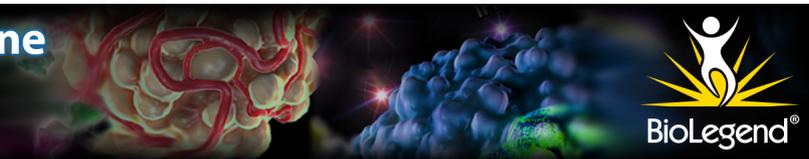


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# Abrogation of CTL Epitope Processing by Single Amino Acid Substitution Flanking the C-Terminal Proteasome Cleavage Site<sup>1</sup>

Nico J. Beekman,\* Peter A. van Veelen,\* Thorbald van Hall,\* Anne Neisig,<sup>†</sup> Alice Sijts,<sup>‡</sup> Marcel Camps,\* Peter-M. Kloetzel,<sup>‡</sup> Jacques J. Neefjes,<sup>†</sup> Cornelis J. Melief,\* and Ferry Ossendorp<sup>2\*</sup>

CTL directed against the Moloney murine leukemia virus (MuLV) epitope SSWDFITV recognize Moloney MuLV-induced tumor cells, but do not recognize cells transformed by the closely related Friend MuLV. The potential Friend MuLV epitope has strong sequence homology with Moloney MuLV and only differs in one amino acid within the CTL epitope and one amino acid just outside the epitope. We now show that failure to recognize Friend MuLV-transformed tumor cells is based on a defect in proteasome-mediated processing of the Friend epitope which is due to a single amino acid substitution (N→D) immediately flanking the C-terminal anchor residue of the epitope. Proteasome-mediated digestion analysis of a synthetic 26-mer peptide derived from the Friend sequence shows that cleavage takes place predominantly C-terminal of D, instead of V as is the case for the Moloney MuLV sequence. Therefore, the C terminus of the epitope is not properly generated. Epitope-containing peptide fragments extended with an additional C-terminal D are not efficiently translocated by TAP and do not show significant binding affinity to MHC class I-K<sup>b</sup> molecules. Thus, a potential CTL epitope present in the Friend virus sequence is not properly processed and presented because of a natural flanking aspartic acid that obliterates the correct C-terminal cleavage site. This constitutes a novel way to subvert proteasome-mediated generation of proper antigenic peptide fragments. *The Journal of Immunology*, 2000, 164: 1898–1905.

Cytotoxic T lymphocytes recognize antigenic peptides derived from endogenously degraded proteins that are presented by MHC class I molecules. Proteasomes, present in the cytosol and the nucleus as multicatalytic protease complexes, play a major role in the generation of these antigenic peptides (reviewed in Refs. 1 and 2). After proteasome-mediated degradation of protein substrates, the resulting peptide fragments are translocated from the cytosol into the endoplasmic reticulum (ER)<sup>3</sup> by TAP molecules, where they can bind to newly generated MHC class I molecules and become transported to the cell surface (reviewed in Refs. 3 and 4). Specific proteasome inhibitors can interfere with, or completely abolish, the generation of CTL epitopes or precursor fragments (5, 6). In IFN- $\gamma$ -stimulated cells, the proteasome appears to be the rate-limiting factor in the cascade of degradation, TAP translocation, and MHC class I peptide loading

(7). Two types of proteasomes are now recognized: the constitutive (household) proteasome, expressing the  $\beta$  subunits X, Y, and Z, and the IFN- $\gamma$ -inducible immunoproteasome, expressing LMP2, LMP7, and MECL-1 (8–10).

Purified 20S proteasomes have been used for digestion analysis of several (small) proteins and long synthetic peptides (9, 11–14), and a direct relationship between fragments generated by the proteasome in vitro and in vivo has been shown (15, 16). In vitro proteasome-mediated digestion analysis (12, 13, 15, 17) and proteasome inhibitor studies (17, 18) have shown that in particular the C terminus of the CTL epitope is precisely determined by the proteasome, whereas the N terminus is often elongated by one or two amino acids. The optimal MHC class I presentable peptide length is most likely generated by N-terminal trimming by ER resident (19–21) or cytosolic amino peptidases, as has recently been suggested for the OVA CTL epitope (17, 22). Several studies have shown that residues either within (13, 23) or in the flanking regions of the CTL epitope can have strong influence on the processing of the epitope (12, 15, 24–27).

Evasion mechanisms that viruses and tumors have exploited to circumvent recognition by CD8 T lymphocytes operate at many different levels of the MHC class I processing and presentation pathways (reviewed in Ref. 28). At the level of proteolytic processing, EBV was found to interfere via an internal repeat of the EBNA-1 protein (29) and adenovirus 12 is able to eliminate expression of both LMP2 and LMP7 (30). We have previously shown that murine leukemia virus variants can evade immune recognition by a single amino acid change within the CTL epitope sequence that resulted in premature destruction of the epitope by proteasome-mediated cleavage (13). This shows that single-point mutations can result in alterations in the primary amino acid sequence that affects viral epitope processing by the proteasome.

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<sup>3</sup> Abbreviations used in this paper: ER, endoplasmic reticulum; MuLV, murine leukemia virus; MS, mass spectrometry; NLVS, 4-hydroxy-5-iodo-3-nitrophenylacetyl-leu-leu-leu-vinylsulfone.

