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Engineering the Folding Pathway of Insect Cells: Generation of a Stably Transformed Insect Cell Line Showing Improved Folding of a Recombinant Membrane Protein

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The baculovirus-insect cell expression system has proven to be a valuable tool for the high level production of a multitude of recombinant proteins. However, production of membrane proteins in infected insect cells is often hampered by incorrect folding and processing which results in the accumulation of non-functional protein. Here, we report the construction of a Sf9 insect cell line stably transformed with the *nina*A gene from *D. melanogaster* (Sfn cell line). The ninaA protein is a membrane bound cyclophilin which acts as a peptidyl-prolyl *cis/trans* isomerase during the folding process of rhodopsin 1 in *D. melanogaster* rhabdomere. Engineered Sfn insect cells infected with a recombinant baculovirus bearing the human dopamine transporter gene under the control of the polyhedrin promoter showed a $\geqslant$5 times enhanced uptake of [^{3}H]dopamine in comparison to similarly infected Sf9 cells. This increase in specific transport activity was not due to an altered K_m value in the Sfn cell line. The uptake in infected Sfn cells was blocked by the peptidyl-prolyl *cis/trans* isomerase inhibitor cyclosporin A which had no effect on infected Sf9 cells. From these results we conclude that the prolyl-*cis/trans* isomerase activity of the ninaA in the stably transformed Sfn cell line was responsible, directly or indirectly, for the improved folding of the heterologously produced human dopamine transporter.

The baculovirus expression system has developed into a valuable and popular tool for the production of foreign proteins in lepidopterian insect cell lines. In most cases the system uses late viral baculovirus promoters (p10, polyhedrin) to drive the expression of foreign genes in insect cells after infection with a respective recombinant baculovirus (1-4). Meanwhile, a multitude of different baculovirus vectors are available and the generation of a recombinant baculovirus is an easy task. The infected insect host cells provide the eucaryotic cellular machinery for the production of the recombinant protein and, therefore, carry out most of the post-translational processing events such as glycosylation, phosphorylation, palmitoylation, myristoylation or addition of glycosyl-phosphatidylinositol (GPI) anchors (4, 5). Nevertheless, despite the great advantages offered by this system its major drawback is, that, due to the dramatic perturbations of the cellular environment caused by the virus infection, the insect cells may not be able to fulfill the post-translational modifications exactly. Especially, in the case of the production of membrane proteins, correct folding of the recombinant protein in the insect cells is often the rate limiting step (6). Therefore, despite the massive production of recombinant membrane protein after onset of the polyhedrin promoter ($>$12 h p.i.), often only low levels of functional protein can be detected. These deficiencies in the folding pathway can result in the accumulation and/or precipitation of the immature protein in or at the ER. Possibilities to circumvent these problems are either to change the promoter for expression of the foreign gene, e.g. selection of viral promoters which initiate transcription earlier in infection and, therefore, allowing a longer period for post-translational processing while host modification pathways are still functional (7-9) 2.), to fuse target or signal peptides to the foreign gene which enforce correct translocation and subsequent folding (7-12) or to construct or to use insect cell lines which are or have been optimized for correct folding and processing of the desired recombinant membrane protein (13-15).

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Nevertheless, there is only limited knowledge about the processes involved in the folding of integral membrane proteins. In general, molecular chaperons prevent the proteins from wrong intra- and/or intermolecular interactions which otherwise would result in aggregation and degradation (16-18). Foldases such as peptidyl-prolyl *cis/trans* isomerases and disulfide-isomerases accelerate the folding of proteins (19). Recently, the ninaA-gene (**nina**: **n**either **i**nactivation **n**or **a**fterpotential) was isolated from *Drosophila melanogaster* photoreceptor cells and found to encode a 237-amino acid protein with striking homology to cyclophilins of other organisms (20-22). Cyclophilins are proteins which catalyze the *cis/trans* isomerization about an Xaa-Pro peptide bond, an activity important for protein folding *in vivo*. The immunosuppressant cyclosporin A (CsA) binds to the active site of cyclophilins with nanomolar affinity and is thus a potent inhibitor of the PPI activity (23, 24). NinaA has been found to directly interact with rhodopsin 1 ensuring correct folding of this membrane bound protein in the rhabdomere of the fly (25).

In this report, we describe the construction of an insect cell line stably transformed with the *D. melanogaster nina*A gene under control of the early baculovirus promoter *ie*1. This insect cell line revealed a time-dependent cyclosporin-inhibitable dopamine transport two days after infection with a recombinant baculovirus bearing cDNA of the human dopamine transporter gene. In comparison to wild-type Sf9 cells this cell line revealed a $\geqslant 5$ fold higher import rate for dopamine and a $\geqslant 5$ fold higher value of WIN 35.428 binding sites due to improved folding of the recombinant protein in the engineered cell line.

MATERIALS AND METHODS

General methods. Recombinant DNA techniques were performed according to established protocols (26,27) and all cloning steps were ensured by DNA sequencing (28).

Cells, cell culture, and infection of cells. *Spodoptera frugiperda* cells (Sf9-cells; ATCC no.:CRL 1711) as well as Sf9 cells stably transformed with the *D. melanogaster* ninaA gene (=Sfn cells) were routinely propagated at 27°C in TNM-FH medium supplemented with 5% fetal calf serum (FCS) and 50 μg/μl gentamycin (29). Suspension cultures of insect cells were maintained in 250 ml spinner flasks (Techne, England) stirred at 70 rpm and 27°C in TNM-FH medium. Infection of cells with baculovirus were carried out when cells were in logarithmic growth phase at a cell density of 1-1.5$\times 10^6$ cells/ml. Virus used for the experiments was titered by the end point dilution method (30) and expression experiments were performed by infecting cells with a *moi* of 10. If not stated otherwise, cells were harvested 40-48 hours after infection.

