Analytical Biochemistry 278, 59–68 (2000)
doi:10.1006/abio.1999.4409, available online at http://www.idealibrary.com on IDEAL®

Protein Expression in *Drosophila* Schneider Cells

Jürgen Benting,*,†,[1] Sandra Lecat,*,† Daniele Zacchetti,‡ and Kai Simons*,†,[2]

**Cell Biology and Biophysics Programme, European Molecular Biology Laboratory, D-69117, Heidelberg, Germany; †Max Planck Institut für Molekulare Zellbiologie und Genetik, Dresden, Germany; and ‡Department of Neuroscience, Dibit, H. San Raffaele Scientific Institute, Milan, Italy*

Received September 3, 1999

We expressed recombinant secreted, membrane, and cytosolic proteins in stably transfected *Drosophila* Schneider (SL-3) cells. To allow easy cloning of N- and C-terminal fusion proteins containing epitope- and His-tags for the detection of recombinant proteins and purification by affinity chromatography we constructed new expression vectors. To exemplify the general applicability of protein expression in Schneider cells we characterized the expression system with respect to inducibility, localization of the recombinant proteins, yields of purified proteins, and presence of posttranslational and cotranslational modifications. Secreted proteins became quantitatively N-glycosylated in SL-3 cells and the *N*-glycan of a Golgi-resident membrane protein was found to be Endo-H-resistant. Myristoylation of AnxXIIIb, a member of the annexin family, could be demonstrated and glycosylphosphatidylinositol-anchored proteins containing their lipid anchor were expressed efficiently in SL-3 cells. Since generation of stable cell lines and mass culture of SL-3 cells is cheap and easy, they provide an attractive eukaryotic expression system. © 2000 Academic Press

Key Words: **protein expression; insect cells; purification; protein modifications.**

Production of recombinant proteins and their purification is an important technique in molecular biology and biotechnology. There is need for eukaryotic expression systems because important eukaryotic posttranslational modifications do not occur in *Escherichia coli*. Therefore, proteins which require posttranslational or cotranslational modifications (i.e., N- and O-glycosylation, myristoylation, palmitoylation, prenylation, and addition of a glycosylphosphatidylinositol (GPI)[3] anchor) for their proper folding, maturation, localization, or function must be produced in eukaryotic cells. Mammalian cells, for example, stably transfected COS cells, are used for protein expression, but costs for growing mass cultures are high. This can be circumvented by the use of insect cells which grow at high densities at room temperature under normal air atmosphere. Insect cells perform comparable post- and cotranslational modifications as compared to mammalian cells except for N-glycosylation. Endogenous *N*-glycans of insect cells and *N*-glycans of most recombinant proteins expressed in lepidopteran cells have been found to be trimmed high-mannose structures that lack terminal galactose and sialic acid (1, 2), although the synthesis of complex *N*-glycans has been reported (3, 4). Another difference to mammalian *N*-glycans is the presence of fucose bound to the asparagine-linked *N*-acetylglucosamine, a modification that has been found in diptera, hymenoptera, and lepidoptera (5–7).

Recombinant baculovirus is the most widespread system to drive overexpression of exogenous proteins in insect cells. However, secretory proteins and plasma membrane proteins are often made in small amounts, probably due to virus production which takes hold of the secretory pathway of the infected cell (8). This may also affect the expression of Golgi-resident proteins. As an alternative inducible expression system we used

[1] Present address: Hoechst Schering AgrEvo GmbH, H872N, Forschung Biochemie, 65926 Frankfurt a.M., Germany.

[2] To whom correspondence should be addressed at EMBL, Meyerhofstrasse 1, 69117 Heidelberg, Germany. Fax: +49 6221 387512. E-mail: Simons@EMBL-heidelberg.de. www.embl-heidelberg.de/ExternalInfo/simons/index.html.

[3] Abbreviations used: ADH pA, alcohol dehydrogenase polyadenylation site; BSA, bovine serum albumin; FCS, fetal calf serum; FXa, factor Xa protease; GPI, glycosylphosphatidylinositol anchor; LPH, lactase-phlorizin hydrolase; MCS, multiple cloning site; Ni-NTA, nickel–nitrolotriacetic acid; PBS, phosphate-buffered saline; PLAP, human placental alkaline phosphatase; PNGase F, peptide–*N*-glycosidase F; PI-PLC, phosphatidylinositol-specific phospholipase C; pMT, *Drosophila* metallothionein promotor; TEV, tobacco etch virus; TX, Triton X.

0003-2697/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

stably transfected *Drosophila* Schneider cells (SL-3 line). This cell line has been used successfully to purify soluble mouse MHC II I-E^d heterodimers (9) and several secretory proteins (10–12). Schneider cells have also been used for the functional analysis of proteins involved in gene regulation or cell adhesion (13). In this paper we present our data on the expression, localization, posttranslational modification, and purification of different proteins in *Drosophila* Schneider cells to document the general applicability of this expression system. As membrane proteins we expressed the vesicular integral membrane protein of 36 kDa (VIP36), a type I transmembrane N-glycosylated Golgi protein with homology to plant lectins (14, 15) and the proteolipid VIP17/MAL (vesicular integral membrane protein of 17 kDa) (16) involved in apical transport in MDCK cells (17, 18). As secretory proteins we expressed a truncated form of VIP36, comprising only the ectodomain of the protein (VIP36 Exo) lacking its cytosolic and transmembrane domains (19), and two forms of the rat growth hormone (rGH), the wild-type nonglycosylated protein (rGHO) and a mutant containing two artificial N-glycosylation sites (rGH12) (20). The capability of SL-3 cells for myristoylation was analyzed by expressing annexin XIIIb (Anx XIIIb) (21), a cytosolic myristoylated Ca^{2+}-dependent phospholipid-binding protein. Finally, the production of GPI-anchored proteins was characterized by expressing a VSV-G epitope-tagged version of human placental alkaline phosphatase (VSV-PLAP). To further develop the expression system we constructed new expression vectors which allow straightforward generation of fusion proteins containing removable epitope- and His-tags for immunodetection and purification by affinity chromatography.

MATERIAL AND METHODS

Materials

Lipofectin, G-418, and SF-900 II medium were from Gibco-BRL. Puromycin, polylysine, pyrogallol, and phosphatidylinositol-specific phospholipase C (PI-PLC) were purchased from Sigma. Endoglycosidase H (Endo H) and peptide-*N*-glycosidase F (PNGase F) were from Boehringer Mannheim. Nickel–nitrolotriacetic acid (Ni-NTA)–agarose was from Qiagen. Protein inhibitor cocktail (CLAP, 1000× concentrated) was chymostatin, leupeptin, antipain, and pepstatin (all from Sigma) at a concentration of 10 mg/ml each in dimethyl sulfoxide. ECL and [9,10(*n*)-^{3}H]myristic acid were from Amersham-Buchler. SDS was from Bio-Rad, Triton X-100 (TX-100) and TX-114 were from Serva, and *N*-octyl-D-glucopyranoside (OG) was from Calbiochem.