We have studied the processing and presentation of a Moloney murine leukemia virus (MuLV) env gp70-encoded CTL epitope SSWDFITV (31) that was shown to be a subdominant epitope in C57BL/6 mice but can induce significant protective immunity upon DNA vaccination (32). The closely related Friend and Rauscher MuLV, but also the distinct, endogenous AKV-type MuLV, share a high degree of sequence homology in the epitope region. The Rauscher MuLV amino acid sequence comprising the epitope is identical to that of Moloney virus and CTL recognize cells infected with both virus types. However, CTL cannot recognize cells that express Friend MuLV. In this study, we show that obliteration of the required precise C-terminal proteasome-mediated cleavage of the homologous Friend MuLV env sequence results in failure of TAP translocation and MHC class I binding. Consequently, the Friend env sequence ceases to serve as an epitope, despite the presence of an intact, strongly MHC class I-binding sequence.

## Materials and Methods

### Peptides and proteasome digestion assay

Peptides were synthesized on a multiple peptide synthesizer (Abimed AMS 422; Abimed Analysen-Technik, Langenfeld, Germany) as described elsewhere (33). Peptides were purified by reversed phase-HPLC in an acetonitrile-water gradient and lyophilized from acetonitrile-water overnight. Before use, peptides were dissolved in proteasome digestion buffer as described previously (15).

The 20S proteasomes were purified from RMA cells as described elsewhere (34). RMA proteasomes contain relatively high amounts of the immunosubunits LMP2, LMP7, and MECL-1 (13). Peptides (26 mer, 20  $\mu\text{g}$ ) were incubated with 1  $\mu\text{g}$  of purified RMA proteasomes in 300  $\mu\text{l}$  at 37°C for 1, 4, and 24 h. Trifluoroacetic acid (30  $\mu\text{l}$ ) was added to stop the digestion. Peptide digestions were stored at -70°C.

### Mass spectrometry (MS)

Electrospray ionization mass spectrometry was performed on a hybrid quadrupole time-of-flight mass spectrometer, a Q-TOF (Micromass, Manchester, U.K.), equipped with an on-line nanoelectrospray interface (capillary tip 20  $\mu\text{m}$  internal diameter \* 90  $\mu\text{m}$  outer diameter) with an approximate flow rate of 250 nl/min. This flow was obtained by splitting of the 0.4 ml/min flow of a conventional high-pressure gradient system using an Accurate flow splitter (LC Packings, Amsterdam, The Netherlands). Injections were done with a dedicated micro/nano HPLC autosampler, the FAMOS (LC Packings), equipped with two additional valves for phase system switching experiments. Digestion solutions were diluted five times in water-methanol-acetic acid (95:5:1, v/v/v), and 1  $\mu\text{l}$  was trapped on the precolumn (MCA-300-05-C8; LC Packings) in water-methanol-acetic acid (95:5:1, v/v/v). Washing of the precolumn was done for 3 min to remove the buffers present in the digests. Subsequently, the trapped analytes were eluted with a steep gradient going from 70% B to 90% B in 10 min, with a flow of 250 nl/min (A, water-methanol-acetic acid (95:5:1, v/v/v); B, water-methanol-acetic acid (10:90:1, v/v/v)). This low elution rate allows for a few additional MS/MS experiments if necessary during the same elution.

Mass spectra were recorded from mass 50–2000 Da every second with a resolution of 5000 FWHM. The resolution allows direct determination of the monoisotopic mass, also from multiple charged ions. In MS/MS, mode ions were selected with a window of 2 Da with the first quadrupole, and fragments were collected with high efficiency with the orthogonal time-of-flight mass spectrometer. The collision gas applied was argon (4\*10<sup>-5</sup> mbar), and the collision voltage ~30 V.

The peaks in the mass spectra were searched in the digested precursor peptide using the Biolynx/proteins software (Micromass, Manchester, U.K.) supplied with the mass spectrometer.

The intensity of the peaks in the mass spectra was used to establish the relative amounts of peptides generated after proteasome digestion. The relative amounts of the peptides are given as a percentage of the total amount of peptide digested by the proteasome. The percentage of the F and Y peptides were used to calculate the ratio between these two types of peptides. We found a ratio between 100- and 1000-fold more F than Y peptide.

### Cell lines and culture conditions

RMA is a Rauscher MuLV-induced T lymphoma cell line; FBL-3 is a Friend MuLV-induced erythroleukemia cell line; RMA-S is an RMA-de-

rived TAP2 mutant cell line; and EL-4 is a chemically induced (non-MuLV) thymoma cell line. B6 m29 is a fibroblast cell line derived from a primary culture of mouse embryo cells. All murine cell lines are of C57BL/6 (H-2<sup>b</sup>) origin. HeLa-K<sup>b</sup> is a stable transfectant of the human HeLa cell line expressing the mouse H-2K<sup>b</sup> MHC class I molecule. CTL clone 10B6 is derived from a Moloney MuLV-immunized B6.CH-2<sup>bm13</sup> mouse and cultured as described elsewhere (31).

All cell lines were cultured in Iscove's modified Dulbecco's medium (BioWhittaker, Verviers, Belgium) supplemented with 8% heat-inactivated FCS (Greiner, Frickenhausen, Germany), 2 mM L-glutamine, 100 U/ml penicillin, and 20  $\mu\text{M}$  2-ME at 37°C in 5% CO<sub>2</sub>.

### CTL bioassays and proteasome inhibition

Chromium-51 release cytotoxicity assays were performed as described (13). The mean percentage of specific lysis of triplicate wells was calculated as follows: % specific lysis = [(cpm experimental release - cpm spontaneous release)/(cpm maximum release - cpm spontaneous release)] × 100.

