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 β_2-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 β_2-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[1]. 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[2–12].

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[2,3]. Although evidence has accumulated that is consistent with this hypothesis[6,7,10,13–15], the mechanisms by which epitopes are generated, transported and complexed 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 tumour cell line that escapes immunological rejection *in vivo* across a minor histocompatibility barrier (Öhlén *et al.*, unpublished observations). It has a known defect in the association of β_2-microglobulin with class I heavy chains, and a reduction of ~95% in the level of assembled class I molecules expressed at the cell surface[16–18]. 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 β_2-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-2^b lymphoma RBL-5 by exposure to the mutagen ethyl methane sulphonate (EMS) and repeated rounds of treatment with antisera against class I molecules and complement[16,17]. It expresses ~1/20 of the amount of H-$2D^b$, K^b and β_2-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 β_2-microglobulin, but most of the heavy chains bear high mannose oligosaccharides, do not associate with β_2-microglobulin and remain intracellular[18].

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-$2D^b$ (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. 1*a*). 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

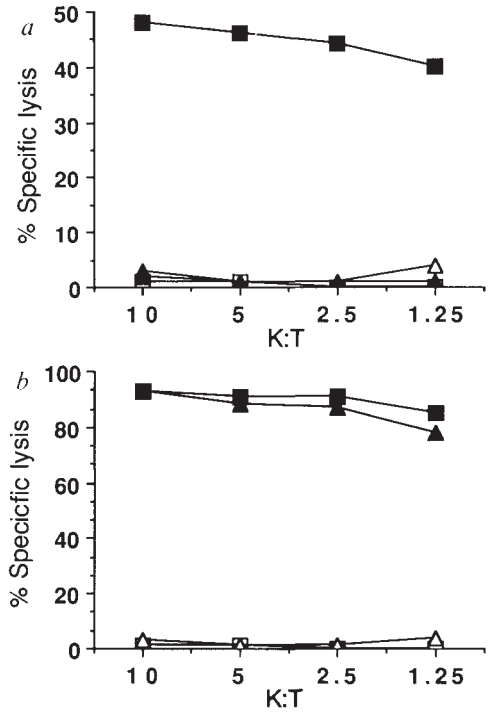


FIG. 1 CTL clone F5 recognizes RMA-S cells treated with peptide NP(1968) 366–379, but not infected with influenza A virus. *a*, Target cells were: ■, RMA infected with influenza E61-13-H17; □, RMA uninfected; ▲, RMA-S infected with E61-13-H17; △, RMA-S uninfected. *b*, Target cells were: ■, RMA treated with NP(1968) 366–379; □, RMA untreated; ▲, RMA-S treated with NP(1968) 366–379; △, RMA-S untreated.

METHODS. Clone F5 was isolated from an influenza infected C57BL/6 mouse as described[2]. It's activity was tested by ^{51}Cr release assay on influenza E61-13-H17 virus-infected or peptide-treated (5×10^{-5} M for 1 h) RMA-S and RMA cells as described[3]. [^{51}Cr]-labelled target cells (2×10^4) were exposed to varying numbers of CTL clone F5 cells to give the ratios of killer to target (K:T) shown. After 4 h of contact the ^{51}Cr released from target cells into the supernatant was measured and per cent specific lysis calculated as: (release by CTL − release in medium alone)/(2.5% triton X-100 release − release in medium alone). Spontaneous ^{51}Cr released in medium was 12–19% of that released by triton X-100.

