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Expression of the mouse Gαq using *Baculovirus* expression system

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Abstract

Baculovirus expression system has been used successfully to over-express eukaryotic proteins in insect cells. This system uses a very strong viral promoter, AcNPV polyhedrin, for high level protein expression. Here a *Baculovirus* expression system was used to express the *Mouse* Gαq protein (mGαq) in SF9 insect cells. The recombinant protein was made at high levels and it was found in the cell membrane where it functions as a signaling molecule.

Keywords: *Baculovirus, G-proteins, expression system, insect cells.*

Introduction

There are several gene expression systems for production of proteins at high levels. Over-expression of proteins in the bacterium, *E. coli*, is very popular. However, due to lack of proper post-translational modifications, it is possible that expression of eukaryotic genes in *E. coli* leads to production of biologically non-functional proteins. To assure as close to wild type protein processing as possible, the system of choice are eukaryotic systems. *Baculovirus* expression system is a powerful tool to produce recombinant proteins in insect cells or insect larvae (Miller 1988, Murhammer 1991, Fraser 1992, Kidd & Emery 1993, Ikonomou *et al*. 2003). The use of recombinant *Baculovirus* as high- level expression system is becoming more and more popular. Numerous examples of the successful high-level expression of biologically active vertebrate proteins have been reported such as: androgen receptor (Janne *et al.* 1993), G-protein coupled receptors (Sarramegna *et al.* 2003, Massotte 2003), insulin receptor (Wente & Rosen 1990), M-CSF receptor (Koths 1997), dbl-oncogene (Graziani *et al.* 1991), kinesin proteins (Hirokawa & Noda 2001), Ras proteins (Lowe *et al.* 1992), murine or human antibodies (Potter *et al.* 1993), drug metabolizing enzymes (Guengerich & Parikh 1997), and recombinant glycoproteins (Jarvis *et al.* 1998, Marchal *et al.* 2001, Jarvis 2003, Tomiya *et al.* 2003). Vertebrate and invertebrate core glycosylation appears to be similar, but terminal glycosylation is different in these two classes of organisms (Tomiya *et al.* 2003, Jarvis 2003). Nevertheless many biologically active glycosylated vertebrate proteins have been ex-pressed using modified *Baculovirus* expression systems (Tomiya *et al.* 2003, Jarvis 2003).

Although *AcNPV* (*Autographa californica Nuclear Polyhedrosis Virus*) does not replicate in vertebrate cells, it expresses foreign genes with levels of expression that are dependent on the strength of the promoter used to drive transcription of the alien gene. Therefore, *AcNPV* has been considered as a potent mammalian cell delivery system for gene therapy (Pieroni & La Monica 2001, Ghosh *et al.* 2002, Huser & Hofmann 2003). Production of viral vaccines (Vlak & Keus 1990) and viral surface display (Grabherr & Ernst 2001) are among other applications of *Baculovirus* expression system.

In a *Baculovirus* expression system, the gene of interest will be inserted downstream of the polyhedrin promoter, a very strong pro-moter belonging to *AcNP* virus. Infection of SF9 insect cells with the recombinant virus leads to expression of the recombinant protein at high levels. In most cases the protein will be modified properly and will be localized to the right place in the cell. Therefore, the recombinant protein usually maintains its normal function. In general *Baculovirus* expression system has four features: 1) Expression levels of the recombinant gene

compared to those of other eukaryotic expression methods are higher (Miller 1988, Fraser 1992), 2) By infecting the cells with multiple recombinant viruses, this system can be used to express hetero-oligomer protein complexes (Griffiths & Page 1997, Harris & Polayes 1997), 3) *Baculoviruses* use specific insect species as host and therefore they are not infectious to human or domestic animals (Pieroni & La Monica 2001, Ghosh *et al.* 2002, Huser & Hofmann 2003), 4) *AcNP* virus proliferates in the cells derived from insects like *Spodoptera frugiperda* and *Trichoplusia ni*. These cell lines can be adapted to grow in suspension and therefore the recombinant protein can be produced in large scales using bio-reactors (Shuler *et al.* 1990).

