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Biochemical characterization of a heterotrimeric G_i-protein activator peptide designed from the junction between the intracellular third loop and sixth transmembrane helix in the m4 muscarinic acetylcholine receptor

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ABSTRACT

Muscarinic acetylcholine receptors (mAChRs) are G-protein coupled receptors (GPCRs) that are activated by acetylcholine released from parasympathetic nerves. The mAChR family comprises 5 subtypes, m1–m5, each of which has a different coupling selectivity for heterotrimeric GTP-binding proteins (G-proteins). m4 mAChR specifically activates the $G_{i/o}$ family by enhancing the guanine nucleotide exchange factor (GEF) reaction with the Gα subunit through an interaction that occurs via intracellular segments. Here, we report that the m4 mAChR mimetic peptide m4i3c(14)Gly, comprising 14 residues in the junction between the intracellular third loop (i3c) and transmembrane helix VI (TM-VI) extended with a C-terminal glycine residue, presents GEF activity toward the G_{i1} α subunit (Gαi1). The m4i3c(14)Gly forms a stable complex with guanine nucleotide-free Gαi1 via three residues in the VTI(L/F) motif, which is conserved within the m2/4 mAChRs. These results suggest that this m4 mAChR mimetic peptide, which comprises the amino acid of the mAChR intracellular segments, is a useful tool for understanding the interaction between GPCRs and G-proteins.

1. Introduction

G protein-coupled receptors (GPCRs) play an important role in a wide variety of physiological processes and pathophysiological conditions by mediating numerous cellular signaling cascades [1,2]. Members of the GPCR superfamily share a molecular architecture comprising seven transmembrane α-helices (TM) connected by three extracellular and three intracellular loops (i1c–i3c), which create binding pockets for extracellular signals, including photons of light, small molecules, hormones, peptides, lipids, and proteins, and enable coupling to heterotrimeric GTP binding-proteins (G-proteins) that are composed of Gα, Gβ, and Gγ subunits [3]. GPCR crystal structures provide important information for enhancing human health as well as advancing fundamental biological science [4]. The crystal structure of the β_2-adrenergic receptor (β_2AR) in complex with the G_s-protein revealed that the coupling interface with G_s-protein is generated by i2c, TM5, and TM6 [5]. However, the consensus sequence for the specificity of G_s-coupling is not clear from the sequence analysis of other GPCRs. Furthermore, the Gα subunit of the G_s-protein in complex with β_2AR is in a guanine nucleotide-free state, with the structure inducing large displacement of the α-helical domain. Although these structural observations were assumed to require a structural change in the Gα subunit for releasing GDP from an inactive state, the precise mechanism by which the GPCR induces structural change in the G-protein remains poorly understood.

Muscarinic acetylcholine receptors (mAChRs) were first GPCRs to be purified from cerebral membranes and reconstituted with purified G-protein in lipid vesicles [6,7]. mAChRs are expressed in both the peripheral (PNS) and central (CNS) nervous systems and comprise five subtypes, m1–m5 [8]. The m1, m3, and m5 subtypes couple with the $G_{q/11}$ family of G-proteins, whereas the m2 and m4 subtypes couple with the $G_{i/o}$ family of G-proteins [9]. The chimeric mAChRs generated from these subtypes that possess different

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selectivities for G-proteins were a useful tool for understanding how GPCRs distinguish between different G-proteins. Indeed, the mutational analysis of the m2 and m3 subtypes of mAChRs led to the identification of two, four amino acid motifs, VTIL and AALS, located at the i3c loop/TM-VI junction, for the specific recognition of the $G_{i/o}$-protein and the $G_{q/11}$-protein, respectively [10,11].