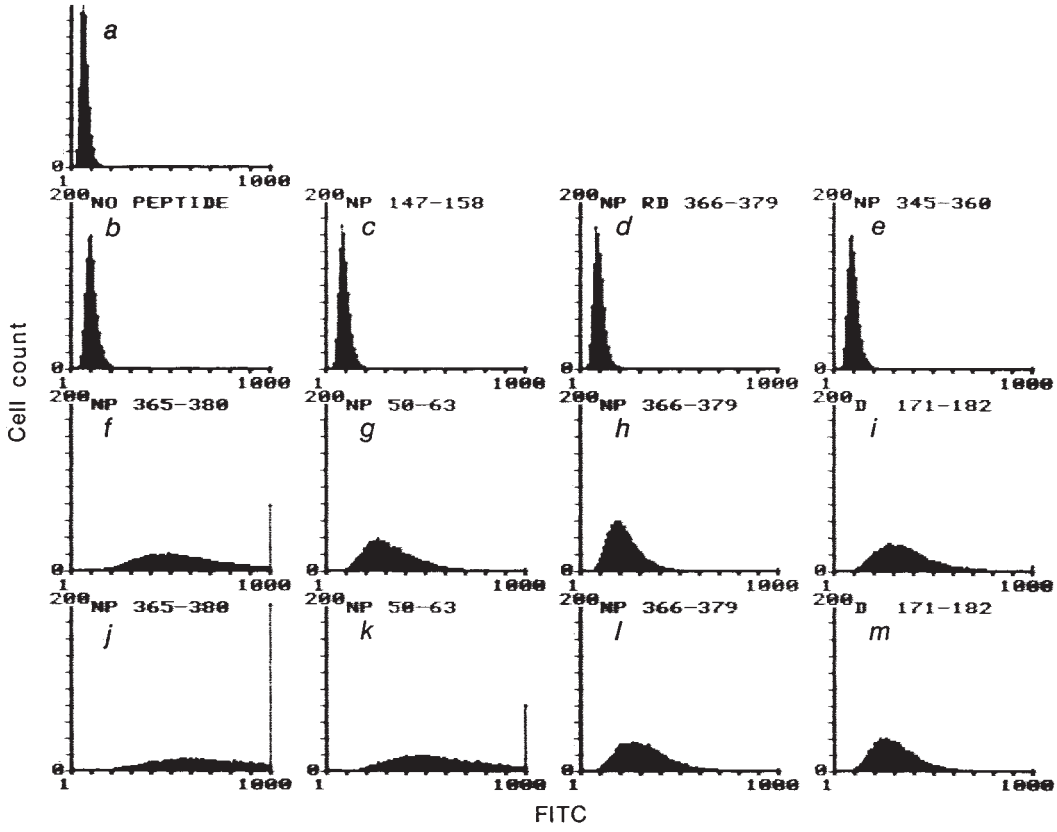


FIG. 2 The indirect immunofluorescence staining of RMA-S cells exposed to various peptides for 5 or 24 h. *a*, Background staining with no first antibody (NFA); *b*, Low level B22.249 (D^b) specific staining detected on untreated RMA-S cells. The remaining panels show RMA-S cells stained with B22.249 after treatment with various peptides: *c*, NP(1968) 147–158 for 5 h; *d*, retro-D isomer of NP(1968) 366–379 for 5 h; *e*, NP(1968) 345–360 for 5 h; *f*, NP(1934) 365–380 for 5 h; *g*, NP(1968) 50–63 for 5 h; *h*, NP(1968) 366–379 for 5 h; *i*, H-2 D^b residues 171–182 (ref. 19) for 5 h; *j*, NP(1934) 365–380 for 24 h; *k*, NP(1968) 50–63 for 24 h; *l*, NP(1968) 366–379 for 24 h; *m*, H-2 D^b 171–182 for 24 h.
METHODS. RMA-S cells (5×10^5) were exposed to medium alone or peptides at 5×10^{-5} M in a total volume of 1.5 ml for the times stated. Cells were then collected and stained by indirect immunofluorescence as described[2]. The first layer was the D^b specific monoclonal antibody B22.249 (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.

(amino acids 366–379 of NP (A/NT/60/68[3]). We found that the two cells were recognized to the same extent after exposure to peptide at concentrations $>10^{-6}$ M (Fig. 1*b*), despite the fact that untreated RMA-S cells normally express ~1/20 the number of H-2D^b molecules as RMA. These results led us to speculate that treatment of RMA-S with the peptide may have increased expression of H-2D^b at the cell surface.

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 NP(1934) 365–380, revealed an increase in D^b expression of between two- and fivefold (Fig. 2*f*, *h*, *j* and *l*). This was detected with an antibody demonstrated to bind only to heavy chains associated with β_2-microglobulin[22].

We then tested two additional peptides that prevent presentation of the sequence NP(1968) 366–379 to the CTL clone F5[19]. The inhibitory effect of these peptides indicates that they bind the D^b molecule[19] 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^b molecule itself (residues 171–182). We chose the latter because of its homology with the equivalent sequence from

