Glycoprotein production for structure analysis with stable, glycosylation mutant CHO cell lines established by fluorescence-activated cell sorting

Sonja Wilke,¹ Joern Krausze,¹ Manfred Gossen,^{2,3} Lothar Groebe,⁴
Volker Jäger,¹ Ermanno Gherardi,⁵ Joop van den Heuvel,¹ and Konrad Büssow^{1*}

¹Division of Structural Biology, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany

²Max Delbrück Center for Molecular Medicine (MDC), 13125 Berlin, Germany

³Berlin-Brandenburg Centre for Regenerative Therapies (BCRT), 13353 Berlin, Germany

⁴Department of Experimental Immunology, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany

⁵MRC Centre and Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom

Received 26 January 2010; Accepted 16 March 2010

DOI: 10.1002/pro.390

Published online 29 March 2010 proteinscience.org

Abstract: Stable mammalian cell lines are excellent tools for the expression of secreted and membrane glycoproteins. However, structural analysis of these molecules is generally hampered by the complexity of N-linked carbohydrate side chains. Cell lines with mutations are available that result in shorter and more homogenous carbohydrate chains. Here, we use preparative fluorescence-activated cell sorting (FACS) and site-specific gene excision to establish high-yield glycoprotein expression for structural studies with stable clones derived from the well-established Lec3.2.8.1 glycosylation mutant of the Chinese hamster ovary (CHO) cell line. We exemplify the strategy by describing novel clones expressing single-chain hepatocyte growth factor/scatter factor (HGF/SF, a secreted glycoprotein) and a domain of lysosome-associated membrane protein 3 (LAMP3d). In both cases, stable GFP-expressing cell lines were established by transfection with a genetic construct including a GFP marker and two rounds of cell sorting after 1 and 2 weeks. The GFP marker was subsequently removed by heterologous expression of Flp recombinase. Production of HGF/SF and LAMP3d was stable over several months. 1.2 mg HGF/SF and 0.9 mg LAMP3d were purified per litre of culture, respectively. Homogenous glycoprotein preparations were amenable to enzymatic deglycosylation under native conditions. Purified and deglycosylated LAMP3d protein was readily crystallized. The combination of FACS and gene excision described here constitutes a robust and fast procedure for maximizing the yield of glycoproteins for structural analysis from glycosylation mutant cell lines.

Keywords: glycoprotein; crystallization; CHO; Chinese hamster ovary cells; LAMP-3; DC-LAMP; CD208; HGF; hepatocyte growth factor; scatter factor

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Helmholtz Association of German Research Centres (Protein Sample Production Facility).

*Correspondence to: Konrad Büssow, Helmholtz Centre for Infection Research, Inhoffenstr. 7, 38124 Braunschweig, Germany.
E-mail: konrad.buessow@helmholtz-hzi.de

Introduction

The crystallization of glycoproteins is challenging, because glycan moieties are flexible, often heterogeneous and generally do not contribute to crystal contacts. Large glycans may mask possible crystal contacts on the protein surface. Some glycosylation sites can be removed by mutagenesis. However, many proteins require glycosylation for folding and transport through the secretory pathway.

Mutant CHO cell lines that synthesize glycoproteins with truncated carbohydrates have enabled the crystallization of many glycoproteins. The CHO Lec3.2.8.1 cell line does not process N-linked glycans beyond the high-mannose type.¹ Its protein products are, therefore, more homogenous and more likely to crystallize.² Moreover, high-mannose N-linked glycans can be truncated efficiently to a single *N*-acetylglucosamine (GlcNAc) by endoglycosidases.

To circumvent establishing stable cell lines, transient transfection of HEK293 cells is used to produce glycoproteins for crystallization.^{3,4} Complex glycosylation of HEK293-produced proteins is prevented by addition of glycosylation inhibitors such as kifunensine to the growth medium. In addition, a mutant HEK293 cell line has been established lacking *N*-acetylglucosaminyl-transferase I (GnTI) activity.⁵ This defect leads to the production of proteins with homogenous N-linked high-mannose sugars.

