The translocation, folding, assembly and redox-dependent degradation of secretory and membrane proteins in semi-permeabilized mammalian cells

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We describe here a semi-permeabilized cell-system which reconstitutes the efficient synthesis, translocation, folding, assembly and degradation of membrane and secretory proteins. Cells grown in culture were treated with the detergent digitonin which selectively permeabilized the plasma membrane leaving the cellular organelles, such as the endoplasmic reticulum (ER) and *trans*-Golgi network intact. These permeabilized cells were added to an *in vitro* translation system, either wheatgerm or reticulocyte lysate, supplemented with RNA coding for either membrane or secretory proteins. Efficient translocation and modification of proteins by these cells was demonstrated by protease protection, photocross-linking of nascent chains to components of the translocation apparatus and by post-translational modifications such as glycosylation or hydroxylation. A comparison was made between the ability of semi-permeabilized cells and microsomal vesicles to fold and assemble proteins. The results show that the intact ER within these cells can assemble proteins much more efficiently than vesicularized ER. Furthermore, the semi-permeabilized cells carried out the redox-dependent degradation of tissue-type plasminogen activator. This system has all the advantages of conventional cell-free systems, including speed and, importantly, the ability to manipulate the components of the assay, while retaining intracellular organelles and, therefore, allowing cellular processes to occur as they would in the intact cell.

INTRODUCTION

Proteins entering the secretory pathway are targeted to and translocated across the membrane of the endoplasmic reticulum (ER) where they are folded, modified and assembled prior to their transport to the Golgi apparatus [1–4]. Studies using a cell-free system which is supplemented with microsomal membranes as a source of ER, suggested that the initial stages of protein translocation are mediated by mammalian Sec61α, and in some cases a second component the TRAM (translocating chain-associated membrane) protein [5–8]. A role for both the mammalian Sec61 complex (α,β and γ-subunits) [9] and the TRAM protein has been confirmed by reconstituting protein translocation from purified components [10]. The translocated protein folds and assembles within the lumen of the ER, a process which is normally a prerequisite for transport to the Golgi, leading to the idea that this organelle exerts some kind of quality control over the proteins destined for further transport through the secretory pathway [11]. Proteins resident within the ER which are thought to facilitate the folding and assembly of proteins entering the secretory pathway include protein disulphide isomerase (PDI). immunoglobulin heavy-chain-binding protein (BiP), endoplasmin and calnexin [12–16].

Optimized cell-free systems which are capable of carrying out the initial stages in synthesis, translocation and folding, have proved particularly valuable in determining the requirements for co- and post-translational disulphide bond formation [17–21]. The advantages of these systems are that they are quick and the individual components can be manipulated both in their redox environment and, in the case of the microsomal membranes, their protein composition. Although this approach has been very successful in determining the initial requirements for protein translocation, folding, disulphide bond formation and assembly, it does have limitations. These include the low yield of correctly assembled complexes and the lack of information on the ability of the assembled protein to be transported to the Golgi apparatus.

Recently a procedure has been described which allows the selective permeabilization of the cellular plasma membrane, resulting in semi-permeabilized cells which retain a functional and morphologically intact ER [22]. These cells were used to reconstitute the transport of pre-synthesized proteins from the ER to the Golgi apparatus. The procedure involves treating virus-infected cells, whose proteins have been radiolabelled, with the detergent digitonin and isolating the cells free of their cytosolic components. Cytosol isolated separately is then added back to these cells along with an energy regeneration system. Specific proteins may then be immunoprecipitated and their transport from the ER to Golgi assayed by their resistance to digestion with endoglycosidase H or sensitivity to endoglycosidase D, which demonstrates modification of oligosaccharide side-chains by Golgi-specific enzymes [23]. There are a number of distinct advantages to using this system. As this is an *in vitro* system the individual components can easily be manipulated, providing a means by which cellular processes can be studied under a variety of conditions and by which reagents such as chemical cross-linkers can be added and directed to proteins within the ER. Also, since the ER remains morphologically intact, the spatial localization of folding and transport processes within the reticular network may be studied.

We have now extended these studies to show that digitonin-permeabilized HT1080 cells can be used in a conventional *in vitro* translation system such as a rabbit reticulocyte lysate or wheatgerm extract. These cells are capable of replacing microsomal membranes as a source of ER in such a system and can efficiently carry out the translocation, processing and modification of a variety of polypeptide chains. Moreover the semi-permeabilized

Abbreviations used: BiP, immunoglobulin heavy-chain-binding protein; β2 m, β-2-microglobulin; DAPI, 4′,6-diamido-2-phenylinimide; DP-cells, digitonin-permeabilized cells; DTT, dithiothreitol; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; HA, haemagglutinin; HLA, human leucocyte-associated antigen; MHC, major histocompatability complex; PPL, preprolactin; PDI, protein disulphide isomerase; t-PA, tissue-type plasminogen activator; TDBA, 4-(3-trifluoromethyldiazirino)benzoic acid; TRAM, translocating chain-associated membrane protein.

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cells proved to be more efficient than microsomal vesicles in carrying out the folding and assembly of newly translocated proteins and in the redox-specific degradation of newly synthesized proteins. This is the first time that the processes occurring within an intact ER during protein synthesis and folding have been studied in a system amenable to biochemical manipulation.

