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Structural Elements of a Protein Antigen Determine Immunogenicity of the Embedded MHC Class I-Restricted T Cell Epitope

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Substantial effort has been invested into optimization of vector structure, DNA formulation, or delivery methods to increase the effectiveness of DNA vaccines. In contrast, it has been only insufficiently explored how the higher order structure of an antigenic protein influences immunogenicity of embedded epitopes in vivo. Potent CD8+ T cell responses specific for a single immunogenic epitope are induced upon electrovaccination with plasmid DNA encoding the full-length heavy chain of the human HLA-Cw3 molecule. Contrary to expectations, a minimal construct, which provoked a substantial release of IFN-γ from specific CTLs in vitro, did not induce a significant response in vivo. Systematically altered variants of the Cw3 molecule were thus tested both in vivo and in vitro to determine which structural parts are responsible for this discrepancy. In complementation experiments the participation of trans-acting helper epitopes was ruled out. Successive C-terminal truncations, human/mouse domain swap variants, and subdomain modifications defined the α3 region of the HLA heavy chain and membrane anchoring as critical elements. Based on these data, refined minimal constructs were engineered that triggered very high in vivo responses. The most advanced variant consisted only of an adenoviral leader, antigenic epitope, α3 domain, and 16 aa of the transmembrane domain. When a tumor Ag epitope was incorporated into one of these high performer minimal constructs, protection against melanoma metastases was attained upon vaccination. Thus, structural elements of the Ag can dominantly influence immunogenicity in vivo. These elements can also markedly improve the immunogenicity of unrelated Ags and may form the basis of a new generation of DNA vaccines. 


The advent of DNA vaccination during the last decade opened an exciting new era of vaccine research. Although there were sporadic reports of in vivo transfection in the 1950s and 1960s (1–3), the potential of these discoveries was largely disregarded until recently. It was only in the early 1990s that two papers showing direct in vivo gene delivery were published. Wolff (4) and Yang (5) with their colleagues showed expression and prolonged biological activity of a reporter gene in murine skeletal muscle, while Tang et al. (6) first reported that a tumor Ag epitope is induced upon electrovaccination with plasmid DNA encoding the full-length heavy chain of the human HLA-Cw3 molecule. Contrary to expectations, a minimal construct, which provoked a substantial release of IFN-γ from specific CTLs in vitro, did not induce a significant response in vivo. Systematically altered variants of the Cw3 molecule were thus tested both in vivo and in vitro to determine which structural parts are responsible for this discrepancy. In complementation experiments the participation of trans-acting helper epitopes was ruled out. Successive C-terminal truncations, human/mouse domain swap variants, and subdomain modifications defined the α3 region of the HLA heavy chain and membrane anchoring as critical elements. Based on these data, refined minimal constructs were engineered that triggered very high in vivo responses. The most advanced variant consisted only of an adenoviral leader, antigenic epitope, α3 domain, and 16 aa of the transmembrane domain. When a tumor Ag epitope was incorporated into one of these high performer minimal constructs, protection against melanoma metastases was attained upon vaccination. Thus, structural elements of the Ag can dominantly influence immunogenicity in vivo. These elements can also markedly improve the immunogenicity of unrelated Ags and may form the basis of a new generation of DNA vaccines.

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Abbreviations used in this paper: CD62L, CD62 ligand; E3 adenovirus, E3 19K protein leader sequence; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; IC, intracytoplasmic; mTRP, murine tyrosinase-related protein; RYLK, H-2Kd-restricted Cw3 epitope; RYLKNKGETL; RYLE H-2Kd-encoded homolog of RYLELGNETL; SVYD, mTRP-2-derived epitope SVYDDFFVWL; TM, transmembrane portion.

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Plasmid constructs harboring the full-length Cw3 coding sequence reliably induced high level, specific CD8+ T cell responses upon electrovaccination. In contrast to that, and to our surprise, we have found that plasmids in which the antigenic epitope was engineered after leader sequences that promote endoplasmic reticulum (ER) delivery provoked only insignificant responses. Nevertheless, the same constructs triggered substantial IFN-γ release from epitope-specific CTL lines in vitro upon transfection into H-2Kd+-presenting cells.

