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Allele-Selective Effect of PA28 in MHC Class I Antigen Processing

Takotoshi Yamano,‡* Hidetoshi Sugahara,‡* Shusaku Mizukami, Shigeo Murata,† Tomoki Chiba,‡ Keiji Tanaka,§ Katsuyuki Yui,¶ and Heiichiro Udono3*

PA28 is an IFN-γ-inducible proteasome activator and its genetic ablation causes complete loss of processing of certain Ags, but not all of them. The reason why this occurs and how PA28 influences the formation of peptide repertoires for MHC class I molecules remains unknown. In this study, we show the allele-specific role of PA28 in Ag processing. Retrovirus-transduced overexpression of PA28α decreased expression of Kd (Dd) while it increased Kb and Ld on the cell surface. By contrast, overexpression of PA28αΔC5, a mutant carrying a deletion of its five C-terminal residues and capable of attenuating the activity of endogenous PA28, produced the opposite effect on expression of those MHC class I molecules. Moreover, knockdown of both PA28α and β by small-interfering RNA profoundly augmented expression of Kd and Dd, but not of Ld, on the cell surface. Finally, we found that PA28-associated proteasome preferentially digested within epitopic sequences of Kd, although correct C-terminal flankings were removed, which in turn hampered production of Kd ligands. Our results indicate that whereas PA28 negatively influences processing of Kd (Dd) ligands, thereby, down-regulating Ag presentation by those MHC class I molecules, it also efficiently produces Kb (Ld) epitopes, leading to up-regulation of the MHC molecules. The Journal of Immunology, 2008, 181: 1655–1664.

Major histocompatibility complex class I ligands are produced mainly by proteasomes (1–3). The proteasome activator PA28 (α and β), which is strongly induced by the major immunomodulatory cytokine IFN-γ (1, 4), has been implicated in the regulation of MHC class I Ag processing (5). PA28 accelerates the production of MHC class I ligands from longer precursor peptides by the 20S proteasome in vitro (6). The C-terminal flanking region is critical for efficient production of the T cell epitope (7). It is possible that PA28 activates the 20S proteasome by opening its α-ring (8) that is usually closed and through which substrates can pass into the core catalytic portion. In vivo analysis has also shown that the processing of several, but not all, Ags is stimulated by overexpression of PA28α and PA28β (9). Likewise, the lack of PA28 impairs the ability to process a melanoma Ag TRP2-derived peptide, but does not apparently result in a deficient processing of other Ags such as OVA (10, 11). This indicates that PA28α/β is not a prerequisite for Ag presentation in general, but plays an essential role for the processing of certain Ags. So far, the reason why PA28 is crucial in the processing of certain Ags remains unknown.

IFN-γ stimulation increases expression of the “homo-PA28 proteasome” and the “hybrid proteasome” (12). The former proteasome is a complex where PA28 is attached to both ends of the central 20S proteasome and the latter comprises the 20S proteasome flanked by PA28 on one side and a 19S cap (alias regulatory particle RP or PA700) on the other, functioning as a new ATP-dependent protease, similar to 26S proteasomes, which have a 19S cap on both sides (13). It has been suggested that hybrid proteasomes play a major role in IFN-γ-induced peptide supply for MHC class I molecules, because they can directly process ubiquitylated proteins into MHC class I ligands or into the shortest precursor peptide (14). Indeed, PA28 deficiency suppressed up-regulation of cell surface MHC class I molecules by IFN-γ, even though immunoproteasomes could be induced (10). Thus, PA28, possibly as a hybrid proteasome, is a prerequisite for IFN-γ-induced enhancement of MHC class I expression. Cascio et al. (14) have shown that the peptide repertoire produced in vitro by a hybrid proteasome from insulin growth factor 1 protein was very different from that produced by the 26S proteasome. Considering the essential role of PA28 in IFN-γ-induced enhancement of MHC class I, it is possible that the repertoire of MHC class I ligands changes in response to IFN-γ; however, the PA28-induced peptide repertoire in vivo has not yet been studied and neither has the role of allelic polymorphism on the activity of PA28.

