

# Expression of Cell Surface GPI-Anchored Human p97 in Baculovirus-Infected Insect Cells

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**Abstract:** The baculovirus/insect cell system (*Autographa californica* multiple nuclear polyhedrosis virus/*Spodoptera frugiperda* Sf9 cells) was used to express the GPI-anchored human melanoma tumor antigen, melanotransferrin or p97. This system served to study the expression and productivity of recombinant GPI-anchored p97 by insect cells. The Sf9 cells expressed a cell surface GPI-anchored form of p97 as well as a soluble form of p97 that did not appear to be derived from the GPI-anchored form of p97. Both recombinant forms, although Endo H resistant, migrated slightly faster (~88 kDa) than the native p97 (~95–97 kDa). The insect GPI-anchored p97 was sensitive to PI-PLC, which exposed a detectable cross-reacting determinant. The Sf9 cell surface p97 expression was similar to that of human melanoma (SK-MEL-28) cells, whereas the Sf9 cell specific secretion rate was 10-fold higher. Also Sf9 cells retained considerably higher levels of p97 within the cell. The Sf9 cell surface expression of p97 varied with time after infection, with the maximum expression, which appeared independent of multiplicities of infection greater than 1, occurring at 48 h. After 48 h, levels of cell surface and secreted p97 fell whereas p97 retained within the cell increased, which possibly reflected the lytic nature of the expression system. The successful expression of GPI-anchored human p97 by the baculovirus/insect cell system not only provides a source of p97 for further research but also is the basis of an alternative method for the commercial production of GPI-anchored proteins. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* 55: 41–53, 1997.

**Keywords:** baculovirus; insect cell; p97; glycosylation; GPI anchor; protein expression

## INTRODUCTION

A fundamental issue in cell biology is the role of post-translational modification in controlling the structure

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and function of proteins. One post-translational modification which is currently being studied is the attachment of preassembled glycosylphosphatidylinositol (GPI) anchors to proteins. This modification allows proteins to become plasma membrane associated and confers on these proteins certain unique properties, such as apical localization in polarized cells and ability to interact with proteins involved in signal transduction and cell protection (Ferguson and Williams, 1988; Lisanti et al., 1990; Low, 1987). The basic GPI anchor structure of ethanolamine-PO<sub>4</sub>-(mannose)<sub>3</sub>-glycosamine-inositol-PO<sub>4</sub>-lipid has been found to be highly conserved throughout eukaryotic evolution. Modifications can be made to this structure by the addition of various side chains, with the most elaborate side chains being found in protozoa (Ferguson and Williams, 1988; Ferguson, 1994; McConville and Ferguson, 1993). Although the biosynthesis and function of GPI anchors have been extensively studied in mammalian cells and protozoa (Ferguson and Williams, 1988; Ferguson, 1994; Low, 1989; McConville and Ferguson, 1993), little attention has been paid to the expression of GPI anchors in lower invertebrates such as insect cells.

The baculovirus/insect cell system has been used to express a wide variety of proteins from many different species. This system takes advantage of the fact that the viral protein, polyhedrin, is produced at very high levels in infected insect cells (up to 50% of total cell protein under optimum conditions) but is not required for viral replication/production (Luckow, 1991). Replacement of the polyhedrin gene with a foreign gene under the control of the polyhedrin promoter results in a virus which will produce high levels of foreign protein upon infection of insect cells. This system is useful for producing mammalian proteins because the proteins are expressed in the correct location within the cell, are usually correctly folded, and undergo some of the posttranslational

modifications that occur in mammalian cells (Luckow, 1991; Miller, 1988).

It was originally considered that insect cells were unable to synthesize GPI anchors and carry out the post-translational modification of GPI anchor addition (Albert-Wolf et al., 1991). However, recent studies have demonstrated that insect cells are able to express fully functional recombinant GPI-anchored proteins on the cell surface (Choudrai et al., 1994; Davies and Morgan, 1993; Longacre et al., 1995; Scheirle et al., 1992). For example, the *Trypanosoma brucei* transferrin binding protein complex (ESAG 6 and 7) (Choudrai et al., 1994), the C-terminal fragments (42 and 19 kDa) of *Plasmodium vivax* merozoite surface protein (Longacre et al., 1995), and human complement-inhibition protein (CD59) (Davies and Morgan, 1993) have been expressed in this manner.

In one other study HLA-DR4Dw4 molecules were expressed as a recombinant GPI-anchored form by replacing the transmembrane and cytoplasmic domains by the carboxy terminal sequence of the decay acceleration factor (Scheirle et al., 1992). This sequence directed the protein to be GPI anchored.

These studies were carried out mainly to express large quantities of the recombinant GPI-anchored protein for further characterization. In all cases the expression of the recombinant GPI-anchored protein was successful and the GPI anchor was identified by phosphatidylinositol phospholipase C (PI-PLC) sensitivity (Choudrai et al., 1994; Davies and Morgan, 1993; Scheirle et al., 1992) exposure of the cross-reacting determinant (CRD) (Choudrai et al., 1994) and labeling with [<sup>3</sup>H] ethanolamine (Longacre et al., 1995).

Soluble forms of the recombinant protein were also found in the supernatant (Choudrai et al., 1994; Davies and Morgan, 1993), although it was not clear whether they were derived from the GPI-anchored form or separately secreted into the media (Longacre et al., 1995). The majority of soluble CD59 that was found in the medium was GPI anchored and was considered to be derived from lysed cells since the medium was not analyzed until 3 to 5 days after infection (Davies and Morgan, 1993).

To further investigate the expression of GPI anchors by insect cells, the GPI-anchored human melanoma tumor antigen, p97, was chosen to be expressed by baculovirus-infected insect cells. The p97 is a 97-kDa monomeric sialoglycoprotein that has recently been shown to be involved in the transferrin-independent transport of iron into mammalian cells (Kennard et al., 1995). The p97 was originally described as a melanoma-associated oncofetal antigen (Brown et al., 1980; Dippold et al., 1980; Woodbury et al., 1980) and was characterized using monoclonal antibodies (MAb) raised against human melanoma cells (Brown et al., 1981; Dippold et al., 1980; Woodbury et al., 1981), including the SK-MEL-28 melanoma cell line.

Structurally, p97 belongs to the group of iron binding proteins that include human serum transferrin, human lactoferrin, and ovotransferrin from egg white (Brown et al., 1982). It is encoded on the same region of the human chromosome 3 as serum transferrin and the transferrin receptor (Seligman et al., 1986).

Based on studies using SK-MEL-28 melanoma cells and Chinese hamster ovary (CHO) cells transfected with human p97, it was shown that p97 was attached to the cell surface by a GPI anchor (Alemany et al., 1993; Food et al., 1994). Furthermore, a distinct secreted form of p97 was also detected in the spent medium (Food et al., 1994; Kennard et al., 1993).

The *Autographa californica* multicapsid nuclear polyhedrosis baculovirus (wild-type AcMNPV) was used to express human GPI-anchored p97 in *Spodoptera frugiperda* insect cells. The expression of the native major envelope protein of the baculovirus, gp64 envelope fusion protein, also known as gp67, was monitored as a control. This protein is a type I integral membrane glycoprotein that plays an essential role in baculovirus infection (Whitford et al., 1989). The insect cell expressed p97 was compared with its native form, and the overall productivity of p97 by the baculovirus/insect cell system was studied. The successful expression of functionally active GPI-anchored p97 by insect cells will provide an abundant source of protein for further investigations into the function and characterization of p97.