The unique combination of a potent plasmid-based immunization protocol together with the fast and accurate detection of specific in vivo responses enabled us to find an answer to this puzzle by broad genetic screens. In a trans-complementation experiment the participation of trans-acting helper epitopes was ruled out. Systematically altered variants of the full-length Cw3 molecule were then constructed and tested both in vivo and in vitro, which identified the membrane-anchored α3-domain as a crucial determinant of in vivo immunogenicity. Based on these data, new, progressively refined minimal constructs were engineered. In vivo responses elicited by one of these constructs were even higher than those produced by the full-length molecule. As a proof of the concept, a melanoma tumor Ag epitope was incorporated into such a high performer minimal construct. Pronounced protective effects were found in the B16 mouse melanoma model when mice were electrovaccinated with this plasmid.

Materials and Methods

Mice

Eight-week-old female DBA/2 mice were purchased from Harlan (Borchen, Germany) and held under specific pathogen-free conditions. Mice were acclimated 1 wk before the start of the study. All animal work was performed in full compliance with institutional and legal guidelines.

Cell lines

The murine colon carcinoma cell line C-26 was a gift from M. Colombo (INT, Milan, Italy). The murine mastocytoma cell line P815 was obtained from American Type Culture Collection (Manassas, VA). The murine mastocytoma cell line P815-Cw3 444/C931, expressing full-length HLA-Cw3, was a gift from J.-C. Cerottini (LICR, Lausanne, Switzerland). P815 123 cells stably expressing the minimal construct 123 were created in our laboratory. P815 cells were transfected with the plasmid 123 by electroporation, and stable clones showing high expression of the fusion protein were obtained by chemical selection, FACS sorting, and limiting dilution. B16F10, a murine melanoma cell line of C57/BL/6 origin, was a gift from the Scripps Institute, La Jolla, CA. The murinized mastocytoma cell line P815-Cw3 444/C931, expressing full-length HLA-Cw3, was a gift from J.-C. Cerottini (LICR, Lausanne, Switzerland). P815 was obtained by chemical selection, FACS sorting, and limiting dilution. B16F10, a murine melanoma cell line of C57/BL/6 origin, was a gift from P. Schreiber (Boehringer Ingelheim Austria, Vienna, Austria), from which the pigmented, tyrosinase-related protein (TRP) 1- and 2-expressing, and in vivo aggressively growing B16F10M subline was subcultured in our laboratory.

Tumor challenge experiment

For the B16 lung metastasis model, groups of C57BL/6 mice (10 animals/group) were electrovaccinated three times at intervals of 1 wk using 50 μg plasmids 789 and 790. Both constructs harbor the dominant SVYD epitope in vivo. We compared three different constructs (Fig. 1a). Plasmid 513, which encodes full-length HLA-Cw3, provoked high CD8+ T cell levels in previous experiments and served as a positive control. The two others were minimal constructs where the antigenic RYLK epitope was preceded by leader sequences to ensure efficient translation and ER delivery of the product (24). Plasmid 540 contains the adenovirus E3 19K leader, and plasmid 557 contains the Ig light chain leader. Plasmids were electrovaccinated on day 2, and PBMCs were analyzed by FACS on day 15. A steady increase in the percentage of PB10+ CD8+ T cells was observed with the full-length construct 513. After a peak of >17% on day 15, the response slowly decayed. These and other data suggested that a period of 13 days between electrovaccination and FACS
analysis of PBMCs was optimal for measurement of the peak response, and this period was used in all other in vivo experiments. Surprisingly the two minimal constructs, 540 and 557, did not elicit CD8/H11001 T cell responses above background.

To determine whether extensive changes in structure would interfere with efficient expression and Ag presentation of minimal constructs 540 and 557 in murine cells, we tested these and other minimal epitope constructs in vitro (Fig. 2). C-26 mouse colon carcinoma cells were transiently transfected with plasmid DNA and cocultivated with HLA-Cw3-specific CTL lines. Supernatants were collected after 4 days, and their IFN-/H9253 content was measured by ELISA. Again, plasmid 513 served as a positive control. In addition to constructs 540 and 557, plasmid 123, encoding the RYLK epitope fused to the C terminus of enhanced green fluorescent protein (EGFP), was introduced as another minimal construct. Plasmids 533 and 536 are respective homologs of plasmids 557 and 540, except that the RYLK epitope was replaced by the SVYD epitope derived from the murine B16 tumor Ag TRP-2. Plasmid 149 encoded EGFP only. In contrast to our findings in vivo, the two minimal constructs, 540 and 557, are comparable to plasmid 513 in transforming cells into CTL targets in vitro. Also, plasmid 123 stimulates strong IFN-/H9253 production that is clearly directed against the RYLK 10 mer, as can be seen by comparison to plasmid 149, which provokes only background stimulation of CTLs. As expected, cells transfected with plasmids 533 and 536, which harbor the SVYD epitope, do not stimulate HLA-Cw3-specific CTLs in vitro.