Generation of a stably transformed ninaA Sf9 insect cell line. The cDNA bearing the *Drosophila melanogaster* ninaA-gene was kindly provided by Prof. Dr. Zuker (University of California). To facilitate subsequent cloning steps, the coding region was amplified with the primers ninaAfor (5′-CCCGGATCCCCGCAAAATCATGAAGTCATTGCTCAATCGGA-TAAT-3′) and ninaArev (5′-GTCGCCGTTCACAATGT-3′). The PCR-fragment was restricted with *Bam*H I/*Nru* I, the plasmid *pKS-ninaA* which bears the complete ninaA cDNA with *Nru* I/*Kpn* I and after purification both fragment were cloned into *Bam*H I/*Kpn* I-linearized vector *pAc-IE1-Tom* (Lenhard, unpublished vector construct). The resulting plasmid *pAc-IE1-Tom-ninaA* was co-transfected into Sf9 insect cells with plasmid *pAc-IE1-neo* which contains the neomycin resistance gene under the control of the IE1-promotor (7). Subsequently, the cells were incubated at 27°C in FCS deficient medium. After 24 h the medium was supplemented with 5% FCS and the transfected cells were incubated for another 48 h. In order to select G418 resistant cells, 20000 cells were seeded into a 24-well dish and propagated in TNM-FH medium supplemented with 1mg/ml Geneticin G418. It has been demonstrated previously (7-9), that about 70 % of the G418-resistant cells are also recombinant with respect of the desired gene. Cells were kept for 3 weeks at 27°C under selective pressure with two changes of selection medium. After that, resistant cells were isolated by dilution and resulting cell lines were tested for the presence of the ninaA gene by Southern blot analysis and PCR. One stably transformed cell clone was selected for further experiments.

Construction of hDAT-recombinant baculovirus. The cDNA encoding for the human dopamine transporter kindly provided by Dr. T. Liu (Hoechst/Roussel; USA) was recloned into pBluescript. A PCR was performed with the oligonucleotides pNKs-ori-for (5′-*CCCGGTACCAAGCTTATAAATATGAGTAAGAGCAAATGCT*-3′) and pNKs-ori-rev (5′-*GGGCTCGAGGTACCTACACCTTGAGCCAGTGG*-3′) (matching sequence underlined) to provide *Kpn*I sites 5′ and 3′ of the coding region. Afterwards this DNA fragment was ligated into the baculovirus transfer vector pVL1393 (3). 1-2 μg of the resulting plasmid pVL93hDAT with 0.4 μg of linearized baculovirus BaculoGold-DNA (*Autographa californica* BaculoGold; Pharmingen) was co-transfected into Sf9 insect cells using cationic liposomes (Lipofectin; GIBCO BRL). After 5 days of incubation at 27°C the virus containing supernatant was collected and recombinant virus clones were screened by plaque assay on Sf9 cells. For verification of the selected virus clones, genomic DNA from infected cells was prepared and examined for the presence of the hDAT by Southern hybridization (31) using a digoxigenine labeled DNA-fragment from the hDAT coding region. From one recombinant virus which was selected for further experiments a stock solution was produced and titered.

SDS-Page and immunoblot analysis. Infected cells were harvested at 600 g for 10 min at 4°C and resuspended to a final concentration of 10000 cells/μl in cold TEN-buffer [50 mM Tris-Cl pH 7.4, 150 mM NaCl, 10 mM EDTA] supplemented with protease inhibitor cocktail [Pefabloc 0.5 mM, Leupeptin 5 μg/μl, Aprotinin 5 μg/μl, Pepstatin 2 μg/μl, EGTA 0.1 mM]. The buffer also contained 1% SDS for solubilization. After a 1:2 dilution with TEN-buffer a benzonase treatment was performed for at least 15 minutes on ice with 3 U/μl benzonase. After that treatment, the proteins were separated on a 10% SDS-polyacrylamide gel (32), transferred to a polyvinylidenefluoride membrane and processed as described (33). The membrane was blocked for 1 hour at room temperature with 5 % non-fat milk powder in PBS-Tween [13.7 mM NaCl, 2.7 mM KCl, 1.5mM KH_2PO_4 13 mM Na_2HPO_4, 1.5 mM NaN_3, 0.02% Tween 20]. Membranes, thereafter, were incubated with either the ninaA-specific antibody (dilution 1:200) or the NET213/230 antiserum (dilution 1:2000) for 1 hour. The membrane was washed for three times and subsequently was incubated with goat-anti-rat alkaline phosphatase conjugate or goat-anti-rabbit alkaline phosphatase conjugate (Sigma; dilution 1:1000), respectively. Protein bands were visualized by enzymatic reaction in alkaline phosphatase buffer [100mM NaCl, 100mM Tris pH 9.5, 5mM $MgCl_2$] with 30 μl 5-bromo-4-chloro-3-indolylphosphate p-toluidinium salt [BCIP-stock: 50 mg/ml DMF] and 30 μl nitroblue tetrazolium chloride [NBT-stock: 50 mg/ml 70% DMF].

