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Novel Features of Amphiphilic Peptide Mas7 in Signalling via Heterotrimeric G-Proteins

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Abstract: Amphiphilic peptide Mas7, a structural analogue of mastoparan is a known activator of heterotrimeric G_i-proteins and its downstream effectors. This study investigated the functional interaction of Mas7 with a plasma membrane protein from CHO cells, the endogenous mono-ADP-ribosyltransferase. The substrate of endogenous mono-ADP-ribosyltransferase was the ADP-ribosylated protein with a molecular mass of 36 kDa, which corresponded to the β subunit of heterotrimeric G-proteins. The effect of Mas7 on endogenous mono-ADP-ribosyltransferase activity was in the micromolar range with a maximal activation of 205% over the basal. In pertussis treated plasma membranes, it was found that the effect of Mas7 on endogenous mono-ADP-ribosyltransferase was partially blocked, which suggests the involvement of G-proteins, such as G_i or G_0. In addition, an immunoassay was developed for the visualization of interaction between the α subunit and the $\beta\gamma$ dimer of G-protein on a Ni-NTA support. The physical interaction was tested of Mas7 with the heterotrimeric G-protein α_{i2} subunit, which was overexpressed together with $\beta_1\gamma_2$–His^6 subunits in sf9 cells. An interaction between G_{i2} heterotrimer and Mas7 was not observed, which was not in accordance with previously reported results of mastoparan obtained for G_i-proteins from bovine brain. In conclusion, the signal is mediated from Mas7 to endogenous mono-ADP-ribosyltransferase via pertussis sensitive G-proteins. Furthermore, it is hypothesized that G_{i2} G-proteins are not involved in the process.

Keywords: heterotrimeric G-proteins; Mas7; immunoblotting; ADP-ribosylation; sf9; CHO cells

INTRODUCTION

Heterotrimeric guanine nucleotide-binding proteins (G-proteins) serve as plasma membrane-bound transducers that connect receptors to effectors and thus to intracellular signalling pathways [1]. G-proteins are made up of three polypeptide subunits, α, β and γ, the α subunit acting as an enzyme that binds and hydrolyses GTP. The β and γ subunits form a dimer which dissociates only when denatured. When G-proteins are signalling, in essence they function as two separate units as the signal can be communicated either by the α subunit or the $\beta\gamma$ dimer. In mammals, there are currently known 20α, 7β and 11γ subunits [2] and on the basis of sequence similarities of their α subunits, G-proteins are grouped into four subfamilies, G_s, $G_{i/0}$, G_q and G_{12} [3].

The functions of G-proteins are primarily regulated by association of GTP with the α subunit, hydrolysis of GTP to GDP and P_i, and dissociation of GDP [4,5]. Besides these low molecular weight regulators of G-protein function, G-proteins are activated by other non-receptor activators such as AlF_4^-, non-hydrolysable analogues of GTP [4], some hydrophobic amines or amphiphilic peptides [6].

Abbreviations: CHO, Chinese hamster ovary; FAK, focal adhesion kinase; PTX, pertussis toxin.

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Among these peptides, only the mastoparan (amino acid sequence: INLKALAALAKKIL**)**, a cationic-amphiphilic tetradecapeptide toxin isolated from wasp venom is well documented in the literature. It has a wide variety of cellular effects, including the induction of insulin [7], serotonin [8] and histamine secretion [9]. All these mastoparan effects have been linked to the activation of heterotrimeric G-proteins [10], small GTP-binding protein rho/rac [11], ATP and GTP-degrading nucleotidases [12], guanylyl cyclase [13], nucleoside diphosphate kinase [14], phospholipase C [15] and phospholipase A_2 [16]. The effect of mastoparan on heterotrimeric G-proteins is well characterized on the molecular level. Therefore, mastoparan functionally and structurally mimics G-protein coupled receptors activation of G_i and G_0 proteins [10] via specific interaction with the carboxyl terminus of the α subunit [17].

To further investigate the actions of amphiphilic mastoparans, Mas7 (amino acid sequence: INLKALAALAKALL), a structural analogue of mastoparan obtained by substituting alanine for lysine in position 12 and leucine for isoleucine in position 13 [10], has been used in this study. These substitutions produced an enhancing effect on GDP/GTP exchange of the G-protein α-subunit, which is five-fold more potent than the effect of mastoparan [10]. Mas7 evokes rapid up-regulation of FAK tyrosine phosphorylation [18] and modulation of K_{ATP} channels [19] via activation of G-proteins, elevates cytosolic Ca^{2+} through membrane pore formation [20] and elicits an alteration in lipid composition [21]. The downstream effectors of Mas7 have been less frequently characterized and are linked to a broad range of effects of mastoparan. This study investigated the functional interaction of Mas7 with a plasma membrane protein from CHO cells, the endogenous mono-ADP-ribosyltransferase [22], and analysed the involvement of PTX sensitive G-proteins in this process. In addition, an immunoassay was developed for visualization of the physical interaction between the α subunit and the $\beta\gamma$ dimer on a Ni-NTA support and tested the interaction of Mas7 with heterotrimeric G-protein of α_{i2} overexpressed together with $\beta_1\gamma_2$-His^6 dimer in sf9 cells.

