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The role of protein disulphide isomerase in the microsomal triacylglycerol transfer protein does not reside in its isomerase activity

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The microsomal triacylglycerol transfer protein (MTP), an $\alpha\beta$ dimer, is obligatory for the assembly of apoB-containing lipoproteins in liver and intestinal cells. The β subunit is identical with protein disulphide isomerase, a 58 kDa endoplasmic reticulum luminal protein involved in ensuring correct disulphide bond formation of newly synthesized proteins. We report here the expression of the human MTP subunits in *Spodoptera frugiperda* cells. When the α subunit was expressed alone, the polypeptide formed insoluble aggregates that were devoid of triacylglycerol transfer activity. In contrast, when the α and β subunits were co-expressed, soluble $\alpha\beta$ dimers were formed with significant triacylglycerol transfer activity. Expression of the α subunit with a mutant protein disulphide isomerase polypeptide in which both -CGHC- catalytic sites had been inactivated also yielded $\alpha\beta$ dimers that had comparable levels of lipid transfer activity relative to wild-type dimers. The results indicate that the role of the β subunit in MTP seems to be to keep the α subunit in a catalytically active, non-aggregated conformation and that disulphide isomerase activity of the β subunit is not required for this function.

INTRODUCTION

The microsomal triacylglycerol transfer protein (MTP), a soluble protein residing within the lumen of the endoplasmic reticulum, catalyses *in vitro* the transfer of triacylglycerol, cholesteryl ester and phospholipid between membranes [1]. The protein is an $\alpha\beta$ dimer [2], the 97 kDa α subunit of which is the abetalipoproteinaemia gene product [3–6]. It is required for the assembly and secretion of very-low-density lipoprotein from hepatic cells and chylomicrons from enterocytes. The 58 kDa β subunit is a highly unusual multifunctional polypeptide, being identical with the enzyme protein disulphide isomerase (PDI; EC 5.3.4.1) [1,7]. PDI also acts as the β subunit of the vertebrate prolyl 4-hydroxylase $\alpha_2\beta_2$ tetramers [8–10] and the *Caenorhabditis elegans* prolyl 4-hydroxylase $\alpha\beta$ dimer [11] that catalyse the formation of 4-hydroxyproline in collagens and related proteins (reviewed in [12–14]). Other suggested functions of this polypeptide are to serve as a chaperone-like protein that non-specifically binds peptides within the lumen of the endoplasmic reticulum [15–20] and to act as a major cellular thyroid hormone binding protein [21], a dehydroascorbate reductase [22], and a developmentally regulated retinal protein termed r-cognin [23] (reviewed in [24,25]).

The functions of the PDI/β subunit in the MTP dimer are poorly understood. MTP itself shows no PDI activity, but dissociation of the dimer leads to appearance of the isomerase activity of the PDI subunit [27]. However, it is not known whether the isomerase activity is necessary for $\alpha\beta$ dimerization. On dissociation, the free 97 kDa α subunit forms insoluble aggregates that are devoid of lipid transfer activity [27].

To elucidate the role of the PDI/β subunit in MTP we expressed the 97 kDa α subunit either alone or together with the PDI/β polypeptide in insect cells. The PDI/β polypeptide has two -Cys-Gly-His-Cys- sequences that represent two independently acting catalytic sites for the isomerase activity [28,29]. The role of the isomerase activity of the PDI/β subunit for the MTP dimer formation and triacylglycerol transfer activity was studied by expressing the 97 kDa α subunit together with a PDI/β subunit in which both catalytic sites have been inactivated [28,29] by converting their sequences into -Ser-Gly-His-Cys-.

EXPERIMENTAL

Construction of the baculovirus transfer vector pVL-αMTP and generation of the recombinant virus

The cDNA for the α subunit of MTP was digested from the plasmid pSV7D-FLMTP [30] with the *Eco*RI and *Bam*HI restriction enzymes. The resulting *Eco*RI–*Eco*RI and *Bam*HI fragments containing 70 bp of the 5′ untranslated sequence, the whole coding region and 155 bp of the 3′ untranslated sequence were cloned into the *Eco*RI–*Bam*HI site of the baculovirus transfer vector pVL1392 [31]. The recombinant baculovirus transfer vector was co-transfected into *Spodoptera frugiperda* Sf9 cells with a modified *Autographa californica* nuclear polyhedrosis virus DNA (PharMingen) by using calcium phosphate transfection, and the recombinant virus (αMTP) was selected and purified [32].