Immunoprecipitation. 3×10^6 cells were infected with recombinant baculovirus in petri dishes (6 cm Ø, Nunc). For deglycosylation

experiments infected cells the medium was supplemented with 10 μg/ml tunicamycin [stock-solution: 1 mg/ml in 0.1 M NaOH]. After 48 h the cells were washed several times with methionine-free TNM-FH-medium and the cells were labeled for 4 h in methionine-free TNM-FH-medium supplemented with 25 μCi/ml [^{35}S]-methionine [NEN, 10.2 mCi/ml; 8.68 μM]. Afterwards cells were rinsed three times with cold PBS-buffer and solubilized on ice in cold radioimmunoprecipitation buffer for 30 min [RIPA: 150 mM NaCl, 1 mM EDTA, 250 μM phenylmethylsulfonyl fluoride, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 10 mM Tris-HCl, pH 7.4]. The extract was centrifuged for 15 min at 8000 g (4°C) and the supernatant was incubated overnight with 10 μl of the hDAT specific NET213/230 antiserum at 4°C (antiserum NET213/230 was a generous gift from Dr. R. Hammermann, University of Bonn (34, 37). The hDAT/antibody complex was pelleted after binding to Protein-A Sepharose CL-4B beads (Sigma) for another 2 h at 4°C. The beads were washed three times with RIPA-buffer and, finally, vortexed for 30 min at 4°C in 50 μl of 1×SDS sample buffer. As judged by scintillation counting, equal amounts of protein were separated by SDS-PAGE. SDS gels were fixed for 10 min in 10% acetic acid, soaked in sodium salicylate for 1 h at 4°C, dried and exposed to X-ray film (Kodak XAR-5) at −70°C for 12 h.

Uptake experiments. Insect cells were infected and harvested as described above. Experiments performed to check the influence of tunicamycin or cyclosporin A on the activity of the transporter were carried out by incubation of the cells in TNM-FH medium supplemented with 10 μg/ml tunicamycin or 20 μM cyclosporin A, respectively. For time course experiments, the cells were grown and infected in spinner flask (Techne). At different time points, cells were harvested and submitted to SDS-PAGE and subsequent immunoblot analysis as well as uptake experiments. The experiments were performed in uptake buffer [90 mM NaCl, 5 mM Tris-HCl, 7.5 mM HEPES, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM D-Glucose, 0.1 mM ascorbic acid]. 10^6 cells were pelleted (RT, 600 g, 10 min), washed once with and resuspended in uptake medium. The assay volume always was 200 μl. Cells were preincubated in 100 μl of either uptake buffer (total transport) or uptake buffer containing 300 μM cocaine (unspecific transport) for 10 min (10 min preincubation in the presence of cocaine ensures equilibrium) at 25°C by slow rotation. The transport was started by the addition of 0.2 μM [^{3}H]-dopamine (NEN; spec. activity: 41.5 - 56.5 Ci/mmol; final assay concentration of 0.1 μM [^{3}H]dopamine in each vial) in 100 μl of uptake-buffer. The cells were incubated for 10 min at 25°C (the steady-state phase of uptake lasts at least 20 minutes; data not shown). Dopamine uptake was terminated by addition of 1 ml cold uptake buffer. Subsequently, cells were pelleted (10 min, 600 g, 4°C), washed once with uptake buffer and lysed in 200 μl 1% SDS. The amount of [^{3}H]-dopamine transported into the cells was determined by scintillation counting (Canberra Packard Tri Carb 1500). Non inhibited transport was carried out in triplicate, cocaine-inhibited transport in duplicate.

RESULTS AND DISCUSSION

Construction of a stably transformed insect cell line Sfn producing the D. melanogaster NinaA. The initial idea was to engineer an insect cell line with an optimized folding apparatus especially suited for the production of G protein coupled receptors. Recently, the *nina*A-gene from *D. melanogaster* has been described to be essential for the folding of the main rhodopsin (Rh1) in the rhabdomere of the fly. NinaA specifically interacts and complexes with Rh1 enforcing correct folding and subsequent targeting. In ninaA mutant flies the Rh1 is retained in the ER and the rhodopsin levels are reduced >100 fold. NinaA is a member of the family of cyclophilins which act as peptidyl-prolyl *cis/trans* isomerases catalyzing the rate limiting step in the folding process. The target of the NinaA is Rh1 which like all rhodopsins belongs to the family of G protein receptors. Therefore, we choose the cDNA of the *D. melanogaster nina*A gene for the construction of a stable transformed Sf9 cell line. For constitutive expression in the insect cells the gene was cloned under the control of a early baculovirus promoter. The immediate early baculovirus vector pAcIE1 which already had been used for the construction of other stably transformed Sf9 based cell lines was utilized (7-9). As shown in figure 1a, the resulting plasmid pAcIE1ninaA contained the complete coding region of the ninaA gene under the control of the *ie*1 promoter, which is a early baculovirus derived promoter recognized by the insect cell polymerase immediately after transfection (7, 35). The 5′- and 3′-untranslated regions of the *nina*A cDNA have been deleted during construction of the plasmid. Sf9 insect cells were cotransfected with pAcIE1ninaA and pAcIE1Neo and G418-resistant cell clones were selected, raised and propagated (7). To ensure that these G418-resistant cell clones had been transformed with the *nina*A-containing plasmid, they were examined for the presence of the *nina*A gene by Southern hybridization as well as PCR (Fig. 1b). Several cell clones scored positive during this analysis and one clone was selected and characterized for further experiments. In figure 1c, an immunoblot analysis of this insect cell clone which was named Sfn, in comparison to Sf9 cells is presented. Only in the Sfn cells the *nina*A-specific antiserum stained a twin protein band with an apparent molecular mass of 33-35 kDa confirming successful production of the NinaA. As revealed during further characterization the growth of the Sfn cells in comparison to Sf9 cells was reduced (Fig. 1d). A similar reduction in growth rate was also detected in the case of a Sf9 line stably transformed with the human β_2 adrenergic receptor (9).

