

The N-terminal Flanking Region of the TRP_{2360–368} Melanoma Antigen Determines Proteasome Activator PA28 Requirement for Epitope Liberation*

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Proteasomes are known to produce major histocompatibility complex (MHC) class I ligands from endogenous antigens. The interferon- γ -inducible proteasome activator PA28 plays an important role in the generation of MHC ligands by proteasomes. Generation of the HLA-A*0201 restricted melanoma antigen TRP_{2360–368} by the proteasome has been shown to be dependent on the function of PA28 *in vitro* and *in vivo* (Sun, Y., Sijts, A. J., Song, M., Janek, K., Nussbaum, A. K., Kral, S., Schirle, M., Stevanovic, S., Paschen, A., Schild, H., Kloetzel, P. M., and Schadendorf, D. (2002) *Cancer Res.* 62, 2875–2882). Here we analyzed the role of the epitope sequence environment in determining this PA28 dependence. Experiments using the melanoma TRP_{2288–296} epitope and the murine cytomegalovirus-derived pp89 epitope precursor peptide for epitope replacement revealed that the TRP_{2360–368} flanking sequences can transfer PA28 dependence onto otherwise PA28 independent epitopes. Moreover, the N-terminal flanking sequence is sufficient to establish PA28 dependence of an epitope by allowing PA28-induced coordinated dual cleavages. These results show that N-terminal flanking sequences strongly influence epitope generation efficiency and that PA28 function is particularly relevant for the generation of normally poorly excised peptide products.

The generation of major histocompatibility complex (MHC)² class I ligands and the recognition of MHC class I-ligand complexes by CD8⁺ T cells is an effective tool for the elimination of infected or disordered cells from organisms. The ubiquitin-proteasome system represents the major source for MHC class I-presented peptides exposed to CD8⁺ T cells (1). Ubiquitin-proteasome system-mediated peptide generation is

influenced by structural changes in the 20S catalytic core (20S proteasome), which occur by exchange of the standard catalytic subunits by immunosubunits. The ATP-dependent 26S proteasome, composed of a 20S core and a 19S regulatory complex, recognizes substrates by a multi-ubiquitin signal. In contrast, 20S proteasomes can accept larger non-ubiquitinated peptides as substrates for ATP-independent degradation (3). However, 20S proteasomes exist in a latent state within the cells. In consequence, proteasome activity and function is regulated in part by modulating substrate entry by the PA28 complex, which attaches ATP-independent to the outer α -rings of the 20S proteasome. Detailed kinetic analysis using fluorogenic peptide substrates has shown that activation of the 20S proteasome by PA28 occurs by facilitating either substrate entry and/or product exit, with no effects on the active sites (3). In support, structural analyses have demonstrated that attachment of the activator to the 20S core leads to the opening of the central gate of the α -ring (4).

The expression of PA28 is constitutively enhanced in cells with specialized antigen-presenting function and is induced by stimulation of cells with interferon- γ . The presentation of a number of viral MHC class I epitopes is enhanced in the presence of PA28 (5, 6). Moreover, the action of PA28 appears to be independent of the presence of immunosubunits in the 20S core (7). *In vitro* digestion experiments performed by incubating epitope-containing peptides with 20S proteasomes in PA28 presence resulted in an immediate liberation of PA28-dependent epitopes (8–10). The conclusion drawn from these experiments was that PA28 changes the cleavage behavior of the 20S core in a characteristic manner; without PA28 the proteasome cuts substrates by consecutive and independent single cleavages, whereas association with PA28 results in a strong enhancement of proteasomal double cleavages and an accelerated liberation of MHC class I ligands or their precursors (8, 9).

A striking example of PA28 action that stand in contrast to the above findings has been provided recently by our analysis of two cytotoxic lymphocyte epitopes, TRP_{2288–296} and TRP_{2360–368}, derived from the melanoma differentiation antigen TRP2 (tyrosine-related protein 2) (11). Melanoma cell lines presented the former epitope, but three of them showed a dramatically diminished presentation of the latter epitope. Interferon- γ treatment restored the presentation of TRP_{2360–368} on all melanoma cell lines but one, Mel18, which was completely unable to present this TRP2-derived peptide. Analysis showed that the

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² The abbreviations used are: MHC, major histocompatibility complex; HPLC, high pressure liquid chromatography; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; rMD, restrained molecular dynamics.

