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Association of class I major histocompatibility heavy and light chains induced by viral peptides

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We describe a cell in which association of a major histocompatibility complex class I heavy chain with β2-microglobulin is induced by a peptide derived from influenza nucleoprotein. Association of antigenic peptides with the binding site of class I molecules may be required for correct folding of the heavy chain, association with β2-microglobulin and transport of the antigen-MHC complex to the cell surface.

The majority of cytotoxic T lymphocytes (CTL) recognize epitopes of viral or other foreign proteins in association with class I major histocompatibility complex (MHC) molecules. Epitopes are generated from protein antigens synthesized in the cytoplasm, and are presented at the surface of the cell in a form that can be mimicked in vitro by incubation with short synthetic peptides.

These results have led to the suggestion that the proteins recognized by class I-restricted CTL are degraded in the cytoplasm, and the peptides derived from them transported to the cell surface in association with class I molecules of the MHC.

Although evidence has accumulated that is consistent with this hypothesis, the mechanisms by which epitopes are generated, transported and presented with class I molecules are not known.

One approach to this problem is to study cell mutants which seem to lack one or more of these functions. We have investigated a tumor cell line that escapes immunological rejection in vivo across a minor histocompatibility barrier (Öhlin et al., unpublished observations). It has a known defect in the association of β2-microglobulin with class I heavy chains, and a reduction of ~95% in the level of assembled class I molecules expressed at the cell surface. This cell has lost the ability to present an epitope from influenza nucleoprotein synthesized in the cytoplasm, but does present the same epitope when exposed to it as a synthetic peptide in the extracellular fluid. This paradox is resolved by showing that exposure of the cell to extracellular peptide induces assembly, transport and surface expression of class I molecules. Association of peptides with the binding site on the class I heavy chain may be required for stable association of the heavy chain with β2-microglobulin, and expression of the peptide-MHC complex at the cell surface.

Endogenous antigen

The mutant cell line RMA-S was derived from the Rauscher virus-induced H-2b lymphoma RBL-5 by exposure to the mutant ethyl methane sulphonate (EMS) and repeated rounds of treatment with antisera against class I molecules and complement.

It expresses ~1/20 of the amount of H-2D, K and β2-microglobulin at the cell surface when compared with RBL-5 cells exposed to EMS but not selected with antibodies (referred to as RMA). RMA-S synthesizes both class I heavy chains and β2-microglobulin, but most of the heavy chains bear high mannose oligosaccharides, do not associate with β2-microglobulin and remain intracellular.

The mutant RMA-S was compared with RMA as a target for recognition by a cytotoxic T-cell clone (F5) specific for influenza nucleoprotein (NP) in association with the class I molecule H-2D (ref. 3). RMA was efficiently recognized and killed by clone F5 after infection with influenza virus, whereas RMA-S was resistant to lysis in identical conditions (Fig. 1a). The inability of this CTL clone to recognize infected RMA-S cells was not due to inefficient infection by the virus, as synthesis and degradation of NP in infected RMA-S were not impaired (data not shown).

RMA-S and RMA were then compared as targets after treatment with the peptide epitope recognized by the CTL clone

![Graph](image-url)
Fig. 2. The indirect immunofluorescence staining of RMA-S cells exposed to various peptides for 5 or 24 h. a: Background staining with no first antibody (NFA); b: Low level B2.2.49 (D\(^2\)) specific staining detected on untreated RMA-S cells. The remaining panels show RMA-S cells stained with B2.2.49 after treatment with various peptides: c: NP5(1968) 147-158 for 5 h; d: retro-D isomer of NP5(1968) 366-379 for 5 h; e: NP5(1968) 345-360 for 5 h; f: NP5(1964) 365-380 for 5 h; g: NP5(1968) 50-63 for 5 h; h: NP5(1968) 366-379 for 5 h; i: H-2\(^D\)\(^\alpha\) residues 171-182 (ref. 19) for 5 h; j: NP5(1934) 365-380 for 24 h; k: NP5(1968) 50-63 for 24 h; l: NP5(1968) 366-379 for 24 h; m: H-2\(^D\)\(^\alpha\) residues 171-182 for 24 h.

METHODS. RMA-S cells (5 x 10\(^4\)) were exposed to medium alone or peptides at 5 x 10\(^{-5}\) M in a total volume of 1.5 ml for the stated times. Cells were then collected and stained by indirect immunofluorescence as described. The first layer was the D\(^\alpha\) specific monoclonal antibody B2.2.49 (ref. 45) as neat culture supernatant (C/S), and the second layer was FITC labelled affinity purified goat anti-mouse antibody (Sigma) at 1:40 dilution. The samples were analysed on an Ortho Cytofluorograf.

