

Folding and Assembly of Major Histocompatibility Complex Class I Heterodimers in the Endoplasmic Reticulum of Intact Cells Precedes the Binding of Peptide

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Summary

Major histocompatibility complex (MHC) class I molecules are heterotrimers consisting of a polymorphic H chain, β_2 -microglobulin (β_{2m}) and peptide. Peptides are thought to associate early during biosynthesis but the order of assembly of class I molecules from their component subunits in intact cells is not settled. We have studied the assembly of MHC class I molecules in intact cells with or without peptide transporters. MHC class I H chain/ β_{2m} heterodimers can be efficiently recovered only 4 min after translation and are preceded by a folding intermediate. Approximately 2 min after their formation, the class I heterodimers are loaded with peptides resulting in stable class I heterotrimers. In these in vivo studies, no evidence was obtained that peptide binding to the H chain preceded the association with β_{2m} . In contrast, nonassembled class I H chains could be recovered immediately after translation, but this pool did not participate in the formation of class I molecules.

The function of MHC class I molecules is to present protein antigens in the form of peptide fragments to the immune system. Peptides are essential for the stability of class I molecules as class I molecules devoid of peptide dissociate in detergents (1, 2) or at the cell surface (3, 4). Furthermore, peptides are required for efficient intracellular transport of class I molecules (5, 6). Class I molecules can best be considered a heterotrimer consisting of a membrane inserted H chain, β_2 -microglobulin (β_{2m})¹, and peptide. The third subunit, the peptide, contains considerable sequence variation. Each of the three subunits influences the final conformation of the heterotrimer. β_{2m} influences the conformation of class I H chains (7–9) and peptide binding has been shown to affect Ab epitopes in the $\alpha 1$ and $\alpha 2$ domains of class I molecules (10, 11). Peptides can also bind to free H chains and modify epitopes (12–14). The kinetics of peptide binding to H chain/ β_{2m} heterodimers suggest the formation of tight fitting pockets around the peptide anchor residue side chain (15). The intimate linkage between the three subunits of MHC class I molecules is furthermore visualized in the three-dimensional structure of class I molecules complexed to peptide (16, 17).

The order of assembly of the three subunits of MHC class

I molecules can, in principle, go along two lines: the H chain can first associate with peptide and then with β_{2m} , or the H chain can first associate with β_{2m} and then with peptide. Experimental evidence has been provided for both pathways since it was found that peptides can bind in vitro to class I H chain/ β_{2m} heterodimers (1, 2) as well as to free H chains (12, 14).

Most of these studies have been performed in detergent lysates. This may not be representative for the physiological situation, since the order of assembly in a cell may be controlled by accessory molecules like calnexin that bind to class I molecules before peptide loading (18, 19) as well as by the local concentrations of the respective class I subunits. In addition, the kinetics of assembly of class I molecules have thus far not been studied immediately after translation, whereas assembly events may be expected to occur rapidly after translation. For example, Braakman et al. (20) showed that proper folding of influenza hemagglutinin occurs within 10 min after translation. In vitro translation of class I subunits has been used to analyze the assembly of class I molecules early after translation (21–23). Under these conditions, β_{2m} associated immediately after translation with the class I H chain. This is followed by the binding of exogenous peptide and does not require the MHC-encoded peptide pump for peptide entrance into microsomes (22). Further experiments showed that the assembly of the class I H chain/ β_{2m} heterodimer is critically dependent on the correct reducing conditions

¹ Abbreviations used in this paper: β_{2m} , β_2 -microglobulin; ER, endoplasmic reticulum; HA, hemagglutinin.