I had previously expressed Gαq in mammalian cells by using lipofectamine-mediated transfection method. However, the percentage of cells transfected with the vector was about 20% and also the expression levels were relatively low (not shown). In this paper, a *Baculovirus* expression system (Bac-to-Bac *Baculovirus* Expression System, Invitrogen) has been used to express *mouse* Gαq in SF9 insect cells. The results of western blotting experiments showed that SF9 cells infected with Gαq recombinant *baculoviruses*, express the protein at high levels. Interestingly the Gαq protein made by this system was localized to proper location (cell membrane) in the cells, suggesting that the recombinant protein maintains its biological functions.

Materials and Methods

Cloning of Gαq cDNA into pFast-Bac-1 plasmid: The mouse *Gαq* cDNA cloned into pCMV vector was a gift from Dr. Peter Klein (HHMI, Department of Medicine, university of Pennsylvania, USA). A fragment of this clone carrying *Gαq* cDNA (digested with BamHI and Hind III) was subcloned into the corresponding sites of the vector, pFast Bac-1 (Invitrogen). The presence of the insert and its correct ori-entation were verified by restriction digestion and DNA sequenceing.

Generation of Gαq recombinant Baculoviruses (recombinant Bacmids): The purified recombinant pFast Bac-1 plasmid was trans-formed into *E. coli* DH10Bac (Invitrogen) as described by the sup-plier. *E. coli* DH10Bac carries the *baculovirus* shuttle vector (bacmid), bMON14272 (136 kb), and also the helper plasmid, pMON 7124 (13.2 kb), which encodes the transposase and confers resistance to tetracycline. The helper plasmid provides the Tn7 transposition function. 10-fold serial dilutions of the transformed bacterial cells were plated on LB agar containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml Blue-gal, and 40 μg/ml IPTG. The plates were incubated for 48 hours at 37^{o}C and then ten large white colonies were selected for further analysis. The selected colonies were re-streaked on fresh LB agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml Blue-gal, and 40 μg/ml IPTG. The plates were incubated overnight at 37^{o}C and then from each re-streaked plate, a single colony (confirmed to have a white phenotype) was grown in a 2 ml liquid culture containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, and 10 μg/ml tetracycline. The recombinant bacmid DNA was isolated by a protocol described by the supplier (Invitrogen) and the presence of gene of interest (*mouseGαq*) in the recombinant bacmid was verified by PCR using M13 forward (5'-GTTTTCCCAGTCAC-GAC-3') and reverse (5'-CAGGAAACAGCTA-TGAC-3') primers flanking the mini-*att* Tn7 site within the LacZ-complementation region. Each PCR reaction was performed in a 50 μl reaction mixture containing 100 ng recombinant bacmid DNA as template, 1 pmol/μl each primer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 2.5 unit of Taq polymerase and 5 μl of 10 X buffer. The reactions were denatured for 3 minutes at 93^{o}C and incubated for 30 cycles (denaturing at 94^{o}C for 45 sec, annealing at 55^{o}C for 45 sec, and polymerizing at 72^{o}C for 1 min). Final polymerization was ex-tended for 7 min.

SF9 cell culture, transfection and viral amplification: SF9 cells were grown and maintained in TNF-FH medium (Invitrogen) supplemented with 10% FBS (fetal bovine serum), 50 units/ml penicillin and 50 μg/ ml streptomycin. For transfection experiments, SF9 cells

were grown in a suspension shaker culture (in a 27°C humidified incubator) until they reached a concentration of 1.5×10^6 cells/ ml. From this suspension culture, 5.0×10^5 cells were seeded in each well of a 6-well (35 mm) plate in TNF-FH medium (containing 10% FBS) and grown for 18-24 hours.

