Baculovirus expression and biochemical characterization of the human microsomal triglyceride transfer protein

Penelope J. RITCHIE*, Anne DECOUT†, Joanna AMEY*, Christopher J. MANN*, Jacqueline READ*, Maryvonne ROSSENEU†, James SCOTT* and Carol C. SHOULDERS*¹

*MRC Molecular Medicine Group, Imperial College School of Medicine, Hammersmith Hospital, London W12 0NN, U.K., and †Department of Biochemistry, Laboratory for Lipoprotein Chemistry, University of Gent, Hospitaalstraat 13, 8-900 Gent, Belgium

The microsomal triglyceride transfer protein (MTP) complexed to protein disulphide isomerase (PDI) is obligatory for the assembly of chylomicrons and very-low-density lipoproteins. The determination of the atomic structure of the MTP–PDI heterodimer has important implications for the treatment of those forms of hyperlipidaemia associated with the overproduction of very-low-density lipoproteins, which predispose to premature coronary heart disease. To perform structural studies of the human MTP–PDI complex it was necessary to produce milligram quantities of pure protein. We chose the baculovirus expression system for this purpose. Insect cells were co-infected with recombinant viruses encoding FLAG-tagged MTP and His-tagged PDI; the resulting heterodimer was purified by affinity chromatography. From 5 litres of insect cells, 4–6 mg of more

than 95% pure recombinant protein was obtained. CD and attenuated total reflection Fourier-transform infrared spectroscopy indicate that the purified protein has around 34% α -helical and 33% β -structure content. The recombinant protein had a comparable triglyceride transfer activity to that of bovine MTP–PDI. The production of polyclonal antibodies raised against the MTP and PDI subunits of the purified protein is described. The present study demonstrates the feasibility of expressing two proteins at high levels in insect cells and describes a transferable methodology for the purification of the resulting protein complex.

Key words: apolipoprotein B, endoplasmic reticulum, lipoprotein, protein disulphide isomerase, protein purification.

INTRODUCTION

Chylomicrons and very-low-density lipoproteins are among the largest macromolecular complexes (50–1000 nm) secreted from eukaryotic cells. The assembly of neutral lipids and phospholipids into chylomicrons and very-low-density lipoproteins is nucleated around a single molecule of apolipoprotein B (apoB) in the endoplasmic reticulum (ER). This process has an absolute requirement for a microsomal triglyceride transfer protein (MTP) complexed to an ER-resident chaperone and disulphide-bond forming enzyme, protein disulphide isomerase (PDI) [1–3]. Defects in the apoB gene and the MTP gene cause hypobetalipoproteinaemia [4] and abetalipoproteinaemia [5–9] respectively. Affected individuals secrete no normal apoB-containing lipoproteins and have malabsorption of dietary fat and of the fat-soluble vitamins [10]. Deficiency in the antioxidant fat-soluble vitamin E leads to the development of spinocerebellar and retinal degeneration [11].

Human MTP comprises 894 amino acid residues and has primary sequence homology throughout its entire length with the N-terminal region of the ancient egg yolk protein vitellogenin [12]. At the gene level, 15 of the 17 MTP gene intron/exon boundaries are conserved with vitellogenin [12]. The relationship between MTP and lipovitellin (LV), the mature product of the vitellogenin gene, is further strengthened by a comparison of the predicted secondary structure of MTP with the recently determined secondary structure of crystalline lamprey LV [12–14]. Lamprey LV comprises three major structural domains: a

globular N-terminal β -barrel, an extended α -helical structure and a substantial C-terminal lipid-binding cavity. We have obtained and tested by extensive site-directed mutagenesis a molecular model of MTP based on the co-ordinates of lamprey LV [15]. Although our results are consistent with the overall conservation of the β -barrel and of the α -helical domain of LV in MTP, such modelling experiments cannot provide the detailed data obtained by X-ray crystallography.

PDI is an abundant ER-resident protein comprising 508 residues. The PDI associated with MTP maintains the MTP complex in a soluble state [3,16]. Moreover, because PDI alone contains the 'KDEL' ER-retention sequence it might also anchor the MTP complex in the ER, at the site of apoB translocation. PDI is proposed to form six structural domains [17]: a, a', e, b, b' and c. The a and a' domains are similar to thioredoxin, a well-characterized 12 kDa cytosolic reducing agent that is required for the catalysis of native disulphide bond formation in the cell [18–20]. The b and b' domains have similarity with one another but not with any other protein. Domain e has similarity with the steroid-binding site of oestrogen receptors, whereas domain c contains a highly acidic calcium-binding site followed by the 'KDEL' retention and retrieval sequence [21].

