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Immunology

Surface appearance and instability of empty H-2 class I molecules under physiological conditions

(major histocompatibility complex/peptide binding/antibody trapping)

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ABSTRACT **Recent evidence suggests that endogenously produced antigenic peptides are required for assembly of major histocompatibility complex class I chains with β_2-microglobulin and transport to the cell surface. The RMA-S mutant cells are thought to be defective in intracellular peptide loading to class I molecules and, therefore, devoid of class I surface expression. Here we report that at physiological temperature (37°C) "empty" class I molecules appear at the cell surface of RMA-S cells where they can be trapped with H-2 antibodies. In the absence of the stabilizing ligand, the class I molecules rapidly alter their conformation but remain at the cell surface as demonstrated with a rabbit antiserum. Such denatured H-2 molecules can also be found on normal wild-type RMA cells. However, their amount is strongly reduced after culture of RMA cells with a class I binding peptide. These findings indicate that empty class I molecules appear at the surface not only on mutant but also on normal cells, suggesting that in normal cells the supply with peptides is limited.**

T lymphocytes do not recognize foreign antigen *per se* but only endogenously derived antigenic peptides bound to the peptide binding groove on major histocompatibility complex (MHC) molecules. The peptides are probably generated in the cytosol by proteolytic degradation. It is assumed that they are transported to the endoplasmic reticulum and bound by MHC class I molecules during the assembly of the class I heavy chain and β_2-microglobulin (β_2m).

Mutant cells, such as the murine RMA-S and the human T2 or 721.174 cells, appear to have a defect in the transport of peptide to the MHC class I molecules resulting in drastically reduced class I surface expression (1–3). The incubation of the mutant T cells with peptide induced a partial restoration of surface expression (1–3). These and other studies (4, 5) indicated that peptide was required for the stabilization of the class I–β_2m complex. It was postulated that in peptide-treated RMA-S cells the peptide would enter the cells, possibly the endoplasmic reticulum, and stabilize the class I–β_2m complex and that, in the absence of peptide, the class I and β_2m molecules would not assemble and, therefore, not reach the cell surface. However, it seemed also possible that at least some class I molecules could associate with β_2m in the absence of peptide and reach the surface. This assumption was supported (but not proven) by the observation that "empty" class I–β_2m complexes devoid of peptide are stable and can reach the surface at low temperature (26°C) but decay at 37°C (6).

We investigated the question whether the empty class I–β_2m complexes can also assemble and reach the cell surface at 37°C, which reflects more physiological conditions. The results show that at 37°C the empty H-2 class I molecules appear indeed at the cell surface, where they denature rapidly and persist in a denatured form.

MATERIALS AND METHODS

Cells, Reagents, and Staining Procedures. The Rauscher virus-transformed mouse lymphoma T cells RMA and RMA-S (7, 8) were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum. For surface staining, the cells were incubated at 4°C with hybridoma supernatants containing anti-K^b or -D^b antibodies (9–11) followed by fluoresceinated goat anti-mouse IgG (Sigma). Flow cytometric analysis was performed with a FACScan (Becton Dickinson). The D^b-binding nucleoprotein peptide (NP) 365-380 (1) was generously provided by A. Townsend, the optimal nonamer NP 366-374 (12, 13) was from H.-G. Rammensee (Max-Planck-Institute Tübingen, F.R.G.), and the K^b-binding peptide NP 345-360 (1) was produced in our laboratory.

Immunoprecipitation. Cells were surface iodinated using lactoperoxidase as described (14), washed three times with phosphate-buffered saline, lysed on ice for 20 min with lysis buffer [0.2% Nonidet P-40/50 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM phenylmethylsulfonyl fluoride/aprotinin (30 μg/ml)]. Debris was removed by centrifugation, and MHC class I antigens were immunoprecipitated using monoclonal antibody (mAb) Y-3 (15) or the exon 8 rabbit serum raised against a peptide from exon 8 of K^b, generously provided by Smith and Barber (16). SDS/PAGE on 15% gels was performed as described (14). Gels were exposed to Kodak XAR-5 films.