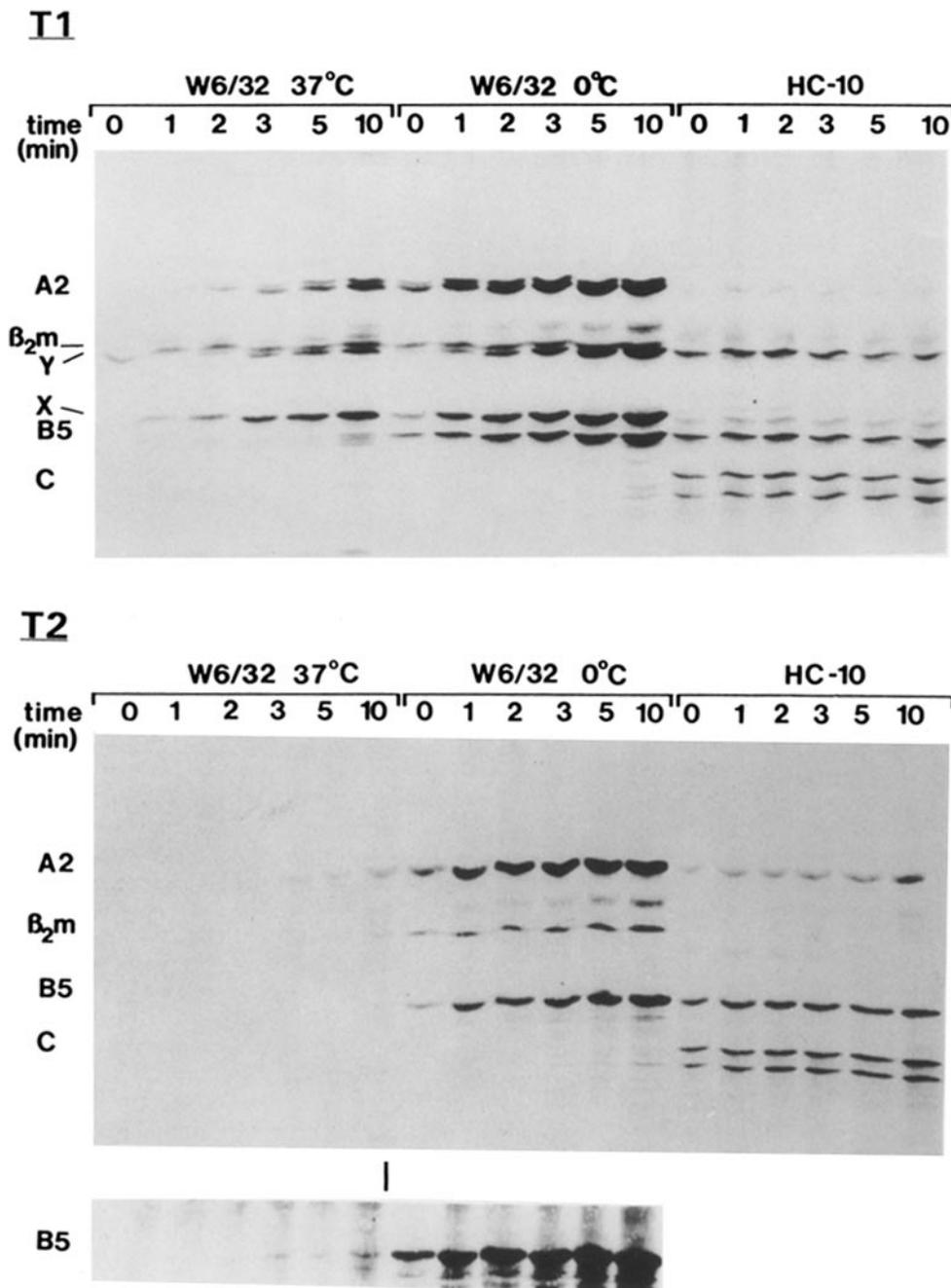


Figure 1. Class I assembly and peptide loading in cells expressing or lacking peptide transporters. Early events in class I assembly were followed by labeling T1, T2, and T2/TAP1+2 cells with [³⁵S]methionine/[³⁵S]cysteine for 2 min. Cells were chased for the times indicated. The resulting lysate was divided in two equal portions: one half was incubated at 37°C (*W6/32 37°C*) and the other half was maintained at 0°C (*W6/32 0°C*), and class I complexes were isolated with the mAb W6/32. Class I H chains were sequentially immunoprecipitated with the mAb HC-10 from the lysates maintained at 0°C (*HC-10*). The class I molecules were separated on 1D-IEF and the position of the different class I subunits is indicated. The C locus product and two HLA-A,B locus products that are not typed in T1 cells are indicated as C, X, and Y, respectively. HLA-B5 molecules are intrinsically unstable and a longer exposure of the relevant part of the autoradiogram is shown for T2 and T2/TAP1+2. Only class I molecules loaded with peptides survive exposure to 37°C, as indicated by the loss of class I molecules in T2 cells and the isolation of temperature-stable class I molecules in T1 and T2/TAP1+2 cells (*W6/32 37°C*). Note the difference in kinetics in the appearance of class I molecules precipitated with W6/32 after 37°C exposure (heterotrimers) and when maintained at 0°C (heterodimers and heterotrimers). The appearance of class I molecules removed at 0°C is similar in the three cell lines. Class I H chains appear with similar kinetics for all class I products. Anode is at the bottom.

during *in vitro* translation (24). It is unclear how this relates to the situation in intact cells.

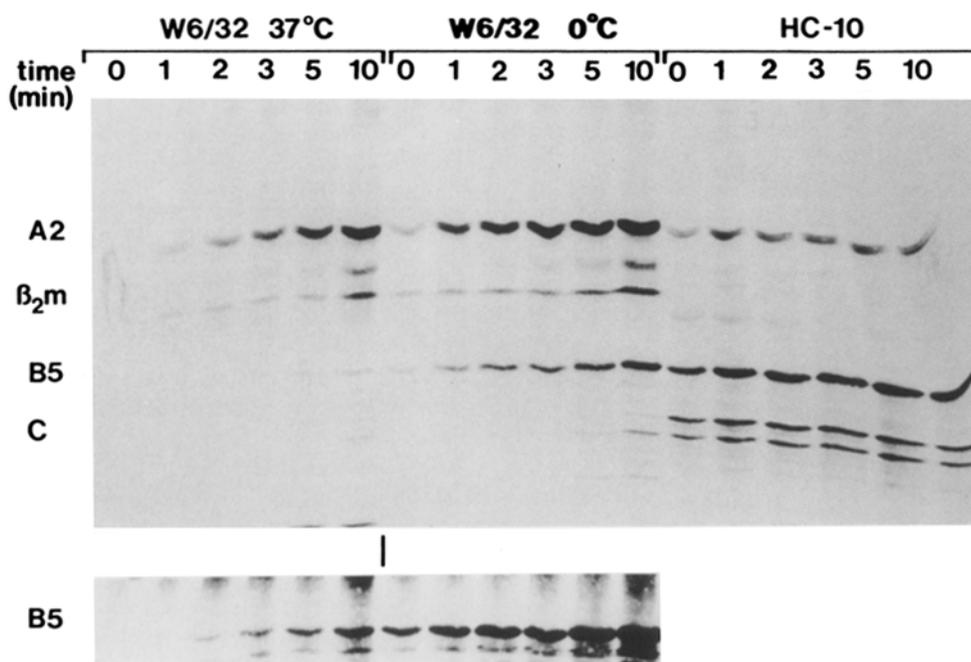
Our experiments focused on the biosynthesis of class I molecules in intact cells during the first 10 min after translation. The results indicate that first class I H chain/ β_2m heterodimers arise from a folding intermediate that is generated cotranslationally. After about 2 min, these class I heterodimers are loaded with peptide. In addition, a pool of free H chains can be isolated. This pool does not participate in the formation of class I heterodimers or heterotrimers.

Materials and Methods

Antibodies

The following mAbs and sera were used: W6/32 (recognizing assembled HLA-A, -B, and -C locus products [25]), BBM.1 (recognizing assembled and free β_2m [26]), and HC-10 (recognizing unassembled class I H chains with a preference for HLA-B and -C locus products but also HLA-A2 and other HLA-A locus products [27]); a rabbit polyclonal antiserum against class I H chains (27, 28). This antiserum recognizes unassembled HLA-A, -B, and -C heavy chains.