Antibodies

A mouse monoclonal anti-HA-tag (clone 12CA5, Boehringer Mannheim), a rabbit anti-FLAG-tag (Santa Cruz Biotechnology), a mouse monoclonal anti-VSV-tag (clone P5D4), an affinity-purified rabbit anti-VIP36 (14), and an affinity-purified rabbit anti-Anx XIIIb antibody (22) have been used in this study. Secondary rhodamine-conjugated goat anti-rabbit antibodies were from Dianova (Hamburg, FRG).

Plasmids

The vectors pBS-PURO and pUChsNEO (23) containing the puromycin-resistance or the neomycin-resistance gene under the control of the *Drosophila* heat-shock promotor and pRmHa-3 (24) were kindly provided by Dr. K. Karjalainen (Institute for Immunology, Basel). The vector pRmHa-3 contains the *Drosophila* metallothionein promotor (pMT) which is activated by Cu^{2+} and a polyadenylation signal. pRmHa-3 was modified by introducing a multiple cloning site downstream of the metallothionein promotor to generate pRmHa-3.1 (Fig. 1). pRmHa-N and pRmHa-C were generated by inserting complementary oligonucleotides into pRmHa-3.1. The complete sequences of the vectors are available on our homepage (www.embl-heidelberg.de/ExternalInfo/simons/index.html). The signal sequence of VIP36 was amplified by PCR with the primers 36Bac-N, 5′-CGCCGAATTCAATATGGCGGCGGAAGGCTGGATTTGGC, and 36SS-AS, 5′-GTCAGTTATGCTAGCAACAACCGGCCCCAGCAA, and cloned as *Eco*RI–*Nhe*I fragment into pRmHa-N to generate pRmHa-Nss. pRmHa-N and pRmHa-Nss contain the Kozak sequences ACC **ATG** G or AAT **ATG** G flanking the start **ATG,** respectively (25, 26). A cDNA construct coding for a C-terminally His-tagged version of VIP36 was generated by PCR with VIP36 cDNA as a template (14) with the primers 5′-CGCCGAATTCAATATGGCGGCGGAAGGCTGGATTTGGC and 5′-TCGGGATCCTCAGTGGTGGTGGTGGTGGTGGTAGAAACGCTTGTTCC and cloned into pRmHa-3.1. The cDNA coding for the C-terminally myc- and His-tagged VIP36-Exo (19) was subcloned into pRmHa-3.1. The cloning of pRmHa-C/Anx XIIIb has been described (22). N-terminally VSV-tagged PLAP (VSV-PLAP) with the signal sequence of lactase-phlorizin hydrolase (LPH) was provided by T. Harder (Institute for Immunology, Basel). Briefly, the VSV-G epitope was introduced upstream of the PLAP cDNA lacking its N-terminal signal sequence by PCR with the primers 5′-GTTCAGATATGCATTACACTGATATCGAAATGAACCGCCTGGGTAAGATCCCAGTTGAGGAG introducing a *Nsi*I-site and 5′-AGCCAGTCTAGAGGGACACTCAGGGAGCAGT introducing a *Xba*I site. The signal sequence of LPH (27) which was kindly provided by P. Keller (EMBL, Heidelberg) was amplified by PCR with

the primers 5′-TAGCTAGCCACCATGGAGCTCTTTTGG and 5′-ATCCTGCAGAGATTCCCAGTCTGA. Subsequently, both PCR products were ligated in frame into pcDNA-3 to generate pcDNA-3/VSV-PLAP. The cDNA coding for the VSV-tagged PLAP with the signal sequence of LPH was subcloned into pRmHa-3.1. Wild-type rGH and a mutant rGH which contains two consensus sites for N-glycosylation (20) were tagged with a C-terminal His-tag by PCR using the primers rGHse 5′-CGCGGATCCGTGGCGATGGCTGCAGACTCTCAG and rGH3′ His 5′-TAAGGATCCTTAGTGGTGGTGGTGGTGGAAAGCACAGCTGCTTTCCGC introducing *Bam*H1 sites and cloned into the vector pRmHa-3.1. The cDNA coding for VIP17 was cut out with *Nco*I–*Bam*HI from pBAT-VIP17 (16), blunted with mung bean nuclease, and ligated in frame into pRmHa-N that was cut with *Xma*I–*Age*I and blunted with T4 polymerase.

Cell Culture

The Schneider cell line SL-3 (28) was grown at 27°C under normal atmosphere in SF-900 II medium (Gibco-BRL) supplemented with 1% fetal calf serum (FCS) at densities between 5×10^5 and 1×10^7 cells/ml. At low densities cells grow adherent but detach at higher densities and grow in suspension. Mass cultures were grown in 2-liter roller bottles in 400 ml of medium. Frozen stocks were kept in 90% FCS, 10% dimethyl sulfoxide at −80°C. Schneider cells can be obtained from the American Type Culture Collection (ATCC No. CRL-1963).

Transfection

Two days before transfection 1×10^7 cells were plated on a 3-cm dish. Cells were washed three times with SF-900 II and cotransfected with 2–4 μg of the respective pRmHa construct and 0.2–0.4 μg of pBS-PURO or pUChsNEO using 10–20 μl lipofectin in SF-900 II (without FCS and antibiotics) in a total volume of 1 ml. After 6 h 1 ml of SF-900 II containing 2% FCS was added. Two days later the cells were resuspended in 24 ml of medium containing either 10 μg/ml puromycin or 1 mg/ml G-418 and split into a 24-well plate. After 2–3 weeks of selection 5–50 clones have developed in each well. These clones were kept together and were used as polyclonal cell lines. The best expressing polyclonal cell lines were identified on Western blots 24 h after induction with 1 mM $CuSO_4$.

Immunofluorescence

SL-3 cells were spotted on a polylysine-coated slide for 20 min, fixed with 4% paraformaldehyde for 15 min, quenched for 10 min with 50 mM NH_4Cl in PBS (phosphate-buffered saline), and permeabilized with PBS containing 0.1% TX-100 for 20 min. After blocking for 30 min in PBS with 0.2% bovine serum albumin (BSA) antigens or epitopes were detected with the primary antibodies diluted 1:150 in PBS with 0.2% BSA for 45 min at 37°C. Unbound antibodies were removed by three washes with PBS for 5 min and the bound antibodies were detected with secondary antibodies diluted 1:150 in PBS with 0.2% BSA for 45 min at 37°C. After four washes with PBS, the cells were mounted in 90% glycerol in PBS containing 4% pyrogallol as an antifading reagent.