The structures of apoB and of the MTP–PDI heterodimer are unknown; this leaves unresolved a number of important questions relating to the nature of the molecular interactions between the MTP–PDI heterodimer and apoB during the lipoprotein assembly process [22,23]. Here we describe a rapid procedure for purifying recombinant human MTP–PDI from the

Abbreviations used: apoB, apolipoprotein B; ATR-FTIR, attenuated total reflection Fourier-transform infrared; ER, endoplasmic reticulum; LV, lipovitellin; m.o.i., multiplicity of infection; MTP, microsomal triglyceride transfer protein; PDI, protein disulphide isomerase; TG, triglyceride.

¹ To whom correspondence should be addressed (e-mail cshoulde@rmps.ac.uk).

insect baculovirus expression system. We show that the resulting protein is biochemically indistinguishable from the bovine wild-type protein, fully active and immunogenic. We also show that the level of recovery and purity of the MTP-PDI heterodimer from the baculovirus system are sufficiently high to allow us to proceed with structural studies directed towards obtaining a crystal structure of the wild-type and mutant forms of MTP in the absence and the presence of lipid.

MATERIALS AND METHODS

Materials

Baculovirus transfer vector pVL1392 was purchased from Invitrogen (Leek, The Netherlands). Human PDI cDNA and rabbit anti-(human PDI) antiserum were gifts from Professor Kari Kivirikko (Biocenter and Department of Medical Biochemistry, University of Oulu, Oulu, Finland) and Dr. Edwige Nicodeme (Glaxo Wellcome, Paris, France) respectively. The full-length human MTP cDNA and the rabbit anti-(MTP peptide) antisera, Ab/MTP/COOH, have been described [2,5]. *Pfu* DNA polymerase was purchased from Stratagene (Cambridge, U.K.). Sf900II medium, penicillin and streptomycin for the maintenance of *Spodoptera frugiperda* (Sf9) cells were obtained from Gibco-BRL (Paisley, Scotland, U.K.). Linearized BacPAK6[®] viral DNA, transfection reagents, and TALON[®] metal-affinity resin were purchased from Clontech (Basingstoke, Hants., U.K.). Sea-plaque[®] agarose was from Flowgen (Shenstone, Staffs., U.K.). Spinner flasks were from Bellco Glass (Vineland, NJ, U.S.A.). Anti-FLAG[®] M2 affinity resin, FLAG[®] peptide, Freund's adjuvant, dimethyldichlorosilane and lipids were supplied by Sigma (Poole, Dorset, U.K.). Protein molecular mass markers, horseradish peroxidase-conjugated anti-rabbit antibody and protein assay kit were from Bio-Rad (Hemel Hempstead, Herts., U.K.). Slide-a-Lyser dialysis cassettes and concentrating solutions were obtained from Pierce and Warriner (Chester, Cheshire, U.K.). Radionucleotides and chemiluminescence reagents were supplied by Amersham Life Sciences (Little Chalfont, Bucks., U.K.). The suppliers of all other specialist reagents were as described [5].

Construction of transfer vectors

PCR and the appropriate restriction sites were used to manipulate the MTP and PDI sequences. PCRs were performed with *Pfu* DNA polymerase in accordance with the manufacturer's instructions. The sequence of the oligonucleotide used to generate the FLAG-tag epitope (Asp-Tyr-Lys-Asp₄-Lys) at the 3' end of the coding region of MTP cDNA was 5'GGATGGGATCCTCACTTGTCGTCATCGTCCTTGTAGTCAAACCATCCGCTGGAAGTACT3'. The sequence of the PDI/His₆ oligonucleotide was 5'ATCGATGGATCCTCAATGGTGGTGATGGTGA-TGGGAACCGCGCATCAGTTCATCTTTCACAGCTTTC3'. The FLAG and His₆ extensions were fused in-frame to the C-termini of MTP and PDI cDNA species respectively and juxtaposed to a terminator codon. The sequences of the MTP/FLAG and PDI/His₆ cDNA species in the final transfer vectors, pVL1392-MTP/FLAG and pVL1392-PDI-HIS6, were confirmed by sequencing.