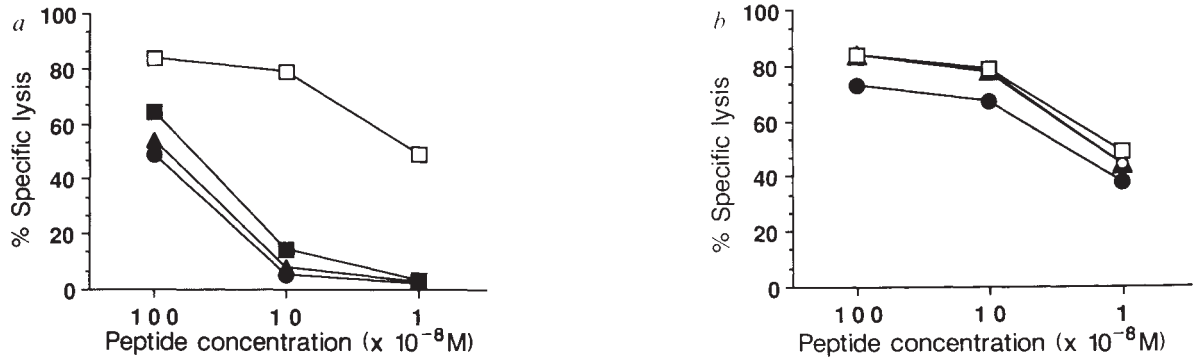


FIG. 3 Recognition by clone F5 of RMA-S cells exposed to NP(1968) 366–379 is inhibited by defined competitor peptides. *a*, Three sequences that compete efficiently: ■, NP(1934) 365–380; ▲, NP(1968) 50–63; ●, H-2 D^b 171–182; □, no competitor. *b*, Three sequences that do not compete: ▲, retro-D isomer of NP(1968) 366–379; ●, NP(1968) 147–158; ○, NP(1968) 345–360; □, no competitor.
METHODS. [^{51}Cr]-labelled RMA-S cells were exposed to the peptide NP(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 NP(1968) 366–379 therefore ranged between 10^4:1 to 10^2.1. The competitor sequences were found by trial and error as described[19] and were derived from published influenza NP sequences[3] except for the peptide comprised of residues 171–182 of H-2 D^b which was chosen on the basis of its relationship to the homologous sequence in HLA Cw3, which has similar inhibitory activity[19–21].