In addition to identification of the motifs underlying the selectivity of G-proteins using mutational analysis, biochemical studies using short synthetic peptides corresponding to the intracellular segments of GPCRs supported the requirement of the intracellular short segments for direct interaction with G-proteins [12–14]. We previously reported the activation of G_q-protein by the peptide fragment m3i3c, a mimic of human m3 mAChR, comprised of the 16 amino acid residue corresponding to the i3c loop/TM-VI junction [15]. Mimetic peptides of the intracellular segment, involved in the coupling of GPCRs with the specific G-proteins, are useful in analyzing the molecular mechanism by which GPCRs recognize the different subtypes of G-proteins. Here, we report the biochemical characterization of the mimetic peptide m4i3c(14)Gly, corresponding to the i3c loop/TM-VI junction in m4 mAChR, which displays GEF activity toward Gαi1. We found that m4i3c(14)Gly forms a stable complex with the guanine nucleotide free state of Gαi1. Furthermore, mutational experiments indicated that two residues in the VTIF motif, Ile402 and Phe403, play an essential role in the complex formation with Gαi1. Our observation provides a novel molecular insight into the selectivity of GPCRs for G-protein, which involves the i3c loop/TM-VI junction.

2. Materials and methods

2.1. Sample preparation

Glutathione *S*-transferase (GST)-fused Gαi1 (GST-Gαi1) was expressed in *Escherichia coli* BL21 Star (DE3) cells and purified as previously described [16]. The expression construct for the Maltose Binding Protein (MBP)-fused Gαi1 (MBP-Gαi1) encoded the Gαi1 amino acid sequence (residues 30–354) and was expressed in pMAL-c2x (New England Biolabs) using the restriction enzyme recognition sites EcoRI and SalI. The nucleotide fragment encoding ubiquitin and the i3c loop/TM-VI junction (residue 394–407) of m4 mAChR was inserted into pET19b (Novagen) using the restriction enzyme sites NdeI and SalI. Twenty milliliters of an overnight culture of *E. coli* BL21 Star (DE3) cells, transformed with pMAL-c2x-Gαi1 and pET19b-His-ubi-m4i3c(14)Gly, was inoculated into 2.5 L Luria–Bertani (LB) medium containing 0.5% glycerol, 0.2% α-lactose, 0.05% D-glucose and 50 μg/ml ampicillin or kanamycin. These recombinant proteins were expressed at 25 °C overnight.

The cells expressing the MBP-Gαi1 were disrupted by sonication at 4 °C. The supernatant was applied onto an amylose resin column (New England Biolabs). The fractions eluted using 50 mM Na HEPES buffer (pH 7.5) containing 150 mM NaCl, 1 mM dithiothreitol (DTT), and 20 mM maltose were loaded onto a HiTrap Butyl sepharose HP column (GE Healthcare) and washed with 20 mM Na HEPES buffer (pH 7.5) containing 1 M $(NH_4)_2SO_4$, 1 mM DTT. MBP-Gαi1 was eluted using a linear $(NH_4)_2SO_4$ concentration gradient (1.0–0 M). His-ubi-m4i3c(14)Gly was solubilized in 20 mM Tris-acetate buffer (pH 8.0) containing 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol and loaded onto the Ni-sepharose column (GE Healthcare). The Ni-sepharose column was washed with 20 mM Tris-acetate buffer (pH 8.0) containing 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol, and 20 mM imidazole. The His-ubi-m4i3c(14)Gly was eluted with 20 mM Tris-acetate buffer (pH 8.0) containing 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol, and 0.5 M imidazole. The purified proteins were concentrated using a Vivaspin 20 (Vivascience).

2.2. Determination of GDP bound to Gαi1

[α-^{33}P]GDP-bound Gαi1 (3.5 μM) was incubated in 20 mM Tris (pH 7.4), 0.1 M EGTA, 0.1 mM DTT, 100 μM GDP, and 10% glycerol, in the absence and presence of 100 μM m4i3c(14)Gly, at 25 °C. At the indicated times, 50 μL aliquots were supplemented with AlF_3 to terminate the nucleotide dissociation reaction, and the bound radioactivity was determined on a liquid scintillation counter [17].