MATERIALS AND METHODS

Reagents

Restriction enzymes, T7 RNA polymerase and nucleotides were obtained from Boehringer (Lewes, East Sussex, U.K.). Nuclease-treated rabbit reticulocyte lysate, amino acids minus methionine, and RNasin ribonuclease inhibitor were purchased from Promega (Southampton, U.K). L-[^{35}S]Methionine was purchased from Amersham International (Amersham, Bucks., U.K.). Chymotrypsin type I-S and trypsin type III were from Sigma (Poole, Dorset, U.K.). HT-1080 human fibrosarcoma cells (A.T.C.C. CCL121) were from the American Type Culture Collection (Rockville, MD, U.S.A.). Tissue culture reagents and anti-[human tissue-type plasminogen activator (t-PA)] monoclonal antibody (clone L172D) [24,25] were purchased from Gibco Life Technologies Ltd. (Glasgow, U.K.). Digitonin was obtained from Calbiochem (Nottingham, U.K.). Goat anti-(human t-PA) polyclonal antibody was purchased from American Diagnostica Inc. (Greenwich, U.S.A.). The antibody to bovine PDI was as described previously [20]. The cDNA clone coding for influenza virus haemagglutinin (HA) from A/Japan/305/57 strain in pGEM-4Z, the cDNA clone coding for t-PA and the polyclonal rabbit antiserum directed against HA were gifts from Dr. M. J. Gething, Howard Hughes Medical Institute, Dallas, TX, U.S.A. The conformational-specific antibody to HA (GY-4) was a gift from Dr. R. G. Webster [26]. The anti-TGN38 (antibody No. 4) [27] was a gift from Dr. George Banting, University of Bristol, Bristol, U.K. The cDNA coding for HLA-A2 was a gift from Dr. Nick Holmes, Addenbrookes Hospital, Cambridge, U.K. Plasmids pGEM4 T7 PPL and pGEM4 T7 Ii encoding preprolactin (PPL) and the invariant chain of major histocompatibility complex (MHC) class II molecules were a gift from Professor Bernhard Dobberstein, ZMBH, Heidelberg, Germany. Antibody to human leucocyte-associated antigen (HLA) heavy chain was a gift from Dr. Hidde Ploegh, MIT, Cambridge, MA, U.S.A. Mouse monoclonal antibody W6/32 was purchased from Serotec, Kidlington, Oxford, U.K. ^{14}C-labelled SDS/PAGE protein molecular-mass standards were prepared by reductive methylation of lysyl residues using the procedure of Dottavio-Martin and Ravel [28]. Microsomal vesicles were prepared from canine pancreas as described previously [29]. All other reagents were purchased from Sigma Chemical Co. (Dorset, U.K.).

Preparation of digitonin-permeabilized cells

These were prepared by a modification of the method described by Plutner et al. [22]. Confluent HT1080 cells from a 75 cm^2 flask were rinsed once with PBS, then incubated with 2 ml of PBS containing 2.5 mg/ml trypsin for 3 min at room temperature. The flask was transferred to ice where 8 ml of ice-cold KHM (110 mM potassium acetate, 20 mM Hepes, pH 7.2, 2 mM magnesium acetate) was added containing 100 μg/ml soybean trypsin inhibitor and the cells released from the plate. Cells were pelleted at 1200 rev./min (280 *g*) for 3 min and resuspended in 6 ml of KHM containing 40 μg/ml digitonin (diluted from a 40 mg/ml stock in DMSO) and incubated on ice for 5 min. To terminate permeabilization 8 ml of KHM was added and cells were pelleted and resuspended in 50 mM Hepes, pH 7.2, 90 mM potassium acetate. After 10 min the cells were pelleted and resuspended in 100 μl of KHM (approx. 2×10^6 cells). Endogenous mRNA was removed by adding $CaCl_2$ to a concentration of 1 mM and staphylococcal nuclease to 10 μg/ml and incubating at room temperature for 12 min. The reaction was terminated by the addition of EGTA to a concentration of 4 mM, and pelleting the cells. Digitonin-permeabilized cells (DP-cells) were resuspended in 100 μl of KHM.

Cell-free transcription

The cDNA coding for HLA-A2 was subcloned as an *Sal*I–*Hin*dIII fragment into pBluescript SK$^+$ and designated pAJM1. For the generation of full-length transcripts pAJM1 was linearized with *Hin*dIII, pGEM4Z HA was linearized with *Bam*HI and both transcribed with T7 RNA polymerase. The cDNA coding for t-PA and type-X procollagen were transcribed as reported previously [21,30]. For the generation of truncated transcripts pGEM4 T7 PPL was linearized with *Pvu*II and pGEM4 T7 Ii with *Nco*I. Both transcripts were transcribed with T7 RNA polymerase.

Transcription reactions were carried out as described by Gurevich et al. [31]. Reactions were incubated at 37 °C for 4 h followed by phenol/chloroform extraction and ethanol precipitation. RNA was resuspended in 50 μl of RNase-free water containing 1 mM dithiothreitol (DTT) and 40 units of RNasin (Promega, Southampton, U.K.).

Immunofluorescence

HT1080 cells were plated on 12 mm × 12 mm coverslips prior to use, fixed with methanol (−20 °C, 10 min), washed with PBS and then blocked with 1% (w/v) BSA in PBS before addition of antibodies. Cell membranes were permeabilized with 0.1% saponin prior to incubation with primary antibody. Rabbit antisera were detected with a fluorescein isothiocyanate (FITC)-conjugated goat anti-(rabbit immunoglobulin) antibody (Dakopatts, Denmark) and cellular DNA stained with 4′,6-diamido-2-phenylinimide (DAPI) as described previously [32].

Cell-free translation

RNA was translated using either a rabbit reticulocyte lysate (FlexiLysate, Promega, Southampton, U.K.) for 1 h at 30 °C or wheatgerm extract at 26 °C for 15 min (see photocross-linking analysis). The translation reaction (25 μl) contained 16 μl of reticulocyte lysate, 1 μl of 1 mM amino acids (minus methionine), 15 μCi of L-[^{35}S]methionine (Amersham International, Bucks., U.K.), 1 μl of transcribed RNA and, where appropriate, 1 μl of nuclease-treated canine pancreatic microsomes. After translation, *N*-ethylmaleimide was added to a final concentration of 20 mM.

Photocross-linking analysis

The ϵ-4-(3-trifluoromethyldiazirino)benzoic acid (TDBA)-Lys-tRNA was prepared as described [33] and the components of the wheatgerm cell-free translation system were prepared and used as described previously [34]. Translations were supplemented with 20 nM purified canine signal recognition particle [35] and were pulsed by the addition of 7-methylguanosine 5′-monophosphate to a final concentration of 2 mM after 10 min. Translations were continued for a further 5 min and then cycloheximide was added to a final concentration of 2 mM to prevent further chain elongation. DP-cells were added to 1.5 μl per 25 μl of translation mix and the mixture incubated at 26 °C for a further 5 min before being chilled on ice and irradiated as

previously described [36]. Following irradiation the membrane-bound components were isolated by centrifugation through a high-salt cushion (0.25 M sucrose, 0.5 M potassium acetate, 5 mM magnesium acetate and 50 mM Hepes/KOH, pH 7.9) by spinning for 5 min at 100000 *g* (at 4 °C) in a Beckman TL100 rotor. To analyse total integral membrane products the obtained pellet was subjected to extraction with 0.1 M Na_2CO_3 as previously described [36] and the resulting pellet resuspended in SDS/PAGE sample buffer and subjected to electrophoresis. For analysis by immunoprecipitation the pellet obtained after purification through a high-salt cushion was resuspended in low-salt buffer (0.25 M sucrose, 0.1 M potassium acetate, 5 mM magnesium acetate and 50 mM Hepes/KOH, pH 7.9) and then 4 vol. of immunoprecipitation buffer A was added and samples processed for immunoprecipitation as described below.

