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Baculovirus expression of mammalian G protein α subunits

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Complementary DNAs encoding three subtypes of the α subunit (α_{i-1}, α_o and α_s) of rat guanyl nucleotide regulatory proteins were used to construct recombinant baculoviruses which direct high-level expression of the corresponding proteins in cultured Sf9 insect cells. The expressed proteins were recognized by polyclonal antisera specific for the different α chains, and co-migrated with the native proteins from rat brain membranes in immunoblotting analyses. Soluble and particulate forms of all three immunoreactive α chains were observed following ultracentrifugation of cell lysates. Biosynthetic radiolabelling of infected cells with [^{35}S]methionine or [^{3}H]myristate showed that both soluble and particulate forms of α_{i-1} and α_o were myristoylated; in contrast, α_s did not incorporate myristate. The soluble fractions from cells expressing α chains showed high levels of GTP-binding activity over that observed in uninfected cells, or in cells infected with wild-type virus. The peak expression levels observed at 72 h post-infection were highest for α_o at ca. 400 pmol of GTP-γ-^{35}S/mg protein, or roughly 2% of the total soluble protein. The results of this work show that the baculovirus system can be employed for high-level production of mammalian G protein α chains which retain GTP-binding activity and are appropriately modified by myristoylation.

GTP-binding protein; Heterologous expression; Baculovirus

1. INTRODUCTION

Cell surface receptors for a diverse group of extracellular signalling molecules function through the intermediary of heterotrimeric ($\alpha\beta\gamma$) guanyl nucleotide regulatory proteins (G proteins) (reviewed in [1–3]). Signal transduction is initiated by formation of a ternary complex composed of agonist, receptor and G protein trimer, and the consequent catalyzed exchange of GDP for GTP on the α subunit. The activated α chain (i.e. with GTP bound) then dissociates from the receptor and the $\beta\gamma$ dimer, and interacts directly with an effector (enzyme, ion channel, etc.) to modulate its activity and thereby transmit the signal to the interior of the cell. Termination of the signal is achieved by hydrolysis of bound GTP to GDP by a GTPase activity intrinsic to the α chain, and reformation of the inactive $\alpha\beta\gamma$ trimer.

The multifunctional α subunit of the G protein thus plays a central role in the transduction process, directing interactions with receptor, effector and the $\beta\gamma$ subunits, as well as the binding and hydrolysis of GTP. Molecular genetic studies have demonstrated the existence of multiple subtypes of the α chain; to date, 17 distinct cDNAs encoding α subunit variants, falling into four homology groups, have been cloned (reviewed in [3]). Biochemical studies with the native proteins and recombinant α chains expressed from cDNAs have demonstrated that the subtypes possess distinct receptor and effector coupling specificities, and show differences in certain enzymatic parameters. The α subunits also show differential susceptibility to ADP-ribosylating bacterial toxins and co-translational modification by *N*-myristoylation [1,2,4–7].

Given the marked heterogeneity in G protein α chains, numerous laboratories have utilized heterologous expression systems to produce identified gene products for biochemical analyses and mutagenesis studies. In vitro transcription/translation of cDNAs has been useful in comparing effector coupling properties of the α chain variants [8], and *E. coli* expression has yielded sufficient quantities of functional α chains, and site-directed mutants, for purification and detailed biochemical characterization [9–11]. The α subunits produced in both systems show 10- to 20-fold reduced apparent affinities for effectors in functional assays as compared to the same proteins isolated from natural sources. A recent study suggests that this deficiency may be due in part to the lack of specific covalent modifications of the expressed proteins [12].

In view of the problems in producing appropriately modified mammalian proteins in bacteria, we set out to examine a eukaryotic expression system for the production of G protein α chains. The baculovirus-insect cell system has enjoyed considerable success as a high-level expression vehicle for numerous functional mammalian proteins (reviewed in [13]). The Sf9 insect cell line has

Abbreviations: GTP-γ-S, guanosine 5′-γ-thiotriphosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; ATP, adenosine triphosphate; PBS, phosphate-buffered isotonic saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; AcNPV, *Autographa californica* nuclear polyhedrosis virus.

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been shown to carry out many co- and post-translational modifications typical of mammalian cells, including *N*-linked glycosylation [14], phosphorylation [15], fatty acylation [16] and polyprenylation [17]. We report here that recombinant baculoviruses containing cDNAs for three α subunit variants from rat (α_{i-1}, α_o and α_s) direct high-level expression of functional proteins which are appropriately modified by myristoylation.