2.3. Complex formation and pull-down assay of Gαi1 with m4ic3(14)Gly

Firstly, His-ubi-m4i3c(14)Gly was refolded in the refolding buffer (50 mM Tris–HCl pH 8.0, 0.2 M NaCl, 1 mM DTT and 0.4 M arginine). To generate the Gαi1/m4i3c(14)Gly complex, a solution containing an equal molar ratio (16 μM) of GST- or MBP-Gαi1 and refolded His-ubi-m4i3c(14)Gly was prepared and incubated overnight at 30 °C in the pull-down buffer (50 mM Tris–HCl buffer, pH 8.0, 0.2 M NaCl, 1 mM DTT), containing 4 mM $CaCl_2$, 0.4 M arginine, and 50 mU apyrase (New England Biolabs). These complexes were precipitated using glutathione sepharose 4B (GE Healthcare) and amylose resin, which had been equilibrated with the pull-down buffer. The complex bound to resin was washed five times with 1 ml pull-down buffer and eluted using the pull-down buffer containing 10 mM glutathione or 20 mM maltose. The amount of GST- or MBP-Gαi1 and bound His-ubi-m4i3c(14)Gly in each eluted solution was determined using SDS-PAGE.

2.4. Purification of the GST-Gαi1/His-ubi-m4ic3(14)Gly complex

Forty milliliters of the GST-Gαi1/His-ubi-m4i3c(14)Gly complex was applied to the glutathione sepharose 4B column and eluted using 50 mM Tris–HCl buffer (pH 8.0) containing 0.2 M NaCl, 1 mM DTT, and 10 mM glutathione. Fractions were pooled and then concentrated using a Vivaspin 20. Further purification of the GST-Gαi1/His-ubi-m4i3c(14)Gly complex was performed using a HiPrep sephacryl S-500 16/60 column (GE Healthcare). The protein concentration was determined using a pierce BCA protein assay kit (Thermo Scientific).

2.5. GDP determination

The concentration of GST-Gαi1 was determined from the band area in SDS-PAGE, measured by imaging software ImageJ [18]. The GDP bound to GST-Gαi1 alone and in complex with His-ubi-m4i3c(14)Gly was released from 80 μg of GST-Gαi1 by thermal denaturation at 95 °C for 20 min. The denatured proteins were removed by centrifugation at 17,400× *g* for 10 min at room temperature. The supernatants were applied to a HiTrap Q HP column, equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing 10 mM NaCl. GDP bound to the anion exchange column was eluted using a liner NaCl concentration gradient (10–500 mM). GDP was detected using ultraviolet absorbance at 254 nm.

2.6. Trypsin protection analysis

GST-Gαi1 (6 μM) alone and in complex with His-ubi-m4i3c(14)Gly was mixed with trypsin to a final concentration of 60 nM and incubated at 4 °C for 5, 10 and 25 min. For each time point, a 10 μL solution was mixed in SDS-PAGE loading buffer and separated using SDS-PAGE. The proteolytic fragments were visualized by Coomassie brilliant blue staining with EzStain Aqua (Atto, Japan).

2.7. Dynamic light scattering analysis

Dynamic light scattering measurements were carried out using a Zetasizer Nano (Malvern Instruments Ltd.). Protein solutions were centrifuged at 17,400× *g* for 10 min to remove debris. All measurements were performed using a 50 μL quartz cuvette filled with the 50 μL protein solution at 20 °C. Size distributions were calculated from the intensity data acquired at regular time intervals.

3. Results

3.1. m4i3c(14)Gly shows GEF activity towards Gαi1

m4i3c(14)Gly comprises 14 residues (residues 394–407), corresponding to the i3c/TM-VI junction of m4 mAChR and an additional C-terminal glycine residue (Fig. 1A). This glycine residue was added to increase the solubility of the peptide without perturbing the GEF activity of the peptide (to be reported elsewhere). The VTIL motif within this region plays a key role in the selectivity of G-protein coupling (Fig. 1B) [10]. m4i3c(14)Gly displays high similarity with m2 mAChR, which specifically activates subtypes of Gαi and Gαo. In contrast, the Gαq specific mAChRs m1, m3 and m5 have an AALS motif in place of the VTI(L/F) motif. Structural modeling of m4i3c(14)Gly, based on the crystal structure of the active form of m2 mAChR (PDB ID: 4MQS), revealed that the VTIF motif creates a hydrophobic surface on one side of the α-helix (Fig. 1C) [19]. To test the GEF activity of m4i3c(14)Gly, we measured the GDP dissociation rate of Gαi1 in the absence and presence of m4i3c(14)Gly (Fig. 1D). The GDP dissociation rate of Gαi1 in the presence of m4i3c(14)Gly was markedly enhanced, 18.3 fold compared to activity in absence of m4i3c(14)Gly. These results suggest that the m4i3c(14)Gly peptide designed from m4 mAChR acts as a mimetic activator for Gαi1.