RESULTS

To investigate the possibility that in the mutant RMA-S cells empty H-2 molecules can reach the cell surface under physiological conditions (37°C), the RMA-S cells were cocultured at 37°C with mAbs against the relevant K^b and D^b molecules. After washing, the cells were then stained with fluoresceinated anti-mouse immunoglobulin. Fig. 1*a* shows that after 1 hr of coculture with antibody some staining could be observed and that a maximum was reached after almost 6 hr. Double staining with anti-mouse immunoglobulin and mouse anti-β_2m demonstrated that these trapped H-2 molecules were associated with mouse β_2m (Fig. 1*c*). This was confirmed in immunoprecipitation studies, which also revealed that these surface molecules were endoglycosidase H-resistant and, therefore, of the mature form (data not shown). The kinetics of surface appearance in the presence of antibody was very similar to the kinetics observed with the D^b-binding influenza peptide NP 365-380 (Fig. 1*b*). These observations suggest that empty H-2 molecules reach the cell surface where they can be trapped or stabilized by specific antibod-

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Abbreviations: MHC, major histocompatibility complex; β_2m, β_2-microglobulin; mAb, monoclonal antibody; NP, nucleoprotein peptide.

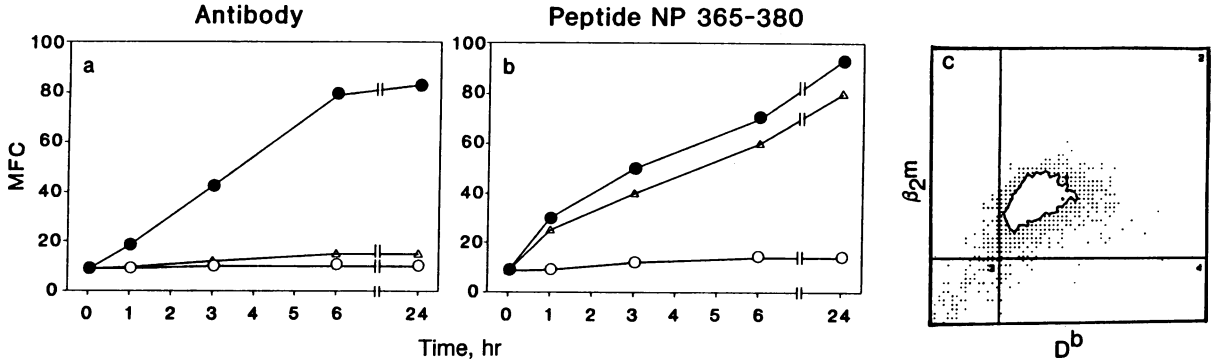

FIG. 1. Trapping of H-2 molecules on RMA-S cells by coculture with antibodies. RMA-S cells were cocultured with a 1:2 dilution of anti-H-2 hybridoma supernatant B22-249 (anti-D^b IgG2a) (●) or 28-14-8 (anti-D^b α3 domain) (△) and stained with fluoresceinated anti-mouse immunoglobulin (*a*) or were incubated after washing with anti-mouse β_2m (Lym 11.2, IgG2b) and double stained with fluorescein isothiocyanate-conjugated anti-IgG2a and phycoerythrin-conjugated anti-IgG2b (*c*). After coculture with 50 μM NP 365-380, cells were stained with B22-249 or 28-14-8 and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin (*b*). Cocultures also were incubated with 0.1% NaN_3 (○), which blocked the surface expression of H-2 molecules. MFC, mean fluorescence channel.

ies. In the absence of a stabilizing ligand, the H-2 molecules appear to rapidly lose their conformational determinants because cells cultured at 37°C in the absence of antibody are only marginal positive. That the trapping with antibody occurred indeed at the cell surface and was not due to endocytosed antibodies was confirmed by visual inspection of aggregates formed with anti-H-2 antibody-coated microspheres (Dynabeads; data not shown).

mAb B22-249 shown in Fig. 1*a* binds to both the α1 and α2 domains of the D^b molecules (17). When a panel of 30 mAbs against K^b or D^b molecules was tested, trapping of empty H-2 molecules was observed with antibodies directed against determinants on the α1 and/or α2 domain, which are known to form the peptide binding site. Less efficient was antibody 28-14-8, which is directed against the D^b α3 domain, and the β_2m-specific antibody Lym 11.2. The same effects were also observed in serum-free medium.