Immunological detection and characterization of the hDAT produced in the baculovirus infected insect cells lines Sf9 and Sfn. After infection of the Sfn cell line with different recombinant baculoviruses bearing different G protein coupled receptors (human β_2 adrenergic receptor, mouse $5HT_{5A}$ receptor, human D_{2S} receptor) no significant enhanced production levels could be detected (data not shown). Unexpectedly, comparison between the Sfn cell line and Sf9 cells, both infected with a recombinant baculovirus containing the human dopamine transporter gene (36), revealed a dramatic effect. As schematically depicted in figure 2, the baculovirus used for infection of the insect cell lines contained the hDAT cDNA under the transcriptional control of the polyhedrin promoter. As expected, infection of Sf9 and Sfn in-

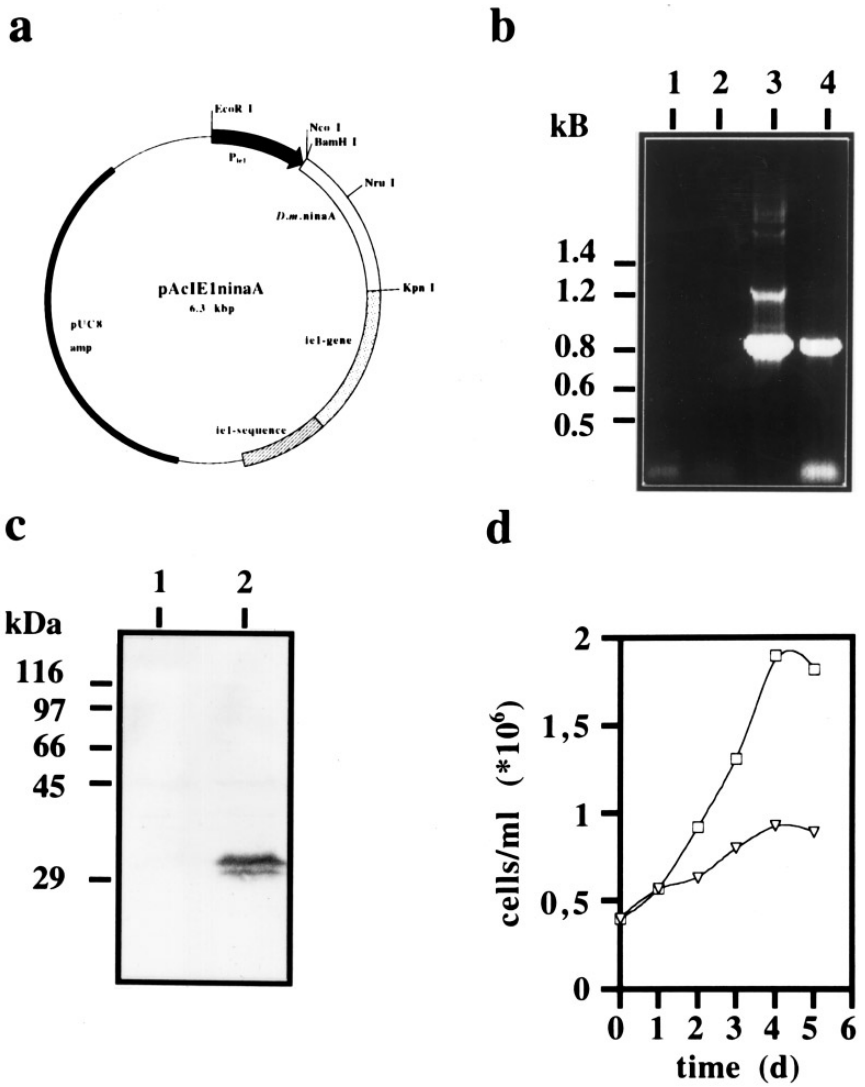

FIG. 1. Construction and analysis of the Sfn cell line. (a) Plasmid pAcIE1ninaA used for establishment of a stable transformed Sf9 insect cell line. The *D. melanogaster nina*A-gene (D.m. ninaA) was cloned under the control of the ie1-promotor (P_{ie1}). amp, ampicillin resistance gene. (b) PCR analysis of the stably transformed Sfn cell line. Genomic DNA from the insect cells was isolated as already described (9). A PCR (1min 94°C, 1min 52°C, 1.5 min 72°C; 30 cycles) with the primers ninaAfor and ninaArev which match to the DNA sequences up- and downstream the *nina*A gene was performed. Lane **1,** Sf9 cells; lane **2,** Sf9 cells infected with BaculoGold virus; lane **3,** plasmid pAcIE1ninaA; lane **4,** Sfn clone. (c) Immunoblot analysis of the Sfn cell line with NinaA-specific antibody. Cells were harvested and 5×10^4 cells were loaded per lane on a 10% SDS polyacrylamide gel. After electroblotting onto a poly(vinylidene difluoride) membrane it was probed with the ninaA specific antiserum. The proteins were visualized as described. Lane **1,** control, Sf9 cells; lane **2,** Sfn cells. (d) Growth curve of the Sfn cell line in comparison to Sf9 cells. Sfn and Sf9 cells were grown in suspension and cell number was determined at the indicated time points. ▽, Sfn cells; □, Sf9 cells.

sect cells with this baculovirus resulted in a time dependent production of the transporter as was revealed by immunoblot analysis with the polyclonal antibody NET 213/230 (Fig. 2c). In both cell lines the glycosylated transporter exhibited an apparent molecular mass of 56 kDa. As can be seen in figure 2b, the Sfn cells showed a slightly more efficient glycosylation of the transporter. Nevertheless, as judged from the Western blots as well as from the results of the immunoprecipitations (Fig. 2b), the production levels for the recombinant transporter were in the same range for both cell lines. Contrary to the immunological analysis, functional analysis of the transporter performed by uptake of [^{3}H]dopamine two days after infection of the cells revealed that the Sfn cells exhibited $\geqslant$ 5 times more functional protein even $\approx$20 fold increase in uptake compared to Sf9 cells. Non-infected as well as the two cell lines infected with the BaculoGold virus did not show specific uptake of

