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Original article

A structure–activity relationship study on position-2 of the $G\alpha_s$ C-terminal peptide able to inhibit G_s activation by A_{2A} adenosine receptor

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Abstract

For some years synthetic peptides corresponding to the C-terminal sequence of Gα proteins represented an useful tool to study the molecular mechanism of the interaction between these proteins and the G protein coupled receptors. Recently, we have focused our attention on the study of the A_{2A} receptor–G_s protein system. We have synthesised a series of 11-mer peptides from the $G\alpha_s$ C-terminus in which residue at position-2 (Leu^{393}) has been alternatively substituted with amino acids having different physico-chemical properties. The aim of our work was to probe the role played by Leu^{393} in the receptor/$G\alpha_s$ interaction. All synthetic peptides were tested for their ability to affect the adenylyl cyclase activity stimulated by agonist activation of A_{2A} adenosine receptors. Our data point out a relevant role played by the side chain of this residue for a correct G protein/receptor coupling, even though the presence of other residues at position-2 of $G\alpha_s$ C-terminus is tolerated. Furthermore, molecular dynamics calculations on the peptides having greater activity show a correlation between the spatial arrangement of the side chain of residue at position-2 and biological activity of synthetic peptides.

Keywords: G protein; Synthetic peptides; Molecular dynamics; A_{2A} receptors

1. Introduction

Many signal molecules, as hormones, neurotransmitters, chemokines and local mediators, induce their physiological effects activating signal transduction pathways by interaction with transmembrane heptahelical receptors [1]. These receptors are coupled with specific cellular effector systems through an appropriate heterotrimeric (αβγ) G protein that functions as transducer of the initial extracellular signal. The signal transduction pathways control numerous essential functions in all tissues and are ubiquitous throughout eukaryotes. They are likely involved in several pathologies. Indeed, within the past 20 years a large number of new drugs, acting as agonists or antagonists for G protein coupled receptors have been registered.

A complete understanding of the molecular basis of the receptor/G protein interaction is essential to have chance to intervene successfully when the signal transduction mechanism is altered in some diseases. Currently, the activation of G proteins is mostly manipulated by targeting their cognate receptor with

Abbreviations: Gα, α subunit of heterotrimeric G proteins; G_s, G protein linked with the activation of adenylyl cyclase; $G\alpha_s$, α subunit of G_s; $G\alpha_i$, α subunit of a G protein (G_i) linked with the inhibition of adenylyl cyclase; $G\alpha_t$, α subunit of the G protein (G_t or transducin) present in rod outer segments; $G\alpha_q$, α subunit of a G protein (G_q) linked with the stimulation of phosphatydylinositol-specific phospholipase C; Fmoc, fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; RMS, root mean square; MD, molecular dynamics; EM, energy minimisation.

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synthetic compounds that behave as receptor agonists or antagonists [2]. A less explored possibility is to intervene at the receptor / G protein interface. Several studies have pointed out specific regions on G_{α} and $G_{\beta\gamma}$ subunits that participate in the interaction with the receptor. Among these, the C-terminus of the G_{α} subunits plays a central role in the selective activation of G proteins by their cognate receptors [3–5], and it appears to be a potential target for drug design [6]. Consequently, studies on synthetic peptides belonging to C-terminal region gain increased importance. Indeed, some G protein-derived peptides, as well as receptor-derived peptides, are known to interfere in receptor/G protein coupling. Peptides corresponding to the last 11-amino acids of Gα C-terminus act as inhibitors of signal transduction and stabilise the high-affinity state of some receptors [7,8]. The receptor-bound conformation of a synthetic peptide from $G\alpha_t$ C-terminus has been analysed by NMR spectroscopy in the presence of dark- and light-activated rhodopsin [9].

Recently, we focused our attention on the study of the interaction between $G\alpha_s$ protein and A_{2A} adenosine receptors [10–12]. For their involvement in dopaminergic transmission at brain level [13], A_{2A} adenosine receptors represent a possible target to develop new therapeutic agents to treat Parkinson's disease and schizophrenia [14–16]. We synthesised several C-terminal fragments of $G\alpha_s$ (from 11 to 21 residues) and tested their ability to affect agonist binding to A_{2A} adenosine receptors and to inhibit receptor-stimulated adenylyl cyclase activity [10]. Furthermore, we carried out extensive structural studies in order to explore the role played by both the backbone conformation and the side chains arrangement in determining the activity of these C-terminal fragments [11].