## MATERIALS AND METHODS

### Cell Lines

*Spodoptera frugiperda* IPLB-Sf21-AE clonal isolate 9 (designated Sf9) cells were maintained in TC100 medium [Grace's medium (Gibco) supplemented with 3.3 g/L TC yeastolate (Difco), 3.3 g/L lactalbumin hydrolysate (Difco), and 10% fetal calf serum (FCS, Sigma)] (Summers and Smith, 1987) at 27°C with no pH control in 100-mL spinner flasks (Bellco) at 60 rpm. Cells were cultured to approximately  $2 \times 10^6$  cells/mL and passaged at  $2 \times 10^5$  cells/mL. The human melanoma cell line SK-MEL-28 was obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FCS, 20 mM N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine at 37°C in a 5% CO<sub>2</sub> humidified environment. Cells were seeded at 1 to  $2 \times 10^5$  cells/mL in 25 mL of growth medium in 75-cm<sup>2</sup> T-flasks (Nunc).

These cells were harvested from the T-flasks using Versene [0.2 g/L ethylenediaminetetraacetic acid (EDTA), 8.0 g/L NaCl, 0.2 g/L KCl, 1.43 g/L Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 0.2 g/L glucose] to lift the cells and centrifuged at 250g for 5 min. Cell number and viability were deter-

mined using a hemocytometer and trypan blue exclusion. The CHO cell line p97aWTBc3, transfected with human p97 (Food et al., 1994), was cultured and harvested in a similar manner to SK-MEL-28.

### Construction of pVL1393/p97 Plasmid

The human p97 cDNA was excised from the pSV2p97a plasmid (provided by Dr. G. D. Plowman and Dr. K. E. Hellstrom of Bristol-Meyers Squibb, Seattle) (Estin et al., 1989), by a HindIII and NruI double digest. The insert was isolated and made blunt ended using Klenow DNA polymerase and inserted into the SmaI site of the baculovirus transfer vector, pVL1393 (provided by Dr. M. Summers) (Luckow and Summers, 1989) using standard recombinant DNA techniques. This plasmid, called pVL1393/p97, was used to insert the p97 cDNA into the baculovirus genome.

### Isolation of p97 Recombinant Virus and Transfection of Sf9 Cells

The Sf9 cells were co-transfected with a mixture of wild-type AcMNPV genomic DNA and p97 construct according to method I described by Summers and Smith (1987). In several rounds of plaque assays, occlusion negative recombinant plaques were detected by visual screening and the purified recombinant virus was designated p97 B-2-1. Virus stock was titered using the endpoint dilution method (Summers and Smith, 1987). The Sf9 were seeded in 50 mL of growth media at  $2 \times 10^5$  cells/mL and grown to approximately  $2 \times 10^6$  cells/mL, which corresponds to the early or mid-exponential growth phase of the culture. This phase of growth has been shown to be optimal for maximum recombinant protein expression (Reuveny et al., 1993). The cell density was chosen to avoid substrate limitation during the period of infection.

The cells were infected with either the wild-type or recombinant virus at a given multiplicity of infection (MOI, virions/cell) by centrifuging the cells at 250g for 5 min and resuspending them in 5 mL of the viral inoculum at 27°C for 1 h with periodic agitation. The cells were returned to the spinner flask and fresh media added to increase the suspension volume back to 50 mL. The infection and cellular expression of p97 was then observed over time.

### Flow Cytometry Analysis and PI-PLC Digestion

The Sf9 cells were infected with wild-type AcMNPV or p97 B-2-1 at an MOI of 1 and harvested at a given hour post-infection (hpi) by centrifuging the cells at 250g for 5 min. An MOI of 1.0 was considered sufficient to result in synchronous infection and to effectively halt cell growth. Confluent SK-MEL-28 cells were used as the positive control. For each sample,  $10^6$  cells were washed

twice with cold FACS buffer (TC100 with 20 mM Na<sub>3</sub>N for infected Sf9 cells or DMEM with 0.5% wt/vol bovine serum albumin, 20 mM HEPES, and 20 mM Na<sub>3</sub>N for SK-MEL-28 cells).

The cells were labeled with the primary MAb, either L235 (ATCC HB8446) or "C" (33B6E4, provided by Dr. Shuen-Kuei Liao, McMaster University, Hamilton, ON). The labeled cells were subsequently stained and fixed with FITC-conjugated goat anti-mouse (GAM) IgG (Sigma) as previously described (Food et al., 1994). Fluorescence intensity was measured using a nonsorting flow cytometer (FACScan, Becton-Dickenson). To determine the effect of PI-PLC on cell surface p97, the cells were incubated with either 100 μL FACS buffer (control) or 100 μL of partially purified (Kennard et al., 1993) PI-PLC in phosphate-buffered saline (PBS) (300 mU/mL) at 27°C for 1 h. PI-PLC is able to specifically cleave the GPI anchor releasing the protein in a soluble form (Ferguson and Williams, 1988; Low, 1987).

The cells were labeled, stained, and fixed as previously described (Food et al., 1994). As a control for PI-PLC treatment the cells were also treated with the primary MAb AcV<sub>1</sub> (provided by Dr. P. Faulkner, Queen's University, Kingston, Ontario) in the form of hybridoma supernatant. The AcV<sub>1</sub> MAb reacts with the wild-type AcMNPV integral membrane protein gp64.

### Biosynthetic Labeling with [<sup>35</sup>S]Methionine: Pulse-Chase Labeling and Immunoprecipitation

The Sf9 cells were infected at  $2 \times 10^6$  cells/mL with p97 B-2-1 at an MOI of 1.0 and harvested at 48 hpi by centrifugation at 250g for 5 min. Then  $4 \times 10^6$  cells were pre-labeled for 1 h at 27°C in 3 mL of methionine-free DMEM (Gibco) supplemented with 20 mM HEPES and 2 mM L-glutamine. This medium was then removed and replaced with 0.6 mL methionine-free DMEM containing 200 μCi [<sup>35</sup>S]methionine/mL (specific activity >1000 Ci/mM for all batches, Amersham Canada Limited).

The cells were pulsed for 30 min at 27°C and chased in 2 mL of TC100 at 27°C for the times indicated. Confluent SK-MEL-28 cells were treated in a similar manner to Sf9 cells at 37°C and chased in DMEM. The spent medium or supernatant was collected and the cells were lysed in 1 mL of lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 40 μg/mL PMSF) on ice for 30 min. The lysates and cell supernatants were cleared by centrifugation at 11,000g for 30 min at 4°C prior to immunoprecipitation.

The MAb, L235, was used to immunoprecipitate p97 according to standard procedures using protein A-sepharose beads (Pharmacia). Immunoprecipitated proteins were eluted from protein A-sepharose by boiling at 95°C for 5 min in 20 μL buffer [50 mM K<sub>2</sub>HPO<sub>4</sub>, 20 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and 2% N-octylglucoside, pH 7.5] prior to analysis by SDS-

polyacrylamide gel electrophoresis (PAGE) (10% w/v) under reducing conditions. Gels were fixed and autoradiographed using Kodak X-OMAT AR film.

### Endoglycosidase H and F Digestion of Immunoprecipitated p97

The [<sup>35</sup>S] methionine labeled p97 immunoprecipitated from cell lysates and spent medium supernatants was digested with 5 mU Endoglycosidase H (Endo H) (Boehringer Mannheim) per sample for 24 h at 37°C. The p97 was then eluted from the protein A beads and analyzed by SDS-PAGE. In the case of Endoglycosidase F (Endo F) (Boehringer Mannheim) digestion, the immunoprecipitated labeled p97 was first eluted from the protein A before incubation with 10 mU Endo F for 24 h at 37°C. This was to denature the protein in order to ensure complete removal of the oligosaccharides from p97.