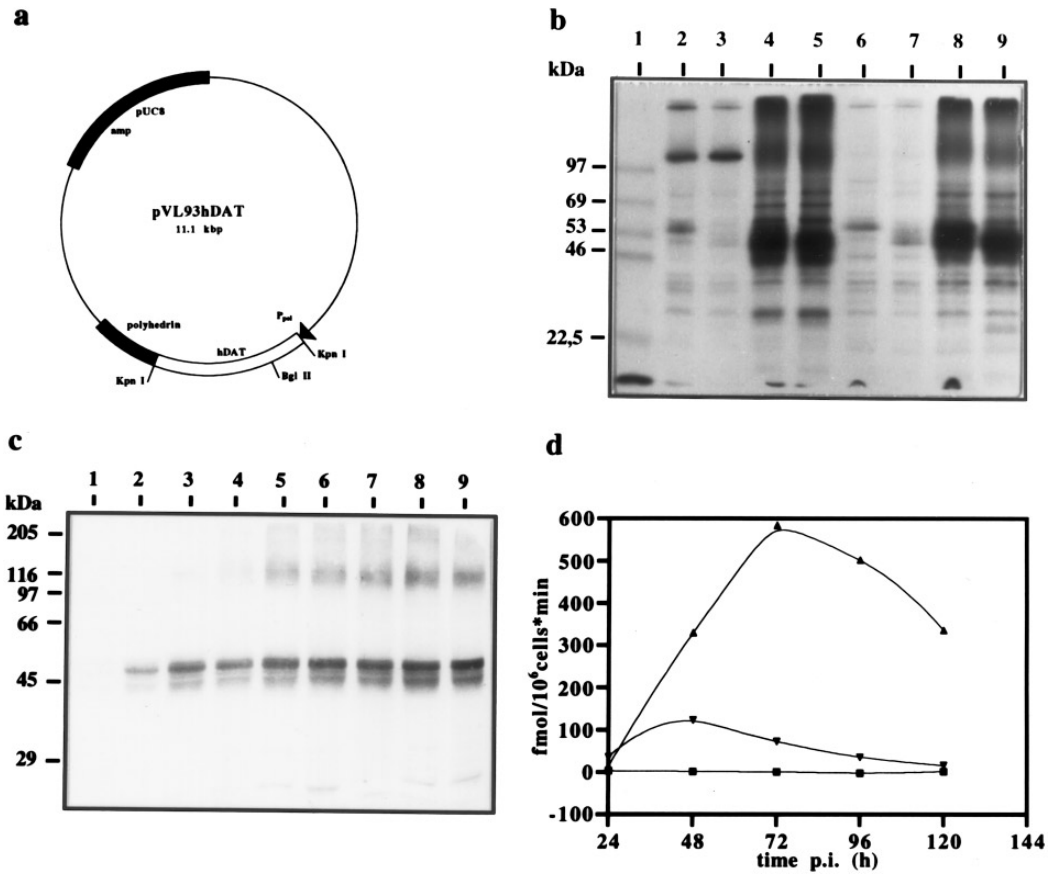

FIG. 2. Production of the human dopamine transporter in Sf9 and Sfn insect cells after infection with a recombinant baculovirus. (a) Plasmid pVL93hDAT was used to generate the recombinant baculovirus VL93hDAT. In the plasmid the cDNA encoding the human dopamine transporter is under the transcriptional control of the polyhedrin-promotor (P_{Pol}). (b) Immunoprecipitation analysis of the hDAT. Sf9 and Sfn cells were infected with a moi of 10 with VL93hDAT virus. After 48 h, infected cells were metabolically labeled and subsequently solubilized in RIPA-buffer. The solubilized proteins were incubated with the transporter specific NET213/230-antiserum and protein was precipitated and analyzed by SDS-PAGE. An autoradiography of the gel is presented. Lane **1,** [^{14}C]-labeled protein marker (DuPont NEN); lane **2,** Sf9 cells infected with BaculoGold virus; lane **3,** tunicamycin treatment of Sf9 cells infected with BaculoGold virus; lane **4,** Sf9 cells infected with baculovirus pVL93hDAT; lane **5,** tunicamycin treatment of Sf9 cells infected with baculovirus pVL93hDAT; lane **6,** Sfn cells infected with BaculoGold virus; lane **7,** tunicamycin treatment of Sfn cells infected with BaculoGold virus; lane **8,** Sfn cells infected with baculovirus pVL93hDAT; lane **9,** tunicamycin treatment of Sfn cells infected with baculovirus pVL93hDAT. (c) Time-dependent expression of hDAT in Sf9 insect cells. Sf9 cells were infected with recombinant baculovirus pVL93hDAT, and every 12 hours after infection, beginning with 24 h p.i., cells were harvested and subjected to immunoblot analysis using the NET213/230-antiserum. Lane 1 represents the immunoreactive band 24 h after infection, lane 9 the band after 120 h. (d) Time course of production of recombinant transporter in Sf9 and Sfn cells as measured by uptake of [^{3}H]dopamine. Sf9 and Sfn cells were infected with pVL93hDAT at a moi of 10 in a 250 ml spinner flask. BaculoGold infected Sf9 cells were measured as control. At the time points indicated, cells were harvested by centrifugation and uptake of dopamine was measured as described under Experimental Procedures.

dopamine (Fig. 2d). Subsequent determination of the K_M in both cell lines revealed that the difference in the uptake of dopamine was not due to a dramatically altered K_M (Fig. 3) but that the V_{max} had changed significantly. Also, two other stably transformed cell lines which scored positive during the subsequent screening of the G418-resistant cell clones revealed enhanced dopamine transport after infection with the DAT-virus. Here, one cell clone showed a slightly lower, the other cell clone a similar increase in uptake of dopamine in comparison to the Sfn cell (data not shown). In agreement with the uptake measurements described above, binding studies with the specific dopamine transporter radioligand WIN 35.428 performed on membranes prepared from infected Sf9 and Sfn cells revealed that in Sfn cells $\geqslant$ 5 times more binding sites occured whereas the K_D (Sf9: $K_D \approx 56.6$ nM; Sfn $K_D \approx 49.5$) was not changed significantly. Also here, non-infected as well as the two mock-infected cell lines showed no specific WIN 35.428 binding sites. From these results, we concluded that in the Sfn cells more functional transporter was produced and targeted to the plasma membrane.