MATERIALS AND METHODS

Materials

Sf9 and Chinese hamster ovary cells (CHO-K1) were obtained from ECACC, UK. [^{32}P]NAD^+, ESL kit and Dextran 500 were supplied from Amersham Pharmacia Biotech, UK. Hank's balanced salt solution (HBSS) was provided by Gibco, UK and fetal bovine serum was from Biochrom (Germany). Dulbecco's modified Eagle's medium (DMEM), acrylamide/bis-acrylamide and other electrophoresis reagents were from Eurobio, France. Nitrocellulose transfer membranes were from Schleicher and Schuell, Germany. Ultima Gold scintillation liquid was from Packard, USA. Polyethylene glycol 4000 was from Merck (USA). The Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose beads were from Qiagen, USA. Rabbit polyclonal anti-α_{i2} and anti-β (T-20) were from Santa Cruz Biotechnology (USA). Goat anti-rabbit IgG horseradish peroxidase conjugates and Mas7 were from Calbiochem, USA. Baculovirus vectors with the alpha subunit of heterotrimeric G-proteins G_{i2}, and $\beta_1\gamma_2$-His^6 dimer were a generous gift from Dr Gilman (Texas, USA). All other chemicals were from Sigma Aldrich Co, USA and were of analytical grade.

Recombinant Viruses

Baculovirus vectors carrying the α subunit of heterotrimeric G-proteins G_{i2}, and $\beta_1\gamma_2$-His^6 subunits tagged by hexahistidine on *N*-terminus of γ_2 are available from various laboratories.

Cell Cultures

Sf9 cells were maintained as a monolayer culture at 28 °C in TNM-FH insect medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.035% $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml gentamicin.

Chinese hamster ovary (CHO) cells were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 0.39% $NaHCO_3$, 58 mg/l proline, 53 mg/l aspartatic acid, 59 mg/l glutamatic acid, 60 mg/l asparagine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere.

Expressing Recombinant Proteins in sf9 Cells

Sf9 cells were co-transfected with recombinant Baculoviruses vectors carrying the α subunit of G_{i2} heterotrimeric G-proteins together with $\beta_1\gamma_2$-His^6 subunits, as previously described [23], but with minor modifications. In essence, 60%–70% confluent cells per 100 cm dish were infected with

high titre recombinant Baculovirus stock solution. After 60 min of incubation at 28 °C the virus stock was diluted 3-fold with a fresh medium, then the cells were incubated for another 48 h. The expression of α_{i2} and $\beta_1\gamma_2$-His^6 in plasma membranes was analysed by 11% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto nitrocellulose membrane and followed by ECL immunoblot analysis using specific anti-α_{i2} and anti-β antibodies (1 : 1000, Santa Cruz).

Plasma Membrane Preparation

After expressing a recombinant G-protein α_{i2} subunit together with $\beta_1\gamma_2$ dimer, sf9 cells were harvested by scraping and processed for plasma membranes according to the protocol [24], but with minor modifications. Cells (approximately 22.5×10^6) were centrifuged at $1000 \times \mathbf{g}$ for 10 min and suspended with 2.25 ml of ice-cold lysis buffer (50 mM NaHepes, pH = 8.0, 0.1 mM EDTA, 3 mM $MgCl_2$, 100 mM NaCl, 10 μM GDP, 10 mM β-mercaptoethanol and protease inhibitors 500 μM O-fenantrolin, 2 μM pepstatin in 1 mM phenylmethylsulphonyl fluoride). Cells were lysed by Teflon/glass Potter homogenizer and followed by sonification for 1 min. Cell lysates were centrifuged at $750 \times \mathbf{g}$ for 10 min. The supernatant was centrifuged at $100\,000 \times \mathbf{g}$ for 60 min. The resultant pellet was then suspended in wash buffer (50 mM NaHepes, pH = 8.0, 3 mM $MgCl_2$, 50 mM NaCl, 10 μM GDP, 10 mM β-mercaptoethanol and protease inhibitors as above) and stored at −80 °C. All operations were carried out at 4 °C. The protein concentration in membrane preparations was 2.0 mg/ml as determined by Biorad Protein Assay at 595 nm.