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expression of PA28 was strongly impaired in Mel18 cells. Consequently, TRP2_{360–368} epitope presentation and specific cytotoxic lymphocyte lysis was completely restored by transfection with PA28 cDNAs. Importantly, *in vitro* experiments with purified 20S proteasomes, PA28, and a synthetic peptide harboring the TRP2_{360–368} epitope were able to mimic the *in vivo* situation and revealed that the presence of PA28 resulted in production of the TRP2 epitope and an N-terminally extended precursor peptide. The generation and presentation of the second epitope, TRP2_{288–296}, did not correlate with the presence of PA28. Thus PA28 in some cases appears to be able to alter the immunological phenotype of a cell by inducing subtle structural changes in the 20S proteasomes that allow the generation and presentation of normally hidden epitopes.

So far it is not known what determines the PA28 dependence of an epitope, raising the question of whether the TRP2_{360–368} epitope and the polypeptide domain that harbors this epitope possess distinct characteristics that determine their complete PA28 dependence. Our experiments now demonstrate that the flanking N-terminal sequences can determine either the PA28 dependence or independence of proteasomal MHC class I epitope generation.

MATERIALS AND METHODS

Peptides—Peptides were synthesized using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) methodology (0.1 mmol) on an Applied Biosystems 433A automated synthesizer. The peptides were purified by HPLC and analyzed by mass spectrometry (ABI Voyager DE PRO). The peptide sequences are shown in Figs. 3A, 4A, and 5.

Proteasome and PA28—20S proteasome was isolated from T2 cells (c20S), and PA28 was isolated from red blood (12, 13).

In Vitro Digest of Peptides—1 μ g of proteasome was incubated with 10 μ g of peptide in a final volume of 100 μ l of 20 mM Hepes, pH 7.8, 2 mM magnesium acetate, 2 mM dithiothreitol without or with PA28 (1 μ g) for the indicated times at 37 °C. Digests were stopped with 0.1 volume of trifluoroacetic acid. All digests were repeated at least three times.

Peptide Analysis and Quantification—Samples were analyzed by reverse-phase HPLC, using an HP1100 system (Hewlett-Packard) equipped with an RPC C2/C18 SC 2.1/10 column (GE Healthcare). Analysis was performed on line with a LCQ ion trap MS equipped with an electrospray ion source (ThermoQuest, Germany) (11). In Figs. 1–4 one representative result is shown for each.

¹H NMR Spectroscopy—Samples of the peptides were dissolved in 10 mM Tris/HCl (pH 7.0), 5 mM NaCl/D₂O (90/10; v/v). Spectra of both peptides (1.5 mg dissolved in 80 μ l) were recorded at 298 K and 600.03 MHz on a Bruker Avance 600 NMR spectrometer equipped with a 2.5-mm micro probe head (Bruker Biospin GmbH). The ¹H spectra were internally referenced to D₄-TSP. TOCSY spectra were recorded with mixing times of 80 ms, NOESY spectra with mixing times of 180 and 250 ms. Each two-dimensional experiment was processed with standard Bruker software (Topspin).

Structure Calculations—The data derived from NMR spectra were analyzed via restrained molecular dynamics (rMD) simulations using XPLOR-NIH on a SUSE linux PC

(14). Distance restraints (220 for TRP2_{350–378} pp89_{ins} and 195 for TRP2_{283–309}pp89_{ins}) were generated from analysis of ¹H NOESY experiments. For each peptide 50 starting structures were generated by randomizing the atom coordinates of peptide models followed by 1000 step Powell energy minimization. All structures were submitted to an 18-ps rMD calculation in conjunction with a simulated annealing procedure. The 10 structures with the lowest total energy were submitted to an additional 90-ps rMD simulation at 300 K.