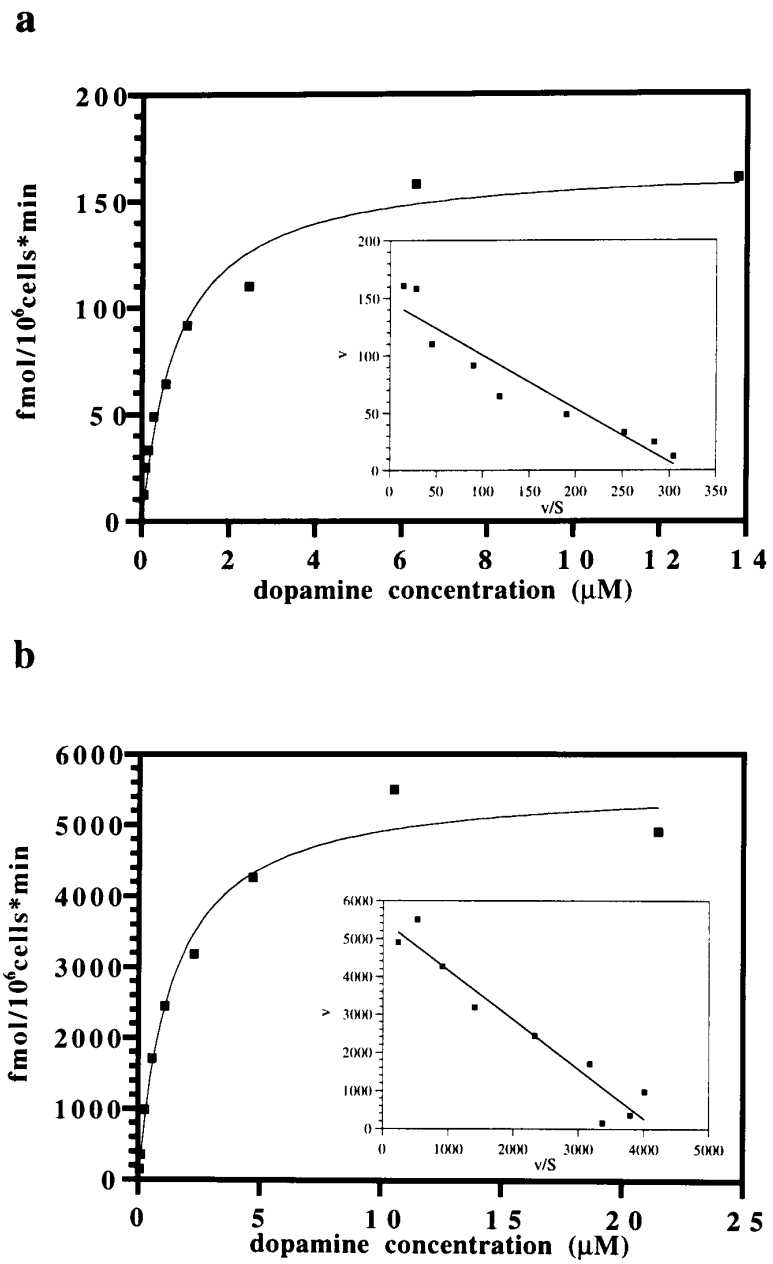

FIG. 3. Kinetics of dopamine uptake in infected Sf9 and Sfn cells. Sf9 (**a**) and Sfn (**b**) cells were grown in suspension in 250 ml spinner flasks and infected with pVL93hDAT virus with a moi of 10. 48 hours after infection cells were harvested and uptake was assayed in the presence of the indicated concentration of [^{3}H]dopamine for 10 min. Eadie Hofstee plots of the data are presented in the insets.

Influence of tunicamycin and cyclosporin A on the transporter activity in infected insect cells. Tunicamycin which inhibits N-linked glycosylation was used to determine the effect of glycosylation on the apparent molecular mass and the function of the hDAT in the Sf9 and Sfn cell lines. Treatment of the two insect cell lines infected with the recombinant baculovirus reduced the uptake of dopamine to about 30-40% of that detected in both non-treated cell lines (Fig. 4). From the immunoprecipitation analysis one can conclude that tunicamycin did not reduce transporter synthesis (Fig. 2b). However, treatment with tunicamycin resulted in a reduction of the molecular mass of the recombinant protein from ≈56 kDa to ≈50 kDa. This shift of about 6 kDa is in agreement with the postulated presence of 3 potential N-glycosylation sites detected in the amino acid sequence. From this we concluded, that glycosylation of the hDAT influenced, directly or