Cell surface expression of class I

A comparison of RMA-S before and after exposure to NP (1968) 366-379, or to the related sequence NP5(1934) 365-380, revealed an increase in D\(^\alpha\) expression of between two- and fivefold (Fig. 2f, j, h and i). This was detected with an antibody demonstrated to bind only to heavy chains associated with \(\beta\)_microglobulin\(^{12}\).

We then tested two additional peptides that prevent presentation of the sequence NP5(1968) 366-379 to the CTL clone F5\(^{10}\). The inhibitory effect of these peptides indicates that they bind the D\(^\alpha\) molecule\(^9\) although this has not been demonstrated directly. One peptide was derived from the NP sequence (residues 50-63). The other is from a conserved region of the D\(^\alpha\) molecule itself (residues 171-182). We chose the former because of its homology with the equivalent sequence from

![Graph](image)

Fig. 3. Recognition by clone F5 of RMA-S cells exposed to NP(1968) 366-379 is inhibited by defined competitor peptides. a: Three sequences that compete efficiently: NP5(1934) 365-380; NP5(1968) 50-63; H-2\(^D\)\(^\alpha\) residues 171-182; no competitor. b: Three sequences that do not compete: retro-D isomer of NP5(1968) 366-379; NP5(1968) 147-158; NP5(1968) 345-360; no competitor.

METHODS. \(^{14}\)C labelled RMA-S cells were exposed to the peptide NP5(1968) 366-379 at the concentrations shown either alone, or in combination with competitor peptides at a fixed concentration of 10\(^{-4}\) M. The ratios of competitors to NP5(1968) 366-379 therefore ranged between 10\(^{-4}\) to 10\(^{-1}\). The competitor sequences were found by trial and error as described\(^{13}\) and were derived from published influenza NP sequences\(^{8}\) except for the peptide composed of residues 171-182 of H-2\(^D\)\(^\alpha\) which was chosen on the basis of its relationship to the homologous sequence in HLA-Cw3, which has similar inhibitory activity\(^{19\text{-}21}\).
HLA Cw3, which has a comparable inhibitory activity. To ensure that the Dα molecules on RMA-S (which had been exposed to a mutagen) retained their specificity for peptides, the inhibitory effect of these peptides was assayed using RMA-S cells (Fig. 3a and b).

The peptides that inhibited the recognition of RMA-S by the Dα-restricted clone F5 also induced expression of Dα at the surface of RMA-S cells (Fig. 3g and i). The three control peptides did not inhibit recognition by clone F5, nor did they induce expression of Dα (Fig. 3c, d and e). There is therefore a correlation between induction of Dα expression on RMA-S and the ability to inhibit presentation of NP(1968) 366–379 in the lysis assay, implying that only peptides that bind Dα induce its expression.

The class I molecule Dα is unusual because it can reach the cell surface in the absence of endogenous β2-microglobulin synthesis in the murine cell line RIE (ref. 22). Most class I antibodies bind MHC class I heavy chains only when they associate with β2-microglobulin but free Dα heavy chains can be detected with the antibody 28148S, which binds the α3 domain of both free and β2-microglobulin-associated heavy chains. Free Dα heavy chains were not detected by an excess of an inhibitory antibody.

**METHODS** Aliquots of 5×10⁶ RMA-S cells in 0.75 ml medium (RPMI 1640/10% FCS) were placed in Costar 24-well plates. At times 0, 1, 2, 3, 4 and 5, 0.75 ml of cell medium containing twice the desired concentration of peptide was added to appropriate aliquots of cells. At 5 h all the cells were collected and stained by indirect immunofluorescence with antibody 2B2.249 as described in Fig. 2. b. All aliquots of cells were incubated with peptide for 6 h at 37°C. The final concentrations of peptides ranged from 1–10024×10⁻⁶ M as shown.
of 28148S binding on the surface of RMA-S cells, either before or after induction with peptides (Fig. 4e and k).

We used additional class I-specific monoclonal antibodies to show that the effect of peptides on RMA-S is H-2 allele-specific (Fig. 4g). Treatment with NP (Fig. 4h) induced D\(^{\alpha}\) (compare Fig. 4a-d with 4i-j), but not K\(^{\beta}\) (Fig. 4e and m). In contrast, NP (1968) 345-360 has the opposite effect, inducing K\(^{\beta}\) (Fig. 4i) but not D\(^{\alpha}\) (Fig. 4f-h). The polyclonal cytotoxic T-cell response to influenza nucleoprotein in H-2\(^{d}\) mice is not restricted through K\(^{\beta}\) (ref. 5). The induction of K\(^{\beta}\) by a peptide is not unexpected; however, as we have found other peptides which induce D\(^{\alpha}\) on RMA-S cells (Fig. 2), and can compete in the CTL lysis assay (Fig. 3), but are not epitopes recognized by CTL from H-2\(^{d}\) mice. The time course and dose response characteristics of the induction of H-2D\(^{\alpha}\) on RMA-S are shown in Fig. 5a, b. The effect is detected after one hour of exposure to peptide, and increases over six hours. The maximum level of D\(^{\alpha}\) induced was ~1/5 of that found on the RMA cell.