Analysis of recombinant proteins in insect cells

Insect cells (Sf9 or High Five, Invitrogen) were cultured in TMN-FH medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (BioClear) at 27 °C. The cells were infected at a density of 10^6/ml at a multiplicity of 5. When producing an MTP dimer, the cells were co-infected with the viruses αMTP and PDI/β [33], coding for the wild-type PDI/β polypeptide, or PDI/β_{NC} [29], coding for the double-mutant PDI/β polypeptide in which the sequences coding for the two Cys-Gly-His-Cys catalytic sites had been converted into those coding for Ser-Gly-His-Cys. The cells were harvested 72 h after infection, washed twice with a solution of 150 mM NaCl and 20 mM phosphate, pH 7.4, homogenized

Abbreviations used: MTP, microsomal triacylglycerol transfer protein; PDI, protein disulphide isomerase.

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in a 10 mM Tris buffer, pH 7.8, containing 100 mM NaCl, 100 mM glycine, 10 μM dithiothreitol and 0.1 % Triton X-100, and centrifuged at 10000 g for 20 min at 4 °C. The resulting supernatants were analysed by denaturing SDS/PAGE (8 % gel) or non-denaturing PAGE (8 % gel) followed by staining with Coomassie Brilliant Blue or Western blotting with a polyclonal antibody to the α subunit of bovine MTP (a gift from Dr. John Wetterau, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, U.S.A.) or to the human PDI/β subunit polypeptide [11]. The cell pellets were further solubilized in 1 % (w/v) SDS and analysed by denaturing SDS/PAGE (8 % gel).

Assay of MTP activity

The triacylglycerol transfer activity was determined by slight modifications of the reported methods [30,34]. Aliquots of cell homogenates were incubated with 5 μl of each of the donor vesicles (4 nmol of phosphatidylcholine, 0.33 nmol of cardiolipin, 0.03 nmol of [^{14}C]trioleylglycerol and 0.01 % butylated hydroxytoluene) and acceptor vesicles (24 nmol of phosphatidylcholine, 0.19 nmol trioleate and 0.01 % butylated hydroxytoluene) in a final volume of 100 μl in 15 mM Tris/HCl buffer, pH 7.4, containing 35 mM NaCl, 1 mM EDTA and 0.02 % NaN_3 at 37 °C for 30 min. The reactions were quenched by mixing with 100 μl of ice-cold 15 mM Tris/HCl buffer, pH 7.4. The negatively charged donor vesicles were removed from the reaction mixture by adsorption onto DEAE-cellulose (0.1 ml of a 1:1 ratio of Whatman DE52 cellulose and 15 mM Tris/HCl buffer, pH 7.4), followed by centrifugation at 15000 g for 4 min at room temperature. The ^{14}C content of acceptors was determined in an aliquot (0.2 ml) of the donor-free supernatant by liquid-scintillation counting. The activity of triacylglycerol transfer is expressed as pmol of triacylglycerol transferred per h per mg of protein.

Other assays

Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad) or as described by Lowry et al. [35].

RESULTS

Expression of the α subunit of MTP in insect cells

A recombinant baculovirus encoding the α subunit of human MTP was generated and used to infect *S. frugiperda* cells. The cells were harvested 72 h after infection, homogenized in a buffer containing 0.1 % Triton X-100, and centrifuged. The cell pellets were solubilized in 1 % (w/v) SDS, and the proteins soluble in 0.1 % Triton X-100 and 1 % SDS were analysed by SDS/PAGE under reducing conditions (Figure 1). No detectable amounts of the recombinant α subunit were extracted from the cell homogenates with a buffer containing 0.1 % Triton X-100, as shown by Western blotting with an antibody to the α subunit of bovine MTP (Figure 1B, lane 2). The minor band (the product with an apparent molecular mass of 97 kDa) visible on the gel stained with Coomassie Brilliant Blue (Figure 1A, lane 2) probably represents some co-migrating polypeptide rather than the α subunit, as it was not recognized by the polyclonal antibody raised against the α subunit and moreover was present in cells expressing the PDI/β subunit alone (Figure 1A, lane 6). In comparison, the recombinant α subunit could be extracted from the cell homogenates with 1 % SDS as shown both by Coomassie staining (Figure 1A, lane 3) and Western blotting (Figure 1B, lane 3). Comparison of the intensity of the Coomassie stained band with those of several proteins suggested that the level of expression was approx. 2–5 μg per 10^6 cells.