RESULTS

To test the PA28 dependence of epitope generation synthetic 29-mer and 27-mer polypeptides harboring either the TRP2_{360–368} or the TRP2_{288–296} MHC class I epitope, respectively, were processed *in vitro* by standard proteasomes (20S proteasomes) in the absence or presence of purified PA28. Generated MHC class I ligands or their precursor peptides were detected by mass spectrometry. In perfect agreement with the situation in melanoma cells (11), generation of the TRP2_{288–296} peptide was completely independent of PA28 presence. In contrast, the TRP2_{360–368} epitope was generated only in PA28 presence (Fig. 1A). We detected both, the TRP2_{356–368} epitope precursor and the TRP2_{360–368} epitope in those experiments, although the latter at very low quantity. Remarkably, generation of both fragments essentially required the presence of PA28. These data raised the question of whether the epitopes themselves and/or their flanking sequences determine the observed requirement for PA28 activity. To answer this query, we performed epitope switching experiments in which TRP2_{360–368} was inserted into the potentially permissive (PA28-independent) TRP2_{283–309} sequence, and vice versa, TRP2_{288–296} was inserted into the potentially nonpermissive (PA28-dependent) sequence of TRP2_{350–378} (Fig. 1B). Insertion of TRP2_{288–296} epitope into in the “nonpermissive” TRP2_{350–378} context resulted in complete PA28 dependence of TRP2_{288–296} generation. In contrast, the PA28-dependent TRP2_{360–368} epitope became PA28-independent when inserted into the “permissive” context of TRP2_{283–309}. Therefore we concluded that PA28 dependence on or independence of the two TRP2 epitopes is determined by their flanking sequences and that by inserting either epitope into a nonpermissive or permissive sequence context, PA28 dependence can be altered.

Although the above experiments suggested that the flanking sequences might harbor the information for PA28 dependence, a possible functional interaction between the epitopes and their surrounding sequence context could not be excluded. Therefore, we replaced both TRP2 epitopes by an entirely different sequence representing the relevant 11-mer precursor peptide of the murine cytomegalovirus IEpp89_{166–176} epitope (15). This well studied antigenic peptide is efficiently generated *in vitro* by c20S and is well detectable by mass spectrometry (8). Integration of the pp89 precursor peptide into the nonpermissive TRP2_{350–378} sequence context (TRP2_{350–378} pp89_{ins}) resulted in complete PA28 dependence of its generation (Fig. 2A). In contrast, insertion of the pp89 sequence into the permissive TRP2_{283–309} (TRP2_{283–309}pp89_{ins}) rendered the generation of the precursor peptide completely PA28-independent (Fig. 2B).

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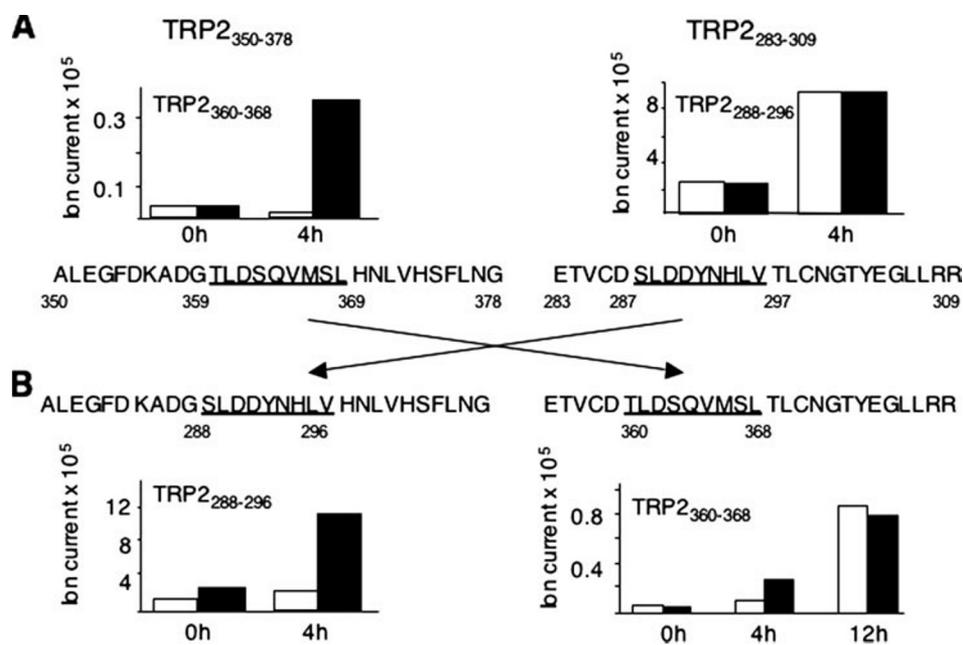


FIGURE 1. **Epitope generation from the nonpermissive TRP2₃₅₀₋₃₇₈ sequence is PA28-dependent.** *A*, peptides derived from two regions of the TRP2 protein were processed *in vitro* for 4 h with c20S proteasomes in the presence (black bars) or absence of PA28 (white bars). The excised epitope sequences (underlined) were analyzed by mass spectrometry. *B*, the antigenic epitope sequences TRP2₂₈₈₋₂₉₆ and TRP2₃₆₀₋₃₆₈ were exchanged between the two TRP2 sequences and processed *in vitro* by 20S proteasomes with or without PA28, respectively.