TX-114 Phase Separation and PI-PLC Treatment

N-terminally VSV-tagged PLAP was subjected to temperature-induced phase separation in TX-114 and PI-PLC treatment as described in Lisanti *et al.* (29). Partitioning of PLAP into the detergent or water phase was analyzed on Western blots using the mouse monoclonal P5D4 antibody.

Protein Purification by Affinity Chromatography on Ni-NTA Agarose

Secreted proteins (VIP36 Exo, rGH) were precipitated from the cell-free medium with 40% ammonium sulfate, sedimented by centrifugation at 9000 rpm for 30 min in a Sorvall GS-3 rotor, and dissolved in PBS (all steps were performed at 4°C). The starting volume was reduced by a factor of 15. To remove Cu^{2+} that interferes with the binding of His-tagged proteins to Ni-NTA agarose, the solution was dialyzed twice against 100 vol of 10 mM Tris/HCl, pH 8, 150 mM NaCl, 10% glycerol (buffer A). After dialysis, the solution was adjusted to 5 mM imidazole and rotated for 4 h with 2 ml Ni-NTA beads per liter of starting volume. Membrane proteins (VIP36 and PLAP) and cytosolic proteins (Anx XIIIb) were first solubilized or released by lysing the sedimented cells in 10 vol buffer A containing 5 mM imidazole, 2% TX-100, and CLAP. The lysate was kept on ice and passed 10× through a syringe with a 22-gauge needle to shear DNA and cleared by centrifugation (20 min, 20,000 rpm, 4°C, Sorvall SS-34 rotor). The supernatant, between 5 and 50 ml, was mixed with Ni-NTA beads and rotated overnight. To avoid unspecific binding of contaminating proteins the amount of Ni-NTA beads added was adjusted to the expected protein yields (i.e., if 5 mg of His-tagged protein was expected 0.5 ml of Ni-NTA beads were added). After adsorption of the proteins the beads were sedimented, the supernatant (flowthrough) was removed, and the beads were transferred into a column and washed extensively with buffer A (containing 0.1% TX-100 for the purification of VIP36 and Anx XIIIb). Subsequently, the beads were washed with

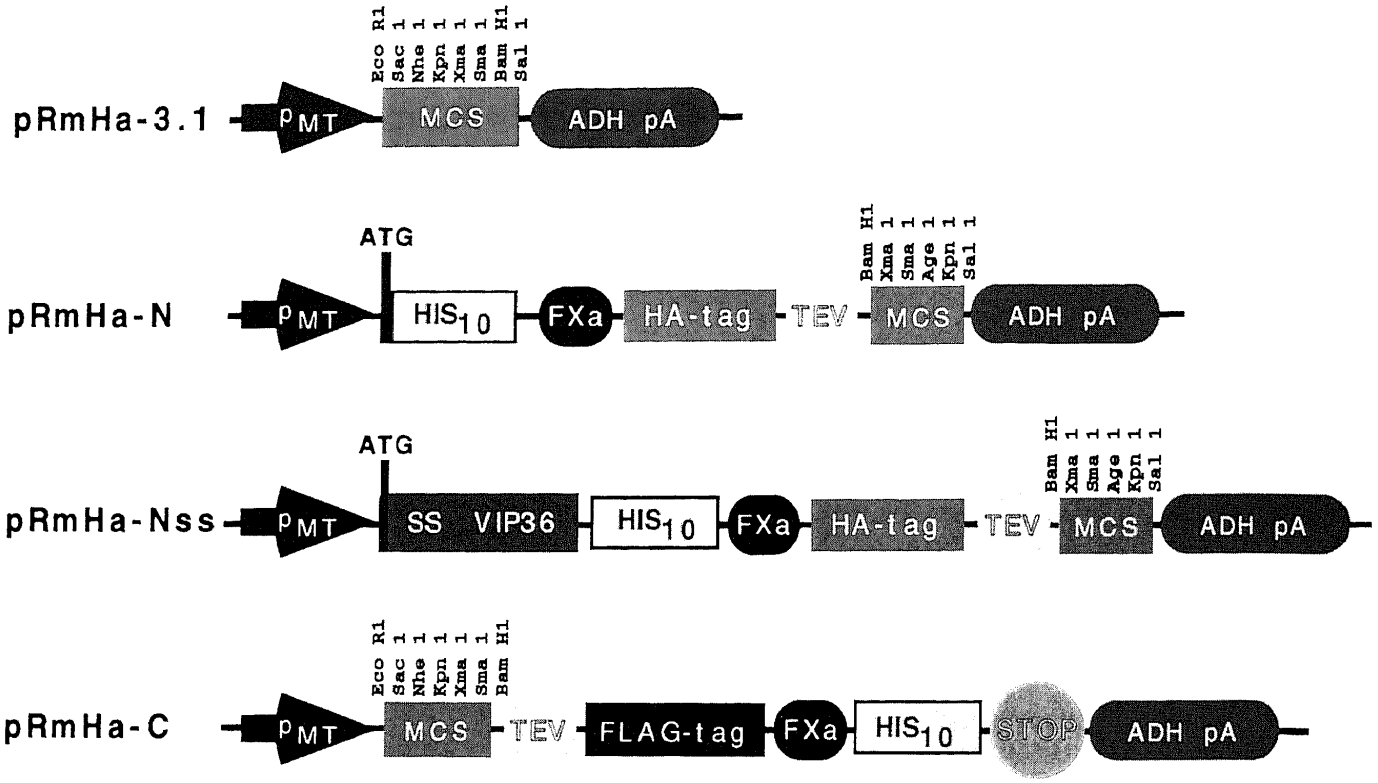

FIG. 1. Schematic drawing of pRmHa-3.1 and pRmHa vectors. Abbreviations: pMT, *Drosophila* metallothionein promotor; MCS, multiple cloning site; His_{10}, DNA sequence coding for a stretch of 10 histidines; ADH pA, alcohol dehydrogenase polyadenylation site; FXa, DNA sequence coding for a cleavage site for the factor Xa protease; HA-tag, DNA sequence coding for the HA epitope recognized by the monoclonal antibody 12CA5; TEV, DNA sequence coding for a cleavage site for tobacco etch virus protease; FLAG-tag; DNA sequence coding for the FLAG epitope recognized by the monoclonal anti-FLAG antibody.

buffer A containing increasing amounts of imidazole (see Results). Proteins were eluted with 100–200 mM imidazole in buffer A. The fractions were analyzed by SDS–PAGE and the gels were stained with Coomassie (0.2%) and Bismarck brown R (0.05%) in 40% methanol, 7% acetic acid. Protein determinations were carried out using the micro BCA kit (Pierce).