In continuing our studies on the A_{2A} receptor–G_s protein system, we report here a study designed to evaluate the role of Leu^{393} in the A_{2A} receptor–$G\alpha_s$ protein coupling in order to obtain new insight on the involvement of this residue in the receptor/$G\alpha_s$ protein interaction. We have synthesised and tested a series of 11-mer peptides from the $G\alpha_s$ C-terminus in which Leu^{393} (position-2) has been alternatively substituted with residues of different physico-chemical properties.

2. Results and discussion

Several studies have pointed out the importance of the α-subunit C-terminus as one the critical points of contact with activated receptors [3,9,17,18]. In fact, C-terminal region is generally conserved within each of the four families of G proteins (G_s, G_i, G_q and $G_{12,13}$) while differences may be found among members of different families. The only exception is represented by Leu at position-2 and -7 that are absolutely invariant in C-terminal regions of all Gα subunits. Mutagenesis studies on C-terminus of $G\alpha_t$ have demonstrated that substitution of Leu at position-2 is detrimental for both rhodopsin and guanine nucleotide binding [19,20]. Martin et al. have reported that in all sequences that bind rhodopsin with high affinity, identified by screening of a combinatorial peptide library, Leu at position-2 is always present except in two peptides in which it is switched to Met [21]. Marshall et al. have synthesised constrained peptides to probe the interaction sites between photoactivated-rhodopsin and transducin [22]. They found that Leu-2 was an invariable residue as confirmed by significantly reduction of activity owing to mutations of Leu-2 to Ile or tert-leucine

To probe the role played by Leu^{393} in the A_{2A} receptor/$G\alpha_s$ interaction, we have synthesised 11-mer peptides (peptides **1**–**6**), corresponding to the C-terminal region of $G\alpha_s$ subunit, in which Leu^{393} has been differently substituted. Table 1 shows the amino acid sequences of the synthetic peptides used in this study.

All peptides were tested for their ability to affect the adenylyl cyclase activity stimulated by agonist activation of A_{2A} adenosine receptors. We used as reference the unmodified 11-mer peptide $G\alpha_s$ (384–394) that we have previously shown to possess inhibitory activity [10]. The results of biological assays, in which we have evaluated the effects of peptides **1**–**6** and $G\alpha_s$ (384–394) as percent of inhibition of cAMP production stimulated by an agonist (10 μM NECA), are also reported in Table 1.

The biological data show that all modified peptides appear to be significantly less active with respect to the reference peptide $G\alpha_s$ (384–394) (inhibition of 24%). However, peptides **5** and **6**, in which Leu^{393} has been replaced by Asp and Thr, respectively, retain a residual activity (inhibition of 16%). Substitution of Leu^{393} with

Table 1
Amino acid sequences and inhibitory activity of $G\alpha_s$ (384–394) and synthetic analogues

Peptide	Amino acid sequence	% cAMP inhibition [a]
$G\alpha_s$ (384–394)	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Leu**-Leu-OH	24 ± 0.3
1	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Ala**-Leu-OH	2.7 ± 0.1
2	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**DLeu**-Leu-OH	9.1 ± 0.8
3	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Phe**-Leu-OH	4.8 ± 0.2
4	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Lys**-Leu-OH	4.3 ± 0.5
5	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Asp**-Leu-OH	16 ± 2.3
6	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Thr**-Leu-OH	16 ± 0.5

D-Leu also yielded a peptide endowed with some residual activity, albeit weak (peptide **2**; inhibition of 9.5%). Finally, the replacement of Leu^{393} with either Ala (peptide **1**; 3.0%), Phe (peptide **3**; 5.0%), or Lys (peptide **4**; 4.5%) have led to peptides practically devoid of inhibitory activity.

The three peptides showing activity, i.e. peptides **6**, **5**, and **2**, were subjected to a preliminary analysis by molecular dynamics calculations in order to explore the spatial region occupied by the side chains of residues at position-2 (Thr^{393}, Asp^{393}, and D-Leu^{393}; respectively). We have used as starting model the $G\alpha_s$ (384–394) structure [12], opportunely modified at position-2, and we have kept the backbone frozen. Fig. 1 shows a comparison among the spatial arrangements of the side chains of residues Thr^{393} (peptide **6**), Asp^{393} (peptide **5**), D-Leu^{393} (peptide **2**), and Leu^{393} (in the unmodified $G\alpha_s$ (384–394)). The Asp^{393} side chain shows the best overlapping with the side chain of Leu^{393} in the reference peptide. D-Leu^{393} side chain have just one methyl group overlapped with a methyl group of Leu^{393} in $G\alpha_s$ (384–394). Finally, Thr^{393} side chain, even though not perfectly superimposable with Leu^{393} side chain of $G\alpha_s$ (384–394), shows its methyl group oriented like the methylene group of Leu^{393}.

