Volume 254, number 1,2, 89-93 FEB 07517 August 1989

Mapping of β-adrenoceptor coupling domains to G_s-protein by site-specific synthetic peptides

Dieter Palm, Gerald Münch, Christian Dees and Mirko Hekman

Dept of Physiological Chemistry, University of Würzburg, Koellikerstraße 2, D-8700 Würzburg, FRG

Received 26 June 1989

Peptides corresponding to the known sequence of turkey erythrocyte β_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 β-adrenoceptor had no effect.

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

1. INTRODUCTION

β-Adrenergic activation of the adenylate cyclase complex involves sequential interaction of the β-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 α-subunit (α_s) of G_s. The GTP-α_s complex subsequently stimulates adenylate cyclase. The intrinsic GTPase activity of α_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 β-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 transmembrane-spanning regions connected by extracellular and intracellular hydrophilic loops. The turkey erythrocyte β_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 α_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 β-receptors. The α-subunits of G_s consist of two different forms ranging from 44.5 to 46 kDa. Turkey α_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 α_2/β_2-chimeric receptors [18]. In

Correspondence address: M. Hekman, Dept of Physiological Chemistry, University of Würzburg, Koellikerstraße 2, D-8700 Würzburg, FRG

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

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

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 β-receptor and have tested them in competition with β-receptor for interaction with α_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]GTPγS (800–1500 Ci/mmol), [α-^{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^- 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^- 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 μl of a phosphatidylethanolamine/phosphatidylserine/cholesterol hemisuccinate (12:8:5) lipid mixture (5 mg/ml) were added to 215 μl 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 × 0.4 cm Sephadex G-25 (fine) column. The amount of G_s incorporated into phospholipid vesicles was determined by binding of 1 μM [^{35}S]GTPγS [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 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. [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 GTPγS (or 10^{-5} M GTP) and 0.5 mM $MgCl_2$ 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 $MgCl_2$ and 10^{-7} M GTPγS.

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 β_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 GTPγS.

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_{50} values of >200 and 15 μM, 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_{50} (μM)	Adenylate cyclase activity at 100 μM peptide (%)
(A) β-Receptor peptides			
T61–71	LVIAAIGRTQR	>200	80
T138–159	DRYLAITSPFRYQSL		
	MTRARAK	15	10
T284–295	REHKALKTLGII	200	130
T338–353	FNPIIYCRSPDFRKAF		100
(B) Control peptides			
T138–159 [Met = O^{153}]	DRYLAITSPFRYQSL		
	(Met = O)TRARAK	20	15
T2–10	GDGWLPPDC		94
H404–418	LDSQGRNCSTNDSPL		95
H404–418 [Nle^{411}]	LDSQGRN(Nle)		
	STNDSPL		98

Peptides indicated with T or H correspond to β_1-turkey or β_2-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