### Phase Partitioning with Triton X-114

The Sf9 cells, infected at a MOI of 1 with wild-type AcMNPV or p97 B-2-1, were harvested at 48 hpi and labeled with [<sup>35</sup>S]methionine for 30 min and chased in TC100 for 16 h. Aliquots of 1 mL were centrifuged to separate the cells and supernatant. The cells were lysed at 4°C in solubilization buffer ( $4 \times 10^6$  cells/mL of buffer) containing precondensated 1% Triton X-114 (Sigma) instead of 1% NP-40. One hundred microliters of 10% Triton X-114 was also added to 900  $\mu$ L cell supernatants. The lysates and cell supernatants were cleared of nuclei and cellular debris by centrifugation at 50,000g for 1 h at 4°C. To obtain phase separation, the samples were then incubated at 37°C for 5 min and centrifuged at 3000g for 3 min at room temperature. The samples were re-extracted three times to improve the separation, and the corresponding phases were pooled. The volumes were equalized by adding lysis buffer without detergent to the detergent phases. The samples were subsequently immunoprecipitated (described above).

### PI-PLC Treatment of Triton X-114 Detergent Phase p97

The p97 samples that had been recovered from the detergent phase of the supernatant and cell lysate of  $1 \times 10^7$  Sf9 pre-labeled and lysed cells (described above) were treated with PI-PLC (300 mU/mL) and incubated at 37°C for 1 h. The PI-PLC treated samples were again Triton X-114 extracted and then immunoprecipitated with L235 MAb as described above. This experiment was repeated for gp64 using the AcV<sub>1</sub> MAb in order to ensure that non-GPI-anchored proteins were not affected by PI-PLC treatment.

### Determination of Cellular p97 Expression

(a) *Secreted p97*: Cultures of Sf9 cells (infected with p97 B-2-1 at MOI of 1), SK-MEL-28, and p97aWTBc3 were established in the appropriate media. After 48 h (48 and 72 hpi in the case of Sf9 cells), cell densities were determined using a hemocytometer and trypan blue exclusion. The spent growth media or supernatant was recovered and the concentration of p97 secreted in the growth media was determined using a modified immunofluorescent assay and fluorescence concentration analysis (Kennard et al., 1993). Briefly, the MAb L235 was immobilized on carboxyl-polystyrene capture particles (0.77  $\mu$ m, Idexx). Soluble p97 binds to L235 and is labeled with the fluoresceinated secondary anti-p97 MAb, 33B6E4, or "C". The fluorescence was read by a Pandex fluorescence concentration analyzer (PCFA, Idexx) and p97 concentration determined according to Kennard et al. (1993). Based on the total cell number and p97 concentration of the growth media, the cellular p97 secretion could be determined.

(b) *Cell surface p97*:  $2 \times 10^7$  cells from Sf9, SK-MEL-28, and p97aWTBc3 cultures were pelleted after 48 h of growth (48 and 72 hpi in the case of Sf9) and washed in PBS. The cells were resuspended in 1 mL of partially purified PI-PLC (Kennard et al., 1993) at 300 mU/mL PI-PLC in PBS and incubated for 1 h at 37°C. To ensure the majority of p97 had been cleaved from the cell surface, the cells were also checked by flow cytometry as previously described. The PI-PLC solution was recovered and the concentration of the cleaved p97 determined according to the previously described method. Based on the total cell number and the total cleaved p97, the amount of cell surface or GPI-anchored p97/cell could be determined. The cell surface p97 expression for all cell lines was also monitored by flow cytometry analysis over a 96-h period. The effect of MOI was studied by comparing the surface expression of p97 by infected Sf9 cells over time for MOIs of 0.1, 1.0, 10, and 100.

(c) *Total cellular p97*:  $2 \times 10^7$  cells from Sf9, SK-MEL-28, and p97aWTBc3 cultures were pelleted after 48 h of growth (48 and 72 hpi in the case of Sf9) and incubated in lysis buffer on ice for 30 min. The mixture was spun and filtered and the supernatant assayed for p97 according to the previously described method. Based on the total number of cells and the total amount of p97 recovered, the total amount of p97/cell could be determined.

### Analysis of the Cross-Reacting Determinant

Secreted and PI-PLC cleaved cell surface p97 was recovered from Sf9, SK-MEL-28, and p97aWTBc3 cells as previously described. Secreted samples of p97 were also treated with PI-PLC at 300 mU/mL and incubated at 37°C for 1 h. All p97 samples were diluted to 10  $\mu$ g/

mL and bound to immobilized L235 (described above). The bound p97 was then labeled with 20  $\mu$ g/mL of FITC labeled anti-CRD rabbit antiserum (specific for the CRD of trypanosome variant surface glycoprotein (VSG), provided by Dr. T. W. Pearson, University of Victoria, B.C.) and incubated at room temperature for 30 min. The fluorescence of the immune complex was read using the PCFA. As a positive control, a sample of VSG (WATAT 1.1, provided by Dr. T. W. Pearson) was bound to immobilized anti-WATAT 1.1 VSG rabbit antiserum (provided by Dr. T. W. Pearson). As a negative control, a sample of human transferrin (Sigma) was bound to immobilized anti-human transferrin sheep antiserum (Sigma). Background fluorescence was based on samples containing no p97.

