High-yield production of functionally active human serum transferrin using a baculovirus expression system, and its structural characterization

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Recently, there has been much interest in expressing recombinant human serum transferrin (HST) and mutants thereof for structural and functional studies. We have developed a baculovirus expression system for the rapid and efficient production of large quantities of HST (> 20 mg/l). Like native HST, the recombinant protein can bind two ferric ions in the presence of bicarbonate, and is actively taken up by receptor-mediated endocytosis. Secondary structure calculations from CD measurements indicate a content of 42 % α-helix and 28 % β-sheet. This is the first reported use of a non-mammalian expression system to produce functional HST, and will provide a practical tool to allow expression of a wide range of HST variants for mutagenesis studies.

INTRODUCTION

Human serum transferrin (HST) belongs to the transferrin family of metal-binding proteins that transport iron and provide bacteriostatic functions in a wide variety of physiological fluids in vertebrates (for reviews, see [1–4]). It is a single-chain glycoprotein of 679 amino acids containing two asparagine-linked glycan chains, with a glycosylation-dependent molecular mass in the range 76–81 kDa [5,6]. X-ray crystallography studies indicate that it is organized into two similar lobes linked by a short flexible spacer peptide, each of which contains a cleft that will bind a metal anion together with a synergistic co-ion [7–10].

Despite its discovery more than 50 years ago, the precise mechanisms of HST function remain to be completely elucidated. Central to further work is the ability to generate mutant transferrin molecules. Site-specific and domain replacement mutants will allow further characterization of iron binding and release both *in vitro* and *in vivo*. Over the past few years, much attention has been focused upon expressing unglycosylated and single-lobed half-molecules for structure/folding analyses by X-ray crystallography, NMR spectroscopy and CD [11–17].

Woodworth and co-workers [11–13] have described a baby hamster kidney (BHK) cell expression system for producing HST, its N-terminal domain and glycosylation-defective mutants. While high levels of expression were obtained (up to 125 μg/ml for the wild type), this method has limited applicability to mutagenesis studies because gene amplification in methotrexate is required; this is labour-intensive and requires several months' work before large-scale protein expression can begin.

Bacterial expression systems have been reported [14–16]. Their primary advantages over the BHK mammalian expression systems are that selection of expression clones is rapid and that large cultures can be rapidly and inexpensively produced. However, *Escherichia coli*-expressed HST is biologically inactive, largely due to incorrect intramolecular disulphide bond formation. Recently, *Pichia pastoris* was used successfully to express the N-terminal domain of HST [17]. Yeasts can form intramolecular disulphide bridges, and possess many of the advantages that simple expression systems have over mammalian ones. However, a non-mammalian expression system for the functional full-length HST molecule has yet to be described.

The baculovirus expression vector system (BEVS) is frequently a method of choice for the expression of recombinant mammalian proteins (see [18]). Apart from the simplicity and cost-effectiveness of this method, the insect host cells possess many of the protein-processing and -folding mechanisms of mammalian cells; therefore functional and antigenic differences are rarely seen. In this paper we report a BEVS for the efficient production of biologically active HST.

EXPERIMENTAL

Construction of the transfer vector

The HST gene was amplified from the plasmid TFR27A (obtained from A.T.C.C.; catalogue no. 53106) by PCR using a proof-reading DNA polymerase (Vent®; New England Biolabs G.m.b.H., Schwalbach, Germany). The two oligonucleotide primers used for this reaction were 5′-CTAGTCTAGAGCGCA-CCCGGAAGATGAGGCTC-3′ (upstream) and 5′-AATCCC-CCCGGGTTAAGGTCTACGGAAAGTGCAGGC-3′ (downstream). The resulting 2132 bp fragment comprised the entire coding region for HST, including its secretion signal leader sequence, plus a termination codon and flanking artificial *Xba*I and *Xma*I enzyme restriction sites for subcloning. This DNA fragment was subcloned into the baculovirus transfer plasmid pBacPAK8 (Clontech Laboratories Inc, Palo Alto, CA, U.S.A.) via the *Xba*I and *Xma*I sites, and its DNA sequence was checked using a commercial sequencing kit (Sequenase version 2; Amersham). This plasmid contains the powerful polyhedrin promoter to drive expression of the HST gene, a polyadenylation site and sequences to allow homologous recombination with a baculoviral vector.

Cell culture and production of recombinant virus

Recombinant baculovirus was generated using the BacPAK baculovirus expression system (Clontech), according to the manufacturers' instructions, using the High Five host insect cell

Abbreviations used: HST, human serum transferrin; BEVS, baculovirus expression vector system; NTA, nitrilotriacetate.

