

## Differences in the Expression and Localization of Human Melanotransferrin in Lepidopteran and Dipteran Insect Cell Lines

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The ability of several lepidopteran and dipteran insect cell lines to express human melanotransferrin (p97), a glycosyl phosphatidylinositol (GPI)-anchored, iron-binding sialoglycoprotein, was assessed. *Spodoptera frugiperda*-derived (Sf9) cell lines, transformed with the p97 gene under control of a baculovirus immediate-early promoter, were able to constitutively express the protein and correctly attach it to the outer cell membrane via a GPI anchor as demonstrated by PI-PLC treatment. In contrast, stable constitutive expression could not be demonstrated with cell lines derived from either *Drosophila melanogaster* (Kc1 or SL2) or *Lymantria dispar* (Ld652Y) despite the observation that p97 could be detected in transient expression assays. This may indicate that the long-term expression and accumulation of p97 is inhibitory to *Drosophila* cells, possibly due to improper localization of the protein and resultant competition for cellular iron. In stably transformed Sf9 cells, p97 was expressed on the cell at a maximal level of 0.18  $\mu\text{g}/10^6$  cells and was secreted at a maximal rate of 9.03 ng/10<sup>6</sup> cells/h. This level was comparable to the amount expressed with the baculovirus system (0.37  $\mu\text{g}/10^6$  cells and 31.2 ng/10<sup>6</sup> cells/h) and transformed CHO cells (0.88  $\mu\text{g}/10^6$  cells and 7.8 ng/10<sup>6</sup> cells/h). Deletion of the GPI cleavage/attachment site resulted in an eight-fold increase in the secretion rate of p97, when com-

pared to the intact construct suggesting that the rate-limiting step involves processing of the GPI anchor. © 1999 Academic Press

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The analyses of complex eukaryotic proteins often necessitates expression in heterologous systems that are free of endogenous factors that might interact with the protein or components in the assay system. In recent years several highly processed mammalian proteins have been successfully expressed in either baculovirus-infected or stably transformed insect cell lines. For example, human G-protein-coupled receptors (GPCRs) were expressed in baculovirus-infected Sf9 insect cells which do not contain the identical GPCRs. This cell line does, however, possess low levels of G-proteins that can be activated by heterologous GPCRs allowing structure/function analyses to be conducted (Tate and Grisshammer, 1996). In another instance, the role of calnexin, a membrane-bound endoplasmic reticulum-associated protein that is not present in *Drosophila*, in the transport and assembly of class I major histocompatibility complex molecules, was firmly established (Jackson *et al.*, 1994). The inherent ability of insect cells to express, process, and localize mammalian proteins, in a manner similar to that of their mammalian counterparts, has been exploited to provide high-quality material for studies involving protein crystallization (Garcia *et al.*, 1996), antibody production (Kirkpatrick *et al.*, 1995), and analysis of receptor/ligand interactions (Ivey-Hoyle *et al.*, 1991). Indeed,

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many of the posttranslational modification pathways typical of mammalian systems also occur in insect cells (Jarvis *et al.*, 1990; Jarvis and Finn, 1995). Similar to their mammalian counterparts, these pathways are often cell line- or tissue-specific (Roth *et al.*, 1992; Davis and Wood, 1995; Wagner *et al.*, 1996). Recently it has been demonstrated that the N-glycosylation pathway present in insect cells can be modified by providing the genes encoding these deficient enzymes (Jarvis and Finn, 1996; Jarvis *et al.*, 1997). This genetic engineering of posttranslational processes is especially significant for those using baculovirus expression systems, since the viruses infect only a limited repertoire of cell lines. The narrow host range or lack of versatility in cell line selection with the baculovirus system has prompted the development of expression systems capable of functioning in a broader range of dipteran (Johansen *et al.*, 1989) and/or lepidopteran cell lines (Shotkoski *et al.*, 1996; Pfeifer *et al.*, 1997; McLachlin and Miller, 1997; Hegedus *et al.*, 1998).

An important posttranslational event that can significantly influence the integrity and function of proteins expressed in heterologous systems is proper cellular localization. Most cell surface-associated proteins are bound to the outer membrane through the physical interaction of a hydrophobic transmembrane domain with the lipid bilayer; however, a relatively small, but important, group of proteins is linked to the membrane via a glycosyl phosphatidylinositol (GPI) anchor. This arrangement consists of a hydrophobic phosphatidylinositol residue embedded within the plasma membrane which is linked to the carboxyl terminus of the protein via a variable glycan chain and phosphoethanolamine intermediary (Ferguson and Williams, 1988). A few GPI-linked proteins have been successfully expressed in insect cells; however, this has been restricted to lepidopteran cell lines susceptible to baculovirus infection (Schierle *et al.*, 1992; Davies *et al.*, 1993; Choudrai *et al.*, 1994; Longacre *et al.*, 1995; Kennard *et al.*, 1997).

Recently, human melanotransferrin (p97), a GPI-anchored sialoglycoprotein, was expressed using the baculovirus system (Kennard *et al.*, 1997). The protein was first described as a melanoma-specific antigen (Brown *et al.*, 1981) and has been expressed as a 738-amino-acid precursor possessing a 19-amino-acid amino-terminal signal peptide as well as a 25-amino-acid hydrophobic carboxyl-terminal region (Rose *et al.*, 1986). Initially, the hydrophobic terminus was presumed to anchor the protein to the outer membrane; however, this was subsequently shown to be a GPI cleavage/attachment signal sequence (Food *et al.*, 1994). The protein consists of two symmetrical domains, each presumed to contain seven disulfide bridges, and several putative N-glycosylation sites and it bears a strong similarity to the transferrin-like iron-

binding proteins (Rose *et al.*, 1986). The role of p97 in mediating a transferrin-independent iron metabolism pathway was confirmed by the observation that iron uptake was significantly increased in transferrin receptor-deficient CHO cell lines transformed with a p97 cDNA construct (Kennard *et al.*, 1995). In addition, phase partitioning revealed that two forms of p97 exist in mammalian cell cultures, a membrane-bound form and a secreted form that may originate from alternate processing of the mRNA (Food *et al.*, 1994) as is the case with the chicken homologue (McNagny *et al.*, 1996). Recently, elevated levels of p97 were detected in amyloid plaques within the brain tissues of Alzheimer's disease patients, suggesting that it may play a role, either direct or indirect, in disease pathology (Jefteries *et al.*, 1996). Here we assess the intrinsic ability of stably transformed lepidopteran and dipteran cell lines to express human melanotransferrin under the direction of constitutive or inducible promoters.

