Expression of the Proteasome Activator PA28 Rescues the Presentation of a Cytotoxic T Lymphocyte Epitope on Melanoma Cells

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ABSTRACT

The proteasome system represents a major source of HLA class I-presented peptides exposed to CTLs. Stimulation of cells with IFN-γ instantly induces the expression of the proteasome immunosubunits as well as the proteasome activator PA28. These proteins have been shown to optimize class I antigen presentation of several viral CTL epitopes; however, their contribution to tumor antigen processing remains poorly understood. Here, we analyzed the generation of an HLA-A*0201-presented epitope derived from the melanoma antigen tyrosinase-related protein 2 (TRP2). Melanoma cells that lacked the IFN-γ-inducible proteasome activator PA28 and immunoproteasomes did not display the TRP2_280–368 epitope to specific CTLs. Our experiments demonstrate that epitope presentation correlated with the presence of PA28 and could be completely rescued by restoration of PA28 expression. In vitro digestion of TRP2 polypeptides with 20S proteasomes confirmed that PA28 is essential for epitope liberation. Thus, our experiments indicate that PA28 provides the threshold for CTL recognition of this epitope. Importantly, processing of a second TRP2-derived epitope, TRP2_290–368, was diminished in IFN-γ-treated cells, even in the absence of immunoproteasome up-regulation. Therefore, the reported IFN-γ-induced self-regulation of epitopes may not necessarily be a consequence of immunoproteasomes as suggested previously.

INTRODUCTION

Cell surface presentation of antigenic peptides by MHC class I molecules is a prerequisite for efficient CTL response induction, effector function, as well as for CTL repertoire selection. To allow binding to MHC molecules, intracellular proteins must be degraded into smaller fragments that are translocated by a specialized peptide transporter, termed TAP, into the endoplasmic reticulum. Finally, 8–11 amino acid long peptides containing an appropriate binding motif can assemble with MHC class I β2-microglobulin dimers for transportation to the cell surface (reviewed in Ref. 1). Investigations into the source of antigenic peptides have revealed that proteasomes, the major proteolytic machinery in the cytosol, are responsible for the generation of most of the MHC class I ligands (2–4). Although proteasomes have been demonstrated to perform the correct COOH-terminal cleavages, many MHC class I-bound peptides are generated as NH2-terminally extended precursors that are trimmed to the right size by amino- and endopeptidases (5, 6). Proteasomal catalytic activity is exerted by the 20S core proteasome, a cylindrical structure composed of four stacked rings of seven α or β subunits each. Enzymatic activity is mediated by three of the seven β subunits, i.e., Y/δ (β1), Z (β2), and X/MB1 (β5). 20S core particles are usually found associated with a 19S regulatory complex (PA700), which binds to the α-rings to form the 26S proteasome and is responsible for substrate capture and the transportation of substrates into the 20S proteolytic channel (1).

Exposure of cells to IFN-γ, a major immunostimulatory cytokine produced during immune responses by activated T lymphocytes and natural killer cells, enhances antigen presentation by up-regulation of the MHC and TAP gene products as well as by the induction of three facultative catalytic proteasome β-subunits. These induced proteasome subunits, i.e., LMP2 or β1i (induced), MECL-1 or β2i and LMP7 or β5i, are constitutively expressed in professional antigen-presenting cells. They replace the constitutive subunits Y/δ or β1, Z or β2, and X/MB1 or β5 in newly assembled 20S proteasomes or so-called immunoproteasomes (1). Incorporation of these subunits alters the proteasomal catalytic properties (7–10), resulting in enhanced liberation of many virus-derived antigenic peptides (11–20). On the other hand, immunoproteasomes were shown recently to impair the generation of several antigenic peptides derived from tumor-associated antigens (21).

IFN- and dendritic cell maturation further increases the expression levels of the proteasome activator PA28 or 11S REG (reviewed in Refs. 1, 22, and 23). PA28 is a heptameric complex composed of α and β subunits that binds to the 20S α-rings, leading to the formation of hybrid proteasome with the 19S complex on the one and PA28 on the other side (24). Analysis of transfectant cell lines and PA28β gene-deficient mice demonstrated that PA28 enhanced antigen presentation of several viral CTL epitopes (13, 25–27). In vitro, PA28 was shown to accelerate proteasome-mediated liberation of MHC class I ligands from polypeptides, however, without changing the proteasomal cleavage specificity (28). Nonetheless, it is of importance to note that in all cases studied thus far PA28 only improved the presentation of epitopes. Thus, all of the effects of PA28 described thus far were of strictly quantitative character, and in no case was PA28 expression shown to result in the presentation of new CTL epitopes.

