T he 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 specific immune response is initiated toward the gene products.

In the following years a series of papers (7–9) demonstrated that plasmid DNA is able to induce potent humoral and cellular immune responses against the encoded Ag in laboratory animals. Genetic immunization was shown to induce protective immunity against bacterial and parasitic infections (10) and seemed to offer new perspectives for the treatment of cancer and even allergy. However, limited efficiency of DNA-based vaccines still precludes their routine use in human prophylaxis and therapy. Vector structures as well as delivery modalities are thus continuously being modified and optimized. In contrast, relatively little work has been invested into understanding the role that protein structure itself plays in the immunogenicity of embedded antigenic epitopes. For example, subcellular localization, speed and mode of degradation, or post-translational epitope modifications (11, 12) can all influence the dominance of a given Ag. Notably, several laboratories have confirmed that shuttling the class I-restricted antigenic epitopes directly into the endoplasmic reticulum results in enhanced levels of specific CD8+ T cells (13, 14).

We have chosen the potent H-2Kd restricted Cw3 response, first described by Maryanski et al. (15), as a model system to compare and optimize various vaccination strategies. They have immunized DBA/2 mice with the heavy chain of the human HLA Cw3 molecule, stably expressed by MHC class I-matched transfectants, which provoked robust CD8+ T cell responses. All the responder T cell clones recognized the dominant epitope (HLACw3.170–179 in its original designation; RYLK for short) presented in MHC H-2Kd context, and showed exclusive usage of the Vβ10 segment in their TCR β-chains. Thus, RYLK-specific CTLs had the unique phenotype of CD8+ CD62 ligand (CD62L)+ TCRβ10+ (16, 17). Later it has been established that 97% of RYLK-H2-Kd tetramer-positive cells from Cw3-immunized animals were VB10+ (18).

We could elicit high level CTL responses in this model system by genetic immunization using the novel technique of electrovaccination (19). Here the administration of a buffered plasmid DNA solution, followed by electroporation at the site of the injection, result in high levels of Ag expression, which, in turn, provoke massive humoral and/or cellular immune responses. Due to the high magnitude of the Cw3 response, these RYLK-specific cells could be exactly quantified in small samples of peripheral blood by flow cytometry.

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2 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; RYLMGKTEL, RYLE MHC-H2-encoded homolog of RYLMGNETL; SVYD, mTRP-2-derived epitope SVYDFFVWL; TM, transmembrane portion.
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-2K¹⁺-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/B16/6 origin, was a gift from R. Kircheis (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. 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/B16/6 origin, was a gift from R. Kircheis (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 defined from the tumor Ag murine TRP-2 (mTRP-2). Control mice (two groups, 10 animals/group) were left untreated. One week after the last vaccination mice were challenged by i.v. injection of 8 × 10⁴ B16F10M cells in 100 μl Dulbecco’s PBS. The number of cells needed was established by previous titration experiments and reproducibly gives a lung surface coverage of 80% after 3 wk. To avoid aggregation of cells, the suspension was kept on ice until injection. On day 22 postchallenge, mice were sacrificed, and lungs were examined for coverage with metastases. The percentage of the surface covered with metastases was estimated.

Epitopes, plasmids, and immunizations

RYLK is the short-hand notation for the DNA sequence that encodes the H-2K²⁻restricted dominant Cw3-derived epitope RYLKNGKETL; RYLE stands for the MHC-H2 encoded mouse homolog RYLELGNETL; SVYD denotes the dominant H-2K²⁻restricted epitope SVYDFFVWL of mTRP-2 expressed by the murine melanoma B16 (20). In keeping with FDA guidelines, all plasmids used for vaccinations carried the neomycin phospho-transferase gene (KanR) for bacterial selection. The CpG content and, consequently, the immunogenicity of the plasmid backbone were therefore lowered (21). Symbolic structures of the plasmids are depicted side-by-side to the results; further details of construction are available upon request. Plasmids were purified on CsCl gradients or were custom manufactured by ELIM Pharmaceuticals (South San Francisco, CA) and contained <5 EU/mg endotoxin. The required amounts of plasmids were diluted into 100 μl 20 mM HEPEs (pH 7.4) buffer, and 50 μl of each was applied to both quadriceps femoris muscle of mice under avertin anesthesia. Immunization was followed immediately by electroporation of the injected area (80 V, three pulses of 60 ms with repoling) using an Electro Sorex Porator device (T820; BTX, San Diego, CA).