## MATERIALS AND METHODS

### *Cell Lines and Baculovirus*

The human melanoma line SK-MEL-28 was obtained from the American Type Culture Collection and maintained on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 20 mM Hepes (Food *et al.*, 1994). The Chinese hamster ovary cell line (p97aWTBc3) transformed with a p97 mammalian expression construct (Food *et al.*, 1994) was maintained on Ham's F12 medium (Gibco) supplemented with 10% FBS, 20 mM Hepes, 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine in a humidified chamber at 37°C under 5% CO<sub>2</sub>. Cell lines derived from the lepidopteran insects *Spodoptera frugiperda* (Sf9) and *Lymantria dispar* (Ld652Y) or the dipteran insect *Drosophila melanogaster* (Kc1 and SL2) were maintained on TC-100 complete medium (20.36 g/L TC-100 medium (Gibco-BRL), 0.35 g/L NaHCO<sub>3</sub>, 3.3 g/L TC-yeastolate (Gibco), and 3.3 g/L TC-lactalbumin hydrolysate; adjusted to pH 6.2 with 5 M KOH) with 10% fetal bovine serum (Gibco-BRL) at 27°C. Cell number and viability were determined using a hemocytometer and staining with a 1/10 vol of 0.4% trypan blue solution.

The recombinant AcMNPV baculovirus expressing p97, designated p97 B-2-1, was propagated as per Kennard *et al.* (1997).

### *Construction of p97 Expression Vectors*

The metal-inducible p2ZMtn97 and constitutive p2ZOp2C97 constructs were generated by cloning a 2.4-kb *EcoRI*-*NruI* fragment from pA3-2 containing the entire open reading frame from the p97 cDNA into the *EcoRI*-*PvuII* site of the mammalian expression

**TABLE 1**  
Comparison of Cellular p97 Expression in Various Cell Types

Clonal line	Cell type	Cell surface-specific p97 <sup>a</sup> ( $\mu\text{g}/10^6$ cells)	Cell-specific p97 secretion rate <sup>b</sup> (ng/ $10^6$ cells/h)
p97aWTBc3	CHO	0.88 (0.88)	6.2 (7.8)
p97B-2-1	Sf9 <i>Spodoptera frugiperda</i> (baculovirus-infected 48hpi) <sup>c</sup>	0.37 (0.37)	31.2 (31.2)
p2ZOp2C97 C.16	Sf9 <i>Spodoptera frugiperda</i> (stable clonal cell line)	0.12 (0.18)	2.96 (9.03)
p2ZOp2F97d C.16	Sf9 <i>Spodoptera frugiperda</i> (stable clonal cell line)	0	58.0 (58.0)
p2ZOp2F97d C.21	Sf9 <i>Spodoptera frugiperda</i> (stable clonal cell line)	0	56.0 (76.9)
p2ZMtn97 C.7 and C.8	SL2 <i>Drosophila melanogaster</i> (stable clonal cell line)	0	0

<sup>a</sup> Based on p97 harvested from PI-PLC-treated cells.

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

<sup>c</sup> p97 expression levels are based on 48-h cultures (maximal expression).

vector pZeoSV (Invitrogen) to generate pZeoSV97. Subsequently, a 2.6-kb *EcoRI*–*BglII* fragment containing the p97 coding region plus the SV40 polyadenylation sequence was subcloned from pZeoSV97 into the *EcoRI*–*BamHI* site of p2ZOp2A to generate p2ZOp2C97 (Hegedus *et al.*, 1998). This vector uses the *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (*OpMNPV*) immediate-early 2 gene promoter (IE-2) to direct constitutive expression in insect cell lines (Pfeifer *et al.*, 1997) and a chimeric IE-2-bacterial synthetic promoter to confer resistance to the antibiotic Zeocin (Invitrogen) in both insect cell lines and *Escherichia coli*. In another series, a 2.6-kb *SpeI*–*BglII* fragment from pZeoSV97 was subcloned into the *XbaI*–*BglII* site of p2ZMtn (Hegedus *et al.*, 1998) to generate p2ZMtn97. This construct places the p97 cDNA under the direction of the heavy metal-inducible *D. melanogaster* metallothionein promoter which is only functional in dipteran cell lines.

A series of nested 3' deletions were generated to eliminate the p97 GPI signal sequence using the exonuclease 3/S1 nuclease method (Sambrook *et al.*, 1989). Approximately 10  $\mu\text{g}$  of the plasmid pA3-2 was digested with *NruI*, which cleaves 87 bp downstream of the stop codon, and subjected to exonuclease 3 treatments ranging from 30 to 180 s to remove the terminal 200 bp (approximately 25 amino acids) from the *NruI* site, after which the ends were made blunt using Klenow DNA polymerase and dNTPs. The plasmid was digested with *HindIII* which cleaves 5' to the start codon and the pooled fragments from each time point cloned into the *HindIII*–*EcoRV* site of p2ZOp2F (Hegedus *et al.*, 1998). This vector possesses stop codons in all three frames to replace the stop codon eliminated when generating the 3' deletions.

### *Insect Cell Line Transformation*

Insect cells were transformed with 2  $\mu\text{g}$  of CsCl-purified DNA using 10  $\mu\text{l}$  of Cellfectin (Gibco) and stable Zeocin-resistant cell lines selected as described previously (Pfeifer *et al.*, 1997). Stable cell lines analyzed in this paper are described in Table 1.