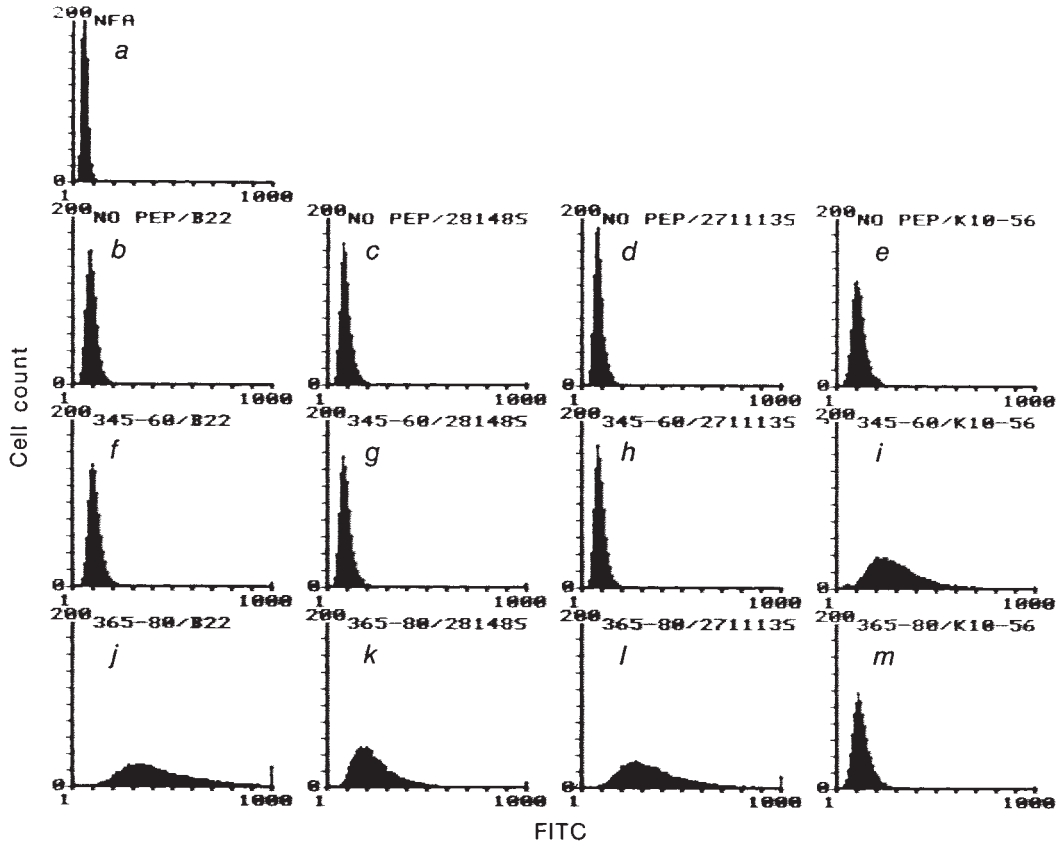


FIG. 4 Induction of class I molecules on RMA-S cells by peptides is H-2 allele specific. *a*, Background staining with no first layer antibody (NFA). *b–e*, Untreated RMA-S cells stained with the following antibodies: *b*, B22.249 specific for the $\alpha 1$ domain of H-2 D^b (refs 22–24); *c*, 28148S specific for the $\alpha 3$ domain of H-2 D^b (refs 22, 23); *d*, 271113S specific for the $\alpha 1$ domain of H-2 D^b (refs 22–24); *e*, K10-56 specific for the $\alpha 1+\alpha 2$ domains of H-2 K^b (refs 23, 46). *f–i*, RMA-S cells treated with 10^{-4} M NP(1968) 345–360 for 6 h and stained with: *f* B22.249 ($\alpha 1$, D^b); *g*, 28148S ($\alpha 3$, D^b); *h*, 271113S ($\alpha 1$, D^b); *i* K10-56 ($\alpha 1+\alpha 2$, K^b). *j–m*, RMA-S cells treated with 10^{-4} M NP(1934) 365–380 for 6 h and stained with: *j*, B22.249 ($\alpha 1$, D^b); *k*, 28148S ($\alpha 3$, D^b); *l*, 271113S ($\alpha 1$, D^b); *m*, K10-56 ($\alpha 1+\alpha 2$, K^b).
METHODS. RMA-S cells were exposed to peptides NP(1934) 365–380 or NP(1968) 345–360 at 10^{-4} M for 6 h and stained by indirect immunofluorescence as described in Fig. 2. The first layer antibodies were either neat culture supernatants (B22.249, K10-56) or ascites purified on protein A Sepharose (28148S, 271113S) as described[47] and used at 5 μg m^{-1} (ref. 2).

HLA Cw3, which has a comparable inhibitory activity[19–21]. To ensure that the D^b 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. 3*a* and *b*).

The peptides that inhibited the recognition of RMA-S by the D^b restricted clone F5 also induced expression of D^b at the surface of RMA-S cells (Fig. 2*f*, *g* and *i*). The three control peptides did not inhibit recognition by clone F5, nor did they induce expression of D^b (Fig. 2*c*, *d* and *e*). There is therefore a correlation between induction of D^b 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 D^b induce its expression.

The class I molecule D^b is unusual because it can reach the cell surface in the absence of endogenous β_2-microglobulin synthesis in the murine cell line R1E (ref. 22). Most class I antibodies bind MHC class I heavy chains only when they associate with β_2-microglobulin but free D^b heavy chains can be detected with the antibody 28148S, which binds the $\alpha 3$ domain of both free and β_2-microblobulin-associated heavy chains[23]. Free D^b heavy chains were not detected by an excess

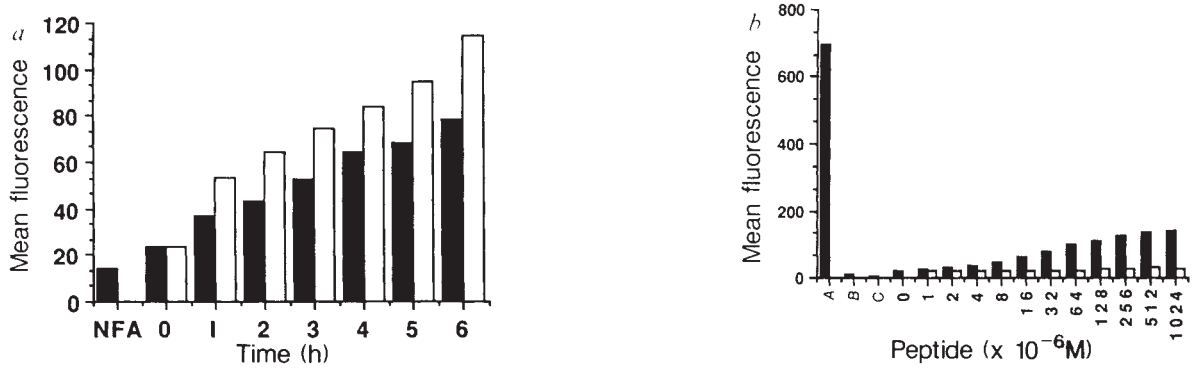


FIG. 5 Time course (*a*) and dose response (*b*) of the induction of H-2 D^b on RMA-S by peptide. *a*, Time course in response to 2.5×10^{-4} M (□); or 0.5×10^{-4} M (■) of NP(1934) 365–380. NFA, background staining with no first layer antibody. *b*, Dose response of the induction of D^b on RMA-S by peptides NP(1934) 365–380 (■); and NP(1968) 345–360 (□). *A*, RMA stained with B22.249 for comparison; *B*, background staining of RMA with no first antibody; *C*, background staining of RMA-S with no first antibody; 0, untreated RMA-S stained with B22.249.

METHODS. Aliquots of 5×10^5 RMA-S cells in 0.75 ml medium (RPMI 1640/10% FCS) were placed in Costar 24-well plates. *a*, At times 0, 1, 2, 3, 4 and 5 h 0.75 ml of pre-warmed medium containing twice the desired concentration of peptide was added to appropriate aliquots of cells. At 6 h all the cells were collected and stained by indirect immunofluorescence with antibody B22.249 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 1–1,024 $\times10^{-6}$ M as shown.

of 28148S binding on the surface of RMA-S cells, either before or after induction with peptides (Fig. 4*c* 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. 4). Treatment with NP(1934) 365–380 induced D^b (compare Fig. 4*a–d* with 4*j–l*), but not K^b (Fig. 4*e* and *m*). In contrast, NP(1968) 345–360 has the opposite effect, inducing K^b (Fig. 4*i*) but not D^b (Fig. 4*f–h*). The polyclonal cytotoxic T-cell response to influenza nucleoprotein in H-2^b mice is not restricted through K^b (ref. 5). The induction of K^b by a peptide is not unexpected, however, as we have found other peptides which induce D^b on RMA-S cells (Fig. 2), and compete in the CTL lysis assay (Fig. 3), but which are not epitopes recognized by CTL from H-2^b mice[19].