Good stable cell lines, although difficult to obtain, have the advantage that protein production can be scaled up easily and repeated indefinitely. Industrial production of therapeutic antibodies and cytokines relies on stable cell lines and considerable optimization has been invested into this system. Usually, stable cell lines are established by transfection with plasmid vectors carrying the gene of interest and a selection marker. Large numbers of the resulting antibiotic resistant cells have to be screened to identify the rare clones that provide high-product yield and genetic stability. Removing antibiotic selection pressure during cell propagation frequently leads to mosaic gene silencing and diminishing protein yields.⁶

Preparative FACS represents an advantageous alternative to antibiotic selection in stable cell line development.⁷ Cell sorting can isolate events of transgene integration into the rare favorable genomic loci where high-level expression is possible and silencing does not occur.^{8,9} The cell sorting approach requires a fluorescent readout that is correlated to the expression of the recombinant product of interest. Internal ribosomal entry sites (IRES) can be used to couple the expression of the gene of interest to a marker that can be readily detected by FACS, such as GFP, or a cell surface antigen.^{10,11} Alternatively, the secreted product itself can be captured on the cell surface by a polymer matrix or low temperature.¹²

A two-step procedure was described by Kaufman *et al.* that combines stable GFP cell line develop-

Figure 1. Establishment of production cell lines. Flow chart of the cell line development strategy that combines cell sorting and site-specific recombination. First, the parental cell line was transfected with a vector containing an EF1 α promoter, a GFP reporter gene flanked by two homospecific FRT F3 sites and a LAMP3d or scHGF gene (gene of interest, GOI). Cells with high and stable GFP expression were selected and cloned by two rounds of cell sorting. By transfection with a Flp expression vector, the GFP gene was excised by recombination of the FRT F3 sites and the transgene expression switched from GFP to the gene of interest. Thus, the new production cell line is established. Cones = FRT F3 sites, P = EF1 α promoter.

ment by cell sorting with site-specific recombination to remove the GFP marker (Fig. 1).¹³ The transgene contains a promoter for transcription of a GFP marker, flanked by recombination target sites. Excision of the GFP marker activates a gene of interest located downstream. This approach is target independent and spares the cells from producing GFP along with the protein of interest.

Here, we established a mammalian protein expression system specifically useful for protein crystallization projects according to the approach of Kaufman *et al.* We show the generation of CHO Lec3.2.8.1 production cell lines for two glycoproteins: a luminal domain of lysosome-associated membrane protein 3 (LAMP3d) and an engineered single chain (sc) variant of hepatocyte growth factor/scatter factor (HGF/SF).

HGF/SF is secreted as a biologically inactive single chain (sc) precursor with four glycosylation sites and is proteolytically cleaved into the mature, biologically active two chain form.¹⁴ Stable CHO Lec production cell lines for wild type HGF/SF and the ligand binding domain of its receptor Met have already been established.¹⁵ We describe here a new CHO Lec production cell line for a noncleavable HGF/SF mutant (scHGF).

LAMP-3 is specifically expressed in lymphoid organs and dendritic cells.¹⁶ The proximal half of the LAMP-3 luminal domain (LAMP3d) has three glycosylation sites and is homologous to the other members of the LAMP family, LAMP-1 and LAMP-2,

Figure 2. Preparative cell sorting. Transfected CHO Lec3.2.8.1 cells were selected by preparative FACS to isolate master cells. All GFP positive cells were selected 7 days post transfection (4% and 50% for scHGF and LAMP3d, respectively). Another week later, the top 3% and 7% fluorescent cells were selected with thresholds 1000 and 1778 arbitrary units for scHGF and LAMP3d, respectively.

which are involved in lysosomal biogenesis, lysosomal fusion of phagosomes and autophagy.^{17,18}

Both proteins were produced and purified in milligrams per liter of culture medium with the new cell lines, followed by enzymatic deglycosylation and crystallization of LAMP3d.

Results

Establishment of GFP master cell lines

CHO Lec3.2.8.1 production cell lines were established in two steps: stable clonal GFP “master” cell lines were generated, followed by excision of the GFP reporter gene by the site-specific recombinase Flp.¹⁹ The latter step placed LAMP3d and scHGF under control of the vector’s promoter.