Treatment with Glycosidases

For digestion with PNGase F, purified VIP36 (1 μg) was boiled for 5 min in 30 μl of 50 mM Tris/HCl, pH 7.4, 0.5% SDS, 2% β-mercaptoethanol, and 10 mM EDTA. After addition of 3 vol 50 mM Tris/HCl, pH 7.4, 0.625% octylglucoside, 10 mM EDTA, the sample was boiled again for 1 min. CLAP was added to a final concentration of 1:250 and the sample was incubated in the presence and absence of 1.5 U of PNGase F for 36 h at 37°C. Digestion with Endo H was carried out after 5 min boiling of 1 μg of VIP36 in 150 mN Na–citrate, pH 5.5, 0.6% SDS and 0.2 M β-mercaptoethanol. A 1.5 vol of 0.75% octylglucoside was added, and the sample was boiled again for 1 min and incubated for 36 h at 37°C with 5 U of Endo H.

Metabolic Labeling with [^{3}H]Myristic Acid

SL-3 cells were labeled for 3 days in a total volume of 2 ml medium with 40 μCi of [9,10(n)-^{3}H]myristic acid (1.89 TBq/mmol with 37 mBq/ml in ethanol). To purify radiolabeled Anx XIIIb, 2×10^7 cells were lysed in 200 μl PBS containing 150 mM NaCl, 1% TX-100, and CLAP for 20 min at 37°C. The solubilized material was cleared by centrifugation (10 min at 14,000 rpm in an Eppendorf centrifuge) and the supernatant was incubated overnight at 4°C with 10 μl of Ni-NTA beads. Subsequently, the beads were washed in the above buffer with 40 mM imidazole, bound proteins were eluted with 200 mM imidazole and separated by SDS–PAGE. Total radiolabeled cellular proteins and purified myristoylated Anx XIIIb were separated by SDS–PAGE. The gels were treated with Entensify (DuPont) and the radiolabeled proteins were visualized by autoradiography.

RESULTS

The expression vector pRmHa-3.1 (Fig. 1) contains the *Drosophila* metallothionein promoter followed by a

multiple cloning site and the polyadenylation signal of the *Drosophila* alcohol dehydrogenase (ADH pA) (24). To facilitate the cloning of fusion proteins containing epitope- and His-tags that are used for their purification, we constructed the vectors pRmHa-N, pRmHa-Nss, and pRmHa-C. pRmHa-N allows the expression of N-terminally His- and HA-tagged fusion proteins. pRmHa-Nss was constructed by inserting a cleavable signal sequence (amino acids 1–44 of VIP36) upstream of the N-terminal His- and HA-tag of pRmHa-N such that the signal peptide is removed after translocation of the fusion protein into the lumen of the endoplasmic reticulum. The protein, now starting with an N-terminal His-tag, is secreted into the medium. Finally, pRmHa-C has been made for C-terminal fusions to a FLAG- and a His-tag. The epitope-tags of the fusion proteins are recognized by commercially available antibodies. All new vectors contain factor Xa and tobacco etch virus (TEV) protease cleavage sites which allow the sequential removal of the tags. Additionally, there are thrombin-cleavage sites (not indicated in the maps) next to the TEV site.

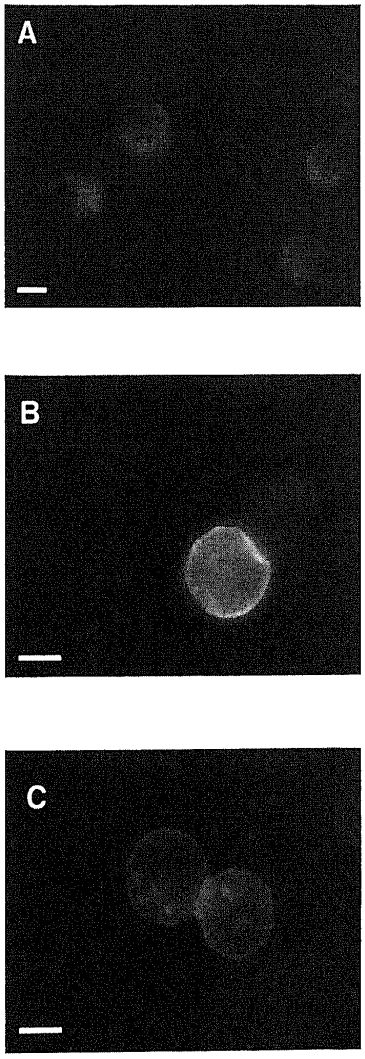

FIG. 2. Immunofluorescence localization of recombinant proteins in SL-3 cells (bar, 5 μm). (A) Detection of full-length VIP36 with a polyclonal anti-VIP36 antibody. The protein is detectable in the perinuclear region of the cells. (B) Detection of HA-tagged VIP17 with the monoclonal anti-HA antibody. The protein is localized at the cell surface. (C) Detection of N-terminally VSV-tagged PLAP with the monoclonal anti-VSV antibody. The protein is localized at the cell surface and in intracellular vacuole-like structures.

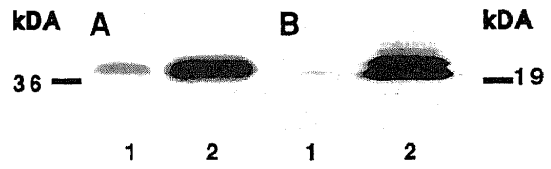

FIG. 3. Background expression levels and inducibility of expression after 24 h of induction with Cu^{2+}. (A) Western blot detected with the anti-VIP36 antibody. 5×10^5 of noninduced (lane 1) and induced (lane 2) stably transfected SL-3 cells expressing VIP36 were loaded per lane. (B) Western blot analysis detecting the expression of HA-tagged VIP17 with the monoclonal anti-HA antibody in 5×10^5 noninduced (lane 1) and induced (lane 2) SL-3 cells.

Note that the cDNAs coding for VIP36 Exo, full-length VIP36, the rat growth hormone and PLAP already contained in-frame epitope- and/or His-tags which have been introduced during previous cloning steps. Therefore, these proteins were expressed using pRmHa-3.1.

Localization of Overexpressed Proteins

One criterion which suggests the production of a functional protein is the proper localization of the overexpressed protein in the same compartment as its endogenous counterpart. In contrast, the mislocalization of a recombinant Golgi or plasma membrane protein to the endoplasmic reticulum would indicate the production of a misfolded protein.