Examples are shown in Table 1. Among the antibodies tested, the anti-K^b mAbs were always more efficient for trapping than the D^b antibodies, although they could stain equally well the wild-type RMA cells with constitutive expression of K^b and D^b molecules. This suggests that at 37°C the K^b molecules appear at the surface of RMA-S cells at a higher rate than the D^b molecules, that the rate of appearance is similar but the total level of D^b molecules is lower in RMA-S cells, or that D^b denatures more rapidly than K^b.

RMA-S cells have been shown to express at lower temperatures (26°C) large amounts of empty K^b and D^b molecules at the cell surface (6). With our panel of K^b antibodies, we did not observe major differences in their ability to bind to the empty K^b molecules on RMA-S cultured at 25°C as compared to K^b molecules on RMA, which are probably occupied by peptides, suggesting that the presence or absence of a peptide in the K^b binding site does not drastically influence antibody binding. Examples are presented in Table 1. For D^b the situation is not clear because all of the D^b antibodies bound less well to RMA-S (25°C) than to RMA cells. This could be due to the absence of peptides, to denaturation of the D^b molecule, or to a low expression rate on RMA-S (25°C).

Table 1. Trapping of empty H-2 molecules on RMA-S with antibodies

		Mean fluorescence channel			
		RMA-S (37°C)		RMA-S	RMA
mAb	Specificity	− mAb	+ mAb	(25°C)	(37°C)
K25-8.7	IA^k	2.5	4.5	2.2	2.5
B22-249	$D^b\alpha1,2$	8.0	55	70	310
T17-433	$D^b\alpha2$	4.0	40	60	300
28-14-8	$D^b\alpha3$	4.5	15	45	290
E3-25	$K^b\alpha1,2$	8.0	75	200	250
K10-56	$K^b\alpha1$	4.0	60	140	220
B8.24	$K^b\alpha1$	4.0	70	200	230
Lym 11.2	β_2m	7.1	8.3	110	144

RMA-S were cocultured at 37°C for 6 hr without or with mAbs and then stained with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin. H-2 expression on RMA-S cells cultured for 12 hr at 25°C and on RMA cells cultured at 37°C also is shown.

Peptide Binding by Empty H-2 Molecules. Next we studied whether the H-2 molecules induced on RMA-S cells by various peptides were as stable as H-2 molecules on normal cells. After coculture for 6 hr with peptide NP 365-380 or NP 366-374, the RMA-S cells were washed, incubated further at 37°C in the absence of additional peptide, and then stained for H-2 expression. Fig. 2*a* demonstrates that about 50% of the H-2 molecules stabilized by coculture with the 16-mer NP 365-380 rapidly lost their conformational determinants in the absence of excess peptide, probably because they released the peptide. However, with the high-affinity peptide NP 366-374 (12), most D^b molecules were permanently stabilized. In contrast, the K^b-binding peptide NP 345-360 was lost at 37°C by most induced K^b molecules (Fig. 2*a*). The nonamer NP 366-374 was also about 100 times more efficient in the "induction" of surface expression of D^b than was NP 365-380 (Fig. 2*b*). These findings suggest that the stabilization of empty molecules at the cell surface depends on the affinity of the respective peptides.