In conclusion, RYLK epitope carrying minimal plasmid constructs that were well expressed and did stimulate HLA-Cw3-specific CTLs in vitro failed to elicit a specific CTL response in vivo upon electrovaccination. Thus, generally the expression of epitope-only minimal constructs is not severely impaired, and it is unlikely that simple variations in expression levels caused the dramatic differences between full-length and minimal constructs in vivo. We therefore reasoned that portions of the full-size Cw3 molecule that lie outside the dominant epitope must be responsible for this remarkable difference, and we performed a genetic complementation experiment.

Epitope-only constructs cannot be trans-complemented in vivo

Trans-complementation was used to address the possible involvement of helper epitopes (25, 26). Plasmid 540, which harbors an RYLK epitope preceded by the adenoviral E3 leader, induced only low levels of specific CD8/H11001 T cell expansion. Groups of DBA/2 mice, four animals per group, were electrovaccinated with 50 /H9262 g/animal of plasmid 513 ( ), 540 (O), or 557 ( ). Control mice ( ) received 584. Vaccine administration is indicated by the arrowhead. The day before vaccination and on days 8, 15, 22, and 37 postvaccination blood samples of 200 l were taken, and lymphocytes were isolated by gradient density centrifugation. Cells were CD8/CD62L/VB10 triple stained. VB10/CD62L/ -cells among CD8/H11001 lymphocytes were quantified by FACS. Samples were treated individually without pooling in all experiments. Note that the percentage of VB10 cells is shown on a logarithmic scale.

FIGURE 1. Anti-Cw3 responses in DBA/2 mice show pronounced differences depending on the context of the dominant epitope. a, Representative structures of plasmids used for immunization. Note that Cw3-derived sequences are depicted with symbols on a white background, whereas structural parts of non-Cw3 origin are on a black background. Plasmid 513, full-length HLA Cw3; plasmid 540, RYLK epitope preceded by adenoviral E3 19k leader; plasmid 557, RYLK epitope preceded by Ig light chain leader; plasmid 584, human growth hormone. b, Time course of RYLK-specific CD8/H11001 T cell expansion. Groups of DBA/2 mice, four animals per group, were electrovaccinated with 50 /H9262 g/animal of plasmid 513 ( ), 540 (O), or 557 ( ). Control mice ( ) received 584. Vaccine administration is indicated by the arrowhead. The day before vaccination and on days 8, 15, 22, and 37 postvaccination blood samples of 200 l were taken, and lymphocytes were isolated by gradient density centrifugation. Cells were CD8/CD62L/VB10 triple stained. VB10/CD62L/ -cells among CD8/H11001 lymphocytes were quantified by FACS. Samples were treated individually without pooling in all experiments. Note that the percentage of VB10 cells is shown on a logarithmic scale.
None of the plasmids injected together with 513 could significantly complement for missing elements in plasmid 540 compared with the robust Cw3 response induced by the positive control 513. Even plasmid 529 that carries all structural elements of HLA-Cw3 except the antigenic epitope induced a response only marginally above background. These data suggest that putative critical structural parts must reside on the same molecule where the epitope is also present; they must be in some ways physically connected.

C-terminal truncations of HLA-Cw3 assign a significant role for the α3/TM region in the induction of potent CD8⁺ T cell responses in vivo

The localization of important structural elements was then started by successive C-terminal truncations of the full-length HLA-Cw3. In plasmid 653 the TM/intracytoplasmic (IC) domains were truncated completely; the resulting molecule can thus be considered soluble. Plasmid 654 encoded an HLA-Cw3 molecule in which the α3 domain was also deleted. Finally, plasmid 655 only encoded the α1 domain and lacked the RYLK epitope. The truncation mutants together with plasmid 513 were then tested in vivo (Fig. 4a). By truncation of the TM/IC domains (plasmid 653) the expansion of VB10⁺ CD8⁺ T cells was reduced by 80% compared with the positive control. This level dropped to background when the α3 domain was also deleted (654). With only the α1 domain left, plasmid 655 did not elicit any expansion of VB10⁺ CD8⁺ T cells.