Expression of an active recombinant human MTP in insect cells

To study whether it is possible to obtain an association of the MTP α subunit with the PDI/β subunit into an MTP dimer, insect cells were co-infected with two recombinant baculoviruses, each encoding one of the two human polypeptides. Analyses of the cell homogenates by SDS/PAGE under reducing conditions indicated a distinct change in the solubility of the α subunit polypeptide when compared with the situation in which the α subunit was expressed alone. In cells infected with both viruses, about half of the α subunit could be extracted with a buffer containing 0.1 % Triton X-100 (Figures 1A and 1B, lane 4), and

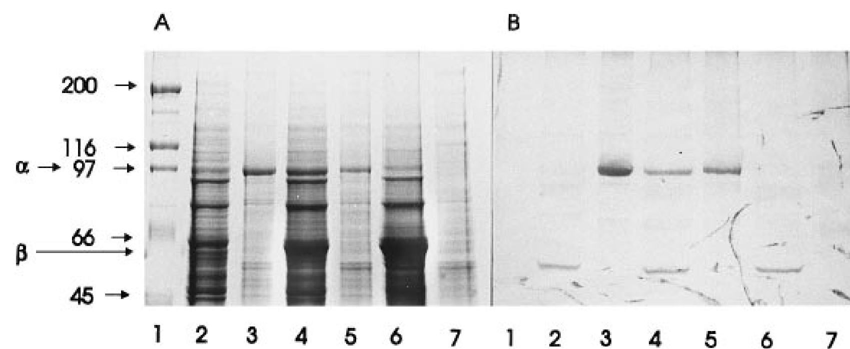

Figure 1 Analysis of the expression of the 97 kDa α subunit of human MTP in insect cells by SDS/PAGE under reducing conditions

Insect cells were infected with a virus coding for the α subunit of MTP either alone (lanes 2 and 3) or together with a virus coding for the PDI/β subunit (lanes 4 and 5), or they were infected with a virus coding for the PDI/β subunit alone (lanes 6 and 7). The cells were homogenized in a buffer containing 0.1% Triton X-100 and centrifuged, and the cell pellets were further solubilized in 1% SDS. The proteins soluble in Triton X-100 (lanes 2, 4 and 6) and SDS (lanes 3, 5 and 7) were separated by SDS/PAGE (8% gel) and analysed by staining with Coomassie Brilliant Blue (**A**) and by Western blotting with an antibody to the α subunit of bovine MTP (**B**). Lane 1 shows molecular mass markers (kDa). The locations of the α and PDI/β subunits are shown by the short and long arrows respectively.

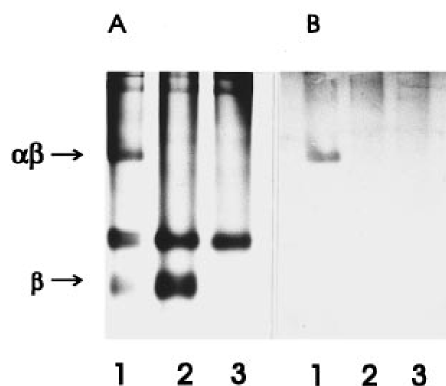

Figure 2 Non-denaturing PAGE analysis of MTP dimer formation from the 97 kDa α subunit and PDI/β subunit expressed in insect cells