We therefore analyzed the intracellular localization of recombinant membrane-associated proteins by immunofluorescence in stable polyclonal SL-3 cell lines selected for high protein expression (Fig. 2). Endogenous VIP36 localizes to the Golgi complex in MDCK cells (15). Recombinant VIP36 was localized in the perinuclear Golgi region of SL-3 cells (Fig. 2A) and therefore shows a comparable intracellular distribution as the endogenous protein in mammalian cells. Recombinant VIP17 was detected in the plasma membrane of SL-3 cells (Fig. 2B) as it has been shown for the endogenous protein in epithelial cells (16, 30). As expected, the GPI-anchored protein PLAP was mainly detected in the plasma membrane (Fig. 2C). We conclude that the overexpressed proteins are properly localized in SL-3 cells. We also found that 20–70% of the cells of a stable polyclonal cell line expressed high amounts of either recombinant protein.

Inducibility of Protein Expression

The expression of the recombinant proteins VIP36 and VIP17, as determined by Western blot analysis, is

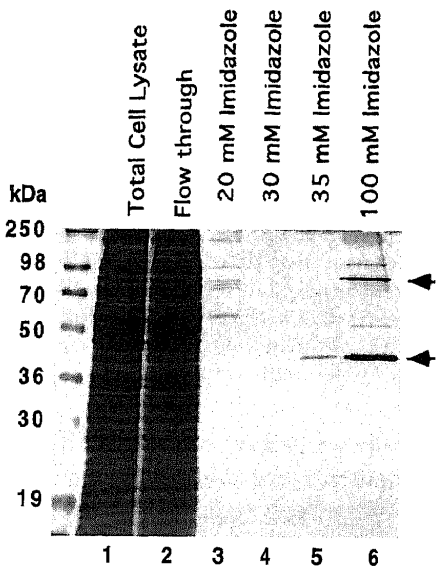

FIG. 4. Purification of recombinant full-length VIP36 from SL-3 cells. Protein pattern of total cellular protein of SL-3 cells stably expressing VIP36 (5×10^5 cells, lane 1) and of the flowthrough after incubation with Ni-NTA agarose (5×10^5 cells, lane 2). Proteins eluted by washes of the column with increasing amounts of imidazole (lanes 3–5) and elution of His-tagged VIP36 with 100 mM imidazole (lane 6). In lanes 3–6, 1% of the wash and the elution volumes was loaded. The arrows mark monomeric and dimeric VIP36. Coomassie staining.

highly inducible by Cu^{2+} (Fig. 3). In the absence of Cu^{2+}, the stable cell line showed a faint background expression of VIP36 (Fig. 3A, lane 1) and of VIP17 (Fig. 3B, lane 1), showing a low but detectable leakiness of the metallothionein promotor. After 24 h induction with 1 mM Cu^{2+} the expression levels of VIP36 (Fig. 3A, lane 2) and VIP17 (Fig. 3B, lane 2) increased drastically.

Recombinant VIP17 was detected on Western blots with the anti-HA antibody as a doublet band. The presence of two isoforms of VIP17 also has been found for endogenous VIP17 in MDCK cells (16). VIP17 did bind to Ni-NTA agarose (data not shown) and could be detected with the anti-HA antibody, indicating the presence of the epitope- and His-tag upon expression from the pRmHa-N vector.

Large-Scale Protein Purification

Prior to large-scale purification of His-tagged full-length VIP36 by affinity chromatography on Ni-NTA agarose, stably transfected SL-3 cells were induced for 3 days. Since the protein is not visible as a prominent band in the total protein pattern (Fig. 4, lane 1), the presence of the protein in total cell lysates, its efficient solubilization in Triton X-100, and the quantitative adsorption to Ni-NTA beads were monitored by Western blotting (data not shown). However, after washes of the column with 35 mM and elution with 100 mM imidazole, VIP36 is visible on a Coomassie-stained gel (Fig. 4, lanes 5 and 6). The purified protein appeared as two bands after SDS–PAGE (Fig. 4, lane 6), the lower 40-kDa band corresponding to the monomer size and the upper 80-kDa band corresponding to the dimer size of VIP36. An average yield of 0.5 mg/liter of cells at 5×10^6 cells/ml was obtained.

The secreted protein VIP36 Exo was purified from the cell culture supernatant of stable cell lines after 5 days induction. A band corresponding to the expected size of VIP36 Exo is present in the protein pattern of the medium (Fig. 5, lane 1). Since Cu^{2+} can interfere with the binding of the His-tagged protein to Ni-NTA agarose, the proteins were first concentrated by ammonium sulfate precipitation to reduce the total volume, and Cu^{2+} was removed by dialysis. VIP36 Exo was efficiently precipitated by ammonium sulfate (Fig. 5, lane 2) and dialyzed (lane 3). The protein bound quantitatively to Ni-NTA beads and was depleted from the flowthrough fractions (lane 4). After washing the column with 100 ml 5 mM imidazole and 15 mM imidazole in buffer A (lanes 5 and 6) VIP36 Exo was eluted in buffer A with 200 mM imidazole (lane 7). From 1 liter of cell culture supernatant (at a cell density of 5×10^6 cells/ml) 20 mg of VIP36 Exo could be purified. Using the same protocol equivalent amounts of rat growth hormone were purified.

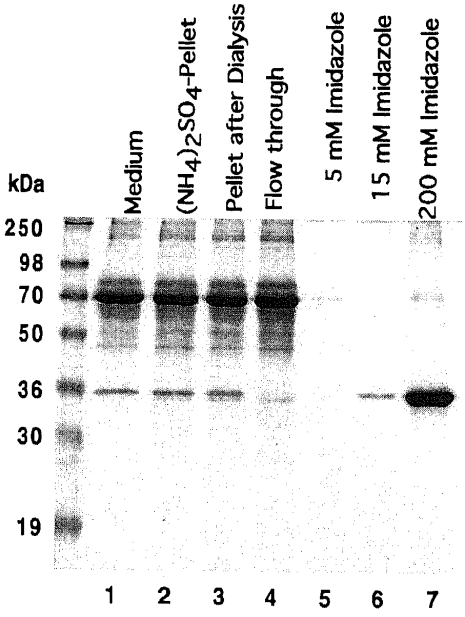

FIG. 5. Purification of VIP36 Exo. SDS–PAGE of 30 μl of cell-free medium after 5 days of induction (lane 1). Protein pattern of $(NH_4)_2SO_4$-precipitated medium (lane 2) and after dialysis (lane 3). Protein pattern of the flowthrough (lane 4), washes with 5 mM imidazole (lane 5) and 15 mM imidazole (lane 6). Lanes 1–6 were loaded with an amount equivalent to 30 μl of cell-free medium. Protein pattern of 0.1% of the total protein eluted with 200 mM imidazole (lane 7). Coomassie staining.