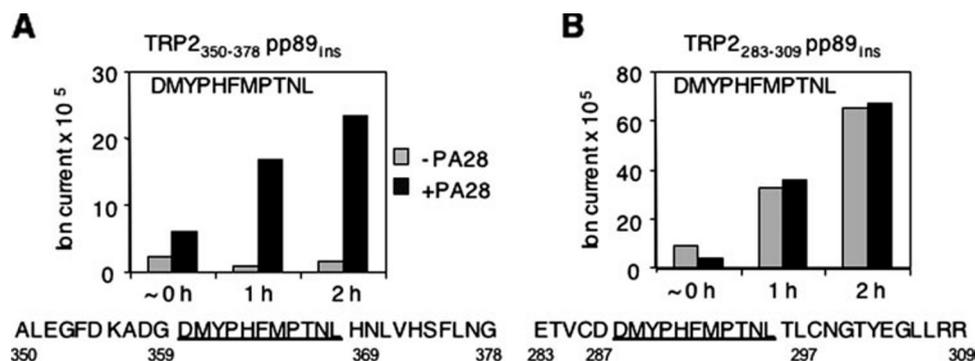


FIGURE 2. **The pp89^{Ld} epitope precursor sequence inserted into the nonpermissive TRP2₃₅₀₋₃₇₈ requires the presence of PA28 for generation.** *A*, the peptide sequence DMYPHFMPNTL was inserted in the nonpermissive TRP2₃₅₀₋₃₇₈ sequence and digested with 20S proteasomes plus (black bars) or minus PA28 (gray bars) for 2 h. *B*, the precursor sequence inserted in the permissive TRP2₂₈₃₋₃₀₉ context was processed and marked as in *A*. Detection of the generated pp89 epitope precursor, DMYPHFMPNTL, was performed by mass spectrometry.

From these experiments we concluded that the information for PA28 dependence can be transferred onto other antigenic peptides and that the flanking sequences of the TRP2₃₆₀₋₃₆₈ epitope determine their PA28 dependence. Furthermore, there exists no obvious functional interaction between the epitope sequence and the surrounding sequence context with regard to PA28 requirement.

To further study the influence of the flanking regions on PA28 dependence for proteasomal processing, the peptide TRP2₃₅₀₋₃₇₈pp89_{Ins} was modified by truncations of the N- and C-terminal flanking regions and analyzed as above (Fig. 3A). Partial truncation of the N-terminal flanking sequence (TRP2₃₅₀₋₃₇₈ΔN₅pp89_{Ins}) preserved PA28 dependence, although the efficiency of epitope liberation was slightly reduced (Fig. 3B). Similarly, partial truncation of the C-terminal flanking region (TRP2₃₅₀₋₃₇₈ΔC₅pp89_{Ins}), or truncation

of both terminal flanking sequences by 5 residues (TRP2₃₅₀₋₃₇₈ΔN₅/ΔC₅pp89_{Ins}), did not affect PA28 dependence. Nevertheless, the efficiency of epitope precursor generation from the latter peptide was diminished in the presence of PA28, most likely because of the shortening of the flanking sequences (9). Neither complete truncation of the C-terminal flanking residues from the TRP2/pp89 hybrid sequence (TRP2₃₅₀₋₃₇₈ΔC₁₀pp89_{Ins}) nor its truncation from the original human TRP2 polypeptide (TRP2₃₅₀₋₃₇₈ΔC₁₀wt) abolished the PA28-dependent liberation of the precursor peptides (Fig. 3C). In both cases PA28 was essential for antigenic peptide liberation. However, Complete truncation of the N-terminal flanking sequence, *i.e.* TRP2₃₅₀₋₃₇₈ΔN₁₀pp89_{Ins}, abolished PA28 dependence and led to a dramatically increased efficiency of the precursor peptide liberation. These data strongly suggest that the N-terminal flanking region of the human PA28-dependent TRP2₃₆₀₋₃₆₈ epitope is predominantly responsible for the PA28-dependent epitope liberation and that the C-terminal flanking sequences have only a minor influence.