2. EXPERIMENTAL

2.1. *Construction of recombinant baculoviruses*

Cloned cDNAs encoding rat olfactory epithelial α_{i-1}, α_o and the 52 kDa form of α_s (kindly provided by R. Reed, Johns Hopkins University) [18] were excised from their cloning vectors by *Eco*RI digestion and subcloned into pTZ18R [19] using established techniques [20]. The complete α_s cDNA was isolated as a product of a partial *Eco*RI digest. Oligonucleotide-directed mutagenesis was performed using the Bio-Rad MutaGene kit to engineer restriction sites immediately flanking the translational start and stop codons. The α_{i-1} sequence had *Bam*HI sites introduced using the oligonucleotides 5′-GCT CAG TGT GCA GCC CAT GGA TCC TCT AGA GTC GAC-3′ (5′-site) and 5′-CGA CTC ACT ATA GGG GAT CCT TAG AAG AGA CCA-3′ (3′-site); the α_o cDNA had *Bgl*II sites introduced using 5′-CGA AGT ACA TCC CAT AGA TCT ACT GGC CGT CGT TTT AC-3′ (5′-site) and 5′-CGA CTC ACT ATA GGA GAT CTT CAG TAC AAG CCA C-3′ (3′-site); *Nhe*I sites were introduced into α_s cDNA with the oligonucleotides 5′-GCC GAG GCA GCC CAT GCT AGC GGC ACT GGC CGT-3′ (5′-site) and 5′-GAC CTG CAG GCA TGC CGC TAG CTT AGA GCA GCT C-3′ (3′-site). Sequences of the mutagenized regions were verified by dideoxy DNA sequencing [21].

The α_{i-1} and α_o coding segments were subcloned into the *Bam*HI site of the baculovirus transfer vector IpDC-126 (provided by D. Tessier, Montréal), while the α_s sequence was cloned into the *Nhe*I site of pJV-ETLZ, a modified transfer vector containing the LacZ gene under control of the baculovirus p10 promoter (provided by C. Richardson, Montréal). The individual plasmids were cotransfected with wild-type AcNPV viral DNA into monolayer cultures of Sf9 insect cells and recombinant viruses isolated by sequential plaque purifications as described [22]; initial screening of α_s recombinants employed early detection procedures based on expression of β-galactosidase [23].

2.2. *Expression of recombinant α subunits*

Cultures of Sf9 insect cells were grown in suspension or as monolayers at 27°C in TNM-FH medium [22] containing 10% fetal calf serum (FCS). For expression studies, cells were seeded in 3-cm plastic dishes (Corning) at 10^6 cells/well and infected with recombinant or wild-type virus at a multiplicity of infection (m.o.i.) of 10. The cultures were maintained at 27°C under 100% humidity for specified periods (see section 3). At the time of harvesting, the plates were cooled on ice for 10 min, the medium removed and the cell monolayers rinsed three times with cold phosphate-buffered isotonic saline (PBS). The cells were then either extracted directly in SDS sample buffer [24] (250 μl/well) for immunoblot analyses, or soluble and particulate fractions prepared (see below).

2.3. *Cell fractionation*

Washed cell monolayers were scraped with a rubber policeman into 0.8 ml of cold lysis buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 25 μM GDP) containing protease inhibitors (10 μM 3,4-dichloroisocoumarin, 1 μM leupeptin, 200 μM TPCK, 20 mg/ml trypsin inhibitor, 0.3 μM aprotinin and 0.1 mg/ml E-64). Cells were lysed by repeated passage (10 strokes) through a 26-gauge needle, and the lysates centrifuged at 43,000 rpm for 45 min at 4°C in a Beckman Tl-100 ultracentrifuge equipped with a Ti-45 rotor. The supernatant (soluble) and pellet (particulate) fractions were separated, the pellets resuspended in lysis buffer and the two fractions either analyzed immediately (GTP-γ-S binding) or stored at −80°C for up to 1 month (immunoblotting).