Insect cell culture and production of recombinant viruses

Stock Sf9 cells were maintained as spinner cultures at densities between 5×10^5 and 3×10^6 cells/ml in Sf900II medium containing 50 µg/ml penicillin and 50 µg/ml streptomycin. Growth was at 27 °C. Recombinant viruses encoding MTP/FLAG and

PDI/His₆ were obtained by liposomal co-transfection of Sf9 cells. Tissue culture dishes (35 mm) were seeded with 1.5×10^6 cells and transfections were with 100 ng of BSU361-linearized BakPAK viral DNA and 100 ng of pVL1392-MTP/FLAG or pVL1392-PDI-HIS6, complexed to 50 µl of transfection reagent. At 5 days after transfection the supernatants were collected and single recombinant viruses [passage 1 (P1)] isolated by one round of plaque purification. Passage 2 viral stocks expanded from P1 stocks typically gave titres in the range $(5-10) \times 10^7$ plaque-forming units/ml. P2 viral stocks were used at a multiplicity of infection (m.o.i.) of 0.1 for the generation of high-titre $[(8-20) \times 10^7$ plaque-forming units/ml] P3 viral stocks. P3 stocks were prepared in 1 litre spinner flasks containing 500 ml of Sf9 cells at a density of 5×10^5 /ml.

Small-scale expression studies

Analysis of expression was undertaken on microsomal fractions, which were maintained at 4 °C at all stages. To obtain microsomes, cells were washed in PBS, homogenized in 0.25 M sucrose containing 20 mM imidazole, pH 7.4, 430 k-i.u./ml Trasylol (Bayer, Newbury, Berks, U.K.) and 4 µg/ml PMSF, and layered on a discontinuous gradient of 1.8 M sucrose in 20 mM imidazole overlaid with 0.5 M sucrose in 20 mM imidazole. Centrifugation was at 100 000 g for 1 h at 4 °C with an SW41Ti rotor (Beckman). The pellicle of microsomes at the 0.5–1.8 M sucrose interface was resuspended in 10 vol. of 10 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.1% deoxycholate. The luminal contents were released by sonication, then clarified of membrane-associated material by centrifugation for 1 h at 100 000 g at 4 °C with an SW41Ti rotor (Beckman). Cell pellets were resuspended by probe-sonication.

Large-scale purification

The MTP-PDI heterodimer was purified from 5 litres of cells. Infections were performed at a total m.o.i. of 5 by premixing equal m.o.i.s of recombinant viruses, MTP/FLAG and PDI/His₆. Cells were infected at a density of 2×10^6 cells/ml, incubated for 1 h and diluted to a density of 10^6 cells/ml with Sf900II medium containing 50 µg/ml penicillin and 50 µg/ml streptomycin. Cells were harvested 43–67 h after infection, as soon as the cell viability decreased to less than 90%. All buffers used in the purification were sterilized and maintained at 4 °C unless stated otherwise. To minimize losses during the purification procedure, all tubes and glassware in contact with the recombinant protein were pretreated with dimethyldichlorosilane. Cells were harvested, washed twice in PBS containing 4 µg/ml PMSF, resuspended in 80 ml of TN1 buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl] containing 430 k-i.u./ml Trasylol, 4 µg/ml PMSF and 3 mM sodium azide. Cell membranes were disrupted with a Polytron homogenizer, diluted to 240 ml with TN1, probe-sonicated and further diluted to 600 ml in TN1. Soluble MTP-PDI heterodimer was isolated by ultracentrifugation at 100 000 g for 1 h at 4 °C, with an SW28 rotor (Beckman), then applied overnight to a prepared 8 ml anti-FLAG M2 affinity gel column at a rate of 40 ml/h at 4 °C. Non-specifically bound proteins were removed by washing with 50–100 ml of TN1 buffer containing 3 mM sodium azide. Protein elution was with 5 mg of FLAG peptide in 10 fractions (1 ml each) of TN1 containing 3 mM sodium azide. Fractions containing the MTP-PDI heterodimer were pooled and dialysed overnight in 2 litres of TN2 buffer [20 mM Tris/HCl (pH 8.0)/100 mM NaCl]. The dialysate was applied and reapplied four times at room temperature to a 4 ml prepared TALON metal-affinity column at a flow rate of 1 ml/min. Weakly bound proteins were removed by washing with 20 ml of TN2 buffer. Elution was with 10 ml of 200 mM imidazole in TN2

Figure 1 Immunoblot analysis of the expression of MTP/FLAG and PDI/His₆ in Sf9 cells

Sf9 cells (2.5×10^7) were infected with recombinant viruses encoding MTP/FLAG and/or PDI/His₆ at a total m.o.i. of 5. Cells were harvested 65 h after infection; microsomes were prepared as described in the Materials and methods section. Equal aliquots (10%) of total (lanes T), soluble (lanes S) and pelleted membrane-associated (lanes P) fractions were size-fractionated by SDS/PAGE and immunoblotted. The three left panels show immunoreactive products detected with rabbit anti-(human MTP peptide) antisera, Ab/MTP/COOH, diluted 1:1000. The right panel was probed with rabbit anti-(human PDI) antibody, diluted 1:500. The positions of molecular mass markers are indicated (in kDa) at the left.

buffer. Peak fractions were pooled and dialysed overnight in 2 litres of TN3 buffer [15 mM Tris/HCl (pH 7.0)/75 mM NaCl] in a 0.5–3 ml capacity Slide-a-Lyser dialysis cassette, then concentrated to 0.5 ml with the 'concentrating' reagent of Pierce and Warriner. Protein yields were estimated with a protein assay kit with BSA as standard.