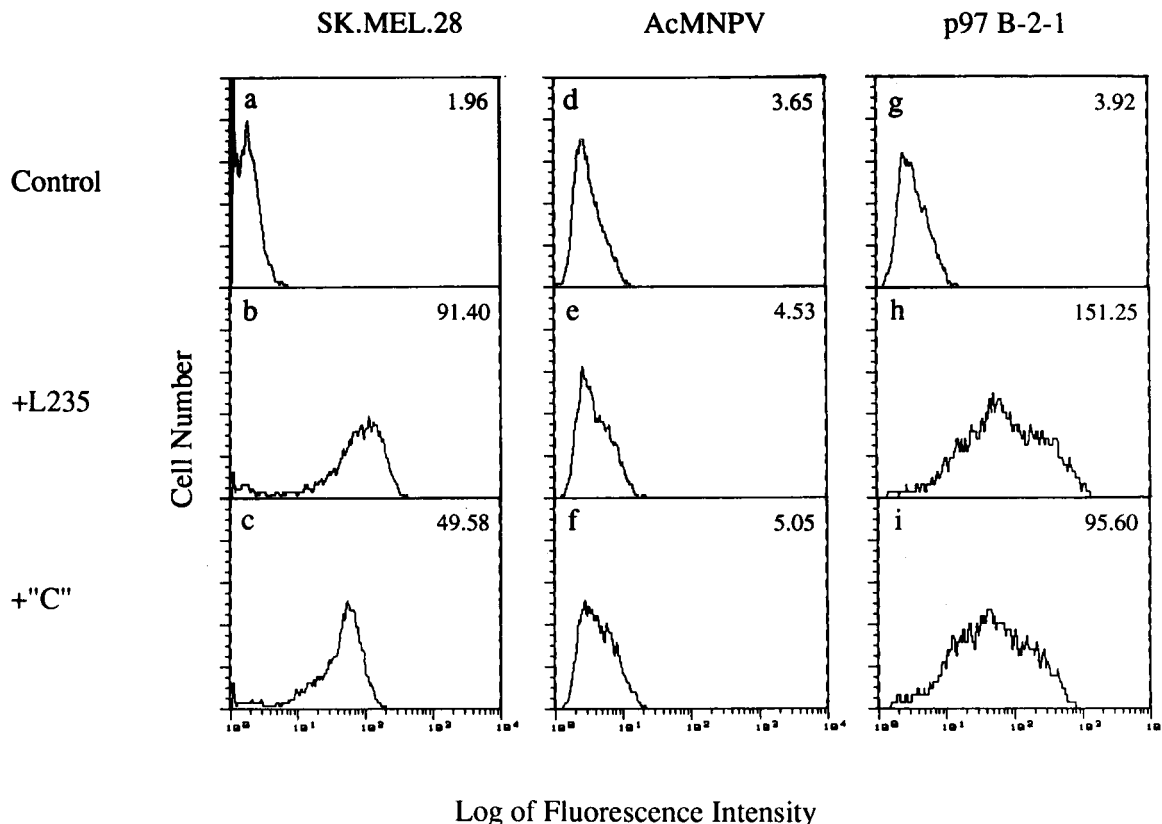
## RESULTS

### Expression of Recombinant p97

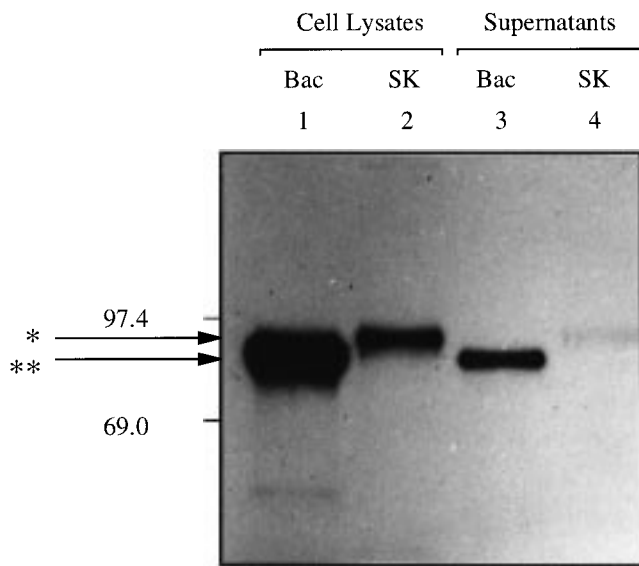
Flow cytometry analysis of p97 B-2-1 infected Sf9 cells revealed that recombinant p97 was expressed at the cell surface (Fig. 1, panels h and i). The presence of p97 was detected using the anti-human p97 MAbs, L235,

and "C". SK-MEL-28, a human melanoma cell line that expresses p97, was used as a positive control, and Sf9 infected with wild-type AcMNPV was used as a negative control. Although the linear mean fluorescence was higher for p97 B-2-1 infected Sf9 cells compared to SK-MEL-28 cells (e.g., 151.25 vs. 91.40 when staining with L235), the population scatter for Sf9 cells was considerably greater than for SK-MEL-28 cells (Fig. 1, panels h and b or i and c). Neither mock infected (data not shown) nor wild-type AcMNPV infected Sf9 cells were stained by either L235 or by "C" (Fig. 1, panels e and f), indicating that they do not express any surface proteins that cross-react with these MAbs and that the increased fluorescence of p97 B-2-1 infected Sf9 cells was due specifically to the presence of p97.

Figure 2 shows that recombinant p97, expressed by infected Sf9 cells, was found in the growth medium as well as associated with the cell surface. This soluble p97 appears to be a single major form and is the same size as the p97 immunoprecipitated from the cell lysate (Fig. 2, lanes 1 and 3). This result was similar to p97 immunoprecipitated from SK-MEL-28 cell lysate and supernatant (Fig. 2, lanes 2 and 4). However, the p97 from Sf9 cells migrates slightly faster (~88 kDa) than p97 from SK-MEL-28 cells (~95 kDa). The difference in size of



**Figure 1.** Analysis of cell surface p97 expression by flow cytometry. SK-MEL-28 cells and infected Sf9 cells (wild-type AcMNPV or p97 B-2-1, MOI of 1, 72 hpi) were labeled with L235 (b, e, h) or "C" (c, f, i) and subsequently stained with FITC-conjugated GAM IgG. The negative controls indicating background levels of fluorescence were generated by no first antibody staining of each cell type (a, d, g). The histograms are plotted on a log scale while the mean linear fluorescence is indicated in the top right-hand corner of each profile.



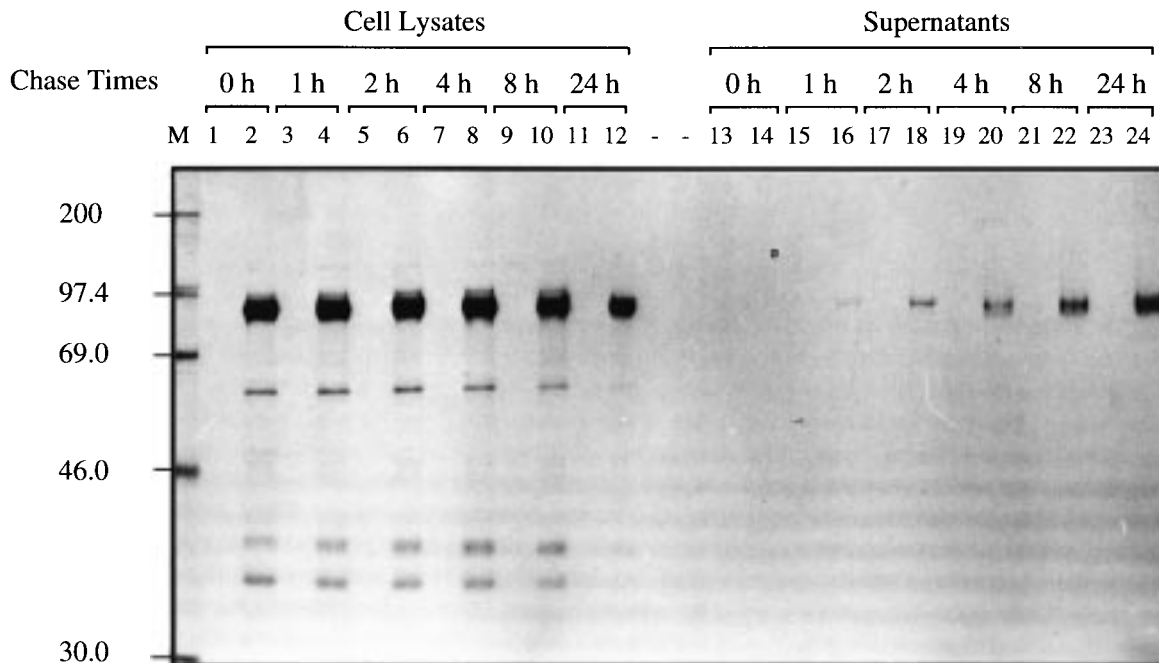
**Figure 2.** Immunoprecipitation of p97 from SK-MEL-28 cells and from p97 B-2-1 infected Sf9 cells. Infected Sf9 cells (p97 B-2-1, MOI of 1, 48 hpi) (Bac) were pulsed with [<sup>35</sup>S]methionine for 30 min and chased in TC100 for 8 h at 27°C. SK-MEL-28 cells (SK) were pulsed with [<sup>35</sup>S]methionine for 30 min and chased in normal cell culture medium for 8 h at 37°C. The labeled p97 was immunoprecipitated from cell lysate (Sf9 Lane 1, SK-MEL-28 lane 2) and supernatant (Sf9 lane 3, SK-MEL-28 lane 4) using L235. The samples were analyzed by SDS-PAGE (10% w/v) under reducing conditions. The molecular weight markers are indicated on the left. The p97 from SK-MEL-28 cells and supernatant is marked with a single asterisk while recombinant p97 from Sf9 cells and supernatant is marked with a double asterisk.

the fully processed forms of p97 is likely due to differences in glycosylation.

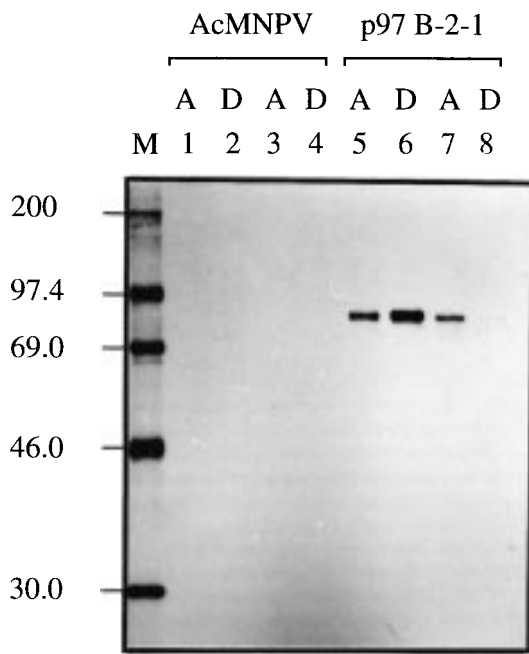
Pulse-chase studies (Fig. 3) showed that the p97 immunoprecipitated from infected Sf9 cell lysate and supernatant migrated as a single major form over the 24-h chase period. This indicated that the p97 was rapidly processed into its final form and that the protein was relatively long lived, based on its continued detection over the 24-h period.

