A Single Residue Exchange Within a Viral CTL Epitope Alters Proteasome-Mediated Degradation Resulting in Lack of Antigen Presentation

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Summary

CTL epitope (KSPWFTTL) encoded by AKV/MCF type of murine leukemia virus (MuLV) differs from the sequence in Friend/Moloney/Rauscher (FMR) type in one residue (RSPWFTTL). CTL experiments indicated defective processing of the FMR peptide in tumor cells. Proteasome-mediated digestion of AKV/MCF-type 26-mer peptides resulted in the early generation and higher levels of epitope-containing fragments than digestion of FMR-type peptides, explained by prominent cleavage next to R in the FMR sequence. The fragments were identified as 10- and 11-mer peptides and were efficiently translocated by TAP. The naturally presented AKV/MCF peptide is the 8-mer, indicating ER peptide trimming. In conclusion, a single residue exchange can cause CTL epitope destruction by specific proteasomal cleavage.

Introduction

Cytotoxic T lymphocytes (CTLs) recognize endogenously derived antigenic peptides presented by major histocompatibility complex (MHC) class I molecules. The proteasome complex plays an important role in the generation of MHC class I–presented peptides (reviewed by Goldberg and Rock, 1992; Howard and Seelig, 1993). Processing of endogenous protein antigens requires targeting to the proteasome complex, in which ubiquitination might be an important mechanism (reviewed by Ciechanover, 1994), and subsequent degradation by the proteasome. The "catalytic core" of this multicomponent complex is the 20S proteasome, a cylindrical particle (Löwe et al., 1995) displaying proteolytic activity with different specificities (Orlowski et al., 1993). Evidence for an essential role of the proteasome complex in the generation of MHC class I–presentable peptides is accumulating. Alterations in presentation of certain antigens have been observed in mice carrying targeted mutations of the LMP genes (Van Kaer et al., 1994; Fehling et al., 1994). Peptide aldehyde inhibitors of the proteasome were shown to block antigen presentation in intact cells (Rock et al., 1994). Highly purified 20S proteasomes have been used to digest whole protein or long synthetic peptides to generate antigenic peptides (Dick et al., 1994; Boes et al., 1994; Niedermann et al., 1995; Eggers et al., 1995). Proteasomal fine-specificity has been suggested to determine epitope hierarchy (immunodominance) in two chicken ovalbumin CTL epitopes (Niedermann et al., 1995).

MHC class I presentation of a proteasome-processed peptide requires translocation into the endoplasmic reticulum (ER). Transporter associated with antigen processing (TAP) is a heterodimer inserted in the ER membrane and is involved in transport of peptides into the ER lumen in an ATP-dependent fashion (Neefjes et al., 1993, Shepherd et al., 1993; Androlewicz et al., 1993). The rules for translocation of a peptide by TAP have been investigated by several groups. Both size and sequence characteristics of the peptide can influence translocation (Momburg et al., 1994a, 1994b; Schumacher et al., 1994; Neisig et al., 1995).

Murine leukemia viruses (MuLV) are oncogenic retroviruses inducing lymphomas in mice, and occur in two major types: endogenous AKV/MCF-type MuLV and exogenous Friend/Moloney/Rauscher (FMR)-type of MuLV. T cell immunity plays an important role in protection against these viruses, as shown by several investigators (reviewed by Zijlstra and Melief, 1986). CTLs raised against MuLV in H-2^b mice are either specific for the AKV/MCF type or for FMR type of MuLV (Plata and Lilly, 1979). Indeed, our MCF virus-specific CTL clones are not able to recognize tumor cells expressing FMR type of MuLV (Sijts et al., 1994a). We have identified the MCF-encoded immunodominant CTL epitope MCF8 (KSPWFTTL) (Sijts et al., 1994a) that was also immunodominant in AKR/Gross MuLV (White et al., 1994). This epitope differs in only 1 aa from the homologous sequence in FMR-type viruses, which have an arginine (R) instead of a lysine (K) at position 1 of the epitope. This does not affect peptide binding to MHC class I K^b molecules (Sijts et al., 1994a). Peptide vaccination showed that the FMR peptide is immunogenic in C57BL mice (Sijts et al., 1994a). However, CTLs raised against the FMR type of peptide were not capable of recognizing the tumor cells endogenously expressing FMR, whereas CTL raised against both the MCF/AKV and FMR peptides were able to recognize MCF-induced tumor cells. This suggested that the epitope is not processed in

FMR-type MuLV. We now address the question whether the difference in amino acid sequence between both types of viruses, either in the epitope itself or in the flanking regions, can influence processing and transport of the epitope. We have used purified 20S proteasomes to perform digestion analysis of 26-mer synthetic peptides, covering the CTL epitope in either AKV/MCF or FMR context. These experiments show that epitope containing fragments are generated from peptides encoding the AKV/MCF sequence, but inefficiently from the FMR sequence. We describe that in the FMR type of MuLV this epitope is destroyed by the presence of a dominant protease cleavage site within the epitope. This shows that a single amino acid change can abolish productive processing of an immunodominant CTL epitope.

Results

MuLV-Type Specific CTLs Suggest Defective Processing

CTLs raised against AKV/MCF MuLV are not cross-reactive with FMR MuLV (Plata and Lilly, 1979). We generated CTL against an MCF virus, derived from C57BL mice (MCF1233) (Zijlstra et al., 1983). These CTLs recognize a K^b-presented epitope KSPWFTTL (M8) present in the p15E–env gene product (Sijts et al., 1994a). The corresponding sequence in FMR type of MuLV differs in only 1 aa at position 1: RSPWFTTL (FMR8). CTLs raised against both M8 and FMR8 synthetic peptides were able to recognize tumor cells (771 lymphoma) endogenously expressing MCF1233 MuLV, but not RMA tumor cells expressing the FMR-type Rauscher MuLV (Sijts et al., 1994a). In this study, we have used a 771-directed CTL clone A9 that was selected to recognize FMR8 synthetic peptide next to the M8 peptide. Despite cross-reactivity at the synthetic peptide level, clone A9 was not able to recognize RMA or RBL-5 cells endogenously expressing Rauscher MuLV, indicating absence of presentation of the FMR peptide. Expression of K^b MHC class I and of the MuLV p15E protein on RMA cells, as detected with specific monoclonal antibodies, was found normal (data not shown). In addition, RMA cells can be recognized by Moloney MuLV-specific CTLs recognizing an env–gp70 encoded K^b-binding peptide (Sijts et al., 1994b), indicating normal expression of MuLV-encoded proteins. These data suggest that this MuLV CTL epitope is not correctly processed in the FMR type of MuLV.