The time course and dose response characteristics of the induction of H-2D^b on RMA-S are shown in Fig. 5*a*, *b*. The effect is detected after one hour of exposure to peptide, and increases over six hours. The maximum level of D^b induced was ~1/5 of that found on the RMA cell.

Association of D^b with β_2-microglobulin

The time taken to double the number of D^b molecules on RMA-S cells treated with peptides was between 1 and 3 hours, depending on the concentration of peptide (Fig. 5*a*). The speed of this effect indicated that peptides could be inducing transport of intracellular D^b molecules to the cell surface, which might occur should peptides induce the association of D^b heavy chains with endogenous β_2-microglobulin.

RMA-S cells were therefore labelled with [^{35}S]methionine for 40 minutes after exposure to peptides, and the D^b heavy chains (both free and β_2-microglobulin associated) were immunoprecipitated with the antibody 28148S which is specific for the $\alpha 3$ domain[22–24]. If association with endogenous β_2-microglobulin is stimulated by peptides, then more β_2-microglobulin should be co-precipitated with D^b heavy chains.

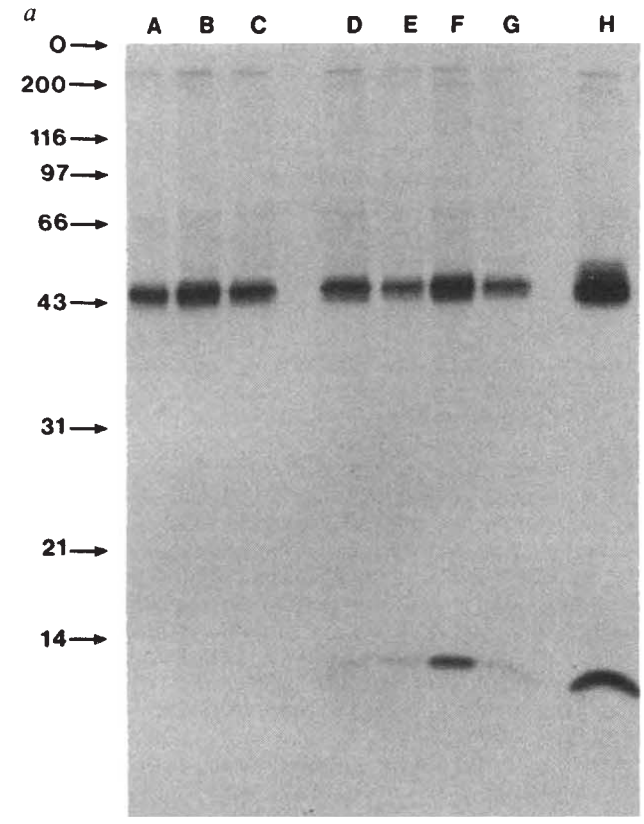
Figure 6*a*, lane A, shows that D^b heavy chains in RMA-S co-precipitated a barely detectable amount of β_2-microglobulin[18]. Lanes B and C show that treatment with two control peptides had no effect on chain association. In contrast, each of the four peptides thought to bind D^b increased the amount of co-precipitated β_2-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^b-restricted T-cells[19] and so may have a higher affinity for D^b.