The pEF_{F3}EGFP_{F3}mcs vector contains a human elongation factor 1 α (EF1 α) promoter and an enhanced GFP reporter gene, flanked by the synthetic variant F3 of the Flp recombination target (FRT) site (Supporting Information Fig. S1).¹³ LAMP3d and scHGF were cloned downstream of GFP (Fig. 1). CHO Lec3.2.8.1 cells were transfected with the resulting vectors pEF_{F3}EGFP_{F3}scHGF and pEF_{F3}EGFP_{F3}LAMP3d. GFP positive master cell clones that integrated the transgene into favorable chromosomal loci were selected by two rounds of preparative FACS (Fig. 2). In the first sorting step, 7 days post transfection, all GFP positive cells were isolated. In the second step, 2 weeks after transfection, those cells expressing GFP most strongly were sorted and cloned. Individual GFP master cell clones (46 and 34) were obtained for LAMP3d and scHGF, respectively. Their GFP expression was monitored over 12 weeks by flow cytometry. After this time, 39% and 35% of the master cell clones for LAMP3d

and scHGF, respectively, were stable and showed homogenous and persistent GFP fluorescence.

Transfection with the LAMP3d vector led to a higher proportion (50%) of GFP positive cells in comparison to scHGF (4.4%) in the first FACS step. This difference may be due to variations in the efficiency of the transfection method or the larger scHGF insert size. Consequently, a higher number of LAMP3d cells than scHGF cells was available for the second round of sorting, and a higher fluorescence threshold was used for sorting (Fig. 2).

GFP excision and characterization of scHGF and LAMP3d cell lines

The GFP reporter of the master cell lines was deleted for expression of the genes of interest. We transfected three stable GFP master cell lines for scHGF with an optimized Flp recombinase expression plasmid.²⁰ The recombinase catalyzed a double-reciprocal crossover of the FRT sites resulting in reporter gene excision (Fig. 1). Thereby, the scHGF transgene was transcriptionally activated. Five days post transfection, 16%, 18%, and 36%, respectively, of the three transfected master cell lines were detected as GFP negative by flow cytometry [Fig. 3(A)]. Nonfluorescent cell clones were isolated for each of the three master cell lines—one or two subclones per master cell line. The reporter gene excision was confirmed on the genomic level by PCR [Fig. 3(B)]. scHGF expression was detected in all tested cell lines by Western blot analysis. Subclones derived from the same master cell line expressed

Figure 3. Flp-mediated reporter gene excision. A: Analytical FACS of a homogenous and stable GFP master cell clone (first plot). The cell line was transiently transfected with a Flp expression vector for GFP excision. Five days later, GFP excision lead to a second, nonfluorescent cell population (second plot). Subcloning resulted in GFP-negative clonal cell lines expressing scHGF (third plot). B: Confirmation of GFP excision by PCR. The chromosomally integrated gene cassette was amplified before (lane 1) and after (lane 2) GFP excision in a scHGF master cell line.

Figure 4. scHGF yield and genetic stability. HGF/SF product titres and specific productivities of stably transfected CHO Lec3.2.8.1 cell lines were quantified by ELISA. The productivity of a stable scHGF cell line established by cell sorting and GFP excision (Fig. 3) was compared with a conventionally established cell line for wild type (wt) HGF/SF. scHGF production was followed over 22 passages. pcd = pg cell⁻¹ day⁻¹.

equal amounts of scHGF, due to their isogenicity (data not shown).

We cultivated the most productive scHGF cell clone SWI4_25a in a spinner flask. Productivity and stability were analyzed by ELISA. An average amount of 2.3 mg/L scHGF was produced in four days, and the mean specific productivity was ~1 pg per cell per day (pcd). A production cell line for wild type human HGF/SF that had been established by conventional antibiotic selection produced only half of that amount under the same conditions (Fig. 4). scHGF production was stable, as product yield and specific productivity did not change significantly over 22 passages (Fig. 4).