In support of this conclusion, the combination of the N-terminal flanking region of PA28-independent TRP2₂₈₈₋₂₉₆ with the C-terminal flanking region of the PA28-dependent TRP2₃₆₀₋₃₆₈ epitope (TRP2_{N₂₈₃₋₂₈₇C₃₆₉₋₃₇₈pp89_{Ins}})

resulted in a strikingly improved liberation of the epitope precursor peptide and abrogated PA28 dependence (Fig. 4). The presence of PA28 affected epitope precursor liberation only slightly. These data therefore complement the experiments shown in Fig. 3C for TRP2₃₅₀₋₃₇₈ΔN₁₀pp89_{Ins} and demonstrate that the N-terminal flanking sequence plays a pivotal role in the determination of PA28 dependence of an epitope. Accordingly, the presence of the permissive N-terminal flanking sequence as in TRP2_{N₂₈₃₋₂₈₇ΔC₁₃pp89_{Ins}} revealed a complete PA28 independence of pp89 precursor peptide liberation (Fig. 4B).

To study the effects of PA28 on proteasomal cleavage site preference within the permissive and nonpermissive peptide substrates, the generated fragments from different processing experiments were analyzed in detail. Frequent cleavage sites or sites used to generate large amounts of specific fragments are marked by *arrows* (Fig. 5). The carboxyl site of the C-terminal

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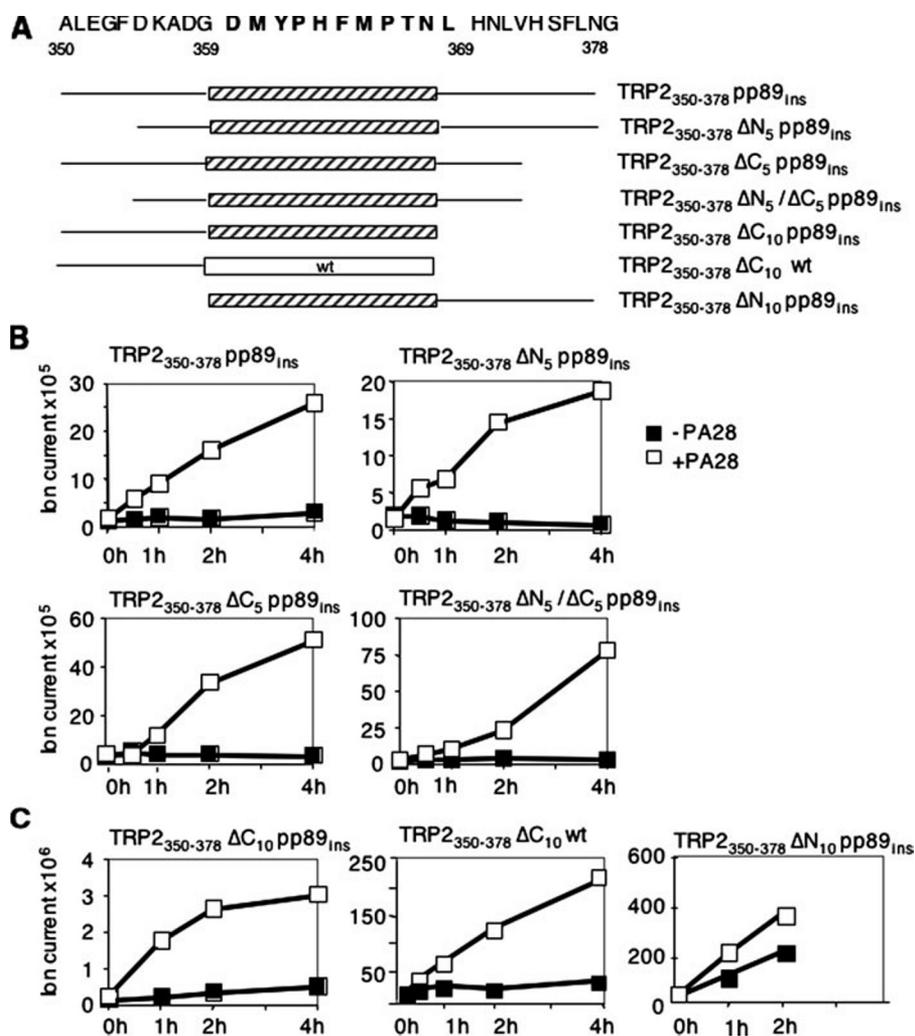


