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NMR structure of a human homologous *methuselah* gene receptor peptide ☆

Tatsuhiko Kikkou [a], Osamu Matsumoto [a,*], Tadayasu Ohkubo [b], Yuji Kobayashi [b], Gozoh Tsujimoto [c]

[a] *Faculty of Pharmaceutical Sciences, Chiba Institute of Science, Choshi, Chiba 288-0025, Japan*
[b] *Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan*
[c] *Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan*

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Abstract

Human APG1 gene is homologous to *Drosophila methuselah* gene associated with extended life span. A peptide (APG1: RNGKRSNRTLREE) corresponding to a predicted region of the intracellular third loop of G protein-coupled receptor coded in human APG1 gene could activate G_i protein α subunit directly. The three-dimensional molecular structure of the peptide in SDS-d_{25} micelles was determined by 2D ^{1}H NMR spectroscopy. APG1 formed an α-helical structure at the C-terminal site and a positive charge cluster at the N-terminal site. The cluster was also found in several other G_i protein-coupled receptor peptides. Therefore, the positive charge cluster on the helical structure might be engaged in G protein activation.

Keywords: APG1 receptor; *Drosophila methuselah*; NMR; Peptide structure; Intracellular loop peptide

The *methuselah* (*mth*) gene in *Drosophila* is known to be associated with life span by a screening test for gene mutation. In *Drosophila*, 35% increase in average life span is shown by knocking out the *mth* gene [1]. The predicted protein from *mth* gene is thought to be G protein-coupled receptor (GPCR) [2]. GPCRs play an important role in intracellular signal transduction [3], and most of GPCRs take a common multiple membrane-spanning conformation in the seven transmembrane domains [4]. It is reported that the intracellular third loop (IC3) peptides of human β2-adrenergic receptor [5] and human prostaglandin EP3α receptor [6] can activate G protein directly. Therefore, the IC3 peptide of GPCR will be a good model for resolving the biological molecular mechanism for G protein activation. In the previous study of an IC3 peptide of prostaglandin EP3α receptor, this receptor peptide forms a helical structure in SDS micelles and the structure of this peptide in SDS micelles consists of a positive charge cluster on the N-terminal side of the chain [6]. In this work, we investigated a peptide (APG1: RNGKRSNRTLREE) corresponding to the predicted IC3 region of APG1 receptor protein derived from APG1 gene, which is a human homolog of *mth* gene (Fig. 1), to clarify the G_i protein activation mechanism and the solution structure.

Materials and methods

Sample preparation. The sequence of IC3 region of APG1 receptor has been identified by the analysis of APG1 sequence using the secondary structure prediction programs, SOSUI [7], TopPred [8], and TMHMM [9]. A peptide (APG1: RNGKRSNRTLREE) corresponding to the region of human APG1 receptor IC3 and a peptide (GABAb IC2: HTGFTKKEEKKEWRKTLEPWK) corresponding to the intracellular second loop (IC2) of human GABAb receptor were synthesized by

☆ *Abbreviations:* COSY, correlated spectroscopy; G protein, Guanine nucleotide-binding protein; NOE, nuclear Overhauser effect, also used for NOESY cross peak; NOESY, NOE spectroscopy; ppm, parts per million; SDS-d_{25}, sodium dodecyl-d_{25} sulfate.

* Corresponding author. Fax: +81 479 30 4740.
E-mail address: osamu@cis.ac.jp (O. Matsumoto).

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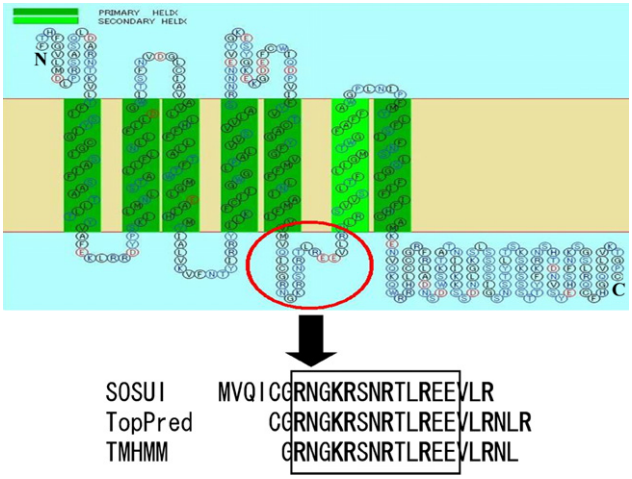

Fig. 1. Schematic drawing of the predicted transmembrane domains of APG1 receptor. Circle region represents the third intracellular loop (IC3) of APG1 receptor. The amino acid sequences of IC3 were predicted with three programs: SOSUI, TopPred, and TMHMM. The square shows a synthesized region in the present experiment.