CHO plasma membranes were prepared as described previously [22], but again with minor modifications. Briefly, cells (approximately 2×10^8) were washed in Hanks' balanced salt solution (HBSS) buffer without Ca^{2+} and Mg^{2+}, detached by hypotonic buffer containing 10 mM TES (pH 7.5) and 1 mM EDTA. Everything was carried out at 4 °C. Detached cells were homogenized in Teflon/glass Potter homogenizer and centrifuged at $300 \times \mathbf{g}$ for 10 min. Crude membranes were collected from the supernatant by a 20 min spin at $42\,000 \times \mathbf{g}$. The pellet membranes were resuspended in 10 mM TES (pH 7.5) containing 0.25 M sucrose, followed by purification of plasma membranes with an aqueous two-phase system with dextran and polyethylene glycol. Crude membranes of 1 ml were mixed with 2.72 g of 20% Dextran 500, 1.156 g of 40% polyethylene glycol 4000, 800 μl of 1 M sucrose, 2.124 ml of deionized water, 200 μl 0.2 M potassium phosphate buffer (pH 7.4). After continuous mixing in dextran and polyethylene glycol two-phase system for 15 min, the crude membranes were centrifuged at $2500 \times \mathbf{g}$ for 20 min. The upper dextran phase was transferred into the aqueous two-phase system and extracted for 15 min. After the second extraction, the crude membranes were centrifuged at $2500 \times \mathbf{g}$ for 20 min, the upper phase containing plasma membranes, was removed and combined with 5 volumes of 10 mM TES (pH 7.5) buffer containing 0.25 M sucrose, and centrifuged at $48\,000 \times \mathbf{g}$ for 35 min. The pellet was suspended in 25 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA and protease inhibitors 500 μM O-fenantroline, 2 μM pepstatine and 1 mM phenylmethylsulphonyl fluoride were added. The concentration of proteins in the plasma membranes was determined by Biorad Protein Assay at 595 nm.

Ni-NTA Assay

Plasma membranes (200 μg of proteins/sample) from sf9 cells were incubated for 15 min at 37 °C with 50 μM GDPβS or 50 μM GTPγS and centrifuged at $100\,000 \times \mathbf{g}$ for 60 min. The rest of the procedure was undertaken at a temperature below 4 °C. Membranes in the pellet were diluted to 5 mg/ml with a wash buffer supplemented with 1% sodium cholate, containing fresh proteinase inhibitors. The membrane proteins were solubilized for 60 min with constant stirring prior to a centrifugation at $100\,000 \times \mathbf{g}$ for 40 min. The supernatant was collected and 40 μl of it was incubated with 20 μl of Ni-NTA beads for 16 h. After incubation the supernatant was removed (30 μl of it was used for further analysis) by centrifugation at $500 \times \mathbf{g}$ for 5 min. Ni-NTA beads were washed three times with 10 volumes of buffer A (20 mM NaHepes, pH = 8.0, 1 mM $MgCl_2$, 100 mM NaCl, 10 μM GDP, 10 mM β-mercaptoethanol, 0.5% $C^{12}E^{10}$, 5 mM imidazole). After the last washing the Ni-NTA beads were diluted in 100 μl Laemmli buffer, boiled and analysed by 11% SDS-PAGE. The proteins contained in the gel were transferred onto nitrocellulose membrane by electroblot. This procedure was followed by ECL immunoblot analysis using specific anti-α_{i2} and anti-β antibodies (1 : 1000, Santa Cruz). In the experiment with Mas7 the protocol was modified: 50 μM of peptide was incubated for 15 min at 37 °C with the plasma membranes, followed by the second incubation for 15 min at 37 °C with 50 μM GDPβS.

Immunoblotting Analysis

After the run on SDS-PAGE (16 cm long), the proteins were transferred from the gel onto nitrocellulose membrane with a constant current of 400 mA for 5 h. The blot was first incubated for 1 h in blocking buffer (2% bovine serum albumin in TBS; 20 mM Tris-HCl, 500 mM NaCl; pH = 7.5). The blocking buffer was then replaced by a buffer TTBS (0.05% Tween 20 in TBS) and incubated with the primary polyclonal rabbit IgG anti-α_{i2} (1 : 1000, Santa Cruz) or anti-β antibodies (T20, 1 : 1000, Santa Cruz) at room temperature for 2 h. The primary antibodies were washed three-times in TTBS buffer for 10 min and incubated with secondary peroxidase-conjugated goat anti-rabbit IgG antibodies (1 : 1000, Calbiochem) at room temperature for another 1 h. After washing in TTBS buffer three times for 10 min and once in TBS for 10 min, the immunoreactive proteins caught on the blot were exposed for 15–60 s to Kodak X-Omat film using an ECL chemiluminescence detection kit (Amersham Pharmacia Biotech, RPN 2106).

