MHC Class I Heavy Chain mRNA Must Exceed a Threshold Level for the Reconstitution of Cell Surface Expression of Class I MHC Complexes in Cells Transformed by the Highly Oncogenic Adenovirus 12*

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In primary embryonal fibroblasts from transgenic mice expressing H-2b genes and a miniature swine class I transgene (PD1), transformation with adenovirus 12 results in suppression of assembly and cell surface expression of all class I complexes. Cell surface expression of PD1 can be recovered by transfecting the cells with peptide transporter genes. However, reconstitution of the H-2Kb gene expression requires, in addition, a 2-fold increase in the steady state level of the H-2Kb mRNA that can be attained by treatment of the cells with interferons or by transfecting them with the H-2Kb gene. A detailed analyses of the biogenesis of class I molecules has revealed the steady state expression of free class I heavy chains that are not converted into conformed complexes even when peptide transporter genes are overexpressed. The fact that class I complex assembly seems to be highly inefficient in certain cell lines might be a major in vivo obstacle for the elimination of transformed or virus-infected cells by cytotoxic T lymphocytes, especially in view of the fact that the level of class I gene transcription is often down-regulated in cancer cells and/or that assembly of class I major histocompatibility complexes can be subverted by virus-encoded proteins.

Major histocompatibility complex (MHC)1 class I molecules are polymorphic, integral membrane proteins that bind a diverse group of peptides derived from endogenous antigens and display these peptides for recognition by cytotoxic T lymphocytes (CTL) (1). This mechanism enables the immune system to control infectious diseases and the growth of tumor cells (2–4).

The biochemistry and cell biology of antigen processing and presentation by MHC class I molecules has been analyzed in detail in the recent years (5, 6). It is well established that the efficient transport of class I molecules to the cell surface depends on the assembly of the heavy chain/β2m dimer with peptides, most of which are generated by cleavage of proteins in the cytosol and are actively transported into the ER by a heterodimeric complex, known as transporter associated with antigen presentation (TAP). The trimeric complex is then transported through the Golgi apparatus to the cell surface. En route to complex formation, the nascent heavy chain transiently associates with the ER resident proteins calreticulin and calnexin, as well as with the adaptor molecule tapasin and with TAP (7–9).

Mutant cell lines (5, 6, 10, 11), “knockout” mice (12, 13), and tumor cells (4, 14, 15), which do not express TAP genes and/or β2m (16–18), are generally devoid of cell surface MHC class I molecules. We have previously shown that in cell lines derived from primary embryonal fibroblasts from transgenic mice expressing both the endogenous H-2 genes and a miniature swine class I transgene (PD1), transformation with the highly oncogenic Ad12 results in a significant reduction in peptide transporter (TAP1 and TAP2) and in proteasome-associated (LMP2 and LMP7) gene expression, and consequently in suppression of cell surface expression of all class I antigens (15, 19–21). The mRNA levels encoded by class I heavy chain and β2m genes in most of these cell lines are reduced by only 2–3-fold (15, 19). Expression of these genes is either normal or up-regulated in cells transformed by the non-oncogenic virus Ad5. Re-expression of TAP and LMP gene products by stable transfection of an Ad12-transformed cell line completely reconstituted the cell surface expression and assembly of PD1 but did not affect the expression of H-2Kb molecules (21). Enhanced expression of β2m in the cells further induced the assembly and expression of PD1 but did not affect the cell surface expression of H-2 molecules (22). These data raised the possibilities that a modest reduction in H-2 mRNA levels critically affects the efficiency of assembly of H-2 complexes, that the H-2 heavy chains are mutated, and/or that Ad12-transformed cells are deficient in a factor(s) that facilitates the assembly of β2m with a particular set of class I heavy chains. We now show that a modest enhancement in the steady state level of class I mRNA, above a threshold level, results in complete reconstitution of assembly and cell surface expression of fully conformed class I complexes. These studies brought to light the existence of a large pool of biologically inactive, free heavy chains in both “normal” and transformed cells that we characterized and whose biological significance we discuss.

Experimental Procedures

Cell Cultures—The Ad12-transformed (VAD12.79, VAD12.42), E1Ad5-transformed (A505), TAP-transfected cell line (VAD12.79/TAP), and the “normal” cell line M1 have been previously described (19–21).
Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 10% fetal calf serum, penicillin, streptomycin, gentamycin, and amphotericin B at the recommended concentrations (23). Media and supplements were purchased from Biological Industries (Bet Haemek, Israel).