### Characterization of the Soluble and Membrane-Bound Forms of p97

The p97 was further characterized by Triton X-114 phase separation of p97 from pulse-chase labeled cells and supernatant. It was found that the majority of p97 from Sf9 cell lysate partitioned into the detergent phase (Fig. 4, lanes 5 and 6), whereas the p97 found in the supernatant partitioned mainly into the aqueous phase (Fig. 4, lanes 7 and 8). This suggests that the soluble p97 found in the supernatant was not GPI anchored and was generated either intracellularly or cleaved from the surface of the cell. The p97 from the cell lysate that was found in the detergent phase was due to the amphiphilic nature of transmembrane and GPI-anchored proteins. The portion of p97 from the cell lysate that partitioned into the aqueous phase reflected the soluble form of p97. These results were in agreement with a previous study that identified a membrane-bound GPI-anchored and a secreted form of p97 expressed by SK-MEL-28 cells (Food et al., 1994).



**Figure 3.** Pulse-chase analysis of recombinant p97 in infected Sf9 cell lysates and corresponding supernatant. Infected Sf9 cells (wild-type AcMNPV or p97 B-2-1, MOI of 1, 48 hpi) were pulsed with [<sup>35</sup>S]methionine for 30 min and chased in TC100 at 27°C for various lengths of time. The p97 was immunoprecipitated from all samples using L235. The samples were analyzed by SDS-PAGE (10% w/v) under reducing conditions. The odd-numbered lanes are from wild-type AcMNPV infected Sf9 cells and the even-numbered lanes are from p97 B-2-1 infected Sf9 cells. M refers to the molecular weight markers indicated on the left.



**Figure 4.** Triton X-114 phase separation of recombinant p97 present in p97 B-2-1 infected Sf9 cell lysates and corresponding supernatant. Infected Sf9 cells (wild-type AcMNPV or p97 B-2-1, MOI of 1, 48 hpi) were pulsed with [<sup>35</sup>S]methionine for 30 min and chased for 16 h at 27°C. Cell lysates and supernatant were collected and all samples were separated into the aqueous (A) and detergent (D) phases by Triton X-114 extraction. Wild-type AcMNPV infected Sf9 cell lysate (lanes 1 and 2) and corresponding supernatant (lanes 3 and 4) as well as p97 B-2-1 infected Sf9 cell lysate (lanes 5 and 6) and corresponding supernatant (lanes 7 and 8) were incubated with L235 to immunoprecipitate recombinant p97. The samples were analyzed by SDS-PAGE (10% w/v) under reducing conditions. M refers to the molecular weight markers indicated on the left.

#### Effect of PI-PLC Treatment of Cell Surface p97

It has been previously demonstrated that p97 is attached to the cell surface membrane of SK-MEL-28, Caco-2, and fetal duodenal cells by a GPI anchor (Alemany et al., 1993; Food et al., 1994). Sensitivity to PI-PLC is a hallmark of GPI-anchored proteins. Figure 5 shows that cell surface recombinant p97 expressed by infected Sf9 cells was cleaved from the surface by bacterial PI-PLC. In this study the PI-PLC treatment removed approximately 75% of the cell surface p97 from SK-MEL-28 cells (Fig. 5, panels a, b, and c) and approximately 87% from p97 B-2-1 infected Sf9 cells (Fig. 5, panels d, e, and f). This suggests that recombinant human p97 expressed by infected Sf9 cells was attached to the surface by a GPI anchor.

To ensure that this was a specific effect, wild-type AcMNPV and p97 B-2-1 infected cells were also treated with PI-PLC and labeled with the AcV<sub>1</sub> MAb, which recognizes gp64, an integral membrane protein expressed at the surface of infected cells (Whitford et al., 1989). PI-PLC had no effect on the surface expression of gp64 in either cell type (Fig. 5, panels g, h, i, j, k, and l). It was also found that when the fraction of p97 from

lysed p97 B-2-1 infected Sf9 cells that partitioned into the Triton X-114 detergent phase was treated with PI-PLC and re-extracted with Triton X-114, the p97 separated mainly into the aqueous phase due to cleavage of the hydrophobic GPI tail. Whereas PI-PLC treatment of detergent phase non-GPI-anchored gp64 from p97 B-2-1 infected cell lysates had no effect on the phase distribution of gp64 (data not shown). These results clearly showed that recombinant p97 expressed by infected cells was attached by a GPI anchor to the cell surface and not simply associated with another GPI-anchored protein.

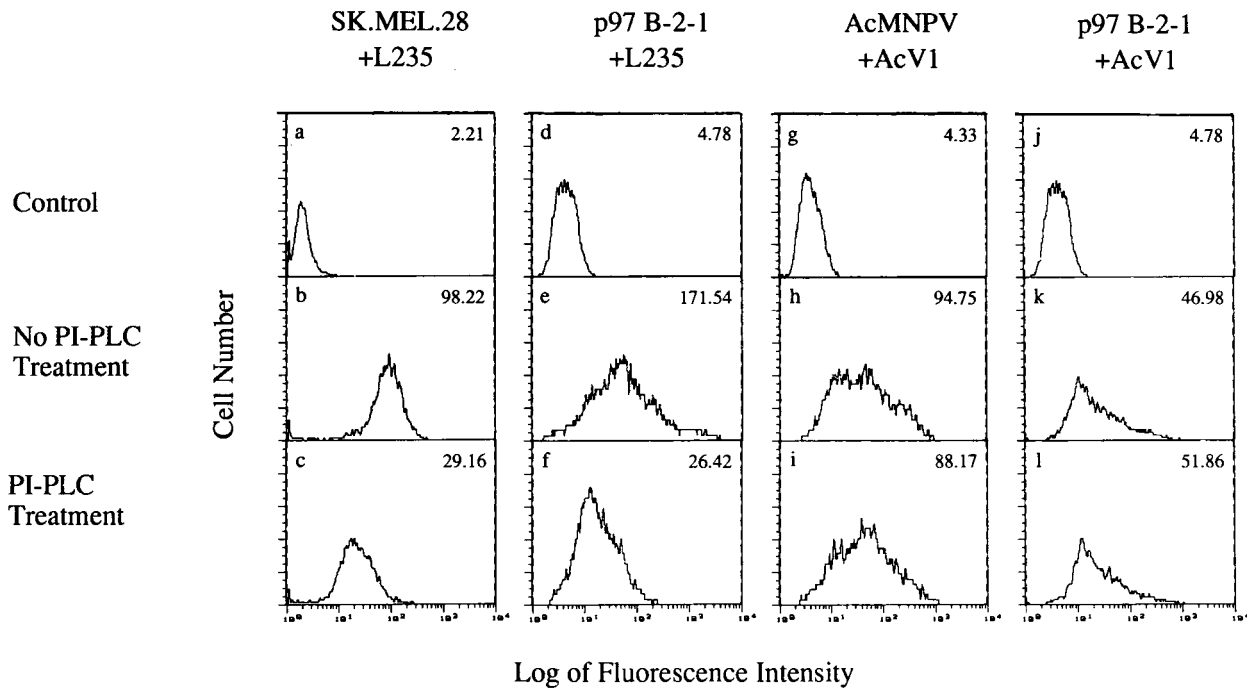
#### CRD Analysis of p97

The properties of the soluble and PI-PLC released membrane-bound forms of p97 expressed in Sf9, SK-MEL-28, and p97aWTBc3 cells were compared using rabbit antibodies specific for the cross reacting determinant (CRD) of soluble trypanosome VSGs (Fig. 6). These antibodies can cross react with other GPI-anchored proteins after the CRD has been exposed by treatment with PI-PLC (Ferguson and Williams, 1988). It can be clearly seen that only PI-PLC cleaved forms of cell surface p97 have a CRD and were, therefore, GPI anchored. The lack of antibody binding to the supernatant p97, even after PI-PLC treatment, shows that it is unlikely that this form of p97 was derived from the GPI-anchored form.