FIGURE 3. Flanking regions are involved in a PA28-dependent mode of peptide excision. *A*, based on the pp89 peptide inserted into the nonpermissive TRP2_{350–378} region (TRP2_{350–378}pp89_{ins}), peptides were synthesized that carry a partial or complete C-terminal deletion (5 amino acids deleted in TRP2_{350–378}ΔC₅pp89_{ins} or complete deletion in TRP2_{350–378}ΔC₁₀pp89_{ins}); were modified on the N terminus (5 amino acids deleted in TRP2_{350–378}ΔN₅pp89_{ins} and complete deletion in TRP2_{350–378}ΔN₁₀pp89_{ins}); or were partially deleted on both termini (TRP2_{350–378}ΔN₅/ΔC₅pp89_{ins}). Additionally, the nonpermissive N terminus was combined with wild type TRP2_{360–368} (TRP2_{350–378}ΔC₁₀wt). *B* and *C*, the different fusion peptides listed in *A* were processed with 20S for 4 h with or without PA28, and the released pp89 insert was analyzed. Liberated precursor peptides, which were generated in the presence of PA28 (open squares) or in the absence of PA28 (filled squares), are shown.

leucine residue of the pp89 insert represents the predominant proteasomal cleavage site, both in the presence and absence of PA28. Remarkably, the N-terminal site required for liberation of the pp89 11-mer precursor was used predominantly in the presence PA28 (Fig. 5, *A–D*). The 11-mer precursor was efficiently generated despite a strong PA28-induced cleavage within the epitope (Fig. 5, *A* and *C*). Thus, in a nonpermissive context (Fig. 5, *A–C*) PA28 appears to favor the additional and preferential usage of a cleavage site at the N terminus of that potential antigenic peptide whereas the already strong C-terminal cleavage was not significantly affected by PA28.

These observations raise the question of whether there are defined structures within the TRP2_{350–378} peptide domain that are responsible for its PA28 sensitivity. Thus both TRP2-derived peptides with the same insert, *i.e.* TRP2_{283–309}pp89_{ins} (Fig. 6*A*) and TRP2_{350–378}pp89_{ins} (Fig. 6*B*), were analyzed by ¹H

NMR spectroscopy. However, the spectra of the two peptides exhibited similar characteristics. The ¹H chemical shift values of most of the amino acids of both peptides correspond to the tabulated proton shifts of random coil proteins (16). Amino acid side chains are obviously exposed to the same solvent environment. The absence of medium- and long-range nuclear Overhauser effects (NOE) indicates that no helices or β -sheets are present. Furthermore, there was no hint for β -turns formed by proline. When energy-preferred states of the 29- and 31-mer peptide, respectively, were calculated and overlaid (shown for each three states), no defined structure elements were found, suggesting that structural features do not play an important determining role in the recognition, binding, and processing of peptide substrates by the proteasome-PA28 complex.

DISCUSSION

Previously we reported that the proteasomal liberation and consequent MHC class I presentation of the TRP2_{360–368} epitope from the tyrosinase-related protein 2 in human melanocytes essentially requires the function of PA28 (11). Here we demonstrate that the flanking sequences of the melanoma TRP2_{360–368} antigen can transfer PA28 dependence onto otherwise PA28-independent human and viral epitopes and, importantly, that the proximal N-terminal flanking residues of the TRP2_{360–368} epitope alone are sufficient to determine PA28 dependence of epitope generation. Fusion of the TRP2_{360–368} epitope with the N-terminal flanking sequence of a PA28-independent TRP2 epitope however rendered the TRP2_{360–368} epitope completely PA28 independent.

Considering what is known about the action of PA28 on the proteasome, this is a surprising and completely unexpected result. X-ray structure analysis had shown that trypanosomal PA26 opens the central gate within the yeast proteasome α -ring (4). Consequently it was proposed that such an open conformation would allow the release of slightly longer N-terminally extended peptides presumed to be more adapted to support antigen presentation. In addition, detailed biochemical analysis of the effect of PA28 on proteasomal cleavage properties in connection with epitope liberation suggested that PA28 induced coordinated, dual cleavages for enhanced epitope gen-

