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Efficient Processing of an Antigenic Sequence for Presentation by MHC Class I Molecules Depends on Its Neighboring Residues in the Protein

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Summary

Processing of endogenously synthesized proteins generates short peptides that are presented by MHC class I molecules to CD8 T lymphocytes. Here it is documented that not only the sequence of the presented peptide but also the residues by which it is flanked in the protein determine the efficiency of processing and presentation. This became evident when a viral sequence of proven antigenicity was inserted at different positions into an unrelated carrier protein. Not different peptides, but different amounts of the antigenic insert itself were retrieved by isolation of naturally processed peptides from cells expressing the different chimeric proteins. Low yield of antigenic peptide from an unfavorable integration site could be overcome by flanking the insert with oligo-alanine to space it from disruptive neighboring sequences. Notably, the degree of protection against lethal virus disease related directly to the amount of naturally processed antigenic peptide.

Introduction

Cytolytic T lymphocytes (CTLs) of the CD8 subset recognize short peptides that are derived by processing from intracellular proteins and presented at the cell surface by major histocompatibility complex (MHC) class I molecules (Townsend et al., 1986b; reviewed in Townsend and Bodmer, 1989). External addition of synthetic peptides to cells has shown that MHC class I molecules can bind and present to CTLs of defined specificity not only one particular peptide but also a series of analogs thereof that differ in length and antigenic potency. As opposed to this experience, naturally processed peptides retrieved from cells that synthesize the respective antigenic protein appear to be of rather limited diversity (Van Bleek and Nathenson, 1990; Rötzschke et al., 1990a, 1990b). The underlying principles are not fully understood, although it seems clear that the MHC molecules themselves play a decisive role in endogenous peptide selection, because a peptide presented by a particular MHC class I molecule cannot be isolated from cells that do not express this MHC molecule (Falk et al., 1990). Earlier steps in the generation of antigenic peptides involving cleavage of proteins by proteases are largely undefined (Yewdell and Bennink, 1990).

There is ample evidence that processing and presentation of antigenic peptides are not dependent on the intracellular destination and the physiologic function of the antigenic protein (Townsend et al., 1986a; Braciale et al., 1987; Volkmer et al., 1987). The antigenic sequence can even be part of a chimeric protein (Chimini et al., 1989; Del Val et al., 1991). Until now the data were in accordance with the view that the sequence of the presented peptide is the only determinative parameter for its presentation. Based on this assumption, algorithms have been proposed for predicting antigenicity from sequence characteristics (DeLisi and Berzofsky, 1985; Rothbard and Taylor, 1987). Yet now the predictive value of these algorithms has proven to be limited.

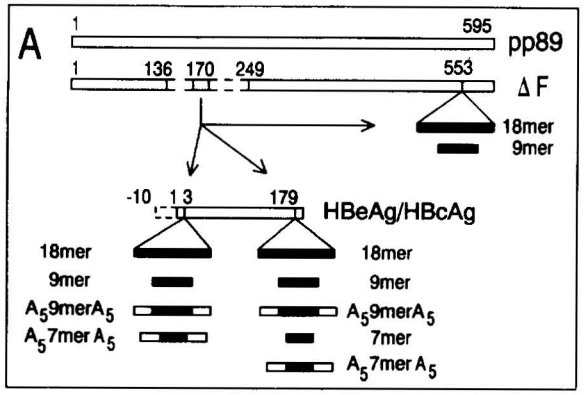
An influence on antigen processing and presentation of residues that flank the finally presented antigenic peptide in the protein would explain failures of antigenicity predictions. We have therefore tested whether the amino acids that flank a sequence of defined antigenic potency have an effect on its processing and presentation. For this purpose we used the nonapeptide sequence ^{168}YPHFMPTNL^{176} (one-letter code), which is presented by the murine MHC class I molecule L^d (Del Val et al., 1988, 1989; Reddehase et al., 1989) and represents for CTLs of the BALB/c strain the only antigenic site recognized in the IE1 protein pp89 (Volkmer et al., 1987; Del Val et al., 1991), a regulatory protein of murine cytomegalovirus (MCMV) (Keil et al., 1987). CD8 T lymphocytes specific for that protein protect against lethal MCMV infection (Jonjić et al., 1988), and protective immunity is also induced by chimeric proteins that contain only the nonapeptide of pp89 as insert (Del Val et al., 1991). By placing this sequence into different positions of an unrelated carrier protein, we tested a possible positional effect on antigen presentation and used protection of mice against lethal virus challenge as a means to estimate its biological relevance.

Two findings are described. First, amino acids that flank an antigenic sequence in the protein can have a profound positive or negative effect on antigen processing and presentation, both in cell culture and in vivo. Second, differences in presentation efficiency reflected differences in the amount of naturally processed peptide.