Cell lines were transfected with 600 units/ml of IFN-α/β (Lee Biomedical Research Inc., San Diego) or 100 units/ml IFN-γ (Boehringer Mannheim, Germany) for the indicated time periods before harvesting.

Stable Transfection—VDAD12.79 and VAD12.79/TAP were transfected by the calcium phosphate-DNA coprecipitation method (21). The transfection mixture contained 10 μg of plasmid DNA (the H-2Kb cDNA expressed from a CMV promoter in pCMV DNA (Invitrogen)). 1 μg of plasmid DNA containing the puromycin gene (pBabe encoding the puromycin resistance gene was a kind gift from P. Murray, Whitehead Institute, Boston), and 5 μg of carrier DNA (sheared salmon sperm DNA, Sigma). 24 h after transfection, the cells were washed with phosphate-buffered saline and fresh medium was added; after 24 h, the medium was supplemented with 110 units/ml puromycin (Sigma). Following selection of puromycin-resistant cells, individual cell colonies were isolated and expanded in culture.

Antibodies—The following antibodies were routinely used for FACS analyses and immunoprecipitations: 20.8.4S (recognizes the α1/α2 epitope on H-2Kb) (24), 27.11.13 (recognizes an α1 epitope on H-2Dα) (24), PT85A (recognizes a public determinant on swine lymphocyte antigen, α1) (25), and rabbit antibodies directed against H-2Kb peptide 8 (26). Rabbit antibodies directed against free heavy chains (H-2Kb + H-2Dα) (27) were a kind gift from Prof. H. Ploegh (MIT). Other antibodies are cited in the text.

FACS Analysis—Cells were harvested by mild trypsinization, followed by washes in media supplemented with 5% fetal calf serum and 0.01% sodium azide. About 106 cells were incubated at 4 °C with the appropriate concentration of the first antibody for 60 min, washed, and then incubated in the dark for another 45 min with the second antibody. Cells were washed with phosphate-buffered saline, and fluorescence intensity was analyzed with a Becton-Dickinson cell sorter (Becton Dickinson, Mountain View, CA).

Metabolic Labeling, Protease Inhibitors, and Immunoprecipitation—Cells were grown to 80% confluence and starved for 60 min in methionine-free medium. They were then labeled in methionine-free medium containing 150 μCi/ml [35S]methionine (Amersham International, Little Chalfont, UK) for 60 min unless otherwise indicated, washed with phosphate-buffered saline, and chased for the indicated intervals. The cells were lysed with buffer containing 0.5% Triton X-100, 50 mM Tris (pH 7.5), and 150 mM NaCl. The immunoprecipitates were washed with buffer containing 0.1% Triton X-100, 50 mM Tris (pH 7.5), and 150 mM NaCl. For Endo H treatment, immunoprecipitates were eluted by adding 25 μl containing 50 mM Tris (pH 8), 1% SDS and boiled for 5 min. The samples were centrifuged, and the supernatant was added to 25 μl of 50 mM citrate buffer (pH 5.5) containing 1000 units of Endo H (New England Biolabs, Beverly, MA). The samples were incubated at 37 °C for 18 h, followed by the addition of sample buffer. All the buffers contained 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin (Sigma). Protein concentration was determined by Bradford reagent (Sigma), and equivalent protein amounts were loaded on the gels.

The protease inhibitor MG-132 at a concentration of 10 μM (Calbiochem) was added to the chase media as indicated.

The immunoprecipitates were fractionated on 10% or 15% SDS-polyacrylamide gel electrophoresis and X-Omat AR x-ray films (Eastman Kodak Co.) were exposed to the dried gels.

Probes and Plasmids—The following probes were previously described (19–21). The PD1 specific probe was a SacI-BamHI genomic fragment containing exons 2–7 of the PD1 gene; the H-2 probe was an EcoRI-HindIII fragment derived from pH-2-33 (H-2Kb); the β2m probe was a PstI-PstI fragment from β2m cDNA; ribosomal RNA probe (rRNA) was a HindIII-HindIII fragment derived from pWES. A plasmid containing H-2Kb cDNA was a kind gift from Dr. L. Eisenbach (The Weizmann Institute of Science, Rehovot, Israel).

Hybridizations—The hybridization solution contained 4× SSC, 50% formamide, 0.2% SDS, 0.1% polyvinylpyrrolidone, and 100 μg/ml salmon sperm DNA. Hybridizations were carried out at 42 °C, followed by washes with 2× SSC, 0.1% SDS at room temperature, and 0.2× SSC at temperatures ranging between 55 and 65 °C. After stripping with a boiling solution of 0.1% SDS, the blots were used for additional hybridizations.