In our recent work [11], we have reported the results of a detailed conformational analysis on synthetic peptides corresponding to the last C-terminal residues of $G\alpha_s$ subunit, performed with the aim to explore the dependence of biological activity from the conformational propensity of these peptides. With particular reference to Leu^{393}, we have reported that its side chain, together with Met^{386} and Ile^{382} side chains, takes part in a hydrophobic surface that seems to have such an orientation to undertake contacts with the C-terminal helix of the cognate receptor.

The results of the present study appear to confirm that residue at position-2 should occupy a well defined spatial region to undergo correct contact with the receptorial counterpart. The replacement of Leu^{393} with D-Leu, in peptide **2**, even though have not caused total loss of activity, have led to very weakly active peptide. It is probable that the different orientation of the D-Leu side chain, as showed in Fig. 1, make unfavourable the interaction of this residue with the receptor. The presence of a hydrophobic interaction between the receptor and the peptide surface in which the side chain of residue at position-2 is involved appears also to be important. Asp side chain, in peptide **5**, displays the best overlapping with Leu^{393} side chain of $G\alpha_s$ (384–394), but its carboxyl group cover the same region of $>CHCH_3$ of Leu^{393}, thus disrupting at least in part the hydrophobic surface observed in the unmodified peptide. Otherwise, Thr side chain, in peptide **6**, is oriented in such way to better guarantee the preservation of such hydrophobic surface.

Finally, it appears reasonable to ascribe the loss of inhibitory activity of peptides **1**, **3**, and **4** to steric and

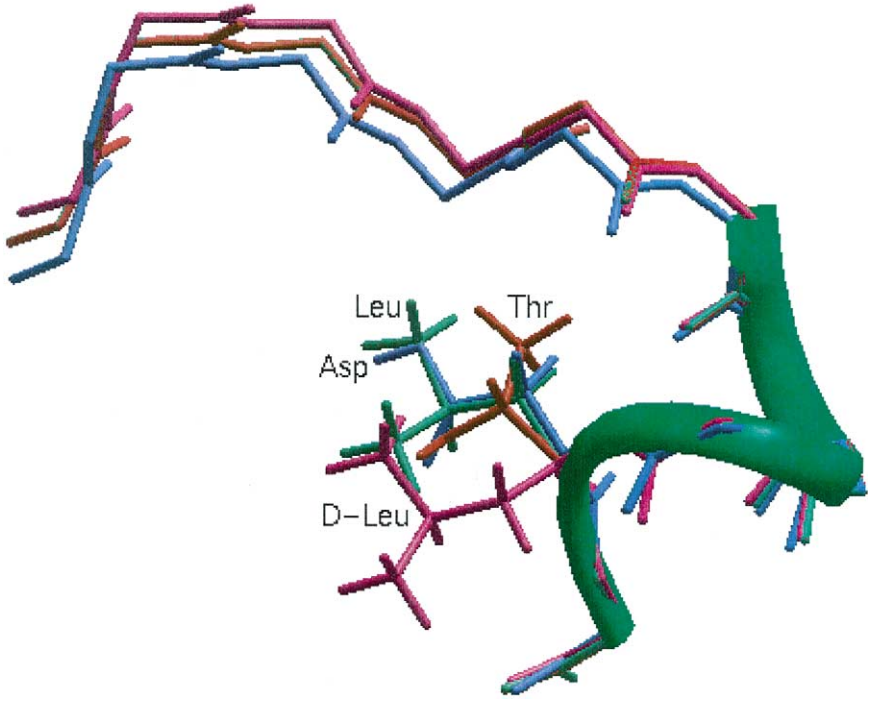

Fig. 1. Superposition of Cα atoms of peptides **6** (red–orange), **5** (blue), **2** (magenta) with Cα atoms of $G\alpha_s$ (384–394) (green). The helical arrangement of last five residues of $G\alpha_s$ (384–394) is highlighted by a green tube ribbon. The spatial orientation of side-chain of amino acids at position-2 is displayed.

chemical characteristic of the residues at position-2 (Ala, Phe, and Lys, respectively), clearly different from those of the corresponding residue in the unmodified $G\alpha_s$ (384–394).