Measurement of secreted TNF- $\alpha$  by stimulated CTL was performed with a bioassay using WEHI 164 clone 13 cells as described previously (35). Percentage of TNF- $\alpha$  release of triplicate wells was calculated as follows: % TNF- $\alpha$  release = [(A<sub>550-650</sub> experimental wells - A<sub>550-650</sub> wells containing medium only)/(A<sub>550-650</sub> wells containing 500 pg/ml TNF- $\alpha$  - A<sub>550-650</sub> wells containing medium only)] × 100.

Production of IFN- $\gamma$  by the CTL was measured by a sandwich ELISA performed in maxisorp plates (Nunc, Roskilde, Denmark) using anti-mouse IFN- $\gamma$ -specific mAbs (clones R4-6A2 and biotinylated XMG1.2; PharMingen, San Diego, CA), streptavidin-conjugated poly-HRP (CLB, Amsterdam, The Netherlands), and 2, 2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (Sigma, St. Louis, MO) as a substrate. In every test titrated amounts of recombinant mouse IFN- $\gamma$  (PharMingen) served as a standard. OD at 415 nm was measured using kinetic 2.12 software in an EL312e Biokinetics ELISA plate reader (Biotek Instruments, Winooski, VT).

The proteasome-specific inhibitors lactacystin (Calbiochem, Breda, The Netherlands) (36) and 4-hydroxy-5-iodo-3-nitrophenylacetyl-leu-leu-leu-vinylsulfone (NLVS; Calbiochem) (37) were used to block proteasome activity. B6 m29 cells were infected with Abelson Moloney MuLV by incubation of the cells for 3–4 days in complete culture medium as described previously (38). These cells were used as targets in CTL cytokine secretion assays in the presence or absence of 20  $\mu\text{M}$  lactacystin or 20  $\mu\text{M}$  NLVS in complete culture medium for 4 h at 37°C. Cells were washed three times with exceeding amounts of culture medium. RMA tumor cells were treated using a peptide-stripping procedure as described elsewhere (18): cells were incubated with 10  $\mu\text{M}$  lactacystin for 2 h at 37°C, followed by a 1–2 min mild acid treatment (pH 3.0) according to Storkus et al. (39) and incubated for another 2 h with lactacystin. After extensive washing, the cells were used as targets in <sup>51</sup>Cr release assays.

### Transient transfections

Plasmid DNA was transfected into HeLa.K<sup>b</sup> cells and seeded onto flat-bottom 96-well plates, using the DEAE-dextran method, as described previously (40). Two days after transfection, culture medium was replaced and 5000 CTL were added in each well. Expression constructs used were mini-gene construct-encoding 8-mer peptide SSWDFITV directly behind the signal sequence of E3 protein of adenovirus type 5 (41); Moloney MuLV p-gag/pol-gtp (*Scal-NaeI* fragment); and Moloney MuLV p-env (*Bg/III-NheI* fragment) driven by the Moloney MuLV long terminal repeat.

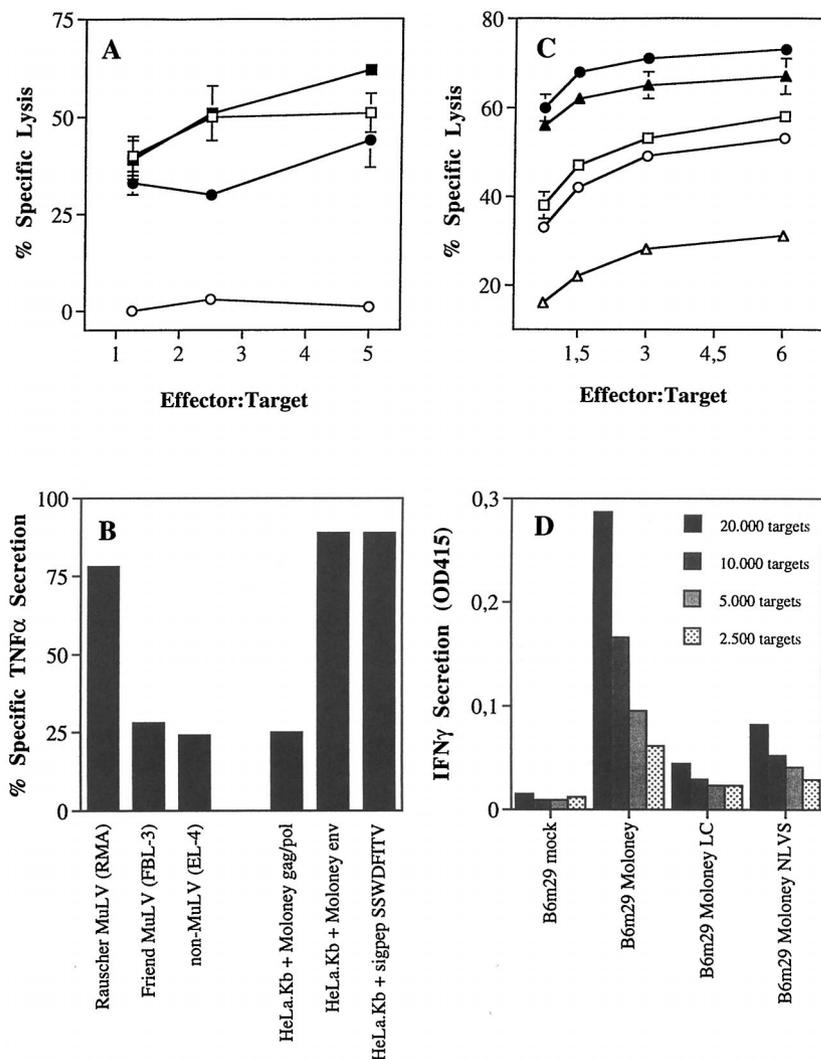
### TAP translocation assays

The TAP-dependent translocation assay was performed as described elsewhere (42). In short, peptides of interest were tested for their ability to compete for TAP-dependent translocation of a <sup>125</sup>I-iodinated model peptide in streptolysin O-permeabilized EL-4 cells.