3.2. Complex formation of Gαi1 with m4i3c(14)Gly

To analyze the effect of m4i3c(14)Gly on the GEF reaction of Gαi1, we attempted to crystallize the complex of Gαi1 with m4i3c(14)Gly. The crystals were obtained from the complex solution mixed in a 1:5 M ratio (0.3 mM GDP-bound form of Gαi1 and 1.5 mM m4i3c(14)Gly) (Supplementary Fig. S1A). Structural analysis based on X-ray diffraction data at a resolution of 3.30 Å revealed no residual electron density corresponding to the m4i3c(14)Gly; however, residual electron density corresponding to the guanine nucleotide, GDP, was observed in the interface between the helical domain and the Ras-like GTPase domain (Supplementary Fig. S1B). These findings suggest that GDP, when dissociated from Gαi1, decreases the stability of the complex containing the nucleotide free form of Gαi1 and m4i3c(14)Gly by inhibiting the Gαi1/m4i3c(14)Gly complex, through rebinding to the nucleotide free form of Gαi1 in complex with the m4i3c(14)Gly. Therefore, the stabilization of the Gαi1/m4i3c(14)Gly complex in solution provides a clue to understanding the binding mode of Gαi1 to the GPCR-mimetic activator peptide.

It has previously been reported that a complex of the nucleotide free form of the Gα subunit with a GPCR can be prepared using the