The excision of the GFP reporter gene in stable LAMP3d master cell lines was performed as for scHGF. Recloning resulted in homogeneously non-fluorescent cell lines derived from two distinct master cell lines. Western blotting showed that three cell clones derived from one of the LAMP3d master clones produced equal amounts of protein, and considerably more than a cell clone derived from the second master cell line.

scHGF protein production and deglycosylation

scHGF (3 mg) was produced from the cell supernatant of a 2.5-L batch cultivation of clone SWI4_25a by heparin and ion exchange chromatography [Fig. 5(A)]. scHGF was mainly obtained as a single protein chain due to the mutations at the protease cleavage site. Small amounts of the cleavage products α - and β -chain were detected by SDS-PAGE and confirmed by mass spectrometry (data not shown), despite these mutations.

To increase the chances of crystallization, we deglycosylated scHGF with endoglycosidase H (endo

H). Masses of the glycosylated and deglycosylated protein, measured by MALDI-TOF mass spectrometry [Fig. 5(B)], corresponded to the calculated masses of threefold glycosylated scHGF before and after deglycosylation (84,129 and 81,090, respectively). Additional ESI-TOF mass spectrometry indicated that position N402 was unglycosylated, whereas the others (N294, N566, and N653) bore GlcNAc₂Man₅ chains.

LAMP3d protein production, deglycosylation, and crystallization

Conditioned medium (20 L) was produced with the most productive LAMP3d cell line by continuous perfusion with a 2.5 L bioreactor. Purified LAMP3d (17 mg) was obtained upon diafiltration, nickel chromatography, and gel filtration [Fig. 6(A)]. Mass spectrometric peptide mapping of the three LAMP3d SDS-PAGE bands of 20–25 kDa [Fig. 6(A)] after in-gel tryptic digestion identified GlcNAc₂Man₄₋₅ at one (predominantly at site N291), two (predominantly at sites N266 and N291) or all of the three predicted N-glycosylation sites. LAMP3d was deglycosylated with endo H [Fig. 6(A)]. Needle-like crystals were obtained under several conditions of a sparse matrix

Figure 5. scHGF purification and deglycosylation. A: scHGF was produced by a 2.5-L batch cultivation and purified with a heparin column (lane 1), followed by ion exchange chromatography (lane 2). Proteins were analyzed by 12% SDS-PAGE and Coomassie staining. B: Confirmation of enzymatic deglycosylation of scHGF by MALDI mass spectrometry. Molecular weights of 84,352 and 81,083 Da were measured for glycosylated and deglycosylated scHGF, respectively. m/z = ion mass/ion charge, $M^{+/2+}$ = molecular ions with one/two positive charges.

Figure 6. Purification, deglycosylation and crystallization of LAMP3d. A: LAMP3d was produced in a bioreactor. The conditioned medium was concentrated 10-fold and exchanged to phosphate buffer by diafiltration. LAMP3d was purified by IMAC and gel filtration. The purified protein (lane 1) was deglycosylated by treatment with endo H_f (lane 2), followed by removal of endo H_f by gel filtration (lane 3). Proteins were analyzed by 12% SDS-PAGE and Coomassie staining. B: Protein crystal of LAMP3d. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

crystallization screen. Crystals were grown under an optimized condition in space group P3 and diffracted to a resolution of up to 2.5 Å [Fig. 6(B)]. A dataset was collected for one crystal (Table I). Additional phasing experiments will be required for structure determination.

Discussion

Establishing highly productive and stable cell lines for structural studies by standard transfection and antibiotic selection procedures requires isolation and characterization of hundreds or thousands of cell clones. We have successfully implemented an alternative strategy to clone stable cell lines derived from the glycosylation mutant CHO Lec3.2.8.1 line. The process took about 4 months from the first transfection to the expression of the protein of interest and did not require the isolation and characterization of larger numbers of cell clones. Thus, time and effort was reduced considerably compared to conventional approaches.

Master cell lines that had a stable reporter expression over 12 weeks gave rise to scHGF and LAMP3d production cell lines that were equally stable. The best scHGF cell line produced twice as much HGF/SF as a wild type HGF/SF cell line that

had been established with antibiotic selection and that had required a considerable effort of screening for good producers. The productivity of the LAMP3d cell line allowed the generation of a sufficient amount of protein for a successful crystallization project. Thus, our approach is able to establish cell lines with higher productivity and avoids the problem of genetic instability.