T2/TAP1+2



Cell Lines and Culture Conditions

The cell lines T1 and T2 (29) and T2 transfected with the rat peptide transporters TAP1 and TAP2^{*} (T2/TAP1+2 [30]) were used and maintained in RPMI supplemented with 10% FCS.

Gel Electrophoresis and Quantitation

One-dimensional IEF was performed as described (1D-IEF [31]). Gels were fluorographed using DMSO/2,5-Diphenyloxazol (PPO; Merck & Co., Rahway, NJ) and exposed to Kodak XAR-5 films. Gels were quantitated by a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

Biochemical Experiments

Pulse-chase experiments with varying pulse and chase times were performed as follows:

Pulse-chase Experiments to Follow Peptide Loading. 3×10^7 T1, T2, or T2/TAP1+2 cells were starved for 0.5 h in 1.2 ml methionine/cysteine-free RPMI-1640 medium supplemented with 10% FCS. Cells were labeled with 250 μ Ci [³⁵S]cysteine/methionine (20/80%; ICN-Flow, Boston, MA) for 2 min. Incorporation of label was stopped by addition of 1 mM methionine and cysteine and cells were chased for 0, 0.5, 1, 2, 5, and 10 min, respectively. At every chase point, 200 μ l of cell suspension was injected into 1 ml ice-cold lysis mix containing NP-40. Nuclei were removed (5 min, 12,000 rpm) and the lysates were precleared by a nonspecific precipitation with normal rabbit serum (NRS) at 4°C for 1 h. The lysates were split into two portions. One portion was left on ice and the other was incubated at 37°C for 1 h. All other steps were performed at 4°C. The nonspecific precipitation was repeated. Class I molecules (with mAb W6/32) and free class I H chains (with mAb HC-10) were immunoprecipitated sequentially. Class I complexes were isolated within 4 h after lysis. However, the same results

were obtained when class I molecules were isolated 24 h after lysis (data not shown).

Pulse-chase Experiments to Follow the Appearance of Class I Heterodimers. 3×10^7 T2/TAP1+2 cells were starved for 0.5 h in 1.2 ml methionine/cysteine-free RPMI medium supplemented with 10% FCS. Cells were labeled with 250 μ Ci [³⁵S]cysteine/methionine mix for 1 min. Incorporation of label was stopped by addition of 1 mM methionine and cysteine or 20 mM iodoacetamide. Cells were chased for 0, 0.5, 1, 2, 5, and 20 min, respectively. At every chase point, 200 μ l cell suspension was injected into NP-40 lysis mix and class I molecules (with the mAb W6/32) and free class I H chains (with the polyclonal anti-H chain serum) were immunoprecipitated sequentially. All steps were performed at 4°C. Alternatively, 3×10^7 T2/TAP1+2 cells were labeled with 250 μ Ci [³⁵S]cysteine/methionine in 1.2 ml methionine/cysteine-free RPMI medium supplemented with 10% FCS for 45 s. Incorporation of label was inhibited with 1 mM methionine and cysteine and cells were chased for 0, 0.5, 1, 1.5, 2, and 4 min. At each chase point, 200 μ l of cell culture was injected into NP-40 lysis mix and class I molecules and β_2m were immunoprecipitated sequentially with mAbs W6/32 and BBM.1, followed by immunoprecipitation of free H chains with the rabbit anti-H chain serum.

Lysates were denatured by boiling for 5 min after addition of a final concentration of 1% SDS and 5% β -ME. Lysates were then precleared with 5 μ l NRS/ml lysate followed by two consecutive additions of 100 μ l (10% vol/vol) Staphylococcus A. Denatured H chains were isolated with the rabbit anti-H chain serum.

Results

Appearance of Class I Heterodimers and Peptide Loading *in Vivo*

To analyze assembly of class I heterotrimers *in vivo*, we utilized the hybrid cell line (.174xCEM)T2 that is deficient

in peptide loading, and the same cells in which peptide loading was reconstituted by transfection of rat MHC-encoded peptide transporters TAP1 and TAP2^a (T2/TAP1+2) (30). T2 lacks one chromosome 6 and has a deletion in the class II region of the MHC on the other chromosome 6 (29). Direct comparison of T2 and T2/TAP1+2 should visualize the role of peptide in the assembly of class I molecules. The parental cell line (.174xCEM)T1, expressing human TAP1 and TAP2 and class I molecules from both fusion partners (29), was included to control for any possible effect on class I assembly of other gene products located in the deletion on chromosome 6.

Cells were labeled biosynthetically for 2 min and chased for the times indicated (Fig. 1). Three different conformations of class I molecules were identified in the lysates; (a) class I H chain/ β_2m heterodimers and H chain/ β_2m /peptide heterotrimers were recovered with the mAb W6/32 from lysates maintained at 0°C. W6/32 recognizes an epitope on the $\alpha 1\alpha 2$ domain of the H chain and requires correct as-

sembly of H chain with β_2m (25); (b) only class I heterotrimers were isolated by W6/32 from lysates exposed to 37°C. Class I heterodimers dissociate under these conditions because they are not stabilized by peptide and are therefore not recognized by W6/32 (1, 2, 32); (c) nonassembled class I H chains were recovered with the mAb HC-10 or a rabbit anti-H chain serum. HC-10 and the rabbit anti-H chain serum recognize epitopes on the $\alpha 1$ domain of free class I H chains and exclusively recognize free H chains under native and fully reducing conditions (27, 33). Analysis of the pulse-chase experiments on 1D-IEF separated the different class I molecules expressed by T1 and T2 and revealed different aspects of the assembly of class I molecules, as discussed below (Fig. 1). For quantitative comparison, the precipitated class I complexes and H chains shown in Fig. 1 were scanned with a phosphorimager. The results were expressed as percentage of the maximal amount obtained within one set of data (Fig. 2).