Sequence Analysis of Epitope-Containing Region

The lack of presentation of the peptide in FMR MuLV–induced tumor cells prompted us to compare the amino acid sequence of the region in proximity of the epitope. The published sequences of Moloney MuLV (Shinnick et al., 1981) and MCF1233 MuLV (Sijts et al., 1994c) revealed next to the R to K difference within the epitope two divergent residues upstream (at positions −10 and −18 away from the epitope) and three divergent residues downstream (positions +10, +13, and + 29) of the epitope (Figure 1A). To exclude the possibility of additional mutations in the RMA and 771 cell lines present in our lab, we performed RT–PCR analysis of cDNA generated from isolated RNA of these two cell lines, using primers surrounding a fragment of 324 bp. Sequencing of these fragments resulted in completely identical nucleotide as well as amino acid sequences as published, including the six type-specific residues (Figure 1A).

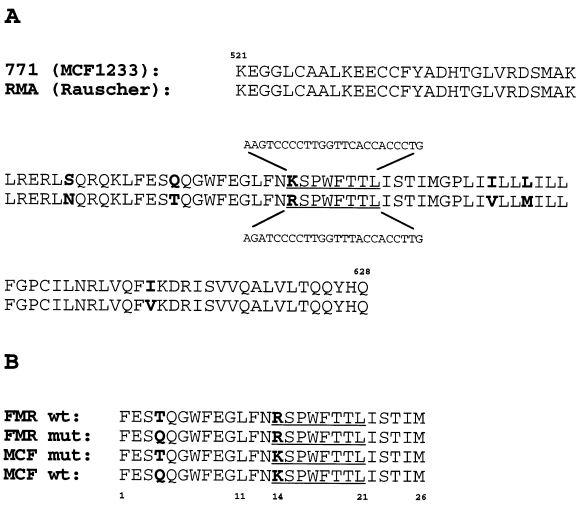

Figure 1. Amino Acid Sequences of the MuLV CTL Epitope-Encoding Region of env–p15E

(A) PCR analysis was performed on cDNA derived from total RNA of MuLV-induced cell lines 771 (MCF1233) and RMA (Rauscher MuLV), as present in our lab. Sequencing of the PCR fragments revealed that both nucleotide and amino acid sequences were identical to those published for both virus types. Amino acid sequences of both 771 and RMA are aligned and differences are depicted in bold. Nucleotide sequences corresponding to the CTL epitope are shown.

(B) 26-mer synthetic peptides used for proteasome digestion analysis. Four peptides were used: two wild-type peptides (FMR wt; MCF wt), and two peptides with one residue changed for the respective amino acid of the other virus type at position 4 (FMR mut; MCF mut).

No Generation of Antigenic Peptides by Proteasomal Digestion of FMR MuLV Sequences

To explore whether the lack of presentation of the FMR8 peptide is due to a processing defect determined by the amino acid sequence, 26-mer synthetic peptides covering the epitope and residues of interest in close proximity to the epitope were synthesized (Figure 1B). Both the natural and specifically changed MCF/AKV and FMR type of peptides were used for in vitro digestion experiments using isolated 20S proteasomes. Solubility problems prohibited the use of C-terminal elongated peptides. These cover the transmembrane region of p15E and thereby contain an extensive stretch of hydrophobic residues.

20S proteasomes were isolated from RMA cells. Figure 2 shows a two-dimensional polyacrylamide gel indicating that all identifiable proteasomal protein components are present. The N-terminally flanked peptides were incubated with isolated 20S proteasomes for 24 hr at 37°C or were mock incubated (without proteasome). Cytotoxicity assays were performed to test the generation of fragments with antigenic potency in the peptide

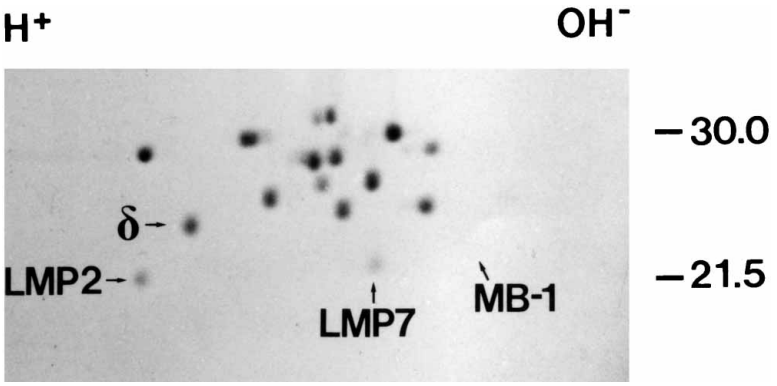

Figure 2. Analysis of 20S Proteasomes Isolated from RMA Cells by Two-Dimensional Gel Electrophoresis

The subunits of highly purified RMA proteasomes (50 μg) were separated on NEPHGE/PAGE gels as described (Groettrup et al., 1995) and visualized by Coomassie stain. Each of the visible protein spots can be assigned to one of the 16 subunits of the mouse proteasome according to their migratory positions (M. G., unpublished microsequence data). For simplicity, we only marked the four exchangeable subunits LMP2, LMP7, δ, and MB-1. Molecular masses are given in kilodaltons.

digests. K^b expressing RBL-5 target cells were loaded with titrated concentrations of 20S proteasome-digested peptides, and tested for recognition by CTL clone A9. Figure 3A shows strong lysis of RBL-5 cells loaded with proteasome-digested K-containing peptides in comparison with mock-treated peptides, indicating the generation of epitope-containing fragments. In contrast, target cells loaded with proteasome-digested R-containing peptides were not susceptible to lysis (Figure 3B). CTL clone A9 was able to recognize both synthetic 8-mer peptides, including the relevant 10- and 11-mer natural length variants (see below) (Figures 3C and 3D).