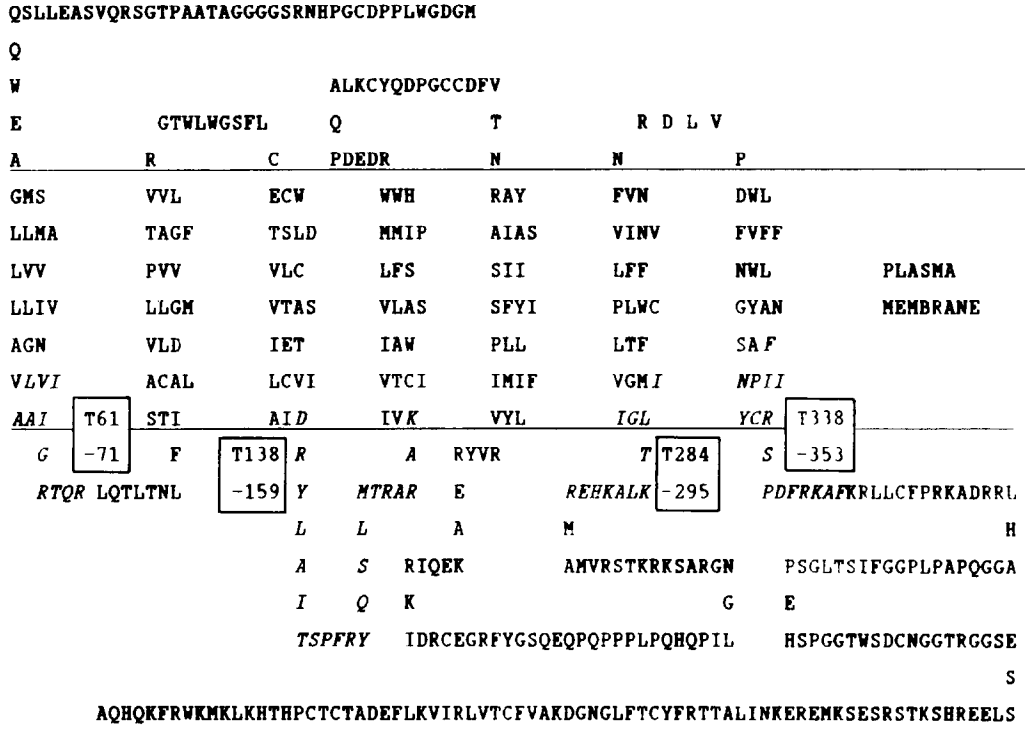

Fig.1. Position of the synthetic peptides in the proposed structure of turkey erythrocyte β_1-adrenergic receptor (cf. [3]). Letters in italics represent the synthetic peptides.

the unrelated control peptides showed no effect up to 200 μM. 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 β-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 GTPγS 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 GTPγS 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 μM). This effect could be explained assuming that the peptide

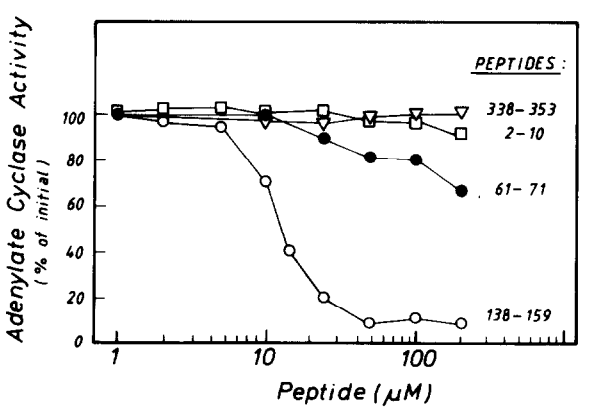

Fig.2. Effects of synthetic β-receptor peptides on hormone-mediated cyclase activtion in turkey erythrocyte membranes. Membranes were preincubated with peptides for 30 min at 4°C and hormone-dependent G_s activation was carried out for 5 min at 37°C with 10^{-7} M GTPγS and 10^{-5} M l-isoproterenol 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.

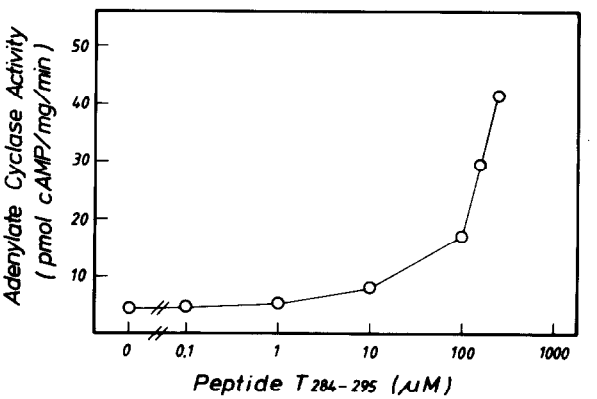

Fig.3. Stimulatory effect of peptide T284–295 on hormone-independent cyclase activity in turkey erythrocyte membranes. The membranes were preincubated with T284–295 for 30 min at 4°C and the cyclase assay was performed for 20 min at 32°C in the presence of 10^{-5} M GTP and 10^{-5} M l-propranolol.

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^{353}) had no effect. Our results are in agreement with those reported with site-directed mutants of the human and hamster β_2-receptor [15–17], however peptide T338–353 did not interfere with receptor-G_s coupling under our experimental conditions.

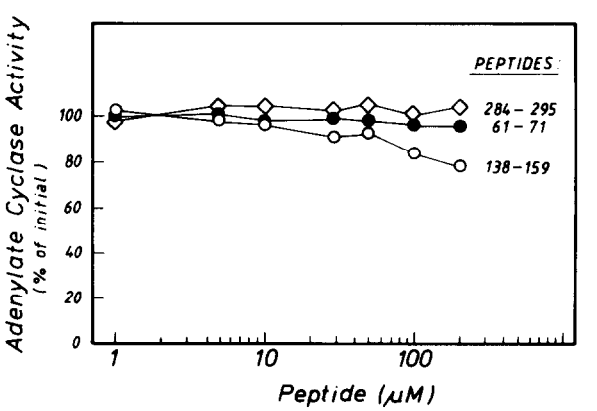

Fig.4. Effects of synthetic β-receptor peptides on G_s-mediated cyclase activation in turkey erythrocyte membranes. AlF_4^--activated membranes were incubated with peptides for 30 min at 4°C and the cyclase assay was carried out for 20 min at 32°C as described in section 2.

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 α-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 α_2- and β_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 β-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.