Heterogeneous protein glycosylation can impede the formation of well-diffracting crystals. LAMP3d and scHGF, expressed by CHO Lec3.2.8.1 cells, were susceptible to deglycosylation by endo H, leading to homogenous protein preparations and successful crystallization of LAMP3d.

Our data show that cell sorting and marker gene excision represents a reliable and efficient method to establish useful glycosylation mutant producer cell lines, directly suitable for large scale production. The method requires a cell sorter, which is available in many research centers as a central service facility.

The production of glycoproteins still represents a bottleneck for the structural analysis of this important class of proteins. The method presented here allows researchers to obtain recombinant glycoproteins with homogenous glycosylation with less time and effort.

Materials and Methods

Plasmid construction

Two mutations (K491D and R494E) were introduced into the coding region of human HGF/SF (Swissprot P14210) that prevents the cleavage of the precursor. The resulting cDNA fragment and a C-terminal His₆-tag sequence were cloned by PCR between the BamHI and NotI sites of the vector pEF_{F3}EGFP_{F3}mcs¹³ (GenBank GU983383, Supporting Information Fig. S1) resulting in the plasmid pEF_{F3}EGFP_{F3}scHGF.

The human LAMP-3 sequence was analyzed with PipeAlign and MACSIMS^{21,22} to identify conserved domains. A sequence encoding a mouse immunoglobulin signal peptide (Swissprot P01750), amino acids 222–381 (VKTG...SDYT) of LAMP-3 (GenBank AAH32940), and a His₆-tag was cloned between the

Table 1. LAMP3d Data Collection Statistics

Beamline	DESY X12
Space group	P3
Unit cell dimensions	
<i>a</i> (Å)	52.2
<i>c</i> (Å)	141
Wavelength (Å)	1.771
Resolution (Å)	45.2–2.5
Mosaicity (°)	0.451
Unique reflections	26,524
Completeness (%)	88.3
Redundance	5.7
<i>I</i> / σ <i>I</i>	11.19
Twinning fraction	0.056

BamHI and NotI sites of pEF_{F3}EGFP_{F3}mcs, resulting in the plasmid pEF_{F3}EGFP_{F3}LAMP3.

Cell culture and transfection

CHO Lec3.2.8.1 were propagated in suspension cultures of ProCho5 medium (Lonza, Cologne, Germany) in spinner flasks at 37°C, 5% CO₂ and 80 rpm. Cell numbers and viability were assayed by trypan blue dye exclusion method.

Plasmid DNA for transfection was purified with the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) and linearized by digestion with SalI followed by purification with the Plasmid Extract II Kit (Macherey-Nagel, Düren, Germany). For stable transfection, 1.5×10^6 cells in 2 mL medium were transfected with linearized plasmid DNA by using program U-24 of the Amaxa nucleofection device according to manufacturer's guidelines (Lonza, Cologne, Germany). Twenty-four hours post transfection, the medium was exchanged and the cells were seeded into six-well plates at 37°C in a humidified atmosphere with 5% CO₂ at 150 rpm on an Incutec K15-500 linear shaker. In the following days, the transfected cell cultures were expanded before entering the stationary phase.

Flow cytometry and preparative FACS

CHO Lec3.2.8.1 cells were transported and sorted at room temperature, not on ice. GFP expression was analyzed with a Guava EasyCyte™ Mini System (Guava Technologies, Hayward, CA). Cells were stained with 50 µg/mL propidium iodide to exclude dead cells from the analysis. Preparative FACS was performed on a MoFlo high-speed cell sorter (Beckman Coulter, Krefeld, Germany). The sorter was equipped with an argon-ion laser tuned to 488 nm with 100 mW of power and an automated cell deposition unit for sorting into 96-well plates. GFP fluorescence was detected in FL1 through a 530/40-nm bandpass filter. Data analysis was performed using CytoSoft™ 4.2 and WinMDI 2.9 software.