Expression of MTP cDNA in Cos-1 cells

Cos-1 cells (2×10^7) were transfected with an expression construct encoding MTP/FLAG as described [2,5]. Cells were harvested 36 h after transfection and extracts were prepared as described [6].

Generation and characterization of rabbit polyclonal anti-(MTP–PDI) antibodies

New Zealand White rabbits were inoculated with 100 µg of FLAG- and His₆-purified MTP–PDI heterodimer. The immunogen was emulsified in complete Freund's adjuvant. Inoculations were repeated at 4-week intervals with a further 100 µg of the immunogen emulsified in incomplete Freund's adjuvant. Serum was screened for the presence of antibodies by immunoblotting of Cos-1 cell extracts expressing MTP and PDI.

Western blotting

Proteins were separated by SDS/PAGE and transferred to pretreated PVDF membrane by wet blotting in 10 mM Caps (pH 11)/10% (v/v) methanol. Non-specific sites were blocked with Blotto [5% (w/v) non-fat milk powder in 10 mM Tris/HCl (pH 7.4)/1 M NaCl/0.1% (v/v) Tween 20] for 1 h at room temperature. The membrane-bound proteins were incubated with primary and secondary antibodies, diluted in Blotto as specified, for 1 h each at room temperature. Washing between incubations was with 10 mM Tris/HCl (pH 7.4)/1 M NaCl/0.1% (v/v) Tween 20. Immunoreactive products were detected with a goat anti-(rabbit IgG) horseradish peroxidase-conjugated secondary antibody, followed by enhanced chemiluminescence, in accordance with the manufacturer's instructions.

CD measurements

Samples were diluted to 0.15 mg/ml in 10 mM phosphate buffer, pH 7.5. CD spectra were recorded at 25 °C with a Jasco 710 spectropolarimeter in cuvettes with a path length of 1 mm. Spectra were recorded from 185 to 260 nm at a scan speed of 50 nm/min and a time constant of 0.125 s, and represent a mean from nine accumulations. Mean residue ellipticity was calculated with a mean residue mass of 111.2 Da. The secondary structure estimates were calculated by curve-fitting of the ellipticity curve in accordance with the variable selection procedure developed by Johnson [24].

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy

ATR-FTIR spectra were collected on a Bruker FTIR spectrophotometer (Equinox 55) equipped with a liquid-nitrogen-cooled mercury cadmium telluride detector. The spectra were obtained at a nominal resolution of 4 cm⁻¹ with an average of 64 scans per spectrum. The internal reflection element was a germanium plate (50 mm × 50 mm × 2 mm) with an aperture angle of 45°, yielding 25 internal reflections. Samples were prepared by slowly evaporating 25 µg of MTP under a stream of N₂ on the germanium ATR prism. To differentiate α-helical components from random structure, the sample was exposed for 2 h to ²H₂O-saturated N₂ at room temperature. The frequency limits for the different structures were as follows: 1662–1645 cm⁻¹ for α-helix, 1689–1682 and 1632–1613 cm⁻¹ for β-sheet, 1644.5–1637 cm⁻¹ for random structure, and 1682–1662.5 cm⁻¹ for β-turns [25].

Lipid transfer activity

MTP activity was measured by a triglyceride (TG) transfer assay *in vitro* adapted from Wetterau et al. [26] and Narcisi et al. [5]. Vesicles were prepared by probe-sonication in 2.5 ml of 15:35 buffer [15 mM Tris/HCl (pH 7.4)/35 mM NaCl/0.5% BSA/3 mM sodium azide/1 mM EDTA]. Donor vesicles comprised 1.7 mg of phosphatidylcholine, 3 µg of [³H]glycerol trioleate, 235 µg of cardiolipin and 0.01% (w/v) butylated hydroxytoluene. Acceptor vesicles comprised 10.4 mg of phosphatidylcholine, 25 µg of triolein, 0.01% (w/v) butylated hydroxytoluene and 7.2 µg of [¹⁴C]dipalmitoyl phosphatidylcholine. MTP (10–100 ng) was incubated with 5 µl of each of the vesicles, in a final volume of 100 µl of 15:35 buffer, at 37 °C for the specified durations. Reactions were quenched by the addition of 400 µl of ice-cold 15:35 buffer without BSA. Donor vesicles were adsorbed on 0.5 ml of pre-equilibrated DEAE-cellulose (Whatman DE-52) maintained in an equal volume of 15:35 buffer without BSA. DEAE-bound vesicles were pelleted by centrifugation at 13 000 g for 10 min. To remove all remaining traces of DEAE, 500 µl of the supernatant was recentrifuged before liquid-scintillation counting. Lipid transfer activity is expressed as percentage of TG transfer per unit time in conditions under which it had been verified that the assay was linear with extract concentration.