ADP-ribosylation Assay

ADP-ribosylation followed the incorporation of [^{32}P] ADP-ribose into the β subunit of heterotrimeric G-protein and was performed with 5 µg of CHO plasma membrane preparation as described previously [25] with minor modification. The 50 µl of plasma membranes in the ADP-ribosylation buffer (50 mM potassium phosphate buffer (pH = 7.5), 0.5 mM $MgCl_2$, 4 mM dithiothreitol, 5×10^6 cpm or 2.3 µCi [^{32}P]NAD^+ (specific activity = 1000 Ci/mmol) and 6 µM β-NAD^+) were incubated at 37 °C for 1 h with and without 50 µM Mas 7.

When the experiments were performed in the presence of pertussis toxin (preactivated in 62.5 mM dithiothreitol at room temperature for 1 h), 50 µl of reaction mixture (10 µg of plasma membranes, 13 µg/ml of pertussis toxin, 50 mM Tris (pH = 8.0), 25 mM dithiothreitol, 0.5 mM EDTA, 1 mM ATP, 5×10^6 cpm[^{32}P]NAD^+, 1 mM β-NAD^+) was incubated at 37 °C for 1 h. After incubation, the reaction mixture was centrifuged at 10 000 × **g** for 15 min. The resulting pellet was washed in 100 µl of 1 M KCl at 10 000 × **g** for 15 min, resuspended in 50 µl of ADP-ribosylation buffer and manipulated as the protocol above describes [25].

The ADP-ribosylation reactions were stopped by diluting the samples with 50 µl Laemmli buffer, then boiled and analysed by 11% SDS-PAGE followed by electroblot onto nitrocellulose membrane. The radioactivity incorporated into the membrane proteins was detected and quantified by Instant Imager (Packard) or probed with specific anti-β antibodies (1 : 1000, Santa Cruz).

Other Methods

Data are expressed as the mean ± SD of at least two independent experiments. The non-linear least squares fitting of the curves and statistical analysis (Student's t-test, $p < 0.05$ was considered significant) were carried out by PRISM3 computer program (GraphPad Software, USA), which was used also for graphical presentation of the results.

RESULTS

The Effect of Peptide Mas7 on Endogenous Mono-ADP-ribosylation of G-protein β-subunit from CHO Cells

The effect of Mas7 on endogenous mono-ADP-ribosyltransferase activity in CHO plasma membranes was in the micromolar range with a maximal activation of 205% over the basal (Figure 1A and 1C). Reduced SDS-PAGE analysis showed ADP-ribosylated protein bands with a molecular mass 36 kDa, which corresponded to the β subunit. ADP-ribosylated protein bands were additionally confirmed by ECL Western blot analysis using anti-β antibodies (Figure 1B).

In pertussis toxin treated CHO plasma membranes, the effect of 100 µM Mas7 on mono-ADP-ribosyltransferase activity was decreased to 43% over the basal (Figure 2A) in comparison with the effect of Mas7 alone (Figure 1A and 1C). This could be explained by an indirect interaction of Mas7 with mono-ADP-ribosyltransferase via G_i/G_0 proteins. The expression of the β subunit during the experiment was unchanged (Figure 2B).

Expression of Recombinant α_{i2} Subunit and $\beta_1\gamma_2$-His^6 Dimer in sf9 Cells

Sf9 cells were co-transfected with recombinant Baculoviruses vectors carrying the α_{i2} subunit together with $\beta_1\gamma_2$-His^6 subunits of heterotrimeric G-proteins. The expression of G-proteins in plasma membranes from sf9 cells was analysed by SDS-PAGE, followed by protein transfer from the gel onto a nitrocellulose membrane. In contrast to the

