The Role of ERp57 in Disulfide Bond Formation during the Assembly of Major Histocompatibility Complex I in a Synchronized Semipermeabilized Cell Translation System*

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We have established a semipermeabilized cell system that reproduces the folding and assembly of a major histocompatibility complex (MHC) class I complex as it would occur in the intact cell. The translation of the MHC class I heavy chain (HLA-B27) in this system was synchronized allowing the folding and assembly of polypeptide chains synthesized within a short time frame to be analyzed. This has enabled us to dissect the time course of interaction of both disulfide and nondisulfide-bonded heavy chain with various molecular chaperones during its assembly in a functionally intact endoplasmic reticulum. The results demonstrate that unassembled, nondisulfide-bonded forms of heavy chain interact initially with calnexin. A later and more prolonged interaction of calreticulin, specifically with assembled, disulfide-bonded heavy chain, highlights distinct differences in the roles of these two proteins in the assembly of MHC class I molecules. We also demonstrate that the thiol-dependent reductase ERp57 initially interacts with nondisulfide-bonded heavy chain, but this rapidly becomes disulfide-bonded and indicates that heavy chain folding occurs during its interaction with ERp57. In addition, we also confirm a direct interaction between MHC class I heavy chain and tapasin, emphasizing the role that this protein plays in the later stages of MHC class I assembly.

Complex and inter-related quality control mechanisms exist within the lumen of the mammalian endoplasmic reticulum (ER)† to ensure correct protein folding. The biosynthesis of multisubunit proteins entering the secretory pathway is regulated at the ER where individual subunits are synthesized and their assembly is co-ordinated. Studies on the biogenesis of major histocompatibility complex (MHC) class I have highlighted intermediate stages during assembly of this multisubunit complex and suggested that a number of ER resident proteins, including calnexin (1), calreticulin (2), tapasin (2, 5, 6), and the transporter associated with antigen presentation (TAP) peptide transporters (3, 4) play a role during the assembly process. Interactions with other proteins such as BiP (7) and Grp94 (8) have also been reported, but it is not clear if these interactions occur during the normal assembly of the MHC class I molecule. Mature MHC class I molecules consist of three subunits, a heavy chain, which assembles with β2-microglobulin within the ER, and an antigenic peptide whose assembly with the β2-microglobulin-heavy chain complex is essential for subsequent transport of a stable and functional class I complex to the cell surface (9). The dynamics of ER chaperone-mediated interactions, the identity of the specific intermediates each ER protein interacts with, and the role these proteins play during assembly remain to be established.

The sequence of events leading to transport of the fully assembled MHC class I complex from the ER is an area of intense study. As the MHC class I heavy chain is translocated into the ER, intra-chain disulfide bonds are formed within the α1 and α2 domains (10). Only the fully disulfide-bonded form is associated with β2-microglobulin, suggesting that the formation of these bonds is a requirement for correct folding and assembly (11). Both nondisulfide- and disulfide-bonded forms of heavy chain have been shown to associate with calnexin, suggesting that this protein plays a role in preventing nonspecific association of heavy chains prior to disulfide bond formation and assembly with β2-microglobulin (10). Once a heavy chain-β2-microglobulin dimer is formed, this then dissociates from calnexin and associates with calreticulin, TAP, and tapasin to form a complex that is required for peptide loading (3, 6, 7). The order of assembly of this complex is unclear, but as soon as the heavy chain is loaded with peptide, the complex dissociates allowing the MHC class I complex to be transported from the ER to the cell surface (3, 4). Mutations within the heavy chain, which prevent this complex from forming (12), or expression of heavy chains in cells lacking tapasin (6) or TAP (13) lead to transport of empty unstable complexes to the cell surface. This indicates that this complex plays a role in ER retention or quality control as well as peptide loading.