The impact of PA28 on tumor epitope processing has not been analyzed thus far. Recently, it was found that immunoproteasomes could negatively affect tumor epitope processing. This observation has important consequences for our understanding of tumor-specific CTL responses. Because thymic dendritic cells, which are responsible for the negative selection of autoreactive T cells, probably possess immunoproteasomes and little constitutive proteasomes (23), this would imply that tumor antigen-specific CTLs specific for antigenic peptides produced by immunoproteasomes and displayed on the dendritic cell surface may be negatively selected. CTLs that recognize peptides produced by constitutive proteasomes may survive and provide immunosurveillance against tumor growth. Nevertheless, a more

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4 The abbreviations used are: TAP, transporter associated with antigen process; PA28, proteasome activator 28; TRP2, tyrosinase-related protein 2; HPLC, high-performance liquid chromatography; LMP, low molecular weight protein; MEC, murine embryonal cell; TET, tetracycline; LDH, lactate dehydrogenase; rHFN, recombinant human IFN; ICAM, intercellular adhesion molecule; TNF, tumor necrosis factor; MECL-1, multicalytic endopeptidase-like 1.
extended analysis of tumor antigen processing will be essential to further validate this hypothesis.

In the present study, we have analyzed the presentation of two HLA-A*0201-restricted antigenic peptides derived from the melanoma differentiation antigen TRP2 (29, 30). Investigating the presentation of these peptides by different melanoma cells, we found that one of the epitopes was presented by each of these cell lines, whereas the second TRP2-derived epitope was recognized only on one of the melanoma cell lines analyzed. The presentation of this last peptide, TRP2\textsubscript{2560–2669}, could be induced by IFN-γ stimulation of the melanoma cells, and the processing of this epitope appeared to require the expression of PA28. Conversely, IFN-γ treatment diminished presentation of the first TRP2 epitope, TRP2\textsubscript{288–296}, surprisingly, also in the absence of induction of immunoproteasome or PA28 expression. Thus, we find that up-regulation of proteasome-related components of the antigen processing machinery enhances the processing of one tumor epitope but seems to play no role in the self-regulation of presentation of another epitope derived from the same tumor antigen.

MATERIALS AND METHODS

Cell Lines and Transfectants. The human melanoma cell lines UKRV-Mel-6a, UKRV-Mel-15a, UKRV-Mel-18a, UKRV-Mel-20b, UKRV-Mel-21a (referred to hereafter as Mel-6a, Mel-15a, Mel-18a, Mel-20b and Mel-21a, respectively, and all HLA-A2** TRP2**, except Mel-20b: HLA-A2** TRP2**), TAP-defective T2 cell line (HLA-A2**), WEHI-164 clone 13 cells, natural killer-sensitive cells K562, and the monkey kidney COS-7 cells were cultured in RPMI 1640 as described previously (30). The stable transfectant MEC clone 29 (MEC29, containing the TET-regulated transcription activator tTak), MEC-PA28 (MEC29 + PA28a and PA28β), and MEC217 cells (MEC29 + LMP2, MECL-1 and LMP7) have been characterized recently (11, 13), with inducible expression of the introduced subunits in the absence of TET. All transfectants were maintained in Isaac’s modified Dulbecco’s medium (BioWhittaker Europe, Berlin, Germany), supplemented with 10% FCS, 2 mM l-glutamine, 20 μM 2-mercaptoethanol (Merck, Darmstadt, Germany), 5 μg/ml polymyxin, 200 μg/ml hygromycin B (Merck), and 500 ng/ml TET (Merck), otherwise indicated.

Peptides and Proteasome Inhibitors. TRP2\textsubscript{2560–2669} nonapeptide TLDSQVMSL, the nonamer TRP2\textsubscript{288–296} TLDSQVMSLHNLVHSFLNG were synthesized in an Applied Biosystems peptide synthesizer (model 432A) using FMOC technology. Peptide purity and structure were confirmed by reverse phase HPLC and mass spectroscopy. Proteasome-specific inhibitor lactacystin and calpain inhibitor I (Reagents and Research.

Flow Cytometry. Immunoﬂuorescence staining was performed according to standard procedures (13). Antibodies used were: W6.32, speciﬁc for HLA class I molecules, BB7.2 (kindly provided by P. Coulie, Ludwig Institute for Cancer Research, Brussels, Belgium), recognizing HLA-A2, L243, speciﬁc for HLA-DR, 23G12, staining ICAM-1 (both purchased from Immunotech) and FITC-conjugated goat-antimouse IgG F(ab), fragments as second antibody. Fluorescence was measured with a FACScalibur flow cytometer using the CELLQuest application program (Becton and Dickinson).