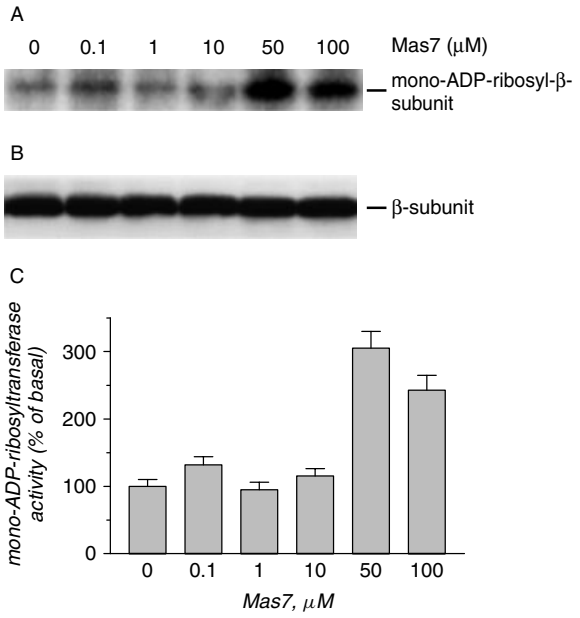

Figure 1 Effect of Mas7 on endogenous mono-ADP-ribosylation of G-protein β-subunit from CHO cells. (A) SDS-PAGE followed by protein transfer from gel onto a nitrocellulose membrane and detection of radioactivity with Instant Imager. (B) Western blot analysis of β subunit using anti-β primary polyclonal rabbit IgG antibodies. (C) Quantification of endogenous mono-ADP-ribosylation. Each point indicated with the standard deviation represents the mean value of two independent experiments. 100% = 8.1 pmol/mg.

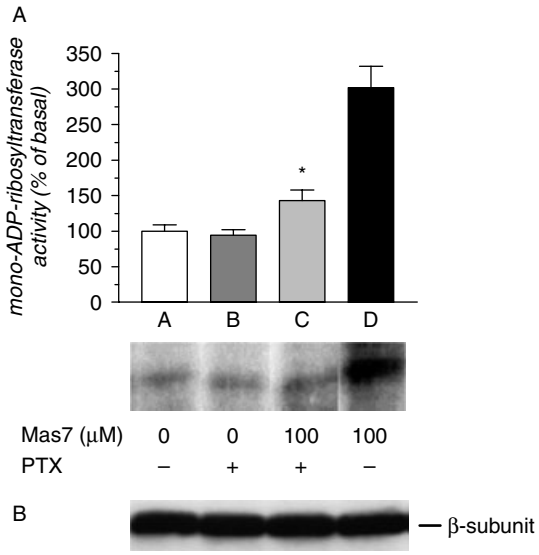

Figure 2 Pertussis toxin inhibits effect of Mas7 on endogenous mono-ADP-ribosylation of G-protein β-subunit from CHO cells. (A) Quantification of endogenous mono-ADP-ribosylation. Bars: A = no PTX, no Mas7 (basal, 100%), B = PTX, no Mas7, C = PTX, 100 μM Mas7, D = 100 μM Mas7. Lower inset: SDS-PAGE followed by protein transfer from gel onto the nitrocellulose membrane and detection of radioactivity with Instant Imager. (B) Western blot analysis of β subunit using anti-β primary polyclonal rabbit IgG antibodies. Each bar with indicated the standard deviation represents the mean value of two independent experiments. 100% = 2.1 pmol/mg. *Significantly different from basal ($p < 0.05$).

control of non-transfected cells (Figure 3A, lane 1), protein staining on nitrocellulose membrane showed strong protein bands (see arrows) with molecular mass of 36 and 41 kDa (Figure 3A, lane 2), which corresponded to the β subunit and α_i subunit of G-proteins, respectively. Overexpressed heterotrimeric proteins $G\alpha_{i2}\beta_1\gamma_2$-His^6 were further examined by immunoblotting, first with the anti-α_{i2}, followed by anti-β primary polyclonal rabbit IgG antibodies (Figure 3B, lane 2). The yield of α_{i2} was increased significantly (Figure 3A and 3B, lane 2) in comparison with non-transfected cells (Figure 3A and 3B, lane 1). The endogenous β subunit was expressed well (Figure 3A, lane 1), but less efficiently than in transfected cells (Figure 3B, lane 2).

α_{i2} Subunit Interacts with $\beta_1\gamma_2$-His^6 Dimer on Ni-NTA Support in GDPβS/GTPγS Dependent Manner

A new technique was developed for following the protein–protein interaction between the α_{i2} subunit and $\beta_1\gamma_2$-His^6 dimer of heterotrimeric G-proteins. Sf9 cells were co-transfected with α_{i2} together with $\beta_1\gamma_2$-His^6 subunits using Baculovirus vectors. Plasma membranes enriched with recombinant $G\alpha_{i2}\beta_1\gamma_2$-His^6 from sf9 cells were subjected to Ni-NTA beads, analysed by SDS-PAGE, electroblotted on to nitrocellulose membrane and followed by immunoblot, using specific anti-α_{i2} and anti-β antibodies. It has been shown that the association or dissociation of the α subunit with $\beta\gamma$ dimer is dependent on guanine nucleotides GDP and GTP, respectively [4]. In normal physiological conditions both nucleotides are relatively unstable, especially GTP which is hydrolysed to GDP and P_i, owing to the intrinsic GTPase activity of the α subunit. In order to minimize the enzymatic and spontaneous hydrolysis of GTP and GDP, their poorly hydrolysable analogues GTPγS and GDPβS were used in this experiment. To activate or deactivate the α_{i2} subunit, maximal effective concentrations of the GTPγS and GDPβS were used throughout