Appearance of Class I Complexes. The total amount of class I heterodimers and heterotrimers recovered with W6/32 at

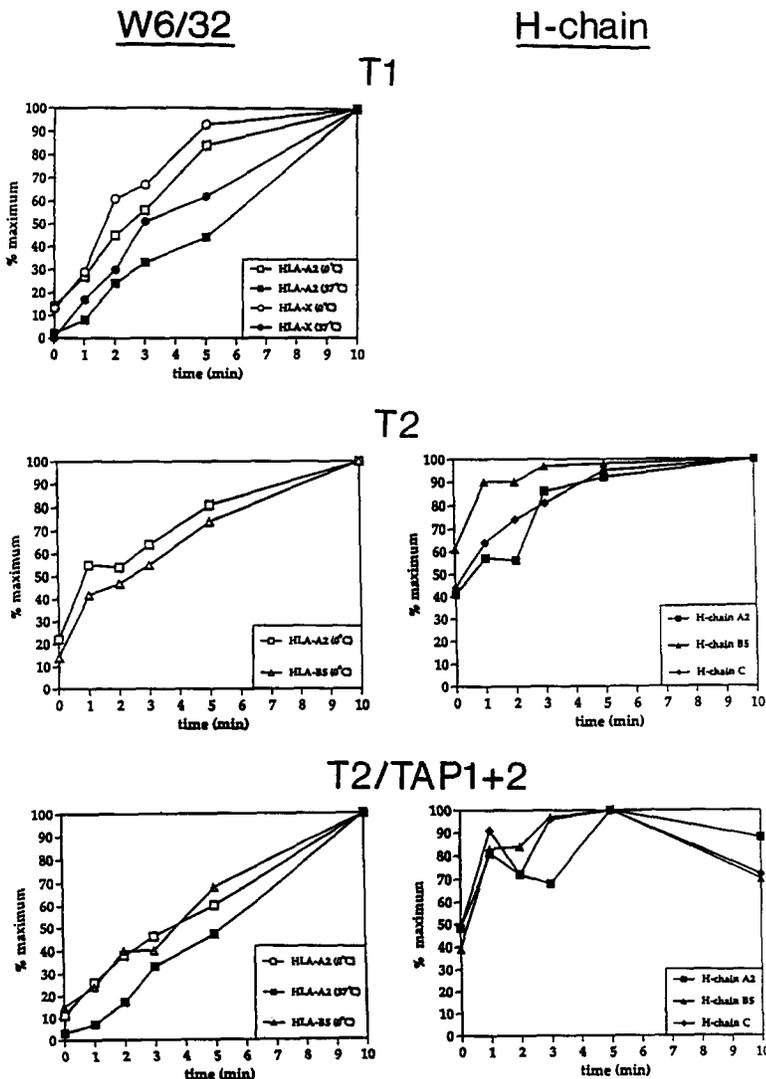


Figure 2. Quantitation of pulse-chase experiments. The gels shown in Fig. 1 were quantitated by phosphorimaging and the intensity of the bands are shown as the percentage of the maximum intensity obtained within one set of data. (W6/32) (Open symbols) Class I molecules recovered from lysates incubated at 37°C; (closed symbols) class I molecules recovered from lysates kept at 0°C. HLA-B5 recovered after exposure to 37°C was only present in minor amounts and did not allow accurate quantitation. HLA-Y is not quantitated because it migrates closely to β_2m . The kinetics of appearance of class I molecules recovered at 37°C followed that of class I molecules recovered at 0°C by 1-2 min. (H chain) The symbols corresponding to the respective class I molecules are shown (inset). The kinetics of appearance of HLA-A2, -B5, and -C H chains is similar.

0°C increased considerably in all three cell lines during the first minutes after a 2-min labeling (Fig. 1, *W6/32 0°C*). The increase of HLA-A2 and HLA-B5 class I complexes isolated with W6/32 occurred with similar kinetics in T2, T2/TAP1+2, and T1 (Fig. 2, *left*). This indicates that peptide supply is not a prerequisite for the formation of the H chain/ β_2m heterodimer.

Loading of Class I Molecules with Peptides. Since the kinetics of appearance of class I complexes recovered with W6/32 in the TAP-deficient cell line T2 was similar to that of T2/TAP1+2 or T1, peptides are apparently not required for the first steps of class I assembly. To follow peptide binding to class I molecules, cell lysates were incubated at 37°C for 1 h (Fig. 1, *W6/32 37°C*). Peptide loading of class I molecules is required to maintain structural integrity upon incubation at 37°C (1, 2, 21, 22). Class I H chain/ β_2m heterodimers will not be recovered by W6/32 after such a treatment. This is illustrated by the finding that hardly any class I complexes can be found in lysates of T2 cells after incubation at 37°C (Fig. 1 *A*). The small amount of HLA-A2 and -B5 molecules isolated from T2 cells are probably loaded with peptides generated in the ER (34, 35). In the cell lines T1 and T2/TAP-1 + 2, class I complexes are recovered after exposure to 37°C, but they appear with different kinetics than the complexes isolated from lysates kept at 0°C (Fig. 1, *B* and *C*; Fig. 2, *left*). The appearance of W6/32-reactive class I molecules recovered at 0°C (heterodimers and heterotrimers) precedes the appearance of class I molecules isolated after incubation at 37°C (only heterotrimers) by some 2 min. This suggests that the class I H chain/ β_2m heterodimer is loaded with stabilizing peptides about 2 min after the formation of the W6/32 epitope. Note that in both T1 and T2/TAP1+2 cells HLA-B5 assembles efficiently into a heterodimer, but the assembled HLA-B5 molecules appear to be less stable at 37°C than the respective HLA-A2 molecules.

Involvement of Free H Chains in Class I Assembly. When free H chains were precipitated with mAb HC-10, the amount of HLA-A, -B, and -C locus-derived H chains followed a similar kinetics: a significant proportion (50–60%) was already present at 0 min of chase, and a plateau was reached after about 3 min of chase (Fig. 1, *HC-10*; Fig. 2, *right*). Most importantly, the amount of free H chains did not decrease with time although there was a concomitant increase of H chain/ β_2m heterodimers precipitated with W6/32 (Fig. 1, *W6/32 0°C*). This suggests that the observed free class I H chains do not participate in the formation of class I H chain/ β_2m heterodimers.

