RESEARCH COMMUNICATION

Production, processing and partial purification of functional G protein $\beta\gamma$ subunits in baculovirus-infected insect cells

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As a result of the inability to resolve the heterogeneous mixture of G protein $\beta\gamma$ subunits present in tissues, it has not been possible to compare different $\beta\gamma$ subunits of the G proteins in terms of their proposed roles in receptor–effector coupling. This study was undertaken to establish the utility of the baculovirus expression system in producing homogeneous $\beta\gamma$ subunits of defined composition for the comparative analysis of these subunits in reconstitution systems. In this study we report the expression, and appropriate post-translational processing, of recombinant β_2 , γ_2 and γ_3 subunits. In addition, we show that the recombinant $\beta\gamma$ subunits can be readily purified, and can functionally interact with the α subunits of the G proteins.

INTRODUCTION

Guanine-nucleotide-binding regulatory proteins (G proteins) couple various types of receptors to specific effectors in cells (for review, see [1]). The G proteins are composed of an α subunit, which is capable of binding and hydrolysing GTP, and $\beta\gamma$ subunits. While structural diversity of the α subunits may provide the requisite specificity of receptor–G-protein and G-protein–effector interactions, a growing body of evidence indicates that structural diversity of the β subunits and, particularly, the γ subunits, also exists, and that this diversity may contribute to the specificity of these interactions. At least three β subunits and three γ subunits have been identified on the basis of their amino acid similarities, as deduced from the sequences of cDNAs. The β_1 [2,3], β_2 [4,5], and β_3 [6] cDNAs have been shown to encode 36, 35 and 36 kDa proteins respectively, while the γ_1 [7], γ_2 [8,9] and γ_3 [10] cDNAs encode 8, 6 and 7 kDa proteins. Although the possible functional diversity of multiple forms of the β and γ subunits has yet to be elucidated, recent evidence suggests that a mixture of $\beta\gamma$ subunits functions to regulate directly the activity of one or more effectors, including phospholipase A_2 [11,12], type II adenylate cyclase [13,14], muscarinic-receptor kinase [15] and Ca^{2+} -ATPase [16]. There is also evidence to indicate that the $\beta\gamma$ subunits function to anchor the G protein in the membrane and that the newly identified post-translational modifications of the γ subunit are required for this function [17,18]. In addition, the $\beta\gamma$ subunits may contribute to the specificity of the G-protein–receptor interaction, since previous studies have shown that the $\beta\gamma$ subunits are required for this interaction [19,20]. Thus the G protein $\beta\gamma$ subunits may play a more active role in receptor–effector coupling than was originally thought.

Because of the lack of a method to resolve the heterogeneous mixtures of $\beta\gamma$ subunits that are present in a wide variety of tissues, it has not been possible to compare the different $\beta\gamma$ subunits in terms of any of these proposed functions. To circumvent this problem, the present study was undertaken to establish the utility of the baculovirus expression system to produce homogeneous $\beta\gamma$ subunits of defined composition that could be used for the comparative analysis of these proteins in reconstitution studies. Of particular interest is that this system

has been reported to carry out a number of post-translational modifications, including prenylation and carboxymethylation [21,22], that are needed for the appropriate processing of the γ subunit. In this study we report the expression of recombinant β_2 , γ_2 and γ_3 subunits in baculovirus-infected insect cells, and demonstrate the appropriate post-translational processing of the recombinant γ subunits. In addition, we report the partial purification of functional recombinant $\beta\gamma$ subunits from these cells.

