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In vitro assembly of MHC class I heterodimers

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In Vitro Assembly of
MHC Class I Heterodimers

by

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Introduction

The Major Histocompatibility Complex (MHC) locus encodes a set of polymorphic cell surface antigens that play a critical role in immune recognition. CD8+ cytotoxic T lymphocytes (CTLs) recognize foreign antigenic peptides derived from such sources as intracellular pathogens and neoplastic transformation in conjunction with MHC class I, whereas MHC class II molecules generally present peptides derived from extracellular sources to CD4+ T helper cells. The class I molecule consists of a heterodimer made up of the MHC-encoded heavy chain and a light chain, β2-microglobulin. It is thought that the subunits associate in the endoplasmic reticulum, and that antigenic peptides also bind to the complex in this compartment. Mutant cells which lack β2m do not express class I molecules on the surface *(Seong et al., 1988)*, demonstrating that formation of the intact heterodimer is required for transport of heavy chain through the secretory pathway.

Solution of the crystal structure of the class I molecule has greatly aided in understanding its characteristics and function *(Bjorkman et al., 1987)*. The extracellular portion consists of three domains, α1, α2, and α3. α1 and α2 form a groove which sits on a beta pleated sheet, and this structure is supported on the membrane surface by the immunoglobulin-like α3 domain and β2m molecule. Both the α2 and α3 domains of the heavy chain and β2m contain paired cysteines which form disulphide bonds in the folded polypeptides, but the significance of these bonds in the structure of the molecules has not been clarified. Peptide fragments of antigenic proteins are bound in the groove and are thus presented to T cells in the context of class I residues making up the groove. It seems that the β2m molecule is critical in inducing and maintaining the functional conformation of the distal domains of the heavy chain, in spite of the fact that β2m itself is not thought to contact the T cell receptor. Furthermore, several recent studies suggest that the peptide may also be an integral component of the correctly structured class I oligomer.

Recently, two cell lines have been described which synthesize both heavy chain and β2m, but which are nevertheless defective in class I surface expression *(Townsend et al.,*

1989; Salter and Cresswell, 1986). One of these is of mouse origin (RMA-S) and the other is human (T2). The vast majority of class I heavy chains in these cell lines are retained in the endoplasmic reticulum, and only a small fraction ever reach the surface. Addition of MHC allele-specific peptide to either intact cells or cell lysates induces formation of stable heterodimers *(Townsend et al., 1989; Townsend et al., 1990; Cerundolo et al., 1990).* The interpretation of these results has been that peptide is a critical component of the class I complex, and that it may in fact induce association of the two subunits. In support of this model, *in vitro* translated heavy and light chains were found to assemble only in the presence of exogenous peptide *(Kvist and Hamann, 1990; Townsend et al., 1990).* On the other hand, addition of excess β2m to RMA-S lysates also allowed detection of heterodimers *(Townsend et al., 1990),* and incubation of these cells at reduced temperature resulted in significant surface expression of class I *(Ljunggren et al., 1990).* These studies indicate that peptide may not be required for the initial assembly of heterodimers but, instead, serves to stabilize an otherwise transient association between heavy chains and β2m.

Proper folding of the heavy chain itself is clearly a prerequisite for formation of a functional complex. While several hypotheses have been proposed regarding the requirements for reaching this correctly folded state, it remains unclear what, if any, factors other than the primary structure of the protein itself are important in guiding heavy chain folding. Mutation of one of the cysteine residues comprising the disulphide bond in the immunoglobulin-like domain, α3, destroys transport to the cell surface *(Miyazaki et al., 1986),* but it is unclear whether the disulphide is necessary for the structure of the domain, or whether inappropriate disulphide bonding with another protein in the ER is responsible for the abnormality.

Some researchers have reported findings suggesting that presence of β2m is necessary during heavy chain synthesis to guide its proper folding *(Williams et al., 1989; Myers et al., 1989).* Furthermore, the possible involvement of a molecular "chaperonin," analogous to

some resident ER proteins of the heat shock family, such as BiP or p95 *(Ellis, 1987)*, is favored by the finding of specific though uncharacterized proteins associated with immature forms of class I early in its biosynthesis *(Degen and Williams, 1991; Townsend et al., 1990)*.

In the present study, use of components synthesized *in vitro* has allowed a more defined examination of the requirements for heavy chain folding. Although translocation of the molecule into the ER lumen aided folding somewhat, significant assembly of the molecule was found even in the absence of ER components. No role for either β2m or peptide could be ascertained for guidance of heavy chain folding. The most striking requirement for attainment of an assembly-competent conformation in this system was an oxidizing environment, implying that formation of disulphide bonds plays a key role in heavy chain biosynthesis.

Results

in vitro translation of HLA-A2.1 and beta-2-microglobulin

The A2.1 allele of the HLA heavy chain was translated in a cell-free system using rabbit reticulocyte lysate and canine microsomal membranes. The proteins were biosynthetically labeled with ^{35}S-methionine, and products were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Figure 1, this mRNA was translated into a protein of approximately 40 kd. Treatment of the translation product with Endoglycosidase H, to remove N-linked oligosaccharides, yielded two bands. The lower band's mobility corresponded to that expected for A2.1 from which the signal peptide has been cleaved, indicating that it has been translocated into microsomes. The upper band does not show a mobility shift upon treatment with Endo H, indicating that it is not glycosylated. This product is most likely A2.1 which has not been translocated into microsomes and therefore retains the signal peptide. Presumably the shift in M_r induced by glycosylation compensates for loss of the signal peptide, causing the two products to

comigrate. Significantly, no band is seen at the size of signal peptide-cleaved A2.1 without treatment with Endo H, demonstrating that virtually all translocated protein is glycosylated. Therefore A2.1 is efficiently translated in this *in vitro* system, and the majority of the translated product is translocated across the ER membranes and glycosylated. Similar results were seen with translation of β2m mRNA, but translocation was even more efficient (data not shown). As β2m is not a glycoprotein, Endo H analysis was not performed.