These results suggest that proteasomal activity in vitro correlates with antigen presentation in vivo.

Proteasomal Digestion Destroys Epitope in FMR MuLV Type

The proteasomal digestion products were analyzed by reverse-phase high performance liquid chromatography (RP–HPLC) and online mass Spectrometry. Figure 4 shows a typical experiment, comparing wild-type FMR (A) and wild-type MCF (B) 24 hr digests. Detectable degradation products (15–20 different fragments, depending on the experiment) were identified by their mass and subsequent sequencing. The abundantly occurring fragments and those relevant for epitope generation are shown. When we evaluated the presence of the optimal 8-mer epitope, this fragment was not detectable in either MuLV-type digest (Figure 4, top row). The C-terminal fragment ISTIM was generated from both sequences (Figure 4, row 2). A 14-mer N-terminal fragment was predominantly present in digests of the FMR type (Figure 4, row 5), indicating a cleavage site after R. Notably, two epitope-containing fragments (a 10-mer and an 11-mer) were present in the MCF-type digest, but in significantly lower amounts in digests of the FMR type of peptides (rows 3 and 4). To confirm peptide degradation as a proteasomal function, the proteolysis was carried out in the presence of inhibitors. Peptide aldehyde inhibitors selectively block proteasome activity in vitro and in vivo (Rock et al., 1994). Both LLnL and MG132 peptide aldehydes were able to inhibit the generation of the relevant fragments in a dose-dependent manner (data not shown).

Next to the K to R difference we also introduced a Q to T mutation in the synthetic peptides as present in the

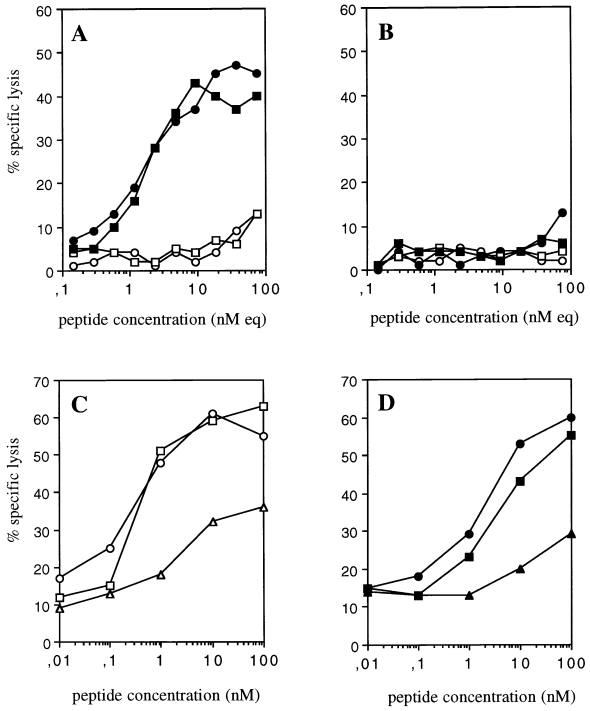

Figure 3. Sensitization of RBL-5 Target Cells with 20S Proteasome Digestion Products of Synthetic Peptides, as Recognized by CTL Clone A9 in a ^{51}Cr Release Assay

26-mer synthetic peptides were digested with purified 20S proteasomes in vitro as described. Digestion products were titrated in 2-fold dilution steps and preincubated with labeled target cells. (A and B) depict the equivalent of the initial concentration of the 26-mer substrate peptide (nM eq). (C and D) depict the actual concentrations of the added synthetic peptides.

(A) MCF-derived peptides MCF wt (squares) and MCF mut (circles), either incubated with proteasomes (closed symbols) or mock (without proteasomes) incubated (open symbols).

(B) FMR-derived peptides FMR wt (circles) and FMR mut (squares), either incubated with proteasomes (closed symbols) or mock (without proteasomes) incubated (open symbols).

(C) Cytotoxicity of clone A9 on RBL-5 target cells sensitized with serial dilutions of synthetic peptides MCF8 (KSPWFTTL, open circle), MCF10 (FNKSPWFTTL, open square) and MCF11 (FNKSPWFTTLI, open triangle).

(D) Cytotoxicity of clone A9 on RBL-5 target cells sensitized with serial dilutions of synthetic peptides FMR8 (RSPWFTTL, closed circle), FMR10 (FNRSPWFTTL, closed square) and FMR11 (FNRSPWFTTLI, closed triangle).

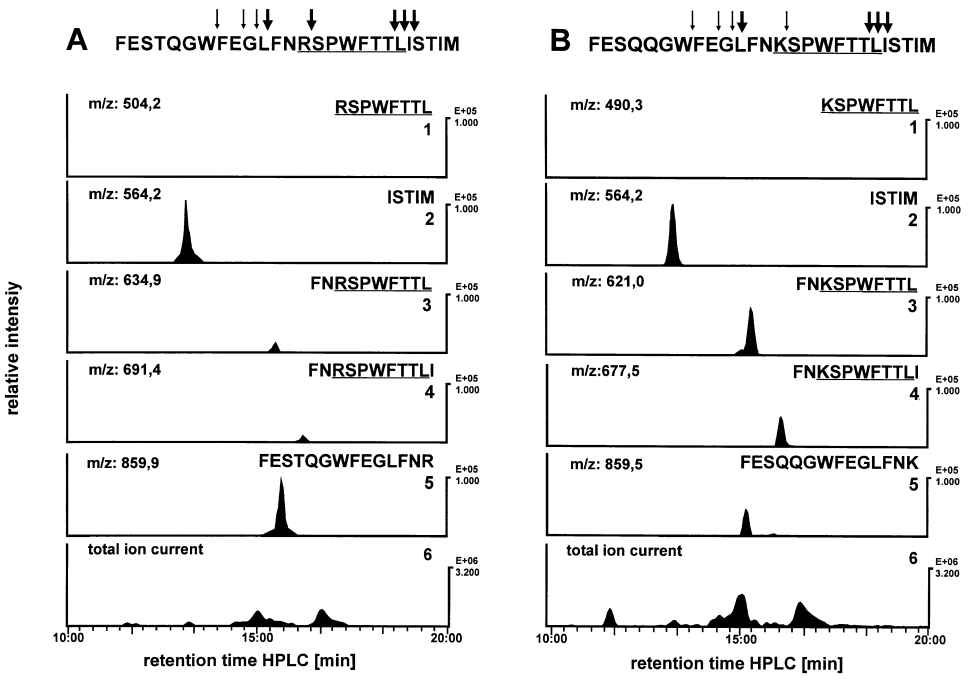

Figure 4. Biochemical Analysis of Proteasome Digestion Products Using HPLC Mass Spectrometry