For each transfection sample 2 μg of purified bacmid DNA and 5 μl of Cellfectin reagent were separately diluted in 100 μl of Sf-900II SFM medium (a serum free medium manufactured by Invitrogen). The diluted bacmid DNA and the diluted Cellfectin were mixed and incubated at room temperature for 45 minutes. While DNA: lipid complexes were incubating, the media from the cells (approximately 1.5×10^6 cells /well) were removed and the cells were washed once with 2 ml of Sf-900II SFM. 0.8 ml Sf-900II SFM was added to each tube containing DNA: lipid complexes and gently mixed (about 1 ml total volume). The washing medium was removed and the cells were covered with the 1 ml medium containing DNA: lipid complexes. The cells were incubated at 27°C for 5 hours and then the medium containing DNA: lipid complexes were removed and 2 ml of complete medium (containing antibiotics) was added to each well. The cells were incubated in a 27°C humidified incubator for 72 hours. The medium containing virus was collected from each well (about 2 ml) and transferred to a sterile 15 ml tube. The tubes were centrifuged at 500 X *g* for 5 minutes to remove cells and large debries. The clarified supernatant was transferred to a fresh 15 ml tube (P1 viral stock) and stored at 4°C protected from light.

Viral amplification and plaque assay were performed by using protocols described by the supplier (Invitrogen).

Expression of the mouse Gαq in SF9 cells, preparation of membrane fraction, and Western blotting: 2 ml of a SF9 suspension culture (1.0 X 10^6 cells/ml) was transferred into each well of a 35 mm dish (6-well dish) and left at room temperature until cells attach to the dish. The medium of cells was changed to Sf-900II SFM and the cells were infected with MOI = 1 and 2 (multiplicity of infection) of *mGαq* recombinant or non-recombinant *baculoviruses*. The cells were harvested 24 and 48 hours post-infection and the remaining steps were all performed on ice. The cells were lysed by passing through a 27G needle in 50 mM Tris-Cl pH= 7.8, 50 mM KCl, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM sodium orthovanadate, 10 nM micro-cystine, 1 mM PMSF, 4 μg/ml leupeptin, and 4 μg/ml pepstatin. The lysate was centrifuged for 30 minutes at 14000 *rpm* and the supernatant was transferred to a new tube. The pellet (membrane fraction) was washed once with lysis buffer, resuspended in this buffer plus 2% triton X-100 and incubated on ice (with shaking) for 45 minutes. The solubilized membrane was centrifuged at 50,000 *rpm* for 30 minutes and the supernatant was stored as membrane proteins at -70°C. About 5μg of membrane proteins was run on a 10% SDS poly acrylamide gel electrophoresis and subjected to western blotting based on a standard protocol described by Sambrook *et al.* (1987). The blot was stained with a specific polyclonal antibody against Gαq (CalBiochem).

Results and Discussion

A physical map of the plasmid pFast Bac-1 and the insert (*mGαq* cDNA) is shown in Fig. 1a. The insert was cloned into the *Bam*HI and *Hind* III sites of the vector (Fig. 1b). In pFast Bac-1, expression of the gene of interest is controlled by a *baculovirus*-specific promoter, P_{PH} (Fig. 1a). The promoter belongs to *Autographa californica multiple nuclear polyhedrosis virus* (AcMNPV) poyhedrin gene. P_{PH} promoter is a very strong promoter which leads to high level expression of the downstream cloned gene in insect cells. The expression cassette is flanked by the left and right arms of Tn7, and also contains a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini Tn7.

The second major component of the system used in this study, is DH10Bac *E. coli* strain that is used as the host for pFast Bac-1 vector. DH10Bac cells contain a *Baculovirus* shuttle vector (bacmid) (Fig. 2a) plus a helper plasmid.