Of the C locus products, mainly free H chains were precipitated with HC-10 and only minor amounts of heterodimers were recovered with W6/32 (Fig. 1, *HC-10* and *W6/32 0°C*). This is in accordance with previous observations that C locus H chains assemble very inefficiently with β_2m (28). The pool of free HLA-A and -B H chains appears with similar kinetics as the pool of HLA-C H chains (Fig. 2, *right*). Since the C locus H chains do not assemble into class I complexes but follow the same kinetics of appearance as HLA-A,B H chains, this emphasizes our contention that the isolated free

HLA-A,B H chains do not participate in the assembly of class I molecules either.

Early Events

In the experiments described above, cells were labeled for 2 min. To study events occurring within these 2 min, T2/TAP1+2 cells were labeled for 45 s and class I molecules were immunoprecipitated by W6/32, followed by the anti- β_2m mAb BBM.1. In a third round of immunoprecipitation, free class I H chains were isolated by mAb HC-10 (Fig. 3 *A*). Class I H chain/ β_2m heterodimers were efficiently recovered only 4 min after translation. However, comparable amounts of β_2m could be isolated throughout the entire chase period. Free class I H chains were already detectable immediately after the 45-s pulse, but incorporation of radio-labeled amino acids into H chains continued during the first minute of chase. As observed in the 2-min pulse (Fig. 1), HLA-A2 and HLA-B5 H chains appeared with kinetics similar to HLA-C chains, and again the increase of H chain/ β_2m dimers was not accompanied by a decrease of the free H chains. Thus, free H chains do not seem to be an intermediate in the assembly of class I complexes. Since β_2m can be recovered throughout the chase period, the observation that the isolated free class I H chains do not participate in the formation of class I complexes cannot be explained by β_2m being present in limiting amounts.

To exclude that the W6/32-precipitable class I complexes are generated from a pool of molecules that is not recognized by either the anti- β_2m Ab BBM.1, the anti-class I H chain Ab HC-10, or the rabbit anti-H chain serum, T1 cells were pulse labeled and chased for the times indicated (Fig. 3 *B*). First, class I heterodimers/heterotrimers were recovered at 0°C. Again, the amount of isolated class I molecules increased during the first 3–5 min of chase. This was followed by three rounds of immunoprecipitation with W6/32 to quantitatively remove class I complexes (Fig. 3 *B*, lane -). Then, free class I H chains were isolated with the rabbit anti-H chain serum. Some increase in the amount of isolated H chains is observed during the first minute of chase. Since this increase is similar for HLA-C (which hardly assemble) and HLA-A,B H chains, the increase is most probably due to the continuing use of tRNA cysteine/methionine during the first minute of chase. No decrease in the amount of class I H chains, which would explain the increase in the amount of class I complexes, is observed. Free H chains were then quantitatively removed by three rounds of immunoprecipitation with the anti-H chain serum (Fig. 3 *B*, lane -). To analyze whether class I molecules exist in a conformation that is not recognized by any of the Abs used (e.g., a H chain-peptide intermediate), we then isolated class I H chains after denaturation and reduction of the lysate. Only small amounts of H chains could be recovered (Fig. 3 *B*, αHC , 100°C, showing an eight times longer exposure). Again, no decrease in the amount of (denatured) class I H chains is observed, suggesting that class I complexes (as isolated with W6/32) are not generated from a detergent-soluble class I conformer. Note that the A2 H