#### Intracellular Transport and Glycosylation of p97 by Sf9 Cells

To investigate the transport and glycosylation of p97 expressed in Sf9 cells, GPI-anchored and secreted p97 was digested with endoglycosidase H (Endo H) at various times during a pulse-chase experiment. Two Endo H sensitive forms were detected in the cell lysate (Fig. 7, lane 1) which were processed to a single higher molecular weight Endo H resistant form (Fig. 7, lanes 2–6) after the 1-h chase, with complete resistance achieved by the 8-h chase. In the untreated cell lysate, there appears to be a single form (Fig. 7, lanes 7–12), which was not processed over the 24-h chase as noted in Figure 4. There is also a single form of p97 found in the supernatant (Fig. 7, lanes 20–24), which was also Endo H resistant (Fig. 7, lanes 14–18) and migrated at the same rate as the fully processed form of p97 from the cell lysate. This result was similar to Endo H treatment of p97 secreted by SK-MEL-28 cells (Food et al., 1994) and showed that the GPI-anchored and soluble forms of p97 use the same secretory pathway for transport to the cell surface.

GPI-anchored and soluble forms of p97, immunoprecipitated from the lysates and supernatants of SK-MEL-28 and recombinant virus infected Sf9 cells, were also treated with Endo F. All forms of p97 were sensitive to Endo F digestion and were reduced to a single form of ~80 kDa (data not shown), which indicated that the



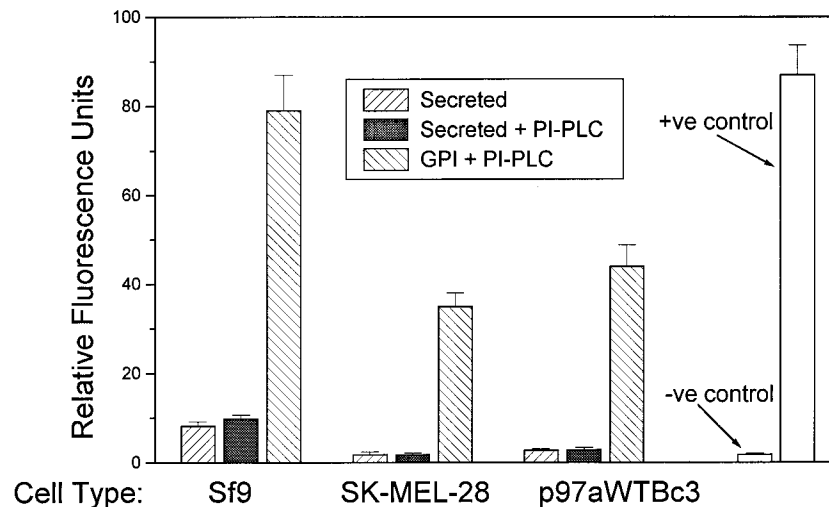
**Figure 5.** Flow cytometry analysis of the effect of bacterial PI-PLC on cell surface p97 expression. SK-MEL-28 cells and infected Sf9 cells (wild-type AcMNPV or p97 B-2-1, MOI of 1, 48 hpi) were incubated with (c, f, i, l) or without (b, e, h, k) bacterial PI-PLC for 1 h at 37°C. Cells were then labeled with either L235 (b, c, e, f) or AcV<sub>1</sub> (h, i, k, l) and subsequently stained with FITC-conjugated GAM IgG. The negative controls indicating background levels of fluorescence were generated by no first antibody staining of each cell type (a, d, g, j). The histograms are plotted in a log scale while the mean linear fluorescence is indicated in the top right-hand corner of each profile.

unglycosylated forms of p97 were the same size for both cell types.

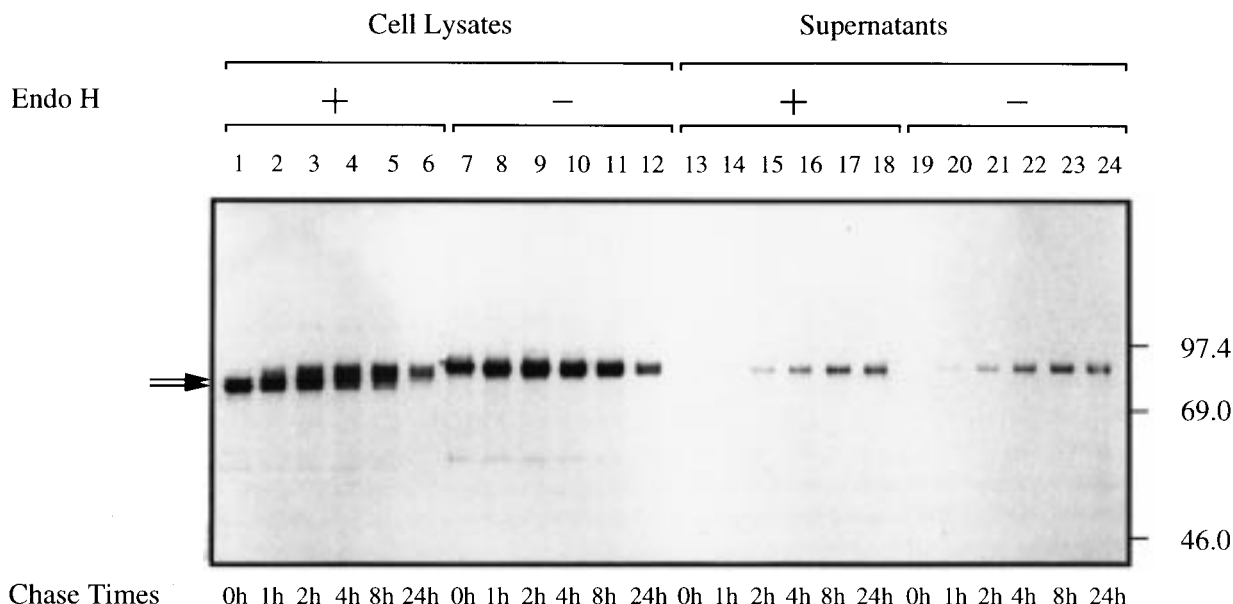
### Comparison of p97 Expression

The following experiments were carried out to compare p97 expression by infected Sf9 cells with that by SK-

MEL-28 and p97aWTBc3 cells. Figure 8 shows that the cell surface expression of p97, as determined by flow cytometry, varied considerably with hpi for infected Sf9 cells, reaching a maximum after 48 hpi, then falling to less than a third of the maximum by 96 hpi. This variability was much greater than for SK-MEL-28 and p97aWTBc3 cells over a similar time period. The fall in



**Figure 6.** Comparison of the binding of anti-CRD antibody to p97. Secreted and PI-PLC released GPI-anchored forms of p97 from infected Sf9 (p97 B-2-1, MOI of 1, 48 hpi), SK-MEL-28 and p97aWTBc3 cells were bound to immobilized L235 and treated with FITC labeled anti-CRD rabbit antisera. Secreted p97 was also treated with PI-PLC prior to the CRD assay. VSG was used as a positive control and transferrin was used as a negative control. Results are averaged from four readings.