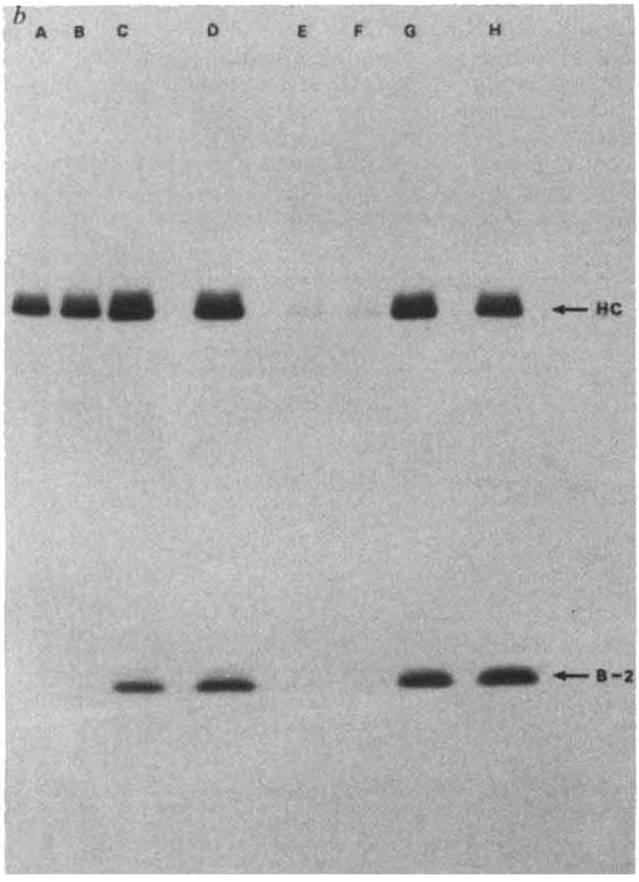
Not only assembly, but folding of the D^b heavy chain is driven by peptide (Fig. 6*b*). The antibody B22.249 reacts with a determinant on the $\alpha 1$ domain of D^b that appears only when D^b is associated with β_2-microglobulin[22]. Lanes A and B of Fig. 6*b* define a cohort of D^b molecules that were labelled in RMA-S during a 15-min pulse with [^{35}S]methionine. The majority of these were neither associated wth β_2-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^b heavy chains became associated with β_2 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[22–24]), precipitates D^b heavy chains more efficiently when they are associated with β_2-microglobulin (compare lane A with C). It may therefore bind to assembled class I molecules with higher affinity than to free heavy chains.

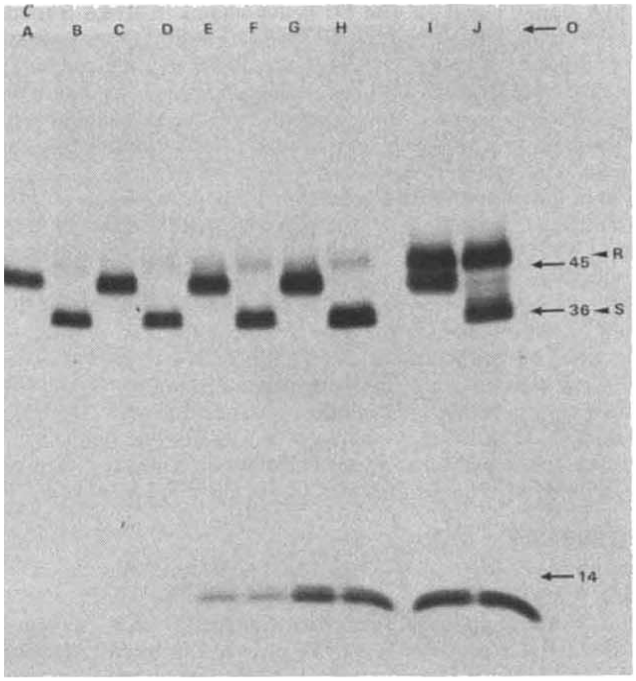
The state of glycosylation of the heavy chains in peptide-treated cells is shown in Fig. 6*c*. RMA-S cells were labelled for 5 hours in the presence of peptides, to allow accumulation of heavy chains that have acquired endo H resistance on passing through the Golgi (refs 25–28). Lanes A–D show that in untreated RMA-S and cells treated with a control peptide, D^b heavy chains are associated with a low level of β_2-microglobulin and acquire minimal endo-H resistance compared with those in

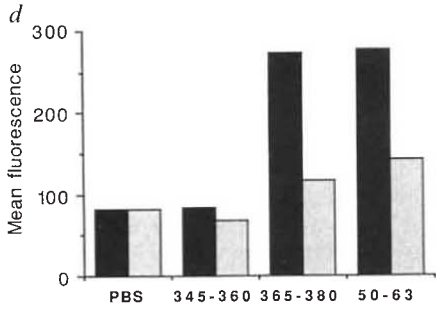
FIG. 6 Effect of peptide on assembly with β_2-microglobulin and movement through the Golgi. *a*, Co-precipitation of β_2-microglobulin with D^b heavy chains from RMA-S treated with peptides for 6 h and labelled for 40 min. Lane A, untreated RMA-S; B, RMA-S + NP(1968) 345–360; C, RMA-S + retro-D isomer NP(1968) 366–379; D, RMA-S + NP(1968) 366—379; E, RMA-S + NP(1934) 365–380; F, RMA-S + NP(1968) 50–63; G, RMA-S + H-2 D^b 171–182; H, RMA untreated. *b*, Peptide induces a conformational change in the $\alpha 1$ domain of the D^b heavy chain in RMA-S cells. The samples in lanes A–D were immunoprecipitated with the antibody 28148S (specific for the $\alpha 3$ domain), and those in lanes E–H with B22.249 (specific for the α_1 domain). Lanes A & E, RMA-S untreated; B & F, RMA-S + NP(1968) 345–360; C & G, RMA-S + NP(1968) 50–63; D & H, RMA untreated. *c*, D^b heavy chains in RMA-S acquire Endo H resistance after treatment with peptides. The samples were immunoprecipitated with 28148S and in lanes A, C, E, G, I were mock digested, and in B, D, F, H, J digested with Endo H. The positions, after digestion, of resistant (R) and sensitive (S) heavy chains are marked to the right of the figure. Lanes A, B, RMA-S + no peptide; C, D, RMA-S + NP(1968) 345–360; E, F, RMA-S + NP(1934) 368–380; G, H, RMA-S + NP(1968) 50–63; I, J, RMA + no peptide. *d*, Brefeldin A blocks the peptide-induced expression of D^b at the cell surface. RMA-S cells were treated with 1 μg ml^{-1} BFA (▨), or mock-treated (■), and exposed to the peptides shown for 5 h. D^b expression was measured with the antibody B22.249. *e*, BFA does not inhibit class I assembly induced with peptides. Lanes A–E, immunoprecipitates from mock treated cells; F–J, immunoprecipitates from BFA-treated cells. A & F, RMA-S + no peptide; B & G, RMA-S + NP(1968) 345–360; C & H, RMA-S + NP(1934) 365–380, D & I, RMA-S + NP(1968) 50–63; E & J, RMA control + no peptide.