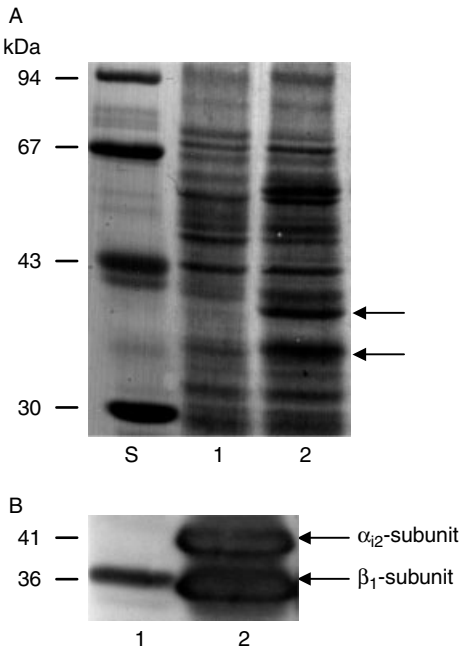

Figure 3 Expression analysis of recombinant α_{i2} and $\beta_1\gamma_2$-His^6 from sf9 cells. (A) SDS-PAGE followed by protein transfer from gel onto the nitrocellulose membrane. (B) Western blot analysis with anti-α_{i2} and anti-β primary polyclonal rabbit IgG antibodies. Lane S: low molecular weight protein markers; lane 1: plasma membranes sf9; lane 2: plasma membranes with $\alpha_{i2}\beta_1\gamma_2$-His^6; $\leftarrow \alpha_{i2}$ or $\beta_1\gamma_2$-His^6 subunits. The amount of protein loaded in each well in SDS-PAGE was 50 μg.

the study. It is clear that dimer $\beta_1\gamma_2$-His^6 binds to Ni-NTA beads (Figure 4, lanes 1 and 2) via a coordinative bond between the nickel and imidazole ring of the histidine. To demonstrate that the immobilized heterotrimer $G_{\alpha i2}\beta_1\gamma_2$-His^6 was properly folded and functional, the samples were treated with 50 μM GTPγS and 50 μM GDPβS, respectively. Depending on GDPβS and GTPγS, respectively, α_{i2} subunit associates and dissociates with $\beta_1\gamma_2$-His^6 dimer (Figure 4, lanes 1 and 2). To be certain that the system works, the amount of the α_{i2} subunit and $\beta_1\gamma_2$-His^6 dimer in the supernatant (the fraction of proteins, which remains unbound on Ni-NTA beads) was analysed. The concentration of $\beta_1\gamma_2$-His^6 dimer in the GDPβS experiment is obviously much higher in comparison with the amount of the α_{i2} subunit. The number of the binding sites for the α_{i2} subunits on Ni-NTA beads is higher than the number of α_{i2} subunits. This explains why the $\beta_1\gamma_2$-His^6 dimer remains free in the supernatant and the α_{i2} subunit disappears from it. The situation in our experiment with GTPγS is inverted. The α_{i2} subunit fully dissociated from $\beta_1\gamma_2$-His^6 dimer on the Ni-NTA beads and it was released from Ni-NTA beads into the supernatant. Therefore, the α_{i2} subunit from plasma membranes treated with GDPβS was not present in the supernatant (Figure 4, lane 3), but it remained in the sample with GTPγS (Figure 4, lane 4). Heterotrimer $G_{\alpha i2}\beta_1\gamma_2$ is functionally active on NI-NTA support, because its α_{i2} subunit interacts with $\beta_1\gamma_2$-His^6 dimer in GDPβS or GTPγS dependent manner.

Peptide Mas7 does not Affect Interaction between α_{i2} Subunit and $\beta_1\gamma_2$-His^6 Dimer

The amphiphilic peptide Mas7, a known activator of G_i G-proteins, was tested in Ni-NTA assay. The experiment was taken under the same conditions as described above, except that Mas7 was added prior to the addition of GDPβS. Interestingly, Mas7 did not affect the association between the α_{i2} subunit and the $\beta_1\gamma_2$-His^6 dimer (Figure 5, lane 4) compared with the control (Figure 5, lane 3). Moreover, in samples without GDPβS, Mas7 did also not alter the interaction of α_{i2} subunit and $\beta_1\gamma_2$-His^6 dimer (Figure 5, lane 2) in comparison with the control (Figure 5, lane 1) in spite of the fact that the α-subunit binds to $\beta\gamma$ more weakly and associates