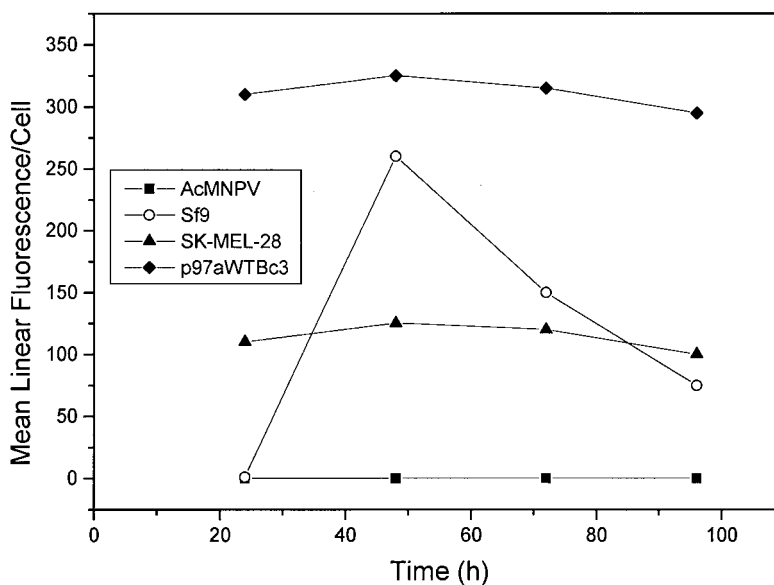
**Figure 7.** Analysis of recombinant p97 glycosylation in Sf9 cells by Endo H digestion. Infected Sf9 cells (p97 B-2-1, MOI of 1, 48 hpi) were pulsed with [<sup>35</sup>S]methionine for 30 min and chased for various times at 27°C. At each time point, cell lysates and supernatants were harvested. All samples were immunoprecipitated with L235 and incubated in the presence (lanes 1–6 and 13–18) or absence (lanes 7–12 and 19–24) of Endo H. The samples were analyzed by SDS-PAGE (10% w/v) under reducing conditions. The two Endo H sensitive forms are indicated with arrows.

expression could be due to breakdown of the processing machinery of the cells as they became further infected and lysed in late infection.

Figure 9 shows the effect of MOI on p97 expression by Sf9 cells. For cells infected with MOIs of 1, 10, and 100 the maximum expression occurred at 48 hpi and then decreased as the infection progressed. For these MOIs, cell growth virtually halted after infection and

the viable cell density increased slightly from  $\sim 2 \times 10^6$  cells/mL to  $\sim 2.5 \times 10^6$  cells/mL by 48 hpi, after which it fell steadily to zero by 144 hpi. For an MOI of 0.1, the maximum, which was similar to that for MOIs of 1 and greater, occurred at 72 hpi.

In this case the viable cell density increased to over  $4 \times 10^6$  cells/mL and did not fall until after 72 hpi. This maximum expression appeared relatively insensitive to



**Figure 8.** Cell surface p97 expression as a function of time and hpi. Cell surface p97 expression of infected Sf9 (wild-type AcMNPV and p97 B-2-1, MOI 1), SK-MEL-28, and p97aWTBc3 cells was monitored as a function of time and hpi by flow cytometry analysis using L235 and stained with FITC-conjugated GAM IgG. Wild-type AcMNPV infected Sf9 cells were used as a negative control.

MOI; however, MOIs of 10 and 100 resulted in a more rapid decrease in p97 surface expression and cell viability after 48 hpi. Therefore, to minimize use of virus stock, it was recommended that Sf9 cells be infected at an MOI of 1.0 and harvested at 48 hpi.

The total cellular production of p97 was compared in Table I. As can be seen, the cell specific expression of cell surface p97 by Sf9 cells was greater than that of SK-MEL-28 and about half that of p97aWTBc3 cells. However, the cell specific secretion rate of soluble p97 by Sf9 cells was 10-fold higher than either SK-MEL-28 or p97aWTBc3 cells. Also, Sf9 retained a much higher portion of p97 within the cell (more than 80% for Sf9 cells vs. 27.5% for SK-MEL-28 cells). It is interesting to note that the cell surface expression and secreted levels of p97 both fell with increasing hpi after about 48 hpi, whereas the retention of p97 within the cell appeared to more than double by 96 hpi. This may indicate, as mentioned previously, that the secretory pathways are breaking down with increased infection, causing interference with normal cell membrane turnover and increased p97 retention.

## DISCUSSION

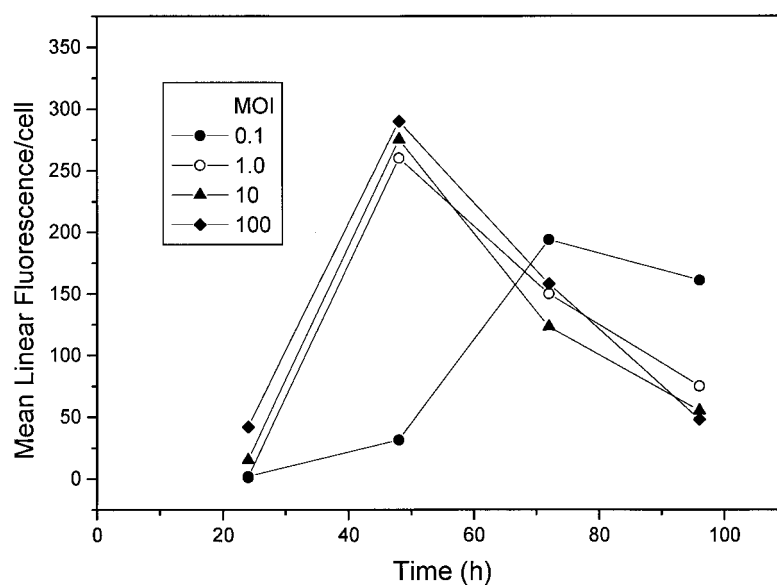
Human p97 was successfully expressed in p97 B-2-1 infected Sf9 cells as demonstrated by flow cytometry analysis using MAbs against two different human p97 epitopes. The expression of recombinant p97 on the surface of the Sf9 cells indicated that the *N*-terminal signal sequence for endoplasmic reticulum translocation was recognized in the insect cells and that the protein had the correct conformation for transport to the cell surface. The fact that both the MAbs, L235 and "C",

which recognize different p97 epitopes (Kennard et al., 1993), bind to p97 found on the surface of infected Sf9 cells suggests that the recombinant p97 is synthesized correctly and properly folded.

It was observed from the flow cytometry analysis that the level of p97 on the surface of recombinant virus infected Sf9 cells was more variable than on SK-MEL-28 cells (Fig. 1). This variability has been noted by other workers (Domingo and Trowbridge, 1988; Jarvis and Summers, 1989; Kupsch et al., 1992; Scheirle et al., 1992) and may reflect the lytic nature of the baculovirus life cycle, which may cause cells which are near death to express lower levels of p97 as a result of reduced transcription/translation and/or the breakdown of the protein transport and membrane turnover machinery. The population of cells could also be mixed due to an MOI of 1.0 resulting in different stages of infection, which would result in a wide range of expression.

To ensure synchronous infection, higher MOIs should be used. This hypothesis was supported by data on gp64, a wild-type viral protein, which also showed heterogeneous expression in wild-type AcMNPV and p97 B-2-1 infected Sf9 cells (Fig. 5). Variability in glycosylation, which often occurs for foreign proteins produced in the baculovirus system (Davidson et al., 1990; Jarvis and Summers, 1989; Luckow, 1991; Miller, 1988), may also affect the transport and stability of p97, resulting in varying levels of p97 at the cell surface.

Overall, for an MOI of 1, the maximum cell surface p97 expression occurred at around 48 hpi but fell to less than a third by 96 hpi (Fig. 8). This maximum level of p97 expression did not appear to be affected by increasing the MOI; however, MOIs of 10 and 100 did result in a more rapid decrease in p97 expression, probably



**Figure 9.** Cell surface p97 expression as a function of MOI. Cell surface p97 expression of infected Sf9 (p97 B-2-1) cells was monitored over time as a function of MOI by flow cytometry analysis using L235 and stained with FITC-conjugated GAM IgG.