Immunoprecipitation

Immunoprecipitation of translation products was carried out at 4 °C in 1 ml of buffer A (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100 and 0.02% sodium azide). Samples were precleared for 30 min with 40 μl of Protein A–Sepharose [10% (v/v) suspension] and immunoprecipitated for 16 h with 1 μl of the appropriate antiserum. The samples were then incubated for 30 min with 40 μl of Protein A–Sepharose suspension before recovery of the immunoprecipitates by centrifugation. Protein A–Sepharose-immobilized immunoprecipitates were then washed twice in 1 ml of buffer A, once in 1 ml of buffer A containing 0.5 M NaCl and finally once in 50 mM Tris, pH 7.5, buffer.

SDS/PAGE

Samples for electrophoresis were resuspended in 30 μl of SDS/PAGE sample buffer [0.25 M Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 20% (v/v) glycerol, 0.004% (w/v) Bromophenol Blue]. DTT (50 mM) was added to reduced samples where indicated. All samples, reduced and non-reduced, were boiled for 5 min prior to electrophoresis. Unless otherwise stated, the cooled samples were loaded into the wells of a 10% SDS/polyacrylamide gel. Gels were stained, fixed, dried under vacuum and visualized by either autoradiography using Kodak XAR-5 film or imaged using a Fujix Bas2000 phosphorimager for quantitative analysis.

Proteinase K treatment

Translation mixtures were assayed for translocation by treating post-translationally with proteinase K as described previously [19].

Chymotrypsin/trypsin digestion

Isolated microsomal vesicles were resuspended in 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 10 mM EDTA and 1% Triton X-100. Aliquots (5 μl or 10 μl) of resuspended microsomal vesicles or translation mixture were made up to a final volume of either 10 μl or 20 μl respectively with the resuspension buffer without Triton X-100 containing chymotrypsin (250 μg/ml) and trypsin (100 μg/ml) and incubated at room temperature for 2 min. The digestion was stopped by the addition of 5 vol. of boiling SDS/PAGE sample buffer and boiling the samples for 3 min.

RESULTS

Preparation and characterization of digitonin-permeabilized HT1080 cells

Previously it has been shown that the detergent digitonin can be used to selectively permeabilize the plasma membrane of cells grown in culture [22]. These semi-permeabilized cells were used to study the transport of pre-synthesized proteins from the ER to the Golgi apparatus. In this study we aimed to prepare digitonin-permeabilized cells (DP-cells) that could replace microsomal membranes in a conventional cell-free translation system so that translocation, folding and assembly of *in vitro*-translated proteins could be studied in an intact ER. Digitonin concentrations were varied and the optimal concentration which allowed selective permeabilization of the plasma membrane determined (results not shown). This optimal concentration of digitonin (40 μg/ml) was used in all subsequent experiments. To demonstrate that the ER and Golgi apparatus remained intact after digitonin treatment we analysed both treated and untreated cells by immunofluorescence microscopy. An antibody to the ER resident protein PDI was used as an ER marker, and an antibody to TGN38/41 was used as a marker for the *trans*-Golgi network [27]. As can be seen, the control cells labelled with the PDI antibody (Figure 1, upper panel) show a reticular staining around the nucleus characteristic of ER, whereas control cells stained with the TGN38/41 antibody (Figure 1, lower panel) show a more compact juxtanuclear staining pattern characteristic of the *trans*-Golgi network. After treatment with digitonin, the structure of both the ER and the *trans*-Golgi network remained intact although there was some swelling of the ER and distortion (see also [22]) which is a likely consequence of the release of cytoplasm.

The DP-cells were further characterized for their ability to carry out the initial stages in the translocation and modification of a number of secretory and membrane proteins when added to a cell-free translation system. Translation of mRNA coding for influenza virus HA and MHC class II invariant chain (Ii) was performed in the absence of added cells and resulted in the appearance of a single major translation product corresponding to the untranslocated, unglycosylated precursor (Figures 2a and 2b, lanes 1). In the presence of DP-cells a second slower migrating band is seen in both cases, indicative of oligosaccharide side-chain addition. This product is protected from proteolysis by proteinase K (Figures 2a and 2b, lanes 2 and 3). The slight increase in mobility of the invariant chain after proteolysis is due to removal of the cytoplasmic domain of this protein (Figure 2b, lane 3). This indicates that the polypeptides have been correctly inserted into the membrane of the ER, leading to glycosylation and protection from proteolysis. Addition of detergent prior to protease digestion demonstrates the susceptibility of the translocated portions of the protein to digestion after membrane solubilization (Figures 2a and 2b, lanes 4). Similar protection from proteolysis was demonstrated for all the proteins translated in this system during this study which include PPL, t-PA, type-X collagen, and the heavy chain of MHC class I (results not shown).

We have used a photocross-linking approach to identify the ER proteins involved in the insertion of nascent proteins into and translocation across the membrane of the ER in DP-cells. The application of this approach to a cell-free system supplemented with canine pancreatic microsomal membranes has identified components of the translocation apparatus such as Sec61α [6,7] and TRAM [5,8]. Here we have translated transcripts encoding truncated versions of the secretory protein PPL and the type-II membrane protein Ii using a wheatgerm cell-free translation system supplemented with TDBA-modified Lys-tRNA.