Metabolic Labeling and Immunoprecipitation. Melanoma cells (2 × 10\textsuperscript{5}) were seeded in flasks and cultured in RPMI 1640 with or without 200 units/ml rhIFN-γ for 2 days. Cells were labeled overnight with tritiated [\textsuperscript{3}H]methionine (100 μCi/ml), washed twice, chased for 4 h in medium without IFN-γ, and then lysed in lysis buffer [1% NP-40, 20 mM Tris (pH 7.5), 10 mM EDTA, and 1% (w/v) NaCl] with 1 mM dithiothreitol, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin as described (11). After centrifugation (5 min, 14,000 rpm at 4°C), the detergent lysates were precleared with 25 μl of protein A-Sepharose (50% slurry) and 25 μl of rabbit preimmune serum overnight at 4°C. 20S proteasomes were immunoprecipitated with 25 μl of protein A-Sepharose and 25 μl of polyclonal 20S proteasome-specific rabbit antisera for 1 h at 4°C. Sepharose beads were washed with NET buffer [50 mM Tris (pH 8), 150 mM NaCl, and 5 mM EDTA] with 0.5% NP40, resuspended in NEPHGE sample buffer and subjected to NEPHGE two-dimensional gel electrophoresis. Gels were exposed for autoradiography.

Western Blot Analysis. Melanoma cells were cultured in the presence or absence of 200 units/ml rhIFN-γ for 4 days and then harvested by trypsinization, washed, and lysed in 50 μl of lysis buffer [0.1% Triton X-100, 20 mM Tris (pH 7.5), 10 mM EDTA, and 100 mM NaCl] containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Cell lysates were centrifuged by centrifugation (5 min, 14,000 rpm at 4°C), and protein contents were determined at an absorbance of 280 nm. Samples containing 100 μg of protein were separated on 12% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Equal loading of individual lanes was confirmed by staining with 1% Ponceau Red. Blots were incubated for 1 h in 10% horse serum/5% (w/v) lowfat dry milk,0.4% Tween 20 in PBS. Expression of PA28a and PA28β-specific polyclonal rabbit antisera diluted in 2% dry milk/0.1% Tween 20 in PBS. Filters were developed with horseradish peroxidase-conjugated antirabbit IgG and enhanced by chemiluminescence (Roche).

PA28 Purification. Frozen cell pellets of 10\textsuperscript{9} induced MEC-PA28 cells were Dounce homogenized in TEAD buffer (20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM NaF, and 1 mM DTE) with 250 mM sucrose, incubated for 30 min on ice, and then centrifuged with a Sorvall SS-34 rotor (30 min, 16,000 rpm at 4°C). Supernatants were incubated with DEAE-Sephaloc for 1 h at 4°C; unbound material was removed by washing with TEAD buffer with 10% glycerol, and bound proteins were then eluted with TEAD/glycerol buffer containing 0.5 M NaCl. Protein-containing fractions, identified by Ponceau S staining, were concentrated by Amicon ultrafiltration using a YM100 membrane and centrifuged on a 15–45% glycerol gradient for 16 h at 40,000 rpm (4°C) in a Beckmann ultracentrifuge (SW Ti rotor). Gradient fractions that contained 20S activity toward Suc–LLVY–AMC (13) were collected, and PA28 was further purified by two successive fast protein liquid chromatography steps using a MonoQ HR5/5 and a phenyl Sepharose column as described (33).

Polypeptide Digestions. One μg of purified proteasomes was incubated with 30 nmol of TRP2 23-mer synthetic peptide for 24 h at 37°C. After terminating the reaction, fragments were separated by high performance liquid chromatography (SMART system; Pharmacia, Freiburg, Germany) equipped with a μRPC C2/C18 SC 2.1/10 column (Pharmacia). Each peptide fraction was analyzed by MALDI mass spectrometry (G2025A; Hewlett Packard, Waldbronn, Germany) and by Edman microsequencing (protein sequencer “procise” 494A; Applied Biosystems, Weiterstadt, Germany) to obtain quantitative results. To analyze the effects of PA28, 26.6 μg of TRP2 29-mer were incubated with 4 μg of 20S proteasomes purified from T2 and T2 LMP2 + LMP7 transfectant cells (12) in a total volume of 400 μl of assay buffer [20 mM HEPES/KOH (pH 7.8), and 1 mM DTT] in the presence or absence of a 4 molar excess of PA28. Samples were removed after 10 and 30 min of digestion, and generated peptide products were analyzed by reverse phase chromatography (HP1100) on a μRPC C2/C18 SC 2.1/10 column [eluent A, 0.05% trifluoroacetic acid in H2O; eluent B, 0.05% trifluoroacetic acid in acetonitrile].
acid in H$_2$O-acetonitrile (3:7 v/v); in 15 min from 5 to 95% eluent B; flow rate, 0.2 ml/min]. Analyses were performed online with an ion trap mass spectrometer (Lcq, ThermoQuest) equipped with an electrospray ion source. Peptides were identified by their molecular masses calculated from the m/z peaks of the single or multiple charged ions and confirmed by tandem mass spectrometry analyses.