### MHC class I peptide-binding assay

The RMA-S peptide-binding assay was performed as described previously (13, 43). In short, RMA-S cells were cultured for 36 h at 26°C and were added to serial dilutions of peptide. After 4 h of incubation at 37°C, cells were washed and stained with an H-2K<sup>b</sup>-specific Ab, and specific fluorescence was determined using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

**FIGURE 1.** CTL specific for the Moloney MuLV CTL epitope do not recognize Friend MuLV-induced tumor cells. **A**, Friend MuLV-induced FBL-3 tumor cells (○) are not recognized by 10B6 CTL in a  $^{51}\text{Cr}$  release assay, but can be lysed by addition of synthetic peptide SSWDFITV (●). Rauscher MuLV-induced lymphoma cells RMA (□) are recognized and killed independently of the presence of exogenous peptide (■). **B**, Specificity of CTL clone 10B6 was measured by TNF- $\alpha$  secretion as detected in the WEHI bioassay. 10B6 cells were incubated with RMA cells; FBL-3 cells; EL-4 cells; H-2K<sup>b</sup> expressing HeLa cells that were transiently transfected with either Moloney MuLV gag/pol construct; Moloney MuLV env construct; and a minigene construct containing the SSWDFITV epitope preceded by the adenovirus E3 signal peptide (sigpep SSWDFITV). **C**, Proteasome-dependent generation of the Moloney MuLV CTL epitope SSWDFITV in tumor cells. RMA cells were pretreated with 10  $\mu\text{M}$  lactacystin for 2 h, stripped by mild acid treatment (pH 3.0), and incubated for another 2 h with lactacystin before the  $^{51}\text{Cr}$  release assay using CTL clone 10B6. □, RMA untreated; ○ and ●, RMA acid stripped;  $\Delta$  and  $\blacktriangle$ , RMA acid stripped and lactacystin treated. Addition of the synthetic peptide SSWDFITV shows the viability of the treated cells (●,  $\blacktriangle$ ). **D**, Proteasome-dependent processing in Moloney MuLV-infected C57BL/6-derived fibroblast cell line B6 m29. 10B6 CTL were incubated with Moloney-infected B6 m29 cells that were treated for 4 h with 20  $\mu\text{M}$  lactacystin (LC) or 20  $\mu\text{M}$  NLVS. After 6 h of incubation with 10B6 CTL, culture medium was harvested and tested for the presence of IFN- $\gamma$  in an ELISA.



## Results

### *Moloney-specific CTL recognize Moloney and Rauscher but not Friend virus-induced tumor cells*

CTL raised against Moloney MuLV are cross-reactive to Rauscher MuLV sharing the epitope sequence SSWDFITV, as described before (31). In contrast, these CTL do not recognize cells transformed by the closely related Friend MuLV, despite the high degree of sequence homology in the epitope SSWDYITV that differs in only one residue that constitutes a well-defined alternative anchor residue at this position (44). Indeed, the Y-containing synthetic peptide can efficiently bind to the MHC class I-K<sup>b</sup> molecule and upon loading onto K<sup>b</sup>-positive target cells can be recognized by the CTL (Ref. 31 and below).

Fig. 1A shows the lack of recognition of Friend MuLV-induced FBL-3 tumor cells by the Moloney-specific CTL clone 10B6. Loading of FBL-3 cells with the synthetic peptide SSWDFITV resulted in efficient recognition, showing that these cells can be lysed by CTL. Even in a more sensitive TNF release assay (Fig. 1B), Friend virus-induced FBL-3 tumor cells are not recognized in contrast to RMA tumor cells endogenously expressing Rauscher MuLV Ags. In the same experiment, CTL can recognize Moloney MuLV env and the minimal epitope sequence introduced as a minigene by transient transfection, whereas Moloney MuLV gag-pol-transfected cells are not recognized. FBL-3 cells have normal levels of MHC class I expression and FBL-3 can be killed by other

MuLV-specific CTL recognizing the endogenously presented gagL CTL epitope (data not shown), indicating normal expression of MuLV-encoded proteins. These data indicate that the potential Friend MuLV-encoded CTL epitope sequence is not properly processed and presented for CTL recognition.

To investigate the involvement of the proteasome machinery in the generation of CTL epitope SSWDFITV, the proteasome inhibitor lactacystin was used to pretreat RMA cells 2 h before and 2 h after mild acid stripping of the cells. Stripping the cells from cell surface presented peptides was found to be essential for effective inhibition of these tumor cells (data not shown). Fig. 1C shows significant inhibition of cytotoxicity of CTL clone 10B6 by lactacystin treatment of RMA cells. CTL recognition could be rescued by addition of the exogenous synthetic peptide SSWDFITV. Similar experiments were performed with B6-derived fibroblast cells that were infected with Moloney MuLV. Both lactacystin (36) and the vinyl sulfone inhibitor NLVS (37) completely inhibited epitope processing in these MuLV-infected cells (Fig. 1D).