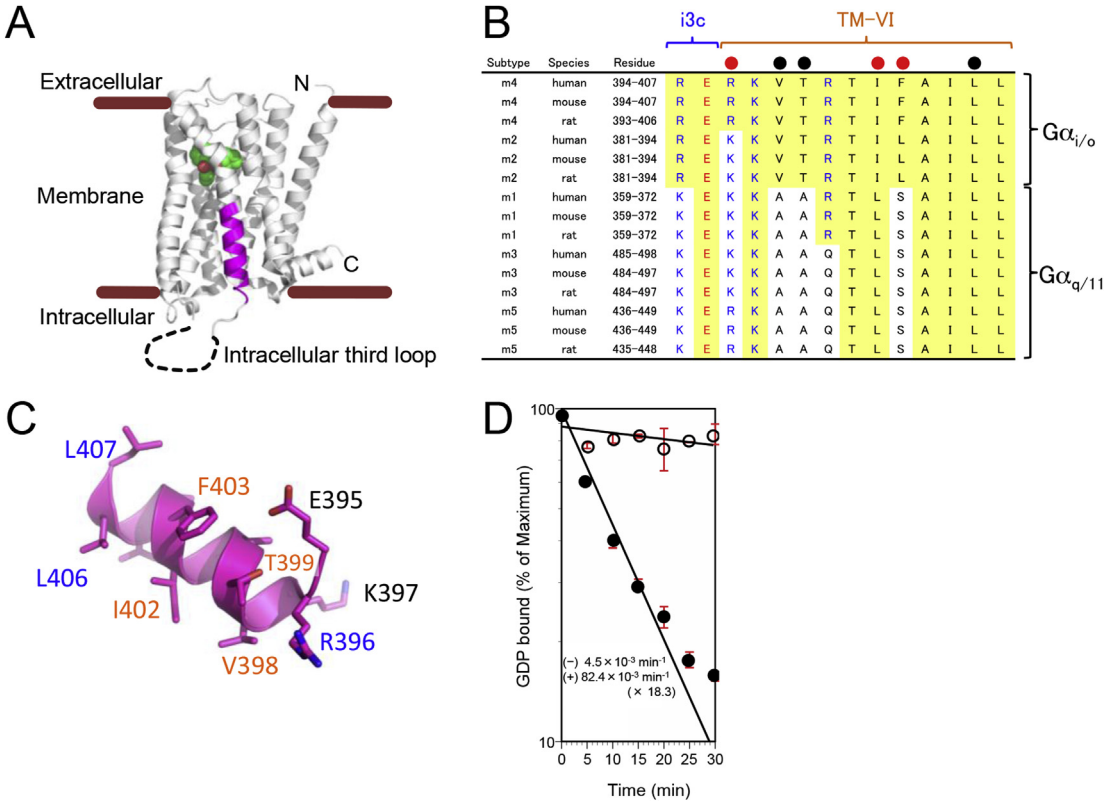


Fig. 1. Structure, amino acid sequence and GEF activity of the m4i3c(14)Gly peptide. (A) Ribbon representation of the structure of m2 mAChR bound to 3-quinuclidinyl-benzilate (QNB). The QNB molecule is drawn in a space-filling model colored in green and red. The i3c/TM-VI junction is colored in magenta. The disordered region of i3c is shown in a black broken line. (B) Amino acid sequence alignment of the i3c/TM-VI junction of mAChRs. The mutations, which caused a reduction in or had no effect on binding affinity to Gαi1, are indicated by red and black circles, respectively. (C) The structure of the m4i3c peptide modeled from the crystal structure of m2 mAChR. The residue numbers colored in orange and blue annotate the VTIF motif and the residues that form a molecular interface with the VTIF motif, respectively. (D) GDP release from Gαi1 in the presence and absence of m4i3c(14)Gly. [α-^{33}P]GDP bound to Gαi1 was exchanged with cold GDP at a release rate of 82.4 × 10^{-3} min^{-1} in the presence of 100 μM m4i3c(14)Gly, whereas the [α-^{33}P]GDP release was not detected in the absence of m4i3c(14)Gly.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nucleotide hydrolase apyrase, which hydrolyzes GDP, releasing it from the Gα subunit bound to the GPCR (Fig. 2A) [5]. However, the Gαi1/m4i3c(14)Gly complex was found to be insoluble when GDP-bound Gαi1 was mixed in an equal molar ratio with m4i3c(14)Gly in the presence of apyrase (Supplementary Fig. S2A and Fig. S2B). To improve the solubility of the Gαi1/m4i3c(14)Gly complex, the large N-terminal tagged proteins, GST- or MBP-Gαi1 and hexahistidine-ubiqutin (His-ubi)-fused m4i3c(14)Gly were used for complex formation. We found that complexes of GST- or MBP-Gαi1 with His-ubi-m4i3c(14)Gly were solubilized in 10 mM HEPES buffer (pH 7.5) containing 150 mM NaCl, 1 mM DTT and 4 mM $CaCl_2$ (Fig. 2B). The complexes between GST- or MBP-Gαi1 and His-ubi-m4i3c(14)Gly could be purified with affinity resin columns, using amylose resin and glutathione sepharose, respectively (Fig. 2C and Supplementary Fig. S2C). These results show that GDP hydrolysis by apyrase in the reactive solution facilitated the formation of a stable complex of Gαi1 with the GPCR-mimetic activator peptide.

3.3. *Physicochemical characterization of the Gαi1/m4i3c(14)Gly complex*

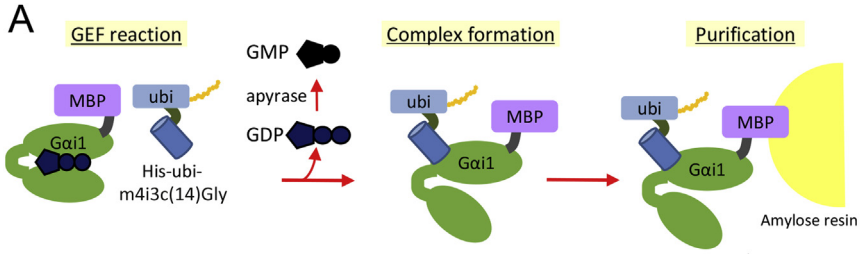
To determine whether Gαi1 is in its nucleotide free form in complex with m4i3c(14)Gly, we performed GDP quantitation using anion exchange chromatography. GDP, which was released as a result of the thermal denaturation of the GST-Gαi1, was eluted as a single peak by a linear gradient, whereas the solution prepared by the thermal treatment of the purified GST-Gαi1/His-ubi-m4i3c(14)Gly complex showed no peak in same eluted volume (Fig. 3A). These results indicate that GST-Gαi1 in complex with His-ubi-m4i3c(14)Gly does not bind the guanine nucleotide.