Depicted is the presence of the most frequently occurring fragments by the ion current of the specific masses in 24 hr digests of FMR wt (A) and MCF wt (B) peptides. Row 6 shows the total ion current profile of all digestion products as separated by HPLC.

respective viral sequence (see Figure 1). Quantification of epitope-containing fragments was performed using standardization with the respective synthetic peptides (data not shown). The generation of these fragments after 24 hr of digestion was predominantly found in digests of the MCF-type peptides (5 to 10-fold higher than in FMR digests), irrelevant of the Q to T mutation at residue 3 of the peptide.

From these results, the major protease cleavage sites can be deduced as indicated by bold arrows on the top of Figure 4. The proteasome cleaves dominantly after the leucine at position 11, after the leucine at position 21, and after the isoleucine at position 22. The most striking difference between the R-containing peptides and the K-containing peptides is the protease cleavage site after the arginine at position 14. This indicates that an important protease cleavage site within a potential CTL epitope is responsible for destruction of the FMR8 but not the M8 epitope.

Kinetics of the Epitope-Containing Fragment Processing

Digestion of synthetic peptides for the relatively long period of 24 hr in the constant presence of proteasome is an artificial situation. If the observed difference represents a biologically relevant phenomenon, the generation of antigenic peptides from the MCF sequence should occur earlier. The kinetics of the generation of antigenic peptides was investigated by mass spectrometric analysis of aliquots of the digestion mixture at early timepoints. As indicated in Figure 5, the epitope-containing MCF fragments were detectable as early as 1 hr and steadily accumulated. The homologous FMR fragments were undetectable at 1 hr and both 10- and 11-mer peptides occurred later and reached significantly lower levels. The difference between both MuLV types is most prominent at early timepoints. The most dominant peptide derived from the FMR sequence was the 14-mer peptide without antigenicity, abundantly present already after 1 hr, suggesting early degradation of the potential epitope. In digests of the MCF 26-mer peptide, the 14-mer was barely detectable after 2 hr and remained far below the level of FMR after 4 hr of digestion. Thus, the different propensity of the two peptides to be cleaved into antigenic peptide precursors was also apparent after short-term degradation.

Epitope-Containing Peptides as Generated by Proteasomal Digestion Are Efficiently Translocated by TAP

In a previous study (Neisig et al., 1995), the MCF8 epitope was shown to be inefficiently transported by TAP in permeabilized RMA cells. However, when the 8-mer epitope was elongated with its natural flanking residues, TAP-mediated translocation was dramatically improved. To a greater or smaller extent, 9 and 10-mer elongated peptides were translocated (also shown in Table 1). In this study, we found two epitope-containing fragments generated by in vitro 20S proteasome digestion from 26-mer peptides. We tested the capacity of these peptides to be translocated by TAP. Both the 10-mer FNKSPWFTTL and 11-mer FNKSPWFTTLI were made synthetically and subjected to the TAP-dependent translocation assay (Table 1). The 10-mer and 11-mer peptides of both the AKV/MCF type as well as the (hypothetical) FMR type were translocated efficiently.

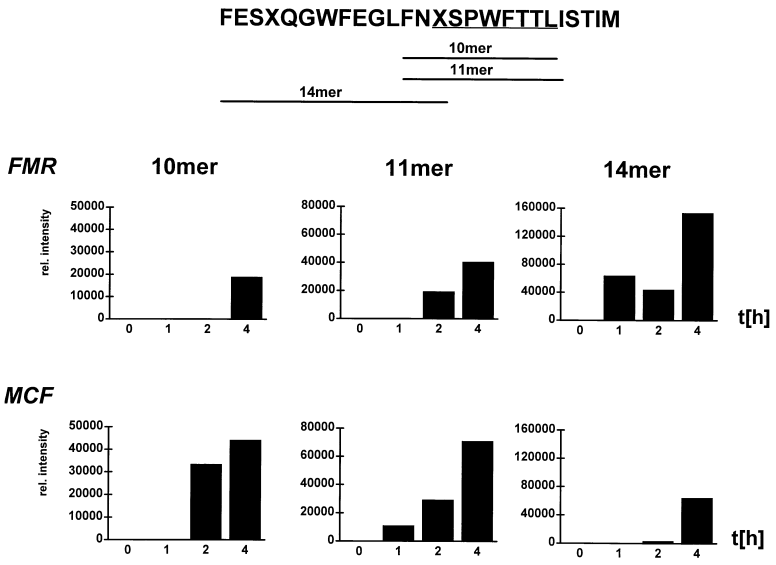

Figure 5. Time Course of the Generation of Epitope-Containing Fragments from the MCF and FMR 26-mer by the 20S Proteasome

Aliquots from different timepoints were analyzed by mass spectrometry. This graph shows the relative amounts of the epitope-containing fragments, 10-mer (FN*XSPWF TTL*), 11-mer (FN*XSPWFTTL*I), and the N-terminal 14-mer (FESXQGWFEGLFNX), produced after 1 hr, 2 hr, and 4 hr of incubation, respectively. X stands for the amino acid residue Q and K in the MCF sequence or T and R in the FMR sequence.

These results indicate that the 20S proteasome generates fragments that can be efficiently translocated into the ER without further processing.