3. Conclusions

The main goal of our work has been to investigate the role of Leu^{393} in the interaction of a $G\alpha_s$ C-terminal undecapeptide with the A_{2A} adenosine receptor. Our data point out a relevant role played by the side chain of this residue for a correct G protein/receptor coupling. However, the presence of other residues at position-2 of $G\alpha_s$ C-terminus is tolerated, as demonstrated by the activity of peptides **6** and **5**. Anyway, further experimental studies are necessary to better understand if the activity of these modified peptides is related to an effect of the amino acid substitutions on the overall structure of C-terminal region of $G\alpha_s$.

4. Experimental

4.1. Materials

N^{α}-Fmoc-protected amino acids, HBTU, HOBt and Wang resin were purchased from Advanced ChemTech (Louisville, KY). For the N^{α}-Fmoc-protected amino acids, the following side chain protecting groups were used: Arg(N^{g}-Pbf); Asp(β-*O*-*t*Bu); His(N^{im}-Trt); Trp(N^{in}-Boc), Gln(Trt), and Tyr(*O*-*t*Bu). Peptide synthesis solvents, reagents, as well as CH_3CN for HPLC were reagent grade and were acquired from commercial sources and used without further purification unless otherwise noted. FAB-MS analyses were performed at the University of Napoli 'Federico II' by MALDI. The purity of the finished peptides was checked by analytical RP-HPLC using a Shimadzu mod. CL-10AD VP system with a built-in diode array detector. In all cases, the purity of the finished peptides was greater than 95% as determined by these methods.

4.2. General method for peptide synthesis and purification

All peptides were synthesised by the solid-phase method of peptide synthesis and purified by RP-HPLC. The peptides were synthesised on 0.15 g each of Wang resin (substitution 0.7 mmol g^{-1}) by manual methods using N^{α}-Fmoc chemistry and an orthogonal side chain protection strategy. The entire synthesis was performed under an atmosphere of Ar. The resin was first swollen in DCM–DMF (1:1) for 2 h and the following amino acids were then added to the growing peptide chain by stepwise addition of N^{α}-Fmoc-Leu-OH, N^{α}-Fmoc-Glu(*O*-*t*Bu)-OH, N^{α}-Fmoc-Tyr(*O*-*t*Bu)-OH, N^{α}-Fmoc-Gln(N^{γ}-Trt)-OH, N^{α}-Fmoc-Arg(N^{g}-Pbf)-OH, N^{α}-Fmoc-Leu-OH, N^{α}-Fmoc-His(N^{im}-Trt)-OH, Fmoc-Met-OH, N^{α}-Fmoc-Arg(N^{g}-Pbf)-OH, N^{α}-Fmoc-Gln(N^{γ}-Trt)-OH, and, using standard solid phase methods. Each coupling reaction was achieved using a threefold excess each of the amino acid, HBTU, and HOBt in presence of a sixfold excess of DIPEA for 1 h. Deprotection of the N^{α}-Fmoc group was carried out by treating the protected peptide resin with 25% piperidine solution in DMF (1 × 4 mL, 5 min, 1 × 4 mL, 20 min). After each coupling and deprotection, the peptide resin was washed with DMF (3 × 4 mL), DCM (3 × 50 mL) and again with DMF. The peptide sequences were thus assembled by alternate cycles of coupling and deprotection. After coupling of the N-terminal amino acid, the N-terminal Fmoc group was deblocked as before and the peptide resin was thoroughly washed with DCM (4 × 25 mL) and dried under an atmosphere of Ar to yield dried peptide resin.

Next, the peptide resin was cleaved by treating with 4 mL of a solution of Et_3SiH (5%), water (5%), *p*-thiocresol–*p*-cresol (0.1%, 1:1) in TFA with shaking at room temperature for 3 h. The resin was then removed from the solution (containing the cleaved peptide) by filtration and the crude peptide was recovered by

Table 2
Physico-chemical properties of peptides **1**–**6**

Peptide	Amino acid sequence	R_f TLC solvents [a]			HPLC [b] k'
		A	B	C	
1	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Ala**-Leu-OH	0.72	0.08	0.33	5.88
2	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**DLeu**-Leu-OH	0.78	0.11	0.35	6.08
3	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Phe**-Leu-OH	0.88	0.19	0.41	6.22
4	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Lys**-Leu-OH	0.65	0.05	0.22	5.61
5	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Asp**-Leu-OH	0.67	0.06	0.25	5.70
6	H-Gln-Arg-Met-His-Leu-Arg-Gln-Tyr-Glu-**Thr**-Leu-OH	0.68	0.08	0.26	5.91

[a] Solvent systems: (A) 1-butanol–HOAc–pyridine–H_2O (5:5:1:4); (B) EtOAc–pyridine–AcOH–H_2O (5:5:1:3); (C) 1-butanol–AcOH–H_2O (4:1:1).
[b] Analytical HPLC performed on a C18 column using a gradient of acetonitrile in 0.1% aqueous TFA at 1 mL min^{-1}.