Results

The Position within a Protein Is Determinative for Presentation of an Antigenic Sequence

If antigen processing is governed only by the antigenic sequence itself, then presentation of a peptide of defined antigenic potency by the restricting MHC molecule should be independent of its location in the antigenic protein from which it is derived by endogenous processing. To test this prediction, chimeric genes were constructed that contained the coding sequence for the antigenic nonapeptide ^{168}YPHFMPTNL^{176} at different positions of the hepatitis B virus core antigen (HBcAg) gene. This peptide was identified previously as the optimal antigenic peptide presented by the MHC class I molecule L^d to clonal (Reddehase et



B

pp89	LGRLM	YDMYP	HFMPT	NLGPS	EKRVW
Δ F/18mer	rsRLM	YDMYP	HFMPT	NLGPS	rsata
HBc/C-end/18mer	srRLM	YDMYP	HFMPT	NLGPS	resqc
HBc/N-end/18mer	gsRLM	YDMYP	HFMPT	NLGPS	rsdpy
Δ F/9mer *	vlqpt	trsYP	HFMPT	NLefr	sataa
HBc/C-end/9mer *	rrrrs	qsrYP	HFMPT	NLsre	sqc.
HBc/C-end/A5 9merA5 *	qsraa	aaaYP	HFMPT	NLaaa	aasre
HBc/N-end/9mer	.m	digYP	HFMPT	NLgdp	ykefg
HBe/N-end/9mer	wlwgi	digYP	HFMPT	NLgdp	ykefg
HBe/N-end/A5 9merA5	digaa	aaaYP	HFMPT	NLaaa	aagdp
HBc/C-end/7mer *	prrrr	sqsrP	HFMPT	Nsres	qc.
HBe/C-end/A5 7merA5	sqsra	aaaaP	HFMPT	Naaaa	asres
HBe/N-end/A5 7merA5	idiga	aaaaP	HFMPT	Naaaa	agdpy

Figure 1. Schematic Representation and Amino Acid Sequence of pp89 and Chimeric Proteins

(A) Proteins are represented by open bars with numbers indicating amino acid positions. pp89-derived antigenic sequences within chimeric proteins are shown as closed boxes not drawn to the same scale as the proteins. Their length and flanking alanine residues are depicted.

(B) Amino acids are given in one-letter code. Capital letters indicate native pp89 sequences. Underlined lowercase letters refer to amino acids encoded by linker sequences for restriction endonucleases introduced during the construction of the chimeric genes. Nonunderlined lowercase letters identify sequences from the carrier proteins. Chimeric proteins are named by citing first the carrier protein, then the location of the insert, and finally the length of the pp89 sequence. Penta-alanine flanking is indicated. Asterisks mark the chimeric proteins described previously (Del Val et al., 1991).

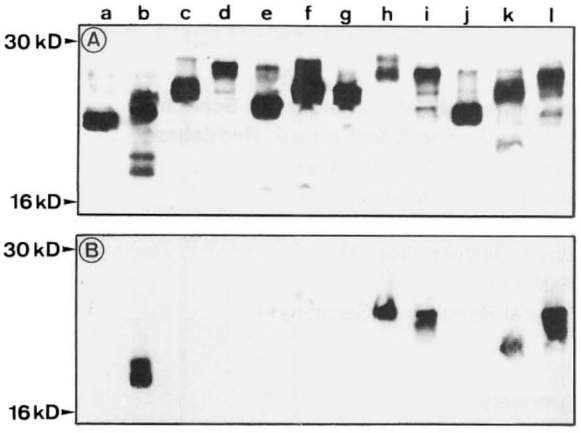


Figure 2. Expression of Chimeric Proteins in Cells Infected with Recombinant Vaccinia Viruses

Western blot analysis with hepatitis B virus–specific antiserum.
(A) Proteins from cells infected with the indicated recombinant viruses.
(B) Proteins secreted into the culture medium. Lane a, HBcAg; lane b, HBeAg; lane c, HBc/C-end/18-mer; lane d, HBc/N-end/18-mer; lane e, HBc/C-end/9-mer; lane f, HBc/C-end/$A_5$9-merA_5; lane g, HBc/N-end/9-mer; lane h, HBe/N-end/9-mer; lane i, HBe/N-end/$A_5$9-merA_5; lane j, HBc/C-end/7-mer; lane k, HBe/C-end/$A_5$7-merA_5; lane l, HBe/N-end/$A_5$7-merA_5. Abbreviations are as in Figure 1.

al., 1989) as well as polyclonal (Del Val et al., 1991) CTLs specific for the IE1 protein pp89 of MCMV. The chimeric genes were recombined into the genome of vaccinia virus. In Figure 1A the constructs are represented schematically, and in Figure 1B the sequences are listed.

In one approach (Figure 1A, top), the nonapeptide sequence (from now on referred to as 9-mer) and an 18-mer, comprising the 9-mer with authentic flanking residues, were translocated from their natural position within pp89 to a position close to the C-terminus. This was accomplished by inserting these sequences into amino acid position 553 of mutant ΔF of pp89, in which the antigenic region is deleted from position 136–249 (Del Val et al., 1988). Presentation occurred from ΔF/9-mer (Del Val et al., 1991) and ΔF/18-mer (Figures 3A and 3B, respectively) with no apparent difference to the presentation from authentic pp89 expressed in cells infected with the recombinant MCMV-*iel*-VAC (Volkmer et al., 1987) (Figure 3A).

In another approach (Figure 1A, bottom), the two peptide sequences were inserted into an unrelated carrier protein, HBcAg, either close to the C-terminus at position 179 (HBc/C-end/9-mer and HBc/C-end/18-mer) or close to the N-terminus at position 3 (HBc/N-end/9-mer and HBc/N-end/18-mer). While presentation occurred from both 18-mer constructs (Figure 3B), a striking difference was seen for the two 9-mer constructs in that C-terminal integration was permissive for presentation of the inserted antigenic sequence, whereas N-terminal integration was unfavorable (Figure 3A).