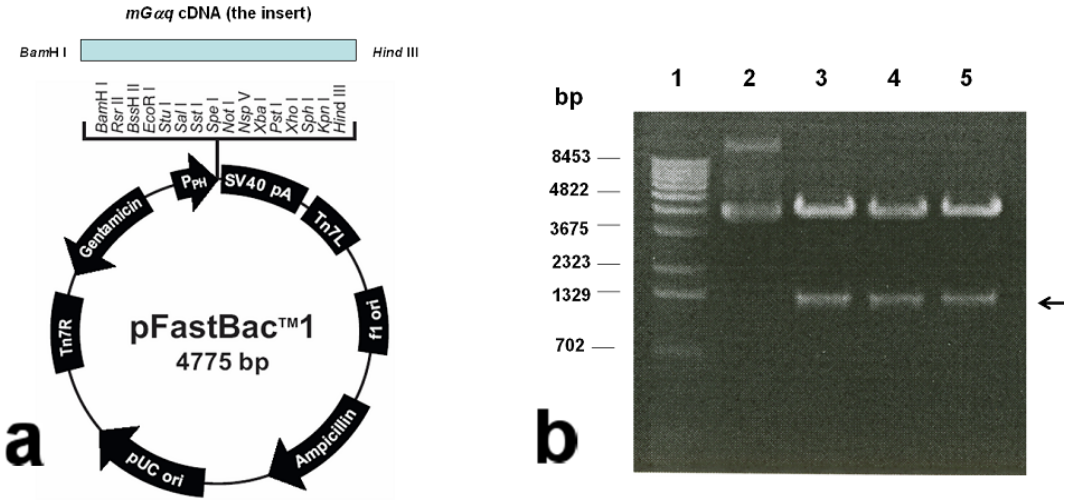

Fig. 1(a) A physical map of the pFast Bac-1 vector. The insert (*mGαq* cDNA) has been cloned into BamHI and Hind III sites of the vector. (b) Three positive recombinant pFast Bac-1 carrying *Gαq* cDNA are di-gested with BamHI and Hind III restriction enzymes and run on a 1% agarose gel electrophoresis (lanes 3-5). The arrow indicates the posi-tion of the insert on the gel (about 1.2 kb). DNA size markers and undigested vector are loaded in lanes 1 and 2, respectively.

The bacmid DNA is a large molecule (about 130 kb) and forms different conformations which appear as several bands on an agarose-gel electrophoresis (Fig. 2a). Bacmid contains a segment of DNA encoding the LacZα peptide into which the attach-ment site for a bacterial transposon, Tn7 (mini-*att*Tn7) has been in-serted. This insertion does not disrupt the reading frame of the LacZα peptide. Once the pFast Bac-1 expression vector is transformed into DH10Bac cells, transposition occurs between the mini-Tn7 element on the pFast Bac-1 vector and the mini-*att*Tn7 target site on the bacmid to generate a recombinant bacmid. This transposition reaction occurs in the presence of transposition proteins supplied by the helper plasmid.

Recombinant bacmid DNA is greater than 135 kb in size. Since restriction analysis is difficult to perform with DNA of this size, PCR analysis was used to verify the presence of the gene of interest (*mGαq*) in the recombinant bacmid (Fig. 2b).

To package *mGαq* recombinant *baculo-viruses*, a cationic lipid (Cell-fectin reagent) was used for transfection of SF9 cells with the recombinant bacmid. The initial viral titer obtained from these transfection experiments was approximately 4 X 10^6 pfu/ml. Amplification of P1 viral stock led to 3-4 fold increase in the viral titer.