We noticed that expression levels of cell surface Kd and Dd, but not Ld, in BALB/c PA28α−/− β−/− cells were slightly higher than those measured on wild-type cells. Knocking down both PA28α and β by small-interfering RNA (siRNA)4 revealed that Kd and Dd, but not Ld, molecules were extremely up-regulated on the cell surface. This implies that PA28 is crucial in the processing of Kd and Dd, but not Ld, molecules, because they can directly process ubiquitylated proteins into MHC class I ligands or into the shortest precursor peptide (14). Indeed, PA28 deficiency suppressed up-regulation of cell surface MHC class I molecules by IFN-γ, even though immunoproteasomes could be induced (10). Thus, PA28, possibly as a hybrid proteasome, is a prerequisite for IFN-γ-induced enhancement of MHC class I expression. Cascio et al. (14) have shown that the peptide repertoire produced in vitro by a hybrid proteasome from insulin growth factor 1 protein was very different from that produced by the 26S proteasome. Considering the essential role of PA28 in IFN-γ-induced enhancement of MHC class I, it is possible that the repertoire of MHC class I ligands changes in response to IFN-γ; however, the PA28-induced peptide repertoire in vivo has not yet been studied and neither has the role of allelic polymorphism on the activity of PA28.

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4 Abbreviations used in this paper: siRNA, small-interfering RNA; LC, liquid chromatography; MS, mass spectrometry; VSV, vesicular stomatitis virus; M6, macrophage; MCMV, murine CMV; CSP, circumsposozite protein; NP, nucleoprotein.

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surface. To clarify the role of PA28, we devised a PA28μ mutant lacking the five C-terminal residues, designated as PA28αΔC5 and capable of competing with the endogenous PA28. Using this mutant PA28αΔC5 together with knocking down PA28 by siRNA and retrovirus-transduced overexpression of PA28α, we examined the role of PA28 on the cell surface expressions of various MHC molecules. Furthermore, we performed digestion assays with the 20S proteasome mixed with recombinant (r)PA28α (or PA28αΔC5) plus PA2β for several synthetic peptides harboring Kk or Kd ligands, and liquid chromatography/mass spectrometry (LC/MS) analysis revealed that whereas the homo-PA28 proteasome is prone to digest within sequences of Kk ligands even with removing correct C-terminal flanking, it was partly able to produce Kd ligands. Our results indicate that the effect of PA28 in Ag processing, be it positive or negative, is allele specific.

Materials and Methods

Cells and cell culture

RL1 is a BALB/c mouse T cell leukemia. EL4 is a methylcholanthrene-induced C57BL/6 mouse thymoma. E.G7 is an OVA-transfected EL4 cell line (15). PA28αΔC5 and BALB/c tumor cells are a methylcholanthrene-induced BALB/c fibrosarcoma. CTLs specific for each peptide were generated and maintained as described previously (16). E.G7 was cultured in RPMI 1640 supplemented with 10% FCS (Invitrogen), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 mM l-glutamic acid, 5 × 10⁻³ M 2-ME, and penicillin-streptomycin supplemented with 400 μg/ml G418. Cells transfected with a retroviral gene (pMSCVpuro encoding murine PA28α, PA28αΔC5) were selected and maintained with 2 μg/ml puromycin.

Abs, peptides, and reagents

Rabbit polyclonal anti-histidine tag was obtained from MBL. Mouse monoclonal anti-20Sα2, Rpt1, PA2β were obtained from BIOMOL. mAb to PA2β was produced from a hybridoma clone 1G11 in our laboratory. Rabbit polyclonal anti-actin Ab was obtained from Sigma-Aldrich. Mouse MHC class I molecules. 25D1.16 mAb specific for OVA257–264-Kb was provided by Dr. R. Germain (17). Abs specific to heat shock protein 90 (LC/MS) analysis revealed that whereas the homo-PA28 proteasome is prone to digest within sequences of Kk ligands even with removing correct C-terminal flanking, it was partly able to produce Kd ligands. Our results indicate that the effect of PA28 in Ag processing, be it positive or negative, is allele specific.

Flow cytometry

C57BL/6 EL4 cells or LPS blasts with or without synthetic peptides (4 nmol) were incubated with the substrates (0.1 mM) at 37°C for 10 min. Peptide-specific antibodies were incubated with the gel to UV light at 360 nm and detected with a 460-nm filter.

Small-interfering RNA

Target sequences for PA28α (5′-AAAGCCAAGGTGGATGTTG-3′) and PA28β (5′-AAAGCCAAGGTGGATGTTG-3′) were selected and maintained with 2 μg/ml puromycin. A series of reverse primers in which the 3′ residues represent the Kb-restricted epitope), OVA 257–269 (SIINFEK) digestion assay with LC/MS was performed as described previously (10). Peptide activity of the proteasome after separation by native PAGE was measured using succinyl-L-Lysine-like activity. After electrophoresis, the gels were incubated with the substrates (0.1 mM) at 37°C for 10 min. Peptide-specific antibodies were incubated with the gel to UV light at 360 nm and detected with a 460-nm filter.