The overall amount of chimeric protein synthesized in cells after infection with the vaccinia recombinants was controlled by Western blot and was found to be comparable in all cases considered (Figure 2), and, specifically, also for construct HBc/N-end/9-mer (lane g). Shifts in electrophoretic mobility of the chimeric proteins in relation to the carrier proteins chiefly correlated with the number of inserted amino acids, except that proteins containing insertions at the C-terminus had a slightly higher mobility than those with insertions at the N-terminus (Figure 2A, compare lanes c and e with lanes d and g, respectively). This might be due to an altered phosphorylation at a C-terminal position of HBcAg close to the integration site (Ganem and Varmus, 1987). In conclusion, the low efficiency of antigen presentation from HBc/N-end/9-mer cannot be explained by a reduced amount of protein available for processing.

To preclude any effect imposed by the vaccinia virus vector system, such as that studied by Townsend et al. (1988), L/L^d cells were transfected with plasmids encoding the chimeric proteins HBc/N-end/18-mer and HBc/N-end/9-mer. Again, N-terminal location of the 9-mer resulted in poor antigenicity (Figure 3C), which clearly indicates that

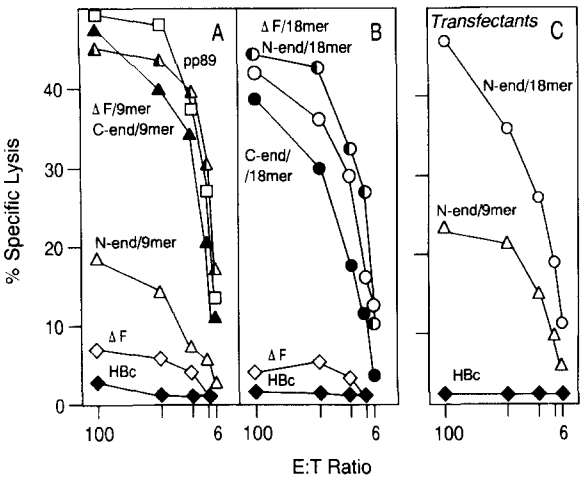


Figure 3. Effect of Flanking Residues on Antigen Presentation from Chimeric Proteins

Recognition by pp89-specific CTLs of BALB.SV cells that were infected with recombinant vaccinia viruses (A and B) or of L/L^d cells that were transfected with recombinant plasmids (C) encoding authentic pp89 or chimeric proteins as indicated: open squares, pp89; half-filled triangles, ΔF/9-mer; filled triangles, HBc/C-end/9-mer; open triangles, HBc/N-end/9-mer; half-filled circles, ΔF/18-mer; filled circles, HBc/C-end/18-mer; open circles, HBc/N-end/18-mer; open diamonds, ΔF; filled diamonds, HBcAg.

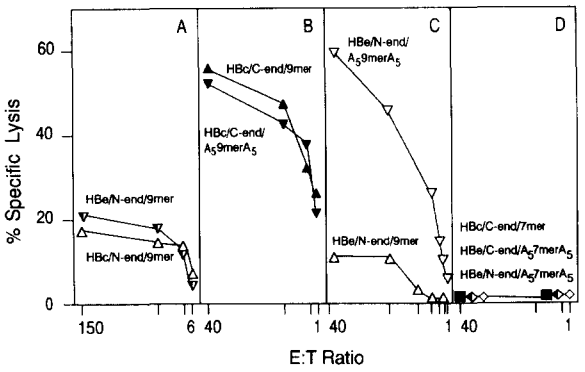


Figure 4. Restoration of Efficient Antigen Presentation by Penta-Alanine Flanking of the Antigenic Sequence

Recognition by pp89-specific CTLs of BALB.SV cells infected with recombinant vaccinia viruses encoding chimeric proteins that contain pp89 sequences: open triangles, HBc/N-end/9-mer; inverted half-filled triangles, HBe/N-end/9-mer; filled triangles, HBc/C-end/9-mer; inverted filled triangles, HBc/C-end/$A_5$9-merA_5; inverted open triangles, HBe/N-end/$A_5$9-merA_5; filled squares, HBc/C-end/7-mer; half-filled diamonds, HBe/C-end/$A_5$7-merA_5; open diamonds, HBe/N-end/$A_5$7-merA_5.

this integration site is unfavorable for presentation of the 9-mer, irrespective of the vector system employed.

Collectively, these findings thus provided an example of a positional effect in antigenic peptide processing and presentation.

Proximity to the N-Terminus Does Not Account for Poor Antigenicity

An apparent difference between HBc/N-end/18-mer and HBc/N-end/9-mer is the distance between the N-terminal methionine of the carrier protein and the first residue of the 9-mer, which is a tyrosine (Figure 1B). If the mere proximity of the N-terminus to the core of the antigenic sequence were incompatible with efficient antigen presentation, for any reason whatsoever, any means of spacing should restore antigenicity. We chose the following approach to the problem: the hepatitis B virus precore protein differs from HBcAg by an additional N-terminal sequence of 29 amino acids, the pre-C region (Ganem and Varmus, 1987), which functions as a signal for secretion and mediates the translocation of the precore protein across the membranes of the endoplasmic reticulum. By removal of the 19 N-terminal pre-C amino acids, and of a portion of the C-terminus, the precore protein is converted into HBeAg, which is then actively secreted (Ou et al., 1986). Thus, the 9-mer, when cloned into hepatitis B virus precore protein, is flanked by 10 additional N-terminal residues in the resulting chimeric HBeAg. As a consequence of the N-terminal insertion, chimeric proteins with lower electrophoretic mobility than expected from the number of inserted amino acids were accumulated and secreted (Figure 2, compare lane b with lane h), which suggests an interference with correct removal of the C-terminal portion of HBeAg. The finding that presentation to CTLs of the antigenic sequence was equally poor from HBe/N-end/9-mer and HBc/N-end/9-mer (Figure 4A) argues against a critical influence of the distance to the N-terminus of the carrier protein. It should be noted that here and in further experiments HBe- and HBc-based constructs proved to be fully interchangeable. This finding is in accordance with previous experience that antigen processing is not dependent on the intracellular destination of the antigenic protein (Townsend et al., 1986a; Braciale et al., 1987; Volkmer et al., 1987).