With these plasmids the in vitro and in vivo results were markedly different (Fig. 4b). Essentially all constructs carrying the RYLK epitope (513, 653, and 654) were similarly effective in vitro regardless of their C-terminal truncations. CTL recognition was lost completely only when the RYLK epitope was missing. These results clearly indicated that structures within the α3/TM domain were key contributors to an efficient in vivo CD8⁺ T cell response.

Membrane anchoring is important for high immunogenicity of HLA-Cw3 in vivo, while the α3 domain can be deleted without considerable loss of antigenicity

To further narrow down critical regions, truncations on the C-terminal side of the α3 domain were introduced. The TM/IC domain was then fused to the remaining molecule (Fig. 5). Together with plasmid 513 as the positive control, these constructs were tested in vivo (Fig. 5a). Two of the four resulting constructs contained parts of the α3 domain extending from aa 203–268 (691) and from aa 203–241 (692), respectively. In plasmid 693 the α3 domain was completely truncated, and in plasmid 694 the α2 domain was deleted additionally. No substantial losses in the expansion of VB10⁺ CD8⁺ T cells were observed, even with α3 completely removed from the molecule (plasmid 693). As expected, plasmid 694 that lacks the RYLK epitope stimulated only background VB10⁺ CD8⁺ T cell expansion.

In vitro these findings could be confirmed (Fig. 5b). Again, removal of the α3 domain did not decrease the antigenicity of the remaining molecule. These data confirmed that membrane anchoring was an important factor for eliciting strong CD8⁺ T cell responses in vivo.
The endogenous transmembrane domain of HLA-Cw3 is critical for the immunogenicity of RYLK-carrying molecules and can be used to engineer a synthetic minimal construct.

Fine mapping of critical regions inside the TM/IC domains was begun with C-terminal truncations in the respective domains. On the basis of plasmid 693 the TM/IC was successively truncated, of which plasmid 707 carries aa 298–350, plasmid 708 carries aa 298–339 and plasmid 709 carries aa 298–314. The final step in this series was the soluble variant 654. The resulting plasmids were tested in vivo. The standard positive control 513 is shown for FIGURE 4. C-terminal truncation of the full-length HLA-Cw3 construct narrows down the region essential for effective in vivo responses, a. Successive truncation of the transmembrane and the α3 domains results in stepwise loss of antigenicity. Groups of four DBA/2 mice were electrovaccinated with 50 μg/animal of plasmid, and blood samples were analyzed 13 days after electrovaccination. b. Stimulation of CTLs in vitro depends on the presence of the epitope, but is otherwise independent from truncation. C-26 mouse colon carcinoma cells were transiently transfected with the indicated plasmids and cocultivated with HLA-Cw3-specific CD8+ T cells, and released IFN-γ was measured by ELISA. Plasmid 513, full-length HLA Cw3; plasmid 540, RYLK epitope preceded by adenoviral E3 19K leader; plasmid 527, α1/α2/RYL from MHC H-2Kd and α3/TM from HLA-Cw3; plasmid 529, full-length HLA-Cw3, except the epitope is mutated to the RYLE homolog; plasmid 530, α1/α2 from HLA-Cw3 and RYLE/α3/TM from MHC H-2Kd; plasmid 610, full-length MHC H-2Kd; plasmid 608, full-length MHC H-2Kd.
showed a significantly decreased capacity of VB10 \textsuperscript{741} for the TM domain of \textit{H2-IA}. Plasmids \textsuperscript{739}, \textsuperscript{740}, and \textsuperscript{741} exchanged: in \textsuperscript{740} for the TM domain of \textit{CD47}, and in plasmid TM/IC. In two other constructs the endogenous TM domain was – \textsuperscript{694} for fusion of the endogenous TM domain to the truncated C-terminal part of \textit{HLA-Cw3}. Plasmid \textsuperscript{691}, aa \textsuperscript{203} plasmids \textsuperscript{691} – 741 of the domain was inserted between RYLK epitope and TM/IC domains. While plasmids \textsuperscript{540} and \textsuperscript{710} induced only low levels of domain was exchanged for TM domain of \textit{HLA-Cw3}; plasmid \textsuperscript{690}, full-length \textit{HLA-Cw3} carrying a – \textsuperscript{203} encoding post-translational GPI link modification. To test the significance of compartment-specific TM anchoring, the endogenous TM domain of \textit{HLA-Cw3} was exchanged for TM domains of different molecules (Fig. 6b). In plasmid \textsuperscript{739} sequences encoding post-translational GPI link modification were fused after the RYLK epitope of the \textsuperscript{a3}-deleted \textsuperscript{693} instead of the endogenous TM/IC. In two other constructs the endogenous TM domain was exchanged: in \textsuperscript{740} for the TM domain of \textsuperscript{CD47}, and in plasmid \textsuperscript{741} for the TM domain of \textit{H2-IA}. Plasmids \textsuperscript{739}, \textsuperscript{740}, and \textsuperscript{741} showed a significantly decreased capacity of VB10\textsuperscript{+}CD8\textsuperscript{+} T cell expansion. Nevertheless, responses provoked by these constructs were still higher than those produced by soluble \textsuperscript{654}. Thus, while the rudimentary endogenous TM domain is sufficient for the induction of strong responses in vivo, it can only be partially substituted with foreign variants. Confocal microscopy of an EGFP-tagged variant of \textsuperscript{709} suggested that this construct retained its membrane anchoring (data not shown).