MATERIALS AND METHODS

Infection of *Spodoptera frugiperda* (Sf9) cells with recombinant baculoviruses

The coding regions of the human β_1 [3], the human β_2 [4], the bovine γ_2 [8] and the bovine γ_3 [10] cDNAs were subcloned into pVL1392 or pVL1393 vectors, the cDNAs from these vectors were transferred to wild-type *Autographa californica* nuclear-polyhedrosis-virus genome, and the resulting recombinant viruses were plaque-purified [23]. The recombinant viruses were used to infect Sf9 cells, grown in monolayers or spinners at 27 °C as described by Summers & Smith [23]. The cells were harvested 72 h after infection, and lysed in 20 mM-Hepes (pH 8.0)/2 mM- $MgCl_2$ /1 mM-EDTA (HME) containing 0.1 mM-phenylmethanesulphonyl fluoride (PMSF) by passage through a 27-gauge needle. The cell lysates were centrifuged at 100 000 g for 30 min to yield cytosolic and particulate fractions. When indicated, the particulate fraction was solubilized with 0.9 % sodium cholate or 0.5 % Lubrol, and then re-centrifuged to yield soluble and insoluble particulate fractions. The cytosolic, soluble particulate and insoluble particulate fractions were monitored for recombinant expression of β and γ subunits by immunoblotting.

Immunoblotting assay

The cytosolic, soluble particulate and insoluble particulate fractions from infected and non-infected cells were resolved by SDS/PAGE followed by transfer to nitrocellulose for immunoblotting [24]. To obtain subunit-specific antibodies, the β -common peptide, KTREGNVRVSREL, the γ_2 -specific peptide, DPLLTVPASENPF, and the γ_3 -specific peptide, KGET-

Abbreviations used: Sf9 cells, *Spodoptera frugiperda* cells; PMSF, phenylmethanesulphonyl fluoride; MVA, mevalonolactone; MM, methylmethionine.

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Fig. 1. Time course of G protein β_2 and γ_2 subunit expression and subcellular distribution in Sf9 cells

A total of 2.5×10^6 cells were infected with the γ_2 and/or β_2 viruses. At 24, 48 or 72 h after infection, the cells were fractionated to yield the cytosolic (C) and particulate (P) fractions. To monitor expression of the recombinant γ_2 and β_2 subunits, 15% of the C and P fractions were resolved by SDS/PAGE and transferred to nitrocellulose for immunoblotting. The blot was exposed to film for 18 h. (a) γ_2 -infected cells, (b) β_2 -infected cells, (c) $\gamma_2\beta_2$ -infected cells.

PVNSTMSIGQAR, corresponding to the predicted sequences for these subunits [2–10], were synthesized. The production and characterization of these antibodies will be described elsewhere (J. C. Cali, E. Balcueva & J. D. Robishaw, unpublished work; details available on application to J.D.R.). The β antibody (GC-4), the γ_2 antibody (B-17) and the γ_3 antibody (B-53) were used at 1:200 dilutions. Antibody binding was detected with ^{125}I -labelled goat anti-rabbit F(ab')₂ fragments.

Prenylation and carboxymethylation assays

To monitor prenylation and carboxymethylation [25,26] respectively, γ_2 - or $\gamma_2\beta_2$ -infected cells were labelled with 200 μCi of [^3H]mevalonolactone ([^3H]MVA) or 100 μCi of [^3H]methylmethionine ([^3H]MM). To monitor incorporation of [^3H]MVA, the cytosolic, soluble particulate and insoluble particulate fractions from infected cells were resolved by SDS/PAGE, transferred to nitrocellulose, and the nitrocellulose was incubated with AMPLIFY solution (New England Nuclear) prior to fluorography. To monitor expression, the nitrocellulose was washed to remove fluor and blotted with the γ_2 -specific antibody. To monitor incorporation of [^3H]MM, the soluble particulate fractions from non-infected and $\beta_2\gamma_2$ -infected cells were resolved on an SDS/polyacrylamide gel. The gel was cut into 2 mm slices prior to fluorography. Subsequently each gel slice was assayed for release of volatile [^3H]methanol following base hydrolysis [27].