RESULTS

Deficient Presentation of a TRP2-derived Epitope, TRP2$_{286–368}$ in a Subset of HLA-A*-02$^+$ TRP$^+$ Melanomas. To identify novel melanoma-specific T-cell epitopes, we previously tested a panel of nonapeptides derived from the melanoma differentiation antigen TRP2 for their capacity to stimulate peripheral blood lymphocytes of melanoma patients and healthy donors in vitro (30). The peptide TRP2$_{288–296}$ was found to induce melanoma-reactive CTLs from peripheral blood lymphocytes of one patient (S.E., referred to as TRP2$_{288–296}$ CTL hereafter) and one donor BFs731931 (30). Meanwhile, another nonapeptide, TRP2$_{296–368}$, was recognized by CTLs from two patients (K.R. and L.C.) and one donor BFs746170 (data not shown). Interestingly, when these TRP2$_{286–368}$ CTLs were further analyzed for reactivity toward melanoma cells, we consistently observed that they reacted to a limited number of TRP2-expressing melanoma cell lines only (Fig. 1, A and B). Whereas TRP2$_{366–368}$ CTLs efficiently killed the Mel-6a and, to some extent, the Mel-21a tumor cell lines, they did not recognize Mel-18a and Mel-15a in a cytotoxicity assay and in an independent cytokine secretion assay. The same was true for COS-7 cells cotransfected with the HLA-A*-0201 and TRP2 genes (Fig. 1C). The addition of synthetic TRP2$_{286–368}$ to these tumor cells resulted in specific CTL activation (Fig. 1B), suggesting the proper expression of HLA molecules. This was further confirmed by analysis of surface expression of HLA-A*-0201 by flow cytometry, which revealed no significant difference between the four melanoma cell lines (data not shown).

To exclude that the phenomenon above was caused by a general antigen-processing deficiency, we tested the melanoma cells for recognition by our recently characterized TRP2$_{288–296}$ CTL (Ref. 30; Fig. 1, D–F). All four A*-02$^+$/TRP$^+$ cell lines as well as the COS-7 cotransfectants were well recognized by TRP2$_{288–296}$ CTL. On the basis of this finding, we conclude that the Mel-15a and Mel-18a tumor cell lines and the COS-7 transfectants are unable to process the TRP2$_{286–368}$ epitope intracellularly.

Production of TRP2$_{366–368}$ Peptide Is Mediated by Proteasomes and Can Be Restored by IFN-γ. The proteasome system has been implicated in the generation of most antigenic peptides presented by HLA class I molecules to CTLs (1–4). To investigate the necessity of proteasomes for TRP2$_{366–368}$ peptide generation, we used the proteasome inhibitors lactacystin and ALLN. Whereas untreated cells elicited a pronounced CTL response (Fig. 2, A and B), preincubation of Mel-6a and Mel-21a target cells with lactacystin and ALLN significantly reduced TNF-β (not shown) and IFN-γ secretion by TRP2$_{560–368}$ CTLs (Fig. 2B). Loading of inhibitor-treated cells with synthetic peptide restored CTL recognition (data not shown), indicating that they were still able to present antigenic peptides. Similar results were obtained for the TRP2$_{288–296}$, CTL epitope (Fig. 2, C and D; and data not shown). We therefore conclude that the generation of both TRP2$_{366–368}$ and TRP2$_{288–296}$ peptides requires proteasome activity. These results also raised the possibility that alteration of proteasomal activity may restore or enhance the TRP2$_{366–368}$ epitope presentation.

Because several IFN-γ-inducible proteins, including three immunosubunits (LMP2, LMP7, and MECL-1) and two PA28 subunits (α/β) modify proteasomal proteolytic activity and have been shown to enhance liberation of several viral CTL epitopes (11–20, 25–27), we tested the effect of IFN-γ on the generation of the TRP2$_{366–368}$ epitope (Fig. 2, A and B). The four melanoma cell lines were cultured in the presence or absence of rhIFN-γ for 8 days and were then used as targets in a 6-h cytotoxicity assay and in a 24-h IFN-γ release assay. As shown in Fig. 2, A and B, IFN-γ treatment enhanced recognition by Mel-6a and Mel-21a by TRP2$_{366–368}$ CTLs. Remarkably, lysis of Mel-15a cells, which were not recognized if untreated, was restored upon IFN-γ treatment. This effect could be blocked by HLA class I-specific antibodies, indicating that peptides were presented by HLA class I molecules (Fig. 2B).

On the other hand, neither untreated nor IFN-γ-treated Mel-18a cells were recognized by TRP2$_{366–368}$ CTLs (Fig. 2, A and B). This is despite the fact that treatment with rhIFN-γ induced a marked increase in cell surface expression of HLA class I molecules in general, of HLA-A*-0201 and HLA-DR in specific, as well as of the costimulatory molecule ICAM-1 on Mel-18a cells (Fig. 3). Moreover, IFN-γ treatment also induced TAP expression in Mel-18a cells (data not shown). These findings suggest that the resistance of Mel-18a cells to TRP2$_{366–368}$ CTL-mediated lysis is not caused by inefficient peptide transportation/presentation or costimulation but may result from deficient processing of these CTL epitopes in cells.