### *Proteasome-mediated digestion indicates altered C-terminal cleavage*

The naturally occurring viruses Moloney, AKV, and Friend MuLV share high sequence homology in the region of the Moloney CTL epitope. Only two residues differ (Table I). The phenylalanine (F) residue within the epitope at the anchor position 5 (Moloney) is

Table I. Synthetic peptides used for digestion experiments<sup>a</sup>

Peptide	Sequence
Moloney	TGRAYWKPSSSWDFITV <u>N</u> NNLTSQA
AKV	TGRASWKPSSSWDYITV <u>S</u> NNLTSQA
Friend	TGRAYWKPSSSWDYITV <u>D</u> NNLTTNQA

<sup>a</sup> Underlined are the variable amino acids flanking the C-terminal V of the potential CTL epitopes.

exchanged for a tyrosine (Y) (AKV and Friend), which is an allowed anchor residue for K<sup>b</sup> binding. The residue just flanking the C-terminal cleavage site of the epitope is different for all three virus types. The Moloney sequence contains an asparagine (N), AKV, a serine (S), and Friend, an aspartic acid (D), at this position. To investigate whether CTL recognition is related to the amino acid sequence differences discussed, the processing of the Moloney, AKV, and Friend MuLV epitopes from their natural context was tested. To this end, we performed in vitro proteasome digestions of the respective 26-mer synthetic peptides harboring the epitope flanked with its natural amino acid sequences. Table II shows the results of the digestion experiments for the three synthetic peptides. The 20S proteasome-mediated digestion of the Moloney peptide results in four major fragments after 1 h of digestion and two major fragments after 4 h. The proteasome precisely determines the C-terminal valine (V) of the epitope. The minimal 8-mer CTL epitope fragment SSWDFITV could not be detected in the digests. The two major fragments were the 7-mer SWDFITV and the 10-mer PSSSWDFITV. The most likely relevant epitope precursor peptide found is the 10-mer fragment, requiring N-terminal trimming to generate the CTL epitope SSWDFITV.

Comparison of the digests of the AKV or Friend MuLV peptide substrates containing an Y instead of an F did not show detectable

cleavage within the epitope. Therefore, lack of presentation of the Friend virus epitope could not be explained by the destruction of the Y-containing epitope sequence. However, a striking difference was found between Moloney/AKV and Friend in cleavage at the C terminus of the epitope. Whereas the Moloney and AKV peptides were precisely cleaved immediately C-terminal of valine, the Friend MuLV peptide substrate showed no detectable cleavage after valine. The cleavage site has shifted dominantly to a site C-terminal of the negatively charged flanking aspartic acid. To exclude the possible influence of the phenylalanine to tyrosine change within the epitope on the cleavage C-terminal of aspartic acid, four analogue synthetic peptide substrates were tested. For this experiment, the sequence of Moloney MuLV was used as backbone. The results in Table III show that the cleavage products generated are not influenced by the presence of either F or Y at position 5 of the epitope core sequence. Instead, cleavage is dominantly influenced by the aspartic acid. The presence of this negatively charged residue next to the valine abolishes the precise C-terminal cleavage site of the epitope.

#### TAP translocation of the precursor peptides generated by the 20S proteasome

We have previously shown that the SSWDFITV peptide is efficiently translocated into the ER in a TAP-dependent translocation assay (42). We now report the efficiency of TAP translocation in vitro of the peptides, as generated by the 20S proteasome. Table IV shows that both the Moloney 8-mer SSWDFITV and the Friend homologue SSWDYITV are very efficiently translocated by TAP as synthetic peptides. However, no detectable TAP translocation was displayed by the peptides flanked by D at the C terminus for all length variants tested of both the Moloney and Friend sequences. Apparently, the negatively charged residue at the C terminus disturbs murine TAP-dependent ER translocation of these

Table II. Major peptide fragments generated by 20S proteasome-mediated digestion of MuLV natural sequences

MuLV Type	Sequence	Digestion Time (h)		
		1	4	24
Moloney	TGRAYWKPSSSWDFITVNNLTSQA			
	SWDFITV	45 <sup>a,b</sup>	64	72
	PSSSWDFITV	23	36	28
	TGRAYWKPSSSWDFITV	18		
	AYWKPSSSWDFITV	14		
AKV	TGRASWKPSSSWDYITVSNLTSQA			
	TGRASWKPSSSWDYITVSNLTSQA	57		
	ASWKPSSSWDYITVSNLTSQA	29		
	TGRASWKPSSSWDYITV	14	12	
	SWDYITV		41	75
	ASWKPSSSWDYITV		30	
	PSSSWDYITV		17	25
Friend	TGRAYWKPSSSWDYITVDNLLTTNQA			
	TGRAYWKPSSSWDYITVDNLLTTNQA	60		
	TGRAYWKPSSSWDYITVD	21	25	
	AYWKPSSSWDYITVD	8	20	
	AYWKPSSSWDYITVDNLLTTNQA	6		
	PSSSWDYITVDNLLTTNQA	5		
	SWDYITVD		35	69
	PSSSWDYITVD		20	31

<sup>a</sup> Intensity is given as a percentage of the total amount of intensity of the peak fragments depicted below.

<sup>b</sup> Fragments with intensities less than three times the noise peak in a spectrum were ignored in the determination of sequences of peptides generated after digestion by 20S proteasomes.