Effect of redox potential on heavy chain conformation

As these experiments determined that the *in vitro* system recapitulates the early events occurring in class I biosynthesis, the translated products were next used to examine oligomerization of the complex molecule. A2.1 and β2m mRNAs were cotranslated in the presence of microsomes, followed by immunoprecipitation and SDS-PAGE analysis. No assembly of the two components could be detected using a complex-specific antibody, W6/32. This lack of assembly could be due to the absence of peptide, as it has been suggested that peptide may be required for association of the two subunits *(Kvist and Hamann, 1990; Townsend et al., 1989)*. Another possibility is that the molecules themselves may not fold into the correct conformation to allow assembly. Alternatively folded forms of class I heavy chain which do not associate with β2m have been reported previously *(Myers et al., 1989; Schnabl et al., 1990)*. Since dithiothreitol (DTT) is routinely added to the reticulocyte lysate during preparation, the reaction mixture was supplemented with oxidized glutathione to neutralize this reducing agent. As can be seen in Figure 2, reversing the reducing state of the reaction had a dramatic effect on assembly of the molecule, resulting in high amounts of product which could be immunoprecipitated by the complex-specific antibody. Furthermore, heavy chain was detected in precipitates of an anti-β2m antibody, BBM.1. An anti-CD4 antibody, OKT4, did not bind any translated protein, showing that precipitation was specific and not due to glutathione-induced aggregation. This virtually complete dependence of

dimerization on an oxidizing environment suggests that disulphide bonding may play a critical role in formation of the class I complex. As two domains of the heavy chain protein contain disulphide bonds, the presence of DTT in the lysate may prevent formation of these bonds and disrupt the folding of the molecule.

In order to better characterize the requirement for oxidized glutathione, several experiments were performed to examine the dependence for assembly on the concentration and timing of addition of the oxidizing agent. A titration experiment was carried out in which varying concentrations of oxidized glutathione were added to the translation reaction. Heterodimer assembly was clearly dose dependent, with the greatest amount of assembly occurring at 2mM oxidized glutathione (Fig 2), a concentration which would titrate out the added DTT. Addition of reduced glutathione did not augment assembly (data not shown), confirming that the observed effect was indeed due to the oxidizing properties of the reagent rather than some other characteristic of glutathione.

The finding that disulphide bonds seem to be important in the proper folding of the molecule provided us with the opportunity to investigate the relative flexibility of class I molecules at different stages of synthesis. Is it critical that the disulphide bonds be formed during synthesis or, alternatively, can they form posttranslationally? If disulphide bridges must form during the actual translation event, it may indicate that the production of properly folded, and thus functional, class I heterodimers is rigorously controlled during synthesis. Once the proper conformation has been disulphide bonded, is it stable or is it sensitive to subsequent reduction? To address these questions, experiments were done to look at the effect of manipulating the oxidation state of the reaction after synthesis of the molecules was complete. As reticulocyte lysate is only competent for translation for 30-60 minutes after thawing, it is possible to examine effects of post-translational manipulations of the system. In one reaction, translation was carried out in the presence of glutathione, followed by addition of DTT to cleave

disulphide bonds. The converse reaction was also performed, in which molecules were synthesized in the presence of DTT and glutathione was added subsequently. It is evident from this experiment that optimal folding of A2.1 requires an oxidative state during translation (Figure 3). While some proper assembly is detected when glutathione is added after translation, it is much less efficient than when glutathione is present throughout translation, occurring at approximately 40% efficiency as determined by densitometric scanning. Furthermore, addition of DTT to the reaction after synthesis did not completely abrogate A2.1's association with β2m, but again, the interaction was much less efficient, with only about 55% remaining assembled as compared to the unreduced sample. Therefore it seems that disulphide bonds play a role both in the initial folding of the molecule as well as in stabilizing its conformation.

Effect of beta-2-microglobulin on heavy chain folding

Having established a system to study conformation and assembly of A2.1, it was of interest to examine other parameters which might affect folding of the molecule. It is known that β2m is required for transport of the heavy chain to the cell surface *(Seong et al., 1988)*. It has been further suggested that, in addition to this transport role, β2m may be important in guiding folding of the heavy chain in the ER *(Williams et al., 1989; Myers et al., 1989)*. That is, if β2m is not present during class I synthesis, the molecule will irreversibly fold into a conformation that is incapable of binding β2m. To examine this possible requirement, heavy chain was synthesized in the absence of β2m. After lysis of the microsomes, purified human β2m was added to the lysates. As shown in Figure 4, heterodimers assemble efficiently even when β2m is added post-translationally. This implies that either β2m is not required for proper class I folding, or that the molecule remains able to attain the correct conformation well after synthesis is complete.

Effect of microsomes on heavy chain folding

Class I is an integral membrane protein. It is translocated into the ER via a signal peptide, which is cleaved, and anchored in the membrane by a hydrophobic

transmembrane sequence. It is therefore possible that membrane anchoring, translocation into the ER, or both are necessary for correct folding. In order to determine whether translocation is required, A2.1 was translated in the absence of microsomes. Excess purified β2m was added to the lysate after translation, since in the absence of microsomes, local concentrations of the two *in vitro* translated subunits does not reach high enough levels to allow association (data not shown). Assembly was then assayed by precipitation with W6/32. As seen in Figure 4, the heavy chain does assemble with β2m when translated in the absence of microsomes. The level of association is considerably diminished, however, when compared to A2.1 which has been synthesized in the presence of microsomes, implying that although translocation is not absolutely required, microsomes do play some role in making the class I molecule competent for assembly.

It is possible that the presence of the signal peptide on the untranslocated heavy chain interferes with folding of the molecule when it is synthesized free of microsomes. An A2.1 variant was employed to investigate this possibility, and assembly was compared in translations carried out in the presence or absence of microsomes. This construct encodes A2.1 lacking the signal peptide, resulting in a cytoplasmic protein, as it fails to be translocated into the ER. Although this variant is translated and recognized by the nonconformation-dependent anti-heavy chain antibody (data not shown), the experiment in Figure 4 shows that assembly with β2m is significantly reduced as compared to the full length A2.1, both in the presence and absence of microsomes. This reduction in the amount of β2m association correlates with that seen when the full length molecule is translated in the absence of microsomes. This result could be due to misfolding of non-membrane anchored molecules, or alternatively, some factor present in the lumen of the ER may play a role in class I folding and oligomerization.