Next, we tested the protease-sensitivity of Gαi1 in complex with m4i3c(14)Gly using trypsin (Fig. 3B). This proteolysis experiment showed the different protease-sensitivity of the GDP-bound form from the nucleotide free form [20]. Two major cleavage products of the GST-Gαi1 GDP bound form were detected with molecular weights of 43 kDa and 14 kDa, which were predicted to correspond to the GST-fused α-helical domain and the Ras-like GTPase domain, respectively. In contrast, GST-Gαi1 bound to His-ubi-m4i3c(14)Gly did not show such major degradation fragment. Weak trypsin degradation fragments were observed, in a molecular weight range of 43–30 kDa, suggesting that GST-Gαi1 in complex with His-ubi-m4i3c(14)Gly has a higher trypsinolysis sensitivity, which is similar to the nucleotide free form of Gαi1.

The nucleotide free form of Transducin (Gtα) was previously characterized as a misfolded, rather than denatured, with a low affinity for the guanine nucleotide and arranged into globular particles approximately 16 nm in diameter with an apparent molecular weight of 2.4 MDa [21]. Using dynamic light scattering (DLS), the molecular weight of the oligomer formed by the GST-Gαi1/His-ubi-m4i3c(14)Gly complex was estimated to be 17.8 MDa, approximately 350 times greater than the molecular mass of the GST-Gαi1/His-ubi-m4i3c(14)Gly complex of 1:1 stoichiometry (Fig. 3C). Similarly, the MBP-Gαi1/His-ubi-m4i3(14)Gly complex forms an oligomer with an apparent molecular weight of 8.5 MDa, indicating that oligomerization of the Gαi1/m4i3c(14)Gly complex cannot be improved by the substitution of the N-terminal GST tag to an MBP tag (Fig. 3C). These results strongly suggest that the nucleotide free form of Gαi1 in complex with m4i3c(14)Gly exists as an oligomer with a misfolded structure, which is similar to the nucleotide free form of Gtα.

3.4. *Determinant residue in m4i3c(14)Gly for Gαi1-binding*

Finally, we attempted to identify the residue in m4i3c(14)Gly involved in Gαi1-binding using a pull-down assay. Based on the α-helical model of m4i3c(14)Gly as shown in Fig. 1C, we posited that the molecular surface of the helix of m4i3c(14)Gly, formed by the VTIF motif and two other residues, Arg396 and Leu406, is involved in Gαi1-binding. Nine mutant proteins were generated in which each residue was replaced with an alanine or aspartate. Compared with the wild-type protein, three alanine mutants (Arg396, Ile402 and Phe403) displayed a significant reduction in Gαi1-binding affinity (Fig. 4). Similar results were obtained for the aspartate mutants of Ile402 and Phe403. In contrast, three alanine mutants (Val398, Thr399 and Leu406) did not show any detectable reduction in binding affinity. However, the Val398 aspartate mutant did



B

	m4i3c(14)Gly	His-ubi-m4i3c(14)Gly
Gαi1	Insoluble	Insoluble
GST-Gαi1	Insoluble	Soluble
MBP-Gαi1	NA	Soluble

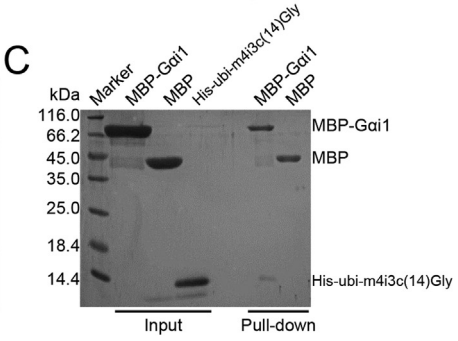


Fig. 2. Pull-down assay of the Gαi1-m4i3c(14)Gly complex. (A) Pull-down assay scheme of the Gαi1-m4i3c(14)Gly complex. Apyrase was added into the reaction mixture to hydrolyze the GDP dissociated from Gαi1. Finally, MBP-Gαi1 bound to the His-ubi-m4i3c(14)Gly was purified using amylose affinity resin. (B) The improvement to the solubility of the Gαi1-m4i3c(14)Gly complex using large affinity tagging proteins. The solubility test suggested that the combination of the GST- or MBP-fused Gαi1 and His-ubi-m4i3c(14)Gly enhances the solubility of the complex. (C) Pull-down assay of MBP-Gαi1 with His-ubi-m4i3c(14)Gly.