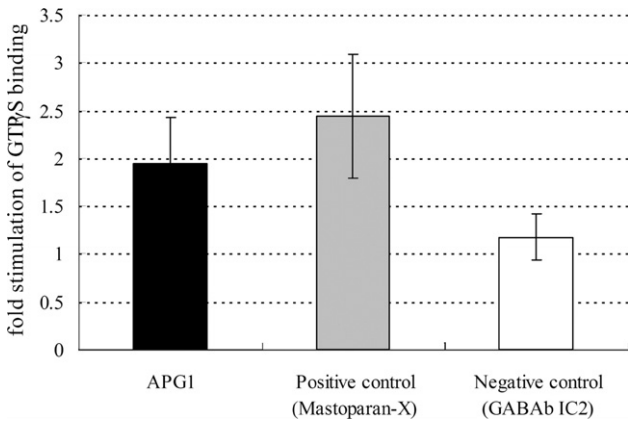

Fig. 2. The effects of specific receptor segments on G_i protein activation of GTPγS binding activity. The GTPγS binding activity was expressed as a ratio of the control buffer that contained 10 nM G_i protein in the absence of sample peptides. GTPγS binding activity of G_i protein in the presence of each 10 μM peptide (positive control: Mastopran-X, negative control: GABAb IC2). The data represent means ± SE of three experiments.

standard solid-phase reaction procedures. The used protected amino acid derivatives, piperidine and *N*-methyl pyrrolidone, were purchased from Wako Pure Chemical Industries. The crude reaction products were purified by the reverse phase HPLC. Purity and molecular weight of the peptide were confirmed by ion-spray mass spectrometry on a Perkin-Elmer SCIEX API III mass spectrometer.

GTPγS binding assay. GTPγS binding to 10 nM G_i proteins was measured in 20 mM Tris buffer (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 25 mM $MgCl_2$, 1 μM [^{35}S]-GTPγS, and peptides at 30 °C. The total amount of G_i protein was determined to be 10 nM, based on the measurement of maximal GTPγS binding in the presence of 1 μM GTPγS and 25 mM $MgCl_2$ at 30 °C [10].

NMR spectroscopy. NMR spectra of 2 mM peptide at 298 K were measured on a Bruker AM-600 (600 MHz) and Varian INOVA 600 spectrometers. The peptide was dissolved in 100 mM SDS-d_{25} micelles in 95% H_2O, 5% D_2O (v/v), and 50 mM phosphate buffer (pH 6.2). Two-dimensional COSY [11], TOCSY [12], and NOESY [13] spectra were recorded. The NOESY spectra were observed in the standard pulse schemes with mixing time of 150 and 500 ms. The mixing times for TOCSY with MLEV-17 spin-lock pulse were 25 and 50 ms. In all experiments, water peaks were suppressed by presaturation. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an external reference of ^{1}H chemical shifts. Typical data size for all experiments was 2048 × 512. All the NMR data were processed with NMR Pipe [14] and analyzed with the PIPP program [15].

Restrained molecular dynamics calculation. NOE restrains were classified into three categories: strong, medium, and weak corresponding to the distance restraints of 1.8–2.8, 1.8–3.5, and 1.8–5.0 Å, respectively. Distance Geometry calculations were carried out with CNS program (version 1.1) [16]. The calculations were performed in three divided stages. In the first stage, 4000 random peptide structures were generated. In the second stage, high-temperature dynamics were carried out to correct residual distance violation. In the third stage, the temperature was decreased from 3000 to 100 K through 20 K steps, and the structure was energy-minimized under the NOE constraints. The final twenty lowest energy structures were analyzed by using the program MOLMOL [17].

Results

GTPγS binding activity of G_i protein induced by the peptide APG1 has been examined *in vitro* in order to investigate how APG1 receptor peptide activates G_i protein directly. Fig. 2 shows the GTPγS binding activity of G_i protein by APG1. Positive control is Mastopran-X [18], which is a wasp venom, peptide and negative control is a peptide corresponding to the IC2 region of human GABAb receptor, GABAb IC2. Results clearly show that the IC3 peptide of APG1 activates G_i protein by increasing the GTPγS binding.