A second A2.1 variant was used to resolve this question. This construct lacks a transmembrane domain, so although it is translocated into the ER, it is a soluble protein.

This transmembrane mutant was also translated in the presence or absence of microsomes, and its conformation was assessed by addition of excess β2m and immunoprecipitation with the complex-specific antibody. Assembly of this molecule paralleled that seen with the full length A2.1: protein that was translocated into microsomes associated with β2m more efficiently than that which was not translocated, but the microsome requirement was not absolute. Densitometric analysis of the precipitated bands revealed that assembly was about 35% as efficient in the nonmicrosomal translations. Therefore, although proper folding and assembly of A2.1 can occur in the absence of microsomes, the process is much more efficient for proteins which have been translocated into microsomes, implying that some factor in the ER lumen aids in folding of A2.1. An attractive hypothesis is that this factor is a molecular chaperonin, such as protein disulphide isomerase (PDI), a prolyl isomerase (PPI), or some other molecule, possibly specific to class I. These resident ER proteins have been shown or suggested to be important in correct folding and oligomerization of several other secreted and cell surface proteins *(Hsueh et al., 1986; Ellis, 1987)*.

Role of peptide in heterodimer stability

All of the above experiments were carried out in the absence of exogenous peptide. Since previous studies have firmly established that peptide is important in stable association of β2m with heavy chain *(Townsend et al., 1989; Ljunggren et al., 1990; Elliott et al., 1991; Townsend et al., 1990)*, it is relevant to ask why assembly is readily seen in this system without addition of peptide. Furthermore, adding peptide to the reaction does not augment association of the subunits to any extent (data not shown). One possible explanation involves temperature. It has been shown that RMA-S cells, when incubated at reduced temperature (below 26°C), are induced to express class I at the cell surface *(Ljunggren et al., 1990)*. As the *in vitro* translations are carried out at 25°C, this may explain the peptide-independent assembly seen here. When translation was carried out at 37°C, however, identical results were seen (Figure 5), discounting this possibility.

An alternative explanation that might account for the results presented here is that the heavy chain/β2m heterodimer is able to form and remains stable in the absence of peptide for the duration of the preclearing step used here (30 minutes to 2 hours). Addition of antibody then further stabilizes the complex, as has been reported previously *(Parham, 1983)*, throughout the course of the experiment. To investigate this possibility, translations were precleared for varying amounts of time, from 2 to 16 hours. W6/32 immunoprecipitated stable heterodimers after as much as 7 hours of incubation at 4°C (data not shown). Allowing the lysates to preclear overnight before addition of antibody, however, completely abrogated any binding of the complex-specific antibody (Figure 6), showing that the heterodimer is not stable for this extended period of time. Similar dissociation kinetics have very recently been shown for peptide-free class I complexes immunoprecipitated from RMA-S cells *(Elliott et al., 1991)*. These findings argue against a direct effect of temperature on the class I heavy chain, and suggest rather that increased temperature may instead decrease the stability of the heavy chain-β2m complex. At 37°C, dissociation of "empty" heterodimers occurs rapidly, either before or immediately upon reaching the cell surface, and before external antibody binding. At 26°C, dissociation kinetics may be slowed, allowing class I to remain associated with β2m long enough to bind antibody at the surface.

To further confirm the physiological relevance of the experiments presented here, it was determined whether class I complexes generated in this *in vitro* system could be stabilized for long periods of time by exogenous peptide. Translation was carried out as above, followed by lysis and addition of 200 uM influenza B nucleoprotein (BNP) peptide 85-93, which has been shown to bind specifically to HLA-A2.1 *(Robbins et al., 1989; Silver et al., 1991)*. Following a 16 hour preclear at 4°C, samples were precipitated with the complex-specific antibody. As shown in Figure 6, presence of the peptide was sufficient to retain the heavy chain/β2m heterodimers in W6/32-reactive form, whereas in the absence of added peptide, no heterodimers were detectable. This effect of

exogenous peptide rules out the possibility that the assembly seen in previous experiments is due to endogenous microsomal peptides, since these endogenous peptides would be expected to have a similar stabilizing effect on the heterodimer. Presence of 2 uM purified β2m also allowed recovery of intact complexes, showing that high concentration of this component was able to push the equilibrium towards association as has been seen with *in vivo* synthesized molecules *(Townsend et al., 1990).* No heterodimers were detected when initial translation was carried out without addition of 2 mM oxidized glutathione. Taken together, these experiments demonstrate that class I heavy chains and β2m synthesized in this *in vitro* system correctly assemble into heterodimers, but only in the presence of an oxidizing environment.

Requirement for disulphide bonding in heavy chain structure

The requirement for oxidation so clearly seen in this system strongly argues that disulphide bonds formed between cysteine residues play a critical role in correct folding of class I. From these experiments it is not clear whether the disulphide bridges are important to the structure of the class I molecule itself or to the putative chaperonin. In order to further clarify this issue, mutagenesis was carried out in which the cysteine residues in the α3, immunoglobulin-like domain of A2.1 were changed to serine. Cotranslation of β2m and the mutant A2.1 was carried out as above, followed by a short (30 minutes) preclearing step and immunoprecipitation with W6/32. Preliminary experiments show no assembly of the mutant with β2m under conditions which result in maximal assembly of the wild type A2.1 (data not shown). The implication of this experiment is that formation of the disulphide bond in the immunoglobulin domain of the heavy chain is absolutely required for its proper conformation and subsequent association with β2m.

Discussion

This study presents a cell-free system in which to study the synthesis of the MHC

class I molecule. Some advantages of this system are that translated proteins can be selectively radiolabeled, and components are easily manipulated. As shown here, the conditions of translation can be modified to examine their effects, and variant protein constructs can be specifically studied. Analysis of *in vitro* translated subunits of the class I heterodimer shows that the proteins are translated and translocated across the microsomal membrane, and the heavy chain is properly glycosylated with high efficiency.