Oligo-Alanine Spacing Can Avoid Unfavorable Flanking and Restores Presentation

At this stage, an influence of the linker and carrier protein sequences directly flanking the antigenic insert (see Figure 1B) appeared to us the most likely explanation for the observed positional effect on antigen presentation. If this were true, creation of a permissive flanking by proper substitutions should be possible. Instead of performing numerous trial and error substitutions, we preferred another approach: it was known from previous experience with synthetic peptides that flanking of the 9-mer with alanines leaves its antigenic potency essentially unchanged (data not shown). Flanking with alanines could therefore serve to space the antigenic sequence from unfavorable neighboring residues. In accordance with previous data (Del Val et al., 1991), penta-alanine flanking in HBc/C-end/$A_5$9-merA_5 did not disturb presentation from a permissive site (Figure 4B). Yet, remarkably, penta-alanine flanking in HBe/N-end/$A_5$9-merA_5 converted the N-terminal site from an unfavorable into a favorable site for antigen processing and presentation (Figure 4C).

Earlier work has shown that the heptapeptide sequence ^{169}PHFMPTN^{175} is not presented from intracellularly synthesized pp89-ΔF- or HBc-based chimeric proteins (Del Val et al., 1991) even though this peptide (Reddehase et

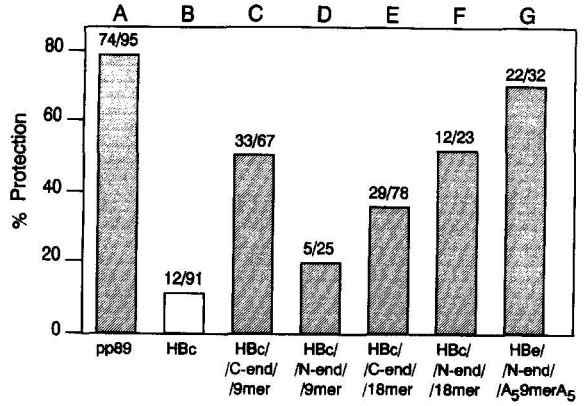


Figure 5. Induction of Protective Immunity by Recombinant Vaccinia Viruses Encoding Chimeric Proteins

Mice were immunized with recombinant vaccinia viruses encoding the indicated proteins and were challenged 3 weeks later with 3 LD_{50}s of MCMV. Protection is expressed on the vertical axis as percentage of surviving mice. The actual number of mice in each group (survivors/total tested) is indicated on top of each column. Data are compiled from seven independent experiments. Significance was calculated by applying Fisher's exact probability test: column B vs. C, significant, $p < 0.0001$; B vs. E, significant, $p = 0.0003$; E vs. F, not significant, $p = 0.1481$; B vs. D, not significant, $p = 0.2865$; D vs. G, significant, $p = 0.0003$.

al., 1989) and the alanine-flanked analog APHFMPTNA (Reddehase and Koszinowski, 1991) are antigenic when externally added to L^d-expressing cells. We therefore wondered whether the strategy of oligo-alanine spacing would also help the heptapeptide sequence to get presented. This was not the case (Figure 4D). The affinity of the heptapeptide to L^d is at least two orders of magnitude lower than that of the 9-mer (Reddehase and Koszinowski, 1991), and, since alanine flanking does not increase the affinity, we speculate that in this case not the flanking residues but the low affinity to L^d of the antigenic sequence itself is responsible for the failure in presentation.

In conclusion, oligo-alanine spacing cannot overcome insufficient antigenicity of a peptide sequence, but it can help avoid negative influences of neighboring residues on an optimal antigenic sequence.

The Efficiency of Antigen Presentation Is Critical for the Induction of Protective Immunity

Thus far, the simian virus 40–transformed cell line BALB.SV was used for studying antigen presentation, and, therefore, one could argue that the data have characterized the processing and presentation properties of this particular cell line rather than having described a principle. No single cell line can a priori be considered representative of the cells that process the chimeric proteins in vivo. It has been shown in previous reports that protection induced by pp89 or the 9-mer thereof is mediated solely by T lymphocytes of the CD8 subset (Jonjić et al., 1988; Del Val et al., 1991). Therefore, an unambiguous way to test the in vivo relevance of the in vitro findings was to probe the quality of the various recombinants in protecting against lethal MCMV infection (Figure 5). In accordance with previous work (Del Val et al., 1991), the 9-mer, when integrated at the C-terminus of HBcAg in the construct HBc/C-end/9-mer, was sufficient to induce significant protective immunity. The two 18-mer constructs induced a similar degree of protection, whereas N-terminal integration of the 9-mer in HBc/N-end/9-mer failed in protection. Induction of pp89-specific T lymphocytes with the latter recombinant could only be detected after selective secondary restimulation in vitro (data not shown). Finally, as was the case with target cell formation, penta-alanine flanking in HBe/N-end/$A_5$9-merA_5 successfully restored also the protective capacity in vivo. In conclusion, the differences in antigen presentation efficiencies as seen in vitro in terms of target cell formation for recognition by CTLs are directly reflected by the quality of the respective recombinants to induce protective CD8 T lymphocytes, which demonstrates that antigen processing and presentation follow the same rules in the cell types that are relevant in vivo.