Excision of the GFP marker gene

Cell lines were transfected with 5 µg of the optimized Flp expression vector pPGKFLPobpA (Addgene plasmid 13793)²⁰ by Amaxa nucleofection. Five days later, the cells were analyzed by flow cytometry and cloned by serial dilution in 96-well plates with CD-Hybridoma medium (Invitrogen, Paisley, UK) containing 5% FCS. After 1 week, the wells were screened for nonfluorescent single colonies on an Axiovert100 fluorescent microscope (Carl Zeiss, Göttingen, Germany) using an LP520 filter for GFP visualization. The detected colonies were expanded and recloned, if necessary. Clonal cell lines were adapted to suspension cultures in serum free ProCho5 medium (Lonza, Cologne, Germany) by adding 10 U/mL

heparin (Sigma, Steinheim, Germany) during the first 2 passages.

Genomic PCR

Genomic DNA was isolated with an AquaGenomic™ kit (MoBiTec, Göttingen, Germany). The absence of the GFP coding sequence following Flp-recombination was confirmed by PCR using two primers flanking the chromosomally integrated gene cassette.

ELISA

HGF/SF product titers were quantified with the human HGF DuoSet® ELISA Development System (R&D Systems, Abingdon, UK). Cells were seeded in 40 mL at 1.5×10^5 cells/mL in spinner flasks. HGF/SF was measured twice, 1 and 4 days after inoculation. Mean values were obtained from three parallel cultures. After counting the cell number, the specific productivity q_p was calculated with the assumption of exponential growth by the equation:

$$p(t) = p(0) + \frac{q_p x_0 (e^{\mu t} - 1)}{\mu}$$

with p = product concentration at time t as inoculation, (µg/mL); x_0 = inoculum cell concentration, (cells/mL); μ = specific growth rate, (h⁻¹).²³

Western blot

Cell culture supernatants containing recombinant proteins were analyzed by 12% SDS-PAGE and Western blotting with a goat anti-HGF antibody (dilution 1:1000, R&D Systems, Abingdon, UK) or an anti-His antibody (dilution 1:1000, Novagen, Darmstadt, Germany) for LAMP3d.

Large-scale cultivation of production cell lines

LAMP3d cells were grown in batch and subsequent perfusion mode in autoclavable stirred tank bioreactors with 2.5-L culture volume. The bioreactors were equipped with a double membrane stirrer for both bubble-free aeration²⁴ via 8 m of hydrophobic polypropylene membrane tubing (Accurel® S6/2, Membrana, Wuppertal, Germany) and perfusion with internal cell retention via 8 m of hydrophilized, microporous membrane tubing of the same type.²⁵ Cells were propagated at 37°C, a stirring speed of 45 rpm, pH 7.4 and a dissolved oxygen (DO) concentration of 40% air saturation. During the production phase, at a cell density exceeding 10⁷ mL⁻¹, temperature was reduced to 32°C. The perfusion rate was adjusted to the metabolic consumption of glucose avoiding a drop below 2.5 g L⁻¹. Samples (2 mL) were taken daily for routine in-process control including quantification of LAMP3d. Harvested cell-free supernatant was concentrated by ultrafiltration followed by diafiltration against PBS8 (50 mM Na₂HPO₄, 0.3M NaCl, pH 8.0) using a Pellicon

2 tangential flow system equipped with two 10-kDa cut-off cartridges (Millipore, Billerica MA). scHGF was produced by a similar process in a 2.5-L culture volume, but without perfusion or diafiltration.

Purification, deglycosylation, and crystallization of LAMP3d

The human LAMP3d was purified by immobilized metal ion affinity chromatography (IMAC) on a 50 mL Ni-NTA superflow (Qiagen) column using PBS8 and an imidazole gradient for elution. The protein was further purified by gel filtration on a 320 mL Superdex 75 pg XK 26/60 column (GE Lifesciences) in GF buffer (10 mM HEPES, pH 7.4, 150 mM NaCl). LAMP3d was deglycosylated at 1 mg/mL over night at 37°C by adding sodium acetate to 100 mM and endoglycosidase H_f to 10,000 U/mL (Endo H_f , NEB). Endo H_f is a fusion protein of endo H and maltose binding protein. Endo H_f and contaminants were removed by gel filtration in GF buffer on a 16/60 size exclusion column of Superdex 75 (GE Healthcare). The protein was concentrated to 22 mg/mL with a 10,000 MWCO Vivaspin membrane concentrator (Sartorius Stedim Biotech, Göttingen, Germany).