Townsend et al. have proposed a three step model for class I assembly in the ER *(Townsend et al., 1990)*. First, newly translated heavy chain must fold correctly to attain a conformation that is competent to bind β2m. Upon reaching this conformation, it can then associate with either peptide or β2m, but these interactions are of relatively low affinity. High local concentrations of the components in the ER allow association to occur more efficiently. Subsequent association of the third component then stabilizes the complex, resulting in a ternary complex which can be transported through the secretory pathway and presented at the cell surface. While the second and third steps, assembly of the components, have been extensively studied, factors involved in the initial folding and conformation of the heavy chain have remained largely undefined. The present study addresses this facet of the process.

Several examples of incorrectly folded class I molecules, which are unable to associate with β2m, have been documented *(Myers et al., 1989; Schnabl et al., 1990)*, demonstrating that proper folding is not intrinsic to the primary sequence of the molecule. Although the presence of β2m cotranslationally has been implicated as being important in this process *(Myers et al., 1989)*, no evidence of a role for β2m could be demonstrated in these experiments. The reason for this inconsistency is not clear, but it is known that certain MHC haplotypes show different conformational characteristics in the absence of β2m *(Williams et al., 1989; Myers et al., 1989)*, indicating that different haplotypes may have distinct folding requirements. Other factors which could

potentially play a role in folding of the heavy chain were also investigated, and no effect of either peptide or membrane anchoring could be demonstrated, as assembly of the complex occurred normally in the absence of these factors.

Translocation across the ER membrane had a significant effect on the amount of assembly-competent A2.1 detected. Although microsomes were not absolutely required, their presence in the assay augmented assembly three-fold. The explanation that glycosylation of the heavy chain is important in heterodimer formation can be ruled out, since previous studies have shown that mutant class I molecules which lack a glycosylation site oligomerize and are transported normally *(Miyazaki et al., 1986; Santos-Aguado et al., 1987; Ploegh et al., 1981).* An alternative hypothesis is that a resident ER protein aids in folding the molecule. Such a chaperonin has been postulated by other investigators who have found specific association of class I with as yet uncharacterized proteins while it is in the ER *(Degen and Williams, 1991; Townsend et al., 1990).*

Assembly of the class I heterodimer was found to be absolutely dependent on the presence of oxidized glutathione, with maximal assembly occurring at concentrations which would neutralize exogenously added reducing agent. Nonspecific aggregation and anomalous effects of glutathione in general have been ruled out by demonstrating that use of reduced glutathione or an irrelevant antibody for precipitation does not result in precipitation of class I. Furthermore, these molecules dissociate with kinetics similar to those of *in vivo* synthesized heterodimers *(Elliott et al., 1991),* and the complexes can be stabilized for extended periods of time by the presence of specific peptide or excess quantities of β2m. These characteristics argue that provision of an oxidizing environment allows A2.1 molecules to correctly fold and assemble into functional complexes.

These results strongly imply that disulphide bonds play a role in proper folding of A2.1. The critical disulphides may be present in the class I molecule itself, since it is known that the heavy chain contains two sets of these bonds. Alternatively, the putative

chaperonin may require disulphide bonds for its structure or function. The fact that some heterodimers still form in the absence of microsomes, whereas assembly is completely abrogated in a reducing environment, suggests that the relevant disulphide bridges are in the class I molecule itself. This hypothesis is supported by the finding that a mutant A2.1 which lacks cysteines in its α3 domain does not appear to assemble into W6/32-precipitable complexes. It has been shown that mutation of one cysteine in the α3 domain of a murine class I, H-2L^d, prevents its transport to the cell surface *(Miyazaki et al., 1986)*, suggesting that the structure of the molecule has been disrupted. The presence of the remaining free cysteine residue may, however, result in anomalous protein-protein interactions, causing its retention in the ER. Interestingly, this mutant was found to associate with β2m *(Miyazaki et al., 1986)*, implying that disruption of α3 structure does not necessarily knock out dimerization, although this association may not be physiologically normal.

Taken together, the findings that disulphide bonds are important to the conformation of A2.1, and that translocation into microsomes aids in assembly, suggest a model in which a resident ER protein directs the formation of the correct disulphide bonds on the nascent A2.1 molecule. This protein could be protein disulphide isomerase or another molecule with similar activity, possibly specific to class I. In the absence of this chaperonin, in the microsome-free translation, A2.1 would form disulphides randomly, therefore attaining the correct conformation at a lower frequency. This model is also consistent with the experiments in which the oxidation state of the reaction was reversed after translation was complete. Addition of the oxidizing agent post-translationally would allow formation of disulphide bonds, but the bonding may be random at this late stage if the putative chaperonin has dissociated from the heavy chain *(Degen and Williams, 1991)*, or if the heavy chain has already folded incorrectly. This would result in the diminished assembly which is observed. On the other hand, once the molecule is synthesized in an oxidizing environment and therefore folded correctly, the disulphide

bonds may no longer be critical, but rather serve as a stabilizing factor. Reduction at this stage would then not completely abrogate assembly. Further support for directed disulphide bonding was shown in a recent report by Silver et al. *(Silver et al., 1991)*, in which purified, denatured class I molecules could be reconstituted *in vitro* with β2m and peptide, but with only partial efficiency.

Kvist et al. *(Kvist and Hamann, 1990)* have also reported study of class I assembly using components synthesized *in vitro*. This group found assembly of heterodimers only in the presence of exogenously added peptide, in contrast to the findings here. This may be due to a longer preclearing step before precipitation than is used in these experiments, although this information is not provided. Another difference in the results of this group is that they see association of heavy chain and β2m without addition of any oxidizing agent. Several possible explanations could account for this difference. First, DTT may not be present in the reticulocyte lysate used. As no negative control antibody is used in their experiments, a trivial explanation is that the peptide causes nonspecific aggregation and precipitation. This phenomenon has been seen in the course of these experiments with an influenza matrix protein peptide, and can not be ruled out. Finally, this group uses a different MHC haplotype, HLA-B27, which could have less stringent oxidation requirements for proper folding. It is important to emphasize that the experiments described here were performed with HLA-A2.1, and may not necessarily be generalizable to all other haplotypes.