Purification of recombinant $\beta\gamma$ subunits expressed in Sf9 cells

The $\beta\gamma$ -infected cells were lysed in HME buffer containing 10 μg of PMSF/ml, and centrifuged to obtain the particulate fraction. After solubilization with 0.5% Lubrol, the soluble particulate fraction was applied to an α_0 -agarose column as described by Mumby *et al.* [28]. Elution of $\beta\gamma$ subunits from the α_0 -agarose column was monitored by silver staining and immunoblotting of SDS/polyacrylamide gels.

RESULTS AND DISCUSSION

Expression of β and γ subunits in Sf9 cells

Elucidation of the role of the G protein β and γ subunits in signal transduction has been hampered by the inability to resolve the heterogeneous mixtures of $\beta\gamma$ subunits that are present in a wide variety of tissues. To circumvent this problem, Sf9 insect

cells were infected with recombinant baculoviruses containing the coding sequences for the bovine γ_2 or the human β_2 gene under the control of the baculovirus polyhedrin promoter. As shown in Fig. 1(a), cells infected with the γ_2 virus expressed an appropriately sized protein of 6.5 kDa, which was specifically recognized by the anti- γ_2 antibody. The γ_2 subunit could not be detected in cells infected with the γ_2 virus at 24 h after infection (Fig. 1), or in non-infected or wild-type-infected cells (results not shown), indicating that this subunit is not normally expressed in insect cells. At 48 h the greatest amount of the recombinant γ_2 subunit was present in the cytosolic fraction, while at 72 h roughly equal amounts of the γ_2 subunit were detected in the cytosolic and particulate fractions. The existence of cytosolic and particulate forms of the recombinant γ_2 subunit represents incomplete processing, as discussed below. As shown in Fig. 1(b), cells infected with a recombinant virus encoding the β_2 subunit expressed an appropriately sized protein of 35 kDa, which was specifically detected by the anti- β antibody. A 27 kDa protein which was detected in the cytosolic fraction with this antibody may represent a proteolytic fragment of this subunit. The recombinant β_2 subunit was expressed predominantly in the particulate fraction, in contrast with the recombinant γ_2 subunit, which was expressed largely in the cytosolic fraction. As shown in Fig. 1(c), cells infected with recombinant viruses encoding the γ_2 and the β_2 subunits expressed 6.5 and 35 kDa proteins respectively, although the level of each of these proteins was somewhat lower in cells infected with both viruses than in cells infected with either virus alone. While the recombinant γ_2 subunit was found in the cytosolic fraction at 48 h after infection, the majority of the recombinant γ_2 and β_2 subunits were present in the particulate fraction at 72 h after infection of cells which both viruses. In all cases, decreased levels of expression of the recombinant proteins were observed in cells at 96 and 130 h after infection (results not shown).

The expression of G protein β and γ subunits in Sf9 insect cells exhibited striking differences from the expression of these subunits in COS mammalian cells transfected with virally driven plasmids [17]. First, the expression of both β and γ subunits was not required for the accumulation of significant levels of either subunit in Sf9 cells, in contrast with COS cells. Second, a significant amount of the γ_2 subunit was found in the cytosolic fraction in Sf9 cells, unlike COS cells.

Prenylation of the γ subunit expressed in Sf9 cells

In addition to demonstrating high levels of expression of recombinant β and γ subunits, it is necessary to show that the recombinant γ subunits undergo the appropriate post-translational modifications, since these modifications appear to be required for the function of the γ subunits. We [29] and others [30,31] have shown that the γ_2 subunit is modified by the attachment of a prenyl group to the C-terminal cysteine residue. To determine if the recombinant γ_2 subunit is prenylated, cells infected with the γ_2 virus were labelled with [^3H]MVA [25]. As shown in Fig. 2(b), these cells expressed two forms of the recombinant γ_2 subunit, as detected by antibody binding. The slower-migrating form was expressed exclusively in the soluble particulate fraction, while the faster-migrating form was expressed in both the cytosolic and soluble particulate fractions. Although these two forms were expressed in roughly equal amounts (Fig. 2b), only the slower-migrating form in the soluble particulate fraction showed incorporation of the [^3H]MVA-derived label (Fig. 2a), indicating that only this form was prenylated. Consistent with earlier studies [17,18], the prenylated form was found exclusively in the particulate fraction, confirming the role of prenylation in promoting membrane interaction.