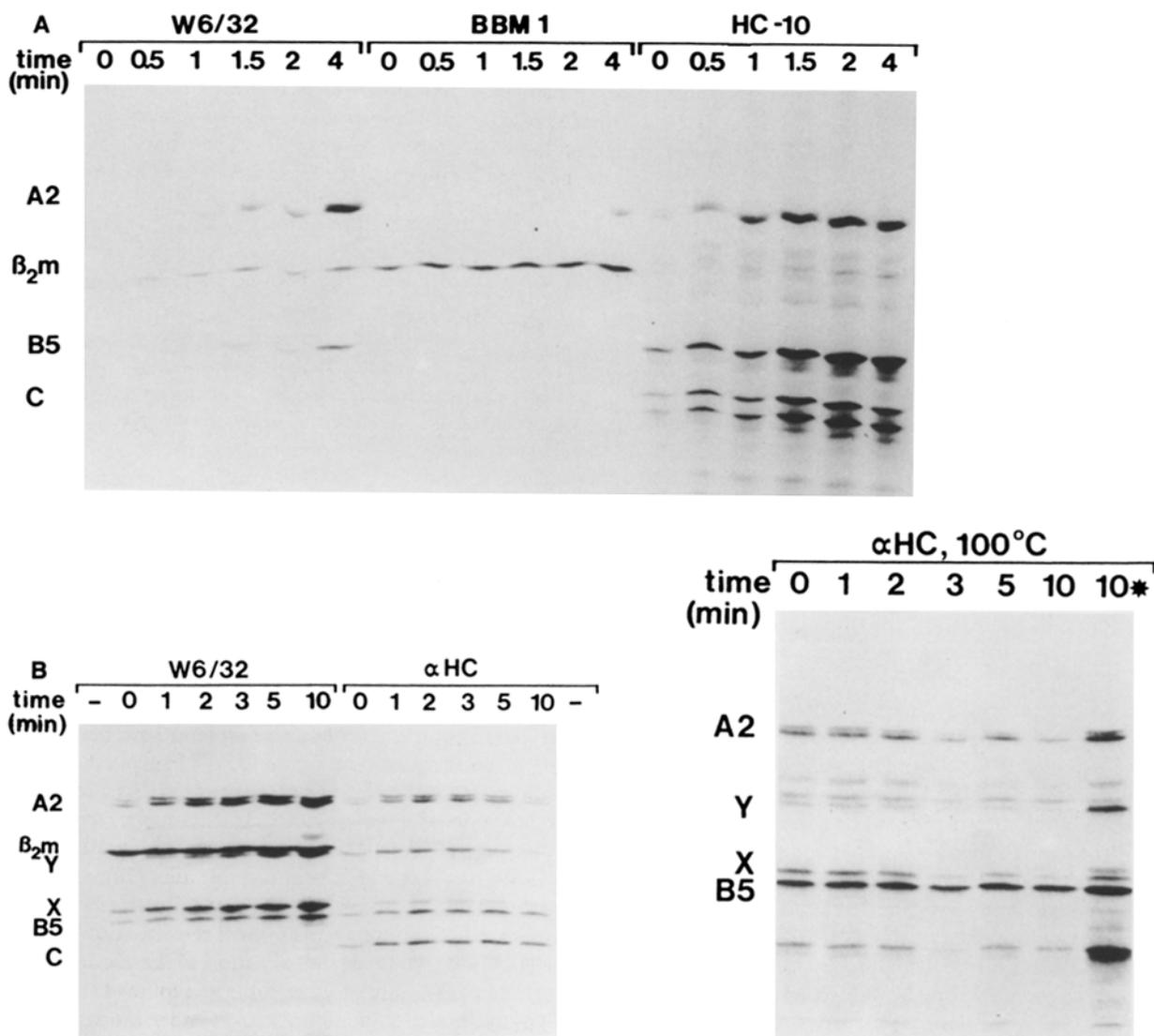


Figure 3. Early events in class I assembly. (A) To follow class I complexes as well as free H chain and β_2m during the early phases after translation, T2/TAP1+2 cells were labeled with [^{35}S]methionine/cysteine for 45 s and chased for the times indicated. Class I complexes (W6/32), β_2m (BBM.1), and free H chains (HC-10) were sequentially immunoprecipitated from the lysates and analyzed on 1D-IEF. The position of the class I alleles is indicated. The amount of radiolabeled H chains increased up to 1.5 min of chase, probably because of continuing incorporation of radioactive amino acids into H chains after the pulse. Class I molecules can be efficiently recovered after 4 min of chase and free β_2m is isolated immediately after the pulse. (B) To analyze the existence of class I conformers early during biosynthesis that escape detection by our Abs, T1 cells were labeled with [^{35}S]methionine/cysteine for 2 min and chased for the times indicated. Class I complexes were first isolated with mAb W6/32. The remaining class I complexes were removed by three rounds of immunoprecipitation with W6/32. (Lane -: class I complexes isolated from 10 min chase point in fourth immunoprecipitation). Then, class I H chains were isolated with the rabbit anti-H chain serum (αHC). Remaining free H chains were removed from the lysate by three rounds of immunoprecipitation with the anti-H chain serum (lane -: H-chains recovered in fourth round of immunoprecipitation from 10 min chase point). The lysate was then reduced and denatured to analyze possible class I conformers that escaped detection with our Abs (αHC , 100°C). Only minor amounts of H chains were recovered and an eight times longer exposure than the other panels is shown. (Lane 10*: H chains isolated after denaturation of a lysate [from 10-min chase time] without prior removal of free H chains). Immunoprecipitates were analyzed on 1D-IEF; the positions of the respective class I H chains are indicated. Class I complexes appear with time, but a corresponding decrease in either free H chains or a soluble class I conformer (that would have been recovered after denaturation) is not observed.

chain is more efficiently recovered with the rabbit anti-H chain serum than with the mAb HC-10 (Fig. 1). This is due to a broader specificity of the rabbit anti-H chain serum (27, 28).

Folding Intermediate in the Assembly of Class I Heterodimers

The observation that W6/32-precipitable heterodimers appear only after 2–4 min of chase suggests the existence of

an early class I conformation that is not detected by HC-10, the rabbit anti-H chain serum, or by W6/32. Since H chains were not recovered with the anti- β_2m mAb BBM.1 within the first 3–4 min after translation, the putative early conformation of class I H chains does not seem to be stably associated with β_2m .

Class I H chains contain two disulfide bridges, one in the

$\alpha 2$ and one in the $\alpha 3$ domain, which are essential for their final conformation (37). It is possible that disulfide bridge formation occurring in a folding intermediate precedes the formation of the H chain/ β_2m heterodimer recognized by W6/32 and, furthermore, is required for stable assembly with β_2m .

To investigate this possibility, we labeled T2/TAP1+2 for 1 min and chased the cells either with iodoacetamide or with excess nonradioactive methionine and cysteine as indicated (Fig. 4). Iodoacetamide alkylates free sulfhydryl groups of cysteines and thereby prevent the formation of disulfide bridges but do not reduce a disulfide bond once formed. Due to its amide group, iodoacetamide is expected to diffuse readily through membranes. Our data support the assumption that the epitope recognized by W6/32 requires disulfide bridge formation, as iodoacetamide clearly inhibited the appearance of this epitope. The increase in W6/32-reactive class I heterodimers observed in the control pulse-chase experiment was

quenched in the presence of iodoacetamide to levels corresponding to the 0-min chase in the control samples (Fig. 4, *top*). The small amount of class I molecules isolated with W6/32 during the chase period probably represents class I molecules with correctly formed disulfide bonds that are present at the 0-min chase time and which are therefore not affected by iodoacetamide. Surprisingly, the appearance at 20 min of the small pool of HLA-C complexes, isolated with W6/32, was not affected by iodoacetamide. Similar amounts of free H chains were recovered in the presence or absence of iodoacetamide (Fig. 4, *bottom*).

Thus, the appearance of class I HLA-A,B complexes recognized by the mAb W6/32 can be inhibited by iodoacetamide. This suggests the formation of a folding intermediate directly after translation with incomplete disulfide bridges.