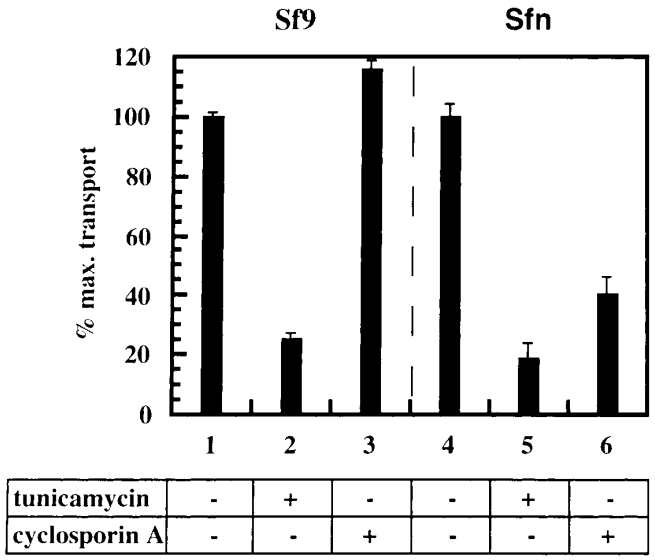

FIG. 4. Effect of tunicamycin and cyclosporin A on the function of recombinant transporter. Cells were seeded in petri dishes and infected with baculovirus pVLhDAT with a moi of 10. The cell culture medium was supplemented with either 10 μg/ml tunicamycin or 20 μM cyclosporin A. Uptake assay was performed as described. Maximal transport rate in each cell lines was normalized to 100%. Column **1,** uptake into infected, non-treated Sf9 cells; column **2,** uptake into infected Sf9 cells after tunicamycin treatment; column **3,** uptake into Sf9 cells after incubation with cyclosporin A; column **4,** uptake into infected, non-treated Sfn cells; column **5,** uptake into infected Sfn cells after tunicamycin treatment; column **6,** uptake into Sfn cells after incubation with cyclosporin A.

indirectly, stabilization, folding and/or targeting of the transporter to the plasma membrane. Nevertheless, glycosylation did not change the functional properties of the transporter. The K_M of the transporter after tunicamycin treatment was not severely affected which is in agreement with results obtained from BHK-21 cells heterologously producing this transporter (37). Similar effects of tunicamycin treatment have been described for the GLYT1 glycine transporter (38), the SERT (39, 40) and the NET (41). Also here, tunicamycin treatment as well as mutations of the potential glycosylation sites led to a progressive decrease in the transport activities and the apparent molecular masses of these proteins. As an other example, the functional production of the GAT in L-cells and HeLa-cells was abolished after deglycosylation (42). In most of the cases it has been shown that the kinetics of transport of the unglycosylated protein was comparable to that of the glycosylated protein. Therefore, referring to glycosylation, Sfn cells behaved like Sf9 cells. Despite the increased uptake found in the Sfn cell line the reduction seen on a percent level is in principal the same as in Sf9 cells. Nevertheless, the Sfn cells behaved different when treated with cyclosporin A. Cyclosporin A has been shown to inhibit the peptidyl-prolyl *cis/trans* isomerase activity of certain cyclophilins (43-46). The cyclophilin NinaA which is constitutively produced in the Sfn cell line is a peptidyl-prolyl *cis/trans* isomerases implicated in catalyzing protein folding of Rh1 in the *Drosophila* compound eye (22, 23, 47). This led us to the question whether the drug cyclosporin A would exert an inhibitory effect on the production of the transporter in the infected Sfn insect cell line. To address this question, we compared the effects of cyclosporin A treatment on the production of functional dopamine transporter in infected Sf9 and Sfn cells. The results presented in figure 4 revealed that dopamine uptake in Sfn cells was reduced to about 40% of that of non-treated cells. Contrary to the tunicamycin treatment which effected both cell lines, cyclosporine A exhibited no inhibitory effect in the infected Sf9 cells. From this we concluded that the increased uptake level in the infected Sfn cells in comparison to infected Sf9 cells was due to the activity of the NinaA. In Sfn cells more correctly targeted transporter is present which as revealed by radioligand binding is due to more correctly folded transporter. Therefore, the NinaA influences, directly or indirectly, the folding process of the nascent transporter in the engineered cell line. The hDAT features 24 proline residues in its amino acid sequence, from which six are located in the second extracellular loop. In a direct way, the NinaA might interact with the transporter catalyzing *cis/trans* isomerization of one or more of the *Xaa-Pro* connections. This kind of isomerization has been shown to be the rate-limiting step in the *in vitro* folding of a number of proteins and might play a essential role *in vivo* also (48-52). Nevertheless, it has been shown that the NinaA only complexes with the Rh1 but not with the other rhodopsins (Rh2-Rh7) in the eye of the fly. This specificity of action would exclude a direct interaction of the NinaA with the transporter. Therefore, other indirect actions of the NinaA have to be taken into account.

In conclusion, the Sfn cell line described in this paper might be a valuable tool for the production of foreign proteins via the baculovirus expression system.

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REFERENCES

1. Luckow, L. V., and Summers, M. D. (1988) *Bio/Technology* **6,** 47–55.
2. Miller, L. K. (1996) *in* Fields Virology (Fields, B. N., Knipe, D. M., Howley, P. M., *et al.*, Eds.), 3rd ed., pp. 533–556, Lippincott-Raven, Philadelphia.