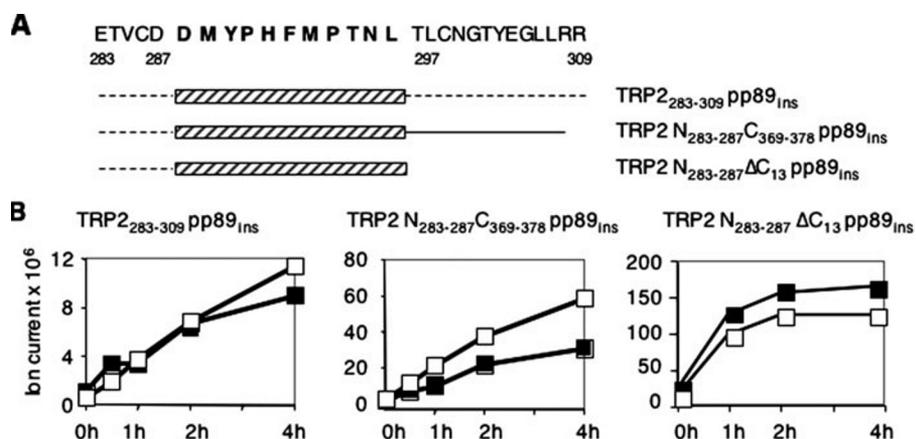


FIGURE 4. The permissive N-terminal flanking sequence is responsible for enhanced peptide liberation, but it abolishes the PA28 dependence of proteasomal cleavage. *A*, the pp89 precursor peptide was inserted in the permissive TRP2₂₈₃₋₃₀₉ region. Additionally, peptides were generated in which the C-terminal flanking sequence of TRP2₂₈₈₋₂₉₆ was exchanged against the C-terminal region of the nonpermissive C-terminal sequence (TRP2₂₈₃₋₂₈₇C₃₆₉₋₃₇₈ pp89_{ins}) or in which the C-terminal sequences were deleted (TRP2₂₈₃₋₂₈₇ΔC₁₀ pp89_{ins}). *B*, the peptides were processed as described in Fig. 3, *B* and *C*, in the presence (*open squares*) and absence of PA28 (*filled squares*).

		PA28
A	A L E G F D K A D G D M Y P H F M P T N L H N L V H S F L N G ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑	+ -
B	D K A D G D M Y P H F M P T N L H N L V H S F L N G ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑	+ -
C	A L E G F D K A D G D M Y P H F M P T N L ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑	+ -
D	E T V C D D M Y P H F M P T N L H N L V H S F L N G ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑	+ -
E	E T V C D D M Y P H F M P T N L ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑	+ -

FIGURE 5. PA28 induces the favored usage of the N-terminal cleavage site by proteasomes. The main cleavage sites that were used by proteasomes with and without PA28 present are indicated by *arrows* in the analyzed peptides: *A*, TRP2₃₅₀₋₃₇₈pp89_{ins}; *B*, TRP2₃₅₀₋₃₇₈ΔN₅pp89_{ins}; *C*, TRP2₃₅₀₋₃₇₈ΔC₁₀pp89_{ins}; *D*, TRP2₂₈₃₋₂₈₇C₃₆₉₋₃₇₈ pp89_{ins}; *E*, TRP2₂₈₃₋₂₈₇ΔC₁₃pp89_{ins}. The *big black arrows* show main cuts, and the *thin arrows* indicate weak cleavage site usage.

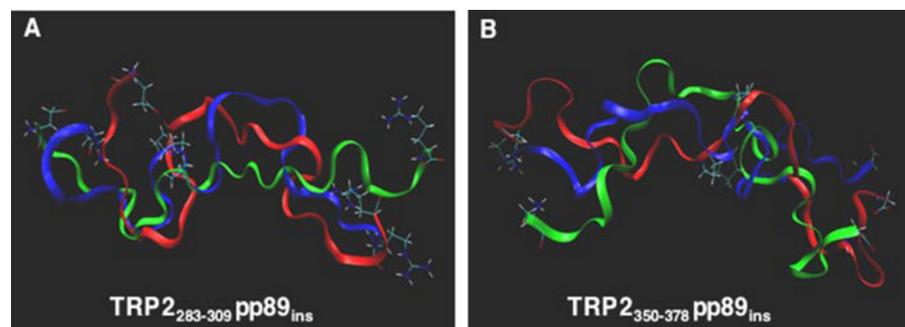


FIGURE 6. The permissive as well as the nonpermissive TRP2 pp89_{ins} peptides were analyzed by ¹H NMR spectroscopy. Three of the calculated energy-favorable structures that were selected as exemplary for the permissive (TRP2₂₈₃₋₃₀₉pp89_{ins}) (*A*) and the nonpermissive peptide (TRP2₃₅₀₋₃₇₈pp89_{ins}) (*B*) exhibited no defined structural elements.