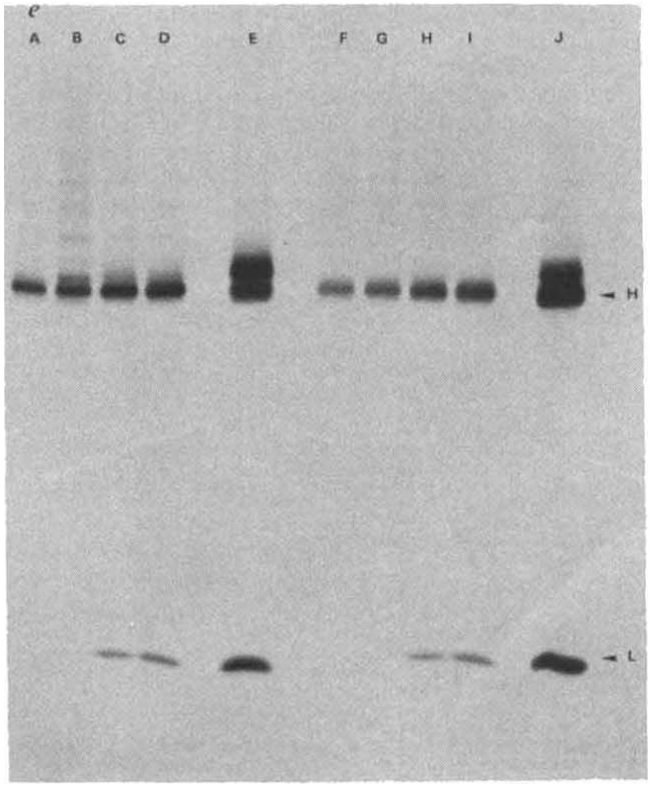
METHODS. *a*, RMA-S and RMA cells were resuspended at 10^6 ml^{-1} in medium (RPMI 1640/10% FCS) alone or containing peptides at 10^{-4} M for 5 h. They were then washed twice in warmed phosphate buffered saline (PBS) and resuspended at 5×10^6 ml^{-1} in methionine-free medium containing peptides at the same concentration for a further hour. [^{35}S]-methionine (120 μCi) was added and the mixture incubated for 40 min at 37 °C. The labelled cells were then washed once in ice-cold PBS and resuspended in 0.5 ml lysis buffer (0.5% v/v NP40, 0.5% mega 9 (ref. 48), 150 mM NaCl, 5 mM EDTA, 50 mM TRIS pH 7.5) containing 2 mM PMSF and 5 mM iodoacetamide. The lysates were precleared with *Staphylococcus A* organisms overnight at 4 °C and immunoprecipitation was performed as described[2] with purified 28148S antibody at a final concentration of 5 μg ml^{-1}. Reduced immunoprecipitates were electrophoresed on a 12% SDS polyacrylamide gel, which was fixed, stained, treated with Amplify (Amersham), dried and exposed to pre-flashed X-ray film. *b* RMA-S and RMA cells were resuspended at 5×10^6 ml^{-1} in medium alone or containing peptides at 2×10^{-4} M for 4 hours. Aliquots of cells (2×10^7) were then washed and resuspended in 1 ml methionine free medium containing peptides at the same concentration for one hour. [^{35}S]methionine (250 μCi) was then added and the mixtures incubated at 37° for 15 minutes. The cells were then washed once in 15 ml of ice cold PBS containing 2 mM of unlabelled L-methionine and lysed as described above. The lysates were divided into two equal parts, ether 28148S or B22.249 added to 10 μg ml^{-1}, and immunoprecipitates prepared as described above. *c*, Aliquots of 1.8×10^7 RMA-S or RMA cells were resuspended in 1 ml of methionine-free medium alone, or containing peptides at 2×10^{-4} M. After incubation for 1 h, 250 μCi [^{35}S]methionine was added and the mixtures incubated for 5 h at 37 °C. The cells were lysed in 1 ml lysis buffer (as above), and immunoprecipitates prepared with the 28148S antibody at 10 μg ml^{-1}. These were divided into two equal portions. One was digested with 0.005 U ENDO H (Boehringer) as described[25], the other was mock-digested. The samples were trichloroacetic acid precipitated with 50 μg Sigma SDS-7 molecular weight markers as carrier, then analysed as in (*a*). *d*, RMA-S cells (5×10^5) were aliquotted in 0.9 ml of medium. BFA (2 μl, 0.5 mg ml^{-1} in methanol), or methanol, were added. (In control experiments we estzblished that methanol at this dilution had no effect on class I expression.) After 45 min incubation at 37 °C, peptides (100 μl in PBS at 1 mM), or PBS, were added and the mixtures incubated at 37 °C for 5 h. The cells were then collected and stained by indirect immunofluorescence with the antibody B22.249 as described in Fig. 2. *e*, In parallel with experiment (*d*), aliquots of 5×10^6 RMA-S or RMA cells were resuspended in 0.9 ml methionine-free medium, and BFA (2 μl, final concentration 1 μg ml^{-1}) or methanol were added as above. After 45 min peptides were added to 10^{-4} M as above, followed by 100 μCi of [^{35}S]-methionine. The mixtures were incubated for 5 h at 37 °C. Immunoprecipitates were prepared and analysed as in (*a*).