Purification of the 20S proteasome and its use for peptide digestion assay with LC/MS

Normal mouse livers (10 ml) were homogenized in buffer A (25 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.25 mM sucrose, 1 mM PMSF), and centrifuged at 10,000 × g for 20 min. The supernatants were further centrifuged at 100,000 × g for 1 h, and then, the resulting supernatant was resolved into AKTA FPLC connected with a RESOURCE Q column (Amersham Biosciences), and the data were analyzed by CellQuest software (BD Biosciences). Flow cytometric analysis was performed on a FACScan (BD Biosciences), and the data were analyzed by CellQuest software (BD Biosciences). The same experiment was performed with cells treated with 1 ng/ml IFN-γ (R&D Systems) for 8 h. The acid-wash recovery assay was performed as described previously (10).

Purification of the 20S proteasome and its use for peptide digestion assay with LC/MS

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Because the C-terminal amino acid residues of PA28 with the action of endogenous PA28 in terms of Ag processing. Various deletion mutants of PA28 for binding to the 20S proteasome (21, 22), we examined how.

\[ \text{Precursor peptides} \] was decreased by PA28 proteins were checked by anti-actin Ab (SDS-PAGE). pon Pharmaceutical).

Two hours later, after introduction, the cell surface K\(\beta\)-OVA257–264 complex was examined with 25D1.16 mAb. Initially, we tried to obtain PA28 variants that could compete with the action of endogenous PA28 in terms of Ag processing.

PA28 deletion mutants capable of interfering with the activity of PA28 in Ag processing. Initially, we tried to obtain PA28 variants that could compete with the action of endogenous PA28 in terms of Ag processing. Because the C-terminal amino acid residues of PA28a are required for binding to the 20S proteasome (21, 22), we examined how various deletion mutants of PA28a influence Ag processing. To this end, we expressed rPA28a mutants whose residues were serially deleted, ranging from 1 to 9 aa at the C terminus, termed PA28aΔC1 to PA28aΔC9, respectively, in Escherichia coli, and purified them to near homogeneity. These mutants were osmotically loaded with OVA248–269, a precursor polypeptide harboring the CTL epitope OVA257–264 of OVA, into EL4 cells (H-2\(^b\)), and the cytolyis of the loaded cells by CTLs specific for OVA 257–264 (Fig. 1A). However, no significant enhancement was observed when 3- or 4-aa deletion mutants of PA28a were used. Interestingly, PA28a mutants lacking five to nine residues from the C terminus markedly inhibited the Ag processing. These effects, i.e., enhancement by PA28a and/or PA28b, and the inhibition by PA28aΔC5 of the OVA epitope processing, were also observed in C57BL/6 LPS blasts (Fig. 1B), indicating that these PA28a variants exert their effects through their association with endogenous PA28a and/or PA28b.

Results

PA28 deletion mutants capable of interfering with the activity of PA28 in Ag processing.

(50 ng), mixed with indicated doses of recombinant PA28a, PA28aΔC5, and PA28β in a total volume of 100 μl, was subjected onto a MultiDetection Microplate Reader, POWERSCAN HT (Dainippon Pharmaceutical).