Differences in Antigen Presentation Efficiency Can not Be Explained by Differences in the Overall Stability of the Chimeric Proteins

In vaccinia virus–infected cells, antigen presentation can be improved by increasing the overall degradation rate of antigenic proteins (Townsend et al., 1988). We therefore studied whether poor presentation from the construct HBc/N-end/9-mer was associated with high stability of the respective chimeric protein. Infected cells were pulsed with [^{35}S]methionine and chased for various periods of time. The HBc/N-end/9-mer chimeric protein had a similar stability as HBc/N-end/18-mer (Figure 6, compare [e] and [c]), regardless of the fact that it was presented much less efficiently to CTLs. Likewise, in the comparison of the two HBe-based constructs, restoration of efficient antigen presentation by penta-alanine flanking in HBe/N-end/$A_5$9-

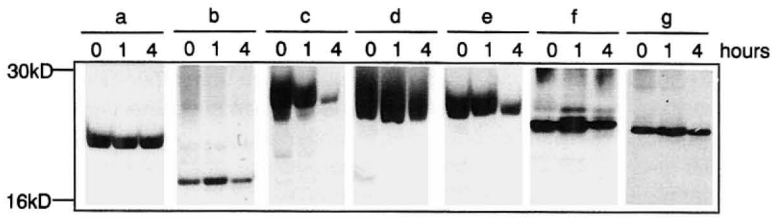


Figure 6. Stability of Chimeric Proteins in Cells Infected with Recombinant Vaccinia Viruses

BALB.SV cells were labeled for 30 min with [^{35}S]methionine 8 hr after infection with the different recombinant viruses and chased in the presence of nonradioactive methionine for 0, 1, and 4 hr, as indicated. Cell lysates were then immunoprecipitated with HBc/HBe-specific antiserum. (a) HBcAg; (b) HBeAg; (c) HBc/N-end/18-mer; (d) HBc/C-end/9-mer; (e) HBc/N-end/9-mer; (f) HBe/N-end/9-mer; (g) HBe/N-end/$A_5$9-merA_5. Abbreviations are as in Figure 1.

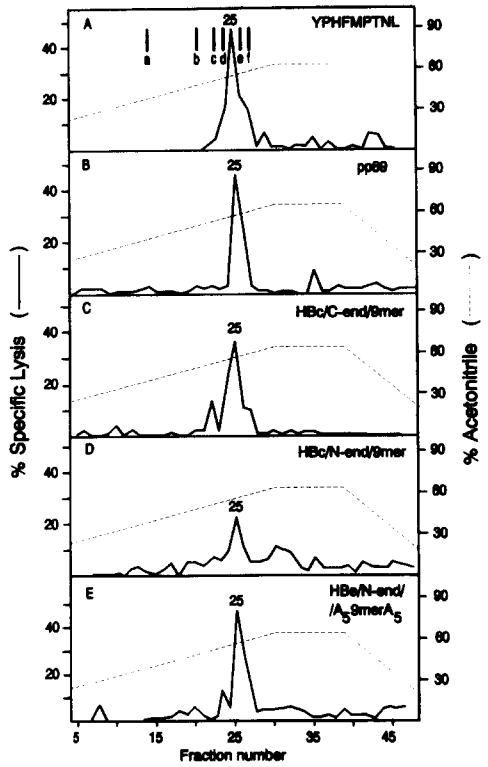


Figure 7. Naturally Processed Peptides from Chimeric Proteins Co-elute with the Synthetic Nonapeptide

(A) The synthetic nonapeptide was subjected to reverse-phase HPLC and eluted in an acetonitrile gradient. Vertical bars indicate the elution positions of related peptides: (a) 7-mer PHFMPTN; (b) 8-mer YPHFMPTN; (c) 8-mer PHFMPTNL; (d) 10-mer YPHFMPTNLG; (e) 10-mer MYPHFMPTNL; (f) 18-mer RLMYDMYPHFMPTNLGPS.

(B–E) BALB.SV cells were infected with recombinant vaccinia viruses expressing the indicated proteins. Acid-soluble molecules were extracted and separated first by gel filtration chromatography and then by reverse-phase HPLC. Fractions were tested in triplicate with pp89-specific CTLs for their content of antigenic peptides.

merA$_5$ was not accompanied by an increased protein degradation rate (Figure 6, [f] and [g]). It was therefore concluded that the overall stability of the chimeric proteins did not account for the differences in efficiency of antigen presentation observed in vitro as well as in vivo.

Processing from an Unfavorable Site Leads to a Lower Amount of Naturally Processed Peptide

What is the molecular explanation for the observed differences in target cell formation and in vivo T lymphocyte sensitization? Antigen presentation requires that the antigenic protein is properly cleaved so as to not destroy the antigenic site and to generate peptide(s) that can bind to the presenting MHC molecules. If natural processing generates peptides that are longer than the inserted optimal antigenic sequence, then processing from the various chimeric proteins, all of which provide their individual flanking residues (see Figure 1B), would lead to different peptides that are likely to differ in their binding affinity for L^d and will thus be presented with different efficiency. Accordingly, reduced antigen presentation from an unfavorable site can have two reasons: processing could generate a lower amount of the optimal antigenic peptide, or could generate longer, suboptimal peptide(s).