Reconstitution of highly effective minimal constructs

Finally, we have assembled only those structural elements of \textit{HLA-Cw3} that were found to contribute to high immunogenicity. Plasmids were progressively refined and tested in vivo. Plasmid \textsuperscript{540} carries the RYLK epitope preceded by the adenoviral leader E3. This construct was fused to the TM domain of \textit{HLA-Cw3} to create plasmid \textsuperscript{710}. Finally, in plasmid \textsuperscript{747} the endogenous \textsuperscript{a3} domain was inserted between RYLK epitope and TM/IC domains. While plasmids \textsuperscript{540} and \textsuperscript{710} induced only low levels of VB10\textsuperscript{+}CD8\textsuperscript{+} T cells, construct \textsuperscript{747} performed better than the positive control \textsuperscript{513} (Fig. 7). In a further step the TM domain of plasmid \textsuperscript{747} was truncated to a rudimentary anchor of 16 aa. As already shown for plasmid \textsuperscript{709}, this short anchor is sufficient for high in vivo immunogenicity of plasmid \textsuperscript{782}.

To test whether the newly defined efficient minimal constructs could be used to boost immunogenicity of CTL epitopes other than RYLK, tumor protection experiments using the B16 melanoma model were performed. The spontaneously arising murine melanoma B16 of C57BL/6 mice became a reference in which Ag-specific vaccination against tumors can be tested (20, 27). Similar to human cancers, B16 cells are very weakly immunogenic, and sublines exist with varying tumorigenic and metastatic capabilities (28, 29). Murine TRP-2 has been identified as the dominant tumor Ag upon expression cloning and screening with B16-reactive cytotoxic T cells. The peptide SVYDFFVWL was defined as the dominant H-2K\textsuperscript{b}-restricted epitope, and was shown to be of therapeutic benefit in the treatment of B16 lung metastasis (20, 30).

For our experiments constructs harboring the SVYD epitope were tested for potential therapeutic effects. Two plasmid variants based on the Cw3 minimal construct \textsuperscript{747} were generated. In plasmid \textsuperscript{789} the RYLK epitope was exchanged for the SVYD epitope, and in plasmid \textsuperscript{790} the SVYD epitope was mutagenized into a SVYD to a signal sequence holds the potential risk of faulty cleavage inside or at the C-terminal side of the antigenic epitope. Since hydrophobic leader signals must be followed by small and neutral residues for correct cleavage by the signal peptidase (31), direct fusion of a highly hydrophobic epitope such as SVYD to a signal sequence holds the potential risk of faulty cleavage inside or at the C-terminal side of the antigenic epitope. Antitumor protection was tested in the B16 lung metastasis model, where previous attempts to induce tumor protection with full-length mTRP-2 electrovaccinations had failed (M. Kalat, Z. Küpçü, S. Schüller, D. Zalusky, M. Zehetner, W. Paster, and T. Schweighoffer, manuscript in preparation). Groups of C57BL/6