Acknowledgements: We are indebted to E.J.M. Helmreich for his interest in and support of this work, and for critical discussions. We thank J. Hoppe for amino acid analysis of synthetic peptides. This work was partly supported by the Deutsche Forschungsgemeinschaft, Grant Pa 92/20 and by SFB 176, Project A1.

REFERENCES

[1] May, D.C., Ross, E.M., Gilman, A.G. and Smigel, M.D. (1985) J. Biol. Chem. 260, 15829–15833.
[2] Feder, D., Im, M.-J., Klein, H.W., Hekman, M., Holzhöfer, A., Dees, C., Levitzki, A., Helmreich, E.J.M. and Pfeuffer, T. (1986) EMBO J. 5, 1509–1514.
[3] Yarden, Y., Rodriguez, H., Wong, S.K.-F., Brandt, D.R., May, D.C., Burnier, J., Harkins, R.N., Chen, E.Y., Ramachandran, J., Ullrich, A. and Ross, E.M. (1986) Proc. Natl. Acad. Sci. USA 83, 6795–6799.
[4] Dixon, R.A.F., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Diehl, R.E., Mumford, R.A., Slater, E.E., Sigal, I.S., Caron, M.G., Lefkowitz, R.J. and Strader, C.D. (1986) Nature 321, 75–79.
[5] Kobilka, B.K., Dixon, R.A.F., Frielle, T., Dohlman, H.G., Bolanowski, M.A., Sigal, I.S., Yang-Feng, T.L., Francke, U., Caron, M.G. and Lefkowitz, R.J. (1987) Proc. Natl. Acad. Sci. USA 84, 46–50.
[6] Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, M., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. and Numa, S. (1986) Nature 323, 411–416.
[7] Kubo, T., Maeda, A., Sugimoto, K., Akiba, I., Mikami, A., Takahashi, H., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Matsuo, H., Hirose, T. and Numa, S. (1986) FEBS Lett. 209, 367–372.
[8] Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28–32.
[9] Ovchinnikov, Y.A. (1982) FEBS Lett. 148, 179–191.
[10] Jürss, R., Hekman, M. and Helmreich, E.J.M. (1985) Biochemistry 24, 3349–3354.
[11] Nukada, T., Tanabe, T., Takahashi, H., Noda, M., Hirose, T., Inayama, S. and Numa, S. (1986) FEBS Lett. 195, 220–224.
[12] Robishaw, J.D., Smigel, M.D. and Gilman, A.G. (1986) J. Biol. Chem. 261, 9587–9590.
[13] Mattera, R., Codina, J., Crozat, A., Kidd, V., Woo, S.L.C. and Birnbaumer, L. (1986) FEBS Lett. 206, 36–42.
[14] Hanski, E., Sternweis, P.C., Northup, J.K., Dromerick, A.W. and Gilman, A.G. (1981) J. Biol. Chem. 256, 12911–12919.
[15] Strader, C.D., Sigal, I.S., Blake, A.D., Cheung, A.H., Register, R.B., Rands, E., Zemcik, B.A., Candelore, M.R. and Dixon, R.A.F. (1987) Cell 49, 855–863.
[16] Strader, C.D., Dixon, R.A.F., Cheung, A.H., Candelore, M.R., Blake, A.D. and Sigal, I.S. (1987) J. Biol. Chem. 262, 16439–16443.
[17] O'Dowd, B.F., Hnatowich, M., Regan, J.W., Leader, W.M., Caron, M.G. and Lefkowitz, R.J. (1988) J. Biol. Chem. 263, 15985–15992.
[18] Kobilka, B.K., Kobilka, T.S., Daniel, K., Regan, J.W., Caron, M.G. and Lefkowitz, R.J. (1988) Science 240, 1310–1316.
[19] Hamm, H.E., Deretic, D., Arendt, A., Hargrave, P.A., Koenig, B. and Hofmann, K.P. (1988) Science 241, 832–835.
[20] Yajima, H., Funakoshi, S. and Akaji, K. (1985) Int. J. Peptide Protein Res. 26, 337–361.
[21] Puchwein, G., Pfeuffer, T. and Helmreich, E.J.M. (1974) J. Biol. Chem. 249, 3232–3240.
[22] Pfeuffer, T., Gaugler, B. and Metzger, H. (1983) FEBS Lett. 164, 154–160.
[23] Hekman, M., Holzhöfer, A., Gierschik, P., Im, M.-J., Jakobs, K.-H., Pfeuffer, T. and Helmreich, E.J.M. (1987) Eur. J. Biochem. 169, 431–439.
[24] Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
[25] Higashijima, T., Uzu, S., Nakajima, T. and Ross, E.M. (1988) J. Biol. Chem. 263, 6491–6494.