2.4. *Immunoblot analyses*

Whole cell extracts in SDS sample buffer, or soluble and particulate fractions (prepared as described above) diluted with an equal volume of 2 × concentrated sample buffer, were heated at 65°C for 10 min and aliquots subjected to SDS-PAGE on a 4% stacking/12.5% running gel [24]. Pre-stained molecular weight marker proteins (Bio-Rad) were run in parallel. Following electrophoresis, the resolved proteins were electro-transferred to BA-85 nitrocellulose membranes (Xymotech) for 90 min at 70 V. The membranes were then processed for detection of immunoreactive α chains essentially as described [25]. Primary incubations were performed overnight at 4°C with 1:3000 dilutions of the polyclonal antibodies AS/7 (for α_{i-1}), RM/1 (for α_s) (both purchased from NEN/Dupont), or $G_{\alpha o}$#4 (for α_o) (a gift from J.K. Northup, Bethesda). Secondary incubations were done for 1 h at room temperature using goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) and bound enzyme visualized with the Vectastain ABC alkaline phosphatase substrate (Vector Laboratories).

2.5. *Biosynthetic radiolabeling*

Monolayer cultures of Sf9 cells (10^6 cells/well in 1 ml TNM-FH/5% FCS) were infected with wild-type AcNPV or recombinant viruses at 10 m.o.i., and incubated at 27°C for 48 h. For methionine labeling, the medium was then replaced with methionine-free TNM-FH/5% FCS supplemented with 0.1 mCi/ml of [^{35}S]methionine/cysteine (sp. act. < 1000 Ci/mmol, Translabel, ICN), and incubations continued for 1 h at 27°C. Incorporation of myristate was achieved by replacing culture medium at 48 h p.i. with fresh TNM-FH/5% FCS containing 0.1 mCi/ml [^{3}H]myristate (10–60 Ci/mmol, NEN/DuPont) and incubating the cells for 6 h at 27°C. At the end of the labeling period, the cell monolayers were washed three times with PBS. These cells were either extracted immediately in SDS sample buffer [24], or soluble and particulate fractions were prepared and then treated with SDS sample buffer as described above. Aliquots of the various extracts were analyzed by SDS-PAGE on 4% stacking/12.5% resolving slab gels and proteins stained with Coomassie blue. Gels of ^{35}S-labeled proteins were dried and exposed for 1 h at −80°C to Hyperfilm-MP (Amersham). For tritiated proteins, the gels were soaked for 30 min in [^{3}H]Amplify (Amersham), dried and exposed to Hyperfilm-ECL (Amersham) for 16 h at −80°C.

2.6. *Assays*

The binding of GTP-γ-^{35}S (1200 Ci/mmol, NEN/Dupont) to soluble fractions of Sf9 cell lysates was measured as described previously [26] with minor modifications. Assays were performed in the presence of 1 mM ATP (to reduce non-specific binding) at 30°C for 1 h, and samples were filtered on 0.45 μm nitrocellulose filters (Sartorius). The filters were air-dried and bound radioactivity determined by liquid scintillation counting in 10 ml of ACS (Amersham) at an efficiency of 96%. Protein concentrations in cell extracts were measured using the Lowry [27] or Bradford (Bio-Rad) techniques.

3. RESULTS

3.1. *Expression of immunoreactive α subunits*

Cultures of Sf9 cells were infected with wild-type AcNPV virus or with recombinant viruses encoding the rat α_{i-1}, α_o or α_s chains, and whole cell extracts prepared at various times post-infection were analyzed by immunoblotting with specific polyclonal antisera. As shown in Fig. 1, the three recombinant viruses directed time-dependent expression of immunoreactive material, detected with the corresponding antiserum, which was