To investigate the structural requirement for G_i protein activation by APG1 peptide, NMR measurements were carried out with APG1 peptide in SDS micelles solution. There are a few NOE cross peaks at the N-terminal part, while $(i, i+3)$ and $(i, i+4)$ NOE cross peaks are observed at the central and C-terminal part. These NOE cross peaks were assumed to reflect the existence of a helical structure in APG1 peptide. The lowest-energy structure of APG1 peptide from CNS calculation is shown in Fig. 3A1. The structural statistics for the twenty lowest-energy structures of 4000 calculated structures are shown in Table 1. The RMSD values were calculated by using the program MOLMOL. This peptide has an α-helix in the C-terminal part. The basic amino acid residues (R1, K4, R5, R8, and R11) are located on one side of the molecules. Electrostatic potential surface of APG1 peptide shows that one side of the molecular surface is covered with the positive charges.

Discussion

From present GTPγS binding assay, we found that G_i protein was directly activated by the peptide corresponding to the predicted IC3 region of human APG1 receptor. The fact of G_i protein activation by the IC3 peptide of APG1 receptor agrees with previously reported results of peptides of human β2-adrenergic receptor [5] and prostaglandin EP3α receptor [6]. These peptides are similar in size.

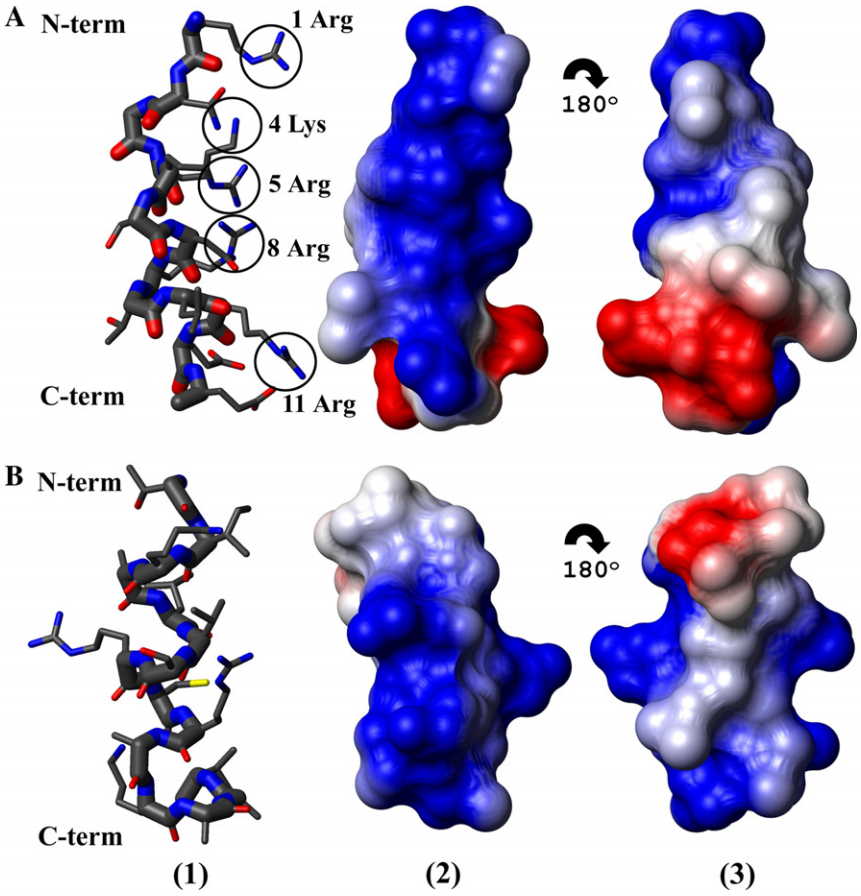

Fig. 3. The lowest-energy structures and electrostatic potential surfaces of (A) APG1 and (B) EP3a peptides. (1) Stick models of peptides. In the model, a thick line represents the backbone of APG1, and thin lines are side chains. The colors in the model are the followings; carbon atoms: dark gray, oxygen atoms: red, nitrogen atoms: blue, a sulfur atom: yellow. In the APG1 structure, side chains of basic amino acid are in the circle. (2) Electrostatic potential model of surfaces of peptides, (3) Hundred and eighty degrees rotation models of (2). Blue parts show positive charge, and red parts show negative charge.

Table 1
Structural statistics for the final 20 structures of APG1

The number of restraints	
All NOE distance restraints	116
Sequential	48
Medium and long range	68
$(i-i+2, i-i+3, i-i+4)$	
Deviations from idealized covalent geometry[a]	
Bonds (Å)	0.0107 ± 0.00012
Angles (°)	1.4191 ± 0.0127
Improper (°)	0.8136 ± 0.0131
Mean coordinate RMSD from mean structure	
Overall structure	
Backbone heavy atoms	0.44 ± 0.16 Å
All heavy atoms	1.23 ± 0.32 Å

[a] The values are given as mean ± standard deviations for the ensemble of the 20 final solution structures.