RMA (lanes I and J). The D^b heavy chains in RMA-S treated with the two active peptides (lanes E–H) bound more β_2-microglobulin, and acquired a degree of endo-H resistance comparable to the level of D^b induced at the cell surface (compare with Fig. 5*b*). On repeating this experiment with a polyclonal serum to immunoprecipitate heavy chains[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[29–32], but endocytosis and protein synthesis are not inhibited[29]. BFA at 1 $\mu g\,ml^{-1}$ blocked the induction of D^o at the RMA-S cell surface by 69–92% in three trials. The same concentration of BFA did not, however, inhibit β_2-microglobulin association with D^b (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^b heavy chains with β_2-microglobulin, and expression of D^b or K^b 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^b 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 high concentration in extracellular fluid can reach a pre-Golgi compartment of RMA-S cells, where they induce the D^b heavy chain to fold and associate with β2-microglobulin. This compartment could be the ER, or possibly an intermediate compartment between the ER and the Golgi[33]. 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 6*a*, *b* and *c*), but induces the equivalent or slightly less D^b at the cell surface (Figs 2 and 6*c*). The assembled D^b 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^b is specific to peptides identified either as epitopes recognized in association with D^b by CTL (refs 3, 7), or as sequences able to prevent the presentation of known epitopes to D^b-restricted CTL (ref. 19), implying that only peptides binding D^b or K^b increase their expression at the surface of RMA-S cells.

The most probable site for peptide binding is the groove formed by the polymorphic α1 and α2 domains of the class I heavy chain[34]. Deletion of these domains prevents binding of β_2-microglobulin to the α3 domain of the D^b molecule[22]. Furthermore, in the crystal structure of HLA-A2, β_2-microglobulin makes multiple contacts with the α1 and α2 domains[35], 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 α1 and α2 domains of newly synthesized class I heavy chains could fold around the peptide to form a three-dimensional structure with affinity for β_2-microglobulin.

This concept does not rule out the exchange 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 β_2 exchange[36]. The observations that fixed cells[37] 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 *in vitro*, argues that it is inefficient[38].

Our hypothesis could explain why class I heavy and light chains synthesized *in vitro* in the absence of peptide fail to associate[39], and also the nature of the mutations that could have given rise to the RMA-S cell and others resembling it[40–42]. Mutations in the heavy chain, or β_2-microglobulin, could interfere with class I assembly[43,44]. 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[32]. Proteins without hydrophobic signal sequences are efficiently presented[2,6]. Entry of peptides into this compartment may therefore involve a specialized mechanism, loss of which could account for the phenotype of RMA-S cells. □

Received 3 March; accepted 16 June, 1989.

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ACKNOWLEDGEMENTS. We thank George Klein for catalysing our collaboration; Jonathon Rothbard and G. Hammerling for peptides and antibodies B22.249 and K10-56 respectively; Nigel Rust and the Nuffield Dept of Surgery for time on the Orthocytofluorograf; Jed Nuchtern, Bill Biddison and Richard Klausner for brefeldin A and for their explanation of its action; Hidde Ploegh for helpful discussion, and John Elvin for editorial assistance. We also thank the MRC (UK), the NCI of the US and the Swedish Cancer Society for support.