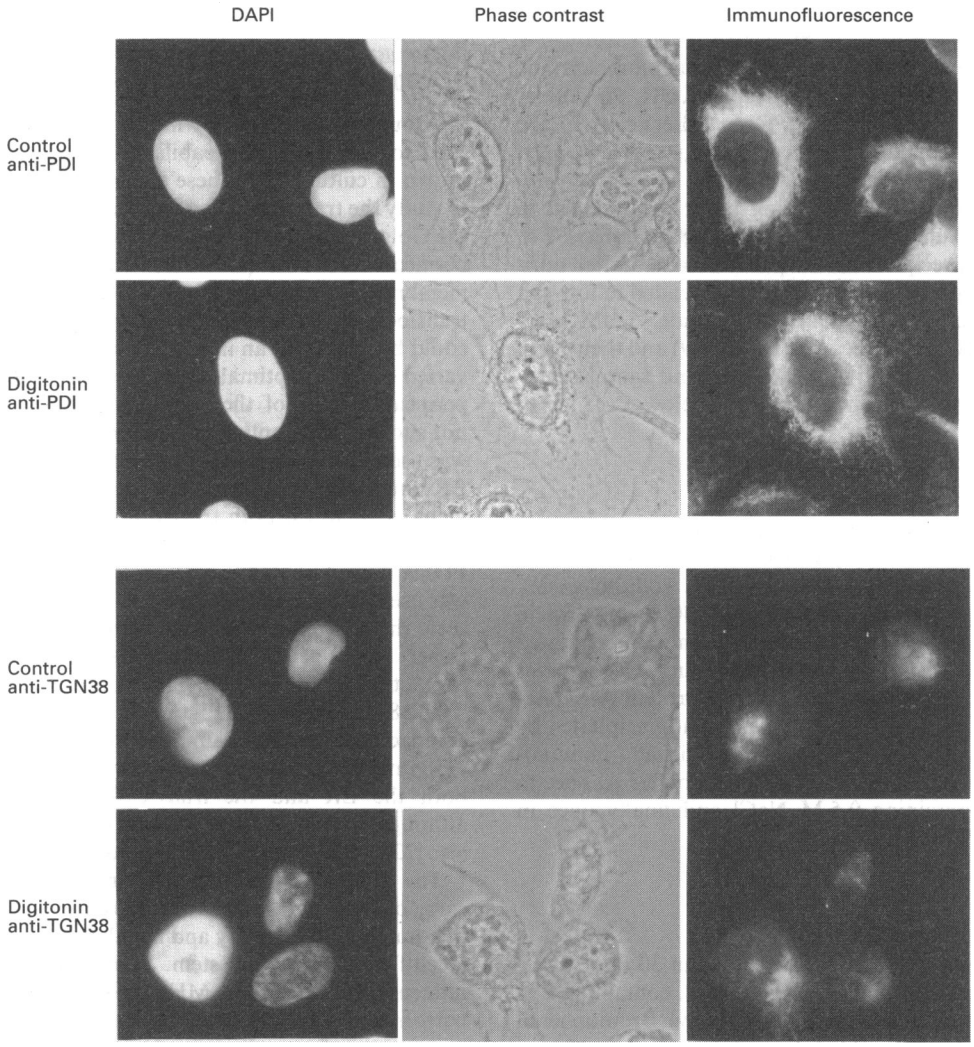

Figure 1 Indirect immunofluorescence of HT1080 cells before and after treatment with digitonin

The distribution of ER (PDI) and Golgi (TGN38) compartments in intact or digitonin-permeabilized (40 μg/ml) cells was determined by indirect immunofluorescence as described. The position of the nuclear staining material was determined by staining with DAPI.

This results in the incorporation of modified lysine residues into the newly synthesized polypeptide chain. The truncated polypeptides synthesized remain attached to the ribosomes and, upon addition of DP-cells, are targeted to the ER membrane. The presence of the ribosome leads to the trapping of the nascent proteins in the translocation complex, allowing interacting components to be identified by photocross-linking to the nascent chain after activation of the TDBA-modified lysine residues.

When an 86-residue N-terminal fragment of PPL was analysed in this way using DP-cells as a source of ER, a 45 kDa photocross-linking product was observed in the membrane pellet after extraction with sodium carbonate buffer, pH 11.5, which removes non-integral membrane-associated proteins (Figure 3a, lane 1). The product required activation of the photocross-linking reagent by irradiation with UV light (cf. Figure 3a, lane 2). Immunoprecipitation identified the cross-linked partner as the TRAM protein (Figure 3a, lane 4) which has previously been identified in canine pancreatic microsomes [5,8]. A similar analysis with the integral membrane protein Ii showed that the major cross-linking partner of a 103-residue N-terminal fragment was the integral membrane protein Sec61α (Figure 3b), as is the case in canine pancreatic microsomes [6,7]. These results indicate that at the molecular level the mechanism of translocation into and across the ER membrane of DP-cells is identical to that already defined in canine pancreatic microsomes.

Folding and assembly of membrane and secretory proteins synthesized in DP-cells

We compared the ability of semi-permeabilized cells and isolated microsomal membranes to translocate and assemble secretory and membrane proteins by translating mRNA coding for either HA, procollagen or the heavy chain of MHC class I in the presence of DP-cells or in the presence of canine pancreatic microsomes. HA is a viral membrane glycoprotein whose folding pathway has been well characterized *in vivo* with homotrimeric structures being formed within the ER prior to export to the Golgi apparatus [13]. HA was translated in a rabbit reticulocyte lysate prepared without the addition of the reducing agent DTT, thus allowing the formation of disulphide bonds during synthesis

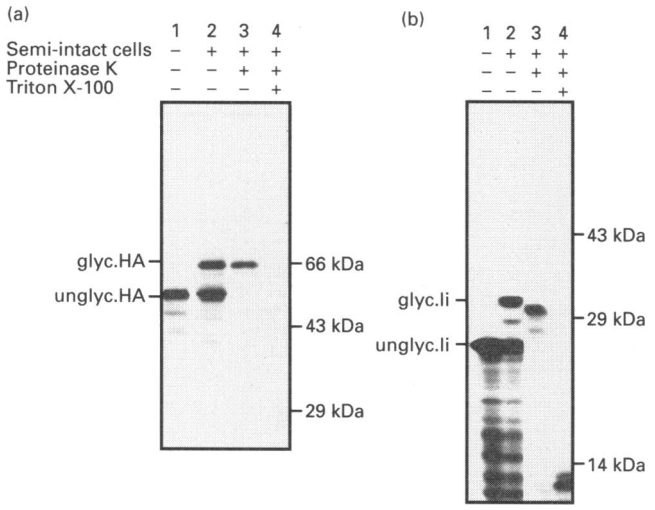

Figure 2 Autoradiograph of proteinase K digestion of products of translation in the presence of DP-cells

Translation was carried out of either RNA coding for influenza virus haemagglutinin (**a**) in a reticulocyte lysate or MHC class II invariant chain (**b**) in a wheatgerm translation system either in the presence (lanes 2–4) or absence (lane 1) of DP-cells. Products of translation were treated with proteinase K post-translationally either in the absence (lane 3) or presence (lane 4) of 1% (w/v) Triton X-100. Proteins were separated by SDS/PAGE through a 10% (**a**) or 12% (**b**) polyacrylamide gel; the gel was dried and developed by autoradiography.