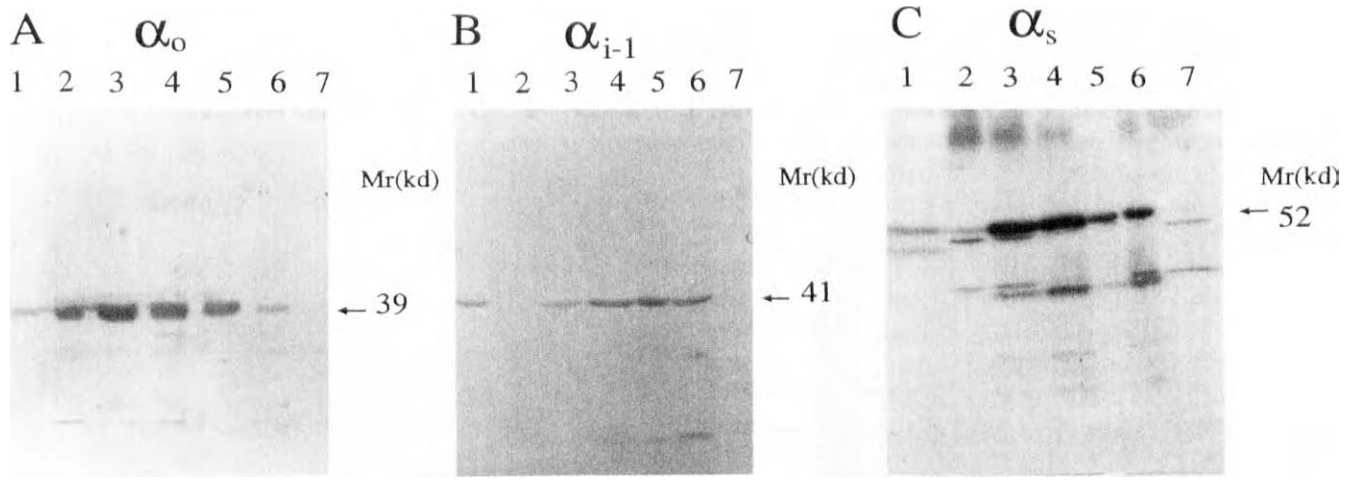

Fig. 1. Immunoblot analysis of recombinant α chains expressed in baculovirus-infected Sf9 cells. Monolayer cultures of Sf9 cells were infected with wild-type or recombinant baculoviruses (m.o.i. = 10), and whole cell extracts or subcellular fractions were prepared and analyzed by SDS-PAGE/immunoblotting as described in section 2. (A) Recombinant α_o analyzed with G_o#4 antisera; (B) recombinant α_{i-1} analyzed with AS/7 antiserum; (C) recombinant α_s analyzed with RM/1 antiserum. Lane 1, 75 μg of rat brain membrane extract; lanes 2–4, 100 μg membrane protein from Sf9 cells extracted at 24, 48 and 72 h p.i., respectively; lanes 5–6, supernatant and pellet fractions, respectively, prepared at 72 h p.i. from Sf9 cells infected with recombinant viruses; lane 7, whole cell extracts of Sf9 cells infected with wild-type AcNPV virus. Arrows indicate the apparent molecular weights (in kDa) of the major immunoreactive species, as compared with molecular weight standards run on the same gel (prestained marker proteins, Bio-Rad).

absent from the wild-type control (Fig. 1, lane 7). The immunoreactive forms were visible at 24 h p.i., and accumulated over 48 and 72 h of infection (Fig. 1, lanes 2–4), consistent with the time course of production of the polyhedrin protein for wild-type virus [22]. In each case, the major immunoreactive species co-migrated on SDS-PAGE with an immunoreactive protein present in rat brain extracts (Fig. 1, lane 1); the apparent molecular weights of the recombinant proteins (41 kDa for α_{i-1}, 39 kDa for α_o and 52 kDa for α_s) are in agreement with those predicted from the deduced primary structures and observed for the native proteins. The α_s antiserum recognized two additional proteins in cells infected with wild-type virus (Fig. 1C, lane 7) and at 24 and 48 h time points following infection with the α_s recombinant virus (Fig. 1C, lanes 2 and 3). Neither of these species co-migrated on SDS-PAGE with the α_s-immunoreactive forms detected in rat brain membranes (Fig. 1C, lane 1) nor with the 52 kDa species expressed from the recombinant α_s virus (Fig. 1C, lanes 2–6). It is not known whether these additional species represent the insect α_s homologues, or merely non-specific cross-reaction with the anti-peptide polyclonal antiserum. These two species were not detected in supernatant fractions from cells expressing the rat α_s (Fig. 1C, lane 5), and thus would not be expected to interfere with the purification of α_s from this fraction (see below). While minor immunoreactive species of lower apparent mass, presumably degradation products of the major form, were also observed for all three recombinants, the results show that the majority of the expressed proteins represent intact α subunits.