The samples were extracted with a buffer containing 0.1% Triton X-100 and subjected to non-denaturing PAGE (8% gel). Lane 1, extract from cells co-infected with viruses coding for the α subunit and PDI/β subunit; lanes 2 and 3, extracts from cells infected with a virus coding for the PDI/β subunit or α subunit respectively. The samples were analysed by staining with Coomassie Brilliant Blue (**A**) and by Western blotting with an antibody to the α subunit of bovine MTP (**B**). The locations of the MTP dimer formed (arrow labelled $\alpha\beta$) and the non-associated PDI/β subunit (arrow labelled β) are indicated. Non-associated α subunit is insoluble in 0.1% Triton X-100 and is not seen. The major band above the band corresponding to the PDI/β subunit in (**A**) is also found in extracts from cells infected with a wild-type *Autographa californica* virus (result not shown).

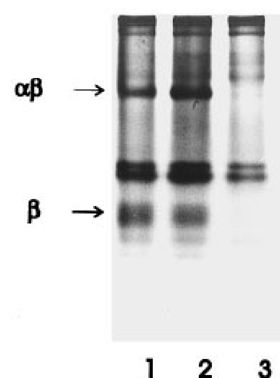

Figure 3 Non-denaturing PAGE analysis of MTP dimer formation from the 97 kDa α subunit and wild-type or mutated PDI/β subunit expressed in insect cells

The samples were extracted with a buffer containing 0.1% Triton X-100, subjected to non-denaturing PAGE (8% gel), and analysed by staining with Coomassie Brilliant Blue. Lane 1, extract from cells co-infected with viruses coding for the α subunit and wild-type PDI/β subunit; lane 2, extract from cells co-infected with viruses coding for the α subunit and the PDI/β subunit in which both catalytic sites for the isomerase activity have been inactivated by converting their first cysteines into serines; lane 3, extract from cells infected with the α subunit-coding virus alone. The locations of the MTP dimer formed (arrow labelled $\alpha\beta$) and the non-associated wild-type or mutated PDI/β subunit (arrow labelled β) are indicated.

Table 1 MTP activity of Triton X-100 extracts from cells infected by various recombinant baculoviruses

The values given are means ± S.D. for four to eight independent samples.

Recombinant polypeptide expressed	n	MTP activity (pmol/h per mg)	MTP activity (%)
α and wild-type PDI/β	8	423 ± 72	100
α and double-mutant PDI/β*	6	425 ± 42	100
α alone	5	49 ± 17	12
Wild-type PDI/β alone	4	0	0
Double-mutant PDI/β alone*	4	17 ± 18	4
None	5	18 ± 5	4

* Both -Cys-Gly-His-Cys- sequences had been converted to -Ser-Gly-His-Cys-.

correspondingly less α subunit was seen in the Triton-insoluble, 1% SDS-soluble fraction (Figures 1A and 1B, lane 5).

Analyses of the proteins soluble in 0.1% Triton X-100 by PAGE performed under non-denaturing conditions indicated the presence in the double-infected cells of a Coomassie-stained protein (Figure 2A, lane 1) that could also be stained by antibodies to the α subunit of MTP (Figure 2B, lane 1) or to the PDI/β polypeptide (result not shown). This product was not seen in extracts from cells infected with the virus coding for either the PDI/β subunit (Figure 2, lane 2) or the α subunit (Figure 2, lane 3) alone. As this protein contains both the α subunit and the PDI/β subunit, it is very likely to be the MTP dimer.

The 0.1% Triton X-100 extracts from cell homogenates containing the recombinant MTP were assayed for the triacylglycerol transfer activity as described in the Experimental section. The MTP activity observed in eight separate experiments ranged from 250 to 1130 pmol/h per mg, which is about 2–8-fold when compared with a value of 140 ± 30 (mean ± S.D.) pmol/h per mg assayed in the same experiments for MTP activity in pig liver homogenates. In control experiments, no triacylglycerol transfer activity was found in homogenates prepared from cells expressing the PDI/β subunit alone, whereas a value of 12 ± 4% was observed for cells expressing the α subunit alone (Table 1).