The role of the thiol-dependent oxidoreductase ERp57 (15) in MHC class I assembly is less clear. It has been suggested that ERp57 may be involved in stabilization of the MHC class I peptide-loading complex, peptide trimming, or the ER-associated degradation of misfolded proteins (30). ERp57 has been shown previously to form a complex with newly synthesized proteins that, like the interaction with calnexin and calreticulin, is glycoprotein-specific. These studies used a cross-linking approach to identify ERp57 as a cross-linking partner that interacted with glycoproteins forming a complex with calnexin or calreticulin (15, 27, 28). Significantly, ERp57 has also been shown to form a complex with MHC class I in intact cells (29, 30). This observation was extended in a kinetic analysis of rodent MHC class I heavy chain interaction with calnexin, ERp57, and TAP (29), which suggested that there is a delay in

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† The abbreviations used are: ER, endoplasmic reticulum; MHC, major histocompatibility complex; TAP, transporter associated with antigen presentation; SP, semipermeabilized; DTT, dithiothreitol; ATCA, aurintricarboxylic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CHAPSO, 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; BMH, a homobifunctional reagent specific free sulfhydryls; NEM, N-ethylmaleimide; β2M, β2-microglobulin.
the interaction of ERp57 with MHC class I in comparison with calnexin. In human cells, ERp57 has been shown to interact with MHC class I heavy chain, β2-microglobulin, calreticulin, and tapasin in a subcomplex that is dependent on the presence of β2-microglobulin and tapasin but not TAP (30). ERp57 may be recruited to this peptide-loading subcomplex to facilitate crucial structural changes by formation or rearrangement of disulfide bonds prior to association with TAP and peptide loading.

To study what is clearly a complex process, one would ideally investigate folding and assembly with a functionally intact ER. To address this aim we have developed techniques that will allow us to delineate the time course of folding, assembly, and interaction of MHC class I molecules with ER resident proteins. This approach makes use of semipermeabilized cells (SP cells) that can be prepared from any cell line grown in culture and then added to an in vitro translation system where they act as a source of ER (14). Thus, folding and assembly can be studied as it would occur in intact cells using a system amenable to experimental manipulation. We have shown that the synthesis of heavy chains can be synchronized allowing the sequential interaction with calnexin, calreticulin, and tapasin to be studied. We also show that the recently discovered glycoprotein-specific chaperone ERp57 (15) interacts with heavy chain during disulfide bond formation.

EXPERIMENTAL PROCEDURES

Transmission in Vitro—The cDNA coding for HLA-B2705 heavy chain was a gift from Dr. Linda Tussey (John Radcliffe Hospital, Oxford, UK) and was subcloned as an EcoRI-BamHI fragment into pH Bluescript SK* (Stratagene, Cambridge, UK). The recombinant plasmid was designated pAJM2. Transcription reactions were carried out as described by Gurevich et al. (16). Recombinant plasmid was linearized with BamHI and transcribed using T7 RNA polymerase (Promega, Southampton, UK). Reactions (100 μl) were incubated at 37 °C for 2 h. Following purification over RNeasy columns (Qiagen, Dorking, UK), the RNA was resuspended in 100 μl of RNase-free water containing 1 mM dithiothreitol (DTT) and 40 units of RNAse (Promega).

Preparation of Semipermeabilized Cells—The human T-lymphoblastoid cell line CEM (17) was maintained in Iscoves’ modified Dulbecco’s medium supplemented with 10% fetal calf serum. SP cells from this cell line were prepared as described previously (18).

Translation in Vitro—RNA was translated using a rabbit reticulocyte lysate (FlexiLysate, Promega) at 30 °C. The translation reaction (per 25 μl) contained 17.5 μl of reticulocyte lysate, 0.5 μl of 1 mM amino acids (minus methionine), 0.5 μl of 100 mM KCl, 15 μCi of [35S]methionine (NEC, Dreieich, Germany), 1 μl of transcribed RNA, and 5.5 μl (approximately 20% of) SP cells. Translations were carried out for 5 min prior to the addition of auranofin/carbacoid acid (ATCA) (75 μM) to prevent re-initiation of translation (19). Translation reactions were incubated for various lengths of time after ATCA addition as described in individual experiments. Translation was terminated by the addition of N-ethylmaleimide (NEM) to a final concentration of 20 mM and by the isolation of the SP cells by centrifugation at 12,000 × g for 2 min. Cell pellets were resuspended in KHM buffer (110 mM KCl, 20 mM Heps (pH 7.2), 2 mM MgOAc) and re-isolated by centrifugation as above. Cell pellets were processed for immunoprecipitation as described below.

