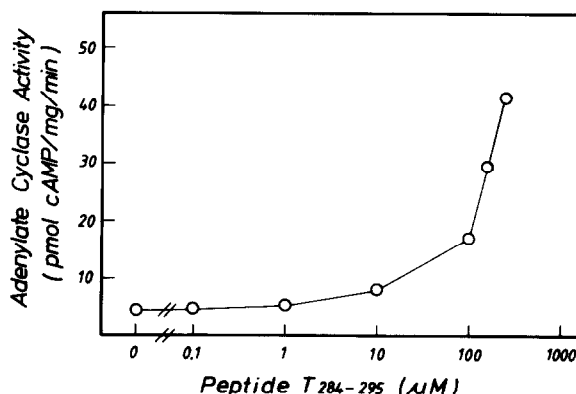


Fig.3. Stimulatory effect of peptide T284-295 on hormone-independent cyclase activity in turkey erythrocyte membranes. The membranes were preincubated with T284-295 for 30 min at 4°C and the cyclase assay was performed for 20 min at 32°C in the presence of  $10^{-5}$  M GTP and  $10^{-5}$  M l-propranolol.

T138-159 when bound to  $G_s$  may cause some sterical constraints resulting in reduced coupling of  $G_s$ -protein with the catalytic subunit.

We conclude from these results that the first and second intracellular loops and the C-terminal portion of the third intracellular loop represented by the peptides T61-71, T138-159 and T284-295 are essential for hormonal signal transmission or its modulation; in contrast, the C-terminal part of the seventh membrane-spanning helix (up to Phe<sup>353</sup>) had no effect. Our results are in agreement with those reported with site-directed mutants of the human and hamster  $\beta_2$ -receptor [15-17], however peptide T338-353 did not interfere with receptor- $G_s$  coupling under our experimental conditions.

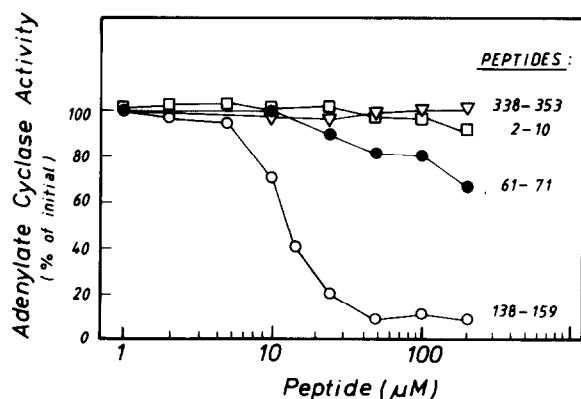


Fig.2. Effects of synthetic  $\beta$ -receptor peptides on hormone-mediated cyclase activation in turkey erythrocyte membranes. Membranes were preincubated with peptides for 30 min at 4°C and hormone-dependent  $G_s$  activation was carried out for 5 min at 37°C with  $10^{-7}$  M GTP $\gamma$ S and  $10^{-5}$  M l-isoproterenol or  $10^{-5}$  M l-propranolol. Agonist-dependent activation is plotted as the difference between the activities in the presence of either isoproterenol or propranolol.

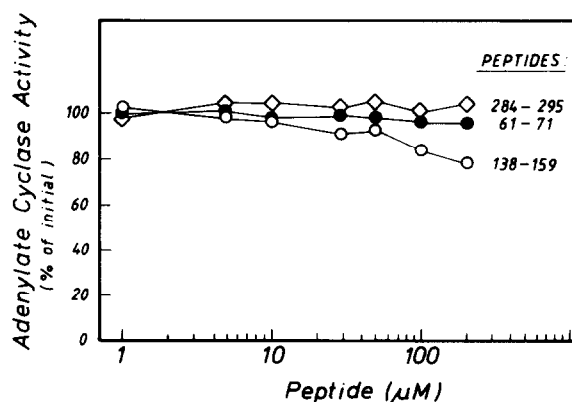


Fig.4. Effects of synthetic  $\beta$ -receptor peptides on  $G_s$ -mediated cyclase activation in turkey erythrocyte membranes.  $AlF_4^-$ -activated membranes were incubated with peptides for 30 min at 4°C and the cyclase assay was carried out for 20 min at 32°C as described in section 2.

Experiments with mutants demand caution, since reduced adenylate cyclase activity as described by O'Dowd et al. [17] may not be the consequence of site-specific interactions, but rather of overall changes in receptor structure and subsequently of receptor anchoring and disposition in the membranes. The ability of peptide T284-295 (C-terminal part of the third loop) to activate  $G_s$  in a hormone-independent fashion can be explained by the assumption that the conformation of T284-295 represents that of an activated receptor. A similar effect has recently been described for mastoparan, a peptide isolated from wasp venom [25], which is capable of mimicking hormone-activated receptor. The amino acid pattern and the secondary structure prediction of mastoparan are very similar to T284-295, since both peptides form a positively charged  $\alpha$ -helix. In addition, the studies of Kobilka et al. [18] on  $G_s$  activation with chimeric receptors point to the importance of the sixth transmembrane helix and its N-terminal portion: all chimeric  $\alpha_2$ - and  $\beta_2$ -adrenergic receptors, which are able to activate adenylate cyclase, contain the segment corresponding to T284-295. Since small peptides like the T284-295 undecamer may adopt any possible conformation of the  $\beta$ -receptor chain, it would be sufficient if a small fraction of the peptide is in the 'active' conformation and is able to replace functionally the receptor. We expect to localize more definitively the functional domains participating in receptor- $G_s$  coupling by studies with additional peptides, especially those corresponding to parts of the third intracellular loop and C-terminal segment of the receptor.

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