To test whether Gαq recombinant viruses can make the recombinant Gαq in SF9 cells, the cells were infected with two MOIs of the virus and were harvested 24 and 48 hours post-infection. The membrane fraction was isolated and the presence of the *mouse* Gαq was ex-amined by western blotting (Fig. 3). Gαq like other alpha subunits of heterotrimeric G-proteins is modified reversibly by palmitoylation on cysteine residues at the N-terminal. This modification has been equated with targeting and anchorage of the Gα subunit to the plasma membrane and influences its interaction with relevant receptors and effectors (Kosloff *et al*. 2003). As shown in Fig. 3, presence of the recombinant Gαq in the membrane fraction of Sf9 cells suggests that the recombinant Gαq gets proper post-translational modifications and folding which is consistent with findings of some workers who have used *Baculovirus* expression system to overproduce receptor proteins (Janne *et al.* 1993, Sarramegna *et al.* 2003, Massotte 2003, Wente & Rosen 1990, Koths 1997).

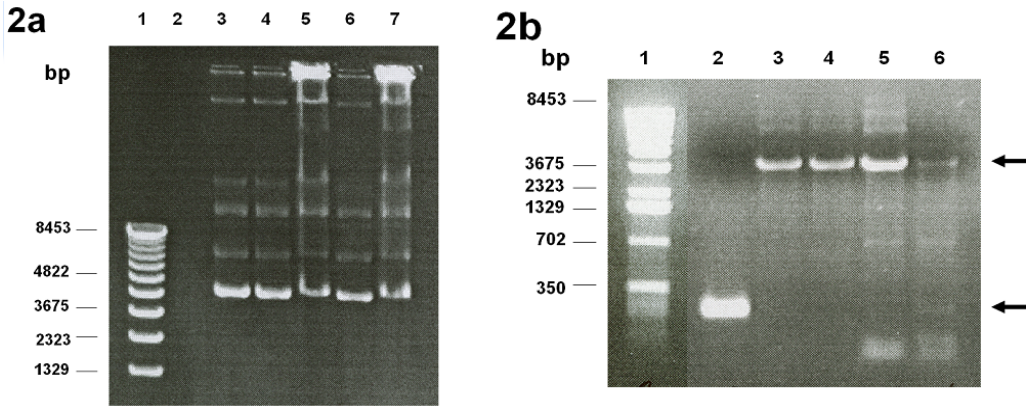

Fig. 2 (a)- A 0.5% agarose gel electrophoresis of a non-recombinant (lane 3) and four recombinant bacmids (lane 4-7). Lane 1 represents DNA size markers and lane 2 is blank. Due to the large size of the bacmid DNA (about 130 kb), the positions of non-recombinant and recombinant bacmids on the gel seem similar. (b) Verification of the presence of *mGαq* in the recombinant bacmid by PCR. The expected PCR products for bacmid alone (lane 2) and the bacmid transposed with *Gαq*-recombinant pFast Bac-1 (lanes 3-6) were ~300 bp and ~3600 bp, respectively. The recombinant bacmid obtained from four different colonies are used as PCR templates (lanes 3-6). The arrows indicate the position of PCR products on the gel.