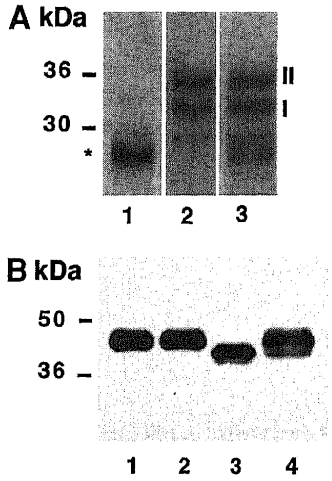

FIG. 6. N-glycosylation of recombinant proteins in SL-3 cells. (A) Comparison of the apparent molecular weight of purified rGH0 and rGH12 by SDS–PAGE. 3 μg rGH0 (lane 1) and 3 μg rGH12 (lane 2) and a mixture of both (lane 3) were loaded. Nonglycosylated rGH is marked by *, monoglycosylated rGH by I and doubly glycosylated rGH by II. Coomassie staining. (B) Treatment of purified full-length VIP36 with glycosidases. Purified VIP36 untreated (lane 1), mock-treated (lane 2), digested with *N*-glycosidase F (lane 3), and digested with endoglycosidase H (lane 4). Western blot detected with a polyclonal anti-VIP36 antibody.

N-Glycosylation

It has been shown qualitatively that proteins expressed in SL-3 cells become N-glycosylated (12). However, whether all the protein produced indeed is glycosylated has not been documented. To address this question we expressed and purified the nonglycosylated wild-type rat growth hormone (rGH0) and mutant containing two artificial N-glycosylation sites (rGH12) following the procedure used for the purification of VIP36 Exo and analyzed their N-glycosylation. N-glycosylated rGH shows a discrete shift in electrophoretic mobility as compared to the wild-type rGH. Thus, the presence of *N*-glycans in rGH can easily be monitored by SDS–PAGE.

As can be seen in Fig. 6A (lane 1) purified His-tagged rGH0, marked by an asterisk, has an apparent molecular weight of 27 kDa. Purified His-tagged rGH12 appears as two bands with slower mobility in SDS–PAGE (lane 2), corresponding to a mixture of monoglycosylated rGH at positions 1 and 2 (I) and doubly glycosylated (II) rGH12 (20). The addition of only one *N*-glycan to rGH12 is characteristic for this protein and not due to an inefficient glycosylation machinery in SL-3 cells, since it has also been observed in MDCK cells (31). To exclude that the faint band below the monoglycosylated rGH12 (Fig. 6, lane 2) is unglycosylated growth hormone, a mixture of purified rGH0 and rGH12 was loaded (lane 3). The faint band does not comigrate with rGH0, showing that all rGH12 secreted from SL-3 cells is N-glycosylated.

The susceptibility of an *N*-glycan to digestion by specific glycosidases allows conclusions about its structure. Purified monomeric His-tagged VIP36 was detected on a Western blot as a single band of ~40 kDa (Fig. 6B, lane 1). Digestion with *N*-glycosidase F (lane 3) but not mock treatment (lane 2) resulted in an increase in mobility as expected after the removal of the single *N*-glycan of the protein. This shift in molecular weight after treatment with PNGase F has been shown for endogenous VIP36 from MDCK cells (19). A modification found in insect cells is an additional fucose at the innermost glucosamine of the *N*-glycan (5–7) which does, if present, prevent deglycosylation by *N*-glycosidase F. The fact that PNGase F was active on VIP36 suggests the absence of fucosylated *N*-glycans in SL-3 cells.

Surprisingly, treatment of VIP36 with endoglycosidase H (Fig. 6B, lane 4) showed that more than 70% of the protein contained endodoglycosidase-H-resistant *N*-glycans. Resistance to Endo H is restricted to *N*-glycans of the hybrid or complex type which have rarely been detected in insect cells.

Myristoylation of Annexin XIIIb

The capability of SL-3 cells for myristoylation was tested by expressing Anx XIIIb cloned in frame into pRmHa-C. On Western blots, the fusion protein can be detected in total cell lysates of transfected SL-3 cells with an anti-FLAG antibody (Fig. 6, lane 1). Metabolic labeling of Anx XIIIb expressing SL-3 cells with [^{3}H]myristate results in the production of a prominent radiolabeled 50-kDa protein (Fig. 7, lane 3), corresponding to the expected size of tagged Anx XIIIb, which is absent in wild-type cells (lane 2). This band can be bound to and eluted from Ni-NTA beads (lane 4), showing that the 50-kDa band is the His-tagged recombinant Anx XIIIb. The C-terminal tags can be removed

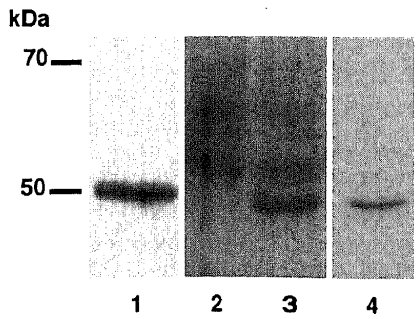

FIG. 7. Characterization of myristoylated Anx XIIIb expressed in SL-3 cells. Detection of Anx XIIIb on a Western blot with the anti-FLAG antibody in total cell lysates of stably transfected SL-3 cells (5×10^5 cells, lane 1). SDS–PAGE and autoradiogram of total [^{3}H]myristate-labeled proteins from wild-type (lane 2) and Anx XIIIb-expressing SL-3 cells (lanes 3) and [^{3}H]myristate-labeled Anx XIIIb purified by adsorption to Ni-NTA beads (lane 4).

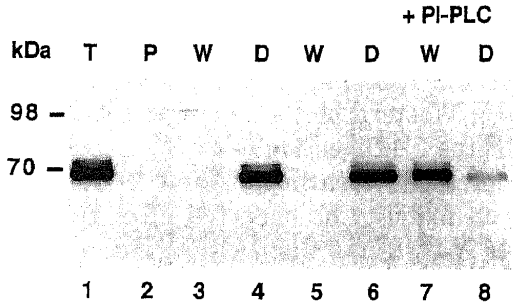

FIG. 8. TX-114 phase separation and PI-PLC treatment of VSV-tagged PLAP. PLAP detected on a Western blot with the anti-VSV antibody in total cell lysates of SL-3 cells stably expressing VSV-PLAP (5×10^5 cells/lane; lane 1). PLAP does not sediment after extraction with TX-114 (P, lane 2). Detection of PLAP in the water (W) and detergent phase (D) after temperature-induced phase separation of TX-114 (lanes 3 and 4), after PI-PLC treatment (lanes 7 and 8), and after mock treatment (lanes 5 and 6). Equivalent amounts of cells were loaded in each lane.

efficiently from purified Anx XIIIb by digestion with TEV protease (data not shown). The myristoylated Anx XIIIb was purified following the protocol for the purification of VIP36 with a yield of 1.6 mg/liter cells.