Crystallization screens were set up in 96-well format with a mosquito nanolitre pipetting machine (TTP LabTech, Melbourn, UK) and various screening suites (Qiagen). Upon optimization, single crystals were obtained in droplets composed of 1 μ L 22 mg/mL protein and 1 μ L of reservoir buffer (0.1M citric acid pH 5.0, 5% PEG 6000).

Crystals were transferred into reservoir supplemented with 30% PEG 6000 and immediately flash frozen in liquid nitrogen. Diffraction data were acquired by an X-ray home source (Rikagu, Seve-noak, UK) and at beamline X12 at the EMBL Out-station (Hamburg, Germany).

Purification and deglycosylation of scHGF

scHGF was purified from cell supernatant by hepa-rin and ion exchange chromatography. Cell superna-tant (2.5 L) were directly loaded on a 25 mL heparin sepharose column. The column was washed with 200 mM Tris-HCl, pH 8.0, and 250 mM NaCl. scHGF was eluted with a linear gradient of 250–1000 mM NaCl. The pooled scHGF-containing fractions were diluted with 200 mM Tris-HCl, pH 8.0 to 250 mM NaCl. The protein was immediately loaded on a 7.9 mL Mono S cation exchange column and, upon washing, was eluted with a linear gradient of 250–1000 mM NaCl. Purified scHGF was deglycosylated in 0.1M sodium acetate by 30 U Endo H_f (NEB) per μ g protein over night at 37°C.

Mass spectrometry

LAMP3d protein bands were excised from a Coomas-ie-stained SDS-PAGE gel, tryptically digested and desalted (Ziptip) using standard protocols. The

(glyco-)peptides then were subjected to MALDI/TOF (Bruker, Ultraflex) and ESI (Micromass QTOF2) mass spectrometric analyses. The identity of relevant (glyco-)peptides was verified by MS/MS analyses.

To prepare desalted samples of scHGF, 50 μ L Ni-NTA superflow beads (Qiagen) were added to samples of His₆-tag scHGF. scHGF was bound by shaking for 1 h and unbound material was removed upon centrifugation. Beads were washed thrice in 5 mM Tris-HCl, pH 8.0 and scHGF was eluted thrice with 100- μ L aliquots of 35% acetonitrile, 0.1% TFA and shaking for 10 min. Eluates were subjected to MALDI-TOF-MS analysis.

Acknowledgments

The authors are greatly indebted to Prof. Pamela Stan-ley for the CHO Lec3.2.8.1 cells, which made this study possible. The authors thank Sarah-Maria Tokarski for excellent technical assistance, Nadine Konisch for operation of bioprocessors, and Uwe Wengler and Jens de Groot for help with protein purification and crystal-lization. They also thank Dr. Manfred Weiss, Dr. Björn Klink, and Dr. Joachim Reichelt for supporting diffrac-tion data collection as well as Dr. Manfred Nimtz for mass spectrometric measurements.

References

1. Stanley P (1989) Chinese hamster ovary cell mutants with multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity. *Mol Cell Biol* 9:377–383.
2. Davis SJ, Puklavec MJ, Ashford DA, Harlos K, Jones EY, Stuart DI, Williams AF (1993) Expression of solu-ble recombinant glycoproteins with predefined glycosy-lation: application to the crystallization of the T-cell glycoprotein CD2. *Protein Eng* 6:229–232.
3. Chang VT, Crispin M, Aricescu AR, Harvey DJ, Nettle-ship JE, Fennelly JA, Yu C, Boles KS, Evans EJ, Stuart DI, Dwek RA, Jones EY, Owens RJ, Davis SJ. (2007) Glycoprotein structural genomics: solving the glycosylation problem. *Structure* 15:267–273.
4. Aricescu AR, Lu W, Jones EY (2006) A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta Crystallogr* 62:1243–1250.
5. Reeves PJ, Callewaert N, Contreras R, Khorana HG (2002) Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogene-ous *N*-glycosylation by a tetracycline-inducible *N*-ace-tylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. *Proc Natl Acad Sci USA* 99: 13419–13424.
6. Liu W, Xiong Y, Gossen M (2006) Stability and homoge-neity of transgene expression in isogenic cells. *J Mol Med* 84:57–64.
7. Mattanovich D, Borth N (2006) Applications of cell sorting in biotechnology. *Microb Cell Fact* 5:12.
8. Bestor TH (2000) Gene silencing as a threat to the suc-cess of gene therapy. *J Clin Invest* 105:409–411.
9. Kito M, Itami S, Fukano Y, Yamana K, Shibui T (2002) Construction of engineered CHO strains for high-level production of recombinant proteins. *Appl Microbiol Bio-technol* 60:442–448.