**FIGURE 1.** Effects of PA28a deletion mutants capable of interfering with PA28 action in Ag processing. A, A series of rPA28a deletion mutants ranging from C-terminal residues 1–9 (designated ΔC1 to ΔC9) were osmotically co-introduced with OVA248–269 (4 nM) into EL4 cells. The cells were used as target cells in a \(^{31}\)Cr-release assay (E:T ratio 10, B: E:T ratio 5, C). B, PA28a deletion mutants affected Ag processing of PA28a\(^{-/-}\)β\(^{-/-}\) cells but not PA28a\(^{-/-}\)β\(^{+/+}\) cells. Intact PA28a, PA28aΔC1, and PA28aΔC5 were co-introduced with OVA248–269 (4 nM) into PA28a\(^{-/-}\)β\(^{+/+}\) or PA28a\(^{-/-}\)β\(^{-/-}\) LPS blasts. The cells were used as target cells in the \(^{31}\)Cr-release assay, as shown in A. C, Ag processing of COOH- but not NH\(_2\)-terminally extended precursor peptides was decreased by PA28aΔC5 in PA28a\(^{-/-}\)β\(^{+/+}\) cells. OVA257–269 and OVA248–264 (4 nM each) were introduced with (Δ) or without (○) PA28aΔC5 into EL4 and LPS blasts. The cells were used as target cells in the \(^{31}\)Cr-release assay. D, PA28aΔC5 associates with the homo-PA28 proteasome as well as with the hybrid proteasomes. Either PA28a or PA28aΔC5 was osmotically introduced into EL4 (upper panel) or PA28a\(^{-/-}\)β\(^{-/-}\) cells (lower panel). Two hours later, cell extracts were separated by native PAGE and chymotrypsin-like activity was examined using suc-LLVY-amc. In addition, the native PAGE gels were subjected to Western blotting with specific Abs to polyhistidine, 20S proteasome as well as with the hybrid proteasomes. Either PA28a or PA28aΔC5 was osmotically introduced into EL4 (upper panel) or PA28a\(^{-/-}\)β\(^{-/-}\) cells (lower panel). Two hours later, after introduction, the cell surface K\(^{0}\)-OVA257–264 complex was examined with 25D1.16 mAb.
We reported previously that PA28 promoted the processing of extended precursor peptides harboring the OVA\textsuperscript{257–264} epitope sequence that had been extended on their C terminus, but not if they had been extended at their only N terminus (10). Therefore, the two peptides, OVA\textsuperscript{257–269} (C-terminal extension) and OVA\textsuperscript{248–264} (N-terminal extension), were loaded into EL4 or LPS blasts from PA28\textalpha+/−/β\textsuperscript{+}/− and PA28\textalpha−/−/β\textsuperscript{−}/− mice with or without PA28\textalphaΔC5. As shown in Fig. 1C, the inhibitory effect of PA28\textalphaΔC5 on cytolytic activity was observed in EL4 and the wild-type LPS blasts loaded with OVA\textsuperscript{257–269} but not OVA\textsuperscript{248–264}. In contrast, PA28\textalphaΔC5 showed no effect on PA28\textalpha−/−/β−/− LPS blasts (Fig. 1C). These results strongly indicate that PA28\textalphaΔC5 blocks the C-terminal processing of the precursor peptide OVA\textsuperscript{257–269} by using endogenous PA28.

To understand the activity of PA28\textalphaΔC5, we subsequently examined its association with the 20S proteasome in the cells. Western blotting using endogenous PA28 revealed its association with the 20S proteasome in the cells. Exogenously introduced PA28\textalpha does not need endogenous PA28 to bind to the 20S proteasome, i.e., the homopolymeric PA28\textalpha complex can associate with the 20S proteasome in vivo. This is consistent with previous findings showing that it functions as an activator in vitro (5). We also confirmed that osmotic coinroduction of OVA\textsuperscript{248–264} with PA28\textalpha but not PA28\textalphaΔC5 into EL4 cells increased the cell surface K\textalpha–OVA\textsuperscript{257–264} complex, compared with OVA\textsuperscript{248–264} alone (Fig. 1E).

PA28\textalphaΔC5 slightly decreased the activity (Fig. 1D, left end panel). Both PA28\textalpha and PA28\textalphaΔC5 associated with the hybrid as well as with the homo-PA28 proteasome in EL4 cells, but only PA28\textalpha associated with these proteasomes in PA28\textalpha−/−/β−/− cells as judged by Western blotting with anti-histidine Ab and other Abs indicated in Fig. 1D. PA28\textalphaΔC5 should not be able to bind to the 20S proteasome because several C-terminal residues of PA28\textalpha are critical for binding. Deletion of only one residue at the C terminus of PA28\textalpha prevents its association with the α-ring of the 20S proteasome, as previously indicated (22). Exogenously introduced PA28\textalpha does not need endogenous PA28 to bind to the 20S proteasome, i.e., the homopolymeric PA28\textalpha complex can associate with the 20S proteasome in vivo. This is consistent with previous findings showing that it functions as an activator in vitro (5). We also confirmed that osmotic coinroduction of OVA\textsuperscript{248–264} with PA28\textalpha but not PA28\textalphaΔC5 into EL4 cells increased the cell surface K\textalpha–OVA\textsuperscript{257–264} complex, compared with OVA\textsuperscript{248–264} alone (Fig. 1E).

PA28 augments the cell surface expression of the K\textalpha–OVA\textsuperscript{257–264} complex and of total K\textalpha.

Subsequently, we prepared stable lines of E.G7 cells expressing a full-length OVA gene transfected with PA28\textalpha, PA28\textalphaΔC5, and mock plasmid (pMSCV empty vector), designated E.G7(PA28\textalpha), E.G7(PA28\textalphaΔC5), and E.G7mock, respectively. Western blot analysis revealed that E.G7(PA28\textalpha) and E.G7(PA28\textalphaΔC5) produced 2- and 3-fold more PA28\textalpha and PA28\textalphaΔC5, respectively, than endogenous PA28\textalpha measured in E.G7mock cells (Fig. 2A).
We also examined the amount of cell surface of Kβ-OVA257-264 and total Kβ for each transfectant cultured with or without IFN-γ. In the absence of IFN-γ, PA28α up-regulated the Kβ-OVA257-264 complex and total Kβ but PA28αΔC5 markedly suppressed expression of both Kβ-OVA257-264 and total Kβ (Fig. 2, B and D). The inhibitory effect by PA28αΔC5 was still visible after 3 days of culture with IFN-γ (data not shown). The down-regulation of Kβ expression was completely restored by a pulse of E.G7 (PA28αΔC5) with OVA257-264 (Fig. 2, C and E), indicating that the diminished supply of endogenous peptides was responsible for the down-regulated expression. Expression of Dα by those transfectants was almost the same as that of Kβ (data not shown).