The recently described method of extracting naturally processed peptides from cells (Rötzschke et al., 1990b) should give the information whether after processing of the chimeric proteins the same peptide or different peptides can be recovered. Naturally processed peptides were acid extracted from infected cells and analyzed by reverse-phase high pressure liquid chromatography (HPLC). As a reference, the optimal synthetic nonapeptide was also subjected to HPLC and was found to elute in an acetonitrile gradient as one dominant peak of biological activity (Figure 7A), corresponding to the only peak detected spectrophotometrically (data not shown). Also indicated in Figure 7A are the elution properties of a relevant set of related peptides of different lengths, none of which coeluted with the nonapeptide. The activity of naturally processed peptides extracted from cells infected with MCMV-*iel*-VAC, encoding authentic pp89, and the vaccinia recombinant HBc/C-end/9-mer, containing the 9-mer at a permissive site of the unrelated carrier protein HBcAg, was of comparable intensity and coeluted in both cases with the synthetic nonapeptide in fraction 25 (Figures 7B and 7C, respectively). Notably, insertion into the unfavorable site in HBc/N-end/9-mer resulted in a lower activity, yet again, in the same fraction (Figure 7D), and rescue of presentation by penta-alanine flanking in HBe/N-end/A$_5$9-merA$_5$ restored the activity retrieved from the cells (Figure 7E).

Based on the identity of the elution characteristics, it appeared to us reasonable to assume that biological activity relates directly to the amount of extracted peptide. On this premise, processing from the unfavorable construct HBc/N-end/9-mer was quantitated and compared with processing from the rescue construct HBe/N-end/A$_5$9-merA$_5$ and from the favorable construct HBc/C-end/9-mer, by diluting fraction 25 of the respective HPLC runs before the cytolytic assay. The difference in cell number needed for peptide extraction to get at comparable activity was about 16-fold between the poorly presented and the two efficiently presented chimeric proteins (Figure 8).

In conclusion, inefficient antigen presentation can be explained by the generation of a lower amount of naturally processed peptide.

Discussion

Insertion of a nonapeptide sequence of defined antigenicity into different positions of an unrelated carrier protein has demonstrated a critical role of flanking residues in determining the efficiency of antigen processing and presentation. This finding has important implications for the predictability of antigenic sites in proteins, for the design of recombinant vaccines, and for the understanding of principles that govern antigen processing and peptide presentation.

From the compilation of amino acid sequences of empirically identified antigenic peptides, algorithms have been formulated to predict antigenic sequences recognized by

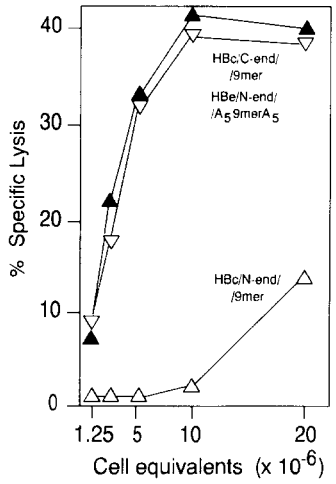


Figure 8. Penta-Alanine Flanking Increases the Yield of Processed Antigenic Peptide

Naturally processed peptides were acid extracted from cells infected with the recombinants HBc/N-end/9-mer (open triangles), HBc/C-end/9-mer (filled triangles), and HBe/N-end/$A_5$9-merA_5 (inverted open triangles) and were separated by gel filtration followed by reverse-phase HPLC. Serial dilutions of fraction 25 of the HPLC runs were tested in the cytolytic assay. The calculated number of infected cells from which peptides were recovered is given on the horizontal axis, and percent specific lysis is given on the vertical axis as a measure of the amount of the relevant antigenic peptide.

T lymphocytes. These algorithms are being used to screen protein sequences for certain motifs. Motifs indicate the propensity of a peptide to adopt a particular sterical configuration (DeLisi and Berzofsky, 1985) or represent a pattern of residues with a typical polarity distribution (Rothbard and Taylor, 1987). Yet, even though successfully employed in some cases, these algorithms have been of limited predictive value. This does not imply that the algorithms are wrong, but may indicate that other parameters in addition to the sequence of an antigenic peptide are critically involved in its generation and presentation. An immediate idea for explaining the failures of antigenicity predictions is that different allelic MHC molecules show subtle preferences for structural features of peptides that cannot be included in a general prediction model. Yet, if this were the only reason, then a peptide with defined affinity for a particular presenting MHC class I molecule should always be presented regardless of the position in the protein from which it is derived by processing. Our results show that this is not the case. Of the two positions chosen at random in the unrelated carrier protein HBcAg, one was permissive for processing and presentation of the antigenic nonapeptide insert, whereas the other was unfavorable. This finding unambiguously documents that proven antigenicity of an amino acid sequence is no guarantee for its adequate processing in the cell. Thus, in the cases of failure, prediction algorithms may have correctly predicted a potentially antigenic sequence, which, however, remained nonantigenic and consequently invisible in assays because an unfavorable position in the protein precluded the generation of the respective peptide.