Immunoblot analyses of soluble and particulate fractions prepared from Sf9 cells at 72 h p.i. revealed the presence of specific immunoreactive species in both fractions (Fig. 1, lanes 5 and 6). The soluble material was comprised almost entirely of the large forms corresponding in size to the intact α chains. The particulate fraction also contained predominantly the intact form, and a small proportion of degradation products. The relative amount of degradation products was highest for α_s, and showed considerable variation between experiments. The appearance of smaller immunoreactive forms does not appear to be related to conditions of cell lysis or storage, since such species were also observed in whole cells extracted directly into SDS sample buffer and analyzed immediately (Fig. 1, lane 4).

The results of the immunoblotting analysis indicate that the relative proportions of soluble and particulate α chains (Fig. 1, lanes 5 and 6, respectively) varied considerably, depending on the specific α subunit. The soluble material was clearly the major form observed for α_o (Fig. 1A), while the soluble and particulate forms were present in roughly equal proportions for α_{i-1} and α_s (Fig. 1B and C, respectively). It is unclear whether the particulate material represents membrane-associated forms of the α subunits, insoluble protein aggregates, or both.

3.2. *Myristate labeling of recombinant α subunits*

The α subunit α_o isolated from natural sources bears a myristoyl fatty acid group in amide linkage to the amino-terminal glycine residue [6,7]. Biosynthetic labeling experiments with mammalian cells expressing recombinant α subunits have shown that α_o and α_{i-1} incorporate [^{3}H]myristate, while α_s does not [4,5]. The