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line (BTI TN 5B1-4; Invitrogen Corp, San Diego, CA, U.S.A.). Essentially, the transfer plasmid was co-transfected with viral DNA into the host cells using lipofection. The viral DNA is a linearized replication-deficient recombinant baculovirus DNA (BacPAK6) derived from *Autographia californica* nuclear polyhedrosis virus. Recombinant virus was isolated, expanded in suspension cultures and titrated using standard methods [18]. High Five cell monolayer cultures were grown at 27 °C in SF900 II serum-free medium (Life Technologies G.m.b.H., Berlin, Germany), supplemented with 1 mM glutamine, 100 units/ml streptomycin and 100 μg/ml penicillin. Suspension cultures included an additional supplement of 10 units/ml heparin (sodium salt; grade 1-A from porcine intestinal mucosa; Sigma-Aldrich Handels G.m.b.H., Vienna, Austria). These cultures were shaken in Erlenmeyer flasks at 80 rev./min at a density of $(0.5–2.0) \times 10^6$ cells/ml.

Large-scale protein expression

High Five cells grown in suspension culture were pelleted by centrifugation at 400 ***g*** for 5 min in a preparative centrifuge, and then resuspended in viral supernatant at a density of 5×10^6 cells/ml at a multiplicity of infection of 1. The viral supernatant was prepared by diluting the viral stock in medium and then adding fresh heparin, glutamine and antibiotics to give the concentrations indicated above. The infection was allowed to proceed for 1 h at 27 °C in an Erlenmeyer flask with shaking at 80 rev./min. The cells were then pelleted as above, resuspended in fresh medium at 1×10^6 cells/ml and then shaken for a further 48 h at 27 °C and 80 rev./min. Cells were removed from the HST-containing supernatants by centrifugation as described.

Purification

Recombinant HST was purified from the expression culture supernatants using hydrophobic interaction chromatography with a Phenyl-Sepharose® column (High Load®; 26/10; Pharmacia, Uppsala, Sweden), essentially as described in [19]. Briefly, an equal volume of 2.4 M ammonium sulphate/0.8 M trisodium citrate (pH 6) was mixed with the supernatant and the precipitate was removed by centrifugation. The supernatant was then loaded on to the column at 10 ml/min, washed with 1.2 M ammonium sulphate/0.4 M citrate (buffer A) and the protein eluted in a gradient of 0–50 % water in buffer A. The HST fractions (identified using SDS/PAGE analysis) were collected and dialysed against 20 mM Tris/HCl, pH 8 (buffer B), for 2 h. As a final 'polishing' step, the protein was chromatographed on a Q-Sepharose® Fast Flow ion-exchange column (50 ml bed volume; Pharmacia). Briefly, after loading, unbound material was washed from the column with buffer B, and the pure HST was eluted in a gradient of 0–100 % 1 M KCl in buffer B. HST-containing fractions were pooled and dialysed against PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.09 mM Na_2HPO_4, pH 7.2).

SDS/PAGE analysis

Electrophoretic separation of proteins by SDS/PAGE was carried out in the presence of β-mercaptoethanol on 10 % polyacrylamide gels [20]. Protein bands were visualized using Coomassie Brilliant Blue stain [21]. Quantities of HST in samples were estimated by comparing their relative staining intensities with those of mass standards prepared using native HST (Boehringer Mannheim).

Sequence determination

Determination of the N-terminal sequence of recombinant HST was carried out using an Applied Biosystems 470A automatic protein sequencer, according to manufacturer's instructions.

CD measurements

CD measurements were performed with a JASCO J710 instrument at 5 °C. Far-UV CD (190–260 nm) spectra of the iron-saturated proteins (prepared as described below) were recorded at a protein concentration of 5.78 μM (as determined from the protein absorbance at 280 nm, using a molar absorption coefficient of 1.4 for a 1 mg/ml solution [22]), in 0.01 cm cells. The spectra, which were averaged over 10 accumulations, were corrected for the PBS baseline. Mean residue ellipticity values ($[\theta]_{\mathrm{m.r.w.}}$) were calculated using the expression:

$$[\theta]_{\mathrm{m.r.w.}}\ (\mathrm{degrees \cdot cm^2 \cdot dmol^{-1}}) = 100\ \theta_{\mathrm{obs}}/lc$$

where θ_{obs} is the observed ellipticity in degrees, c is the molar residue concentration and l is the light path in cm [23]. Calculation of the percentages of secondary structure was performed using the program SELCON [24].