Southern blot analysis of resistant transformed cell lines was conducted as described previously (Pfeifer *et al.*, 1997).

### *Protein Analysis*

(1) *Western blot analysis.* In transient transfection assays, insect cells were harvested 48 h after transformation, pelleted at 4000 *g* in a microcentrifuge and resuspended in 50  $\mu\text{l}$  of cell lysis buffer (20 mM Tris-HCl (pH 7.2), 0.15 M NaCl, 2 mM EDTA, 1% NP-40, and 0.5 mM PMSF) for protein determination and analysis. To detect secreted p97, culture supernatant was concentrated under vacuum using a SpeedVac (Savant) and resuspended in 1/10 vol of lysis buffer.

Western blot analysis was conducted by separating 10  $\mu\text{g}$  of protein on 10% nondenaturing SDS-PAGE gels and transferring to nitrocellulose membranes. The p97 protein was detected using the anti-p97 monoclonal antibody L235 (ATCC HB8446), as the primary antibody at a 1/10 dilution of hybridoma culture supernatant in phosphate-buffered saline and horseradish peroxidase-conjugated goat anti-mouse antibody (Bio-Rad, Richmond, CA) as the secondary at a 1/20,000 dilution followed by detection using the ECL chemiluminescent system (Amersham).

(2) *Pulse-chase labeling and immunoprecipitation.* Sf9 cell lines transformed with p97 expression constructs were grown to mid log phase (approximately

$3-4 \times 10^6$  cells/ml) in TC-100 complete medium supplemented with 10% fetal bovine serum and 250 mg/ml Zeocin in a 50-ml spinner flask at 27°C. The cells were harvested by centrifugation at 2000 *g* for 10 min and incubated for 1 h in methionine-free, cysteine-free DMEM (Gibco). Cellular protein was pulse-labeled in 5 ml of methionine-free, cysteine-free DMEM supplemented with 500 mCi/ml [<sup>35</sup>S]methionine/cysteine (Promix, Amersham) for 45 min. The cells were harvested by centrifugation, washed twice with TC-100 medium, and then chased with 3.0 ml of TC-100 supplemented with 10% FBS. Cell culture aliquots of 1.0 ml were removed after 0, 4, and 24 h and harvested by centrifugation and the cells were lysed in 1.0 ml of solubilization buffer [20 mM Tris-HCl (pH 7.2), 0.15 M NaCl, 2 mM EDTA, 1% Triton X-114, and 0.5 mM PMSF]. Five microliters of 100 mM PMSF was added to the cell-free culture supernatant and the samples stored at -80°C.

Prior to immunoprecipitation, the p97 samples were first centrifuged at 11,000 rpm for 20 min at 4°C to remove cellular debris. Aliquots of 100  $\mu$ l (cell lysate) or 200  $\mu$ l (cell supernatant) were precleared at 4°C for 45 min with the addition of 3  $\mu$ l of normal rabbit serum at which time 50  $\mu$ l of protein A-Sepharose (Sigma), that had been washed three times with solubilization buffer, was added and the mixture was incubated at 4°C for an additional 45 min prior to brief centrifugation. To the supernatant 200  $\mu$ l of undiluted L235 monoclonal antibody hybridoma culture supernatant was added and this was incubated for 1 h at 4°C at which time 50  $\mu$ l of protein A-Sepharose coated with rabbit anti-mouse IgG (Jackson) was added and incubated for 1 h at 4°C. The beads were pelleted by brief centrifugation and washed twice in 0.2% NP-40, 150 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl (pH 7.4), once with 0.2% NP-40, 500 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl (pH 7.4) and once with 10 mM Tris-HCl (pH 7.4).

(3) *Two-dimensional gel electrophoresis.* Isoelectric focusing pH gradient two-dimensional gel electrophoresis was conducted according to Celis *et al.* (1990). First-dimension gels were prepared by dissolving 8.24 g of urea in 3.0 ml 10% NP-40, 3.0 ml dH<sub>2</sub>O and 1.95 ml acrylamide solution (28.38% acrylamide; 1.62% bisacrylamide) and then adding 600  $\mu$ l (pH 5-7) and 200  $\mu$ l (pH 3.5-10) carrier ampholytes. The gels were allowed to polymerize for 2 h after the addition of 10  $\mu$ l TEMED and 15  $\mu$ l 10% ammonium persulfate; the tops were washed three times with dH<sub>2</sub>O and prerun with 40  $\mu$ l lysis solution (9.8 M urea, 2% NP-40, 2% carrier ampholytes (pH 7-9), 100 mM DTT) overlaid with 20  $\mu$ l overlay solution (8 M urea, 1% carrier ampholytes (pH 7-9), 5% NP-40, 100 mM DTT) for 15 min at 200 V, 30 min at 300 V, 60 min at 400 V using 20 mM NaOH

in the upper cathode chamber and 10 mM H<sub>3</sub>PO<sub>4</sub> in the bottom anode chamber. A 50- $\mu$ l volume of lysis solution was added to the beads; this was mixed and centrifuged and 40  $\mu$ l of the supernatant was applied to the gels, which had been washed three times with dH<sub>2</sub>O, overlaid with 20  $\mu$ l overlay solution diluted one half in dH<sub>2</sub>O and run for 20 h at 400 V. The gels were removed, placed in equilibrium solution [0.6 M Tris-HCl (pH 6.8), 2% SDS, 100 mM DTT, 10% glycerol], and subjected to SDS-PAGE using a 10% separating and 5% stacking gel. [<sup>14</sup>C]-methylated molecular weight markers (Amersham) were prepared by boiling in 1 $\times$  sample buffer. After electrophoresis the gels were removed, fixed in a solution of 40% methanol; 10% acetic acid for 1 h, and placed in DMSO with 7.5% diphenyloxazole for 1 h. The gels were washed twice with dH<sub>2</sub>O, dried, and subjected to autoradiography for 30 days at -80°C.