RESULTS

Expression of MTP/FLAG and PDI/His₆

To facilitate the isolation of recombinant human MTP–PDI, the C-termini of human MTP and PDI were FLAG- and His₆-tagged respectively. Sf9 cells were infected with recombinant

Figure 2 Coomassie Blue-stained gel of the purification of recombinant human MTP/FLAG-PDI/His₆ heterodimer

Lane 1, 1 μ g each of five representative marker proteins; lane 2, crude soluble cell extract prepared from 0.5 ml of Sf9 cells [note that neither MTP/FLAG nor PDI/His₆ (PDI/HIS) can be readily identified]; lane 3, 0.2% of the FLAG-purified material; lane 4, 0.5% of metal-ion affinity-purified MTP-PDI; lanes 5 and 6, 0.6 and 2% respectively of the purified protein after concentration 5-fold. This gel shows the results of a single representative experiment. Similar results were obtained on at least five occasions. The positions of molecular mass markers are indicated (in kDa) at the left.

viruses encoding MTP/FLAG and PDI/His₆, then analysed for the presence of MTP and PDI (Figure 1). In cells infected with MTP/FLAG alone, the expressed protein was largely recovered in the membrane pellet. In cells co-infected with MTP/FLAG and PDI/His₆, soluble MTP was recovered (Figure 1). The fraction containing soluble MTP was tested for lipid transfer activity and was found to be active (results not shown). These results reproduce the findings of Lamberg et al. [16] and Rehberg et al. [6], reporting the expression of non-tagged MTP and PDI in insect cells. We conclude that the modifications to the C-termini of MTP and PDI did not impair the production of the MTP-PDI heterodimer in a soluble and active conformation.

Large-scale preparation of recombinant human MTP/FLAG-PDI/His₆

MTP and PDI were expressed in 5 litres of Sf9 cells; the resulting MTP-PDI heterodimer was purified to homogeneity by FLAG-immunoaffinity chromatography, followed by metal-ion-affinity chromatography as described in the Materials and methods section. Samples from each step of the purification were analysed by SDS/PAGE (Figure 2) and protein recovery was determined by assaying for lipid transfer activity (Table 1). Table 1 gives the results from a typical purification, and shows that protein was purified 120–140-fold from the soluble cell extract after both purification steps. The FLAG-immunoaffinity chromatography

step purified the MTP-PDI heterodimer to near-homogeneity (Figure 2, lane 3). Minor contaminants were removed by the metal-ion-affinity chromatography step (Figure 2, lane 4) with minimal loss of MTP-mediated lipid transfer activity, as indicated by the total activity values (Table 1). The yield of the recombinant MTP-PDI heterodimer varied from one preparation to another, but was typically between 4 and 6 mg. The final concentration of the protein to 5–8 mg/ml incurred losses of between 10% and 30% as determined by protein content.

Characterization of the recombinant human MTP/FLAG-PDI/His₆

To compare the structural stability of the recombinant human MTP-PDI heterodimer with that of the bovine protein, the molar ellipticity of the recombinant protein was determined at different guanidinium chloride concentrations. The midpoint for the denaturation transition of the recombinant protein was 2.4 M guanidinium chloride (Figure 3A), which was remarkably similar to that reported for the bovine protein [27]. The structural similarity between the recombinant human MTP-PDI protein and the bovine counterpart was further evident from far-UV CD and ATR-FTIR analyses (Figures 3B and 3C). The estimated amounts of α -helical and β -structure (Table 2) were comparable, as reported previously for bovine MTP-PDI [27].

Next we examined the TG transfer activity of the recombinant human MTP-PDI heterodimer. As a control, recombinant MTP-PDI was heat-inactivated at 100 °C for 10 min. No transfer activity was observed in the control. The specific activity of purified MTP-PDI ranged between 3% and 6% TG transfer/min per μ g. This compares with 3% TG transfer/min per μ g for the MTP-PDI heterodimer isolated from bovine liver [28]. These results suggest that the purification of recombinant MTP-PDI heterodimer from the baculovirus expression system will be an invaluable source of material for structural and functional studies.