Expression of GPI-Anchored Placental Alkaline Phosphatase

As an example for a GPI-anchored protein, we expressed N-terminally VSV-tagged PLAP in SL-3 cells. The protein remained cell-associated and could not be detected in the medium (data not shown). By immunofluorescence, the protein could be detected with the anti-VSV antibody at the surface of SL-3 cells (Fig. 2C). To analyze whether the protein is membrane-associated via a GPI anchor, VSV-PLAP was subjected to TX-114 phase separation and digestion with PI-PLC which removes the diacylglycerol moiety of the GPI anchor (Fig. 8). On Western blot VSV-PLAP was detected as a 70-kDa protein, corresponding to the expected size of the fusion protein (Fig. 8, lane 1/T). PLAP-expressing cells were lysed in 1% TX-114 and incubated for 10 min at 37°C and for 10 min on ice. To remove all nonsolubilized material the sample was spun for 15 min at 4°C at 13,000 rpm. PLAP was not present in the resulting pellet (Fig. 8, lane 2/P), showing that the protein was efficiently solubilized in TX-114. After temperature-induced phase separation at 37°C, the protein partitioned completely into the detergent phase (Fig. 8, lane 4/D). After PI-PLC treatment and phase separation, 90% of the protein was detectable in the water phase (Fig. 8, lane 7/W). The shift from the detergent to the water phase upon PI-PLC treatment indicates the presence of a GPI anchor in the protein. Mock treatment did not result in a shift from the detergent to the water phase (Fig. 8, lanes 5 and 6).

DISCUSSION

We have described the production of secretory, membrane, cytosolic, and GPI-anchored proteins in SL-3 cells. The discussion will focus on the presence of post- and cotranslational modifications and protein yields compared to published results obtained using recombinant baculovirus.

Protein Yields

Expression of secreted proteins in SL-3 cells yields high protein amounts. We purified 20 mg of protein from 1 liter of medium, a number that previously has been reported (10, 12). The yield for the cytosolic protein annexin XIIIb was 1.6 mg/liter of cells. Membrane proteins are generally expressed at lower levels. However, 0.5 mg of VIP36 could be purified from 1 liter of cells. These results show that protein expression in stably transfected SL-3 cells allows the preparation of proteins sufficient for biochemical analysis and crystallography.

The proteolipid VIP17 was also expressed at detectable levels in SL-3 cells, but the yields of purified protein were low. Recent results have shown that overexpression of VIP17 in polarized kidney cells seriously affects cell morphogenesis (18), indicating that VIP17 overexpression has toxic effects. Possibly, the background expression of VIP17 inhibits the proliferation of high expressing SL-3 cells. However, VIP17 and the structurally related protein PLP (proteolipid protein) were overexpressed and purified successfully (at 6 mg/liter of cells) with the baculovirus system (32) (K. Ekroos and K. Simons, unpublished results), suggesting that baculovirus-based expression is the better system for the production of toxic proteins.

N-Glycosylation

Secretory and membrane proteins become N-glycosylated in SL-3 cells with high efficiency, shown by the fact that all rGH12 secreted was at least monoglycosylated. For the full-length transmembrane protein VIP36, we found that the majority of the protein was resistant to treatment with the endoglycosidase Endo H. This enzyme only cleaves high mannose-type *N*-glycans but not *N*-glycans that have been modified in the Golgi complex (trimming of mannose and addition of GlcNAc). This result suggests that the *N*-glycan on VIP36 has been modified to a hybrid or complex form. It has been proposed that insect cells have the capability to synthesize complex *N*-glycans, but due to the presence of sialidase, β-galactosidase, and *N*-acetylglucosaminidase (33) complex oligosaccharides are continuously trimmed back (2). VIP36 has been shown to cycle in the early secretory pathway between the Golgi and the ER (15). It is possible that VIP36 is perma-

nently exposed to the glycosylation enzymes in the early Golgi and keeps its modified *N*-glycan, whereas the *N*-glycans of cell surface and secreted proteins are trimmed back.

Lipid Modifications on Proteins

Myristoylation is a cotranslational modification in which the fatty acid is added to N-terminally located glycines. Posttranslational myristoylation occurs neither *in vivo* nor *in vitro*. Currently, the most frequently used system to produce myristoylated proteins consists of coexpression of the target protein in *E. coli* with the *N*-myristoyltransferase cloned from yeast. However, the yield of fatty-acid-modified protein in *E. coli* is generally only about 1% of the total protein produced. In the particular case of annexin XIIIb, no myristoylation was obtained in *E. coli*. In contrast, Anx XIIIb expressed in SL-3 cells becomes myristoylated. In addition to myristoylation, we have evidence for palmitoylation of caveolin 1, a 21-kDa membrane protein, expressed in SL-3 cells (data not shown). Palmitoylation and myristoylation of proteins has also been reported in Sf9 cells (34, 35). Thus, both stably transfected SL-3 and Sf9 cells infected with baculovirus are appropriate for the production of lipid-modified proteins.

GPI-Anchored Proteins

Expression of GPI-anchored proteins using the baculovirus system is not frequently documented in the literature. The GPI proteins human β_2-glycoprotein and CD59 were expressed in Sf9 cells (36, 37). Both proteins and mutants thereof were released completely or in considerable amounts into the cell culture supernatant of Sf9 cells, indicating an inefficient addition of the GPI anchor. Recently, it has been found that upon baculovirus infection, the synthesis of GPI anchors shuts down (Azzouz, N., Keedes, M. and Schwarz, R. T., personal communication). In contrast, the GPI-anchored protein PLAP expressed in SL-3 cells remained cell-associated and was not secreted into the medium. PLAP was sensitive to treatment with PI-specific phospholipase C as indicated by its shift from the detergent phase to the water phase after TX-114 phase separation. By immunofluorescence, PLAP was localized to the cell surface of SL-3 cells. Together, this shows that addition of GPI anchor to the overexpressed protein was apparently complete. Successful expression of a GPI-anchored cadherin in the *Drosophila* cell line S2 has been reported (38). Therefore, stably transfected insect cells seem to be optimal for the production of GPI-anchored proteins.