PA28αΔC5 was less efficient in production of TRP2181-188 from C-terminally longer precursors, compared with intact PA28α.

PA28α enhanced the expression of Kβ-OVA257-264 and total Kβ, in contrast, PA28αΔC5 suppressed those, as shown in Fig. 2. Therefore, we investigated in the vitro effect of PA28α plus PA28β and PA28αΔC5 plus PA28β together with the 20S proteasome on production of TRP2181-188 from C-terminally longer precursors whose processing was previously shown to be dependent on PA28 (13). The 20S proteasome (50 ng) purified from mouse liver was mixed with graded doses of recombinant PA28α (or PA28αΔC5) and PA28β in a volume of 20 μl, and then added with 1 μg of TRP2181-193, followed by incubation for 3 h at 37°C. A total of 40 μl 0.2% trilluorooacetic acid was added to the mixture and a 10 μl total volume was injected into LC/MS to detect exact Kβ-epitope TRP2181-188. Simultaneously, hydrolysis activity for succ-LLVY-amc was examined. The results showed that both hydrolysis activity and production of TRP2181-188 by PA28αΔC5 were significantly lower than those by PA28α (Fig. 3).

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PA28 attenuates cell surface expression of Kβ and Dβ but not Ld

We next examined the effects of PA28α and PA28αΔC5 on the expression of MHC class I molecules other than Kβ and Dβ. The cell surface expression of Kβ and Dβ on peritoneal macrophages (Moβ) of BALB/c PA28αΔββ mice was significantly higher than on Moβ of wild-type mice, whereas, in contrast, Ld expression was slightly lower in Moβ of PA28αΔββ mice (Fig. 4A). To assess directly the effects of PA28α and PA28αΔC5, we established BALB/c RLδ1(H-2d) expressing PA28α, PA28αΔC5, and mock plasmid, designated RLδ1 (PA28α), RLδ1 (PA28αΔC5), and RLδ1mock, respectively, and cell surface MH C class I molecules of those transfectants were examined. Surprisingly, expression of Kβ was down-regulated but that of Ld was enhanced by PA28α, whereas PA28αΔC5 induced the opposite effects (Fig. 4B). The expression of Kβ was restored to normal level by a pulse with CSP281–289 derived from P. yoelii (23) (Fig. 4C). In an acid-wash recovery assay, PA28α delayed the recovery of Kβ, whereas...
FIGURE 5. Depletion of PA28α and β by siRNA augments cell surface expression of K^d, D^d, but not L^d. A, MEF/3T3 cells were transfected with siRNA against PA28α and β, or with a vector not encoding siRNA. After 48 h, expressed proteins were examined by SDS-PAGE followed by Western blotting with the indicated Abs. The peptidase activity for succ-LLVY-amc and the structure of the proteasome were examined. B, Expression of MHC class I K^d, D^d, L^d of MEF/3T3 cells in A was examined by FACS analysis. C, Mean fluorescence intensities of K^d, D^d, and L^d in B are shown as bar graphs.

PA28αΔC5 accelerated the recovery (Fig. 4D). A pulse with CSP_{191–209} restored the delayed recovery of K^d (Fig. 4D). These results clearly show that PA28α is responsible for the shortage of K^d ligands in the cells. By contrast, expression of L^d was restored by a pulse with an exact L^d epitope, pRL1a (19) (Fig. 4D). In acid-wash recovery, PA28α accelerated, whereas PA28αΔC5 delayed, the recovery of L^d (Fig. 4D). The delayed recovery of L^d was restored by a pulse with pRL1a (Fig. 4D). The results indicate that PA28α stimulates the production of L^d ligands in cells, in contrast to its effect on the production of K^d ligand.