Table III. Major peptide fragments generated by 20S proteasome-mediated digestion of Moloney sequence analogues

Analogues	Sequence	Digestion Time (h)		
		1	4	24
Y, N	TGRAYWKPSSSWDYITVNNLTSQA			
	TGRAYWKPSSSWDYITVN	22 <sup>a,b</sup>	16	
	TGRAYWKPSSSWDYITV	20	15	
	TGRAYWKPSSSWDYITVNNL	12		
	TGRAYWKPSSSWDYITVNN	12	7	
	TGRAYWKPSSSWD	9	14	9
	SWDYITV	7	15	31
	TGRAYWKPSSSWDYITVNNLTSQA	7		
	AYWKPSSSWDYITVN	5		
	KPSSSWDYITV		9	10
	AYWKPSSSWD		8	9
	PSSSWDYITV	4	8	14
	AYWKPSSSWDYITV			7
	KPSSSWDYITVN			6
	SWDYITVNNL	2	2	
SWDYITVN			14	
Y, D	TGRAYWKPSSSWDYITVDNNLTSQA			
	TGRAYWKPSSSWDYITVD	43	9	
	SWDYITVD	23	40	60
	SSWDYITVD	10		
	PSSSWDYITVD	9	12	22
	AYWKPSSSWDYITVD	8	7	
	TGRAYWKPSSSWD	7	9	
	KPSSSWDYITVD		14	18
	AYWKPSSSWD		9	
F, S	TGRAYWKPSSSWDFITVSNLTSQA			
	TGRAYWKPSSSWDFITV	48	13	
	SWDFITV	17	35	57
	TRGAYWKPSSSWD	14	10	5
	KPSSSWDFITV	12	19	13
	PSSSWDFITV	9	18	20
SSWDFITV		5	5	
F, D	TGRAYWKPSSSWDFITVDNNLTSQA			
	TGRAYWKPSSSWDFITVD	68		
	SWDFITVD	16	46	64
	AYWKPSSSWDFITVD	11		
	KPSSSWDFITVD		22	21
	TGRAYWKPSSSWD		20	
PSSSWDFITVD	5	12	15	

<sup>a</sup> Intensity is given as a percentage of the total amount of intensity of the peak fragments depicted below.

<sup>b</sup> Fragments with intensities less than three times the noise peak in a spectrum were ignored in the determination of sequences of peptides generated after digestion by 20S proteasomes.

Table IV. TAP-dependent translocation of Moloney and Friend MuLV CTL epitope peptides with naturally flanked residues

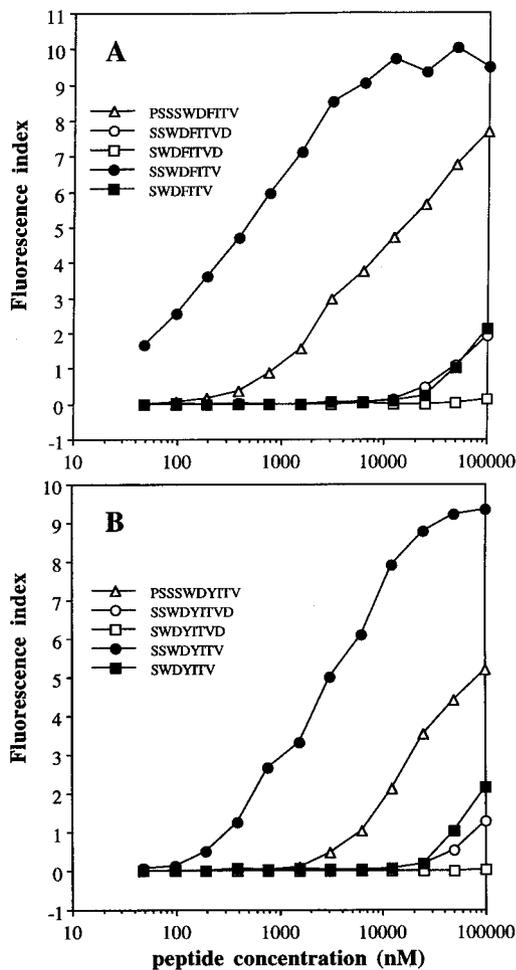
Competitor	IC <sub>50</sub> (μM) <sup>a</sup>
Moloney	
SWDFITV	>100
SSWDFITV	12.0 ± 5.2
PSSSWDFITV	>100
SWDFITVD	>100
SSWDFITVD	>100
PSSSWDFITVD	>100
Friend	
SWDYITV	>100
SSWDYITV	15.7 ± 4.0
PSSSWDYITV	>100
SWDYITVD	>100
SSWDYITVD	>100
PSSSWDYITVD	>100

<sup>a</sup> Mean of three experiments ± SD.

peptides (45). No detectable translocation was measured with the 7-mer variants SWDFITV and SWDYITV, nor with the 10-mer length variants PSSSWDFITV and PSSSWDYITV. Thus, the most likely candidate for TAP transport is the efficiently translocated exact MHC-binding sequence SSWDFITV (or SSWDYITV, if generated).