10. Gaines P, Wojchowski DM (1999) pIRES-CD4t, a dicistronic expression vector for MACS- or FACS-based selection of transfected cells. *Biotechniques* 26:683–688.
11. Mancia F, Patel SD, Rajala MW, Scherer PE, Nemes A, Schieren I, Hendrickson WA, Shapiro L (2004) Optimization of protein production in mammalian cells with a coexpressed fluorescent marker. *Structure* 12:1355–1360.
12. Pichler J, Hesse F, Wieser M, Kunert R, Galosy SS, Mott JE, Borth N (2009) A study on the temperature dependency and time course of the cold capture antibody secretion assay. *J Biotechnol* 141:80–83.
13. Kaufman WL, Kocman I, Agrawal V, Rahn HP, Besser D, Gossen M (2008) Homogeneity and persistence of transgene expression by omitting antibiotic selection in cell line isolation. *Nucleic Acids Res* 36:e111.
14. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF (2003) Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* 4:915–925.
15. Gherardi E, Sandin S, Petoukhov MV, Finch J, Youles ME, Ofverstedt LG, Miguel RN, Blundell TL, Vande Woude GF, Skoglund U, Svergun DI. (2006) Structural basis of hepatocyte growth factor/scatter factor and MET signalling. *Proc Natl Acad Sci USA* 103:4046–4051.
16. de Saint-Vis B, Vincent J, Vandenabeele S, Vanbervliet B, Pin JJ, Ait-Yahia S, Patel S, Mattei MG, Banchereau J, Zurawski S, Davoust J, Caux C, Lebecque S. (1998) A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment. *Immunity* 9:325–336.
17. Eskelinen EL, Saftig P (2009) Autophagy: a lysosomal degradation pathway with a central role in health and disease. *Biochim Biophys Acta* 1793:664–673.
18. Huynh KK, Eskelinen EL, Scott CC, Malevanets A, Saftig P, Grinstein S (2007) LAMP proteins are required for fusion of lysosomes with phagosomes. *EMBO J* 26:313–324.
19. Andrews BJ, Proteau GA, Beatty LG, Sadowski PD (1985) The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences. *Cell* 40:795–803.
20. Raymond CS, Soriano P (2007) High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells. *PLoS ONE* 2:e162.
21. Plewniak F, Bianchetti L, Brelivet Y, Carles A, Chalmel F, Lecompte O, Mochel T, Moulinier L, Muller A, Muller J, Prigent V, Ripp R, Thierry JC, Thompson JD, Wicker N, Poch O. (2003) PipeAlign: a new toolkit for protein family analysis. *Nucleic Acids Res* 31:3829–3832.
22. Thompson JD, Muller A, Waterhouse A, Procter J, Barton GJ, Plewniak F, Poch O (2006) MACSIMS: multiple alignment of complete sequences information management system. *BMC Bioinformatics* 7:318.
23. Pirt SJ (1975) Principles of microbe and cell cultivation. Oxford: Blackwell Scientific Publications Ltd.
24. Lehmann J, Piehl GW, Schulz R (1987) Bubble free cell culture aeration with porous moving membranes. In: Karger S, Ed. *Develop Biol Standard* 66:227–240.
25. Blasey HD, Jäger V (1990) Strategies to increase the efficiency of membrane aerated and perfused animal cell bioreactors by an improved medium perfusion. In: Sasaki R, Ikura K, Eds. *Animal cell culture and production of biologicals*. Dordrecht, The Netherlands: Kluwer Acad. Pub., pp 61–73.