Table 3
Amino acid analysis of peptides **1**–**6**

Peptide	Gln	Arg	Met	His	Leu	Arg	Gln	Tyr	Glu	Xaa	Leu
1	0.98	1.02	0.97	0.98	0.95	0.91	0.96	1.01	0.93	0.98	0.98
2	0.96	0.96	0.95	0.99	0.90	0.97	0.95	0.92	0.99	1.10	0.95
3	0.97	0.98	1.01	0.89	0.89	0.98	0.93	0.91	0.92	0.93	0.98
4	1.01	1.0	0.99	0.93	0.93	0.99	1.05	0.97	0.94	0.98	0.95
5	0.97	0.95	0.88	1.02	0.95	0.98	0.95	0.94	1.02	0.99	0.91
6	0.98	0.97	0.91	0.90	0.99	0.89	1.03	0.98	0.94	0.97	0.93

precipitation with cold anhydrous ethyl ether. Centrifugation at 2000 rpm for 3 min followed by decantation of the supernatant ether and air-drying of the residue yielded the crude peptide as a white to pale beige coloured amorphous solid.

Final peptide purification was achieved using a preparative RP-HPLC Vydac C18 (218TP1520, 15 μm). The peptides were injected onto the column at a concentration of 20–30 mg mL^{-1} in 20% aq. CH_3CN and were eluted with a CH_3CN gradient (0–55%) over 35 min at a flow rate of 15.0 mL min^{-1}, with a constant concentration of TFA (0.1% v/v). The separations were monitored at 230 and 280 nm and integrated with a Shimadzu diode array detector mod. SPD-M10A VP dual wavelength absorbance detector model UV-D. Fractions corresponding to the major peak were collected, pooled, and lyophilised to yield the final peptides as pure (> 95%) white solids. Amino acid analyses were carried out using a Pico-Tag Work Station. Lyophilised samples of peptides (50–1000 pmol) were hydrolysed in heat-treated borosilicate tubes (4 × 50 mm) using the Pico-Tag Work Station (Waters-Millipore, Waltham, MA) for 1 h at 150 °C with 200 mL 6 N HCl containing 1% phenol: a Pico-Tag column (3.9 × 15 mm) was employed to separate the amino acid derivatives. The analytical data for each compound is presented in Table 2 and the amino acid analysis data in Table 3.

4.3. Adenylyl cyclase assay

Adenylyl cyclase activity was assayed by monitoring the conversion of [α-^{32}P]ATP to [α-^{32}P]cAMP, as previously reported [23]. The method involved addition of [α-^{32}P]ATP to rat striatal membranes in the presence of an A_{2A} adenosine receptor agonist (NECA or CGS21680) and GTP or forskolin (FSK) to stimulate adenylyl cyclase and papaverine as aphosphodiesterase inhibitor. Peptides were used at a concentration of 300 μM. Values reported in Table 1 are mean ± S.E. of at least three independent experiments.

4.4. Molecular dynamics calculations

All molecular dynamics and energy minimisation calculations were performed using the CVFF force field as implemented in Discover module of Insight II v.98.0 [24]. We used as starting model the $G\alpha_s$ (384–394) structure [12] that was opportunely modified at position-2, to obtain peptides **6**, **5**, and **2**, using the Builder module of Insight II. After a preliminary short EM, a MD simulation was carried out on each peptide at 310 K for 600 ps after 300 ps equilibration time. During all calculations the peptide backbone was kept frozen.

Conformations were stored each 10 ps and classified in two families on the basis of the RMS of side chain of residues 390–394 that were resulted structured in the unmodified $G\alpha_s$ (384–394). The two families corresponded to two different ranges of RMS, 0.00–2.00 (low RMS) and 2.00–4.00 (high RMS), respectively. The lowest energy conformation was selected from the most populated family of low RMS and the geometry optimised (steepest descent algorithm) adopting a RMS derivative of 0.5 kcal mol^{-1} $Å^{-1}$ as convergence criterion.

Fig. 1 shows a superimposition of final structure of peptides **6**, **5** and **2**, with the structure of the unmodified $G\alpha_s$ (384–394). The alignment was obtained overlapping the C*a* atoms of the five C-terminal residues of peptides **6**, **5** and **2** to the corresponding residues of $G\alpha_s$ (384–394).

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