eration (8). Interestingly, these and other studies also emphasize that PA28 action, while enhancing epitope liberation, does not induce the usage of entirely new cleavage sites by the proteasome. The two models, *i.e.* gating and dual cleavage, may in fact complement each other and are sufficient to explain the enhanced PA28-induced liberation of MHC class I epitopes or their precursor peptides, which are already generated in the absence of PA28 from a permissive sequence context, albeit in lower amounts. However, neither model alone or in combination appears to offer a solution for the PA28 effect observed in connection with the TRP2₃₆₀₋₃₆₈ epitope. TRP2₃₆₀₋₃₆₈ epitope liberation appears to be exceptional in comparison with all of the other PA28-induced epitopes described thus far in that it is embedded into a nonpermissive sequence context that prohibits its liberation by the proteasome in the absence of PA28.

Our epitope switching experiments now have shown for the first time that the flanking sequences of TRP2₃₆₀₋₃₆₈ can be used to transfer PA28 dependence onto another TRP2 epitope as well as to the viral murine cytomegalovirus-derived pp89 epitope. These data are interesting for three reasons. First, they exclude the previously likely possibility that PA28 dependence is due to a cross-talk between a specific epitope sequence and the surrounding sequence environment. Secondly, they demonstrate that PA28 dependence is determined by the flanking sequence, and thirdly they show that this nonpermissive sequence context may be used more generally for experiments in which epitope generation occurs only under PA28-controlled conditions.

A considerable number of examples show that the sequence environment of the epitope can influence its relative generation efficiency by proteasomes (17, 18). Also, it is well established that predominantly the C-terminal flanking residues or the flanking

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sequences of an epitope determine the efficiency of proteasomal antigen generation. Hence, for viral HCV NS3 and murine leukemia virus (MuLV)-derived epitopes as well as for a human p53 tumor epitope it has been demonstrated that the C-terminal flanking residues determine the epitope liberation efficiency by the proteasome (19, 20). On the other hand, it is generally accepted that proteasomal cleavage at the N terminus of an epitope is less well defined and of limited importance. In this context, our experiments in which the C-terminal flanking sequences were deleted as in TRP₂₃₅₀₋₃₇₈ΔC₁₀pp89_{ins} and TRP₂₃₅₀₋₃₇₈ΔC₁₀ wild type are striking in that they demonstrate that PA28 dependence is solely provided by the proximal N-terminal residues of the TRP₂₃₆₀₋₃₆₈ epitope. In support, fusion of the permissive N-terminal residues from the TRP₂₂₈₈₋₂₉₆ epitope, as in TRP₂₂₈₃₋₂₈₇C₂₃₆₉₋₃₇₈ pp89_{ins}, resolves PA28 dependence and results in an extremely efficient generation of the epitope precursor peptide.

How then can the functional predominance of the nonpermissive or the permissive N-terminal flanking sequences be explained? A possibility to consider is that the flanking N-terminal regions provide specific structural features that either prohibit or allow proteasomal cleavage. However, NMR studies of the different polypeptides provided no evidence for structural specificities either in the C-terminal or the N-terminal flanking regions of the inserted epitope. In contrast, cleavage product analysis revealed that proteasomal cleavage near the correct N terminus of the epitope or epitope precursor is strongly impaired in the presence of a nonpermissive N terminus, whereas the opposite is true for the permissive N terminus. In consequence, the function of PA28 may be 2-fold. First of all, our data demonstrate that PA28 supports the efficient usage of a cleavage site in the nonpermissive N-terminal region of a substrate, and thus they extend the previous dual coordinated cleavage hypothesis formulated for PA28 action epitope generation in a permissive sequence environment. Furthermore, our experiments essentially demand that binding of PA28 to the 20S proteasomes not only opens the gate but also induces subtle structural changes onto the 20S proteasome. These could affect the channeling of the substrate within the catalytic cavity and allow docking of the N-terminal residues of the TRP₂₃₆₀₋₃₆₈ epitope close to an active site within the inner β-rings, thus

permitting the use of new cleavage sites within the otherwise nonpermissive sequence context of the proteasome.

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