Iron-binding assays

To determine the number of ferric ions bound by the recombinant HST, the method of Ali et al. [25] was used. Briefly, apo-HST was incubated with increasing concentrations of ferric nitrilotriacetate (ferric-NTA) in the presence of bicarbonate to generate partially and fully iron-saturated isofoms. These isoforms were then separated by multi-zone electrophoresis using polyacrylamide gels containing 6 M urea, 0.1 M Tris, 0.01 M boric acid and 0.05 M EDTA, with an electrode buffer of 0.88 M β-alanine and 0.25 M Tris, pH 8.8, and the proteins were visualized by silver staining [26].

Cell uptake assay

Preparation of holo- and apo-HST

After purification, the recombinant HST was iron-free. For iron saturation, 500 μg of the purified protein (0.5 mg/ml) was first mixed with 0.5 ml of 90 mM $NaHCO_3$ in 1 M Tris/HCl, pH 8.5. To this was added 110 μl of ferric-NTA, prepared by dissolving $FeCl_2$ (anhydrous salt; Sigma) in 123 mM disodium NTA (Sigma) to a final concentration of 41 mM. The reaction mixture was held on ice for 30 min, and then dialysed extensively against PBS.

Native HST (Boehringer Mannhein) was supplied in a partially iron-saturated form. Complete saturation was achieved using the conditions described above. To generate the iron-free isoform, the protein (10 mg/ml) was combined with an equal volume of 100 mM sodium acetate, 1 mM EDTA, 1 mM NTA, pH 4, for 30 min on ice, and then dialysed extensively against PBS.

Incubation of cells with HST

CEM cells (A.T.C.C. no. CCL119) were cultured in RPMI 1640 medium supplemented with 2 g/l $NaHCO_3$, 10 % (v/v) fetal calf serum (BioWhittaker U.K. Ltd.) and glutamine, penicillin and streptomycin at the concentrations indicated above, in a humidified atmosphere at 37 °C. At 2 h prior to the study, cells were serum-starved in RPMI-Mops medium (RPMI 1640 containing 165 mM Mops, adjusted to pH 7 with NaOH, 0.1 % BSA and glutamine plus antibiotics as indicated above). For the assay, 5×10^6 cells were resuspended in 0.5 ml of RPMI-Mops containing 300 nM recombinant or native HST in either the apo- or

holo- form and incubated in a 37 °C water-bath for 30 min. Cells were washed three times in ice-cold PBS to remove free HST and then resuspended in 200 μl of PBS. Cells were analysed for HST uptake by immunofluorescence as described below.

Immunofluorescence analysis

An aliquot of 20 μl of washed cell suspension (5×10^5 cells) was dropped on to an adhesion field of an adhesion slide (Bio-Rad) pre-chilled on ice. These slides provide a convenient means of covalently binding cells to slides without the requirement of precoating or using a Cytospin. After 5 min, when all the cells had settled on to the slide, excess fluid was washed off with ice-cold PBS and the cells were fixed in 3 % paraformaldehyde in PBS for 30 min at 4 °C. Slides were then washed in PBS/10 mM glycine for 5 min. For cells to be permeabilized, the slides were immersed in permeabilization solution (0.1 % Triton in PBS) for 5 min at 22 °C, and then in methanol at −20 °C for 3 min. Subsequent steps were performed at room temperature unless indicated. All slides were washed again in PBS/10 mM glycine for 5 min, and then in PBS/25 mM glycine for 30 min. Blocking was performed overnight at 4 °C in 1 % BSA in PBS, followed by a further three washes in PBS/10 mM glycine (5 min each). Antibodies for detection were prepared in 1 % BSA/0.5 M NaCl in PBS, with the addition of 0.05 % Tween 20 for permeabilized cells. The first antibody was a rabbit polyclonal anti-HST antibody (BioGenix Laboratories, San Ramon, CA, U.S.A.). The second was a rhodamine (TRITC)-conjugated goat anti-rabbit antibody (Accurate Chemical and Scientific Company, Westbury, NY, U.S.A.). The antibodies were applied as a single drop on to the adhesion fields and incubated for 30 min at room temperature, then removed by washing five times in PBS/10 mM glycine. Cells were mounted in Bacto FA Mounting Fluid (Difco Laboratories, Detroit, MI, U.S.A.) and overlayed with a microscope slide. Fluoresence was visualized using a fluoresence microscope (Axiovert 10; Zeiss G.m.b.H., Jena, Germany).