The fact that class I molecules present primarily, if not exclusively, endogenously derived peptides suggests some mechanism by which the cell prevents expression of empty heterodimers which are capable of binding exogenous peptides at the cell surface. This may be due to an inherent instability of the heavy chain-β2m association, causing the molecule to fall apart in the time required to transport it through the secretory pathway at physiological temperature. The effect observed here of destabilization under reducing conditions suggests another possible point of regulation. It has been suggested

that the lumen of the Golgi apparatus is a reducing environment *(Feener et al., 1990)*. Therefore, empty heterodimers which enter this compartment in transit to the cell surface would be further destabilized, whereas complexes containing a peptide would be resistant to this destabilization. The Golgi then may act as a gate to screen out empty complexes from the transport pathway.

To summarize, HLA-A2.1 translated in a cell-free system was used to examine requirements for heavy chain folding. Membrane insertion and presence of β2m or peptide were not found to be necessary during heavy chain synthesis. The observed requirement for an oxidizing environment showed, however, that disulphide bonds are critical for correct folding, and an unknown factor present in the lumen of the endoplasmic reticulum, possibly a chaperonin molecule such as protein disulphide isomerase, seems to aid in this process.

METHODS

Mutagenesis: Oligonucleotide directed site-specific mutagenesis was performed essentially as described by Zoller and Smith *(Zoller and Smith, 1984a)*. In brief, the full-length A2.1 cDNA cloned into M13mp19 was used as a template for primer extension from two primers simultaneously. One primer was the M13 universal primer and the second was a phosphorylated mutagenic oligonucleotide of 20-25 nucleotides in length. Competent TG-1 cells were then transformed with the double-stranded product and mutant clones were detected by hybridizing plaque lifts with ^{32}P-end labelled mutagenic oligonucleotide. Miniprep single-stranded DNA was amplified by PCR and cloned into an appropriate expression vector.

Plasmids: cDNAs for full-length and signal peptide-deleted HLA-A2.1 and human β2m were inserted into a modified SP64 vector bearing the 5' untranslated region of Xenopus laevis β-globin. cDNAs for transmembrane-deleted and cysteine mutant HLA-A2.1 were inserted into pcDNA-neo (Invitrogen).

Transcription: RNA synthesis was carried out according to Promega protocol. Briefly, 5 ug of linearized plasmid DNA was transcribed in the presence of 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 100 ug/ml bovine serum albumin, 0.6 mM each ATP, CTP, UTP, and M^7GpppG (cap analogue), 60 μM GTP, 1 unit/μl RNase inhibitor, and 1 unit/μl SP6 or T7 RNA polymerase. Reactions were carried out for 60 minutes at 37°C, after which 0.6 mM GTP was added for 5-10 minutes. The DNA template was removed by treatment with DNAse 1 (Promega) for 15 minutes. RNA was phenol/chloroform extracted, precipitated with sodium acetate/ethanol, and resuspended in RNase-free water.

Translation: RNA was translated in rabbit reticulocyte lysate supplemented with 7.2 units per reaction of canine microsomal membranes per 50 µl reaction (all reagents from Promega). 2 mM oxidized glutathione (Sigma) was added to the reaction where indicated, and proteins were labeled with ^{35}S-methionine (translation grade, NEN). Reactions were carried out for 60-90 minutes at 25°C unless otherwise indicated. Translation was stopped by dilution and solubilization of membranes in ice cold lysis buffer (1% Triton-X 100, 10 mM HEPES, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 200 mM NaCl, 0.1 mM leupeptin, 2 mM PMSF).

Antibodies: All of the antibodies used in this study are of murine origin and, unless otherwise specified, were prepared from hybridomas obtained from the American Type Culture Collection. W6/32 is a murine anti-human class I complex antibody which recognizes a monomorphic determinant found only in heavy chain/β2m heterodimers and not free heavy chain *(Brodsky and Parham, 1982)*. C7 is a murine anti-human anti-LDL receptor antibody *(Beisiegel et al., 1981)*. It was used as a negative control, but seemed to give a low level of cross-reactivity with class I. This may be due to the fact that the antibody is peptide-specific, and a similar peptide may be present in class I. Later experiments were done using OKT4 as a negative control antibody. OKT4 is specific for human CD4 and gave cleaner results than C7. The anti-heavy chain antibody (Olympus) binds human class I heavy chain (A,B,or C) either free or complexed with β2m. It is not known whether any structural features of class I are important in binding of this antibody. BBM.1 is a murine antibody directed against human β2-microglobulin.

Protein Analysis: After translation, membranes were solubilized by addition of 300 µl lysis buffer per 50 µl translation. Samples were equilibrated to 50 mM Tris, 2% Triton X-100, 0.5% deoxycholate, 100 mM NaCl, and 50 µl 50% Protein A Sepharose was added to each. 20 µg/ml β2m or 200 µM influenza B virus nucleoprotein (BNP) 85-93

peptide *(Robbins et al., 1989)* was added where indicated. Lysates were then either analyzed directly by SDS-polyacrylamide gel electrophoresis or immunoprecipitated. Immunoprecipitation samples were precleared for 30-60 minutes unless otherwise stated. Lysates were incubated with the indicated antibody for 2 hours to overnight with rotating. Protein A Sepharose was again added along with 150 μg/ml bovine serum albumin. Sepharose beads were washed three times, reduced, and run on 15% SDS-polyacrylamide gels. Gels were processed for fluorography and exposed to Kodak Royal X-Omat film.

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FIGURES

Figure 1. Heavy chains translated in the presence of microsomes are translocated and glycosylated. RNA encoding HLA-A2.1 was translated *in vitro* in the presence of microsomal membranes. Total reaction products were mock-treated (lane 1) or treated with endoglycosidase H (lane 2), and subsequently separated by SDS-PAGE. The band which is not shifted by Endo H treatment is presumably untranslocated A2.1 which lacks a sugar residue but retains the signal peptide.

Figure 2. Heterodimer formation requires an oxidizing environment. RNAs encoding A2.1 and β2m were cotranslated in the presence of microsomes. A. Translation was carried out in the presence of 2 mM oxidized glutathione. Products were immunoprecipitated with, lane 1, the class I complex-specific antibody W6/32; lane 2, nonconformation-dependent anti-heavy chain antibody; lane 3, BBM.1, specific for β2m; or lane 4, an irrelevant antibody, C7, which is directed against the LDL-receptor. B. Translation reactions were supplemented with 0, 0.5, 1.0, or 2.0 mM oxidized glutathione, as indicated. Products were immunoprecipitated with W6/32 and analyzed by SDS-PAGE.