The negative impact of PA28α on K^d expression was further examined in the context of IFN-γ treatment which strongly induces endogenous PA28. IFN-γ treatment appeared to starve endogenous K^d ligands in RL.Δ1 (PA28α) more than in RL.Δ1 (PA28αΔC5) because the relative restoration level of K^d by a pulse with the CSP epitope was much larger in RL.Δ1 (PA28αΔC5) (Fig. 4E, left panel). Exactly the opposite effect was seen for expression of L^d, and IFN-γ-treated RL.Δ1 (PA28αΔC5) starved L^d ligands relative to RL.Δ1 (PA28α) (Fig. 4E, right panel). An acid-wash recovery assay supported the negative effect of PA28 in the recovery of K^d molecules; indeed, PA28αΔC5 helped the rapid recovery of L^d molecules whereas it suppressed the recovery of L^d molecules (Fig. 4F).

Next, we knocked down the expression of both PA28α and β of MEF/3T3 by siRNA to confirm the effects of PA28. As shown in Fig. 5A, expression of both PA28α and β was specifically repressed (80 ± 90%) but expression of heat shock protein 90α and the 20Sα2 subunit was not altered, and hydrolysis activity to the substrate succ-LLVY-amc was decreased especially in the homoproteasome. Native-PAGE followed by Western blotting with Abs to PA28α, β, and 20Sα2 showed a decrease of the hybrid proteasome as well as the homo-PA28 proteasome but not of the singly capped 26S proteasome (RC) and the probably empty 20S proteasome (C) (Fig. 5A). Intriguingly, expression of K^d and D^d but not L^d was enhanced by depletion of PA28α and β (Fig. 7B) and those alterations are shown in Fig. 5C, which confirms the negative effect of PA28 on the expression of K^d and D^d.

The PA28-20S proteasome improperly processes epitopes of K^d but produces K^b ligands

So far, our results consistently suggest that for the ligands used in this study, PA28 augments the generation of ligands for D^b, K^b, and L^d, while it attenuates the production of K^d and D^d ligands. It is crucial to know the reason why PA28 negatively influences K^d and D^d ligand production. To this end, in the next series of experiments, we investigated whether PA28 has a different influence on in vitro digestion using several precursor peptides. For this, we prepared four K^b, four K^d, and one L^d ligands extending C-terminal (and N-terminal) flanking regions, although these peptides are not necessarily the relevant or native precursors. We digested them with the 20S proteasome alone or plus recombinant PA28α and PA28β, or plus PA28αΔC5 and PA28β.

We observed exact K^b ligands produced from OVA_{252–269}, TRP2_{181–193}, HSV 16 glycoprotein B_{493–512}, VSV NP_{47–66}, and also observed N-terminal extended L^d precursor peptide from murine CMV pp8_{163–182} (Figs. 6 and 7), although some of these epitopes were also partly digested. The 20S proteasome alone did not produce epitope fragments from OVA_{252–269}, TRP2_{181–193}, and VSV NP_{47–66}, but in the presence of PA28, exact epitopes were cleaved. An epitope from HSV glycoprotein B_{493–512} was produced by the 20S proteasome alone and the quantity was further enhanced by PA28 (Fig. 7). Thus, in these cases, PA28 positively influenced cleavage of the K^d and L^d epitopes. In contrast, exact K^d ligands or its precursors were not recovered by the same proteasome preparations from L. monocytogenes p60_{212–231}, Tum-P198_{14–28}, influenza A HA_{513–532}, P. yoelii CSP_{278–295}.
Correct C-terminal flanking was not removed from *L. monocytogenes* p60212–231, which resulted in improper processing of the epitope. Even when correct C-terminal flanking was removed from Tum-P19814–28, influenza A HA513–532, and *P. yoelii* CSP278–295, strong digestion within the epitopes occurred in the presence of PA28, which might result in destruction of the epitopes. Importantly, even the 20S proteasome alone efficiently digested within the epitope of influenza A HA513–532, but the presence of PA28 diminished production of those fragments, while intact PA28 could stimulate it. Although there was a number of reason(s) for the improper processing, this evidence accounted for the reason why PA28 negatively influenced production of Kd ligands by the proteasome.