Denaturation of Empty H-2 Molecules at the Cell Surface. The observation that empty H-2 molecules could reach the cell surface but could not be detected in the absence of stabilizing ligands raised the question about the fate of the H-2 molecules at 37°C. This could include denaturation, shedding, proteolytic degradation, rapid endocytosis, or a combination thereof. RMA-S and RMA control cells were cultured for 12 hr at 25°C and then immediately surface iodinated at 4°C or iodinated after exposure to 37°C for 15 or 60 min. Immunoprecipitation of K^b molecules was done with the Y-3 antibody, which binds only to K^b molecules with an intact confirmation, or with a rabbit antiserum produced against a K^b peptide derived from the cytoplasmic exon 8. This antiserum detects also denatured K^b molecules (not D^b), but it cannot be used for surface staining (14). Fig. 3, lanes 1–3, shows that antibody Y-3 could not precipitate K^b from RMA-S cultured at 37°C, but it precipitated large amounts of K^b from RMA-S cells cultured at 25°C. Three rounds of precipitation with Y-3 were required for complete removal of all K^b molecules (lanes 4–6). When the RMA-S (25°C) cells

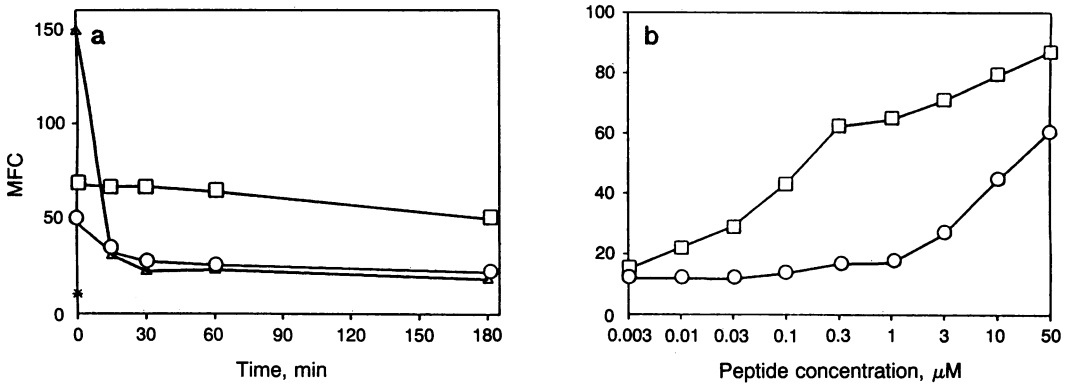

FIG. 2. Binding of peptide to empty H-2 molecules. RMA-S cells were cocultured at 37°C for 6 hr with 50 μM NP 365-380 (○), 50 μM NP 366-374 (□), or 50 μM NP 345-360 (△). Unbound peptide was removed by washing and cells were cultured at 37°C in the absence of additional peptide (*a*). Staining was done with anti-D^b (B22-249; ○ or □) or anti-K^b (E3-25; △). In *b* RMA-S cells were cultured at 37°C with increasing amounts of NP 365-380 or NP 366-374 and stained with B22-249. The asterisk indicates staining in the absence of peptide. MFC, mean fluorescence channel.

were first exposed to 37°C for 15 or 60 min and then surface iodinated, less K^b material could be recovered with antibody Y-3 (e.g., compare lanes 5 and 8 showing the second round of precipitation).

The lysates from which all K^b molecules with an intact conformation had been removed by three rounds of precipitation with Y-3 (Fig. 3, lanes 3, 6, 9, and 12) were then precipitated with the exon 8 antiserum. It can be seen that exon 8 antiserum-reactive (denatured) K^b molecules could be precipitated from RMA-S cells cultured at 37°C (lane 13). The respective amount was higher on RMA-S (25°C) cells, from which the Y-3-reactive material had been removed (lane 14). However, after exposure to 37°C for 15 or 60 min prior to iodination, the amount precipitable with the exon 8 serum was drastically increased (lanes 15 and 16). Note that no β_2m was coprecipitated, which is in contrast to the precipitation with Y-3. These observations suggest that the empty K^b molecules denature rapidly at 37°C and dissociate from β_2m and that the denatured class I heavy chains persist at the cell surface for at least 1 hr. Attempts to detect rapid endocytosis of the denatured H-2 molecules were not successful (data not shown), which is an agreement with the above observation that most of the denatured H-2 molecules could still be found at the cell surface.