Discussion

The assembly of MHC class I molecules is of special interest because it is intimately linked to their function: the presentation of antigenic peptides to CD8⁺ T cells. Class I molecules consist of a polymorphic glycosylated H chain containing the peptide binding groove, a nonglycosylated polypeptide β_2m , and a peptide of varying sequence of 8–10 amino acid residues. Two possible pathways for the binding of peptide to class I molecules in cell lysates have been proposed: either first binding of peptide to the H chain followed by binding of β_2m , or binding of peptide to assembled H chain/ β_2m heterodimers (1, 2, 12, 14). However, since endoplasmic reticulum (ER)-located accessory molecules may play a role in the assembly of class I molecules (18), it is obvious that assembly of class I molecules in lysates or at the cell surface does not necessarily reflect the situation in the ER. Furthermore, compartmentalization of the processes involved in class I assembly may determine the route of assembly (38). The assembly of class I molecules under more physiological conditions has been studied by *in vivo* translation using microsomes (21–24, 39). Under these conditions, β_2m rapidly assembled with the class I H chain after translation but a folding intermediate, as observed in our study, was not identified. The study of the assembly of the class I heterotrimer under *in vitro* translation conditions has a few drawbacks. It is unclear whether the concentration of the respective *in vitro* translated class I subunits correspond to that of intact cells. Furthermore, peptide translocation into microsomes did not require the MHC-encoded peptide transporter (22), and it is unclear whether the concentration of peptides in microsomes is similar to that in the lumen of the ER in intact cells. Others were able to show ATP-independent peptide translocation in microsomes but failed to show peptide binding to *in vitro* translated class I heterodimers (39). Another problem with the interpretation of the results from *in vitro* translation, is the observation that the assembly of class I molecules after *in vitro* translation is critically dependent on the correct reducing conditions (24, 39). It is unclear what the exact reducing conditions are in *in vitro* translation that mimic the ER of intact cells.

In this study, we have overcome these problems by per-

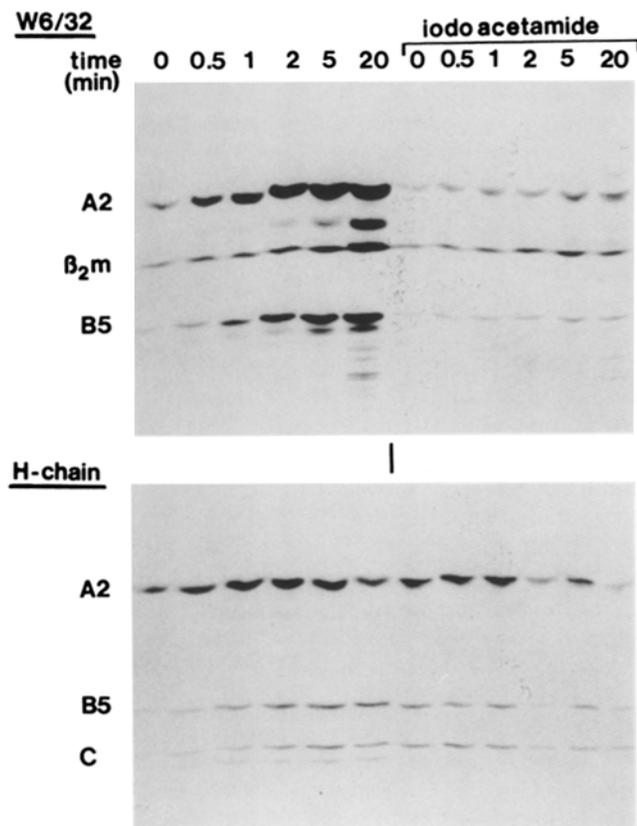


Figure 4. An early folding intermediate in class I assembly. T2/TAP1+2 cells were labeled for 1 min with [³⁵S]methionine/cysteine and chased in the absence or presence of iodoacetamide as indicated. Class I complexes were immunoprecipitated with the mAb W6/32 (*top*) followed by the precipitation of free H chains with the rabbit anti-H chain serum (*bottom*). Immunoprecipitates were analyzed on 1D-IEF. The positions of the different class I subunits are indicated. The control pulse-chase experiment shows the appearance of class I complexes recovered with W6/32. This is suppressed by addition of iodoacetamide at the beginning of the chase. Inclusion of iodoacetamide does not affect the recovery of class I H chains. Note that HLA-C complexes slowly appear in the presence of iodoacetamide.

forming short pulse-chase experiments to follow the kinetics of class I assembly and peptide binding to intact cells. Our experiments therefore describe the assembly of the class I heterotrimer under the most physiological condition possible.

Class I assembly was studied in cells with intact transport (T2/TAP1+2 and T1) and cells lacking the peptide transporters (T2). In all three cell lines, class I complexes that are stable at 0°C assembled with similar kinetics. Our finding that H chain/ β_2m heterodimers are isolated in similar amounts from TAP-deficient T2 cells and T2/TAP1+2 cells indicates that class I heterodimers are apparently stable in the ER of T2 cells and do not dissociate under our experimental conditions. ER-located accessory molecules probably stabilize these class I heterodimers that would have dissociated at the cell surface (3, 4). Salter and Cresswell (36) hardly observed any class I complexes in T2 cells. It should be noted that class I complexes can only be efficiently isolated in T2 cells when the immunoprecipitates are carefully prepared at 0°C. Higher temperatures will result in dissociation of class I heterodimers (1, 6, 32). We had previously observed that after 10 min of labeling, a pool of free class I H chains existed that was not chased into a class I complex (28). However, we could not exclude that another fraction of free H chains was used in the assembly of class I complexes during the first 10 min of translation. In that study (28), the differences in the amount of class I H chains, isolated at later chase times, probably reflect the half-life of the respective H chains in the ER. In this study, we followed the H chains immediately after translation in intact cells. Equal amounts of free H chains were observed in T2 and T2/TAP1+2 cells, but these H chains apparently do not form a reservoir for the generation of fully assembled class I molecules. It should be mentioned that the isolation of C locus H chains (which do not efficiently assemble into class I complexes [28]) serves as an ideal internal control for the recruitment of H chains for class I complexes. HLA-A,B H chains appear with identical kinetics as the HLA-C H chain, indicating again that the isolated H chain pool does not participate in the formation of class I complexes.