RNA Analysis—Cytoplasmic RNA was prepared using a modification of the White and Bancroft method (28) as described previously (21). RNA was denatured and fractionated on a 1.2% formaldehyde/formamid
Reconstitution of Class I Expression in Transformed Cells

To examine if increased class I expression was due to increase in mRNA steady state levels, the effect of IFNs on class I heavy chain, \( \beta_{2m} \), and peptide transporter genes was tested for Ad12-transformed cell lines and compared with that of M1 and A505 cells. A representative experiment is shown in Fig. 2A, and the results of the individual experiments are summarized in Fig. 2B. In Fig. 2B, the PSL values for each mRNA have been normalized to those of rRNA, and the expression of H-2, PD1, and \( \beta_{2m} \) genes in untreated Ad12-transformed cells has been arbitrarily defined as 100% (Fig. 2B panels a and c). Because we could not detect a hybridization signal for TAP genes in untreated cells (see also Ref. 15), the hybridization signal values for TAP1 and TAP2 in treated cells were normalized to those of rRNA and are presented as 100 \times PSL units (Fig. 2B panels b and c). The figure shows that the expression of \( \beta_{2m} \) following treatment with \( \gamma \)-IFN is induced by 2-fold (a and c) and the expressions of TAP1 and TAP2 are induced from zero to levels that are equal or higher than those expressed by M1 and A505 cells (compare panels b and c to panel d), whereas the expression of H-2 class I heavy chain mRNA increases by a maximum of 1.5-fold, and the expression of PD1 is not induced at all (a and c). Quantitative reverse transcriptase-polymerase chain reaction was utilized to distinguish H-D\(^b\) from H-2K\(^b\) mRNA (22) and demonstrated that both were comparably induced (data not shown). The compiled data demonstrate that the transcription of \( \beta_{2m} \) and TAP genes is more effectively induced by IFN-\( \gamma \) then by IFN-\( \alpha/\beta \) and that both IFNs affect to the same extent (1.5-fold induction) the steady state level of H-2 heavy chain mRNA.

2-Fold Increase in the Steady State Level of Class I Heavy Chain mRNA Increases Dramatically the Cell Surface Expression of Class I Complexes—Previous analyses of several individual Ad12-transformed cell lines revealed the following: (a) there is only 1.5–3-fold decrease in the H-2 steady state mRNA levels of Ad12-transformed cells as compared with the “normal” or E1Ad5-transformed cell lines, yet Ad12-transformed cells did not express any cell surface H-2 complexes (15, 19, 21, 22; see also Figs. 1–3); and (b) TAP-transfected Ad12-transformed cells express normal levels of PD1 but very low or undetectable levels of H-2 molecules, whereas the steady state level of PD1 is only 2-fold higher than that of H-2 mRNA (15, 21, 22; see also Fig. 3). These observations, in addition to the dramatic effects of IFNs on the cell surface expression of H-2 complexes relative to the weak enhancement in class I heavy chain mRNA levels, suggested that a small increase (1.5–2-fold) in the level of class I heavy chain mRNA can dramatically affect the cell surface expression of class I complexes. To directly test this hypothesis,
transfected cells. The E1Ad5-transformed cell line A505 expresses all class I molecules (the data for H-2Kb are not shown), whereas VAD12.79 expresses neither, as was demonstrated before (15, 21, 22). VAD12.79/TAP cells are reconstituted for the expression of PD1 but do not express H-2Kb complexes as was described previously (15, 21, 22). The re-expression of normal levels of H-2Kb mRNA induces high cell surface expression of H-2Kb complexes only in VAD12.79/TAP cells (VAD12.79/TAP/Kb). Hence, nearly complete reconstitution of H-2 expression in Ad12-transformed cells can be achieved by re-expression of peptide transporters coupled with a 2-fold induction in H-2Kb mRNA level. Thus, the level of cell surface expression of class I complexes increases exponentially when mRNA steady state levels are enhanced beyond a certain threshold. These data clearly show that class I heavy chain mRNA must exceed a certain threshold to generate efficient assembly of class I complexes for cell surface expression. The potential significance of this phenomenon for antigen presentation by tumor cells and virus-infected cells in vivo led us to perform a detailed analysis of class I heavy chain biogenesis in "normal" and transformed cells.