Identification of the Naturally Presented Peptide Epitope

Our previous studies (Sijts et al., 1994a) strongly indicated that the 8-mer peptide, with a so-called classic K^b binding motif, was the naturally presented peptide. By comparison of synthetic length variants, it was shown that the 8-mer was the optimally binding and recognized peptide. In the present study, we found that the proteasome generates longer (10- and 11-mer) peptides than the 8-mer and even that TAP efficiently translocates the longer peptides rather than the 8-mer. This raises the question of what is actually presented in the K^b molecule at the cellular membrane. Making use of the knowledge that the 10- and 11-mers most likely enter the ER, we tested all synthetic length variant peptides in an RMA-S MHC class I binding assay. The peptide transporter–deficient cell line RMA-S can be loaded with exogenous peptides, thereby stabilizing the MHC class I molecules that were up-regulated for expression at 26°C. K^b expression was detected using a specific monoclonal antibody and the fluorescence is depicted in Figure 6. The data show that the AKV/MCF 8-mer peptide is the strongest binding peptide. Even at low peptide concentrations, it shows similar binding affinity as a control 9-mer Sendai virus CTL epitope S9 (Figure 6A). The 10- and 11-mer, but also both 9-mer variants (NKSPWFTTL and KSPWFTTLI) and the 7-mer (SPWFTTL) are poorly binding peptides. The FMR peptide binding study (Figure 6B) shows comparable results, and confirms that the difference between the two virus types is not due to differential MHC binding capacity.

To identify the naturally presented peptide, 771 cells and RMA cells were treated at low pH to elute MHC-bound peptides and the eluate was subjected to HPLC separation. The individual fractions were incubated with target cells and tested for recognition by clone A9 in a cytoxicity assay. Figure 7 shows that only fraction 17 of the 771 eluate was recognized by the CTL, indicating the presence of the natural peptide. The RMA-derived fractions were not recognized, again showing that the FMR peptide is not detectably presented. Coelution of the synthetic peptide variants indicated that only the 8-mer synthetic peptide elutes in fraction 17, whereas the related synthetic length variants elute in different fractions, as indicated with arrows. These findings prompted us to conclude that the peptide precursors resulting from proteasomal digestion are trimmed in the ER after translocation.

Table 1. TAP-Dependent Translocation of M8 and FMR8 Peptides with Natural Flanking Residues in Streptolysin O–Permeabilized RMA Cells

Competitor	IC_{50} (μM)
M8	>100
FMR8	>100
N-M8	>100
M8-I	20
M8-IS	>100
N-M8-I	2
Peptides identified by proteasomal digestion	
FN-M8[a]	3
FN-FMR8	3
FN-M8-I	6
FN-FMR8-I	4

Competition experiments were performed using a radiolabeled model peptide as described (Neisig et al., 1995). The IC_{50} value was calculated for each competing peptide based on titration curves, and is defined as the concentration of competitor peptide at which 50% inhibition of translocation of the model peptide is reached.
[a] Owing to a synthesis error, this peptide has been abusively annoted to an IC_{50} > 100 μM in a previous report (Neisig et al., 1995).

Discussion

In this study, we report the differential processing of a CTL epitope sequence from two related naturally occurring MuLV types. The peptide in these two virus types

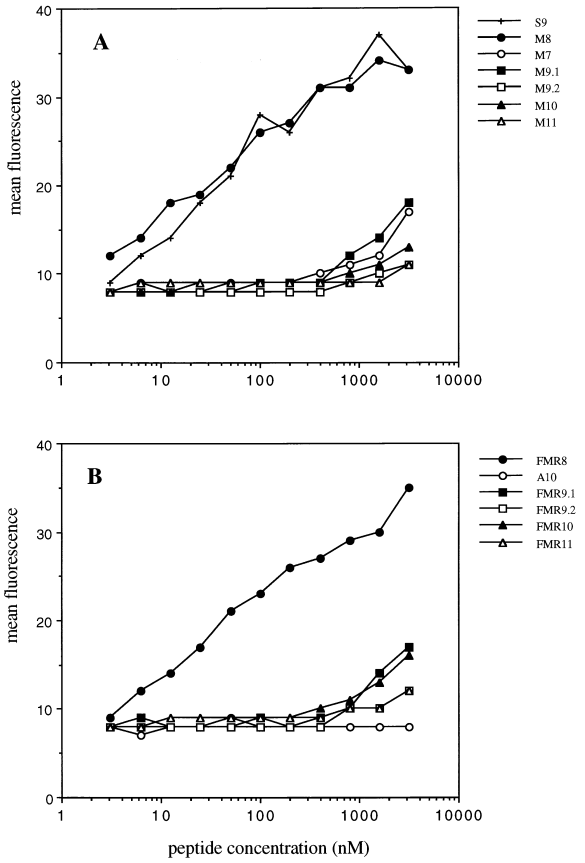

Figure 6. The 8-mer Peptide Is the Optimal K^b Binding Peptide

RMA-S peptide binding assay to compare the relative binding capacity of all possible length variants of the MCF (A) and FMR (B) peptide sequence of the naturally presented epitope. High mean fluorescence values indicate high levels of cell surface expressed MHC class I K^b molecules as stabilized by the added peptide. High affinity binding peptides can stabilize class I molecules even at relatively low concentrations of peptide.

(A) S9 (FAPGNYPAL) is a Sendai virus CTL epitope K^b-binding control peptide. M7 (SPWFTTL) is a 7-mer peptide expected to be generated by proteasomal degradation of the FMR sequence. Length variants of MCF8 (M8), (KSPWFTTL); M9.1, (NKSPWFTTL); M9.2, (KSPWFT TLI); MCF10 (M10), (FNKSPWFTTL); and MCF11 (M11), (FNKSPWFT TLI).

(B) A10 (SGPSNTPPEI) is an Adeno virus type 5 CTL epitope D^b-binding control peptide. Length variants of the FMR8 (RSPWFTTL) are identical as for the MCF8 except they contain an R instead of a K.

only differs at a single amino acid, the closely surrounding sequences are almost identical, and no difference in binding affinity for the class I K^b-binding groove has been found. Both optimally binding peptides are poorly transported by TAP. However, if they are elongated N-terminally, their transport is equally improved. The only difference found in this system is the cleavage specificity of the 20S proteasome in vitro. In digests of the FMR substrate, the first cleavage product that can be detected is the 14-mer FESTQGWFEGLFNR generated by a cleavage product that destroys the antigenic epitope. In contrast, the first cleavage products of the MCF-type sequence are a 10-mer (FN*KSPWFTTL*) and an 11-mer (FN*KSPWFTTL*I) containing the optimal epitope sequence. In the FMR digest, likewise, the 10- and 11-mer precursors are only detectable after extended times of degradation. Whether cleavage products that occur only after long-term degradation in vitro are generated at all in vivo remains open, considering the fact that proteasomes are probably constantly recharged with new polypeptides.