(4) *Immunohistochemistry.* Transformed cells were allowed to adhere to glass coverslips that had previously been coated with a solution of 1 mg/ml poly-L-lysine (400,000 MW) and allowed to dry for 30 min. The slides were rinsed in phosphate-buffered saline (PBS) and fixed for 5 min in freshly prepared 4% paraformaldehyde followed by a 45-s incubation in a 1:1 solution of methanol:acetone. The slides were then rinsed three times in PBS, and in some cases incubated in 0.5% Triton X-100 in PBS for 10 min followed by three additional rinses in PBS. The cells were blocked for 20 min in FATS (20% FBS, 0.5% Tween 20 in PBS) followed by a 60-min incubation with either the L235 or C anti-p97 monoclonal antibodies (used as undiluted hybridoma supernatants) in a humidified chamber. The slides were washed three times in PBS over the course of 10 min and then incubated with the secondary antibody consisting of a 1/30 dilution of FITC-conjugated goat anti-mouse-Fab fragments (Jackson) for 60 min. The slides were washed three times with PBS, mounted, and viewed using either an epifluorescence or confocal microscope.

#### *Quantitation of p97 Expression*

Thirty milliliters of TC-100 complete medium supplemented with 10% FBS in a 50-ml spinner flask was inoculated at an initial density of  $1 \times 10^6$  viable cells/ml. Aliquots of 1.0 ml were removed daily to determine total and viable cell number and to quantify cell-associated and secreted p97 levels.

The amount of p97 produced was determined using a modified immunofluorescence assay (Kennard *et al.*, 1993). Cell-free samples were assayed by binding the p97 to L235 immobilized on carboxyl-polystyrene capture particles (0.77  $\mu$ m, Idexx). The bound p97 was then labeled with a fluoresceinated secondary antibody "C" (33B6E4, provided by Dr. S.-K. Liao, McMaster

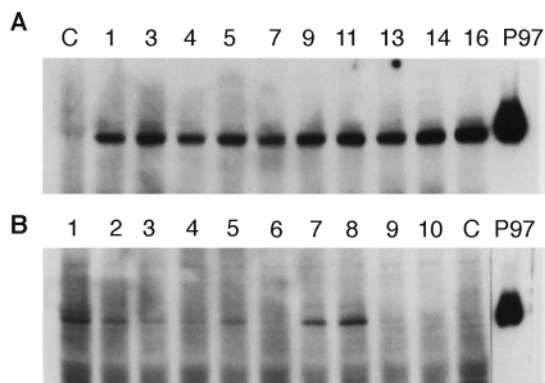
University, Hamilton, Ontario, Canada). The fluorescence of the complex which corresponded to the original concentration of soluble p97 in the supernatant was read using a Pandex fluorimeter (PCFA, Idexx) and compared to purified p97 standards. In the case of the cell membrane-associated GPI-anchored p97, the protein was first cleaved from the cell surface using partially purified phosphatidylinositol phospholipase C (PI-PLC). Approximately  $2 \times 10^7$  cells were pelleted and resuspended in 1.0 ml of 300 mU/ml PI-PLC in PBS and incubated for 1 h at 37°C. The PI-PLC solution was recovered and the concentration of the cleaved p97 was determined according to the previously described method. Based on the total cell density and p97 concentration, GPI-anchored or secreted p97 per cell could be determined.

## RESULTS

### *Stable Expression of Human p97 in Insect Cell Lines*

Previous Western blot analysis of transiently transfected insect cell lines showed that Sf9, *Drosophila*, and to a lesser extent Ld652Y cell lines were capable of expressing p97 (Hegedus *et al.*, 1998). In all cases the molecular weight of the protein was less than that of the mammalian counterpart with *Drosophila* cell lines producing the smallest protein, Sf9 producing an intermediate-sized but larger product, and Ld652Y producing a protein only slightly smaller than the mammalian p97 (Hegedus *et al.*, 1998).

Initially, various insect cell lines were transformed with the constitutive expression construct, p2ZOp2C97, and stable Zeocin-resistant clones were selected and analyzed for expression of p97 (see Table 1). All of the Zeocin-resistant Sf9 clones expressed p97, albeit at varying levels, with a molecular weight identical to that observed using the baculovirus expression system in Sf9 cells (Fig. 1A). The transformed Sf9 lines were stable and showed no overall decline in p97 expression after 12 passages over the course of 3 months in the presence or absence of antibiotic selective pressure. Conversely, stable p97 expression was not detected in any resistant clonal or polyclonal cell lines derived from either *Drosophila* (Kc1 or SL2) or Ld652Y. These cell lines were capable of transiently expressing p97, indicating that long-term constitutive expression of p97 may in some way be deleterious to cell physiology. To test this hypothesis *Drosophila* cell lines were transformed with the inducible expression construct, p2ZMtn97, and selected for Zeocin resistance. When induced with 500 mM CuSO<sub>4</sub> for 24 h prior to protein isolation several individual clones exhibited detectable levels of p97 expression (Fig. 1B). The effect of p97 expression on clonal *Drosophila* cell lines was examined by measuring the growth rate and cell viability using the two highest expressing SL2