**Turnover Rate of Conformed and Free Heavy Chains in TAP-expressing and TAP-deficient cells**—Cell lysates prepared from the cell lines described above were immunoprecipitated with the conformation-dependent antibody 20.8.4S and the antihuman class I heavy chain antiserum (anti-HC) that immunoprecipitates the conformation-dependent antibody 20.8.4S and the anti-H-2Kb antibodies. Two experiments are shown in Fig. 4, A and B, demonstrating that the "normal" cell line M1 (Fig. 4A) and the E1Ad5-transformed cell line A505 (Fig. 4B) express, in addition to the fully conformed H-2Kb complexes, a large pool of free heavy chains. VAD12.79 cells (Fig. 4, A and B) do not express a significant level of conformed complexes but express a small amount of free heavy chains. The level of free heavy chains depends both on the amount of newly synthesized class I heavy chains and on the expression of TAP genes, as both VAD12.79/Kb (Fig. 4A) and especially VAD12.79/TAP (Fig. 4B) express higher levels of free heavy chains than the parental non-transfected cells (VAD12.79). VAD12.79/TAP/Kb cells (Fig. 4B) express more conformed H-2Kb complexes than VAD12.79/Kb, as expected, and these cells also express very high levels of free class I heavy chains. Because VAD12.79/TAP/Kb cells over-express TAP1 and TAP2 (15), the data suggest that there is a stable pool of free heavy chains in the cells even when peptide transporter genes are over-expressed; thus, presumably, a sufficient amount of class I binding peptides exists in the ER.

To test directly whether under such steady state conditions these free class I heavy chains can be converted to conformed class I complexes, the turnover rate of free heavy chains and of conformed class I complexes was analyzed in pulse-chase experiments using A505 cells. A505 cells were utilized because they express peptide transporter and LMP genes and, as shown in Fig. 4, both conformed class I complexes and free heavy chains. The results of a representative experiment are shown in Fig. 5A. Quantitation of these results as shown in Fig. 5C reveal that the free heavy chain pool (H-2Kb+H-2Db) is relatively stable with a half-life of about 2 h. By utilizing lower percentage acrylamide gels, the free H-2Kb and H-2Db nascent chains could be separated, and the half-life of each was determined and compared with that of the conformed H-2Kb complexes. The compiled data of three individual experiments are summarized in Fig. 5D. The data show that nascent H-2Kb and H-2Db chains have a half-life that ranges between 60 and 100 min, respectively, and that both have a shorter half-life than the half-life of conformed H-2Kb complexes. Hence, nearly complete reconstitution of H-2 expression in Ad12-transformed cells can be achieved by re-expression of peptide transporters coupled with a 2-fold induction in H-2Kb mRNA level. Thus, the level of cell surface expression of class I complexes increases exponentially when mRNA steady state levels are enhanced beyond a certain threshold. These data clearly show that class I heavy chain mRNA must exceed a certain threshold to generate efficient assembly of class I complexes for cell surface expression. The potential significance of this phenomenon for antigen presentation by tumor cells and virus-infected cells in vivo led us to perform a detailed analysis of class I heavy chain biogenesis in "normal" and transformed cells.

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and peptides and are converted into conformed complexes. However, treatment of the cells with the proteasome inhibitor MG-132 during the chase prevents the degradation of at least 50% of the free heavy chains such that their half-life is about 150 min (Fig. 6, A and B). The concentration of MG-132 used in these experiments does not affect the assembly and transport of class I complexes (data not shown), indicating that the enhanced expression of free heavy chains following MG-132 treatment does not result from the dissociation of fully conformed complexes. Moreover, the fact that the degradation of free heavy chains could be prevented, and yet the level and the half-life of conformed complexes were not affected, indicates that at steady state, free heavy chains are not converted into conformed class I complexes. Thus, a pool of free heavy chains is present in fibroblast-derived cell lines, it is slowly degraded intracellularly, apparently by the proteasome, and it does not serve as a major reservoir for the establishment of conformed class I complexes.

Despite the fact that A5O5 cells express TAP and LMP genes, we could not rule out the possibility that inefficient assembly of free heavy chains into conformed complexes results from a limiting amount of class I binding peptides in these cells. Thus, we postulated that if the multiple components of the antigen presentation machinery were in excess (as in IFN-γ-treated cells), free heavy chains might be induced to assemble into complexes. IFN-γ enhances the expression of TAP and LMP genes but does not enhance the expression of class I heavy chain genes in A5O5 cells. Consequently, if the free heavy chains have the potential to convert into conformed class I complexes following IFN-γ treatment, an increase in the level of conformed class I complexes in parallel to a decrease in the level of free heavy chains should be observed. Therefore, we next studied the effect of IFN-γ treatment on steady state level and maturation of conformed class I complexes and free class I heavy chains in A5O5 cells. Labeled lysates from A5O5 cells were first immunoprecipitated twice with antibodies directed against conformed H-2Kb complexes (20.8.4S) and then immunoprecipitated twice with anti-heavy chain antibodies (αHC) followed by immunoprecipitation twice with anti-peptide 8 antibodies (αpe8) that recognize both conformed and free heavy chains. These sequential immunoprecipitations enabled the quantitative estimation of each of these class I heavy chain populations in the cells. Fig. 7 shows that IFN-γ treatment (B)
The results of the final precipitation as indicated in the lowest lane. The cells were treated with Endo H as indicated in the figure. Each 20.8.4, anti-HC, and anti-peptide 8 and were either left untreated or were treated with Endo H as indicated in the figure. Each lane shows the results of the final precipitation as indicated in the lowest (+) in the lane.