Production of rabbit polyclonal anti-(human MTP-PDI) antibodies

To test the specificity of the antiserum raised against purified recombinant human MTP/FLAG-PDI/His₆ protein, full-length MTP was expressed in Cos-1 cells, which do not normally express MTP [2]. The antisera recognized a 97 kDa protein product that was present only in Cos-1 cells transfected with a eukaryotic expression construct encoding human MTP/FLAG (Figure 4). A ubiquitously expressed protein that had a molecular mass consistent with the size of PDI was also detected (Figure 4).

DISCUSSION

In the present study we describe a simple procedure for isolating pure recombinant human MTP-PDI in relatively large quantities

Table 1 Purification of recombinant human MTP-PDI heterodimer expressed in the baculovirus expression system

The figures refer to a 5-litre culture of Sf9 cells as the starting material. The initial cell homogenate was clarified by centrifugation and the amount of MTP-mediated triglyceride activity was determined. This figure provides the base from which the percentage yield of the preparation was calculated. After FLAG-purification, incomplete removal of FLAG-peptide at this stage of the preparation might have contributed to the low specific activity of the TG transfer protein.

Step	Volume (ml)	Total protein (mg)	Specific activity (% TG transfer/min per μ g)	Total activity (% TG transfer/min)	Enrichment (fold)	Yield (%)
Soluble cell extract	500	1300	0.05	61 250	1	—
After FLAG-purification	15	15	3.5	43 750	70	71
After His ₆ -purification	3	6	6.7	40 200	134	66

Figure 3 Secondary-structure analysis of recombinant human MTP-PDI heterodimer

(A) The relationship between secondary structural stability of recombinant MTP-PDI and guanidinium chloride concentration. The molar ellipticity at 222 nm of 0.15 mg/ml MTP, diluted in 10 mM phosphate buffer, pH 7.5, was measured at 25 °C in increasing concentrations of guanidinium chloride. (B) ATR-FTIR absorption spectrum of MTP in the amide I region. Experimental results are shown by the solid line; the curve fit spectra (broken line) were obtained after Fourier self-deconvolution of the experimental spectrum. The final positions of the amide I components (dotted lines), after least-squares curve fitting, were 1621, 1627, 1634.5, 1641.5, 1650, 1658.5, 1666, 1673.5 and 1681 cm^{-1} . (C) CD spectrum of recombinant MTP-PDI. The molar ellipticity at 222 nm was measured at 25 °C and plotted as a function of wavelength. Protein concentrations were 0.15 mg/ml in 10 mM phosphate buffer, pH 7.5.

from the baculovirus expression system. This was made possible by the co-infection of insect cells with two different viruses and the subsequent purification of the resulting heterodimer by two

Table 2 Estimate of the secondary structure content of human MTP/FLAG-PDI/His₆ from CD and ATR-FITR analysis

The values shown are means $\pm$ S.E.M.

Technique	Content (%) α -Helix	β -Sheet	β -Turn	Random
ATR-FITR	33.5 $\pm$ 2.0	34.0 $\pm$ 1.5	13.5 $\pm$ 1.0	19.0 $\pm$ 1.5
CD	35.0 $\pm$ 1.8	32.0 $\pm$ 1.0	11.0 $\pm$ 2.0	22.0 $\pm$ 1.0

Figure 4 Western blot showing the specificity of antiserum raised against recombinant MTP-PDI

Extracts from untransfected (UT) Cos-1 cells, Cos-1 cells transfected with an expression construct encoding human MTP (MTP) and from baculovirus-derived human recombinant (r)MTP-PDI, were size-fractionated by SDS/PAGE and immunoblotted. Immunoreactive products were detected with a rabbit anti-(human MTP-PDI) antiserum, diluted 1:500, raised against baculovirus-derived MTP-PDI. Left panel: pre-immune serum did not contain non-specific antibodies against MTP or PDI. Middle panel: antibodies raised after immunization recognized expressed MTP and endogenous PDI in Cos-1 cells. Right panel: confirmation that antibodies raised recognized the antigen used for immunization. The positions of molecular mass markers are indicated (in kDa) at the left.

affinity chromatography steps. Although the dual infection of two recombinant viruses into Sf9 cells has been described by us [16] and others on an analytical scale [6,7,29], this is the first description of a simple and robust methodology for the large-scale purification of the expressed heterodimer. This protocol supersedes the more traditional approach of isolating liver-derived MTP-PDI, involving a series of five column chromatography steps [3,30], and provides a means for isolating human, as opposed to bovine, material for crystallization studies. In addition, our results show that a purification system based on FLAG and metal-ion-affinity chromatography could be used for the purification of other protein complexes involving two or more different subunits.