Proteasome digestion of synthetic peptides covering the AKV/MCF region generates 10- and 11-mer epitopes containing peptide fragments, which are, in addition, selected by the TAP transporter. Longer precursor peptides were generated by the proteasome, indicating the importance of flanking regions. Thus, the proteasome can directly generate peptide fragments that are optimal molecules to be recognized and translocated by the transporter complex. Indeed, modified flanking can increase the generation of antigenic peptides (Del Val et al., 1991; Eggers et al., 1995). For MCF8 epitope presentation, longer precursor peptides are essential, since the minimal 8-mer peptide is not transported by TAP (Neisig et al., 1995). Longer precursor peptides suggest additional N-terminal trimming of the epitope. Our data suggest that additional trimming of the 10/11-mer peptide takes place in the ER compartment. Evidence for the occurrence of peptide trimming in the ER has been obtained by others (Roelse et al., 1994; Snyder et al., 1994; Elliot et al., 1995: Hughes et al., 1996). Trimming seems to take place primarily N-terminally, which fits for the MCF8 precursors representing mainly N-terminally elongated peptides.

Peptide binding studies performed using the RMA-S MHC class I stabilization assay strongly suggest that the 8-mer peptide is the optimal K^b-binding peptide. Both 10- and 11-mer peptides poorly stabilized the MHC complex. However, the CTL assay (Figures 3C and 3D) shows comparable recognition of the 10-mer and 8-mer. Although both assays are based on different principles that might explain the contrasting outcome, e.g., degradation of 10-mers to 8-mers during the cytotoxicity assay, an alternative explanation might be differential K^b–peptide complex dissociation. We found that dissociation rate is a good predictive value for potential CTL epitopes (Van der Burg et al., 1996). We have determined the offrate of the relevant length variants of the epitope by loading RMA-S cells with high concentrations of peptide and measuring the dissociation kinetics at 37°C by specific MAb (data not shown). The 10-mer peptide slowly dissociates from the K^b complex, similar to the 8-mer peptide. In contrast, the 11-mer peptide rapidly dissociates, most likely because the additional isoleucine disturbs the proper fit of the leucine anchor residue in its pocket of the K^b groove.

The routing of transmembrane molecules like p15E–env proteins before targeting to the 20S proteasome in the cytosol is not known. However, the TAP dependence of processing of this transmembrane molecule suggests that the protein is cytosolically degraded and its fragments translocated by TAP, similar to what has been observed for measles virus fusion protein (Van Binnendijk et al., 1992). How this is achieved is unclear, but recent data indicate translocation mechanisms of ER proteins into the cytosol (Wiertz et al., 1996).

From our AKV-related model virus MCF1233 (Zijlstra et al., 1983; Sijts et al., 1994c), we have gained insight in the immune response against MCF1233-induced T

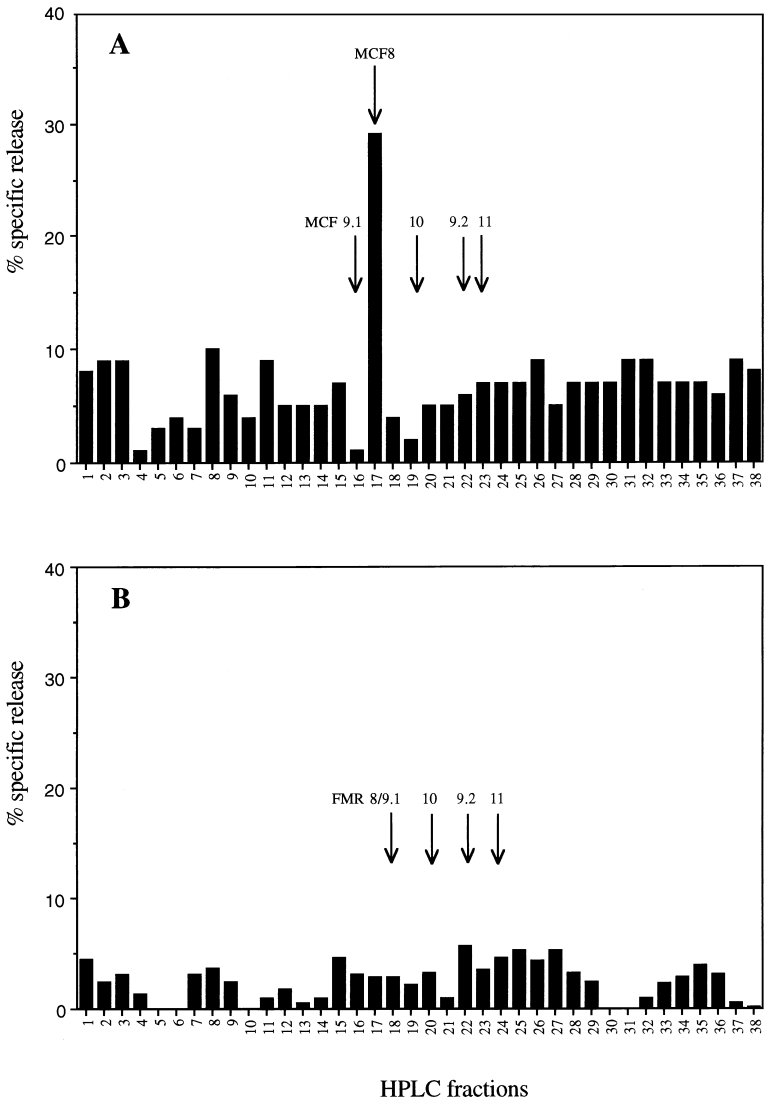

Figure 7. The MCF 8-mer Peptide Is the Naturally Presented Peptide

Acid elution of naturally presented peptides from 771 (A) and RMA (B) cells. Of both cell lines, 10^9 cells were 0.1% TFA eluted and the low molecular mass fraction was subjected to HPLC chromatography, resulting in 38 fractions. All fractions were incubated with target cells and tested for recognition by CTL clone A9. Coelution of the synthetic length variants in the respective fractions is indicated by arrows.