for 48 h induces dramatically the quantity of both conformed and free heavy chains (compare panels A and B). Enhanced signals are observed immediately after the pulse and following a 3-h chase. Thus, IFN-γ treatment affects similarly the levels of conformed complexes and free heavy chains. As the quantity of free class I heavy chains increases in parallel to the increase in quantity of conformed class I complexes, it can be safely concluded that (a) free heavy chains are not converted to conformed complexes following IFN-γ treatment, (b) the efficient assembly of class I complexes results mainly from an increased amount of class I binding peptides in the ER, and (c) the enhanced expression of TAP or LMP genes or both results in an increased free heavy chain pool.

**DISCUSSION**

The expression of MHC class I molecules is essential in the immune response against viruses and tumor cells because they present antigenic peptides to CTL. In accordance with this key role in antigen presentation, MHC class I molecules are expressed in most cells, and their basal level of expression can be induced by a number of cytokines (29–31). The IFN-stimulated response element is the DNA binding site for factors of the IFN regulatory factor family, and it mediates the induction of MHC class I expression by type I and type II IFNs (32). In particular, IFN-γ, a product of activated T lymphocytes and natural killer cells, regulates the expression of different components in the pathway of MHC class I-restricted antigen presentation and is a potent inducer of class I heavy chain, β2m, TAP1, TAP2, LMP2, LMP7, and the regulator PA28 genes (33–37).

Certain cells, such as some tumor and normal cells (e.g., trophoblast cells), are down-regulated for the expression of either class I heavy chain genes or peptide transporter and LMP genes or both (2, 4, 38–44); consequently, these cells cannot be targets for killing mediated by CTL (45). IFNs can often enhance the cell surface expression of MHC class I complexes in such cells; however, despite the extensive information about IFNs and their functions, little information exists as how this is accomplished. Specifically, it is surprising that although IFNs dramatically stimulate class I expression, previous studies (44, 46) revealed only a 1.5–2-fold increase in the steady state mRNA levels encoded by class I heavy chain genes. This moderate effect of IFNs on class I heavy chain mRNA levels agrees with chloramphenicol acetyltransferase-transient transfection assays (47, 48) that showed a small effect on heavy chain gene transcription. Because IFN-induced class I expression is accompanied by much larger increases in TAP and LMP in comparison with class I heavy chain gene expression, it has been assumed that the availability of class I binding peptides is the limiting factor for assembly of class I complexes (49).

We describe a set of “normal” E1Ad5- and Ad12-transformed cell lines that respond to IFN treatment. Ad12-transformed cells are down-regulated for the cell surface expression of class I molecules, but this expression can be induced by IFNs such that it reaches normal levels for all class I molecules (Fig. 1, compare panels A–C with panel D). A detailed analysis of mRNA steady state levels encoded by class I heavy chain, β2m, TAP1, and TAP2 genes revealed that although class I heavy chain gene expression levels are induced by a maximum of 1.5-fold, the induction of peptide transporter gene expression is more dramatic going from zero to nearly normal levels (Fig. 2B, compare panels a and b). Previous studies had shown that the protein expression of LMP subunits was also induced to nearly normal levels (21). The compiled data are in accordance with the concept that the most significant effect of IFNs, and in particular of IFN-γ, is on peptide production and their transport to the ER. Consequently, we anticipated that the reexpression of TAP genes in Ad12-transformed cells would reconstitute the expression of class I complexes. However, in Ad12-transformed cells that have been stably transfected with TAP genes or with TAP+LMP genes, the assembly and cell surface expression of the transgene product (PD1) is reconstituted, whereas the expression of H-2Kb remains undetectable or very low (15, 21, 22). One possible explanation for this could be that in Ad12-transformed cells the moderate suppression in the steady state level of H-2Kb mRNA and, consequently, in the amount of newly synthesized class I heavy chains (21, 22) significantly reduces the efficiency of assembly of H-2Kb complexes. This explanation is in accordance with the fact that the expression of PD1 mRNA in all the cells (including VAD12.79/TAP cells in which PD1 cell surface expression, but not H-2, is reconstituted) is 2–3-fold higher than that of the H-2 genes.