PDI activity of the PDI/β subunit is not required for the formation of a soluble and active MTP dimer

To study the role of the PDI activity of the PDI/β subunit for MTP dimer formation, insect cells were co-infected with two recombinant baculoviruses, one coding for the wild-type α subunit and the other for a double-mutant PDI/β subunit in which both catalytic sites for the isomerase activity had been inactivated by converting their -Cys-Gly-His-Cys- sequences into -Ser-Gly-His-Cys- [28,29]. Analyses of the proteins soluble in 0.1% Triton X-100 by PAGE performed under non-denaturing conditions indicated that the double-mutant PDI/β subunit formed a MTP dimer with the α subunit (Figure 3, lane 2) as efficiently as the wild-type PDI/β subunit (Figure 3, lane 1).

Assays of the 0.1% Triton X-100 extracts of the cell homogenates for triacylglycerol transfer activity indicated that the protein containing the double-mutant PDI/β subunit had comparable levels of MTP activity to the wild-type (Table 1).

DISCUSSION

Four proteins, the MTP dimer [2], the vertebrate type I [8,9] and type II [10] prolyl 4-hydroxylase tetramers and the *C. elegans* prolyl 4-hydroxylase dimer [11] are now known to contain PDI

as their β subunit. The α subunits of the three different types of prolyl 4-hydroxylase show a considerable degree of amino acid sequence identity [10]. All three α subunits form an active prolyl 4-hydroxylase in baculovirus expression with the human PDI/β subunit [10,11,33]. No similarity is found between the amino acid sequence of the α subunit of MTP [3,4] and the α subunits of prolyl 4-hydroxylases [10,11], but as shown here the MTP α subunit readily forms a protein dimer with the human PDI/β polypeptide.

When the α subunit of MTP was expressed in insect cells alone, the polypeptide formed insoluble aggregates that required 1% SDS for solubilization and showed no significant triacylglycerol transfer activity. This result is similar to that observed by Wetterau et al. [27]; on dissociation of the MTP dimer with chaotropic agents or low concentrations of guanidine hydrochloride, the α subunit was rendered insoluble and inactive. The result also agrees with those obtained in expression studies of the various α subunits of prolyl 4-hydroxylases in insect cells that indicate that an α subunit expressed alone, without the PDI/β subunit, forms insoluble aggregates with no prolyl 4-hydroxylase activity [10,11,33]. Thus a main function of the PDI/β polypeptide in all proteins now known to contain this subunit seems to be to keep the α subunit in a catalytically active, non-aggregated conformation. An additional function of the PDI/β subunit in all these proteins is probably to retain the protein within the endoplasmic reticulum, because none of the α subunits has a C-terminal KDEL-like motif [3,4,10,11], and deletion of this sequence from the human PDI/β subunit led to secretion of considerable amounts of a recombinant prolyl 4-hydroxylase tetramer from insect cells [29].

Recent observations suggest that the PDI/β polypeptide catalyses protein folding by acting both as an isomerase and a chaperone [15–20,24]. The non-specific binding of various peptides, which is believed to be related to the chaperone function [15,16,19], has been localized to a single 26 amino acid sequence in the C-terminal region of the PDI/β polypeptide [16]. This site is distinct from the two -Cys-Gly-His-Cys- catalytic sites, and it has also been demonstrated that modification of the thiol groups in the two catalytic site sequences destroys the isomerase activity but has no effect on peptide binding [16]. The present data with the double-mutant polypeptide indicate that isomerase activity of the PDI/β subunit is not needed for association of the α and PDI/β subunits into the MTP dimer; neither is this activity required for MTP activity of the dimer. This result is similar to that obtained in studies on the role of the PDI/β subunit in a vertebrate prolyl 4-hydroxylase tetramer [29]. Very recently it has been demonstrated that deletions introduced into the 26 amino acid peptide binding sequence prevent prolyl 4-hydroxylase tetramer formation (P. Koivunen, T. Helaakoski, A.-P. Kvist, T. Pihlajaniemi and K. I. Kivirikko, unpublished work). It thus seems that the role of the PDI/β polypeptide in all proteins containing this subunit is related to the chaperone-like peptide-binding function of the polypeptide. The role appears to be similar to that thought to be played in certain other proteins by some molecular chaperones such as Hsp90 [25].

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