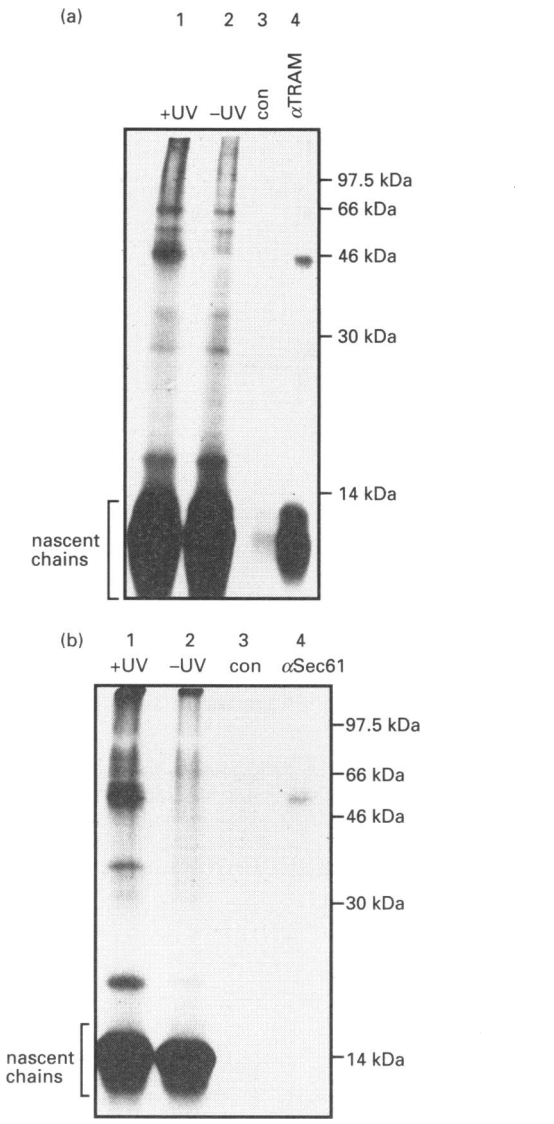

Figure 3 Interaction of preprolactin and invariant chain translocation intermediates with ER proteins involved in protein translocation

The SRP-arrested fragments of preprolactin (**a**) or invariant chain (**b**) synthesized in a wheatgerm translation system and incorporating ε-TDBA-modified lysines were allowed to interact with DP-cells in the presence of cycloheximide and then irradiated with UV light. To identify cross-linked partners, products were immunoprecipitated either with control antisera (lane 3) or with antibodies specific to TRAM (αTRAM; **a**, lane 4) or Sec 61α (αSec61; **b**, lane 4). Proteins were separated on 12% polyacrylamide gels; relative molecular masses are as indicated (kDa).

(Figure 4) [19]. The translation products were immunoprecipitated using a polyclonal antibody to HA and immunoprecipitated proteins separated under reducing (lanes 1 and 2) or non-reducing (lanes 3 and 4) conditions. Under reducing conditions the glycosylated HA synthesized in the presence of DP-cells migrated as a sharper band than that synthesized in the presence of microsomes (compare lanes 1 and 2). This is probably due to more efficient trimming of the terminal glucose residues in the presence of DP-cells. When the products were separated under non-reducing conditions this difference is more pronounced (compare lanes 3 and 4). This could reflect the formation of incomplete or incorrect disulphide bonds in the presence of canine pancreatic microsomes. The HA synthesized in the presence of DP-cells migrates faster under non-reducing conditions (Figure 4, lanes 2 and 4) due to the formation of intramolecular disulphide bonds. There are also some slower migrating bands which probably represent non-dissociated dimers and trimers. Although HA trimers do not form interchain disulphide bonds, they are still partially resistant to dissociation, even after boiling in SDS. This result suggests that trimers of HA are forming when translation is carried out in the presence of DP-cells but not in the presence of microsomes. To confirm that the slower migrating bands are trimers we carried out an immunoprecipitation of translation products synthesized in the presence of DP-cells with a conformational-specific antibody (GY4) that only recognizes native HA [26]. The result shown in Figure 5 indicates that a portion of the molecules recognized by the polyclonal antibody was also recognized by GY4, demonstrating that native trimers had formed. Thus the folding, disulphide bond formation and assembly of HA could be reconstituted in the presence of DP-cells, yielding native trimers.

We translated the RNA coding for type-X collagen in the presence of DP-cells to demonstrate the ability of this system to reconstitute the folding, assembly and hydroxylation of this molecule. Type-X collagen polypeptides form homotrimeric molecules which contain a long triple-helical domain. For the trimer to remain stable at physiological temperature, this triple-helical domain must be stabilized by hydrogen bonding between hydroxyproline residues. Thus, the formation of a stable triple helix is indicative of proline hydroxylation, a modification which occurs within the ER [37]. Furthermore, the stable triple helix once formed is resistant to digestion with trypsin and chymotrypsin, providing a simple test for assaying correct assembly [38]. Figure 6 illustrates that the type-X collagen synthesized in the presence of DP-cells migrates as a diffuse band which is indicative of proline hydroxylation (compare lanes 1 and 2). The collagen

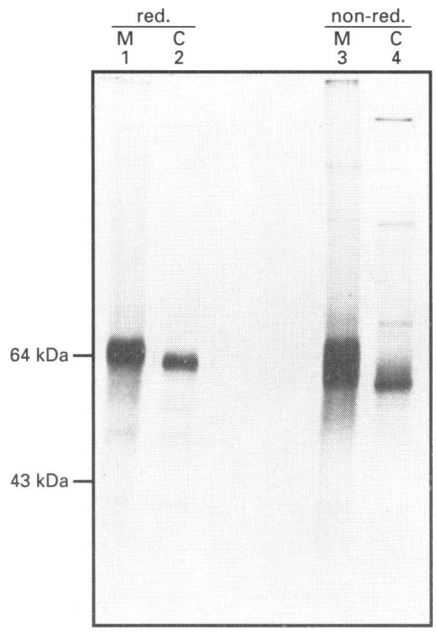

Figure 4 Comparison of HA synthesized in the presence of DP-cells or microsomal membranes

RNA coding for HA was translated in a reticulocyte lysate in the absence of added DTT in the presence of DP-cells (C) or canine pancreatic microsomes (M). Products of translation were treated with *N*-ethylmaleimide (20 mM), immunoprecipitated with antibodies specific for HA and separated without (lanes 1 and 2) or with (lanes 3 and 4) prior reduction with DTT (50 mM). Products were separated by SDS/PAGE through a 10% gel and visualized by autoradiography.