**Association of D\(^{\alpha}\) with \(\beta\)-microglobulin**

The time taken to double the number of D\(^{\alpha}\) molecules on RMA-S cells treated with peptides was between 1 and 3 hours, depending on the concentration of peptide (Fig. 5a). The speed of this effect indicated that peptides could be inducing transport of intracellular D\(^{\alpha}\) molecules to the cell surface, which might occur should peptides induce the association of D\(^{\alpha}\) heavy chains with endogenous \(\beta\)-microglobulin.

RMA-S cells were therefore labelled with \(^{35}\text{S}\)methionine for 40 minutes after exposure to peptides, and the D\(^{\alpha}\) heavy chains (both free and \(\beta\)-microglobulin associated) were immunoprecipitated with the antibody 28148S which is specific for the \(\alpha_3\) domain\(^{24,25}\). If association with endogenous \(\beta\)-microglobulin in intracellular D\(^{\alpha}\) peptides, then more \(\beta\)-microglobulin should be co-precipitated with D\(^{\alpha}\) heavy chains.

**Figure 6a,** lane A, shows that D\(^{\alpha}\) heavy chains in RMA-S co-precipitated a barely detectable amount of \(\beta\)-microglobulin\(^{24}\), Lanes B and C show that treatment with two control peptides had no effect on chain association. In contrast, each of the four peptides thought to bind D\(^{\alpha}\) increased the amount of co-precipitated \(\beta\)-microglobulin (lanes D-G and H, respectively). Of these, NP (1968) 50-63 (lane F) seemed to be the most effective. This correlates with the observation that NP (1968) 50-63 is a more potent inhibitor of recognition by D\(^{\alpha}\)-restricted T-cells\(^\text{24,25}\) and so may have a higher affinity for D\(^{\alpha}\). Not only assembly, but folding of the D\(^{\alpha}\) heavy chain is driven by peptide (Fig. 6b). The antibody B22.249 reacts with a determinant on the \(\alpha_1\) domain of D\(^{\alpha}\) that appears only when D\(^{\alpha}\) is associated with \(\beta\)-microglobulin\(^{24}\). Lanes A and B of Fig. 6b define a cohort of D\(^{\alpha}\) molecules that were labelled in RMA-S during a 15-min pulse with \(^{35}\text{S}\)methionine. The majority of these were neither associated with \(\beta\)-microglobulin (lanes A and B), nor reactive with the \(\alpha_1\) domain specific antibody B22.249 (lanes E and F). However, in cells exposed to NP (1968) 50-63, the D\(^{\alpha}\) heavy chains became associated with \(\beta\) and folded into a conformation detected by B22.249, within 15 minutes of synthesis (compare lanes A, B, C with E, F, G). We have also noted that the antibody 28148S (specific for the \(\alpha_3\) domain\(^{24,25}\)) precipitates D\(^{\alpha}\) heavy chains more efficiently when they are associated with \(\beta\)-microglobulin (compare lane A with C). It may therefore bind to assembled class I molecules with higher affinity than to free heavy chains.

**The state of glycosylation of the heavy chains in peptide-treated cells is shown in Fig. 6c.** RMA-S cells were labelled for 5 hours in the presence of peptides, to allow accumulation of heavy chains that have acquired endo-H resistance on passing through the Golgi with NP (1934) 365-380 induced D\(^{\alpha}\) (Fig. 4g-i). Lanes A-D show that in untreated RMA-S and cells treated with a control peptide, D\(^{\alpha}\) heavy chains are associated with a low level of \(\beta\)-microglobulin and acquire minimal endo-H resistance compared with those in...
RMA (lanes I and J). The D\textsuperscript{d} heavy chains in RMA cells treated with the two active peptides (lanes E–H) bound more \(\beta\)-microglobulin, and acquired a degree of endo-H resistance comparable to the level of D\textsuperscript{d} induced at the cell surface (compare with Fig. 5B). On resting after exposure with a polyvalent serum to immunoprecipitate heavy chains\textsuperscript{18}, we obtained the same result (data not shown).