Cross-linking—Cross-linking was performed using BMH, a homobifunctional reagent specific free sulfur hydride. Following in vitro translation, the membrane fraction was incubated for 20 min on ice in the presence of 50 μM BMH added from a 10 mM stock in MeSO4. Control reactions were carried out in the presence of MeSO4 alone. The reaction was quenched by the addition of 0.1 volume of 100 mM DTT, and the samples were incubated for 10 min on ice. After cross-linking, samples were subjected to denaturation in immunoprecipitation buffer containing 1% SDS for 5 min at 100 °C. This was followed by cooling on ice, removal of 10% of the sample to provide a total cross-linked sample, and addition of 10 volumes of immunoprecipitation buffer without added SDS. Samples were subjected to immunoprecipitation with specific antibodies as described below.

Immunoprecipitation—Isolated cell pellets were made up to a final volume of 1 ml with ice-cold CHAPS immunoprecipitation buffer (50 mM HEPES (pH 7.4), containing 2% CHAPS, 200 mM NaCl, 0.02% (w/v) sodium azide) and pre-incubated with 50 μl of protein A-Sepharose (10% (w/v) in phosphate-buffered saline) for 60 min at 4 °C to preclot the samples of protein A binding components. Insoluble cell debris was removed by centrifugation at 12,000 × g for 5 min after preclotting. Precleared samples were incubated for 2 h with 50 μl of protein A-Sepharose and either 10 μl of monoclonal antibody W6/32 (Serotec Ltd., Oxford, UK), or 1 μl of polyclonal antibody to β2-microglobulin (AMS Biotechnology Uk, Oxon, UK), 5 μl of calnexin (12), 1 μl of calreticulin (Cambridge Biosciences, Cambridge, UK), 1 μl of tapasin Rgp48C (Dr. Peter Cresswell, Howard Hughes Medical Institute, Yale University Medical School), or 1 μl of ERp57 (Dr. T. Wileman, IAH, Firbright, UK). The samples were washed three times with immunoprecipitation buffer then prepared for analysis by SDS-polyacrylamide gel electrophoresis (PAGE) as described below.

SDS-PAGE—Samples were prepared for electrophoresis by the addition of SDS-PAGE loading buffer (0.0625 x Tris/HC1 (pH 6.8), SDS (2% w/v), glycerol (10% v/v), and bromphenol blue) in the presence or absence of 50 mM DTT and boiled for 5 min. After electrophoresis, the gels were dried, processed for autoradiography, and exposed to Kodak X-Omat AR film or image intensities were quantified by phosphorimage analysis.

RESULTS AND DISCUSSION

We have previously demonstrated that, when HLA-A2 heavy chain was translated in the presence of SP cells derived from the human cell line HT1080, a heavy chain/β2-microglobulin heterodimer could be formed as judged by immunoprecipitation with the conformation-specific antibody W6/32 (14). This assembly was inefficient, probably due to a limited amount of β2-microglobulin or peptide in this cell line. To improve efficiency of assembly we use here the human cell line CEM, which is lymphoblastoid in origin and has been used previously to study MHC class I assembly (20). For these studies we translated HLA-B27 heavy chain, but it should be noted that HLA-A2 also assembled efficiently with SP cells prepared from this cell line (results not shown).