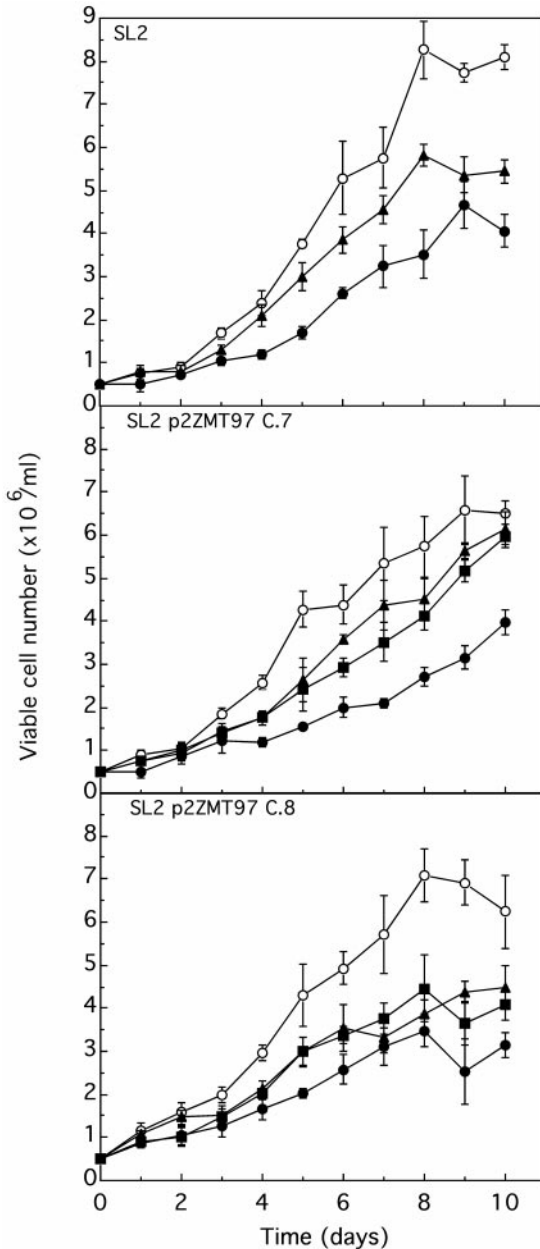


**FIG. 1.** Western blot analysis of selected stable clonal cell lines expressing p97. (A) Sf9 cell lines transformed with the constitutive expression construct p2ZOp2C97; (B) stable *Drosophila* SL2 cell lines transformed with the metal inducible construct p2ZMT97 and induced with 500  $\mu$ M CuSO<sub>4</sub>. The numbers above the lanes indicate the clone number. Lane C contains protein from nontransformed cells and the positive control lane (p97) contains baculovirus-expressed p97 protein.

clones (p2ZMtn97 C.7 and C.8) induced with 0–1000 mM CuSO<sub>4</sub> over a 10-day period (Fig. 2). Both transformed cell lines exhibited a significant reduction in growth rate when induced with CuSO<sub>4</sub>. However, this reduction did not differ appreciably from that observed when the nontransformed SL2 control cell line was exposed to the same concentration of CuSO<sub>4</sub>. Western blot analysis confirmed that at the end of the 10-day period p97 was present in the induced cultures and was associated with the cellular phase (Fig. 3); no p97 was detectable in the culture supernatant. This would indicate that the observed inhibition is in response to CuSO<sub>4</sub> stress and that p97 expression has no “immediate” toxic effects in these cell lines.

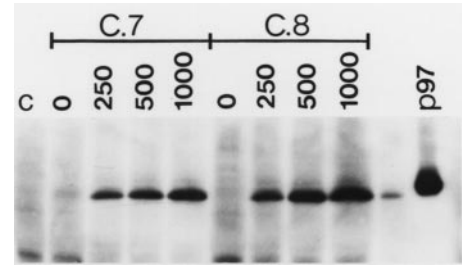
### *Localization of Recombinant p97 in Transformed Insect Cells*

Two forms of the p97 occur naturally in mammals: a membrane-bound form which is attached to the cell surface via a GPI anchor covalently linked to the carboxyl terminus of the protein and a soluble form that is secreted into the medium (Food *et al.*, 1994). Immunofluorescence was used to determine the precise cellular localization of the p97 expressed in insect cell lines. In p97-transformed stable Sf9 cell lines, p97 was found to be localized specifically to the plasma membrane of the cell when viewed using confocal microscopy (Fig. 4). Treatment of these cells with PI-PLC significantly reduced the immunofluorescent intensity, indicating that the p97 is properly attached to the membrane via a GPI anchor. Conversely, transformed *Drosophila* (SL2 or Kc1) cell lines did not exhibit any fluorescence on the cell surface despite producing substantial



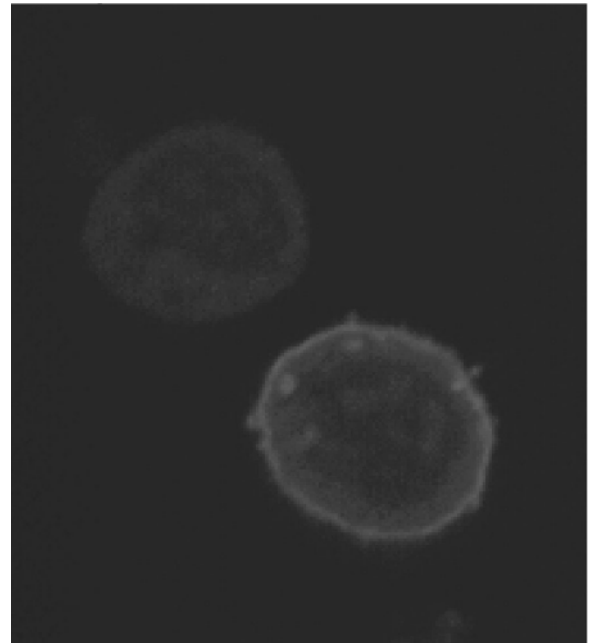
**FIG. 2.** Effects of  $\text{CuSO}_4$  on the growth of the untransformed *Drosophila* SL2 cell line and p2ZMT97 transformed clonal cell lines C.7 and C.8 (indicated). 0 ( $\circ$ ), 250 ( $\blacktriangle$ ), 500 ( $\blacksquare$ ), or 1000 ( $\bullet$ )  $\mu\text{M}$   $\text{CuSO}_4$ .

amounts of cell-associated p97, as revealed by Western blot analysis. Using confocal microscopy, in which 0.1-nm slices were taken through the cell, it was clear that p97 is not found on the cell surface of transformed *Drosophila* cell lines. Often, punctate staining was observed within the cell in p97-transformed SL2 and Kc1 cell lines; however, this staining was not localized to a specific region or organelle and suggests that the protein was present in the cytoplasm. Permeabilization of the cells by treatment with Triton X-100 or use of the