In summary, protein expression in SL-3 cells is an attractive expression system, in particular for secretory, GPI-anchored and membrane proteins. Protein expression can be monitored at the analytical level after transitional transfection of SL-3 cells and within 2–3 days considerable amounts of proteins are produced. Expression of toxic proteins, as VIP17, may be inefficient probably because of constitutive expression from the metallothionein promotor. Finally, it is worthwhile mentioning that coexpression of two or even more proteins at the same time can be achieved easily by cotransfection of two expression plasmids (J.B., unpublished results, and (9)). The new expression vectors are available upon request.

ACKNOWLEDGMENTS

We thank Dr. Klaus Karjalainen for generous gift of SL-3 cells and the pRmHa-3 vector and support during the establishment of the system in our lab. We thank Anton Rietveld and Marino Zerial for critical reading of the manuscript and Iris Ansorge for help during preparation of the manuscript. J.B. was supported by an EMBO long-term fellowship. D.Z. was supported by a Human Capital and Mobility EC grant and a Telethon fellowship. This work was supported by SFB 352 and a TMR network grant.

REFERENCES

1. Kubelka, V., Altmann, F., Kornfeld, G., and März, L. (1994) *Arch. Biochem. Biophys.* **308,** 148–157.
2. Jarvis, D. L., and Finn, E. E. (1995) *Virology* **212,** 500–511.
3. Davidson, D. J., and Castellino, F. J. (1991) *Biochemistry* **30,** 6689–6696.
4. Altmann, F., Kornfeld, G., Dalik, T., Staudacher, E., and Glossl, J. (1993) *Glycobiology* **3,** 619–625.
5. Williams, P. J., Wormald, M. R., Dwek, R. A., Rademacher, T. W., Parker, G. F., and Roberts, D. R. (1991) *Biochim. Biophys. Acta* **1075,** 146–153.
6. Staudacher, E., Altmann, F., März, L., Hard, Kamerling, J. P., and Vliegenthart, J. F. G. (1992) *Glycoconj. J.* **9,** 82–85.
7. Staudacher, E., Kubelka, V., and Marz, L. (1992) *Eur. J. Biochem.* **207,** 987–993.
8. King, L. A., and Possee, R. D. (1992) Chapman and Hall, London.
9. Wallny, H. J., Sollami, G., and Karjalainen, K. (1995) *Eur. J. Immunol.* **25,** 1262–1266.
10. Li, B., Tsing, S., Kosaka, A. H., Nguyen, B., Osen, E. G., Bach, C., Chan, H., and Barnett, J. (1996) *Biochem. J.* **313,** 57–64.
11. Bernard, A. R., Kost, T. A., Overton, L., Cavegn, C., Young, J., Bertrand, M., Yahia, C. Z., Chabert, C., and Mills, A. (1994) *Cytotechnology* **15,** 139–144.
12. Culp, J. S., Johansen, H., Hellmig, B., Beck, J., Matthews, T. J., Delers, A., and Rosenberg, M. (1991) *Biotechnology* **9,** 173–177.
13. Berndorff, D., Gessner, R., Kreft, B., Schnoy, N., Lajous, P. A., Loch, N., Reutter, W., Hortsch, M., and Tauber, R. (1994) *J. Cell Biol.* **125,** 1353–1369.
14. Fiedler, K., Parton, R. G., Kellner, R., Etzold, T., and Simons, K. (1994) *EMBO J.* **13,** 1729–1740.
15. Fullekrug, J., Scheiffele, P., and Simons, K. (1999) *J. Cell Sci.* **112,** 2813–2821.
16. Zacchetti, D., Peranen, J., Murata, M., Fiedler, K., and Simons, K. (1995) *FEBS Lett.* **377,** 465–469.
17. Puertollano, R., Martin-Belmonte, F., Millan, J., de Marco, M. C., Albar, J. P., Kremer, L., and Alonso, M. A. (1999) *J. Cell Biol.* **145,** 141–151.

18. Cheong, K., Zacchetti, D., Schneeberger, E. E., and Simons, K. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6241–6248.
19. Fiedler, K., and Simons, K. (1996) *J. Cell Sci.* **109,** 271–276.
20. Guan, J.-L., Machamer, C. E., and Rose, J. K. (1985) *Cell* **42,** 489–496.
21. Fiedler, K., Lafont, F., Parton, R. G., and Simons, K. (1995) *J. Cell Biol.* **128,** 1043–1053.
22. Lafont, F., Lecat, S., Verkade, P., and Simons, K. (1998) *J. Cell Biol.* **142,** 1413–1427.
23. Steller, H. a. P., V. (1985) *EMBO J.* **4,** 167–171.
24. Bunch, T. A., Grinblat, Y., and Goldstein, L. S. (1988) *Nucleic Acids Res.* **16,** 1043–1061.
25. Cavener, D. R. (1987) *Nucleic Acids Res.* **15,** 1353–1361.
26. Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8148.
27. Mantei, N., Villa, M., Enzler, T., Wacker, H., Boll, W., James, P., Hunzicker, W., and Semenza, G. (1988) *EMBO J.* **7,** 2701–2713.
28. Schneider, I. (1972) *J. Embryol. Exp. Morphol.* **27,** 353–365.
29. Lisanti, M. P., Sargiacomo, M., Graeve, L., Saltiel, A. R., and Rodriguez-Boulan, E. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9557–9561.
30. Martin-Belmonte, F., Kremer, L., Albar, J. P., Marazuela, M., and Alonso, M. A. (1998) *Endocrinology* **139,** 2077–2084.
31. Scheiffele, P., Peranen, J., and Simons, K. (1995) *Nature* **378,** 96–98.
32. Fukuzono, S., Takeshita, T., Sakamoto, T., Hisada, A., Shimizu, N., and Mikoshiba, K. (1998) *Biochem. Biophys. Res. Commun.* **249,** 66–72.
33. Licari, P., Jarvis, D. L., and Bailey, J. E. (1993) *Biotechnol. Prog.* **9,** 146–152.
34. Busconi, L., and Michel, T. (1995) *Mol. Pharmacol.* **47,** 655–659.
35. Kunz, B. C., Muczynski, K. A., Welsh, C. F., Stanley, S. J., Tsai, S. C., Adamik, R., Chang, P. P., Moss, J., and Vaughan, M. (1993) *Biochemistry* **32,** 6643–6648.
36. Davies, A., and Morgan, B. P. (1993) *Biochem. J.* **295,** 889–896.
37. Igarashi, M., Matsuura, E., Igarashi, Y., Nagae, H., Ichikawa, K., Triplett, D. A., and Koike, T. (1996) *Blood* **87,** 3262–3270.
38. Kreft, B., Berndorff, D., Bottinger, A., Finnemann, S., Wedlich, D., Hortsch, M., Tauber, R., and Gessner, R. (1997) *J. Cell Biol.* **136,** 1109–1121.