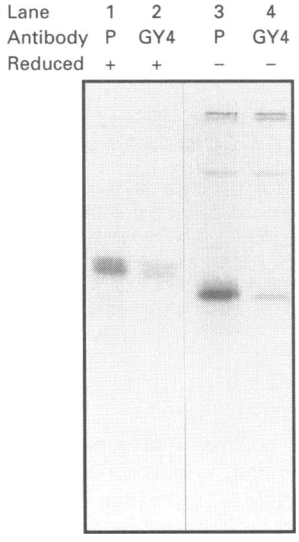

Figure 5 Autoradiograph of translation products after immunoprecipitation with a conformation-specific antibody

Translations were carried out in the presence of DP-cells as described in Figure 4. Products of translation were immunoprecipitated with either a polyclonal antibody (P) which recognizes all forms of the protein (lanes 1 and 3), or a monoclonal antibody (GY4) which only recognizes HA native trimers (lanes 2 and 4). Immunoprecipitates were separated by SDS/PAGE under either reducing (lanes 1 and 2) or non-reducing (lanes 3 and 4) conditions.

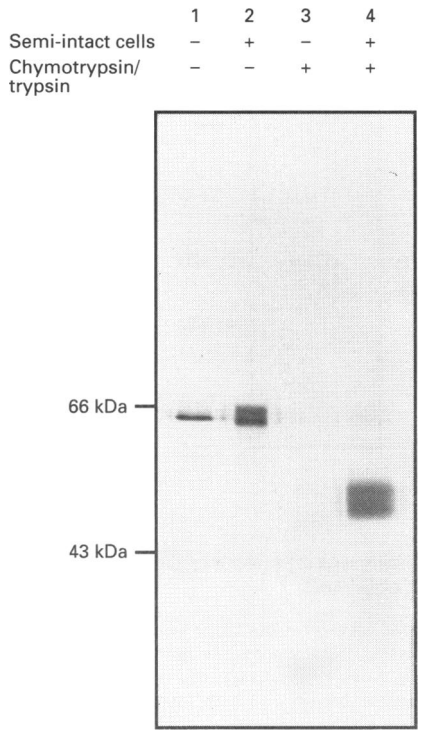

Figure 6 Autoradiograph of products of translation of type-X collagen RNA in the presence of DP-cells

RNA coding for type-X collagen was translated in a reticulocyte lysate either in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of DP-cells. Products of translation were immunoprecipitated with antibody specific for type-X collagen and were separated by SDS/PAGE directly (lanes 1 and 2) or after digestion with a combination of chymotrypsin and trypsin (lanes 3 and 4) as described. Immunoprecipitated proteins were visualized by autoradiography.

was assembled and a stable triple helix formed as demonstrated by the presence of a protease-resistant peptide (non-triple-helical domains are digested) seen after translations were carried out in the presence of cells (Figure 6, lane 4). Thus, type X polypeptides were translocated, hydroxylated and assembled into correctly aligned triple helices, a process which does not take place after translation in the presence of canine pancreatic microsomes [21].

To demonstrate further the ability of DP-cells to fold and assemble protein subunits, we reconstituted the assembly of the MHC class I complex. Class I molecules consist of three subunits, a heavy chain which assembles with β2 microglobulin (β2 m) within the ER, and an antigenic peptide whose assembly with the β2 m–heavy chain complex is essential for subsequent transport of a stable and functional class I complex to the cell surface [39]. We translated the RNA coding for the heavy chain of HLA-A2 in the presence or absence of DP-cells or canine pancreatic microsomes and immunoprecipitated the translation products with either a polyclonal antibody which recognizes all forms of the protein or a conformational-specific monoclonal antibody (W6/32) which only recognizes fully assembled class I molecules [40] (Figures 7a and 7b). In the absence of added cells or microsomes a single product was immunoprecipitated with the polyclonal antibody which corresponds in molecular mass to the HLA-A2 heavy chain (Figures 7a and 7b, lanes 1). However, this product was not recognized by the W6/32 antibody, demonstrating that no assembly had occurred (Figure 7, lanes 2). In the presence of both DP-cells and microsomes a band was

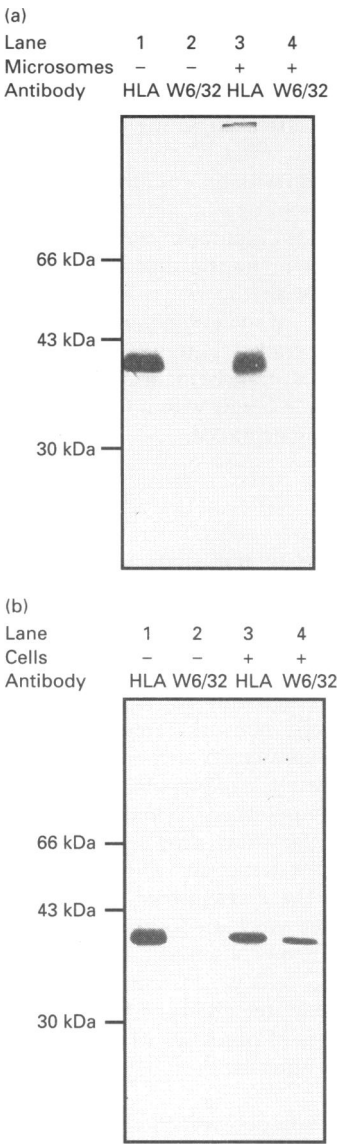

Figure 7 Autoradiograph of products of translation of HLA-A2 RNA in the presence of DP-cells or microsomal membranes

RNA coding for HLA-A2 RNA was translated in a reticulocyte lysate either in the absence (lanes 1 and 2) or presence of canine pancreatic microsomes (**a**) or DP-cells (**b**) (lanes 3 and 4). Products of translation were immunoprecipitated with either an antibody which recognizes all forms of HLA heavy chain (HLA) or with an antibody that only recognizes HLA heavy chain when it is assembled with β2 m and peptide (W6/32). Immunoprecipitates were separated by SDS/PAGE and visualized by autoradiography.

immunoprecipitated with the polyclonal antibody to HLA (Figure 7, lanes 3). This product did not assemble when microsomes were present (Figure 7a, lane 4) but did assemble to form MHC class I molecules in the presence of DP-cells as determined by immunoprecipitation with W6/32 (Figure 7b, lane 4). This demonstrates that the subunits required for class I assembly, i.e. β2-m and endogenous peptide are present in DP-cells.