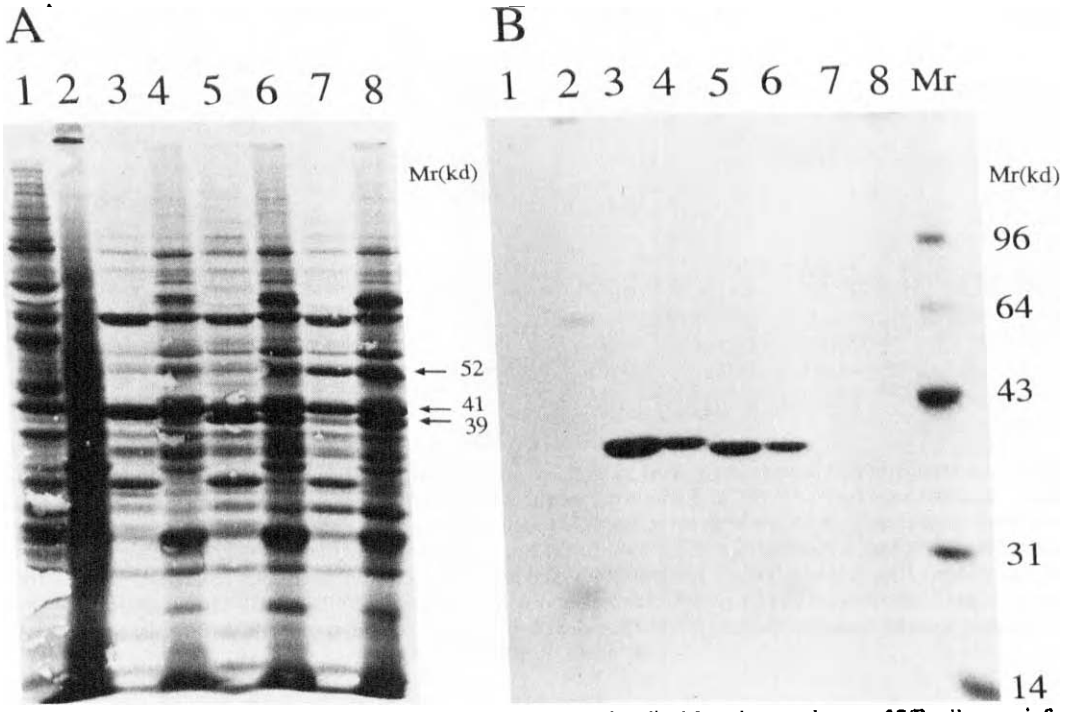

Fig. 2. Biosynthetic radiolabeling of proteins expressed in baculovirus-infected Sf9 cells. Monolayer cultures of Sf9 cells were infected with wild-type or recombinant baculoviruses (m.o.i. = 10). At 48 h p.i., the cells were incubated with [^{35}S]methionine/cysteine (Translabel) for 1 h, or with [^{3}H]myristate for 16 h. Soluble and particulate fractions were prepared from cell lysates, and aliquots (100 μg) analyzed by SDS-PAGE/autoradiography as described in section 2. (A) ^{35}S-labeled proteins; (B) ^{3}H-labeled proteins. In both autoradiograms, the order of samples is soluble (lanes 1, 3, 5, 7) and particulate (lanes 2, 4, 6, 8) fractions from Sf9 cells infected with wild-type AcNPV virus (lanes 1, 2), or recombinant baculoviruses expressing α_{i-1} (lanes 3, 4), α_o (lanes 5, 6) or α_s (lanes 7, 8). Arrows in A indicate positions and apparent molecular weights (in kDa) of the ^{35}S-labeled recombinant α subunits. The last lane (M_r) in B shows the migrations of ^{14}C-labeled molecular weight standards (Pharmacia), and the corresponding molecular weights (in kDa).

ability of Sf9 cells to myristoylate recombinant α chains was examined by labeling infected cells with [^{3}H]myristate in parallel with [^{35}S]methionine and comparing the incorporation of radioactivity in the expressed proteins. As shown in Fig. 2A, [^{35}S]methionine was incorporated into diverse proteins by Sf9 cells following infection with wild-type and recombinant viruses. Close inspection of the autoradiograms for recombinant vs. wild-type viruses reveals the presence of labeled proteins corresponding in size to α_{i-1}, α_o and α_s. When the same experiment to assess [^{3}H]myristate incorporation was carried out in parallel, the α_{i-1} and α_o proteins were clearly labeled (Fig. 2B, lanes 2–6), to a much greater extent than endogenous Sf9 cell proteins, while the recombinant α_s showed no detectable labeling (Fig. 2B, lanes 7 and 8). The insect cell system thus is able to myristoylate mammalian α chains, and exhibits the same specificity (i.e. α_{i-1} and α_o, but not α_s) as reported for mammalian cells. The data do not permit an estimation of the proportion of the recombinant α chains which bear the myristoyl group, due to lack of quantitation of total α chain production and specific activity of the myristate pool. The results clearly demonstrate, however, that the modification does occur in the Sf9 cells, and that both the soluble and particulate pools of intact α_{i-1} and α_o bear the covalently-attached fatty acid.

3.3. *GTP-γ-S binding by recombinant α chains*

As a first step in assessing the functional integrity of the recombinant α subunits, soluble and particulate fractions prepared from infected Sf9 cells were tested for GTP binding activity using the non-hydrolyzable analogue GTP-γ-^{35}S. The particulate material containing α chain immunoreactivity was solubilized with cholate under conditions for extraction of native G proteins from mammalian cell membranes [28]. While this treatment effectively extracted the immunoreactive species, the solubilized protein showed no GTP-γ-S binding activity over that observed for wild-type virus (data not shown). This result suggests that the particulate forms of the α chains may represent denatured, inactive subunits; this material was not characterized further.

Soluble fractions from Sf9 cells infected with recombinant viruses showed a time-dependent increase in GTP-γ-S binding activity over that measured for uninfected or wild-type infected cells (Fig. 3). The time course for appearance of specific binding roughly paralleled that for α chain immunoreactivity, with minor differences being observed for the different α subtypes.

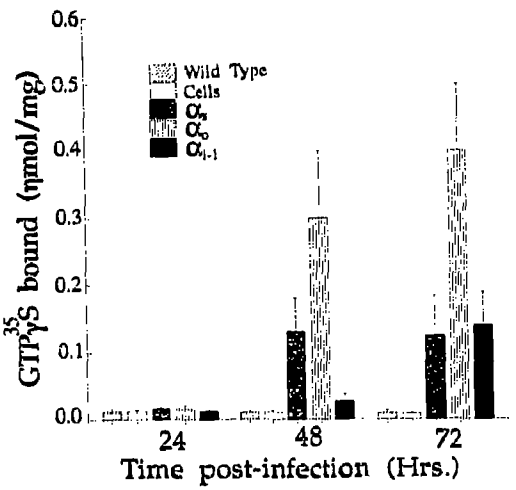

Fig. 3. GTP-γ-^{35}S binding activity in soluble fractions from baculovirus-infected and uninfected Sf9 cells. Monolayer cultures of Sf9 cells were infected with wild-type or recombinant baculoviruses (m.o.i. = 10), and soluble cell fractions prepared at various times following infection were assayed for GTP-γ-^{35}S binding as described in section 2. The bar graph shows the level of GTP-γ-^{35}S binding in pmol/mg soluble protein measured in the five different samples at each time point. The samples (in order) are derived from cells infected with wild-type AcNPV virus, uninfected cells, and cells infected with recombinant viruses expressing α_s, α_o and α_{i-1}. Each time point shows the mean ± S.E.M. for four different experiments, each conducted in triplicate.