Empty Class I Molecules on Normal Cells. Next we investigated whether at 37°C empty H-2 molecules might also reach the cell surface of normal RMA cells. RMA cells cultured at 37°C for 6 hr with peptide NP 365-380 or NP 345-360 showed in several experiments a 20–30% increase in surface expression of D^b (from channel 280 to 347) and K^b (from channel 249 to 338), suggesting the presence of empty class I molecules in or on these cells. A comparable increase in surface H-2 expression was observed for RMA cells cultured at 25°C, as noted by Schumacher *et al.* (4), and for other cells such as EL4 and lymph-node T cells. Immunoprecipitation studies were performed to substantiate this assumption. Fig. 4 shows that, after preclearance of conformationally intact K^b molecules from RMA cells by four rounds precipitation with Y-3 (lanes 1–4), denatured K^b molecules could be recovered with the exon 8 serum (lane 9). To prove that these denatured K^b molecules could be derived from empty K^b/β_2m complexes, the RMA cells were cultured for 6 hr in the presence of the K^b-binding peptide NP 345-360. It was assumed that under these conditions the excess peptide would force more K^b molecules to bind peptide and thereby reduce the amount of empty (and subsequently denatured) K^b at the cell surface. Fig. 4 demonstrates that this is indeed the case. Again, K^b was removed by four precipitations with Y-3 (lanes 5–8). The amount of denatured K^b that could be recovered by the exon 8 serum from cells fed with peptide was clearly lower (lane 10) when compared to RMA cultured in the absence of peptide (lane 9). Since the additional bands of 67 kDa and about 110 kDa (lane 9) disappeared also after feeding of the cells with peptide, it can

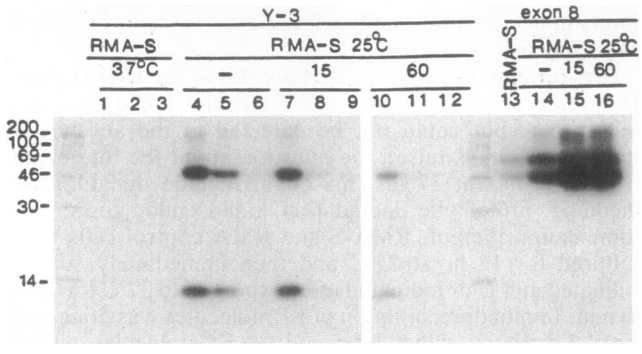

FIG. 3. Denatured K^b molecules on RMA-S cells. RMA-S cells were grown at 37°C (lanes 1–3) or 25°C (lanes 4–6) and precipitated once, twice, or three times with the anti-K^b antibody Y-3. In lanes 7–9 and 10–12, RMA-S cells at 25°C were exposed to 37°C for 15 and 60 min, respectively, prior to iodination. Note the decrease of K^b precipitable with Y-3. Lysates precleared three times with Y-3 (from lanes 3, 6, 9, and 12) were then precipitated with the exon 8 serum for the detection of denatured K^b (lanes 13–16, respectively). Molecular masses (in kDa) are shown to the left.

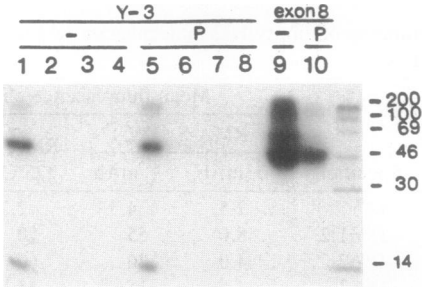

FIG. 4. Denatured K^b molecules on RMA. Lysates of surface-iodinated RMA cells (37°C) were precleared four times with Y-3 (lanes 1–4) and then precipitated with exon 8 serum (lane 9). RMA cells were cultured for 6 hr with 50 μM NP 345-360 and then washed. Lysates were precleared four times with Y-3 (lanes 5–8) and then precipitated with the exon 8 serum (lane 10). Note that the amount of exon 8 serum-precipitable material was reduced in the presence of peptide (lane 10). Molecular masses (in kDa) are shown to the right.

be assumed that the respective unknown molecules were associated with the denatured K^b molecules.