As a control, we examined the presentation of TRP2$_{288–296}$ by IFN-γ-treated and untreated melanoma cells (Fig. 2, C and D). Sur-
Fig. 2. Cytotoxic activities and cytokine responses of TRP2<sub>288–296</sub> CTLs and TRP2<sub>360–368</sub> CTLs toward different melanoma cell lines pretreated with or without IFN-γ. A and C, melanoma cells were cultured in the presence or absence of 200 units/ml rhIFN-γ for 8 days. After an extensive washing step, 5000 viable targets were tested for lysis by TRP2<sub>360–368</sub> CTLs (A) and TRP2<sub>288–296</sub> CTLs (C), respectively, in a 6-h LDH-release assay. Results shown represent mean values of triplicates. B and D, in cytokine secretion experiments, washed melanoma cells (3 × 10<sup>5</sup>/well) were incubated in 50 μl of medium with W6/32 (1:20) or the proteasome inhibitor lactacystin (200 μM) for 1-h at 37°C. 2 × 10<sup>5</sup>/well specific TRP2<sub>288–296</sub> CTLs (B) and TRP2<sub>360–368</sub> CTLs (D) were then added to microwells to give a final volume of 300 μl. IFN-γ content in the supernatants was measured 24-h later using an IFN-γ ELISA kit. Note that in any case, W6/32 monoclonal antibody and lactacystin significantly inhibited IFN-γ release. The lactacystin-treated target cells were stimulated comparable levels of IFN-γ after being loaded with respective exogenous peptides (data not shown).

Surprisingly, although all untreated melanoma cell lines were recognized, IFN-γ treatment dramatically reduced CTL recognition of Mel-6a and Mel-15a and completely abolished recognition of Mel-18a and Mel-21a (Fig. 2, C and D). Thus, IFN-γ treatment up-regulates HLA-A2 presentation of TRP2<sub>360–368</sub> whereas presentation of TRP2<sub>288–296</sub> derived from the same tumor antigen is self-regulated.

Fig. 3. IFN-γ-induced up-regulation of cell surface molecules on Mel-18a cells. Flow cytometric analysis of cell surface expression of MHC antigens and the costimulatory molecule ICAM-1 on IFN-γ-treated (black lines) and untreated (thin black lines) Mel-18a cells is shown. Gray lines, isotype controls.

Incorporation of Immunosubunits Does Not Support TRP2<sub>360–368</sub> Epitope Generation. Proteasome immunosubunits have been shown to enhance MHC class I antigen processing of multiple viral CTL epitopes. To investigate whether immunosubunits are involved in the generation of TRP2<sub>360–368</sub>, we examined the proteasome subunit composition of our melanoma cell panel. Cells were grown in the presence or absence of rhIFN-γ, metabolically labeled with [<sup>35</sup>S]methionine, and 20S proteasomes were immunoprecipitated. Two-dimensional gel analyses (Fig. 4A) showed that unstimulated cells incorporated the constitutively expressed β1/βi subunit in their 20S complexes and, as expected, lacked the inducible subunit β1i/LMP2. Interestingly, both β5 and β5i/LMP7 were found in 20S proteasomes of unstimulated Mel-6a, Mel-15a, Mel-21a, and Mel-18a cells, indicating that these cell lines express this immunosubunit constitutively. However, the expression levels of β5i/LMP7 were rather low and resulted in only partial substitution of constitutive β5/MB1. Exchange of β2i/MECL-1 for β2 could not be evaluated under the electrophoresis conditions used because of the similar pH and molecular weights of these two subunits. Nevertheless, because β2i and β1i incorporate interdependently (34, 35), β2i, similar to β1i, can be considered absent from 20S complexes of unstimulated melanoma cell lines.

IFN-γ stimulation caused an ~50% replacement of constitutive β1 for β1i and a nearly complete replacement of β5 for β5i in Mel-6a, Mel-21a, and Mel-15a (Fig. 4A). Remarkably, neither β1i nor β2i were detected in 20S immunoprecipitates of Mel-18a, suggesting that IFN-γ treatment failed to induce the expression of these subunits in this melanoma cell line. Thus, although Mel-6a, Mel-21a, and Mel-15a cells formed immunoproteasomes after IFN-γ stimulation, the proteasome population in IFN-γ-treated Mel-18a cells remained unchanged.

Taken together, because unstimulated Mel-6a and Mel-15a as well as IFN-γ-stimulated Mel-6a, Mel-21a, and Mel-15a cells present TRP2<sub>360–368</sub>, we infer from these analyses that proteasome subunit composition cannot explain the differential processing of this epitope in our melanoma cell panel.

To confirm this conclusion, we used the recently described MEC217 mouse embryonal cell line (11). This cell line expresses the
immunosubunits under the control of a TET-regulated promoter and has recently been proven a useful tool to trace the contribution of these molecules to MHC class I antigen processing. To examine their capacity to process and present the TRP2\(^{360-368}\) epitope, MEC217 cells were cultured in the absence of TET to induce transcription or in the presence of TET to suppress transcription for 2–3 days and then transiently transfected with cDNAs encoding HLA-A2 and TRP2. After 24 h, the cells were used as targets for TRP2\(^{360-368}\) CTLs in a TNF-\(\gamma\)/H9252 release assay. Results presented in Fig. 5A demonstrate that neither the control MEC29 cells expressing the TET-regulated transcription activator only nor uninduced and induced MEC217 cells were able to stimulate TNF/LT\(\gamma\)/H9251 secretion by TRP2\(^{360-368}\) CTLs as measured by the WEHI-164 cytotoxicity assay.