The finding that residues that flank an antigenic insert have a critical influence on its proper processing complicates the design of recombinant vaccines. One either has to test several insertion sites by trial and error or to learn the rules that make flanking residues permissive or nonpermissive, which may be cumbersome as the role of flanking residues may depend on the sequence of the insert. A sort of "neutral spacing" to separate the insert from disadvantageous influences of its neighboring residues could be an easier solution to the problem. In our example, unfavorable insertion was overcome by penta-alanine flanking of the antigenic insert. Systematic analysis will have to define whether or not oligo-alanine flanking is generally applicable, that is, for other antigenic sequences, other carrier proteins, and other presenting MHC molecules.

Which are the peptides generated by natural processing? Are they different for the different chimeric proteins, or is the antigenic insert itself retrieved in all cases? The main biological activity extracted from cells infected with four different recombinants eluted in all four cases in the same fraction and, moreover, coeluted with the synthetic nonapeptide. If peptides longer than the 9-mer were generated by natural processing from the chimeric proteins, they all should differ from each other, because in all of them the 9-mer insert is provided with essentially different flankings (see Figure 1B). In accordance with others (Rötzschke et al., 1990a) we have had the experience that longer or shorter peptides elute in different fractions. This was true for the 18-mer and 7-mer, for two octapeptides, and even for the two decapeptides in which the nonapeptide is flanked on one side by only one authentic residue. Even though one cannot exclude the possibility that, by chance, two different peptides coelute, the probability that four different longer peptides from the four recombinants all coelute with the nonapeptide is very low. This strongly argues against longer peptides being the finally presented ones.

The idea that peptides shorter than the 9-mer are presented can also be refuted, because the 7-mer, which is the most antigenic peptide among the shorter analogs (Reddehase et al., 1989), not only eluted as synthetic peptide in a different fraction, but also was not presented from chimeric proteins in which it constituted the insert. The multitude of proteins synthesized in the vaccinia virus expression system precluded the approach of natural peptide sequencing employed by Van Bleek and Nathenson (1990), but the evidence is close to proof that the inserted nonapeptide is, in fact, the dominant natural antigenic peptide. In light of recent emphasis given to an observed discrepancy between a synthetic peptide and the corresponding naturally processed peptide (Rötzschke et al., 1990a), the concordance seen in our case was not a priori predictable. The example shows that a discrepancy between natural peptides and predictions obtained with synthetic peptides cannot be taken for a principle. We rather think that accurate definition of the optimal peptide by systematic biterminal shortening of an antigenic sequence (Reddehase et al., 1989) can correctly predict the naturally processed peptide. It is also worth mentioning that the few

other natural peptides described so far (Van Bleek and Nathenson, 1990; Rötzschke et al., 1990a; Falk et al., 1991) are also nona- or octapeptides.

How do flanking residues influence the efficiency of endogenous processing and what can our example contribute to the understanding of the still unknown processes of antigenic peptide generation and presentation? The current opinion is that antigen processing and presentation include at least four steps: first, partial degradation of the antigenic protein, supposedly in the cytosol; second, transport of peptides into the endoplasmic reticulum; third, loading of MHC class I molecules to form a trimolecular complex of MHC class I heavy chain, peptide, and β_2-microglobulin; and, fourth, transport of the complex to the cell surface (Yewdell and Bennink, 1990; Parham, 1990; Townsend et al., 1989). Poor presentation from the unfavorable integration site in HBc/N-end/9-mer was found to be associated with a low amount of recoverable antigenic peptide. We see four possibilities for explanation. First, the reduced amount could result already from an inefficient overall protein cleavage as in the example given by Townsend et al. (1988). In the case reported here, insertions in HBcAg led to slightly different stability of the chimeric proteins, but there was no correlation between protein stability and amount of naturally processed peptide.

Second, the type of residues flanking the antigenic sequence may codetermine the local protein conformation at the antigenic site, and thereby affect the local accessibility for proteases, or may directly account for the presence or absence of cleavage signals for proteases. In both instances, flanking can be favorable or unfavorable for the efficient release of the correct and intact antigenic sequence from the protein. A putative conformational effect on local cleavage efficiency is difficult to prove or refute, and from our examples of efficient presentation from chimeric proteins we do not recognize a consensus in the flanking sequences that could promote cleavage. Future tests will have to show which of the flanking residues, alone or in combination, are responsible for the poor presentation from HBc/N-end/9-mer. One could consider, for instance, the two-sided flanking by glycines and negatively charged residues, as is the case in this construct.

The third possibility to explain poor presentation is that the recently discussed peptide transporters (Parham, 1990) might select peptides by unknown criteria.

The fourth possibility is that longer peptides are excised from the protein in the first place, get transported, associate with L^d, and are then trimmed to the finally presented peptide, a mechanism discussed as one possibility to explain how MHC class I molecules select and protect peptides from complete degradation (Falk et al., 1990). That MHC class I molecules play a decisive role in peptide selection is based on the finding that a particular antigenic peptide can only be extracted from cells that express the corresponding presenting MHC molecule (Falk et al., 1990). The same is true for the peptide derived from pp89, since no biological activity was recovered from infected cells of the mutant mouse strain BALB/c-H-2^{dm2}, in which the L^d gene is deleted (unpublished data). According to this mechanism, longer peptides, in which flanking residues modulate the binding affinity to L^d, will be protected to different degrees from degradation. The bound fraction is then trimmed, thereby yielding different amounts of only one dominant peptide from the various chimeric proteins.