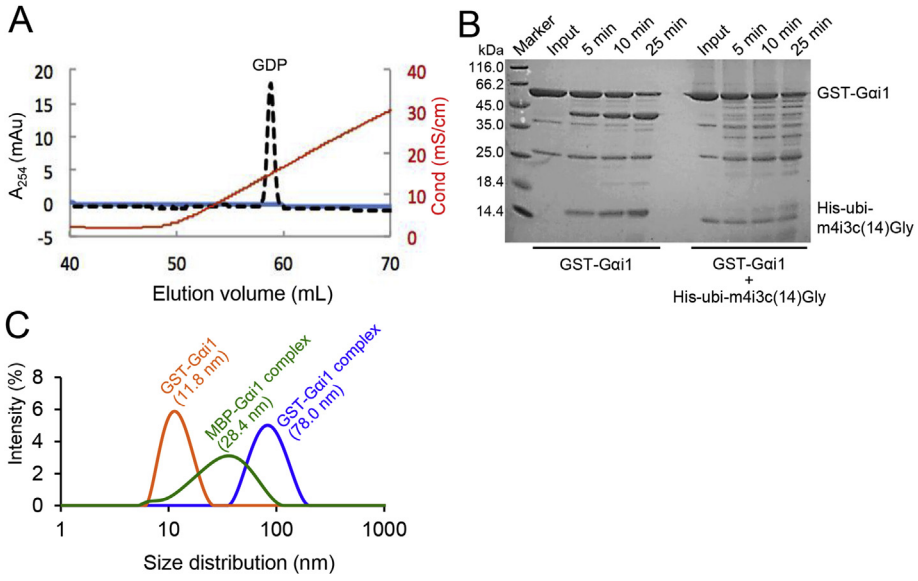


Fig. 3. GDP quantitation, trypsin protection and DLS analysis of the Gαi1-m4i3c(14)Gly complex. (A) GDP quantitation of GST-Gαi1 alone and in complex with His-ubi-m4i3c(14)Gly using anion exchange chromatography. The blue solid and black broken lines of the chromatogram show the GDP elution profiles from GST-Gαi1 alone and in complex with His-ubi-m4i3c(14)Gly, respectively. The red line indicates conductivity of the eluate. (B) Trypsin protection analysis of the GST-Gαi1 alone and in complex with His-ubi-m4i3c(14)Gly. The samples of three different time points were subjected to SDS-PAGE analysis. (C) The DLS measurement of GST-Gαi1 alone and in complex with His-ubi-m4i3c(14)Gly.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

show a reduction in Gαi1-binding, suggesting that this residue is located close to the Gαi1-binding interface. Overall, these results indicate that three residues in m4i3c(14)Gly, Arg396, Ile402 and Phe403, have a critical role in stable complex formation with Gαi1. Interestingly, two residues (Thr399 and Leu406), when replaced with alanine, showed a slight increase in Gαi1-binding affinity. Therefore, suggesting that these residues play a key role in the specific selection of Gα subtypes.

4. Discussion

Biochemical analysis of the m4 mAChR mimetic peptide m4i3c(14)Gly has provided novel molecular insight into the binding preferences of GPCRs with heterotrimeric G-proteins. Our findings, which show that m4i3c(14)Gly forms a stable complex