Therefore, the IC3 loop region of several G protein-coupled receptors would have relevance to G protein activation.

The present NMR structures showed that APG1 peptide in SDS micelles could take a helical conformation (Fig. 3A). At the N-terminal part, basic amino acids are located on one side of the helical surface, and positive charge cluster is formed. A peptide EP3a (TIKALVSRCRAKAAV), corresponding to the N-terminal region in IC3 of prostaglandin EP3α receptor, has also similar positive charge cluster on one side of the helical surface [6] (Fig. 3B).

APG1 is a basic amino acid-rich peptide. Five residues of basic amino acids are contained in 13 residues of amino acids of APG1 (38%). Also in EP3a, four residues of basic amino acids are contained in 15 amino acids (27%). These basic side chains form positive charge cluster on one side of the helical surfaces of receptor IC3 peptides. On the other hand, the surface of G protein has been reported to be covered with negative charge [19]. Therefore, the observed positive charge cluster of the both peptides will interact with the negative charge region of G protein by electrostatic complementation.

The APG1 peptide takes a helical structure. Similarly Mastopran-X and EP3a, which also activates G_i protein, take a fixed helical structure at the N-terminal (Fig. 4). A peptide derived from rat angiotensin II AT_{1A} receptor also forms a short helical region [20]. In the human adrenergic receptor G protein activation site was specified at the third intracellular loop and helical positive charged cluster was

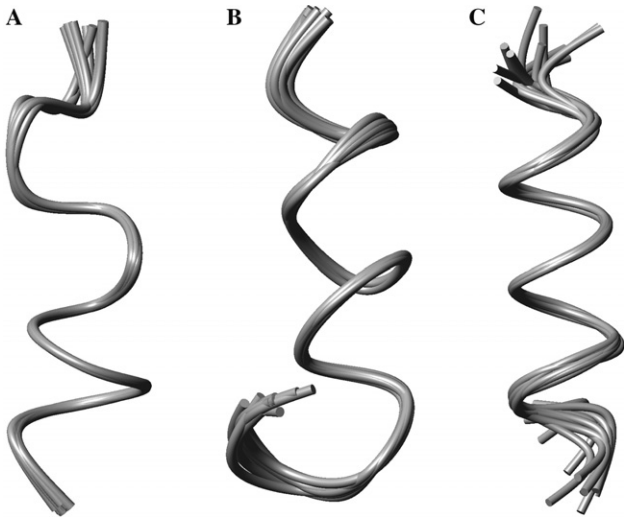

Fig. 4. Overlay tube representation of 20 lowest-energy structures of three peptides: (A) APG1, (B) EP3a, and (C) Mastopran-X.

formed [21]. These results imply that the activation sites of receptors are the limited small region. The next problem is to consider the molecular mechanism for regulation of the receptor-induced G protein activation. Corresponding to our study, when Negishi et al., truncated the long C-terminal loop, G protein was activated by the receptor constitutively without any ligand stimulation [22–25]. The results will make us to imagine that the C-terminal loop structure would prevent the G protein activating site of a receptor, such as IC3 region in our study, from G protein activation during resting stage. Our study may help to clarify the molecular mechanism of G protein activation induced by GPCRs.