To directly test this hypothesis, an Ad12-transformed cell line (VAD12.79) and the TAP-reconstituted VAD12.79 cell line (VAD12.79/TAP) were stably transfected with a vector expressing H-2Kb from a CMV promoter. Individual clones that expressed H-2Kb mRNA in comparable levels with those of the “normal” (M1 cells) or E1Ad5-transformed (A505) cells were isolated. The FACS analyses of the transfected cells demonstrated that indeed VAD12.79 cells expressing peptide transporters coupled with 2-fold higher levels of H-2Kb mRNA (VAD12.79/TAP/Kb) are completely reconstituted for the cell surface expression of H-2Kb (Fig. 3). Collectively, the data show that when peptide transporters are expressed, and thus, presumably, peptides are available for assembly, a 1.5–2-fold induction in the amount of newly synthesized class I heavy chains results in a dramatic increase in cell surface expression of class I complexes. Thus, assembly of class I molecules and consequently expression seems to occur once a threshold level of class I heavy chain expression is surpassed—a phenomenon that might significantly impact upon the ability of cells (especially non-lymphoid cells that normally express low levels of class I molecules) to function in peptide presentation in vivo. Specific examples are primary human tumors and metastases of both human and murine tumors that demonstrate down-regulation of class I heavy chain gene expression (2–4) and cells infected with adenoviruses of subclass C or with human.
CMV that express viral proteins such as the gp19 (adenoviruses) and US3 (human CMV) that bind class I heavy chains and thus interfere with assembly of class I complexes (50, 51). In these cases, a modest increase in the amount of newly synthesized class I molecules might reverse significantly host immune responses in vivo.

To delineate the factors that might enhance assembly of class I complexes, we have analyzed the synthesis and maturation of class I heavy chains at steady state levels in “normal” cells and tumor cells that have been reconstituted for the expression of either peptide transporter or class I heavy chain genes or both, with and without treatment with IFN-γ. Our data demonstrate that assembly of class I complexes is inefficient in fibroblast-derived cell lines and that a large and relatively stable pool of free heavy chains that directly depends on the expression level of both class I heavy chain and TAP genes exists in these cells (Fig. 4, compare the signal obtained in VAD12.79 with that obtained in VAD12.79/Kb and VAD12.79/TAP). For instance, VAD12.79/TAP cells express a significant amount of free heavy chains but only low levels of confirmed H-2Kb molecules. The latter are probably highly unstable, as (a) associated β2m molecules are not detectable in communoprecipitates (see Fig. 4B, 12.79/TAP) and (b) these cells do not express cell surface H-2Kb molecules (Fig. 3). A significant amount of confirmed H-2Kb complexes can be detected only when the expression of both peptide transporter and class I heavy chain genes is reconstituted (Fig. 4B, compare VAD12.79/TAP and VAD12.79/TAP/Kb). The VAD12.79/TAP/Kb cells are similar to the “normal” and E1AD5-transformed cells in that they express not only confirmed H-2Kb complexes but a large pool of free heavy chains. Our data suggest that the expression of peptide transporters is essential not only for the assembly of confirmed class I complexes but also for the maintenance of free heavy chains. Whether the physical presence of peptide transporters is important for the stabilization of associated free heavy chains or the transported peptides can bind free heavy chains in the absence of associated β2m and stabilize them has yet to be determined. Solheim et al. (7) demonstrated that free human heavy chains can be found associated with peptide transporters in β2m-deficient cell lines, supporting the possibility that the direct association of free heavy chains with TAP might stabilize at least some of the free heavy chains.

In view of the data described above, one could speculate that a pool of free heavy chains needs to be established in the cells prior to the assembly of the heavy chains into confirmed complexes. In accordance with this hypothesis are data demonstrating the appearance of fully confirmed heavy chains in parallel to a rapid decrease in free heavy chains in RMA cells following a 1-min pulse with [35S]Met (26), suggesting that free heavy chains are directly converted to confirmed complexes. In contrast, our pulse-chase experiments performed with E1AD5-transformed cells that express high levels of class I molecules on the cell surface showed that the free heavy chains observed at steady state are relatively stable with a half-life of 60–100 min (Fig. 5D) and that they remain Endo H sensitive throughout the chase period (Fig. 5B). They are slowly degraded within 3 h (Fig. 6) and are not converted into fully confirmed class I complexes. Hence, our results support a model in which a variable percentage of newly synthesized heavy chains are not assembled into class I complexes and are eliminated from the cells as part of a quality control mechanism. The relative percentage of free heavy chains does not change even in cells pretreated with IFN-γ (Fig. 7). In fibroblast-derived cell lines, the biologically inactive heavy chain pool might be larger and more stable than in lymphoid cells because of reduced amounts of another molecule(s), such as tapasin, which assembles assembly of class I complexes, or from a slower degradation process of misfolded molecules.