and B cell lymphomas of different MHC backgrounds (Vasmel et al., 1988). By immunization with an MCF1233-induced B cell lymphoma in C57BL/10 mice, we were able to isolate specific CTL lines and clones and we have identified the MCF1233-encoded CTL epitope M8 (KSPWFTTL) (Sijts et al., 1994a). Early studies in the MuLV field already showed that CTLs apparently have fine specificity for the two main types of MuLV, the AKV/MCF and FMR types (Plata and Lilly, 1979). In general, no cross-reactivity is found when CTLs are raised against one type or the other in the H-2^b haplotype. The identification of MuLV CTL epitopes helped in explaining this selectivity (Sijts et al., 1994a, 1994b). The AKV/MCF peptide sequence differed in one residue (K to R) from the FMR sequence. The lack of presentation of this epitope might be explained at several levels. We tested expression levels of MHC class I and p15E–env viral proteins, binding capacity of the peptide to K^b molecules, and the in vivo presence of CTLs capable of recognizing the peptide. None of these parameters could explain the observation. Here, we show that the presence of an R in this sequence leads to selective degradation of the potential CTL epitope in FMR viruses already at the proteasome level, precluding further processing and presentation. Niedermann et al. (1995) compared two independent CTL epitopes of chicken ovalbumin, using Edman degradation to analyze the proteasomal digestion products. In their case, the 8-mer immunodominant epitope appeared to be exactly cleaved from its surrounding sequences. In addition, a number of proteasomal cleavage sites were found within a subdominant ovalbumin CTL epitope, which could explain the subdominance of the epitope.

We demonstrate, using online mass spectrometry and sequencing, that one crucial cleavage site apparently defines the antigenicity of the sequence. Thus, differences in the sequence of naturally occurring viruses can determine the presence or absence of an immunodominant CTL epitope, not only at the level of binding to MHC or T cell receptor (TCR), but also at the processing level by formation of a proteolytic site. Although the nucleotide difference of the K (AAG) to R (AGA) exchange in the two MuLV types involves two nucleotides, theoretically only one (A to G) nucleotide change (K is AAA or AAG; R is AGA or AGG) can lead to the formation of this proteolytic site.

Viral immune escape mechanisms have been suggested to occur at various levels as reviewed by Melief (1992), including down-regulation of MHC, down-regulation of antigen, and secretion of immunosuppressive factors. Specific mutations in T cell epitopes can result in either nonbinding to MHC (anchor replacement) or nonrecognition by the TCR (interaction residue replacement). Here, we demonstrate a new potential immune evasion mechanism; namely, the potential of generating a proteasomal cleavage site within the epitope. Frequent amino acid changes have been observed in CTL epitopes in HIV gag (Phillips et al., 1991) and EBV EBNA (De Campos-Lima et al., 1994). These have been suggested to represent immune escape variants, only some of which are clearly MHC nonbinding peptides. Two EBV EBNA4 CTL epitope mutations (V to L; K to R) did not affect binding to HLA-A11, and CTLs recognized the mutant peptide but not cells expressing the mutated EBNA4 protein endogenously (De Campos-Lima et al., 1994). These findings could indicate a defect at the processing level. Also, in the MuLV model, the possibility exists that the single residue difference between AKV/MCF and FMR MuLV has been selected for by immune pressure. Our MCF1233 MuLV has been isolated from B10.A (H-2^a) mice expressing K^k, indicating a lack of pressure on K^b-presented peptides. On the other hand, tumor cell lines like RBL-5, expressing FMR type of MuLV, have been maintained by serial in vivo passage in H-2^b-expressing mouse strains (C57BL/6) in the past (Plata and Lilly, 1979) that might have resulted in selective pressure on class I (K^b and D^b)–presented CTL epitopes.

MHC class I binding motifs (Falk et al., 1991) have enabled us to predict potential CTL epitopes within a given sequence. Indeed, we have identified CTL epitopes by screening all motif-based class I–binding peptides with tumor-specific CTL (Sijts et al., 1994a, 1994b). However, it is still difficult to select further the immunologically relevant CTL epitopes among high affinity MHC class I–binding peptides, since the rules for processing of T cell epitopes are poorly understood. This report shows that the identification of major proteasomal cleavage sites is feasible and can be highly relevant to epitope prediction. Further elucidation and charting of motifs predicting proteasome cleavage sites can be expected to improve the accuracy of CTL epitope prediction from primary amino acid sequences.

Experimental Procedures

Cell Lines/Culture Conditions

Tumor cell lines: 771 is an MCF1233-induced B cell lymphoma cell line from a C57BL/10 mouse neonatally inoculated with MCF1233 MuLV as described (Sijts et al., 1994a); RBL-5 and RMA are Rauscher MuLV-induced T lymphoma cell lines from C57BL/6 origin (Ljunggren and Kärre, 1985).

CTL clone A9 was isolated from bulk spleen cell culture derived from mice immunized with irradiated MCF1233-induced 771 tumor cells as described (Sijts et al., 1994a). Spleen cells were restimulated with the same 771 tumor cells. After 1 week, bulk cultures were seeded under limiting dilution conditions and clones were randomly isolated and tested for cytotoxicity. Of 12 clones, 3 showed cross-reactivity with the FMR analog peptide RSPWFTTL next to reactivity to the immunodominant epitope KSPWFTTL. Clone A9 was selected for further studies. All cells were cultured in Iscove's modified Dulbecco's medium (GIBCO Biocult, Glascow, United Kingdom) supplemented with 5% fetal calf serum (tumor cells) or 10% fetal calf serum (CTL), penicillin (100 IU/ml) and 20 μM 2-mercaptoethanol. T cell clones were cultured in the presence of 20 IU/ml hIL-2.

Cytotoxicity Assays

CTL assays were performed as described (Sijts et al., 1994a). In short, 2×10^3 $Na_2{}^{51}CrO4$-labeled target cells were incubated with effector cells for 5–6 hr at 37°C. The culture supernatant was harvested and counted for released radioactivity. The percentage of specific ^{51}Cr-release was calculated as a ratio of 100 × (cpm experimental release − cpm spontaneous release): (cpm maximal release (1% Triton X-100) − cpm spontaneous release). All assays were carried out in triplicate. For target cell sensitization, ^{51}Cr-labeled target cells were incubated with peptide or proteasomal digests of peptides in concentrations indicated, 1 hr prior to addition of the CTL. Peptides remained present during the assay.