Since class I complexes, which are stable at 0°C, appear in similar amounts in T2 and T2/TAP1+2 cells, peptide supply apparently does not affect the rate of assembly of class I H chain/ β_2m heterodimers (32). In fact, class I H chain/ β_2m heterodimers are loaded with proper peptide only 2 min after their assembly and within 10 min after their translation. The resulting class I heterodimers were resistant to prolonged incubation at 37°C, which is a parameter for proper peptide loading (1, 2, 32). The 2-min time lag between the conversion of the class I heterodimers into the heterotrimers indicates that stable peptide binding requires some time and that the class I heterodimer is a physiological intermediate in the formation of class I complexes. Several possibilities, which are not mutually exclusive, could account for the 2-min lag required for proper loading of the class I H chain/ β_2m heterodimer. The supply with peptide could be limited by the activity of MHC-encoded transporters and/or by the presence of suboptimal peptides that may bind with lower affinity and have to be exchanged by optimal peptide. It is also possible that long peptides have to be trimmed after binding

to the class I heterodimer to achieve high affinity binding. Such a trimming process has been proposed by Falk et al. (40).

Interestingly, class I H chain/ β_2m heterodimers appear rather slowly in pulse-chase experiments, as assessed by immunoprecipitation with Ab W6/32 recognizing the class I complex (see Fig. 4). When the generation of disulfide bridges was inhibited by culturing the cells in the presence of iodoacetamide, the formation of class I complexes (with the exception of HLA-C) isolated with mAb W6/32 was suppressed. These observations suggest that class I HLA-A,B complexes are preceded by a folding intermediate that still requires proper disulfide bridge formation. The observation that, under *in vitro* translation conditions, class I assembly only occurs under the correct reducing conditions (24, 39), is in line with this. The nature of this folding intermediate is unclear. It is not recognized by either the mAb HC-10 or the rabbit anti-H chain serum. Furthermore, it is not stably associated with β_2m and does not have the epitopes recognized by W6/32 (present on class I heterodimers/heterotrimers). We assume that a folding intermediate is assembled cotranslationally into a H chain/ β_2m heterodimer that still requires disulfide bridge formation for the generation of the W6/32 epitope, but is not stable enough to allow recovery by an anti- β_2m antibody (BBM.1). Since we were unable to recover a class I H chain from such an intermediate after denaturation and reduction of the lysate (Fig. 3 B), this intermediate is probably not soluble under our lysis conditions.

The folding of influenza hemagglutinin (HA) was also studied in intact cells and followed a similar pathway as described here for class I molecules (20). Proper disulfide bridge formation of HA started 1–2 min after translation, was completed within 10 min, and could be inhibited by addition of DTT during the chase. Disulfide bridge formation of class I heterodimers may be slightly faster than that observed for HA. Formation of HA-trimers occurs within 20 min after translation and appears to be linked to the rate of intracellular transport. Class I H chain/ β_2m heterodimers, on the other hand, are assembled within 4–5 min after translation followed some 2 min later by peptide loading. Peptide loading (resulting in the formation of heterotrimers) is required for efficient intracellular transport, possibly by release from accessory molecules like calnexin (19). These accessory molecules are usually slowly synthesized and therefore cannot be visualized with the labeling protocol used in this study. Whether the kinetics of peptide loading is the only factor determining the rate of intracellular transport, and whether this explains the differences in intracellular transport of different allelic forms of class I molecules (28, 41) remains to be determined.

The scheme summarizing the different steps in the assembly of class I molecules in intact cells is shown in Fig. 5. It differs partially from previously proposed pathways (2, 12, 14) because in our experiments no evidence was found for the existence of H chain/peptide complexes as intermediate forms during assembly of class I heterodimers but a folding intermediate appears to precede the formation of the class I heterodimer. However, there clearly exists a pool of free class I H chains, as visualized with our anti-H chain Abs, but which

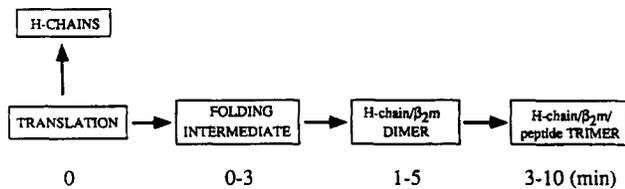


Figure 5. Schematic representation of assembly of class I molecules in the ER. Cotranslationally or shortly after translation a folding intermediate is generated that matures into a class I H chain/ β_2m heterodimer. This heterodimer is loaded with proper peptide some 2 min later. A pool of class I H chains is apparent immediately after translation that does not participate in the formation of class I complexes. The approximate periods of time at which the different class I conformers appear are given.

does not participate in class I assembly. At first sight, this observation is in conflict with the observations that class I H chains can reassociate with β_2m at the cell surface or in vitro (2, 42). In our experiments, β_2m is present in excess in the ER which excludes the possibility that H chains fail to assemble because of the lack of β_2m . It is possible that the free class I H chains are misfolded such that they cannot enter the "folding intermediate state" leading to class I complexes. Alternatively, some H chains may fail to enter a specialized assembly complex (in case such a complex exists), lose the possibility to reenter it, and may be slowly degraded.

In this respect, the assembly of HLA-C locus products is of special interest. We had previously shown that they assemble inefficiently in class I heterotrimers (28). Here, under controlled conditions, we show that HLA-C does not form W6/32-recognizable complexes that are stable at 0°C. Although we cannot exclude that HLA-C H chain/ β_2m heterodimers are intrinsically more unstable in detergent lysates than HLA-A,B heterodimers, it suggests that the low expression of HLA-C is not due to the absence of correct peptides but to inefficient formation of the HLA-C H chain/ β_2m heterodimer. Iodoacetamide does not quench the appearance of HLA-C complexes (Fig. 4), suggesting that the assembly of HLA-C is essentially different (and as a result inefficient) from that of HLA-A and -B molecules. Why HLA-C locus products are largely nonfunctional and whether they have a special, not yet defined function is unclear.

We have followed the assembly of class I molecules in intact cells and have provided evidence that the assembly of class I H chain/ β_2m heterodimers precedes the association with peptide. Immediately after translation class I molecules are found in a folding intermediate that still requires proper disulfide bridge formation. Since both β_2m and peptide are essential for the conformation and the structural integrity of class I molecules, class I H chains undergo at least three steps of consecutive folding and assembly events (Fig. 5) before the resulting class I heterodimers are transported to the cell surface.

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