To more directly examine whether and how the incorporation of immunosubunits influences TRP2\(^{360-368}\) production, we studied proteasomal cleavage site usage in a TRP2 23-mer polypeptide harboring this CTL epitope \textit{in vitro}. The TRP2 23-mer was incubated with purified constitutive or immuno-20S proteasomes; the resulting digests were separated by reverse phase-HPLC and identified and quantitated by on-line electrospray mass spectrometry. Our analyses showed that proteasomes containing constitutive subunits cut the polypeptide mainly at the Leu-368/His-369 peptide bond, thereby generating the correct epitope COOH terminus (Fig. 6A). Surprisingly, the incorporation of the immunosubunits did not significantly change proteasomal cleavage site usage. Although there was a minor cleavage difference at the polypeptide NH\(_3\) terminus, both types of proteasomes preferentially cleaved the Met-366/Ser-367 peptide bond within the CTL epitope and the Leu-368/His-369 epitope flanking peptide bond. Most importantly, although both constitutive and immunoproteasomes liberate the epitope COOH terminus, analysis of the peptide fragments produced did not reveal the generation of the TRP2\(^{360-368}\) epitope or of potential epitope precursor peptides.

**PA28 Deficiency Correlates with Impaired TRP2\(^{360-368}\) CTL Epitope Generation.** To investigate whether the observed recognition of melanoma cells by TRP2\(^{360-368}\)-specific CTLs correlated with expression of PA28, the other IFN-\(\gamma\)-inducible proteasome component, we examined PA28\(\alpha\) and PA28\(\beta\) expression in the four IFN-\(\gamma\)-treated and untreated melanoma cell lines by immunoblot analysis...
Fig. 6. PA28 induces the production of a TRP2 epitope precursor by 20S proteasomes in vitro. A, TRP2356–378 polypeptides were digested with purified 20S constitutive and immunoproteasomes for 24 h as described in “Materials and Methods.” Digestion products were identified by mass spectrometry and quantitated by Edman sequencing. Frequencies of cleavage site usage were calculated as described (44). Thick arrows, dominant cleavage sites; thin arrows, minor cleavage sites. const., constitutive proteasomes; imm., immunoproteasomes. The most prevalent cleavage products are indicated below the polypeptide sequence: c, fragments generated by constitutive proteasomes; i, fragments generated by immunoproteasomes. Note that the correct COOH terminus of TRP2360–368 was created by both proteasome species. TRP2360–368 or NH$_2$-terminally extended epitope precursor peptides were not detected among the cleavage fragments. B, synthetic TRP2360–368 was digested with purified 20S constitutive or immunoproteasomes in the presence or absence of purified PA28 or was incubated with PA28 only. Cleavage products were analyzed by reverse phase-HPLC/mass spectrometry.

PA28 RESCUES THE PRESENTATION OF A TUMOR EPITOPE

PA28 Expression Rescues TRP2360–368 Processing in Malignant Cells. To directly prove the essential role of PA28 in the processing of the TRP2360–368 tumor epitope, we tested whether transfection of the PA28 subunits can restore TRP2360–368 presentation by Mel-15a and Mel-18a cells. Mel-21a was included as a positive control. Cells were transiently transfected with murine cDNA(s) encoding PA28$\alpha$ or PA28$\beta$, or both. Twenty-four h later, the cells were tested for recognition by specific CTLs (Fig. 5B). Whereas transfection with either PA28$\alpha$- or PA28$\beta$-encoding cDNAs failed to restore CTL recognition, the introduction of both PA28$\alpha$ and PA28$\beta$ rendered Mel-15a and Mel-18a sensitive to recognition by TRP2360–368 CTLs. Transfection of PA28$\beta$ but not of PA28$\alpha$ into Mel 21a seemed to enhance basal TNF/LT$\alpha$-$\beta$ release measured by the WEHI-164 cytotoxicity assay; however, introduction of both PA28$\alpha$ and PA28$\beta$ resulted in a more pronounced (2) cytokine secretion. Thus, reconstitution of PA28$\alpha$/$\beta$ complexes within tumor cells restored TRP2360–368 CTL epitope processing and presentation.