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References

[1] Y.J. Lin, L. Seroude, S. Benzer, Extended life-span and stress resistance in the *Drosophila* mutant *methuselah*, Science 282 (1998) 943–946.
[2] J. Bockaert, J.P. Pin, Molecular tinkering of G protein-coupled receptors: an evolutionary success, EMBO J. 18 (1999) 1723–1729.
[3] A.G. Gilman, G proteins: transducers of receptor-generated signals, Biochemistry 56 (1987) 615–649.
[4] D.R. Sibley, J.L. Benovic, M.G. Caron, R.J. Lefkowitz, Regulation of transmembrane signaling by receptor phosphorylation, Cell 48 (1987) 913–922.
[5] T. Okamoto, Y. Murayama, Y. Hayashi, M. Inagaki, E. Ogata, I. Nishimoto, Identification of a Gs activator region of the beta 2-adrenergic receptor that is autoregulated via protein kinase A-dependent phosphorylation, Cell 67 (1991) 723–730.
[6] T. Kikkou, O. Matsumoto, T. Ohkubo, Y. Kobayashi, G. Tsujimoto, NMR structure of an intracellular loop peptide derived from prostaglandin EP3alpha receptor, Biochem. Biophys. Res. Commun. 345 (2006) 933–937.
[7] T. Hirokawa, S. Boon-Chieng, S. Mitaku, SOSUI: classification and secondary structure prediction system for membrane proteins, Bioinformatics 14 (1998) 378–379.
[8] G. von Heijne, Membrane protein structure prediction, hydrophobicity analysis and the positive-inside rule, J. Mol. Biol. 225 (1992) 487–494.
[9] E.L. Sonnhammer, G. von Heijne, A. Krogh, A hidden Markov model for predicting transmembrane helices in protein sequences, Proc. Int. Conf. Intell. Syst. Mol. Biol. 6 (1998) 175–182.
[10] J.K. Northup, M.D. Smigel, A.G. Gilman, The guanine nucleotide activating site of the regulatory component of adenylate cyclase. Identification by ligand binding, J. Biol. Chem. 257 (1982) 11416–11423.
[11] K. Nagayama, A. Kumar, K. Wüthrich, R.R. Ernst, Experimental techniques of two-dimensional correlated spectroscopy, J. Magn. Reson. 40 (1980) 321–334.
[12] A. Bax, D.G. Davis, MLEV-17 based two-dimensional homonuclear magnetization transfer spectroscopy, J. Magn. Reson. 65 (1985) 355–360.
[13] G. Bodenhausen, H. Kogler, R.R. Ernst, Selection of coherence transfer pathways in NMR pulse experiments, J. Magn. Reson. 58 (1984) 370–388.
[14] F. Delaglio, S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, A. Bax, NMR pipe: a multidimensional spectral processing system based on UNIX pipes, J. Biomol. NMR 6 (1995) 277–293.
[15] D.S. Garrett, R. Powers, A.M. Gronenborn, G.M. Clore, A common sense approach to peak picking two-, three- and four-dimensional spectra using automatic computer analysis of contour diagrams, J. Magn. Reson. 95 (1991) 214–220.
[16] A.T. Brunger, P.D. Adams, G.M. Clore, W.L. DeLano, P. Gros, R.W. Grosse-Kunstleve, J.S. Jiang, J. Kuszewski, M. Nilges, N.S. Pannu, R.J. Read, L.M. Rice, T. Simonson, G.L. Warren, Crystallography & NMR System, a new software suite for macromolecular structure determination, Acta Crystallogr. D 54 (1998) 905–921.
[17] R. Koradi, M. Billeter, K. Wüthrich, MOLMOL: a program for display and analysis of macromolecular structures, J. Mol. Graphics 14 (1996) 51–55.
[18] K. Wakamatsu, A. Okada, T. Miyazawa, M. Ohya, T. Higashijima, Membrane-bound conformation of mastoparan-X, a G-protein-activating peptide, Biochemistry 31 (1992) 5654–5660.
[19] D.G. Lambright, J. Sondek, A. Bohm, N.P. Skiba, H.E. Hamm, P.B. Sigler, The 2.0 A crystal structure of a heterotrimeric G protein, Nature 379 (1996) 311–319.
[20] L. Franzoni, G. Nicastro, T.A. Pertinhez, M. Tato, C.R. Nakaie, A.C.M. Paiva, S. Schreier, A. Spisni, Structure of the C-terminal fragment 300–320 of the rat angiotensin II AT1A receptor and its relevance with respect to G-protein coupling, J. Biol. Chem. 272 (1997) 9734–9741.
[21] A. Okuda, O. Matsumoto, M. Akaji, T. Taga, T. Ohkubo, Y. Kobayashi, Solution structure of intracellular signal-transducing peptide derived from human-adrenergic receptor, Biochem. Biophys. Res. Commun. 291 (2002) 1297–1301.
[22] H. Hasegawa, M. Negishi, A. Ichikawa, Two isoforms of the prostaglandin E receptor EP3 subtype different in agonist-independent constitutive activity, J. Biol. Chem. 271 (1996) 1857–1860.
[23] A. Ichikawa, M. Negishi, H. Hasegawa, Three isoforms of the prostaglandin E receptor EP3 subtype different in agonist-independent constitutive Gi activity and agonist-dependent Gs activity, Adv. Exp. Med. Biol. 433 (1997) 239–242.
[24] M. Negishi, H. Hasegawa, A. Ichikawa, Prostaglandin E receptor EP3gamma isoform, with mostly full constitutive Gi activity and agonist-dependent Gs activity, FEBS Lett. 386 (1996) 165–168.
[25] H. Hizaki, H. Hasegawa, H. Katoh, M. Negishi, A. Ichikawa, Functional role of carboxyl-terminal tail of prostaglandin EP3 receptor in Gi coupling, FEBS Lett. 414 (1997) 323–326.