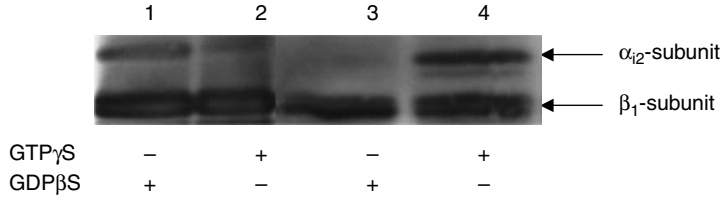

Figure 4 Western analysis of recombinant α_{i2} and $\beta_1\gamma_2$-His^6 from sf9 cells using anti-α_{i2}, and anti-β primary polyclonal rabbit IgG antibodies. Lane 1: plasma membranes with $\alpha_{i2}\beta_1\gamma_2$-His^6 plus 50 μM GDP$\beta$S on Ni-NTA beads; lane 2: plasma membranes with $\alpha_{i2}\beta_1\gamma_2$-His^6 plus 50 μM GTP$\gamma$S on Ni-NTA beads; lane 3: plasma membranes with $\alpha_{i2}\beta_1\gamma_2$-His^6 plus 50 μM GDP$\beta$S in supernatant; lane 4: plasma membranes with $\alpha_{i2}\beta_1\gamma_2$-His^6 plus 50 μM GTP$\gamma$S in supernatant.

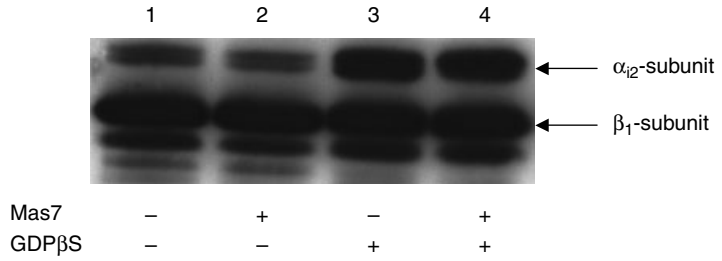

Figure 5 Effect of Mas7 on recombinant $\alpha_{i2}\beta_1\gamma_2$-His^6 from sf9 cells on Ni-NTA support followed by Western analysis using anti-α_{i2}, and anti-β primary polyclonal rabbit IgG antibodies. Lane 1: plasma membranes with $\alpha_{i2}\beta_1\gamma_2$-His^6; lane 2: plasma membranes with $\alpha_{i2}\beta_1\gamma_2$-His^6 plus 50 μM Mas7; lane 3: plasma membranes with $\alpha_{i2}\beta_1\gamma_2$-His^6 plus 50 μM GDP$\beta$S; lane 4: plasma membranes with $\alpha_{i2}\beta_1\gamma_2$-His^6 plus 50 μM GDP$\beta$S plus 50 μM Mas7.

with its dimer less efficiently (Figure 5, lane 1 versus 3).

DISCUSSION

Amphiphilic peptide Mas7, a structural analogue of mastoparan has been linked to the activation of some cellular proteins such as heterotrimeric G-proteins [10], focal adhesion kinase [18] and K_{ATP} channels [19]. The latter two effectors are up-regulated by these G-proteins and not by direct interaction of Mas7 with an effector. The study tested interaction of Mas7 with endogenous mono-ADP-ribosyltransferase, a plasma membrane enzyme from CHO cells. It has been reported that enzymatic mono-ADP-ribosylation involves the transfer of the ADP-ribose moiety from NAD^+ to arginine residue of β-subunit of heterotrimeric G-proteins in CHO cells [22].