In conclusion, residues that directly flank the antigenic sequence in a protein critically influence the amount of naturally processed and presented antigenic peptide. The demonstration that the degree of protection against lethal disease achieved with the recombinants relates directly to the amount of correctly processed peptide underlines the importance of the findings.

Experimental Procedures

Mice

BALB/c mice (H-2^d haplotype) were bred in our colony under specific pathogen-free conditions.

Cells

BALB.SV, a continuously growing fibroblast cell line, was isolated from primary cultures of BALB/c mouse embryo fibroblasts that were infected with wild-type simian virus 40.

Recombinant Vaccinia Viruses

The recombinant vaccinia virus MCMV-*iel*-VAC encodes pp89 (Volkmer et al., 1987). The recombinant MCMV-*iel*(ΔF)-VAC, also referred to as ΔF, encodes a deletion mutant of pp89 that lacks amino acids 136–249 and therefore does not contain any epitope recognized by BALB/c CTLs (Del Val et al., 1988).

Insertion of pp89 Antigenic Sequences into Hepatitis B Virus Genes

An HBcAg gene was used, the 5′ end of which is derived from a synthetic gene (Nassal, 1988) and therefore contains a ClaI site 1 codon downstream of the HBcAg initiation codon. This restriction site was used for the insertion of synthetic oligonucleotides encoding pp89 antigenic sequences. For insertions in the HBeAg gene (Ganem and Varmus, 1987), the same restriction site was used. In addition, the initial AUG codon for HBcAg was converted into AUA by site-directed mutagenesis, in order to prevent translation initiation at this position (Schlicht and Schaller, 1989). For insertions at the 3′ end of the HBcAg gene, a natural AvaI site located 5 codons upstream of the termination codon was used. All constructs were confirmed by sequencing before their recombination into vaccinia virus, under the control of the early-late P7.5K promoter, as described (Volkmer et al., 1987).

Insertion of pp89 Antigenic Sequences into Deletion Mutant ΔF

To reintegrate pp89 sequences at a different position of pp89, deletion mutant ΔF was chosen. A unique BglII site in the 3′ end of the ΔF reading frame (Keil et al., 1987; Del Val et al., 1988), at codon number 553, was used as insertion site for synthetic oligonucleotides. All constructs were sequenced and then recombined into vaccinia virus.

Expression of the chimeric proteins in cells infected with the vaccinia recombinant viruses was tested by Western blot as described (Del Val et al., 1991).

Transfection

L/L^d cells, which are derived from mice of H-2^k haplotype and were transfected with the gene for the MHC class I molecule L^d (Ponta et al., 1985), were supertransfected with plasmids encoding the chimeric protein HBc/N-end/18-mer or HBc/N-end/9-mer under the control of the metallothionein promoter (Junker et al., 1987). For selection of transfected colonies, a plasmid carrying the gene for neomycin resistance was cotransfected (Colbere-Garapin et al., 1981).

Synthetic Peptides and Oligonucleotides

Peptides were synthesized in an Applied Biosystems peptide synthesizer model 431A and analyzed by reverse-phase HPLC. Oligonucleotides were synthesized in an Applied Biosystems oligonucleotide synthesizer model 381A.

Cytolytic Assays

BALB.SV cells were used for infection with recombinant vaccinia viruses to produce target cells as described (Del Val et al., 1988). pp89-specific CTLs were generated by in vitro restimulation with purified MCMV of spleen cells from mice that had been infected with MCMV-*iel*-VAC and were used as effector cells in a standard 3 hr cytolytic assay (Del Val et al., 1988). Graded numbers of cytolytic effector cells in 2-fold or 3-fold dilution steps were used. Data represent the mean percentage of specific lysis from three replicate cultures.

Pulse-Chase Analysis

BALB.SV cells (5×10^6) were labeled for 30 min with 0.5 mCi of [^{35}S]methionine (1200 Ci/mmol) in methionine-free medium 8 hr after infection with recombinant vaccinia viruses. Incorporation was terminated by the addition of nonradioactive methionine to a final concentration of 0.45 mM. Cells were then chased for 0, 1, and 4 hr in normal culture medium. At each chase time, samples were taken and subjected to two rounds of immunoprecipitation with an antiserum recognizing both HBcAg and HBeAg (Schlicht, 1991).

Isolation of Naturally Processed Peptides

BALB.SV cells were infected in parallel with all mentioned recombinant vaccinia viruses at a multiplicity of infection of 3 pfu per cell, and 20 hr later, naturally processed peptides were extracted in parallel from whole cells with trifluoroacetic acid as described (Rötzschke et al., 1990b). Briefly, trifluoroacetic acid–soluble material was separated first on a Sephadex G25 column and subsequently on an HPLC reverse-phase column. Lyophilized fractions were resuspended, diluted in RPMI 1640 culture medium, and dispensed into microtiter plates. ^{51}Cr-labeled BALB.SV cells were then added and, after 1 hr of incubation, pp89-specific cytolytic effector cells were added for a standard 3 hr cytolytic assay. Naturally processed peptides from cells infected with all recombinant viruses were tested in parallel.

Protection In Vivo

Mice were immunized i.v. with 1×10^8 pfu of recombinant vaccinia viruses and challenged i.p. 3 weeks later with 3 LD_{50}s of MCMV, strain Smith (VR-194; American Type Culture Collection, Rockville, MD), isolated from the salivary glands of infected mice (Jonjić et al., 1988).

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