Almost all fragments or epitopes produced by PA28 plus PA28 are lower in quantity than that by PA28 alone, which was a reasonable outcome because of its undermined enzyme activity, although there were some exceptions. One N-terminal extended a precursor of MCMV pp89163–182 and the exact epitope from HSV glycoprotein B493–512, which were evaluated as areas by [M+2H] were higher in quantity by PA28 plus PA28— and FIGURE 6. Digesting patterns of precursor peptides harboring Kd, Kc, and Ld ligands by the proteasome. One microgram of each peptide, A–E, was incubated with the 20S proteasome (50 ng) mixed with 100 ng each of PA28 plus PA28β, or PA28αΔC5 plus PA28β in 20 μl total volume for 3 h at 37°C. A, OVA252–269; B, TRP2181–193; C, *L. monocytogenes* p60212–231; D, Tum-P19814; E, MCMV pp89—182. The digestion mixture was subjected onto LC/MS analysis to detect peptide fragments. Amino acid sequences, their retention time (RT), and area determined by m/z values of [M+H] and [M+2H] of digested fragments were indicated. Horizontal lines under peptide sequences indicated the recovered fragments after digestion. Quantity of each fragment produced by the 20S proteasome and PA28α and PA28β is visualized as follows: >1,000,000 (thickest black bar), 100,000–999,999 (second thickest black bar), 10,000–99,999 (third thickest black bar), 1,000–9,999 (thinnest black bar). Cleavage points by the proteasome were also indicated by inverted triangles (▼) whose sizes paralleled with obtained quantity (area) of the digested fragments. The exact MHC class I epitope is depicted with bold (and/or underlining). Note that each retention time (RT) of the exact epitope produced by digestion of A, B, and E was precisely the same as that of the synthetic corresponding peptide. None of the exact epitope was observed among the digested materials of C and D. N.D., Not detected or 100.
the reason was unknown. However, we observed no significant
difference in cleavage pattern of all peptides used in this study
between PA28/H9251 plus PA28/H9252 and PA28/H9251/H9004
C5 plus PA28/H9252. PA28-
20S proteasome-mediated improper processing of Kd ligands
tested here might be compromised by PA28/H9251/H9004,
which in turn
prevents overdigestion of C-terminally elongated precursors. It is
noted that the cleavage pattern of all peptides after incubation for
1, 3, and 6 h with the proteasome was nearly comparable, although
the longer reaction showed larger quantity of each fragment (data
not shown).

Discussion
Ag processing and presentation is crucial for the initiation of the
immune response. Over the past decade, there is growing evidence
that the proteasome, a large multisubunit protein degradative
machinery in eukaryotes, plays an important role as a processing en-
zyme responsible for the generation of MHC class I ligands (1–3).

This processing system is elaborately regulated by various immu-
nomodulatory cytokines. In particular, IFN-γ induces the forma-
tion of the immunoproteasomes, in which three IFN-γ-inducible
subunits (i.e., β1i, β2i, and β5i) can replace the constitutive cat-
alytic 20S subunits (i.e., β1, β2, and β5) during proteasome bio-
genesis (24). Furthermore, IFN-γ also induces PA28, producing
the homo-PA28-20S proteasome and the hybrid proteasome,
which contributes importantly to efficient production of MHC class
I ligands (6, 12). Furthermore, it has been shown that the immu-
noproteasome and the PA28-containing proteasome in concert or
independently play a critical role in the generation of the MHC
class I ligands (1). However, the molecular mechanisms underly-
ing the correct generation of CTL epitopes by those different types
of proteasomes remain a mystery.

In the present study, we tried to clarify the role of the IFN-γ-
inducible proteasome activator PA28 in the Ag-processing and
-presentation pathway, and surprisingly found different effects of

FIGURE 7. Digesting patterns of precursor peptides harboring K*®, K®, and L® ligands by the proteasome, the same as in Fig. 1, except peptides were
digested by the proteasome. A, HSV glycoprotein B 493–512; B, VSV NP 47–66; C, Influenza A HA 513–532; D, P. yoelii CSP278–295. The exact MHC class I
epitope is depicted with bold (and/or underlining). Note that each retention time (RT) of the exact epitope produced by digestion of A and B, was precisely
the same as that of the synthetic corresponding peptides. None of the exact epitope was observed among the digested materials of C and D. N.D., Not
detected or <100.
PA28 on the MHC class I epitope generation depending on the allelic polymorphism. Indeed, whereas PA28 is unable to produce many of K^b ligands and thereby attenuates cell surface expression of those MHC class I molecules, it is able to produce most (if not all) K^b (also L^d) epitopes, leading to up-regulation of the corresponding MHC molecules on the cell surface. Our previous observation that IFN-γ-induced up-regulation of K^b was canceled in PA28-deficient cells (10) is consistent with the present findings and indicate that PA28 plays a prominent role in IFN-γ-stimulated peptide supply. Conversely, knockdown of both PA28α and PA28β by siRNA significantly increased the expression of K^d and D^d (Fig. 5, B and C), indicating that PA28 negatively influences the presentation of those ligands. Indeed, constitutive expression of K^d and D^d in peritoneal Mφ from the BALB/c PA28α^-/-β^-/- mouse was slightly higher than on wild-type Mφ, whereas that of L^d was lower (Fig. 4A). Acid-wash recovery of cell surface K^b of RL.1 cells was accelerated in the presence of PA28αΔC5, especially upon IFN-γ treatment in which endogenous PA28 was extremely induced; in contrast, recovery of L^d was mostly retarded by PA28αΔC5 but accelerated by intact PA28α (Fig. 4F). These results clearly support the negative and positive influence of PA28 on the processing of K^b and L^d ligands, respectively.