Our initial experiments were designed to demonstrate that the translated heavy chain was targeted to, translocated across, and integrated into the ER membrane of SP cells. In the absence of added cells, HLA-B27 heavy chain is translated (Fig. 1A, lane 1) but remains sensitive to proteinase K treatment (Fig. 1A, lane 2). Translocation could be demonstrated by protection of the translation product synthesized in SP cells from proteolysis by added proteinase K (Fig. 1A, lane 3). The increase in mobility observed after proteinase K treatment is caused by the removal of the cytoplasmic tail, demonstrating that the translated heavy chain was translocated and integrated into the ER membrane (Fig. 1A, compare lanes 3 and 5). We also investigated whether the translocated heavy chain became N-linked-glycosylated. Such a post-translational modification was suggested by the decrease in mobility of the translation product synthesized in the presence of cells as compared with the translation product synthesized in the absence of cells. Glycosylation was confirmed by sensitivity of the translation product to digestion with endoglycosidase H (Fig. 1A, lanes 6). We also demonstrate that, when heavy chain was translated in the presence of SP cells derived from CEM cells, assembly occurs as judged by immunoprecipitation with the conformation-specific antibody W6/32 (Fig. 1B). The HLA-B27 complex has been shown to be relatively stable in the presence of peptide (22), and we confirmed these results by showing that the complex formed in our system was stable up to 55 °C (Fig. 1B).

This would suggest that the majority of complexes were loaded with peptide. These experiments demonstrated when B27 heavy chain RNA was translated in the presence of SP cells derived from CEM cells, assembly of the translocated, glycosylated product with endogenous β2-microglobulin and peptide resulted in the formation of a stable MHC class I complex.

In these initial experiments, the HLA-B27 mRNA was translated for 60 min, which resulted in a heterologous mixture of
polypeptide chains at various stages of synthesis, folding, and assembly. To follow the folding and assembly of a more homogenous population of molecules, we attempted to synchronize the synthesis of polypeptide chains by only allowing a single wave of translational initiation. We incubated our translation reactions for 5 min to allow initiation to occur and then added aurantricarboxylic acid (ATCA), a compound that inhibits initiation but does not inhibit chain extension or cause dissociation of mRNA-ribosome complexes (19). Using this approach, in the absence of added SP cells, we observed the first full length chains 8 min after initiation of synthesis with no further increase of translation product 12 min after synthesis (Fig. 2, A and B). Hence, we could follow a wave of translation products with the time difference between synthesis of the first and last chains being 3–4 min. Such an approach has previously been used to study the sequential interaction of cytosolic chaperones with newly synthesized proteins (24).

Having established this synchronized translation system, we next investigated the time course of assembly of heavy chains with β2-microglobulin in our SP cells. Translation reactions were incubated for 5 min prior to the addition of ATCA and allowed to proceed for a further 5, 10, 15, 25, or 40 min. Cells were isolated from the translations and solubilized, and immunoprecipitation was carried out with antibodies to β2-microglobulin, or antibodies to heavy chain, W6/32 and HC10. The amount of heavy chain assembling with β2-microglobulin was still increasing after 40 min, whereas the amount of heavy chain immunoprecipitated by W6/32 did not increase after 30 min (Fig. 3B), indicating that the supply of peptide to these cells is limiting. The results demonstrate that a population of heavy chain molecules synthesized within SP cells can assemble with endogenous β2-microglobulin and peptide and that we can follow the assembly of these complexes within a synchronized translation system.

When the same translation products were separated without prior reduction, the disulfide status could be evaluated. The rabbit reticulocyte lysate used in these experiments contains no added DTT and supports the formation of disulfide bonds in proteins translated across ER membranes (14). All the heavy chain precipitated with W6/32 and β2-microglobulin antibodies migrated with a faster mobility than the reduced samples, thus indicating the formation of intra-chain disulfide bonds as would be expected for assembled chains (Fig. 3A, top panel). The unassembled full length chains were assembling post-translationally with β2-microglobulin. The amount of heavy chain assembling with β2-microglobulin was still increasing after 40 min, whereas the amount of heavy chain immunoprecipitated by W6/32 did not increase after 30 min (Fig. 3B), indicating that the supply of peptide to these cells is limiting. The results demonstrate that a population of heavy chain molecules synthesized within SP cells can assemble with endogenous β2-microglobulin and peptide and that we can follow the assembly of these complexes within a synchronized translation system.