The proportion of the prenylated form was increased from

Fig. 2. Comparison of [^3H]MVA labelling and immunoblotting of the γ_2 subunit in Sf9 cells

A total of 2.5×10^6 cells were infected with the γ_2 and/or β_2 viruses. (a) To monitor incorporation of [^3H]MVA label, infected cells were labelled from 67 to 72 h after infection with medium containing 200 μCi of [^3H]MVA. After rinsing, infected cells were fractionated to yield cytosolic (C), cholate-soluble particulate (SP) and cholate-insoluble particulate (IP) fractions. Portions (45 %) of the C, SP and IP fractions were resolved by SDS/PAGE and then transferred to a nitrocellulose for fluorography. (b) To monitor expression of the recombinant γ_2 subunit, the nitrocellulose blot from (a) was washed to remove the fluor, and then immunoblotted. Exposure times for fluorography of the [^3H]MVA-labelled protein and for autoradiography of the immunoblot labelled with ^{125}I -goat anti-rabbit second antibody were 14 and 3 days respectively.

Fig. 3. [^3H]MM labelling of the γ_2 subunit in Sf9 cells

Non-infected or $\gamma_2\beta_2$ -infected cells (2.5×10^6) were labelled with 100 μCi of [^3H]MM from 67 to 72 h after infection; 33 % of the soluble particulate fractions from these cells were resolved by SDS/PAGE. To monitor incorporation of [^3H]MM-derived label into proteins, the gel was dried, amplified and subjected to fluorography for 2 days. (The fluorographic exposure of one lane of the gel, corresponding to the $\gamma_2\beta_2$ -infected cells, is shown.) Subsequently, 2 mm slices of gel lane containing both the infected (solid line) and non-infected (broken line) samples, in the 6–60 kDa range, were assayed for release of volatile radioactivity following base hydrolysis, which is characteristic of carboxymethylation.

40 % in γ_2 -infected cells to 90 % in $\beta_2\gamma_2$ -infected cells, as indicated by the increase in the amount of the slower-migrating form in the soluble particulate fraction and the decrease in the amount of the faster-migrating form in the cytosolic and soluble particulate fractions (Fig. 2b). The increase in the prenylated form was also reflected by the increase in [^3H]MVA-derived label incorporated into the slower-migrating form (Fig. 2a). The basis for the effect of β_2 subunit expression in increasing the proportion of the prenylated form of the γ_2 subunit is not clear. While it is possible that β_2 subunit expression resulted in a shift in a prenylated form of the γ_2 subunit from the insoluble to the soluble particulate fraction, this explanation does not appear likely, since only a trace amount of the prenylated form could be detected in the insoluble particulate fraction (Fig. 2a).

Fig. 4. SDS/PAGE of the partially purified recombinant $\beta\gamma$ subunits

The $\beta_2\gamma_3$ subunit was purified from the Lubrol-soluble fraction of infected Sf9 cells by affinity chromatography on α_0 -agarose. Lane A, silver stain of partially purified $\beta\gamma$ subunits eluted from an α_0 -agarose column; lane B, partially purified $\beta\gamma$ subunits immunoblotted with the anti- β antibody (top half) or they anti- γ_3 antibody (bottom half). Numbers indicate the molecular masses of standards.

Identification of the prenyl group

To determine the identity of the prenyl group, the [^3H]MVA-derived label was released from the γ_2 subunit in a Raney-nickel-catalysed reaction, and subjected to chromatography on a gel sizing column with different-length hydrocarbon standards, as described previously [32]. The [^3H]MVA-derived label migrated with the C_{20} standard (results not shown), identifying the prenyl group as a geranylgeranyl moiety. Thus the γ_2 subunit expressed in insect cells is prenylated in an analogous fashion to that in mammalian cells [30,31].