Mas7 increased mono-ADP-ribosylation (Figure 1A) of the β-subunit (Figure 1B) for more than three-fold. Similar binding affinity of Mas7 in micromolar range to endogenous mono-ADP-ribosyltransferase as obtained in our case (Figure 1C) was also observed with other effector systems linked with $[Ca^{2+}]_i$ mobilization and formation of IP_3 [20]. The activation effect of Mas7 could be due to the direct interaction of the peptide with endogenous mono-ADP-ribosyltransferase or the indirect one via G-proteins as observed in other tested effector systems. To test this hypothesis additional experiments were performed with pertussis toxin treated CHO plasma membranes. In this experiment Mas7 was able to increase endogenous mono-ADP-ribosyltransferase activity only up to 1.5-fold in comparison with the basal activity without a ligand (Figure 2A). This could explain the indirect interaction of Mas7 with the endogenous mono-ADP-ribosyltransferase via pertussis toxin-sensitive G-proteins, such as G_i or G_o. However, it is notable in our study that the effect of Mas7 on endogenous mono-ADP-ribosyltransferase was not completely attenuated by pertussis toxin (Figure 2A), suggesting that the residual component was mediated by G_q/G_{11}-proteins or even by the direct interaction of Mas7 with the effector. The idea of G_i mediated activation of endogenous mono-ADP-ribosyltransferase is additionally supported by a report of Higashijima *et al.* Therefore, the effect of Mas7 with a similar binding affinity to endogenous mono-ADP-ribosyltransferase as obtained in our study correlated with the binding affinity of Mas7 to heterotrimeric G_i-proteins [6]. In conclusion, we hypothesize the model in which Mas7 interacts with the heterotrimeric $\alpha\beta\gamma$ form of G_i/G_0 proteins. Heterotrimeric complex dissociates into the α subunit and the $\beta\gamma$ dimer. Free $\beta\gamma$ dimer and its β subunit is now the best substrate for the modification with endogenous mono-ADP-ribosyltransferase from CHO cells. In spite of the fact that Mas7 affects downstream effectors via G-proteins [18, 19] its direct interaction with mono-ADP-ribosyltransferase is not excluded.

Mastoparan has been widely used to prime G-protein activation in a receptor-independent manner with various cell types [see reference 20]. Although the priming mechanism is still unclear, this agent appears to interact with the carboxyl terminus of the α subunit [17] of G_i and G_0 proteins [6]. To our knowledge, the molecular mechanism of interaction between Mas7 and the heterotrimeric G-proteins has not yet been reported. Therefore, the interaction of Mas7 with the heterotrimer $G_{\alpha i2}\beta_1\gamma_2$ was tested. Functional interaction between α_{i1}, but not α_{i2}, subunit and $\beta_1\gamma_2$ dimer has already been shown on

anti-FLAG affinity column using an overexpressed system of sf9 cells [26]. In the Ni-NTA assay with overexpressed alpha subunit α_{i2} and $\beta_1\gamma_2$-His^6 dimer (Figure 3A and 3B), it was demonstrated that the heterotrimer is functional and properly folded because its subunits, α and $\beta\gamma$, interact with each other in a GDPβS/GTPγS dependent manner (Figure 4). Interestingly, Mas7 did not affect the dissociation of α_{i2} subunit from $\beta_1\gamma_2$-His^6 (Figure 5, lane 3 versus 4). The inability of the peptide to accelerate dissociation of α_{i2} from $\beta_1\gamma_2$-His^6 could be due to a very strong association between both subunits in a GDPβS-liganded heterotrimer. To examine that hypothesis, an identical experiment to that shown in Figure 5, lanes 3 and 4, was prepared adding a low concentration of GDP (Figure 5, lanes 1 and 2). The rate of association between α_{i2} and $\beta_1\gamma_2$-His^6 was lower in GDP-liganded compared with GDPβS-liganded heterotrimer. In the GDPβS free system, Mas7 was unable to promote dissociation of α_{i2} from $\beta_1\gamma_2$-His^6 (Figure 5, lane 1 versus 2). The result was very surprising since its relative mastoparan effects GTPase activity of different G_i purified from bovine brain [17] and stimulates GDP/GTP exchange from different α subunits of G_i from rabbit liver [6]. Prior kinetic reports document the ability of Mas7 to stimulate GDP/GTP exchange from the mixture of α subunits of G_{i1} and G_{i3} heterotrimers [6]. There has been no kinetic or proteomic data about the interaction of Mas7 with $G_{\alpha i2}\beta_1\gamma_2$ heterotrimers so far. However, we are well aware that additional experiments, such as positive control with α subunits of G_{i1} or G_{i3}, should be performed in Ni-NTA assay, and are needed to insure the fact that Mas7 does not affect the $G_{\alpha i2}\beta_1\gamma_2$. It is likely that Mas7 does not affect the G_{i2} in comparison with mastoparan, in spite of the homology in their primary and secondary structures [6]. There are some peptides with completely different primary structures, such as peptitergent PD1, but show similar effects on G-proteins as mastoparan [27]. On the contrary, there are peptides, such as Mas17, with nearly identical amino acid sequences as mastoparan, but with totally different physiological effects [6].

In summary, the ability of Mas7 to activate endogenous mono-ADP-ribosyltransferase from CHO cells via pertussis sensitive G-proteins has been shown. However, the interaction between G_{i2} heterotrimer and Mas7 was not observed. Further studies are needed to clarify the complex action of Mas7 on different types of G_i-proteins and its effect on endogenous mono-ADP-ribosyltransferase *in vivo*.

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