In summary, our data suggest that class I complex formation in fibroblasts and probably in other non-professional antigen-presenting cells is highly inefficient and, indeed, does not occur to a significant degree until a threshold level of expression of peptide transporter and class I heavy chain proteins is exceeded. Even in the presence of optimal levels of peptide transporter and class I heavy chain molecules, about half of the newly synthesized class I heavy chains are not assembled into mature class I complexes and are degraded intracellularly. In cells where heavy chains are not limiting, IFN-γ treatment dramatically enhances the expression of both confirmed class I complexes and free heavy chains. It is likely that the induction results from the enhanced transcription of the LMP and peptide transporter genes and, consequently, the increase in class I binding peptides in the ER. It will be interesting to determine whether free heavy chains are incapable of binding β2m and peptides as was previously suggested (52–54) or if they are located in a subcompartment that is inaccessible to peptides and/or β2m or both. Studies are currently underway to answer these questions and to determine what factors can facilitate assembly of class I complexes in both normal and transformed cells.

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REFERENCES

1. Zinkernagel, R., and Doherty, P. C. (1979) Adv. Immunol. 22, 51–177.
2. Goodenough, B. S., Vogel, J. M., and Linsk, R. L. (1985) Science 229, 777–783.
3. Hammerling, G. J., Klar, D., Pulm, W., Momburg, F., and Moldenhauer, G. (1987) Biochem. Biophys. Acta 979, 245–259.
4. Ehlich, R. (1995) Immunol. Rev. 14, 77–97.
5. Yewdell J. W., and Bennink, J. R. (1992) Adv. Immunol. 52, 1–123.
6. Germain R. N., and Margulies, D. H. (1993) Annu. Rev. Immunol. 11, 403–450.
7. Solheim, J. C., Harris, M. R., Kindle, C. S., and Hansen, T. H. (1997) J. Immunol. 158, 2226–2241.
8. Andreolewicz, M. J., Ortsman, B. M., Spies, T., and Cresswell, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12716–12720.
9. Ortsman, B., Andreolewicz, M., and Cresswell, P. (1994) Nature 368, 864–867.
10. Spies, T., and DeMars, R. (1991) Natural 351, 323–324.
11. Attaya, M., Jameson, S., Martinez, C. K., Hermel, E., Aldrich, C., Forman, J., Fischer Lundahl, K., Bevan, M. J., and Monaco, J. J. (1992) Nature 355, 647–649.
12. Van Kaer, L., Ashton-Rickardt, P. G., Ploegh, H. L., and Tonegawa, S. (1992) Cell. 71, 1205–1214.
13. Arnold, D., Driscoll, J., Andreolewicz, M., Hughes, E., Cresswell, P., and Spies, T. (1992) Nature 360, 171–174.
14. Restifo, N. P., Esquivel, F., Kawakami, Y., Yewdell, J. W., Mule, J. J., Rosenberg, S. A., and Bennink, J. (1993) J. Exp. Med. 177, 265–272.
15. Rotem-Yehudar, R., Vinod, S., Kolter, B. H., and Ehrlich, R. (1994) J. Exp. Med. 180, 477–488.
16. Williams, D. B., Barber, H. B., Flavell, R. A., and Allen, H. (1989) J. Immunol. 142, 2576–2586.
17. Zijlstra, M., Bix, M., Simister, N. R., Loring, J. M., Raulet, D. H., and Jaenic, R. (1990) Nature 344, 742–746.
18. Kolter, B. H., Marrack, P., Kappler, J. W., and Smithies, O. (1990) Science 248, 1227–1230.
19. Shemes, J., Rotem-Yehudar, R., and Ehrlich, R. (1991) J. Virol. 65, 5544–5548.
20. Shemes, J., and Ehrlich, R. (1993) J. Biol. Chem. 268, 15704–15711.