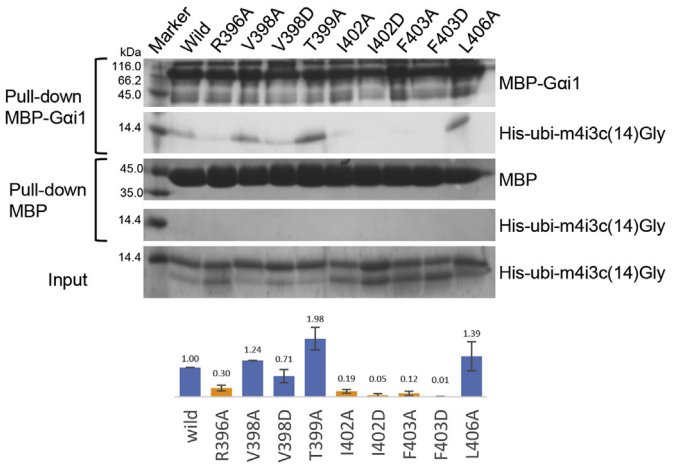


Fig. 4. Pull-down assay of Gαi1 with m4i3c(14)Gly mutants. The pull-down assay of mutant His-ubi-m4i3c(14)Gly with MBP-Gαi1 suggests a critical role for Arg396, Ile402 and Phe403 in complex formation with Gαi1. The relative ratio of His-ubi-m4i3c(14)Gly to precipitated MBP-Gαi1 is shown at the bottom of the figure.

with the nucleotide free state of Gαi1, suggest that the intracellular segment corresponding to m4i3c(14)Gly in m4 mAChR has a central role in promoting the structural change necessary for GDP release in the GEF activity to Gαi1, through direct interaction. However, compared with the complete m4 mAChR molecule, m4i3c(14)Gly displays low GEF activity to Gαi1, which is consistent with the GEF activity of other synthetic peptides derived from intracellular segments of GPCRs, such as α_2AR, β_2AR, and m3 mAChR [12,13,15]. These results highlight the importance of other intracellular segments, such as i2c, in the coupling selectivity of mAChRs. In fact, substitution of the intracellular segments or amino acid residues of m3 mAChR with those from m2 mAChR has revealed that four hydrophilic residues in the i2c of m3 mAChR are required for the selective activation of the Gq-protein [11].

Our binding study using mutants of m4i3c(14)Gly revealed that two residues in the VTIF motif, Ile402 and Phe403, which are homologous to m2 mAChRs, play an important role in complex formation with Gαi1. In the structures of agonist and antagonist-bound m2 mAChR, the residues of m2 mAChR, corresponding to Ile402 and Phe403 of m4 mAChRs, contact the hydrophobic residues of TM-V [19,22]. Comparison with β_2AR in complex with G_s-protein suggests that the Ile389 side chain of m2 mAChR, corresponding to Ile402 of m4 mAChR, creates a coupling interface with the C-terminal helix of Gα [23]. In contrast, Leu390 of m2 mAChR, corresponding to Phe403 of m4 mAChR, does not participate in the cytoplasmic interface with Gαi1, as it is located on the surface and its side chain is exposed to plasma membrane. Although the role of the VTIF motif of m4 mAChR in directly binding to Gαi1 is unclear, our results suggest that the VTIF motif, included in the mimetic peptide structure, has a crucial role in GEF activity towards Gαi1 and complex formation.

In the present study, we have shown that Gαi1 in complex with m4i3c(14)Gly is in its nucleotide free form, creating a high molecular weight oligomer. The oligomerization of Gαi1 in the nucleotide free state is consistent with a previous report, in which the nucleotide free form of the Gα subunit misfolded and formed a high molecular weight polymer in solution [21]. Although we can not exclude the possibility that His-ubi-m4i3c(14)Gly is involved in the

oligomerization of the complex with Gαi1, the oligomer of the nucleotide free Gαi1 bound to His-ubi-m4i3c(14)Gly may be formed using the complementary surface of the Ras-like GTPase domain with the α-helical domain of Gαi1 in another complex.

Single-particle electron microscopy analysis of the β_2AR-Gs complex has shown the position of the α-helical domain of nucleotide free Gαs in complex with β_2AR is highly variable [24]. However, the association between the two domains of Gαs in complex with β_2AR and Gβγ may be prevented by interactions with β_2AR and Gβγ, restricting the spatial arrangement of the subdomains of the Gα subunit. Thus, our observations suggest that the oligomerization of the nucleotide free Gαi1 with His-ubi-m4i3c(14)Gly may be a result of inhibition of the steric effect of GPCR and Gβγ.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.018.

Transparency document

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