FACS analysis

Blood (200 μl) was sampled from the retro-orbital sinus using heparinized capillaries and tubes. Lymphocytes were isolated by gradient density centrifugation (Lymphoprep; Nycomed, Oslo, Norway). Mononuclear cell yield after micro-Lymphoprep was 3–5 × 10⁹/ml. Blood samples from each animal were processed and analyzed individually, without pooling. Normal rabbit Ig and anti-CD16/32 mAb were added to block unspecified sites first, followed by a mixture of anti-CD62L-FITC, anti-CD8-PE, and anti-VB10-biotin (all Abs from BD Pharmingen, San Diego, CA). Allophycocyanin- or CyChrome-conjugated streptavidin (both from BD Pharmingen) were used as second-step reagents. Cells were analyzed on a FACSort or BD Biosciences LSR cytometer (Franklin Lakes, NJ), and >300,000 events gated on PBL were collected. Data were analyzed using WinMDI (J. Trotter, The Scripps Institute, La Jolla, CA), and the percent specific CD8⁺ cells was calculated in two steps. First, live CD8⁺ cells were electronically gated. Then on a CD62L (x-axis) vs VB10 (y-axis) dot plot displaying these gated cells only, four quadrants were set that fined high and low expressing cells. To obtain precise, unbiased values, the low level spontaneous loss of CD62L was corrected by employing the following equation (22, 23):

\[
\frac{(\text{CD62L}^- \times \text{VB10}^-) - (\text{CD62L}^- \times \text{VB10}^+) \times \text{CD62L}^+ \times \text{VB10}^-}{\text{CD62L}^+ \times \text{VB10}^-}
\]

The background measured on naive and control-treated mice using this calculation was 0.3 ± 0.1%.

IFN-γ ELISA

C-26 murine colon carcinoma cells were grown overnight in 96-well plates at 15,000 cells/well and transiently transfected with 500 ng DNA/well using the DEAE-dextran/chloroform method. After 3.5-h incubation the transfection mixture was removed from the cells, and fresh medium was added. The day after transfection 15,000 HLA-Cw3 specific CTLs were added per well. After 4 days of cocultivation supernatants were analyzed for their IFN-γ content by ELISA. For quantification of IFN-γ in cell culture supernatants the OPTEIA mouse IFN-γ Ab set (BD Pharmingen) was used.

Results

Not all epitope-expressing constructs that transform cells into CTL targets in vitro elicit a T cell response in vivo

In an initial time-course experiment we analyzed the kinetics of CD8⁺ T cell expansion against the dominant H-2K¹⁺-restricted HLA-Cw3 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 adenosine 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 days 1 (prevaccination), 8, 15, 22, and 37 (Fig. 1b). A steady increase in the percentage of VB10⁺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-γ 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-γ 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⁺ T cells in vivo (Fig. 3). This construct was then cojected with one of the following chimerical human/mouse plasmid variants: plasmid 527, which encodes α1/α2/RYLE from MHC H-2Kd and α3/transmembrane portion (TM) from HLA-Cw3; plasmid 529, which encodes full-length HLA-Cw3, except that the epitope is mutated to the RYLE homolog; plasmid 530, which encodes α1/α2 from HLA-Cw3 and RYLE/α3/TM from MHC H-2Kd; plasmid 610, which encodes full-length MHC H-2Kd; and plasmid 608, which encodes full-length MHC H-2Kb.
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. Finally, plasmid 655 only encoded the α1 domain and lacked the RYLK epitope. The truncation mutants together with plasmid 513 were then first 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.
showed a significant for the TM domain of H2-IA. Plasmids 739, 740, and 741 exchanged: in 740 for the TM domain of CD47, and in plasmid TM/IC. In two other constructs the endogenous TM domain was – plasmids 691 VB10 While plasmids 540 and 710 induced only low levels of domain was inserted between RYLK epitope and TM/IC domains. 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+ T cells. Released IFN-γ was measured by ELISA. Plasmid 513, full-length HLA Cw3; plasmid 690, full-length HLA Cw3 carrying a BstEII restriction site at the α3/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 HLA-Cw3. Plasmid 691, aa 203–268 of the α3 domain; plasmid 692, aa 203–241 of the α3 domain; plasmid 693, α3 domain completely truncated; plasmid 694, α2 domain deleted additionally.