![FIGURE 5. Membrane anchoring is crucial for effective in vivo responses, while the a3 domain is dispensable. a. Successive truncations of the a3 domain with the TM domain still fused to the remaining molecule. Groups of four DBA/2 mice were electrovaccinated with 50 μg/animal of plasmid, and blood samples were analyzed 13 days after electrovaccination. b. Stimulation of CTLs in vitro. C-26 mouse colon carcinoma cells were transiently transfected with the indicated plasmids and cocultivated with HLA-Cw3-specific CD8\textsuperscript{+} T cells. Released IFN-γ was measured by ELISA. Plasmid \textsuperscript{513}, full-length \textit{HLA-Cw3}; plasmid \textsuperscript{690}, full-length \textit{HLA-Cw3} carrying a BstEII restriction site at the a3/TM domain interface. This site was also used for plasmids 691–694 for fusion of the endogenous TM domain to the truncated C-terminal part of \textit{HLA-Cw3}. Plasmid \textsuperscript{691}, aa 203–268 of the a3 domain; plasmid \textsuperscript{692}, aa 203–241 of the a3 domain; plasmid \textsuperscript{693}, a3 domain completely truncated; plasmid \textsuperscript{694}, a2 domain deleted additionally.](http://www.jimmunol.org/.../by guest on April 20, 2017)
mice were electrovaccinated three times at intervals of 1 wk using plasmids 789 and 790. Control mice were left untreated. One week after the last vaccination mice were challenged by i.v. injection of 8 × 10⁵ B16F10M melanoma cells. On day 22 postchallenge mice were sacrificed, and lungs were examined for coverage with metastases. Both constructs conferred significant protection to vaccinated animals (Fig. 7b), especially plasmid 790, which caused a 7-fold decrease in coverage of lung surface with metastases.

**Discussion**

By using an electrovaccination protocol for immunization we could elicit high level CTL responses. The unusually tight correlation between the phenotype of the responder T cell population and the immunogen in DBA/2 mice immunized with P815-Cw3 cells has been described (17). All responding CD8 T cells directed against a single immunodominant 10-mer epitope (the RYLK epitope) share a unique Vβ10 phenotype. This enables their exact enumeration in small samples of peripheral blood by flow cytometry.

In initial experiments a dramatic expansion of HLA-Cw3-specific VB10+CD8+ T cells in electrovaccinated DBA/2 mice could be shown. Peak responses with >17% specific CTLs in the peripheral blood were reached at day 13 postelectrovaccination. This can be taken as striking proof of the effectiveness of our DNA vaccination approach on the HLA-Cw3 system. The powerful expansion of CTLs was achieved by an extremely simplified, cell-free immunization system using a minimalist vector with low CpG content and no additional adjuvant.

To our surprise experiments showed considerable differences between in vivo and in vitro immunogenicity of several constructs. Some minimal constructs did turn cells into CTL targets in vitro, but were unable to elicit a T cell response in vivo. With this powerful and clearly defined system at hand, we have asked for the molecular basis of this pronounced CTL response. Based on the structurally well-characterized, full-length HLA-Cw3 molecule we created numerous variants of the molecule that were all tested in vivo.

It has been described that ER targeting improves immunogenicity, especially of subdominant class I epitopes (13, 32). Surprisingly and in sharp contrast to the full-length construct 513, ER-targeted epitope-only constructs failed to stimulate CD8+ T cells in vivo. This is clearly not a matter of inefficient expression or liberation of the epitope, as the same constructs did transform cells into targets for RYLK-specific CTLs in vitro. These findings indicate the importance of a correct structural environment of the antigenic RYLK epitope for the induction of potent CTL responses in vivo. Apparently the antigenic epitope encoded by the minimal constructs is expressed, processed, and presented on the cell surface efficiently in vitro, but not in vivo. Professional APCs have been shown to play a major role during DNA vaccination (33, 34). It could well be that the highly active Ag processing machinery of these cells effectively destroys the shorter RYLK epitope-only Ags, while longer constructs have a prolonged existence as Ag depots.