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main nondisulfide-bonded or form non-native disulfides and remain unassembled. This indicates that for heavy chain the process of disulfide bond formation and assembly with β2-microglobulin is connected as has been suggested previously (10).

To investigate the interaction of the translocated heavy chains with the glycoprotein-specific chaperones calnexin and calreticulin we carried out a similar time course and immuno-precipitated the products of translation with antibodies specific for these proteins. The results are shown in Fig. 4. A, translations were carried out as described in Fig. 3. Solubilized cells were immunoprecipitated with antibodies to calnexin, calreticulin, and ERp57 as indicated. Translated heavy chain interacting with these proteins was characterized by carrying out SDS-PAGE under reducing and nonreducing conditions as indicated. These time courses were repeated three times with similar results using different preparations of SP cells. B, band intensities were quantified for each time point by phosphorimage analysis using a FujiBas 2000 phosphorimager and AIDA image analysis software.

We also investigated the interaction of ERp57 with heavy chains during assembly using the synchronized SP cell translation system. Here we show that ERp57 interacts with heavy chains during assembly using a co-immunoprecipitation ap-
in preventing disulfide reduction. Alternatively, the co-immunoprecipitation of heavy chains with antibodies to ERp57 could simply reflect a stable interaction with calreticulin. A role for ERp57 also has been suggested in the degradation of misfolded proteins (31). It is possible that the population of heavy chain molecules that do not assemble with β2-microglobulin or peptide remain associated with ERp57 to facilitate disulfide reduction prior to unfolding and dislocation from the lumen of the ER for proteasome-mediated degradation.

The kinetics of MHC class I interaction with tapasin was also analyzed. The anti-tapasin antibody Rgp48C was utilized to precipitate tapasin-heavy chain complexes. This antibody is only functional under denaturing conditions, and, therefore, it was necessary to perform a cross-linking reaction prior to immunoprecipitation. Conditions for BMH-mediated cross-linking were determined (Fig. 5A), and these were used to isolate heavy chain complexes in the synchronized MHC class I assembly assay (Fig. 5B). Tapasin-specific heavy chain cross-links were clearly isolated after 25 min and suggest a 1:1 stoichiometry between tapasin and heavy chain, because the size of the cross-link was approximately 90 kDa (heavy chain has a molecular mass of 43 kDa and that of tapasin is 48 kDa).

The interaction of tapasin with MHC class I at later time points is in agreement with the suggestion that the heterodimeric complex of heavy chain-β2-microglobulin must form prior to the assembly of a peptide-loading subcomplex which also comprises calreticulin. The discrete nature of the tapasin-specific cross-linking product is also suggestive of a direct interaction between heavy chain and tapasin and emphasizes the crucial role that this protein plays in the association between heavy chains and the TAP peptide transporter (31).

In summary, we have developed a synchronized SP cell translation system that has allowed us to evaluate the timing of interaction of calnexin, calreticulin, ERp57, and tapasin with unassembled and assembled chains. The ability to add any cell line to this system will give us the opportunity to determine the folding, assembly, and interaction of MHC class I molecules with ER resident proteins in cells deficient in specific proteins thought to be involved in this process. Using this approach, we have clearly demonstrated distinct roles for calnexin and calreticulin during the assembly of the MHC class I complex. In addition, we have confirmed that tapasin interacts directly with heavy chain and that this interaction is probably dependent on the formation of a heavy chain-β2-microglobulin heterodimer. We have also shown that ERp57 interacts with both unassembled and assembled heavy chains and suggest that this protein plays a role in initial MHC class I heavy folding, the final stages of assembly, and possibly, the degradation of misfolded heavy chains.

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