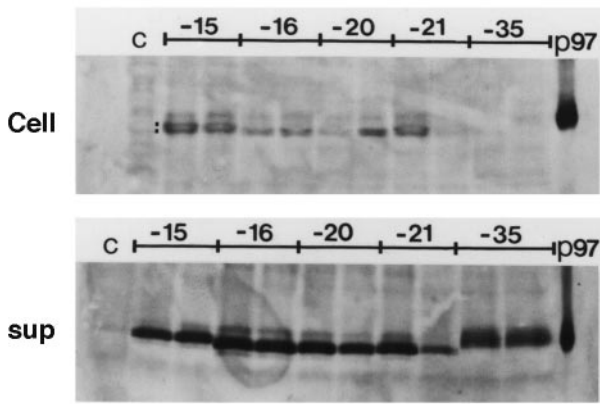
**FIG. 3.** Western blot analysis of *Drosophila* SL2 p2ZMT97 transformed clonal cell lines C.7 and C.8 induced with 0, 250, 500, and 1000  $\mu\text{M}$  of  $\text{CuSO}_4$  for 10 days. The negative control lane (C) contains protein from nontransformed cells and the positive control lane (p97) contains baculovirus-expressed protein.

“C” monoclonal antibody, which recognizes an epitope different from that of the L235 monoclonal, did not result in antibody binding to the cell membrane in the *Drosophila* cell lines, indicating that the lack of staining was not due to the inability of the antibody to access or recognize the epitope within the cell. Collectively, these observations suggest that p97 is synthesized in *Drosophila* cell lines and accumulates in the cytoplasm, but is not transported to the plasma membrane.



**FIG. 4.** Confocal image showing the immunolocalization of p97 on the surface of an Sf9 cell transformed with p2ZOp2C97 (bottom right) compared to a control nontransformed cell (top left). Each cell population was uniform when analyzed separately and were mixed for the purpose of the photograph.





**FIG. 6.** Western blot analysis of stable Sf9 cell lines transformed with p97 GPI-deficient deletion series (indicated numbers refer to deletions shown in Fig. 5). Supernatant (sup) and cell-associated (cell) samples from two individual clones are shown for each construct. The negative control lane (C) contains protein from nontransformed cells and the positive control lane (p97) contains baculovirus-expressed protein. Dots in lane margin indicate location of p97 bands.

tein folding. Both of these critical amino acids have been eliminated in constructs  $-35$  and  $-37$ .

Transformation of Sf9 cells with deletion constructs under the control of the constitutive promoter resulted in many resistant p97-expressing clones. Western blot analysis of cell pellets and the corresponding amount of concentrated culture supernatant revealed that although variable, the majority of the p97 was secreted into the culture medium as expected (Fig. 6). Two forms of p97 with slightly different molecular weights were observed in the cellular pellet of cell lines transformed with constructs  $-15$ ,  $-16$ ,  $-20$ , and  $-21$ . A prominent p97 band was observed in samples of culture medium. Construct  $-35$ , which does not contain the terminal cysteine residue, was also actively secreted but appeared as a larger diffuse band on the Western blots. The epitope that is recognized by the antibody contains a disulfide bond, and thus nondenaturing conditions are required to keep the p97 protein partially intact. The  $-35$  construct does not encode the terminal cysteine residue and thus the carboxyl portion of the protein remains free to bind additional SDS, resulting in a larger but more diffuse band. Nonetheless, the protein is secreted into the culture medium.

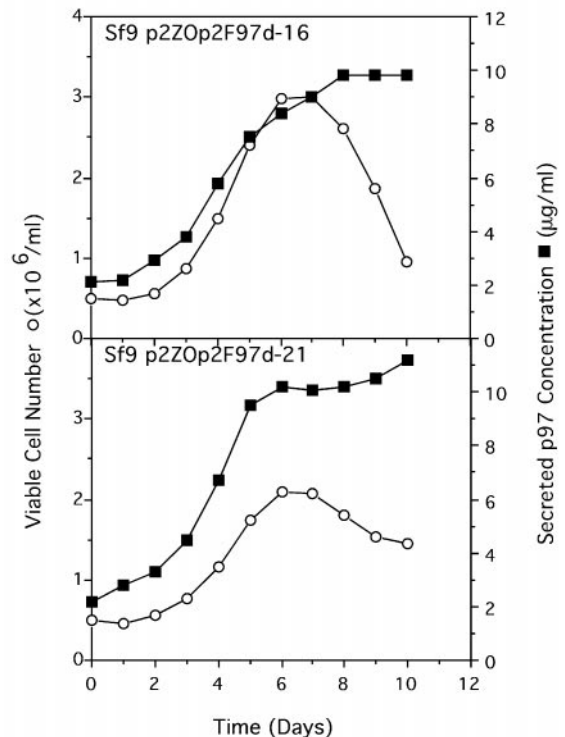
A time-course experiment was conducted to determine if removal of the GPI signal sequence, which resulted in no cell surface expression of p97, also increased the rate of p97 secretion (Fig. 7). The highest overall rate of expression occurred in early-mid log phase but p97 continued to accumulate well into the stationary phase with synthesis ceasing only with the onset of cell death. Total accumulation (both intra- and extracellular) in the culture after 8 days approached 10

$\mu\text{g/ml}$ , corresponding to a maximal secretion rate of approximately 58 and 76.9  $\text{ng}/10^6$  cells/h for Sf9 p97-16 and p97-21 cells, respectively. This represents a 6- to 10-fold increase in secretion when compared to the full-length GPI-anchored form expressed in transformed Sf9 cells and is over double that of the baculovirus expressed full-length p97 (Table 1). No attempts were made to express the GPI-deficient form in either mammalian cells or using the baculovirus system, but a similar increase in productivity might be expected. Western blot analysis revealed that the protein remained physically intact for several days in the culture medium despite the onset of cell death and lysis (Fig. 8).