To exclude the possibility that the observed rescue of TRP2360–368 CTL epitope processing and presentation by PA28 transfection is attributable to unknown, tumor-specific effects and to confirm the essential role of PA28 in TRP2360–368 production, we used the MEC-PA28 cell line, which expresses the PA28$\alpha$ and PA28$\beta$ subunits in a TET-regulated fashion (13). To examine TRP2360–368 processing, MEC-PA28 cells were cultured in the absence or presence of TET to induce or silence transcription, transiently transfected with cDNAs encoding HLA-A2 and TRP2 and then used as targets for a TNF-$\beta$ release-WHEL-164 cytotoxicity assay with TRP2360–368-specific CTLs. Although uninduced MEC-PA28 cells were not able to stimulate cytokine secretion by TRP2360–368 CTLs, coculture of CTLs with induced MEC-PA28 cells resulted in the secretion of a substantial amount of TNF/LT $\alpha$ (Fig. 5A). Taken together, these data demonstrate that PA28 activity is essential for the processing and presentation of the TRP2360–368 epitope.

PA28 Induces the Generation of a TRP2360–368 Precursor Peptide in Vitro. For a considerable number of MHC class I-presented peptides, it has been shown that their generation in vitro, from polypeptide substrates by 20S proteasomes, closely reflects their generation in intact cells both with regard to quantity and quality (11, 14, 36). Hence, we decided to examine the effect of PA28 on TRP2360–368 generation in vitro. A 29-mer TRP2 polypeptide was digested with constitutive and immuno 20S proteasomes in the absence and presence of purified PA28. Because reuptake of processing intermediates into the 20S proteasome during prolonged reactions may result in “overdigestion,” which would interfere with data interpretation, and because PA28 has been shown to effect an immediate liberation of CTL epitopes (28), very short incubation periods were used. Analysis of the cleavage products after 10 and 30 min of digestion showed that in the absence of PA28, neither immuno nor constitutive proteasomes produced any detectable amounts of peptides exhibiting the correct epitope COOH terminus and harboring the complete TRP2360–368 sequence (Fig. 6B and data not shown). In contrast, the addition of PA28 to the digestion reactions resulted in an almost immediate generation of a peptide with the correct epitope COOH terminus but extended at the NH$_2$ terminus. TRP2356–368 (Fig. 6B), a likely epitope precursor. PA28 supported TRP2356–368 generation by both constitutive and immunoproteasomes. The ion currents measured for this peptide in digestion reactions without PA28 corresponded to those obtained in reactions without 20S proteasomes (Fig. 6B and data not shown). Because other potential, NH$_2$-terminally extended, epitope precursors nor the exact CTL epitope were detected in these experiments, we infer that TRP2360–368 is generated as TRP2356–368 and then cleaved to exact size by cytosolic or estrogen receptor-located peptidases. Thus, in agreement with our data obtained with intact cells (Fig. 5), the in vitro data demonstrate a complete dependence of TRP2360–368 epitope generation on the presence of PA28.

DISCUSSION

Here we show for the first time that in specific tumor cells, i.e., Mel-18a cells, the proteasome activator PA28 is essential for the processing and presentation of the TRP2 antigen-derived TRP2360–368 epitope.
epitope. Recognition of TRP2-expressing melanoma cells by TRP2<sub>360–368</sub>-specific CTLs directly correlated with the presence of PA28, and impaired epitope presentation on Mel-18a cells could be rescued by the transfection of PA28 encoding plasmids. In striking contrast to previous reports that showed that PA28 enhanced the generation of epitopes that were already generated in its absence, we show here that PA28 can change the immunological quality of a cell with respect to CTL recognition. We also provide first evidence that IFN-γ can induce self-regulation of tumor epitope presentation, even in the absence of immunoproteasomes and PA28, pointing at the existence of an IFN-γ-inducible, proteasome-independent factor involved in this process.

Morel et al. (21) reported recently that immunoproteasomes are unable to produce several self antigen-derived CTL epitopes, including an important CTL epitope derived from another melanoma differentiation antigen, Melan-A. These data have been the first example of CTL epitopes that are generated by constitutive but not by immunoproteasomes and have a large impact on our understanding of tumor immunology. Because it is likely that thymic dendritic cells, involved in T-cell selection, predominantly contain immunoproteasomes, the authors suggested that their observations could explain how CTLs against self antigens escape destruction in the thymus. These T cells could be recruited for tumor therapy using vaccination strategies.

Our data, however, deviate from the proposed hypothesis. PA28 is highly expressed in dendritic cells (23); nevertheless, it is required for TRP2<sub>360–368</sub> epitope production, indicating that absence of negative selection of HLA-A2-TRP2<sub>360–368</sub> cannot be explained by the incapacity of dendritic cells to process this antigenic peptide. In agreement with the findings by Morel et al. (21), presentation of the second TRP2 epitope, TRP2<sub>288–296</sub>, decreases upon treatment of melanoma cells with IFN-γ (Fig. 2). However, this reduction of antigenic peptide presentation is found on all IFN-γ-treated melanoma cells analyzed, including Mel-18a cells in which immunosubunit expression and PA28 expression are not up-regulated after IFN-γ treatment. Therefore, these data suggest that except for immunosubunits (21), other IFN-γ-induced factors that are not necessarily correlated with the proteasome system may contribute to the diminished presentation of antigenic peptides on tumor cells. Hence, probably not all observations of IFN-γ-induced self-regulation of tumor epitope presentation are explained by immunoproteasome formation.