The production of biologically active human MTP-PDI in insect cells was undertaken with two recombinant viruses: MTP with a FLAG-epitope at its C-terminal end and PDI with a C-terminal extension of six histidine residues. Analysis of infected cells revealed that reproducible production of the MTP-PDI heterodimer could be attained by using premixed equal quantities of high-titre viral stocks of MTP and PDI at the infection stage. Although the C-terminal ER retention and retrieval sequence of PDI was flanked by six histidine residues, we found that substantial quantities of the MTP-PDI heterodimer were retained

in the microsomal fraction. In the traditional MTP-PDI purification procedure involving bovine liver, the preparation of a good microsomal fraction was central to its success. In the present study we found that this purification step could be omitted because virtually pure recombinant MTP-PDI was isolated from crude cell extracts by using a two-step purification procedure. Routinely 4–6 mg of MTP-PDI was purified from a 5-litre culture of insect cells (wet cell weight approx. 50 g) within 1 week of cell infection. This compares favourably with the traditional purification procedure in which Wetterau and colleagues were able to isolate between 0.5 and 3.0 mg of the heterodimer from 600 g of bovine liver [3].

To compare the overall stability, structure and biological function of the baculovirus-derived recombinant MTP-PDI with the native bovine protein, its biochemical and lipid transfer properties were determined. The pattern of the molar ellipticity of the recombinant protein at different guanidinium chloride concentrations was identical with that previously described for bovine MTP-PDI [25], as was the amount of TG transferred between vesicles in a lipid transfer assay *in vitro* [26]. The major difference observed between the two proteins related to their estimated percentages of α -helical and β -sheet structure. In the present study this was determined by far-UV CD and ATR-FTIR analysis; the results were in good agreement. The estimated α -helical content ranged from $33.5 \pm 2\%$ to $35 \pm 1.8\%$, as compared with the previous estimate of $27.3 \pm 1.3\%$ for bovine MTP-PDI [25]. Similarly, the estimate for β -structure was slightly higher. This ranged from $32 \pm 1\%$ to $34 \pm 1.5\%$, as compared with $28.2 \pm 1.5\%$ for the bovine material. These differences might be related to species variation or to differences in experimental detail or sample purity. Whichever is true, we note that the ratios of α -helical to β -pleated sheet structure in both the recombinant and the bovine proteins are remarkably similar, consistent with a common folding.

We report the generation of polyclonal anti-(human PDI) antibodies, which has not previously been described. These will provide a useful reagent in expression studies. Our initial attempts to generate PDI antibodies by using PDI-HIS₆ only as an antigen failed to induce an immune response in rabbits (results not shown). To circumvent this difficulty we used the purified MTP-PDI heterodimer as an immunogen. The fact that this was successful suggests that the immunogenicity of PDI was increased by its association with MTP.

In conclusion, the establishment of a rapid purification procedure for biologically active MTP-PDI, and the production of rabbit polyclonal anti-(MTP-PDI) antibodies, pave the way for analysing the structure and function of wild-type and mutant forms of this key lipid transfer protein.

We thank Jeff Herbert (Immunological Medicine, Imperial College School of Medicine) for help with antibody production. We gratefully acknowledge financial support from the British Medical Research Council. J. S. also thanks the Bristol-Myers Squibb Corporation for a cardiovascular research award.

Note added in proof (received 7 January 1999)

In collaboration with Professor Leonard J. Banaszak, Dr. James R. Thompson and Ms. Judy M. Bratt (University of Minnesota

Medical School, 435 Delaware Street SE, Minneapolis, MN 55455-0326, U.S.A.), we have now obtained crystals from our purified recombinant human MTP-PDI protein.