Figure 3. Effect of oxidation state is partially reversible. A2.1 and β2m were cotranslated in the presence of microsomes in reticulocyte lysate supplemented with 2 mM oxidized glutathione (lanes 1 and 2) or not supplemented (lanes 3 and 4). After translation for 60 minutes, the following additions were made and incubation continued for an additional 60 minutes before lysis: lanes 1 and 4, nothing; lane 2, 5 mM DTT; lane 3, 2 mM oxidized glutathione. Samples were immunoprecipitated with W6/32 and analyzed by SDS-PAGE.

Figure 4. Optimal assembly requires translocation into microsomes. RNAs encoding A2.1 (Full), signal peptide-negative A2.1 (Sp-), or transmembrane-negative A2.1 (Tm-) were translated in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of microsomes. 2 mM

oxidized glutathione was present in all reactions. 2 μM purified β2m was added to each reaction upon dilution and membrane solubilization. Samples were precleared overnight and subsequently immunoprecipitated with W6/32.

Figure 5. Temperature of translation does not affect heterodimer assembly. A2.1 and β2m were translated in the presence of microsomes and 2 mM oxidized glutathione. The reactions were carried out at either 25°C (lanes 1 and 2), or 37°C (lanes 3 and 4). Products were immunoprecipitated by either W6/32 or the nonconformation-dependent anti-heavy chain antibody (αHC), as indicated.

Figure 6. Heterodimers are unstable in the absence of peptide or excess β2m. A2.1 and β2m were cotranslated in the presence of microsomes in reticulocyte lysate containing 2 mM oxidized glutathione (lanes 1-3) or no glutathione (lanes 4-6). Upon dilution and membrane solubilization, the following additions were made: lanes 1 and 4, 200 μM BNP 85-93; lanes 2 and 5, no additions, lanes 3 and 6, 2 μM purified β2m. Samples were precleared overnight, followed by immunoprecipitation with W6/32 and SDS-PAGE analysis.

Figure 7. A schematic diagram of class I folding and assembly.

Figure 1

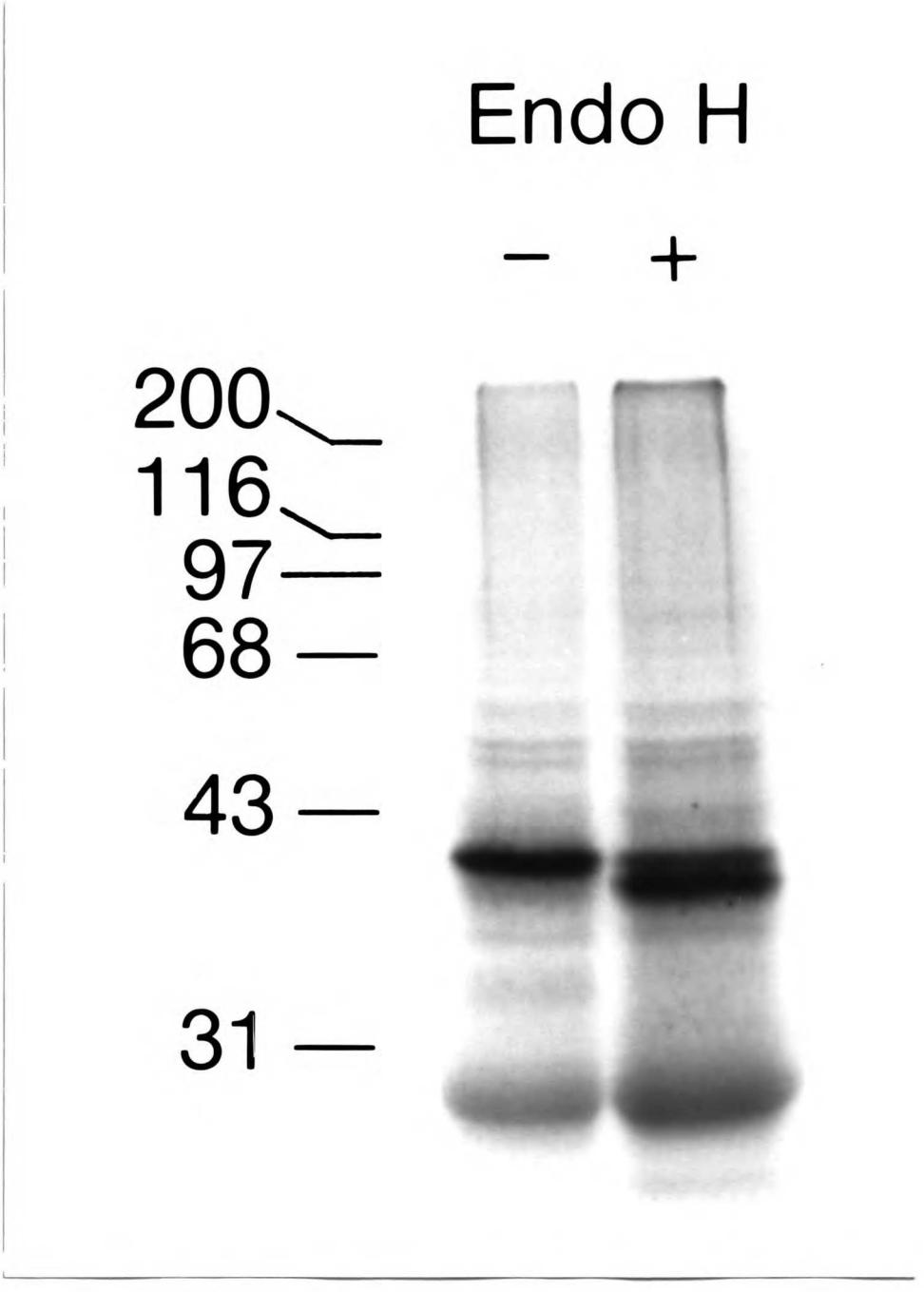

Figure 2

A.