The analysis of IFN-γ-inducible proteasome components in Mel-18a cells revealed that in these cells, the expression of both the immunosubunits and PA28 is impaired and cannot be restored by IFN-γ stimulation. This concerted suppression of five cytokine-inducible proteasome components, which are located on different chromosomes, is remarkable. In most tumors and tumor cell lines analyzed thus far, only the self-regulation of either β1i (LMP7) and β5i (LMP7) or PA28α and PA28β was observed (37–40), suggesting an independent, separate regulation of these gene pairs. In addition, in these tumor cells, IFN-γ was able to overrule the suppressive effects and to restore immunosubunit or PA28 expression. Our observation that IFN-γ does not overcome the suppression of these genes, although Mel-18a cells can be sensitized by this cytokine (Fig. 2, C and D, and Fig. 3), points at the existence of a thus far unknown regulatory factor that governs the coordinated expression of PA28 and the proteasome immunosubunits and which is not functional in Mel-18a cells.

One other remarkable aspect of our experiments is the finding that PA28 can have qualitative effects on MHC class I peptide complexes on the cell surface but did not change the repertoire of MHC class I-presented peptides (13, 25–27). This enhanced peptide presentation, as found for several viral epitopes, was independent of the presence of immunosubunits in the 20S proteasome (13, 26) and occurred in the absence of increased overall or viral protein turnover. This is in agreement with the notion that PA28 associates with 20S-19S complexes and thereby influences the degradation of substrates that are captured and fed into the 20S proteasome by the 19S complex, which limits the rate of protein degradation.

Corroborating the above notion, the generation of TRP2<sub>360–368</sub> also did not require the proteasome immunosubunits. Mel-18a cells, which lack immunoproteasomes, process and present TRP2<sub>360–368</sub> upon transfection of PA28α and PA28β. Moreover, MEC-PA28 cells process this epitope when PA28 expression is induced, in the absence of immunosubunits (13), and show that the rescuing effect of PA28 is not attributable to other tumor-specific factors. Consistent with this are our in vitro digestion experiments with constitutive and immunoproteasomes using TRP2 polypeptide encompassing TRP2<sub>360–368</sub> as a substrate. Analysis of the generated peptide fragments showed that neither constitutive nor immunoproteasomes produced detectable amounts of TRP2<sub>360–368</sub> or of potential epitope precursors, whereas the addition of PA28 led to an instantaneous generation of TRP2<sub>356–368</sub> precursor peptide by both types of proteasomes.

Because 20S proteasomes by themselves fail to process TRP2<sub>360–368</sub> the question arises how PA28 influences the liberation of this antigenic peptide. According to models proposed by Stohwasser et al. (41) and Whitby et al. (42), PA28 may facilitate the exit of digestion products out of the catalytic chamber of the 20S complex, which is likely to rescue larger fragments from further digestion (42). This model implies that PA28 may alter the quality of products generated by proteasomal cleavage. Accepting this as the only possible explanation and the fact that degradation of TRP2 most likely requires the formation of 19S-20S-PA28 hybrid proteasomes, one would have to conclude that epitope-specific facilitated product export is responsible for the observed effect on the PA28-induced TRP2<sub>360–368</sub> generation. This model implies that PA28 may alter the quality of products generated by proteasomal cleavage. Nevertheless, our mass spectroscopic analyses did not reveal any major shifts in cleavage site dominance in the presence of PA28 (data not shown). Instead, the usage of specific cleavage sites, which in the absence of PA28 are hardly used, appears dramatically enhanced at early time points of the processing reaction, resulting in the immediate processing of the TRP2<sub>356–368</sub> precursor peptide. Furthermore, our finding that two tumor epitopes that derive from the same protein are generated with such dramatically differing efficiencies in the absence of PA28 suggests that an altered product release cannot be the only reason for PA28-induced effects. Instead, we suggest that the binding of PA28 to the 20S proteasomes induces subtle conformational changes in the 20S proteasome that alter the accessibility of the active site pockets or their binding affinity for certain epitope-harborring protein stretches. Such a view is partly supported by a recent report by the group of Li et al. (43), which suggests that through inducing changes in proteasome conformation, PA28 may also affect the processing characteristics of proteasomes.

The finding that PA28 can play a fundamental role in antigenic peptide production and can govern the recognition of melanoma cells at least by TRP2<sub>360–368</sub>-specific CTLs may be of great practical importance. It may be possible to incorporate PA28 in antimalanoma T-cell vaccines to ensure an efficient production of this and perhaps of other tumor antigen-derived epitopes. Whether PA28 inclusion indeed leads to a higher efficacy of induced CTL responses is the subject of additional investigations.
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REFERENCES

Expression of the Proteasome Activator PA28 Rescues the Presentation of a Cytotoxic T Lymphocyte Epitope on Melanoma Cells

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