RESULTS AND DISCUSSION

Expression and purification

We have generated a recombinant baculovirus for the production of HST by High Five insect cells. The virus was made by homologous recombination between baculovirus DNA and a transfer vector containing the HST gene. The HST transcription unit codes for all 679 amino acids of the mature protein, plus the 19-amino-acid signal sequence for secretion.

Recombinant virus was isolated and titrated by end-point dilution, as described previously [18]. Expansion of virus in suspension cultures generated large volumes of high-titre stocks ($> 10^7$ infectious units/ml). These viral supernatants were then used for large-scale protein production.

For large-scale protein expression, we carried out the infection of cells at very high density (5×10^6 cells/ml) for 1 h at a multiplicity of infection of 1 in the presence of heparin, followed by removal of the infectious supernatant and replacement with fresh heparin-containing medium. The rationale for this method is several-fold. First, replacement of the infectious supernatant with fresh medium prevents the expression culture from being contaminated with HST carried over from earlier virus-passage infections (such HST is partially degraded by proteases released into the medium by the insect host cells). Furthermore, addition of fresh medium post-infection may also have the advantage of boosting protein expression levels [27]. A common problem with the suspension culture of insect cells in serum-free medium is cell aggregation. The addition of heparin to the growth medium prevents this. Finally, infection at high cell densities reduces the volumes of supernatant required, therefore saving on medium costs and reducing the manual work involved in large-volume culture infection. After infection, cells are still > 95 % viable, as determined by the Trypan Blue exclusion method (see [18]). Expression cultures generated in this way produced > 20 mg/l HST, constituting the major protein in the supernatant as determined by SDS/PAGE analysis (Figure 1).

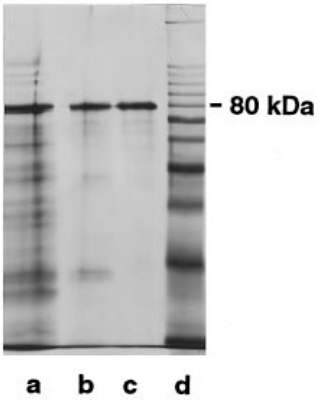

Figure 1 Purification of recombinant HST: SDS/PAGE analysis

Lane a, cell supernatant; lane b, HST fraction after chromatography on Phenyl-Sepharose; lane c, HST fraction after chromatography on a Mono-Q column; lane d, molecular mass markers. Samples containing approx. 200 ng of HST were loaded in each lane.

Purification of transferrin using hydrophobic interaction chromatography has been described as a single-step rapid purification procedure [19]. This published method details the conditions for isolating transferrin from chicken blood and egg yolk in the presence of ammonium sulphate and citrate using a Phenyl-Sepharose® column. We have applied this successfully to separate recombinant HST from the BEVS cell supernatant. The HST was eluted from the column in a gradient of decreasing salt concentration (at about 20 % buffer A; 240 mM ammonium sulphate/80 mM citrate) as a relatively pure protein (> 95 % as determined by SDS/PAGE analysis; Figure 1). Further purification of the protein was achieved using a Q-Sepharose® ion-exchanger. The entire purification process is rapid, and can be completed within 1 day. This method is an efficient alternative to established protocols that typically involve ultrafiltration, ion-exchange chromatography, concentration, gel filtration and then purification to homogeneity using a further ion-exchange step (see [2,11–13]).

Protein characterization

The cloned HST gene used to express recombinant HST contains the native HST leader sequence for secretion. N-terminal sequence analysis of the recombinant protein was used to determine whether this signal sequence was correctly cleaved from the protein. Analysis yielded the amino acid sequence VPDKTVRWCA. This matches that of native HST [5] and is identical to that of the correctly processed protein as predicted from the DNA sequence of the cloned gene used for expression. Therefore the expressed protein is correctly cleaved upon secretion from the insect cells.

In order to verify whether or not the recombinant HST has the correct structural conformation, its CD spectrum was compared with that of native HST. The two CD spectra were found to be

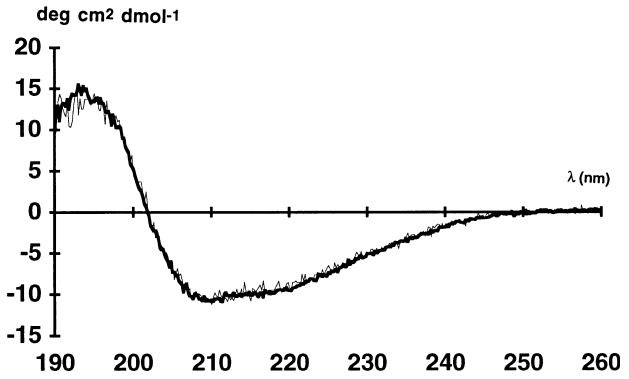

Figure 2 CD spectra of iron-saturated recombinant (thick line) and native (thin line) HST between 190 and 260 nm