Redox-dependent degradation of t-PA in DP-cells

When the mRNA coding for t-PA was translated in a rabbit reticulocyte lysate in the presence of DP-cells, the appearance of a translocated, glycosylated product was found to be dependent

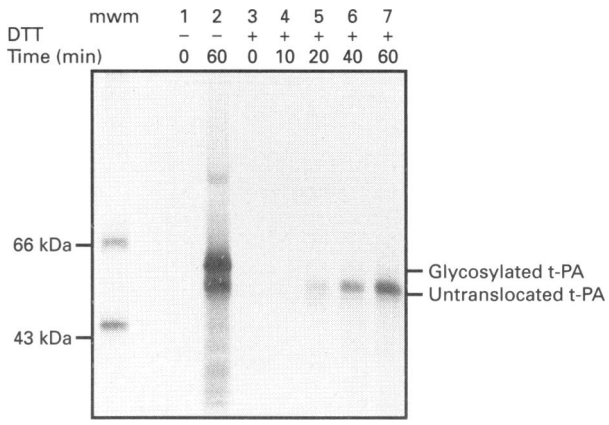

Figure 8 The presence of DTT during translation affects the appearence of translocated, glycosylated t-PA

RNA coding for t-PA was translated in a reticulocyte lysate either in the absence (lanes 1 and 2) or presence (lanes 3–7) of exogenously added DTT (5 mM). Translation reactions were supplemented with DP-cells and products of translation were immunoprecipitated with a polyclonal antibody recognizing all forms of t-PA. Immunoprecipitated products were separated by SDS/PAGE and visualized by autoradiography. Glycosylated t-PA and unglycosylated, untranslocated t-PA are indicated.

upon the absence of exogenously added DTT. Thus, when DTT was added to a final concentration of 5 mM in the translation system during protein synthesis, no translocated product was observed, whereas in the absence of added DTT a glycosylated, translocated translation product was detected (Figure 8). This is in contrast to translation of the same RNA in the presence of canine pancreatic microsomes. Here, translocated protein was detected when DTT was present or absent during translation (Figure 9a, lanes 1 and 3). The untranslocated material is not present in this gel as the microsomes were isolated prior to immunoprecipitation. By use of a conformational-specific monoclonal antibody (M) we could also show that the translation product only folded correctly when DTT was absent (Figure 9a, lanes 2 and 4) and as previously shown was enzymically active [30].

To investigate whether the absence of translation product when DTT was present during synthesis in the presence of DP-cells was due to protein degradation, we first synthesized t-PA in the absence of DTT and then treated the translation products post-translationally with DTT. Translation was carried out for 60 min in the presence of DP-cells and cycloheximide added to prevent further protein synthesis. DTT was then added to a final concentration of 5 mM and translation products immunoprecipitated at various time points with either a polyclonal antibody, which recognizes all forms of the protein (Figure 9b, lanes 8–14), or a monoclonal antibody, which only recognizes the correctly folded, translocated protein (Figure 9b, lanes 1–7). When post-translational incubation was carried out in the absence of added DTT for 60 min no reduction was seen in the amount of t-PA immunoprecipitated with either antibody (Figure 9b, lanes 1,2 and 8,9). There was an increase (to approx. 195%) in the amount of t-PA immunoprecipitated with the conformational-specific antibody (lane 2), which shows that some folding occurred during this time period. This increase was not due to protein synthesis as there was no difference in the amount of t-PA immunoprecipitated with the polyclonal antibody during this time. When post-translational incubation was carried out in the presence of DTT a steady decrease in the amount of t-PA

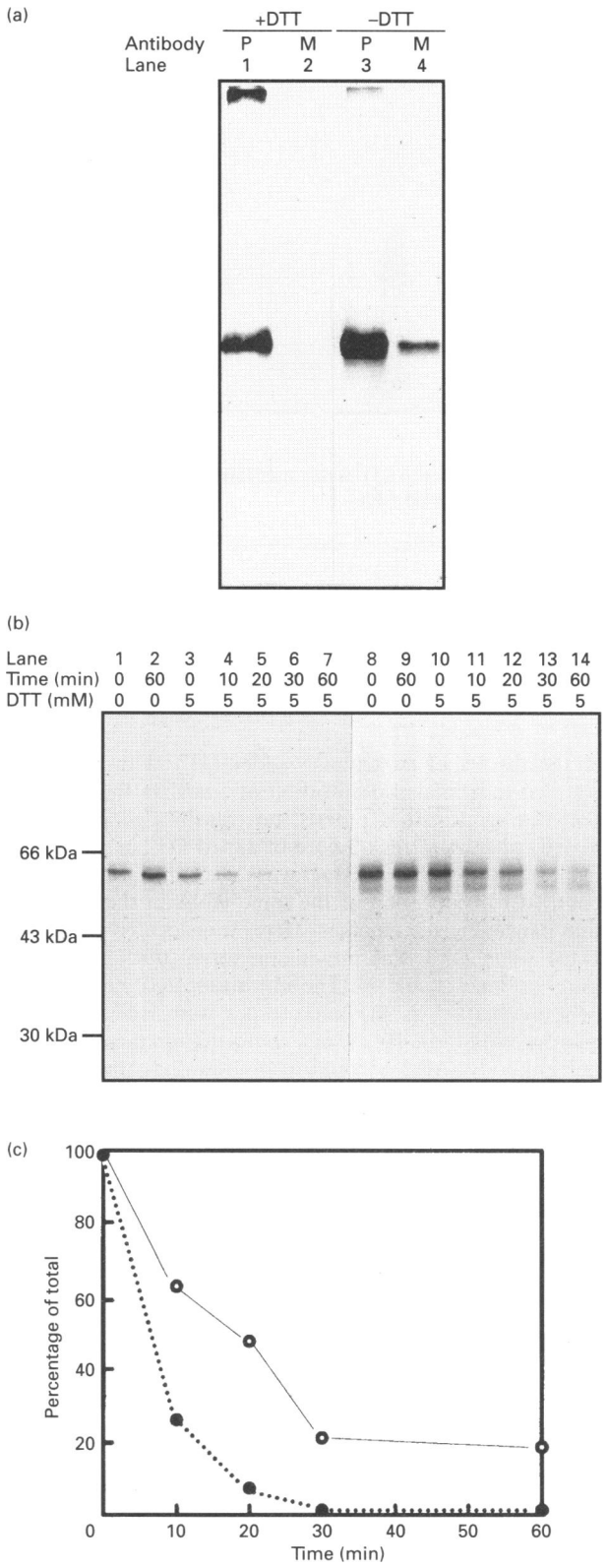

Figure 9 Translation and degradation of t-PA in the presence of DP-cells or microsomes