21. Rotem-Yehudar, R., Groettrup, M., Soza, A., Kloetzel, P. M., and Ehrlich, R. (1996) J. Exp. Med. 183, 499–514.
22. Movat, S. V., Schechter, C., and Ehrlich, R. (1997) J. Biol. Chem. 272, 353–361.
23. Jacoby, W. B., and Pastan, I. H. (1979) Methods Enzymol. 58, 110–116.
24. Ozato, K., and Sachs, D. H. (1981) J. Immunol. 126, 317–321.
25. Davis, W. C., Marusz, S., Lewis, H. A., Splitter, G. J., Perryman, L. E., McGuire, T. C., and Gorham, J. R. (1987) Vet. Immunol. Immunopathol. 15, 337–376.
26. Schindler, C., and Darnell, J. E., Jr. (1995) Ann. Rev. Biochem. 64, 621–625.
27. Gussow, D., Reim, B., Ginjaar, I., Hochstenbach, F., Seemann, G., Kottman, A., and Ploegh, H. L. (1987) J. Immunol. 139, 3132–3138.
34. Yang, Y., Walters, J. B., Frueh, K., and Peterson, P. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4928–4932
35. Wright, K. L., White, I. C., Kelly, A., Beck, S., Trowsdale, J., and Ting, J. P.-Y. (1995) J. Exp. Med. 181, 1459–1471
36. Min, W., Pober, J. S., and Johnson, D. R. (1996) J. Immunol. 156, 3174–3183
37. Reale, C., Dubiel, W., Pratt, G., Ferrell, K., and Rechsteiner, M. (1994) J. Biol. Chem. 269, 20727–20732
38. Restifo, N. P., Esquivel, F., Kawakami, Y., Yewdell, J. W., Mule, J. J., Rosenberg, S. A., and Benink, J. (1995) J. Exp. Med. 181, 1459–1472
39. Cromme, F. V., Airey, J., Heemels, M.-T., Ploegh, H. L., Keating, P. J., Stern, P. L., Meijer, C. J. L. M., and Walboomers, J. M. M. (1994) J. Exp. Med. 179, 335–340
40. Kanna, R., Burrows, S. R., Arguet, V., and Moss, D. (1994) Int. Immunol. 6, 639–645
41. Cromme, F. V., Meijer, C. J. L. M., Snijders, P. J. F., Uyterlinde, U., Kenemans, P., Helmerhorst, Th., Stern, P. L., van der Brule, A. J. C., and Walboomers, J. M. (1993) Br. J. Cancer. 67, 1372–1380
42. Gavioli, R., De Campos-Lima, P.-O., Kurilla, M. G., Kieff, E., Klein, G., and Masucci, M. G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5862–5866
43. Cromme, F. V., van Bommel, P. F. J., Walboomers, J. M. M., Gallee, M. P. W., Stern, P. L., Kenemans, P., Helmerhorst, Th. J. M., Stukart, M. J., and Meijer, C. J. L. M. (1994) Br. J. Cancer. 69, 1176–1181
44. Rodriguez, A.-M., Mallet, M. V., Lenfant, F., Arnaud, J., Girr, M., Urlinger, S., Benussan, A., and Le Bouteiller, P. (1997) Eur. J. Immunol. 27, 45–54
45. Geginat, G., Ruppert, T., Hengel, H., Holmappels, R., and Koszinowski, U. K. (1997) J. Immunol. 158, 3303–3310
46. Ehrlich, R., Sharrow, S. O., Maguire, J. E., and Singer, D. S. (1989) Immunogenetics 30, 18–26
47. Israel, A., Kimura, A., Fournier, A., Fellous, M., and Kourilsky, P. (1986) Nature 322, 743–746
48. Korber, B., Mermod, N., Hood, L., and Stroynowski, I. (1988) Science 239, 1302–1305
49. Villanueva, M. S., Fischer, P., Feen, K., and Pamer, E. G. (1994) Immunity 1, 479–489
50. Andersson, M., Paabo, T., Nilsson, T., and Peterson, P. A. (1985) Cell 43, 215–222
51. Jones, T. R., Wiertz, E. J. H. J., Sun, L., Fish, K. N., Nelson, J. A., and Ploegh, H. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11327–11333
52. Kozlowski, S., Takeshita, T., Boehncke, W. H., Takahashi, H., Boyd, L. F., Germain, R. N., Berzofsky, J. A., and Margulies, D. H. (1991) Nature 349, 74–77
53. Boyd, L. F., Kozlowski, S., and Margulies, D. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2242–2246
54. Otten, G. R., Bikoff, E., Ribaudo, R. K., Kozlowski, S., Margulies, D. H., and Germain, R. (1992) J. Immunol. 148, 3723–3732