REFERENCES

- Gordon, D. A., Jamil, H., Sharp, D., Mullaney, D., Yao, Z., Gregg, R. E. and Wetterau, J. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7628–7632
- Leiper, J. M., Bayliss, J. D., Pease, R. J., Brett, D. J., Scott, J. and Shoulders, C. C. (1994) *J. Biol. Chem.* **269**, 21951–21954
- Wetterau, J. R., Combs, K. A., McLean, L. R., Spinner, S. N. and Aggerbeck, L.-P. (1991) *Biochemistry* **30**, 9728–9735
- Linton, M. F. and Farese, R. V. J. (1997) *J. Lipid Res.* **34**, 521–541
- Narcisi, T. M. E., Shoulders, C. C., Chester, S. A., Read, J., Brett, D. J., Harrison, G. B., Grantham, T. T., Fox, M. F., Povey, S., de Bruin, T. W. A. et al. (1995) *Am. J. Hum. Genet.* **57**, 1298–1310
- Rehberg, E. F., Samson-Bouma, M.-E., Kienzle, B., Blinderman, L., Jamil, H., Wetterau, J. R., Aggerbeck, L. P. and Gordon, D. A. (1996) *J. Biol. Chem.* **271**, 29945–29952
- Ricci, B., Sharp, D., O'Rourke, E., Kienzle, B., Blinderman, L., Gordon, D., Smith-Monroy, C., Robinson, G., Gregg, R. E., Rader, D. J. and Wetterau, J. R. (1995) *J. Biol. Chem.* **270**, 14281–14285
- Sharp, D., Blinderman, L., Combs, K. A., Kienzle, B., Ricci, B., Wager-Smith, K., Gil, C. M., Turck, C. W., Bouma, M. E., Rader, D. J. et al. (1993) *Nature (London)* **365**, 65–69
- Shoulders, C. C., Brett, D. J., Bayliss, J. D., Narcisi, T. M., Jarmuz, A., Grantham, T. T., Leoni, P. R. D., Bhattacharya, S., Pease, R. J., Cullen, P. M. et al. (1993) *Hum. Mol. Genet.* **2**, 2109–2116
- Kane, J. P. and Havel, R. J. (1989) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 1139–1164, McGraw-Hill, New York
- Muller, D. P. R., Lloyd, J. K. and Woolf, O. H. (1983) *Lancet* **i**, 225–228
- Shoulders, C. C., Narcisi, T. M. E., Read, J., Chester, S. A., Brett, D. J., Scott, J., Anderson, T. A., Levitt, D. G. and Banaszak, L. J. (1994) *Nat. Struct. Biol.* **1**, 285–286
- Anderson, T. A., Levitt, D. G. and Banaszak, L. J. (1998) *Structure* **6**, 895–909
- Raag, R., Appelt, K., Xuong, N.-X. and Banaszak, L. (1988) *J. Mol. Biol.* **200**, 553–569
- Mann, C. J., Anderson, T. A., Read, J., Chester, S. A., Harrison, G. B., Köechl, S., Ritchie, P. J., Bradbury, P., Hussain, F. S., Amey, J. et al. (1999) *J. Mol. Biol.* **285**, 391–408
- Lamberg, A., Jauhainen, J., Metso, J., Ehnholm, C., Shoulders, C., Scott, J., Pihlajaniemi, T. and Kivirikko, K. I. (1996) *Biochem. J.* **315**, 533–536
- Freedman, R. B., Hirst, T. R. and Tuile, M. F. (1994) *Trends Biol. Sci.* **19**, 331–336
- Chivers, T., Laboisserie, M. C. A. and Raines, R. T. (1996) *EMBO J.* **15**, 2659–2667
- Jeng, M. F., Campbell, A. P., Begley, T., Holmgren, A., Case, D. A., Wright, P. E. and Dyson, H. J. (1994) *Structure* **2**, 853–868
- Katti, S. K., LeMaster, D. M. and Eklund, H. (1990) *J. Mol. Biol.* **212**, 167–184
- Munro, S. and Pelham, H. R. B. (1987) *Cell* **48**, 899–907
- Patel, S. and Grundy, S. (1996) *J. Biol. Chem.* **271**, 18686–18694
- Wu, X., Zhou, M., Huang, L.-S., Wetterau, J. and Ginsberg, H. N. (1996) *J. Biol. Chem.* **271**, 10277–10281
- Johnson, W. C. (1990) *Protein Struct. Funct. Genet.* **7**, 205–214
- Krimm, S. and Bandekar, J. (1986) *Adv. Protein Chem.* **38**, 181–364
- Wetterau, J. R., Combs, K. A., Spinner, S. N. and Joiner, B. J. (1990) *J. Biol. Chem.* **265**, 9800–9807
- Wetterau, J. R., Aggerbeck, L. P., Laplaud, L. and McLean, L. R. (1991) *Biochemistry* **30**, 4406–4412
- Jamil, H., Dickson, Jr., J. K., Chu, C.-H., Lago, M. W., Rinehart, J. K., Biller, S. A., Gregg, R. E. and Wetterau, J. R. (1995) *J. Biol. Chem.* **270**, 6549–6554
- Meister, M., Dietrich, A. and Gierschik, P. (1995) *Eur. J. Biochem.* **234**, 171–177
- Wetterau, J. R. and Zilversmit, D. B. (1985) *Chem. Phys. Lipid* **38**, 205–222

Received 14 September 1998/6 November 1998; accepted 8 December 1998