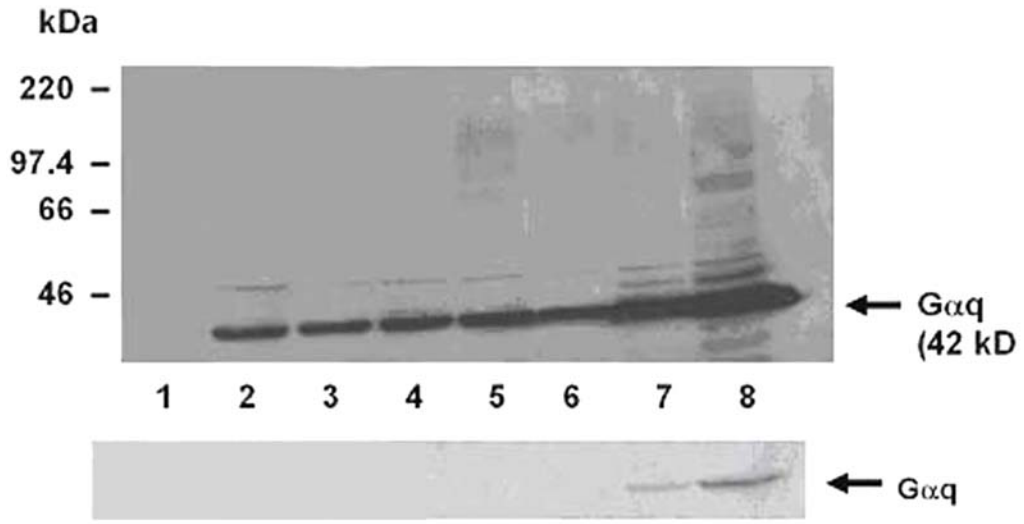

Fig. 3- A western blotting experiment showing the production of recombinant mGαq in SF9 insect cells. The cells were infected with MOI = 1 or MOI = 2 of the recombinant virus and harvest 24 and 48 hours post-infection. The membrane proteins of the cells are shown on a 8% SDS-PAGE. The proteins in the gel are transferred to nitrocellulose and blotted with a polyclonal antibody against Gαq. Equal amount of protein is loaded into each well. The lanes represent the membrane proteins obtained from: lane 2, the cells harvested immediately after infection (T_0), lane 3, non-infected cells harvested 24h post-infection, lane 4, cells infected with MOI = 1 and harvested 24h post-infection, lane 5, cells infected with MOI = 2 and harvested 24h post-infection, lane 6, non-infected cells harvested 48h post-infection, lane 7, cells infected with MOI = 1 and harvested 48h post-infection, lane 8, cells infected with MOI = 2 and harvested 48h post-infection. Pre-stained protein markers are loaded in lane 1. In the upper panel, the weaker bands are probably the result of cross reaction of the antibody with nonspecific proteins. The lower panel is a shorter exposure of film with the same blot. The arrows indicate the position of mGαq in the gel. MOI stands for Multiplicity of Infection.

A fraction of the recombinant Gαq protein was found in the cytoplasm (not shown). It has been reported that G alpha subunits translocate from the membrane to the cytosol as a result of depalmitoylation (Kosloff *et al*. 2003). Therefore, the recombinant Gαq found in the cytosol of Sf9 cells might represent depalmitoylated or non lipid-modified forms of Gαq.

The expression level of the recombinant Gαq 24 hours post-infection is not very high but after 48 hours Sf9 cells produce signi-ficant levels of the recombinant Gαq (Fig. 3). Also Sf9 cells express endogenous Gαq which has the same molecular weight as recombinant protein. Measuring the intensity of bands from western blotting experiments showed that under appropriate conditions one can get about 0.5 mg Gαq/liter of SF9 suspension culture infected with a MOI=2 of the recombinant viruses.

In the experiments whose results have not been shown in this paper, I cross linked [α^{32}P]-GTP to the membrane proteins of SF9 cells (infected with Gαq recombinant viruses) and following immunopre-cipitation of Gαq, I found that this protein was heavily loaded with GTP, suggesting that the Gαq protein made by *Baculovirus* expres-sion system was functional.

In conclusion *Mouse* Gαq (mGαq) has been expressed in SF9 insect cells using a *Baculovirus* expression system. The protein was produced at high level and was localized to the expected position in the cell (cell membrane). These two features are the basic characteristics of an appropriate expression system. In addition, ease of infection of SF9 cells with the recombinant virus, infection of high percentage of cells with the virus, incubation of SF9 cells at room temperature in an ordinary humidified incubator (25^{o}C), and growing SF9 cells in suspension cultures are some features that make the *Baculovirus* expression system an excellent choice for overproduction of proteins, especially in large scales. In this work the *Bac-to-Bac Baculovirus Expression System* manufactured by Invitrogen (*www. invitrogen.com*) has been used for Gαq expression. Today different companies make *Baculovirus* expression systems that might be slightly different from each other but the principles are similar for all of them.

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