Peak levels of production were highest for α_o, at 400 pmol/mg protein, and were approximately three-fold lower for the α_{i-1} and α_s proteins (140 and 120 pmol/mg protein, respectively). These values are from 6- to 20-fold higher than those obtained for wild-type viruses or untreated cells. The recombinant α subunits recovered in soluble fractions from infected Sf9 cells thus exhibit one of the characteristic activities of this group of proteins, the ability to bind guanyl nucleotides.

4. DISCUSSION

The present study shows that mammalian G protein α chains can be expressed in high yield from recombinant baculoviruses in the Sf9 insect cell line. Soluble fractions from cells expressing rat α_{i-1}, α_o and α_s contained predominantly intact α chains, as assessed by immunoblotting with specific antisera; furthermore, the proteins were appropriately modified (α_{i-1} and α_o) or not (α_s) by myristoylation and they bound the guanyl nucleotide analogue GTP-γ-S. The recombinant α chains expressed in soluble form in the baculovirus system could thus serve as a high-level source of these proteins for biochemical and biophysical analyses.

The production of various G protein α chains, including those studied here, has been achieved using a prokaryotic expression system [9–11]. As in the present study, both soluble and particulate forms of the recombinant proteins were present in cell lysates, the insoluble material showing little or no GTP-γ-S binding activity and a high degree of proteolytic degradation. The *E. coli*-expressed proteins have been purified to homogeneity and shown to interact with other components of the signal transduction apparatus, i.e. receptor, $\beta\gamma$ dimer and effector [10,11]. In those studies, the affinities of the recombinant material for $\beta\gamma$ and effector components were 10- to 20-fold lower than those observed for the native α chains isolated from natural sources. Recent work suggests that this lower intrinsic activity is due, at least in part, to the absence of *N*-myristoylation which does not normally take place in prokaryotes. The levels of active α chains recovered in soluble fractions (120–400 pmol/mg protein) are also 2- to 6-fold higher than those reported for the same proteins in the bacterial expression system [9–11].

The soluble pool of α subunit is well-suited to purification of the recombinant proteins, since they are obtained without the necessity of detergent extraction required for the native trimers and should be free of contamination by endogenous trimeric G proteins presumably localized in membrane fractions from the insect cells. The nature of the recombinant α subunits associated with particulate fractions is not clear. We were unable to detect any GTP-γ-S binding activity over background in these fractions, suggesting that they contain denatured, aggregated α chains. Similar findings were reported for bacterially-expressed α subunits [9], and may reflect the consequences of over-expression. It is significant that no correlation was observed between myristoylation and association with membrane fractions, since earlier studies have suggested a role for this modification in localizing α subunits to the membrane bilayer [4,5].

While this work was in progress, Graber et al. [29] reported the production of G protein α subunits in the baculovirus expression system. As in the present study, the authors observed that the recombinant α chains recovered in high yields from soluble fractions exhibited functional activity and were appropriately modified by myristoylation. The present findings indicate that roughly one-half of the recombinant α_s chain was recovered as active material in soluble form, compared to one-tenth in the previous work [29]. In addition, the results of biosynthetic labeling experiments in the two studies suggest that the extent of myristoylation of α_{i-1} and α_o may be considerably higher in the present work. The sources of these apparent differences could lie in the conditions of culture, experimental protocols for labeling, or differences in the specific cDNA constructs employed to generate the α_s-expressing virus.

The study of Graber et al. [29] further shows that the baculovirus-expressed α subunits can be purified to homogeneity and exhibit functional interactions with $\beta\gamma$ dimers and a typical G protein-coupled receptor, the angiotensin II receptor from rat liver. The further appli-

cation of this highly efficient expression system, using recombinant viruses developed in this work and that of Graber et al. [29], should provide the material required for biochemical and biophysical studies of the structures of G protein α chains and for detailed analysis of their functional characteristics in reconstituted signal transducing systems.

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