**Table I.** Comparison of cellular p97 expression.

Cell type	Cell surface specific p97 <sup>a</sup> ( $\mu\text{g} \times 10^{-7}/\text{cell}$ )	Molecules p97 per cell surface ( $\times 10^6/\text{cell}$ )	Total cell specific p97 <sup>b</sup> ( $\mu\text{g} \times 10^{-7}/\text{cell}$ )	p97 on cell surface (%)	Cell specific p97 secretion rate <sup>c</sup> ( $\mu\text{g} \times 10^{-9}/\text{cell}/\text{h}$ )
Sf9 (48 hpi)	3.7	2.3	20.0	18.1	31.2
Sf9 (72 hpi)	1.96	1.22	51.0	3.8	10.5
SK-MEL-28	1.11	0.67	1.53	72.5	2.7
p97aWTBc3	8.3	5.25	9.9	84.1	3.54

Note: SK-MEL-28, p97aWTBc3 and infected Sf9 (p97 B-2-1, MOI of 1) were assayed for expression of cell surface, secreted and total cellular p97 after 48 h of growth (48 and 72 hpi for Sf9 cells).

<sup>a</sup>Based on PI-PLC treated cells.

<sup>b</sup>Based on lysed cells.

<sup>c</sup>Based on p97 secreted into the growth media.

as a result of increased loss of cell viability (Licari and Bailey, 1991). The optimal time for protein expression of 48 hpi and relative insensitivity of maximum protein expression to MOI has been noted in other studies (Licari and Bailey, 1991; Maiorella et al., 1988). However, it is likely that the time from post-infection for maximum expression of recombinant proteins will vary depending on the protein and its cellular location, since post-translational modifications and transport rates will also vary (Luckow, 1991).

It is interesting to note that during this decline of cell surface expression and secretion of p97 the total p97 retained within the cell actually doubled (Table I). This also supports the proposal that the transport of p97 and membrane turnover in late infection breaks down and may even result in protein degradation (Licari and Bailey, 1991). Although the cell specific surface expression of GPI-anchored p97 by Sf9 cells was similar to that of SK-MEL-28 and transfected CHO cells, the cell specific secretion rate of soluble p97 (equivalent to  $\sim 4 \mu\text{g}$  p97/mL after 48 hpi) was nearly 10-fold higher. This level of secretion is similar to that observed in other studies (Reuveny et al., 1993) and could possibly be improved by modifying the growth media, culture conditions, and harvest method (Licari and Bailey, 1991; Maiorella et al., 1988; Reuveny et al., 1993). This suggests that Sf9 may also be useful for producing the soluble form of p97.

Although it was originally considered that Sf9 cells were unable to synthesize GPI anchors (Albert-Wolf et al., 1991), this study (based on flow cytometry, Triton X-114 phase separation, PI-PLC treatment, and CRD analysis) conclusively demonstrated that the recombinant p97 expressed on the cell surface of infected Sf9 cell was GPI anchored and supports the findings of four other studies (Choudrai et al., 1994; Davies and Morgan, 1993; Longacre et al., 1995; Scheirle et al., 1992). In addition to the GPI-anchored form, a soluble form of p97 was detected in the medium of Sf9 infected cells. Based on Triton X-114 phase separation and CRD analysis, it was also shown that the soluble form of p97, which was shown to be the same size as the GPI-anchored form, appeared to be processed differently and

was probably secreted into the media as opposed to being derived from GPI-anchored p97.

Because such a large number of p97 molecules are being synthesized in the Sf9 cells, it is possible that there are not enough GPI precursors available for attachment. Therefore, the C-terminal hydrophobic signal sequence of a newly synthesized p97 polypeptide would be recognized and cleaved by a transamidase or peptidase, but the protein would not be attached to a GPI anchor. Instead, the hydrophilic p97 would be transported out of the cell via a secretory pathway. This is in agreement with a study on the secreted form of gp63 from *Leishmania major* (Mensa-Wilmot et al., 1994) and is also supported by a study of class E mutant mouse lymphoma cells, which cannot synthesize GPI anchors (Fatemi and Tartakoff, 1986).

The size difference between p97 expressed by Sf9 cells and SK-MEL-28 cells may have been due to differences in glycosylation, as is often the case for foreign proteins expressed in the baculovirus system (Domingo and Trowbridge, 1988; Jarvis and Summers, 1989; Luckow and Summers, 1989; Miller, 1988). Recombinant GPI-anchored proteins expressed by Sf9 cells (Choudrai et al., 1994; Davies and Morgan, 1993; Scheirle et al., 1992) were also shown to be slightly smaller than their native forms.

To analyze the glycosylation and transport of p97 in Sf9 cells, an Endo H digestion was performed on immunoprecipitated p97 from a pulse-chase experiment. At the 0-h chase time, p97 from Sf9 cell lysates was found in two Endo H sensitive forms approximately 80 and 82 kDa in size (Fig. 7, lane 1). After a 1-h chase, Endo H resistant forms of p97 are already present, indicating that transport of p97 through the Golgi has already occurred. The highest molecular weight Endo H resistant form is present by the end of the 2-h chase, indicating that p97 is transported to the surface within 2 h (Fig. 7, lane 3). It is not clear, however, why two Endo H sensitive forms of p97 were derived from the cell lysate. The soluble form of p97 found in the supernatant of p97 B-2-1 infected Sf9 cells is Endo H resistant at all chase times, including the 1-h chase time point when it was first detected (Fig. 7, lane 14 on a longer

exposure), indicating that it is released in a fully processed form. Although the soluble form of p97 in SK-MEL-28 cells is also Endo H resistant, it was not detected in the media of [<sup>35</sup>S]methionine labeled cells until after a 4-h chase, possibly due to slower transport of p97 in SK-MEL-28 cells than in p97 B-2-1 infected Sf9 cells.

Endo F digestion of recombinant p97 (data not shown) resulted in a single band which migrated at the same rate as the lower of two bands produced by Endo H digestion and Endo F digestion of p97 from SK-MEL-28 cells. This indicates that the unglycosylated form of p97 is the same size in p97 B-2-1 infected Sf9 cells and SK-MEL-28 cells, which is around 80 kDa. This is similar to the size of the unglycosylated native form of p97 predicted from the cDNA sequence (Rose et al., 1986).

In conclusion, we have shown that recombinant human p97 can be successfully expressed on the surface of infected insect Sf9 cells and that the cells have the ability to carry out the post-translational modification of GPI anchor attachment. In addition to the GPI-anchored form of p97, Sf9 cells were shown to be able to produce a soluble form of p97, which did not appear to be derived from the GPI-anchored form or from cell lysis. The expression of recombinant p97 by baculovirus infected Sf9 cells provides a cheaper alternative to conventional mammalian cell culture for the production of relatively large amounts of either GPI-anchored or soluble forms of p97. Especially since Sf9 cells produce 10-fold higher levels of soluble p97 compared to SK-MEL-28 or transfected CHO cells. Knowledge of variability in recombinant protein expression will be important when considering optimizing recombinant protein production in baculovirus infected insect cell systems.

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## NOMENCLATURE

AcMNPV	<i>Autographica californica</i> multicapsid nuclear polyhedrosis baculovirus
"C"	anti-p97 monoclonal antibody (33B6E4)
CHO	Chinese hamster ovary cells
CRD	cross-reacting determinant
GAM	goat anti-mouse
GPI	glycosylphosphatidylinositol
L235	anti-p97 monoclonal antibody (ATCC HB8446)
MAB	monoclonal antibody
p97aWTBc3	CHO cells transfected with p97
PCFA	Pandex fluorescence concentration analyzer
PI-PLC	phosphatidylinositol phospholipase C
Sf9	<i>Spodoptera frugiperda</i> insect cells
SK-MEL-28	human melanoma cells

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