DISCUSSION

A major finding of the present report is that under physiological conditions (37°C) in the mutant RMA-S cells a fraction of K^b and D^b molecules can associate with β_2m in the absence of peptide and reach the cell surface. The kinetics of the trapping of the empty class I molecules with antibodies was parallel to the effect observed with peptide (Fig. 1). These observations indicate that at least some of the induction of surface class I expression with peptide (1) took place at the cell surface. In the absence of a stabilizing ligand, the empty H-2 molecules seem to denature rapidly and dissociate from β_2m resulting in loss of the conformational determinants recognized by the allo H-2 antibodies. The observation that low temperature (26°C) stabilizes empty H-2 molecules has led to the suggestion that they also appear on the cell surface at 37°C, but direct experimental evidence was missing (4, 6).

Interestingly, so far only antibodies against the α1 or α2 domains could efficiently trap the empty H-2 molecules on RMA-S whereas the antibody 28-14-8 directed against the α3 domain was less effective. It is possible that antibody 28-14-8 can bind to the class I molecules emerging at the cell surface of RMA-S at 37°C but that the denaturation of the α1 and α2 domains also affects the α3 domain causing the rapid release of the antibody bound to α3. Unfortunately, no more antibodies against the α3 domain of K^b or D^b were available to test this hypothesis.

The intracellular forces stabilizing the empty class I–β_2m complexes are not known but they could involve putative chaperon molecules. Alternatively, the stabilizing mechanism could be a relatively high intracellular concentration of β_2m, as suggested by Townsend *et al.* (5). At the cell surface the relative concentration of β_2m is probably lower, leading to dissociation of the β_2m. It is also likely that some empty class I–β_2m complexes dissociate already during their transport to the cell surface, so that altogether from the total number of class I–β_2m complexes produced in the endoplasmic reticulum only a small fraction appears at the cell surface as empty molecules.

Attempts to stabilize the empty class I molecules at the surface of RMA-S cells (37°C) with peptides demonstrated that this depended very much on the optimal size and, therefore, affinity of the peptides. The optimal nonamer NP 366-374 (12) induced 100 times more efficient surface expression of D^b (Fig. 2*b*) and permanently stabilized almost 100% of the D^b molecules whereas with the longer peptide NP 365-380 only partial stabilization was observed after further culture at 37°C. The most likely interpretation for the biphasic decay curve shown in Fig. 2*a* is that about 50% of the D^b molecules had bound a low-affinity peptide, which was rapidly released, whereas the other 50% had been strongly stabilized by an optimal peptide, probably the nonamer, which is known to be present as a contaminant in the NP 365-380 preparation (12). The loss of peptide was even more dramatic with the K^b binding peptide NP 345-360 of which the optimal peptide is not known.

Our data imply that the presence of empty K^b molecules is not restricted to RMA-S mutant cells but that they can also be found on normal cells at 37°C. This was evident from the immunoprecipitation studies (Fig. 4) and from the feeding of RMA cells with specific peptides that results in an about 25% increase of the K^b and D^b expression. Alternatively, such empty molecules on normal cells could be derived from H-2 molecules associated with low-affinity peptides that were lost at the cell surface (see above). In another report, feeding cells with peptide led to a strong increase of the generally low expression of the L^d molecule (18). All these observations indicate that for the various MHC class I alleles in normal cells the supply with endogenous high-affinity peptides is not excessive but limited, thus increasing the chance after viral infection for virus-derived peptides to associate with class I molecules.

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