#### MHC class I binding and CTL recognition of the precursor peptides

The peptides found to be generated by the proteasome were tested for MHC class I binding and CTL recognition. Fig. 2 shows the results of MHC-binding experiments using synthetic peptides of different length variants. Fig. 2A shows the F-containing peptides (Moloney) and Fig. 2B the Y-containing peptides (Friend). The 8-mer peptide SSWDFITV, previously defined as the optimally binding and recognized peptide (28), was reproducibly the peptide with the most optimal MHC class I-K<sup>B</sup>-binding ability. In most

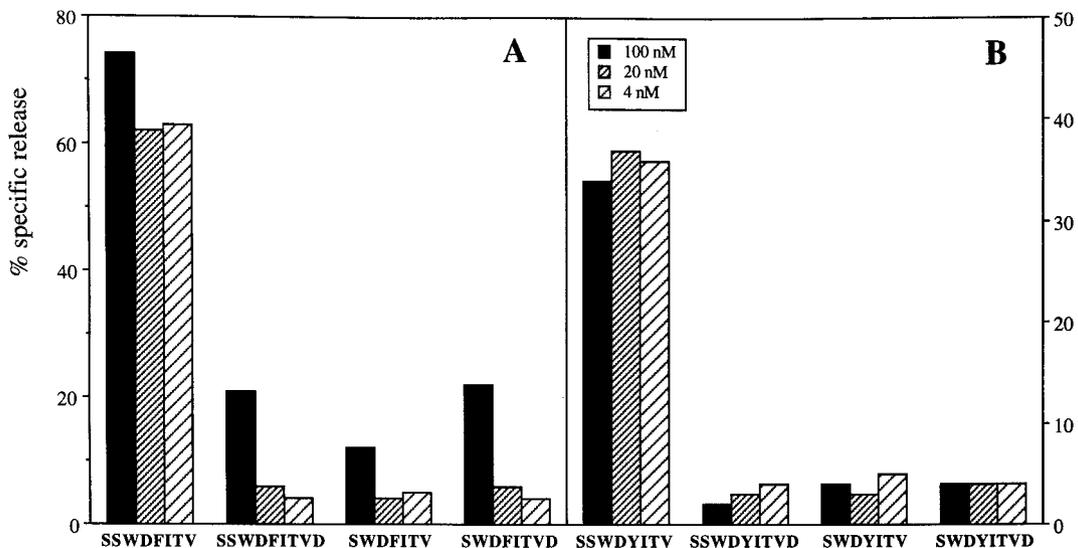


**FIGURE 2.** Peptide binding of synthetic peptides to MHC class I-K<sup>b</sup> molecules is disrupted by the aspartic acid residue flanking the C-terminal valine. Peptide length variants of the MuLV CTL epitope were tested for K<sup>b</sup>-binding capacity using the RMA-S MHC class I stabilization assay. *A*, Peptides of the Moloney MuLV sequence. *B*, Peptides of the Friend MuLV sequence.

proteasome digestion experiments, the amount of this peptide was below the detection level (Tables II and III). The most abundantly generated 7-mer peptide SWDFITV did not exhibit significant binding to the MHC class I-K<sup>b</sup> molecule. The precursor peptide fragment PSSSWDFITV binds to the MHC class I-K<sup>b</sup> molecule, but with significantly less efficiency than the 8-mer. The presence of an aspartic acid as the C-terminal residue of the peptides strongly decreases the binding capacity to the MHC class I-K<sup>b</sup> molecule. Similar results were obtained with the Y-containing peptides (Fig. 2*B*). The same synthetic peptides were used in cytotoxicity assays with CTL clone 10B6 (Fig. 3). The recognition by the CTL is completely abolished when the D is present at the C terminus. We conclude that the presence of this negatively charged residue next to the C-terminal anchor of the CTL epitope strongly disrupts TAP translocation as well as binding to the MHC class I groove and thereby CTL recognition.

**Discussion**

Appropriate C-terminal cleavage of a CTL epitope by the proteasome is a crucial step in the formation of precursor peptides leading to MHC presentable TCR ligands. A single residue exchange, flanking the C-terminal amino acid, obliterates accurate proteasome-mediated cleavage. This leads to the generation of peptide precursors that are neither suitable for TAP translocation nor for MHC class I binding. In this case an N to D exchange is caused by a single nucleotide mutation (N = AAC or AAT codon; D = GAC or GAT codon). An alternative explanation for the lack of epitope presentation of the Friend homologue could be the lower MHC class I-binding affinity. The Friend sequence harbors an alternative anchor residue Y instead of F, the binding affinity of the peptide for the MHC class I-K<sup>b</sup> molecule has been studied in detail and published in our previous paper (31). The binding affinity of the Y peptide is ~5-fold lower than that of the F peptide. However, the affinity of this peptide is still very high and within the range of other well-defined K<sup>b</sup>- and D<sup>b</sup>-presented CTL epitopes as published earlier (46). The affinity of the CTL clone used (10B6, originally raised against the F-containing epitope) for the Y-containing peptide is lower than that for the F-containing peptide, but still within the range of the difference in MHC-binding affinity (31). Our results also show no measurable difference between F and Y



**FIGURE 3.** CTL recognition of EL-4 target cells loaded with a synthetic peptide is disturbed by a D flanking the C-terminal V. Target cells were labeled with <sup>51</sup>Cr, loaded with the respective peptides in three concentrations, and tested for recognition using the specific CTL clone 10B6. *A*, Peptides of the Moloney MuLV sequence. *B*, Peptides of the Friend MuLV sequence.

peptide in TAP translocation (Table IV). We have calculated the difference in F- vs Y-containing epitope generation from Moloney vs Friend 26-mer peptide substrates in the proteasome-digestion assays. In these digests between 100- and 1000-fold more F than Y peptide can be detected. The much greater difference in peptide formation by the proteasome indicates that the proteasome fine specificity rather than the difference in MHC binding is causally related to the lack of epitope presentation of the Friend virus epitope.