**FIGURE 6.** Alterations in transmembrane domain have profound effects on antigenicity of HLA-Cw3 in vivo. a, A rudimentary endogenous TM domain is sufficient to promote high antigenicity. Successive truncations of the endogenous TM domain were fused to the first two domains plus RYLK epitope. Plasmid 513, full-length HLA Cw3, TM/IC truncation mutants are based on 693; plasmid 707, aa 298–350; plasmid 708, aa 298–339; plasmid 709, aa 298–314; plasmid 654, TM deleted completely. b, The endogenous TM domain can only be partially substituted for by foreign TM anchors. Plasmid 513, full-length HLA Cw3, TM/IC exchange mutants are based on 693. Plasmid 739, sequences coding for post-translational GPI link modification; plasmid 740, complete TM portion of CD47; plasmid 741, TM portion of H-2Ia β-chain. Groups of four DBA/2 mice were electrovaccinated with 50 μg/animal of plasmid, and blood samples were analyzed 13 days after electrovaccination.
Short minimal epitope constructs apparently are missing important structural elements. Besides their obvious structural function, these elements could also act in trans, providing T cell help. There are indeed reports on trans-acting helper epitopes, processed and presented by the same APC as the antigenic epitope itself (25, 26). The participation of trans-acting helper epitopes was directly ruled out by a trans-complementation experiment. None of the constructs provided could supplement for missing structural parts of minimal construct 540 in trans, not even plasmid 529, despite its being full-length HLA-Cw3 with the immunogenic epitope replaced by the RYLE homolog of MHC H2-Kd. These results indicate that all structural elements necessary have to be located on the same stretch of amino acids (in cis) as the antigenic RYLK epitope. Possible cis structural requirements for enhanced in vivo antigenicity could be correct folding for efficient processing, organelle-specific trafficking, membrane retention, or interaction of various domains with different molecules.

Dissection of the structural requirements was begun by in vivo testing of domain-wise C-terminal truncation mutants. C-terminal truncation variants showed a two-step pattern in their loss of immunogenicity. The first major loss occurred upon truncation of the TM/IC domain, when α3 was truncated additionally, and the percentage of VB10+ CD8+ T cells returned to background levels. To further characterize the contributions of these two regions, mutant plasmid variants with internal truncations of the α2/α3 domains were designed. Only minor losses in immunogenicity could be linked to the truncation of α3 in vivo, whereas in vitro these constructs performed equally well. As all these constructs were membrane anchored, the endogenous HLA-Cw3 TM/IC domain was considered the most likely determinant of the HLA-Cw3 molecule for high in vivo responses against the RYLK epitope.

Surprisingly, even additional stepwise C-terminal truncations of TM/IC showed no adverse effect until complete removal of the domain. Plasmid 709, with a rudimentary TM anchor of 16 aa, produced a response as high as the positive control 513. These findings clearly indicate that membrane anchoring, most likely in an organelle-specific manner, without intracellular domains is essential.
encoding for post-translational GPI link modification failed to stimulate considerable amounts of CTLs. For the GPI-linked constructs it is obvious that exclusive sorting to the plasma membrane (35) accompanied by different lateral mobility removes much of the Ag from the processing/presentation machinery.

In summary, we were able to design a series of progressively refined minimal constructs. Adding the endogenous TM/IC domain to the minimal epitope construct 540, which reportedly did not stimulate CD8+ T cells in vivo, did significantly boost in vivo immunogenicity. As CTL levels still were not comparable to those elicited by full-length HLA-Cw3, the α3 domain, identified as the second most important structural part, was additionally reinserted. By doing so, a construct even superior to plasmid 513 could be obtained. Thus, besides the importance of TM/IC, α3 also apparently plays a role in efficient processing of HLA-Cw3. This finding comes as a surprise, since the α3 domain had undoubtedly been shown previously to be of only minor importance. On the other hand, the previous results had been obtained in the more complex full-length environment with the domains α1 and α2 still on the molecule. In contrast to a short and minimalist construct, these two domains in the multidomain molecule could compensate for the missing α3 domain. Thereby potential truncation effects were masked.

In conclusion, we have learned that short ER-targeted epitope-only constructs do not work in vivo. An endogenous minimal TM anchor and the α3 domain restores antigenicity completely. An explanation for this structure dependence could be that efficient Cw3 constructs behave similarly to endogenous class I molecules. A small fraction of class-I molecules has been shown to recirculate from the plasma membrane to endosomal compartments (36). It can now be hypothesized that this happens with the Cw3 molecule in APCs, thereby providing the cell with a long-term Ag reservoir. Minimal constructs do not have access to this pathway and are therefore highly prone to rapid proteasomal degradation. The additional requirement for the α3 domain implicates interaction with receptor structures, since this Ig fold domain is contacted, among other major APCs. Such contact with professional APCs could be shown to transfer of membrane vesicles containing the Ag (37).

What remains to be shown is whether the structure-function relationship failed to narrow down the epitopes by endoplasmic reticulum insertion of the minimal peptides. Is enhanced by endoplasmic reticulum insertion and suggests a novel pathway for processing of membrane proteins. J. Exp. Med. 181:1465.


References