RNA coding for t-PA was translated in a reticulocyte lysate either in the absence [(**a**) lanes 3 and 4] or presence [(**a**) lanes 1 and 2] of exogenously added DTT (5 mM). Translation reactions were supplemented with either canine pancreatic microsomal membranes (**a**) or DP-cells (**b**). Microsomal membranes were isolated through a sucrose cushion prior to immunoprecipitation. Products of translation were immunoprecipitated with a polyclonal antibody (P) recognizing all forms of t-PA (**a**, lanes 1 and 3; **b**, lanes 8–14) or a monoclonal antibody (M) that only recognizes correctly folded t-PA (**a**, lanes 2 and 4; **b**, lanes 1–7). For (**b**), DTT (5 mM) was added post-translationally and samples incubated for various lengths of time before immunoprecipitation as indicated. Immunoprecipitated proteins were separated by SDS/PAGE and visualized by autoradiography. The amount of immunoprecipitated material from (**b**) was quantified by phosphorimage analysis and the percentage of material remaining after incubation in the presence of DTT plotted as a function of time (**c**). Open circles represent material precipitated with the polyclonal antibody, closed circles represent material immunoprecipitated with the monoclonal antibody.

immunoprecipitated with either antibody was observed (Figure 9b, lanes 3–7 and 10–14). Note that after immunoprecipitation using the polyclonal antibody, two products are observed, the more slowly migrating of these is the glycosylated, translocated t-PA whereas the faster migrating product is untranslocated, unglycosylated t-PA. After 60 min no t-PA was detected after immunoprecipitation with the conformational-specific antibody, indicating that the epitope for this antibody was lost, and the protein was unfolded (Figure 9c, closed circles). Similarly, after 60 min the amount of translocated and glycosylated t-PA immunoprecipitated with the polyclonal antibody decreased to approximately 20% of the original material, indicating that this protein had been degraded (Figure 9c, open circles). This suggests that the effect of post-translational addition of DTT was to first reduce the disulphide bonds within t-PA, leading to the protein unfolding and being degraded.

DISCUSSION

The main objective of this study was to establish a cell-free translation and translocation system that mimicked as closely as possible the processes that occur within the intact cell. To achieve this aim we used a previously published technique to selectively permeabilize the plasma membrane of cells grown in culture using the detergent digitonin [22]. Apart from some swelling, the ER and *trans*-Golgi network remained intact, as shown by indirect immunofluorescence, demonstrating the success of this approach. These results confirm similar results obtained both by indirect immunofluorescence [22] and by immuno-electronmicroscopy [41]; the latter study showing that the Golgi apparatus remains intact after treatment with digitonin. Previously the source of ER in the conventional cell-free systems has been microsomal vesicles which are formed upon breakage of the cell and subsequent fragmentation of the ER [42]. Having prepared semi-permeabilized cells which retained an intact ER we were now able to compare the ability of intact or fragmented ER to carry out a number of processes which occur during the initial synthesis, translocation and folding of secretory or membrane proteins.

Such a comparison demonstrated that the interaction of the nascent polypeptide chains with the translocation machinery was identical for both sources of ER. Translocation intermediates were found to be photocross-linked to two known components of the ER translocation apparatus, Sec61α and TRAM, consistent with a role for these components in this process [1,4,43]. There were, however, clear differences in the ability of canine pancreatic microsomes and DP-cells to fold and assemble proteins both co- and post-translationally. This was demonstrated by studying the assembly of three different secretory and membrane proteins. The ability to form intramolecular disulphide bonds within the influenza virus HA was initially demonstrated for both sources of ER, yet it was clear from these results that this process was more efficient in DP-cells and led to the formation of SDS-resistant trimers. A simple explanation for this difference

could be a greater possibility for interactions to occur between HA monomers within an intact ER than between HA monomers in different microsomal vesicles.

We have previously shown that microsomal vesicles isolated from HT1080 cells are capable of reconstituting the folding pathway of collagen type X when added to a cell-free system. This demonstrated that this cell-line contains all the modifying enzymes required for the hydroxylation of proline and lysine residues which is essential for stable triple-helix formation [21]. We now show that digitonin-permeabilized HT1080 cells are also capable of carrying out this reaction. The folding of collagen within these cells was found to be more reproducible and more efficient than within HT1080-derived microsomal vesicles (results not shown). The potential to use any cell-line as a source of DP-cells means that the folding and assembly of proteins such as procollagen, which require specific modification or interactions with ER proteins, can be studied. A further example of this requirement is the MHC class I complex. Translation of the heavy chain of MHC class I within canine pancreatic microsomes did not lead to assembly of the complex, possibly due to the absence of endogenous β2 m or peptide within these microsomal vesicles. Alternatively, the human HLA heavy chain used in this study is unable to assemble with canine β2 m. However, in the presence of DP-cells a MHC class I complex was formed, as judged by immunoprecipitation with a conformational-specific antibody [40], illustrating that both β2 m and endogenous peptide must be present within these cells.

The most striking difference between the different sources of ER came in their ability to carry out the ER-specific degradation of t-PA. It is now clear that a degradation pathway exists within the ER to remove polypeptides which are either misfolded or are components of multi-subunit complexes and are synthesized in excess [44]. Recently it has been reported that this degradation is redox-dependent in that it may require the reduction and subsequent unfolding of the protein substrate [45]. When RNA coding for t-PA was initially synthesized in a reticulocyte lysate which contained exogenously added DTT, no translocated product was detected, which was in contrast to experiments carried out with microsomal vesicles. However, when the same experiment was carried out in the absence of exogenously added DTT, translocated and glycosylated t-PA was detected. This suggests that either the translocation machinery is affected by the presence of DTT or that the translocating protein cannot form disulphide bonds and is rapidly degraded. We demonstrated that the latter explanation was more likely to be correct by adding DTT post-translationally and showing that upon reduction and unfolding the synthesized t-PA was degraded.

Thus, the DP-cells are capable of carrying out the initial stages in the translocation, folding and assembly of secretory and membrane proteins and offer a number of advantages over the microsomal vesicles normally used in cell-free translation/translocation systems. The ability to carry out these processes efficiently and under similar conditions to those found in the intact cell will enable manipulative experiments to be carried out to ascertain which resident ER proteins are interacting with the polypeptide chain during translocation and folding.

This work was supported by grants from The Royal Society, The Wellcome Trust No. 39078 to N. J. B. and the BBSRC GR/H/89227 and the Human Frontier Science Program Organization to S.H.

Received 27 September 1994/15 December 1994; accepted 23 December 1994

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