As another test of the induction by peptides of new class I molecules at the cell surface, we investigated the effect of brefeldin A (BFA). In cells treated with BFA, movement of newly synthesized membrane proteins from the endoplasmic reticulum (ER) to the Golgi apparatus is blocked\textsuperscript{26,27}, but endocytosis and protein synthesis are not inhibited\textsuperscript{22}. BFA at 1 \(\mu\)g/ml blocked the induction of D\textsuperscript{d} at the RMA-S cell surface by 69–92% in three trials. The same concentration of BFA did not, however, inhibit \(\beta\)-microglobulin association with D\textsuperscript{d} (Fig. 6 d and e). This indicates that peptides in BFA-treated cells can still enter the cell and induce chain association, but transport of assembled class I molecules out of the ER is blocked.

**Discussion**

We have shown that exposure of the RMA-S mutant cell to certain peptides partially restores association of D\textsuperscript{d} heavy chains with \(\beta\)-microglobulin, and expression of D\textsuperscript{d} or D\textsuperscript{k} molecules at the cell surface (Fig. 2, 4). We have also observed that both RMA and L cell fibroblasts, or the NS-0 myeloma transfected with the D\textsuperscript{d} gene, respond with a modest increase in class I expression (33–235%) after exposure to appropriate peptides (data not shown). Normal cells might therefore exhibit a less exaggerated form of the same phenomenon.

The evidence in Fig. 6 favours the interpretation that peptides at certain concentration in extracellular fluid can reach a pre-Golgi compartment of RMA-S cells, where they induce the D\textsuperscript{d} heavy chains to fold and associate with \(\beta\)-microglobulin. This compartment could be the ER, or possibly an intermediate compartment between the ER and the Golgi\textsuperscript{27}. The assembled complexes are then transported to the cell surface. The peptide NP (1968) 50–63 appears to induce chain association more effectively than NP (1934) 365–380 (Figs 6a, b and c), but induces the equivalent or slightly less D\textsuperscript{b} at the cell surface (Figs 2 and 6c). The assembled D\textsuperscript{d} complexes formed by these two peptides may therefore differ in their stability, or rate of transport to the surface of the cell.

The effect on D\textsuperscript{d} is specific to peptides identified either as epitopes recognized in association with D\textsuperscript{d} by CTL (refs 3, 7, or as sequences able to prevent the presentation of known epitopes to D\textsuperscript{d}-restricted CTL (ref. 19), implying that only peptides binding D\textsuperscript{b} or D\textsuperscript{k} increase their expression at the surface of RMA-S cells.

The most probable site for peptide binding is the groove formed by the polymorphic a1 and a2 domains of the class I heavy chain\textsuperscript{28}. Deletion of these domains prevents binding of \(\beta\)-microglobulin to the a3 domain of the D\textsuperscript{d} molecule\textsuperscript{22}. Furthermore, in the crystal structure of HLA-A2, \(\beta\)-microglobulin makes multiple contacts with the a1 and a2 domains\textsuperscript{23}, but almost certainly would not make direct contact with bound peptide. Chain association may therefore depend on correct folding of these domains. Our results indicate that folding may depend on the availability of peptide ligands. The a1 and a2 domains of newly synthesized class I heavy chains could fold around the peptide to form a three-dimensional structure with affinity for \(\beta\)-microglobulin.

This concept does not rule out the existence of one bound peptide for another after the class I molecule has assembled and is exposed at the cell surface. Peptide exchange is no less conceivable than \(\beta\)-exchange\textsuperscript{24}. The observations that fixed cells\textsuperscript{25} and cells treated with BFA (ref. 32) can present extracellular peptide to class I-restricted T-cells, implies that peptide exchange can occur at the surface of non-mutant cells. But the extremely low level of saturation (0.3%) of class I achieved with peptides suggests that the peptide exchange has saturated the RMA-S cell and others resembling it\textsuperscript{46,47}. Mutations in the heavy chain, or \(\beta\)-microglobulin, could interfere with class I assembly\textsuperscript{24,48}. This is unlikely in RMA-S, however, because fusion with L cell fibroblasts restores class I assembly, expression, and antigen presentation (C. O. et al., manuscript in preparation).

On the other hand, if assembly of class I molecules depends on peptide binding, a defect reducing the concentration of peptides during folding would prevent chain association. BFA blocks presentation of a cytoplasmic antigen to class I-restricted T-cells, suggesting that peptides derived from cytoplasmic proteins in normal cells may be transported into a pre-Golgi compartment\textsuperscript{24}. Proteins without hydrophobic signal sequences are efficiently presented\textsuperscript{24}. Entry of peptides into this compartment may therefore involve a specialized mechanism, loss of which could account for the phenotype of RMA-S cells.