The proteasome determines the C terminus of the epitope, but frequently the N terminus of the peptide fragments generated is a few residues longer than the optimal MHC-presented peptide. The need for precise C-terminal cleavage of CTL epitopes by the proteasome has been reported for different epitopes (17, 18), and its relevance is illustrated in our study. Next to MuLV, we have performed 20S proteasome digestion analysis on long synthetic peptides harboring several defined CTL epitopes such as Sendai virus (47) and human papilloma virus epitopes (43). Although the relevance of in vitro proteasome digestion analysis has been discussed (2), we have applied this method on several epitope-containing sequences and found very distinct cleavage specificities and a strong correlation of the cleavage products with the presented epitopes. In most cases tested, the proteasome cleaved immediately next to the C terminus of the epitope, whereas the N terminus was not precisely defined (our unpublished results). This indicates that the generation of the correct C terminus is a crucial step in CTL epitope generation as also deduced from studies of the OVA K<sup>b</sup>-presented epitope SIINFEKL (17), and suggests that cytosolic carboxypeptidase activity cannot compensate for imprecise C-terminal anchor residue cleavage.

The presentation of the SSWDFITV epitope requires TAP translocation (48) and has been reported previously (42) to be optimally translocated as the minimal 8-mer, although the 9-mer SSSWD FITV is also translocated quite efficiently. However, these fragments are not generated by the 20S proteasome. We could not detect any of the 8-mer or 9-mer fragments. The most abundant epitope-containing precursor peptide found was the 10-mer PSSS WDFITV. However, this 10-mer peptide was not efficiently translocated by TAP (Table IV). The formation of the optimal MHC class I-binding 8-mer peptide apparently requires N-terminal trimming by aminopeptidases. These enzymes are present in both the cytosol and in the ER lumen. Both activities can play a role in the generation of MHC class I-presented peptides. ER trimming has been shown to be important for other epitopes (19–21). For example, peptides with proline at position 3 are poorly translocated by TAP (42, 13) and therefore require N-terminal extensions for proper translocation to the ER. For these epitopes, N-terminal aminopeptidase trimming in the ER is most likely required to generate the CTL epitope. However, for epitopes inefficiently translocated by TAP, like in our study, the Moloney 10-mer precursor peptide PSSSWDFITV, cytosolic N-terminal trimming is most likely the enzymatic activity required to generate peptides that will be efficiently translocated by TAP. Recently, a cytosolic leucine aminopeptidase was described to be IFN- $\gamma$ -inducible and involved in the generation of the OVA CTL epitope SIINFEKL under in vitro conditions (22). The role of cytosolic peptidases in vivo has not been firmly established yet. On the other hand, we cannot firmly exclude that low numbers of 10-mer precursor peptides of the Moloney epitope enter the ER that after N-terminal trimming by ER-resident aminopeptidases yields sufficient epitope formation for CTL recognition. MHC class I-dependent N-terminal trimming is the major activity required to generate the proper MHC class I binding minimal peptide of the OVA epitope, as recently shown by Paz et al. (49).

The proteasomal cleavage specificity could be a potential target mechanism for viral or tumor immune escape. In an earlier study, we showed that a single residue exchange within another MuLV CTL epitope led to premature proteasome-mediated destruction of the potential CTL epitope (13). This mechanism finally led to a similar outcome, namely, nonpresentation of the epitope and thereby evasion on the immune system. Recently, a similar finding was shown for a p53 CTL epitope, in which a tumor-associated single residue mutation (R to H) C-terminally flanking the CTL epitope led to the absence of epitope-containing peptides (50). In this study, the exact mechanism of the lack of epitope generation was not elucidated, although it appears that the flanking residue not only influenced the generation of precursor peptides but also the formation of the minimal epitope. This might indicate that in this case the flanking R influences cleavage inside the epitope, leading to destruction of the epitope. In our study, the flanking D apparently does not influence cleavage within the epitope. The presence of D rather causes a shift in the location of the cleavage site. As a result, precursor peptides are generated that cannot enter the ER because of the negatively charged D that abolishes TAP translocation. A single residue exchange at a site crucial for epitope excision can be a way to evade CTL recognition. In general, these findings represent potential CTL evasion mechanisms at the processing level defined by only a single amino acid mutation.

This MuLV epitope is a subdominant CTL epitope in C57BL/6 mice, whereas the dominant epitope is the recently described D<sup>p</sup>-presented gagL epitope (51). The underlying mechanism(s) determining the dominance or hierarchy of CTL epitopes has not been clarified yet, but the relationship with processing mechanisms leading to lower epitope densities at the cell surface has been suggested in several studies (12, 22). The subdominance of the Moloney K<sup>b</sup>-presented epitope SSWDFITV might be regulated by processing events, involving either the suboptimal formation of TAP-transportable precursor peptides by the proteasome, or by the efficiency of aminopeptidase activity in cytosol or ER. These arguments are underlined by our observations that CTL killing of RMA cells, endogenously expressing the epitope, is always intermediate and can be increased by adding exogenous synthetic peptide (Fig. 1C). This suggests that cell surface presentation of this epitope is not very high and might be suboptimal for inducing high frequency CTL responses.

This study shows that a single amino acid, directly flanking the C-terminal residue of a CTL epitope, can strongly influence epitope formation. Precise definition of the C terminus of CTL epitopes by proteolytic machineries such as the proteasome is an essential step in proper peptide generation. Our findings suggest that abolition of precise C-terminal cleavage by a single point mutation can be a highly efficient mechanism of immune escape.

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