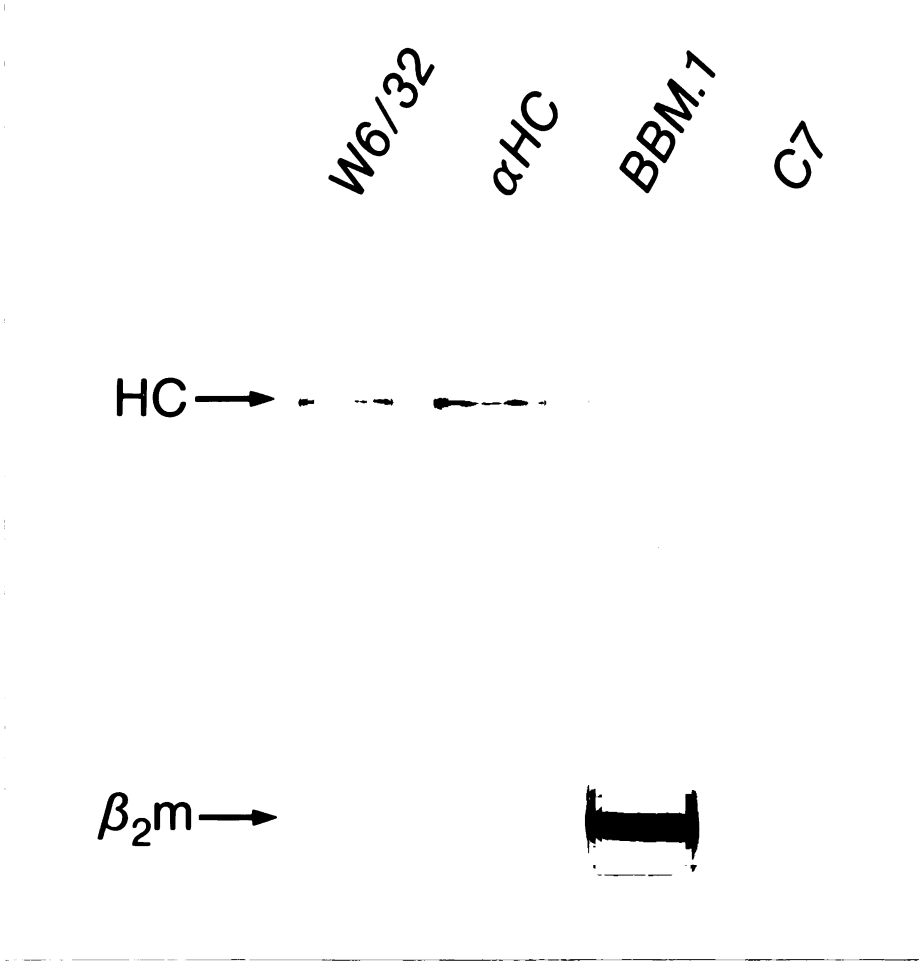

Figure 2

B.

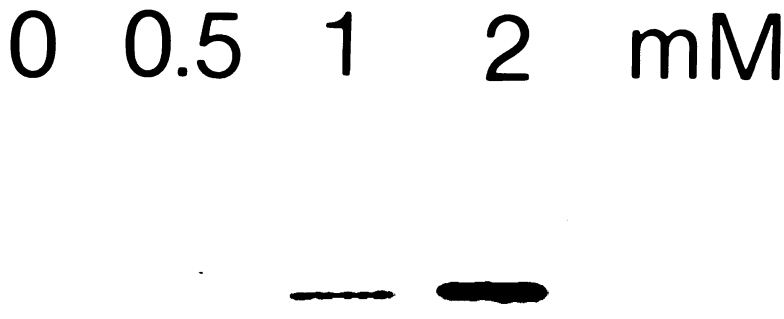

Figure 3

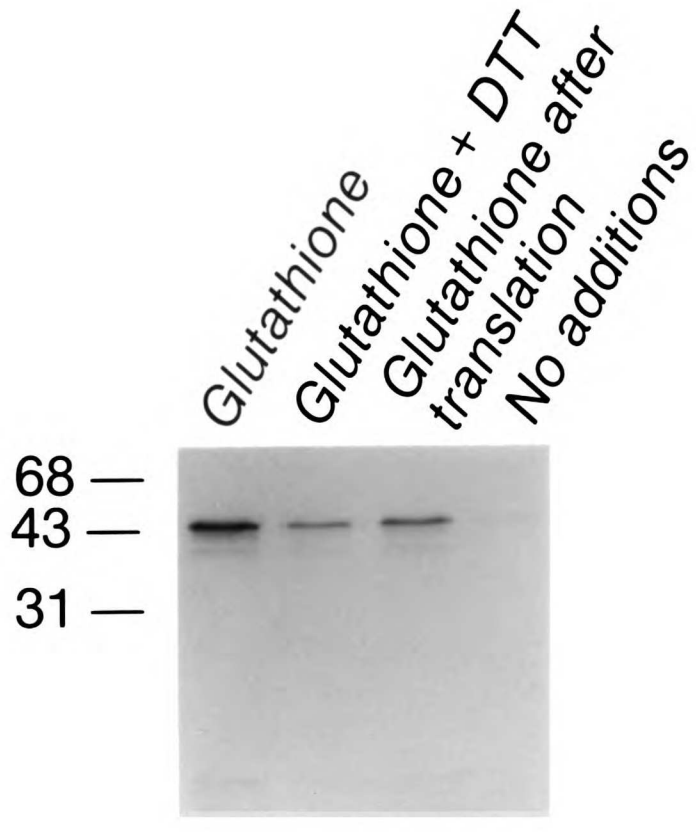
Glutathione
Glutathione + DTT
Glutathione after translation
No additions
68
43
31