Carboxymethylation of the γ subunit expressed in Sf9 cells

To determine if the prenylated form of the γ_2 subunit undergoes carboxymethylation, cells infected with the β_2 and γ_2 viruses were labelled with [^3H]MM. Incorporation of [^3H]MM-derived label into several proteins was observed, including a 6.5 kDa protein identified as the γ_2 subunit based on its presence in infected cells and on its absence from non-infected cells. As shown in Fig. 3, volatile radioactivity was readily released from the 6.5 kDa protein in the $\beta_2\gamma_2$ -infected cells, but not from the other prominently labelled proteins in the $\beta_2\gamma_2$ - or non-infected cells. Since base lability is characteristic of carboxymethylation, the release of volatile radioactivity indicated that a significant portion of the γ_2 subunit expressed in insect cells was carboxymethylated, as in mammalian cells.

Combined expression of $\beta\gamma$ subunits and partial purification of these subunits from Sf9 cells

As for the γ_2 subunit (Figs. 1–3), the γ_3 subunit was also expressed and processed in the baculovirus system, yielding similar results (results not shown). Having established the utility of the baculovirus expression system in producing relatively high levels of appropriately processed β_2 , γ_2 and γ_3 subunits, we sought to demonstrate that the combination of recombinant $\beta\gamma$ subunits produced in this system can be easily purified, and can functionally interact with the α subunits of the G proteins. Previously, Sternweis and colleagues [28] described a procedure for isolation of $\beta\gamma$ subunits from tissues based on their affinity for the agarose-immobilized α subunit of G_o . Using this procedure we have partially purified recombinant $\beta\gamma$ subunits from the particulate fraction of baculovirus-infected insect cells (Fig. 4).

The Lubrol-soluble portion of the particulate fraction was applied to a column of agarose-immobilized α_o . After washing the column, the $\beta_2\gamma_3$ subunits bound to the agarose-immobilized α_o were eluted with aluminium fluoride. As shown in Fig. 4, the β_2 and γ_3 subunits appeared as prominent silver-stained bands of 35 and 7 kDa respectively (lane A), which were recognized by the appropriate antibodies (lane B). The identity of a third prominent silver-stained band of ~ 60 kDa, which was not recognized by the β -subunit-specific antibody, is not known (results not shown). Based on the relative intensities of the silver-stained bands and on the protein concentration, about 1 mg of recombinant $\beta_2\gamma_3$ subunits that are at least 50 % pure can be easily purified from 1 litre of cells. In addition to its simplicity, the purification procedure has the added advantage of selecting $\beta\gamma$ subunits that are functional, since this procedure requires that the recombinant $\beta\gamma$ subunits are able to associate selectively and reversibly with agarose-immobilized α_o . The functionality of the recombinant $\beta_2\gamma_3$ subunits was also confirmed by their ability to support pertussis-toxin-dependent ADP-ribosylation of $G_o\alpha$ (results not shown). With the ability to obtain recombinant $\beta\gamma$ subunits that are appropriately modified and functional from baculovirus-infected Sf9 cells, reconstitution studies to compare the functions of different $\beta\gamma$ subunits of defined composition are now possible. Such studies have not been possible in the past, due to the lack of a method to resolve the heterogeneous mixtures of G protein $\beta\gamma$ subunits that are present in tissues, and due to the probable existence of as yet undescribed β and γ subunits in these tissues. With the growing awareness that G protein $\beta\gamma$ subunits may play an active role in receptor-effector coupling, it will be of particular interest to analyse the ability of different $\beta\gamma$ subunits to regulate adenylate cyclase type II, phospholipase A_2 and agonist-dependent receptor kinase activities.

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