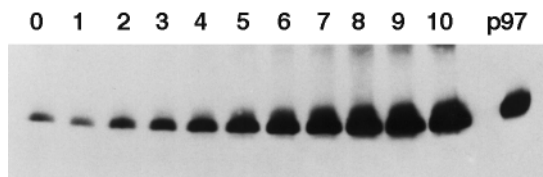
When transformed with the same constitutively expressed GPI cleavage/attachment signal deletion constructs, none of the stably-transformed *Drosophila* cell lines exhibited detectable levels of p97 expression. In transient transfection assays, p97 expression was detected in both the cell pellet and supernatant at approximately equivalent ratios (data not shown).

#### Two-Dimensional Gel Electrophoresis of Recombinant p97

The extent of posttranslational modification of the wild-type p97 isolated from SKMEL-28 cells and re-



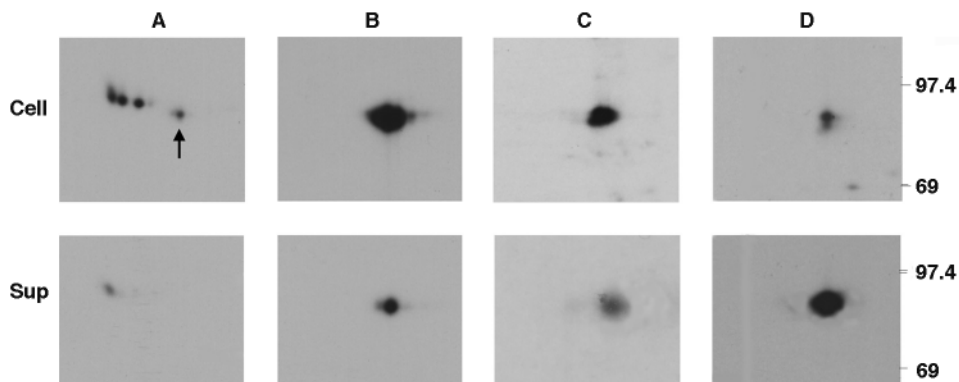
**FIG. 7.** Time-course analysis of GPI-deficient p97 expression in stable Sf9 cell lines transformed with either the p2ZO p2F97d-16 or p2ZO p2F97d-21 constructs (indicated).



**FIG. 8.** Western blot analysis of samples taken over the course of 10 days (indicated) from the culture medium of stable Sf9 cell lines transformed with the p97 GPI-deficient deletion construct p2ZOp2F97d-16. The positive control lane (p97) contains baculovirus-expressed protein.

combinant p97 expressed in Sf9 cells using both stably transformed and baculovirus-infected cells was examined using two-dimensional gel analysis. SKMEL-28 cells, Sf9 cells transfected with recombinant baculovirus (Kennard *et al.*, 1997) and stably transformed Sf9 cells expressing the recombinant p97, were pulse labeled for 15 min with radioactive methionine and cysteine and further chased with an excess of cold amino acids for 0, 4, and 24 h. Figure 9 shows the 4-h chase time point after immunoprecipitation and 2D gel analysis.

Analysis of the SKMEL-28-produced wild-type p97 during intracellular transport (data not shown) indicates that the glycoprotein is first synthesized as the protein indicated by an arrow in Fig. 9A. After the 4-h chase, associated p97 migrates as four different spots on the 2D gel, mostly indicative of a posttranslational modification of the glycans occurring in the Golgi apparatus. Most of the intermediate forms accumulate as the acidic form after the 24-h chase, likely indicative of sialic acid addition. The secreted form corresponds to the most acidic form, indicating a similar posttranslational modification and appears after 4 h with increasing amounts found after 24 h (data not shown).



**FIG. 9.** Two-dimensional gel analysis of p97 expressed in human and insect cells. SKMEL-28 (A), recombinant baculovirus-infected Sf9 cells (B), Sf9 cells constitutively expressing wild-type p97 (C), and Sf9 cells constitutively expressing the p97 120.6 deletion construct (D) were pulse-labeled and chased for 4 h as indicated under Materials and Methods. The immunoprecipitated p97 was analyzed by 2D gel electrophoresis. p97 from cell lysates (Cell) and from the supernatant (Sup) are shown for each cell line with acidic pH on the left side and basic pH on the right. Molecular weight markers are indicated on the right side of the figure. The arrow indicates the first synthesized form of the glycoprotein.

In contrast, pulse-chase of wild-type and recombinant p97 expressed in insect cells (Sf9) show a much lower level of posttranslational modification during intracellular transport to the cell surface (Figs. 9B and 9C). The p97 present in the cellular fraction of the Sf9 cells transformed with the GPI cleavage/attachment signal deletion construct (p2ZOp2F97d-16) exhibited a molecular weight identical to that of the wild-type p97 expressed in insect cells, despite removal of the carboxyl-terminal 16 amino acids (Fig. 9D). The secreted form of p97 is very similar in molecular weight and in isoelectric point as the form synthesized internally (Figs. 9B–9D). The p97 produced in insects does not show the higher acid form seen when p97 is produced in SKMEL-28 cells.

## DISCUSSION

In this study we have demonstrated that inherent differences exist in the ability of insect cell lines to express, process, and localize human GPI-anchored melanotransferrin (p97). Sf9, *Drosophila*, and Ld652Y cell lines were capable of transiently expressing p97; however, the protein was slightly smaller than its native, human counterpart (Hegedus *et al.*, 1998). The p97 protein is not only highly processed but also highly folded, possessing two symmetrical iron-binding domains and 14 disulfide linkages (Rose *et al.*, 1986). Since the protein is expressed, the reduced molecular weight most likely reflects either incomplete posttranslational processing resulting from incomplete glycosylation or inability to process and attach the GPI anchor. It has been reported that other GPI-linked proteins expressed in Sf9 cells using the baculovirus system also exhibit molecular weights slightly less than the native forms (Davies *et al.*, 1993; Choudrai *et al.*, 1994). Removal of carbohydrate modifications from baculovi-

rus-expressed and human p97 using endoglycosidase F and H digestion gives rise to proteins of identical size, approximately 80 kDa, indicating that the molecular weight difference is due to differences in glycosylation (Kennard *et al.*, 1997).