3. O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) Baculovirus Expression Vectors: A Laboratory Manual, Freeman, New York.
4. Bishop, D. H. L. (1994) *in* Membrane Protein Expression Systems. A Users' Guide (Gould, G. W., Ed.), pp. 83–124, Portland Press, London.
5. Fraser, M. J. (1992) *Curr. Top. Microbiol. Immunol.* **158,** 131–172.
6. Grisshammer, R., and Tate, C. G. (1995) *Quart. Rev. Biophys.* **28,** 315–422.
7. Jarvis, D. L., Fleming, J. A., Kovacs, G. R., Summers, M. D., and Guarino, L. A. (1990) *Bio/Technology* **8,** 950–955.
8. Joyce, K. A., Atkinson, A. E., Bermudez, I., Beadle, D. J., and King, L. A. (1993) *FEBS Lett.* **335,** 61–64.
9. Kleymann, G., Boege, F., Hahn, M., Hampe, W., Vasudevan, S., and Reiländer, H. (1993) *Eur. J. Biochem.* **213,** 797–804.
10. Lenhard, T., Maul, G., Haase, W., and Reiländer, H. (1996) *Gene* **169,** 187–190.
11. Gimpl, G., Klein, U., Reiländer, H., and Fahrenholz, F. (1995) *Biochemistry* **34,** 13794–13801.
12. Grünewald, S., Haase, W., Reiländer, H., and Michel, H. (1996) *Biochemistry* **35,** 15149–15161.
13. Tsu, T. A., Eiden, J. J., and Betenbaugh, M. J. (1994) *Ann. NY Acad. Sci.* **721,** 208–217.
14. Tsu, T. A., Watson, S., Eiden, J. J., and Betenbaugh, M. J. (1996) *Protein Expression Purif.* **7,** 281–288.
15. Jarvis, D. L., and Finn, E. E. (1996) *Nature Biotechnol.* **14,** 1288–1292.
16. Gething, M.-J., and Sambrook, J. (1992) *Nature* **355,** 33–45.
17. Bergeron, J. J. M., Brenner, M. B., Thomas, D. Y., and Williams, D. B. (1994) *Trends Biochem. Sci.* **19,** 124–128.
18. Hartl, F.-U., Hlodan, R., and Langer, T. (1994) *Trends Biochem. Sci.* **19,** 20–25.
19. Fischer, G., and Schmid, F. (1990) *Biochemistry* **29,** 2205–2212.
20. Stamnes, M. A., Shieh, B.-H., Chuman, L., Harris, G. L., and Zuker, C. S. (1991) *Cell* **65,** 219–227.
21. Shieh, B.-H., Stamnes, M. A., Seavello, S., Garris, G. L., and Zuker, C. S. (1989) *Nature* **338,** 67–70.
22. Schneuwly, S., Shortridge, R. D., Larrivee, D. C., Ono, T., Ozaki, M., and Pak, W. L. (1989) *Proc. Natl. Acad. Sci.* **86,** 5390–5394.
23. Ondek, B., Hardy, R. W., Baker, E. K., Stamnes, M. A., Shieh, B.-H., and Zuker, C. S. (1992) *J. Biol. Chem.* 267, 16460–16466.
24. Stamnes, M. A., Rutherford, S. L., and Zuker, C. (1992) *Trends in Cell. Biol.* **2,** 272–276.
25. Colley, N. J., Baker, E. K., Stamnes, M. A., and Zuker, C. S. (1991) *Cell* **67,** 255–263.
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., CSH Press, NY.
27. Ausuble, F. M., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience, New York.
28. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci.* **74,** 5463–5467.
29. Summers, M. D., and Smith, G. E. (1987) A Manual of Methods for Baculovirus Vectors and Culture Procedures, Texas Agricultural Experiment Station, College Station, TX.
30. Dougherty, R. M. (1964) Animal Virus Titration Techniques (Harris, R., Ed.), New York.
31. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
32. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
33. Towbin, H., Staehlin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci.* **76,** 4350–4354.
34. Brüss, M., Hammermann, R., Brimijoin, S., and Bönisch, H. (1995) *J. Biol. Chem.* **270,** 9197–9201.
35. Huh, N. E., and Weaver, R. F. (1990) *J. Gen. Virol.* **71,** 195–201.
36. Giros, B., Mestikawy, S. E., Godinot, N., Zheng, K., Han, H., Yang-Feng, T., and Caron, M. G. (1992) *Mol. Pharmacol.* **42,** 383–390.
37. Lenhard, T., Marheineke, K., Lingen, B., Haase, W., Hammermann, R., Michel, H., and Reiländer, H. (1997) *Cell. Mol. Neurobiol.*, in press.
38. Olivares, L., Aragón, C., Giménez, C., and Zafra, F. (1995) *J. Biol. Mol.* **270,** 9437–9442.
39. Tate, C. G., and Blakely, R. D. (1994) *J. Biol. Chem.* **269,** 26303–26310.
40. Sur, C., Betz, H., and Schloss, P. (1996) *Neuroscience* **73,** 217–231.
41. Melikian, H. E., McDonald, J. K., Gu, H., Rudnick, G., Kimberly, R. M., and Blakely, R. D. (1994) *J. Biol. Mol.* **269,** 12290–12297.
42. Keynan, S., Suh, Y.-J., Kanner, B. I., and Rudnick, G. (1992) *Biochemistry* **31,** 1974–1979.
43. Handschuhmacher, R. E., Harding, M. W., Rice, J., and Drugge, R. J. (1984) *Science* **226,** 544–547.
44. Harding, M. W., Handschuhmacher, R. E., and Speicher, D. W. (1996) *J. Biol. Chem.* **261,** 8547–8555.
45. Schreiber, S. L. (1991) *Science* **251,** 283–287.
46. Takahashi, N., Hayano, T., and Suzuki, M. (1989) *Nature* **337,** 473–475.
47. Baker, E. K., Colley, N. J., and Zuker, C. S. (1994) *EMBO J.* **13,** 4886–4895.
48. Lin, L.-N., and Brandts, J. F. (1983) *Biochemistry* **22,** 559–563.
49. Lang, K., Schmidt, F. X., and Fischer, G. (1987) *Nature* **329,** 268–270.
50. Bächinger, H. P. (1987) *J. Biol. Chem.* **262,** 17144–17148.
51. Davis, J. M., Boswell, B. A., and Bächinger, H. P. (1989) *J. Biol. Chem.* **264,** 8956–8962.
52. Kiefhaber, T., Quaas, R., Hahn, U., and Schmid, F. X. (1990) *Biochemistry* **29,** 3053–3061.