Peptides

Peptides were synthesized on a multiple peptide synthesizer (Abimed AMS 422) as described (Gausepohl et al., 1990). Peptides were analyzed for purity by RP–HPLC, lyophilized, and dissolved in phosphate-buffered saline. Stock solutions of 5 mg/ml were stored at −80°C. The MCF peptide for the kinetic study was synthesized on a ABI-synthesizer 433A.

PCR Analysis

RT–PCR analysis was performed as described (Hawes et al., 1993). Total RNA was isolated from 771 and RMA tumor cell lines using the RNAzol method (Cinna Biotecx) and cDNA was generated using AMV reverse transcriptase (Promega). Oligonucleotide primers were selected up- and downstream of the M8 CTL epitope, based on the sequence of MCF 1233 (Sijts et al., 1995) and Rauscher MuLV (Shinnick et al., 1981).

MCF 5′ primer (7201–7225): GCC ACT CAG CAG TTC CAA CAA CTC C

MCF 3′ primer (7687–7711): ATT CAC GCG ATT CTA CTT CTT CTG G

Rauscher 5′ primer (7348–7371): AAA GAA GGA GGA CTG TGT GCT GCC

Rauscher 3′ primer (7648–7672): GCT GGT GGT ATT GTT GAG TCA GGA C

PCR fragments after 40 cycles were cloned and sequenced using the Circumvent Kit (Circumvent Thermal Cycle Dideoxy DNA sequencing with VentR (exo^-) DNA polymerase; New England Biolabs, Incorporated).

Proteasome Digestion Assays

20S proteasomes were isolated from RMA and 771 cells as described (Groettrup et al., 1995). 26-mer peptides (20 μg) were incubated with 1 μg of purified 20S proteasome at 37°C for 24 hr in 300 μl assay buffer as described (Eggers et al., 1995). Digestion products were separated by RP–HPLC SMART-System equipped with a μRPC C2/C18 SC 2.1/10 column (Pharmacia) and eluted with a gradient of 20%–95% of eluent B (70% acetonitrile in 0.09% TFA) in eluent A (0.1% TFA) in 15 min with a flow rate of 50 μl/min, and analyzed online by a tandem quadrupole mass spectrometer TSQ 7000 (Finnigan MAT) equipped with an electrospray ion source. To determine the mass of the generated peptides, mass spectra were acquired over the range m/z 300–1300 each 2 s during the HPLC separation. From these spectra the intensities of all detected ions, the total ion current, and the intensity of specific peptide ions, the ion current of specific masses, are extracted. The specific peptide ions detected with intensities three times above background were sequenced by MS/MS analysis after fragmentation with Argon atoms (2.8 mTorr) using three different collision energies. The peptide sequence was derived from the masses of the generated fragment ions. The amount of peptides was determined by the intensity of the ion current of the double or single charged ions after calibration with the synthetic peptides using 10 pmol of an irrelevant peptide (SYPHFMPTNL) as internal standard. In two experiments, this standard peptide was added to the digest directly before HPLC/MS analysis.

Kinetic study was performed by sampling of 45 μl aliquots from the digestion mixtures at each timepoint, followed by separation on

HPLC (eluent A, 0.05% TFA; Eluent B, 70% acetonitrile containing 0.05% TFA; gradient, 20%–90% B in 18 min; flow rate, 65 μl/min) and analyzed online by mass spectrometry.

Proteasome inhibition studies were done with 1 μg proteasome using 20 μg MCF wild-type peptide in 300 μl assay buffer in the presence of 5–50 μM of the aldehyde inhibitors N-acetyl-L-leucinyl-L-norleucinal (LLnL) (Sigma) or N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal (MG-132, similar to MG-115; Rock et al., 1994) (Myogenics, Cambridge, Massachusetts). After 24 hr at 37°C, the samples were subjected to mass spectrometric analysis. The percentage of inhibition was calculated from the intensity of the relevant degradation products (FNKSPWFTTLI and FNKSPWFTTL).

TAP Translocation Assays

TAP-dependent translocation assays were performed as described (Neisig et al., 1995). In short, peptides of interest were tested for their ability to compete for TAP-dependent translocation of a ^{125}I-iodinated model peptide in Streptolysin O-permeabilized RMA cells.

RMA-S MHC Class I Peptide Binding Assay

The RMA-S assay was performed as described previously (Feltkamp et al., 1993). In short, RMA-S cells were cultured for 36 hr at 26°C and were added to serial dilutions of peptide in serum-free medium. After 4 hr incubation at 37°C, cells were washed and incubated with the K^b-specific monoclonal antibody 28.14.8S (Ozato and Sachs, 1981) for 30 min on ice. After washing, the cells were incubated with fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin G F(ab′)$_2$ fragments for 30 min on ice and immunofluorescence was analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, California).

Acid Elution of Natural Peptides

771 and RMA cells (10^9) were washed with serum-free medium and 150 mM NaCl solution to remove serum proteins and buffer. The cells were incubated for 15 min in 0.1% TFA/H_2O on ice. Cells were spun down for 15 min at 3000 rpm; the supernatant was collected and subjected to size exclusion chromatography to remove proteins. The fractions containing low molecular mass material (<5000 kDa) were concentrated by solid phase extraction on C18 reversed phase material. After elution with 50% and 90% acetonitrile, the organic solvent was removed by speed Vac concentration to get a final volume of 2 ml. HPLC separation was performed as described above, collecting 38 fractions of 50 μl each. From each fraction, 1 μl was diluted in 50 μl medium and tested in triplicate in a cytotoxicity assay. ^{51}Cr-labeled RBL-5 target cells were added in 50 μl and after 1 hr preincubation CTL clone A9 was added (E:T = 5) and incubated for 5 hr.

A coelution experiment was performed using the same HPLC conditions to separate synthetic peptides to determine the fraction at which the individual length variants elute.

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