Figure 4

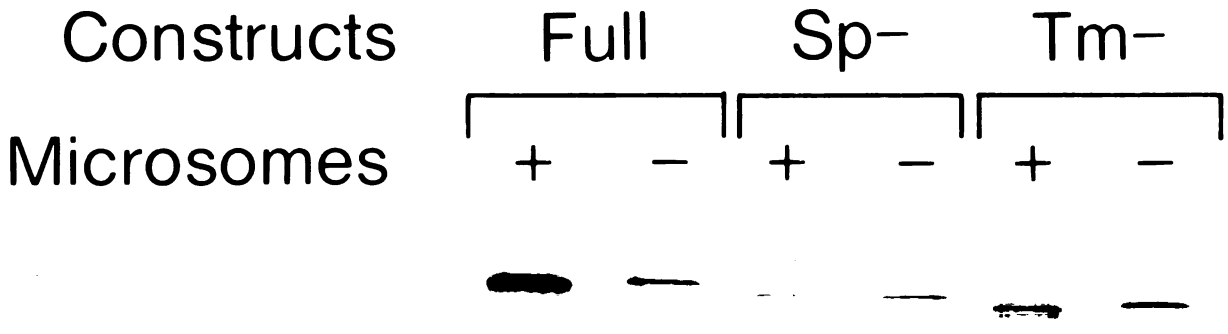

Figure 5

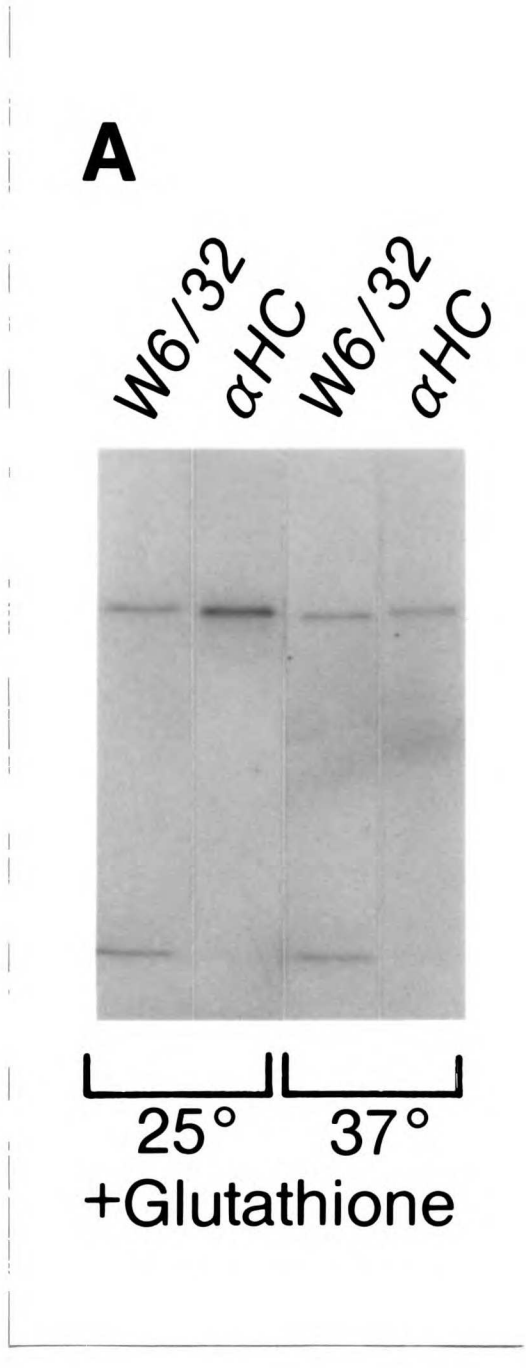

Figure 6

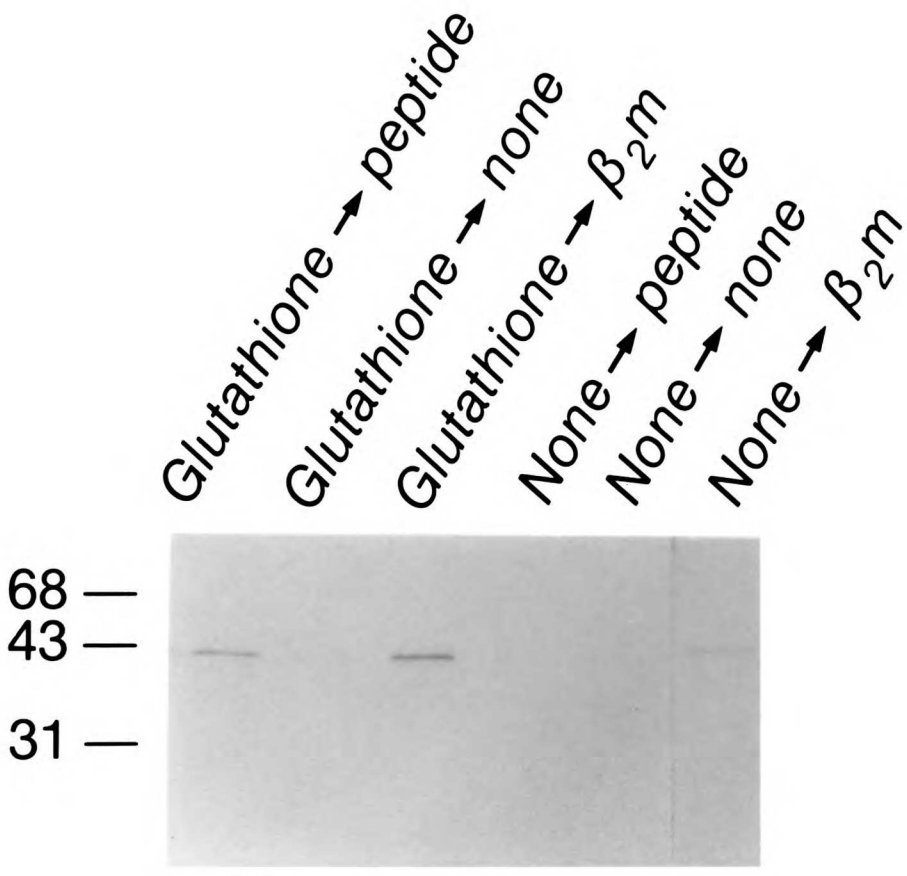
Glutathione → peptide
Glutathione → none
Glutathione → β2m
None → peptide
None → none
None → β2m
68
43
31

Figure 7