Despite a slight reduction in molecular weight of p97 expressed in Sf9 cells, the cells are apparently capable of conducting sufficient core modifications to allow proper transport and localization. In contrast, while both *Drosophila* Kc1 and SL2 cell lines transformed with the same construct produced p97, neither were able to attach the p97 to the outer membrane in a form detectable by confocal microscopy. This may be related to the reduced size of the p97 expressed in either of the *Drosophila* cell lines when compared to Sf9, since the posttranslational addition of complex carbohydrate moieties to proteins while in the endoplasmic reticulum is associated with correct cellular localization. The observation that the p97 expressed in *Drosophila* is only slightly smaller than that produced by Sf9, and certainly larger than the 80 kDa reported for the unprocessed polypeptide (Kennard *et al.*, 1997), indicates that some form of modification is occurring and that the N-terminal signal peptide is most likely directing the protein to the endoplasmic reticulum. However, if one or more of the three putative N-glycosylation signals is unrecognized, or if the modifications are carried out incorrectly, the protein may remain in the endoplasmic reticulum and be shuttled back to the cytoplasm or cytoplasmic organelles. This in itself may confer a significant advantage, in that downstream purification of cytoplasmic proteins is much easier than for proteins that must first be dissociated from membrane components.

In several attempts, we were unable to isolate any *Drosophila* or Ld652Y stable cell lines constitutively expressing p97. Previously, we reported that clones expressing  $\beta$ -galactosidase could be isolated for each of these cell lines. The levels of  $\beta$ -galactosidase production were consistently very high with the *Drosophila* cell lines, whereas the levels of production with Ld652Y and Sf9 clones were lower (Hegedus *et al.*, 1998). The failure to obtain stable transformed Ld652Y cell lines that effectively express p97 suggests that the accumulation of the protein may be toxic. The inability of *Drosophila* cell lines to constitutively express p97 might be explained in terms of the iron-binding function of p97 and the improper cellular localization.

On a per cell basis the stably transformed Sf9 cell lines generated during the course of this work produced levels of membrane-associated p97 approximately one-fifth that of an amplified CHO cell line and one-half that of the baculovirus system (Table 1). When the relative size and surface areas of the two cell lines are taken into consideration, the levels of expression with the transformed Sf9 and CHO cell lines are vir-

tually equivalent. However, the Sf9 cell lines can be grown to higher densities than most mammalian cell lines, suggesting that the expression vectors and the transformed insect cell lines in their current form can produce as much, if not more, total p97 than the selected CHO cells. We emphasize that no attempt was made to optimize the expression of p97 with the Sf9 cell lines nor was any attempt made to select cell lines expressing higher levels of p97. This could be accomplished by screening larger number of clones, by fluorescent cell-sorting selection, by increasing the expression cassette copy number through modified transformation protocols, by analyzing expression throughout the growth phase to determine the optimal time for cell harvest, or by a combination of these.

We did attempt to produce p97 in a form that was not attached to the cell membrane and would be secreted into the culture medium. This would greatly enhance the ease with which the protein could be isolated and makes possible the opportunity for fed-batch and continuous fermentations. Previous experiments to express extracellular proteins in insect cells using the baculovirus system, such as the thyrotropin receptor (Chazenbalk and Rapoport, 1995) and tissue plasminogen activator (Jarvis *et al.*, 1993), indicated that the levels of secreted product were dependent upon factors other than processing of the secretion signal peptide or glycosylation. Deletion of the GPI cleavage/attachment signal from the carboxyl terminus of p97 resulted in over a 10-fold increase in total p97 secreted from transformed Sf9 cell lines. This clearly indicates that processing of the GPI signal peptide and/or attachment of the GPI anchor is limiting secreted expression. An additional advantage of engineering the protein so that it is secreted and not attached to the cell surface was that the secreted protein was highly uniform as indicated by two-dimensional gel electrophoretic analysis. The homogeneity of the product is likely due to the fact that all posttranslational modifications are completed prior to secretion. Furthermore, the secreted form of the protein can be easily isolated from the culture medium without having to disrupt the cells thus allowing optimization of production, downstream processing, and purification.

Two-dimensional gel analysis of p97 produced in SK-MEL-28, recombinant baculovirus, and stably transformed insect cells allowed comparison of posttranslational modifications performed by these systems. The SKMEL-28 stable cell line demonstrated modification of p97 resulting in a dramatic acidic shift of the protein during processing, resulting in secretion of a slightly higher molecular weight protein than from the insect systems. This acidic shift may be due to sialylation of the protein-associated glycans. In neither the recombinant baculovirus nor the stable insect cell system was this acidic shift of p97 observed. The lack of sialylation

would explain the slight difference seen in molecular weight between insect and SKMEL-28 produced p97. The 2D gels also demonstrate the high levels of secretion by the cells containing the truncated p97 (Fig. 9D).

GPI cleavage/attachment signal peptides are identified on the basis of similar sequence motifs, usually an array of carboxyl-terminus hydrophobic amino acids (Kennard *et al.*, 1998), and not individual amino acid identities. It is conceivable that heterologous GPI cleavage/attachment signal peptides are not recognized or processed as efficiently as those derived from native proteins. According to Caras and Weddell (1989) both the sequence and length of the hydrophilic/hydrophobic regions are critical for proper GPI processing. We suggest that this be an important consideration when expressing GPI-anchored proteins, especially if the observed production is less than anticipated. The results presented here clearly underscore the need for a universal series of insect protein expression vectors which function in a broad variety of cell lines derived from diverse genera. The system we have described allows for the assessment and exploitation of the inherent, and possibly unique, protein processing characteristics of individual cell lines depending on the requirements of the specific protein being expressed.

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