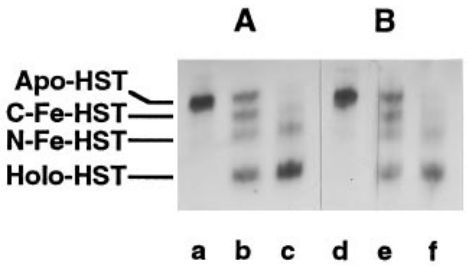

Figure 3 Urea/PAGE separation of native (A) and recombinant (B) HST isoforms generated during the iron-binding process

Partially saturated isoforms containing iron bound at either the N-terminus (N-Fe-HST) or the C-terminus (C-Fe-HST) are shown in lanes b and e. Lanes a and d, apo-HST; lanes c and f, holo-HST.

Table 1 Estimated secondary structure content of holo-HST and lactoferrin

The CD data for native HST were recorded at room temperature [29], whereas those for recombinant HST were recorded at 5 °C (the present paper). Data for human lactoferrin are from Anderson et al. [28].

		Structure (%)	
Protein	Method	α-Helix	β-Sheet
Native HST	CD	23	62
Recombinant HST	CD	41.5	28.4
Human lactoferrin	X-ray	41	24

superimposable (Figure 2), consistent with recombinant and native HSTs having the same structural conformation. In addition, the far-UV CD spectrum was used to experimentally quantify the degree of secondary structure. Analysis of the spectrum, which reflects well defined secondary structure, using the program SELCON indicated that the secondary structure is largely α-helical (42 %), with 28 % β-sheet (Table 1). Although no X-ray structure is presently available for HST, the above CD analysis agrees very well with the X-ray structure of the closely related human lactoferrin protein [28] (Table 1).

The CD data presented here are in conflict with those obtained previously by Mazurier and co-workers [29], who reported 23 % α-helix and 62 % β-sheet for HST (Table 1). It is possible that the different programs used for secondary structure determination could account for this difference. Another explanation could be that the latter work was performed at room temperature, whereas the data given here were recorded at 5 °C. It has been shown in several studies that decreasing the temperature from 25 °C to 5 °C can have a dramatic effect on protein stability and therefore on the secondary structure content as measured by CD [30].

Urea/PAGE analysis of the HST isoforms generated during the iron-binding process is shown in Figure 3. Four clearly distinguishable species are visible, representing iron-free and iron-saturated HST, and HST containing iron bound at either the N- or C-terminal lobe. Iron binding is reversible; acidification causes iron release, regenerating the apo- isoform. Given that the complexation of iron and its bicarbonate co-ion occurs in a deep cleft within each lobe, involving gross conformational changes across the whole protein (iron binding is not simply dependent upon a few surface-exposed residues) [7–10], the ability to reversibly bind iron therefore indicates that the recombinant HST does indeed possess the correct tertiary structure.

To analyse the biological activity of the recombinant HST, immunofluorescence studies were carried out to observe cellular uptake. Cells were incubated with either recombinant or native holo-HST at a 300 nM concentration (approximately one-hundredth of the normal serum concentration of HST in humans [2]). The presence of HST was visualized using fluorescently labelled antibodies. Labelling of permeabilized cells showed punctate fluorescence around the periphery of the cell, characteristic of endocytosed ligand localization (results not shown). This fluoresence appeared similarly intense for both recombinant and native proteins; i.e. the recombinant protein was taken up to the same degree as native HST. In the absence of HST, no non-specific labelling was seen.

Surface labelling of cells incubated with apo-HST showed lower fluorescence intensities than with the iron-saturated protein, for both recombinant and native material (results not shown). One of the functions of HST is to deliver iron into cells. To facilitate this process, the holo-isoform has a much higher binding affinity for the transferrin receptor than does apo-HST [31,32]. That recombinant HST, like the native protein, exhibits reduced surface binding in the apo- form is consistent with correct protein folding and tertiary structure organization necessary for biological activity.

From the data presented here, we conclude that a BEVS using suspension-cultured High Five cells provides an efficient and practical means of expressing HST that can be easily purified from a defined serum-free supernatant. The establishment of this expression technology is the first report of a non-mammalian expression system for biologically active HST. It will provide the basis for a research programme to study the effects of mutations on HST structure and function, generated using the novel PCR–ligation–PCR mutagenesis protocol described by Ali and Steinkasserer [33,34].

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