To test the significance of compartment-specific TM anchoring, the endogenous TM domain of HLA-Cw3 was exchanged for TM domains of different molecules (Fig. 6b). In plasmid 739 sequences encoding post-translational GPI link modification were fused after the RYLK epitope of the α3-deleted 693 instead of the endogenous TM/IC. In two other constructs the endogenous TM domain was exchanged: in 740 for the TM domain of CD47, and in plasmid 741 for the TM domain of H2-IA. Plasmids 739, 740, and 741 showed a significantly decreased capacity of VB10+CD8+ T cell expansion. Nevertheless, responses provoked by these constructs were still higher than those produced by soluble 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 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 HLA-Cw3 that were found to contribute to high immunogenicity. Plasmids were progressively refined and tested in vivo. Plasmid 540 carries the RYLK epitope preceded by the adenoviral leader E3. This construct was fused to the TM domain of HLA-Cw3 to create plasmid 710. Finally, in plasmid 747 the endogenous α3 domain was inserted between RYLK epitope and TM/IC domains. While plasmids 540 and 710 induced only low levels of VB10+CD8+ T cells, construct 747 performed better than the positive control 513 (Fig. 7). In a further step the TM domain of plasmid 747 was truncated to a rudimentary anchor of 16 aa. As already shown for plasmid 709, this short anchor is sufficient for high in vivo immunogenicity of plasmid 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-2Kb-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 747 were generated. In plasmid 789 the RYLK epitope was exchanged for the SVYD epitope, and in plasmid 790 the SVYD epitope was mutagenized into a moderately homologous hydrophobic region of the α3 domain. This was done to preserve the structure of the molecule and to prevent the signal recognition machinery from destroying 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. Küpcü, S. Schüller, D. Zalusky, M. Zehetner, W. Paster, and T. Schweighoffer, manuscript in preparation). Groups of C57/BL6...
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 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 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.

That organelle specificity plays an important role was confirmed by TM/IC domain replacement variants. Plasmids with TM domains of HLA H2-IA (741) and CD47 (740) and with sequences

FIGURE 7. Newly defined, efficient minimal constructs can be used as carrier for an unrelated, weakly immunogenic CTL epitope. a, Four generations of progressively refined minimal constructs. Groups of four DBA/2 mice were electrovaccinated with 50 μg/animal of plasmid, and blood samples were analyzed 13 days after electrovaccination. Plasmid 513, full-length HLA Cw3; plasmid 540, RYLK epitope preceded by adenoviral E3 19K leader; plasmid 710, 540 with endogenous TM domain fused; plasmid 747, 540 with endogenous α3/TM domains fused; plasmid 782, TM domain of 747 truncated to rudimentary 16 aa. b, Strong antitumor protection in the B16 lung metastasis model conferred by optimized minimal constructs. Groups of DBA/2 mice, 10 animals/group, were electrovaccinated three times at intervals of 1 wk using 50 μg plasmids 789 and 790; control mice were left untreated. One week after the last vaccination mice were challenged by i.v. injection of 8 × 10⁶ B16F10M melanoma cells. Mice were sacrificed on day 22 postchallenge, and melanoma nodules were scored as the percent coverage of lung surface. Compared with the untreated group, electrovaccination results in significant protection (p < 0.003 for plasmid 789; p < 0.001 for plasmid 790; by t test). Plasmid 789, RYLK substituted by SVYD; plasmid 790, SVYD inserted into the α3 domain. Both constructs are based on 747. Note that Cw3-derived sequences are depicted with symbols on a white background, whereas structural parts of various murine origin are on a black background.
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 of the Ag itself is important. Often it can now be hypothesized that this happens with the Cw3 molecule. In contrast to a short and minimalist construct, these two domains in the multidomain molecule could compensate for the missing Cw3 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 Cw3 domain restore 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 Cw3 domain implicates interaction with receptor structures, since this Ig fold domain is contacted, among others, by CD8 and the Ag loading machinery. Perhaps special characteristics of the Cw3 Cw3 domain allow atypical interactions with intracellular factors or receptor structures on professional APCs when present on the plasma membrane of transfected factor cells. Such contact with professional APCs could finally lead to transfer of membrane vesicles containing the Ag (37).

While many of the previous studies focused on optimization of vector backbone and delivery strategy, we have successfully shown that protein structure of the Ag itself is important. Often reported to be relatively inefficient in inducing immune responses, DNA vaccination in our system stimulated tremendous CD8+ T cell responses. This was accomplished by using a minimalist vector with low CpG content and without using adjuvant for injection. What remains to be shown is whether the structure-function relationship deduced on the basis of the exceptional HLA-Cw3 system can be exploited in a more general manner for vaccine development. The first promising data in the B16 mouse melanoma model using the TRP-2 tumor Ag in a Cw3 minimal construct have been presented. This approach is superior to previous DNA-based vaccines used in the B16 model, and it is therefore likely that Cw3-based DNA vaccines could boost the immunogenicity of other inefficient CTL epitopes in the future.

References