Why PA28 contributes differently to the MHC class I ligand generation in an allelic polymorphism-dependent fashion is largely unknown. We therefore analyzed a series of synthetic peptides harboring various epitopes whose in vitro digestion was conducted by the PA28-20S proteasome to evaluate from which types of peptides correct epitopes are produced. The PA28-20S proteasome was unable to process any of the K^d ligands tested in this study by the PA28-20S proteasome (6). In fact, we confirmed the enhancing effect of PA28 on processing of this epitope in vivo by osmotically loading the precursor peptides and rPA28α into P815 (H-2^{b}) cells and also in vitro peptide digestion assay with PA28-20S proteasome (data not shown). The reason for this discrepancy is unknown at present. Nonetheless, considering the clear negative effect of siRNA-mediated knockdown of PA28α and β on K^d (D^d) expression, we emphasize that the majority of those ligands, if not all, are likely to be improperly or inefficiently processed by the PA28-20S proteasome. In vitro production of mouse T cell epitopes from longer precursors by the PA28-20S proteasome was mainly observed in the context of L^d and K^b, although only the JAK1 kinase-derived epitope was K^b restricted as described (6). For example, the mouse leukemia peptide pRL1a (7, 25) and the MCMV pp89-derived peptide (6) were for L^d, and the Moloney murine leukemia virus gag-derived peptide (9) and the melanoma Ag TRP2 peptide (10, 11) are K^b ligands. Our current observation that PA28 stimulates the processing of K^d and L^d is consistent with those reports, especially results of in vitro peptide digestion supporting the positive influence of PA28 on the production of K^d ligands by the PA28-20S proteasome (Figs. 6 and 7).

It should be pointed out that production of K^d ligands in vivo was absolutely dependent on the proteasome because acid-wash recovery of K^d as well as L^d was significantly delayed by treatment with the proteasome inhibitor lactacystin (data not shown), suggesting that the 26S proteasome rather than the PA28-20S or another type of proteasome flanked with the newly identified regulatory particle like PA200 (26, 27) and/or the immunoproteasome might be responsible for processing of K^d ligands. To this point, for the processing of P. yoelii CSP_{278–295} and Tum-P198_{14–28}, we observed that a fraction of the 26S proteasome purified from the liver of the PA28α^-/-β^-/- mouse could exactly produce the K^d epitopes, from the same precursors, although these epitopes were not efficiently produced by the PA28-20S proteasome purified from a wild-type mouse liver (data not shown). The other two K^b ligands used in this study were not processed by the 26S proteasome (data not shown), which might suggest the involvement of the aforementioned different proteasomes or of other so far unidentified molecule(s) in the processing of those peptides. However, the processing mechanisms mediated by the proteasome are intriguing in general, because while it is generally accepted that the immunoproteasome is able to dominantly generate a diverse array of epitopes (1), the processing of some Ags is catalyzed specifically by the standard proteasome (alias constitutive proteasome), but not the immunoproteasome (28). Yet, no one knows the mechanistic reason(s) for those different processing profiles.

Our results clearly demonstrate the allele-specific effect of PA28 on the expression of MHC class I, regardless of the fact that these effects are positive or negative. Pool sequencing of peptides eluted from MHC class I molecules of cells, or large sequencing of individual peptides, would provide more direct information on the effect of PA28 in epitope generation. We could not identify a common reason for the inefficient processing of K^d ligands by the PA28-20S proteasome in this study. However, anchor residues of MHC class I ligands (29) might be, at least in part, involved in the allele-specific effect of PA28. Thus, it is possible that tyrosine anchor residue of K^d ligand is responsible for inefficient processing of many of K^b ligands. We are currently focusing on this issue. Mouse K^b-binding peptides have motif XYXXXXXL. Because human HLA-A24 ligands also contain the same binding motif, it will be interesting to investigate whether processing of human HLA-A24 ligands is also down-regulated by the PA28 proteasome. Should these findings apply also to Ag processing and MHC class I expression in humans, they would have a great impact on our understanding of the immune system, and have practical implications especially for vaccination strategies in cancer and infectious diseases. Further studies are necessary to fully demonstrate the role of PA28 in allele-specific Ag processing.

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Disclosures
The authors have no financial conflict of interest.

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