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The proteasome regulator PA28α/β can enhance antigen presentation without affecting 20S proteasome subunit composition

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PA28α/β is a regulatory complex of the 20S proteasome which consists of two IFN-γ inducible subunits. Both subunits, α and β, contribute equally to the formation of hexa- or heptameric rings which can associate with the 20S proteasome. Previously, we have shown that overexpression of the PA28α subunit enhanced the MHC class I-restricted presentation of two viral epitopes and that purified PA28α/β accelerated T cell epitope generation by the 20S proteasome *in vitro,* indicating a role for PA28α/β in antigen presentation. This conclusion was recently confirmed in PA28β gene targeted mice which were severely deficient in MHC class I-restricted antigen presentation. These mice displayed a defect in the assembly of immunoproteasomes, suggesting that a lack of the proteasome subunits LMP2, LMP7, and MECL-1 may account for the deficiency in antigen presentation. In this study we investigated whether the effect of PA28α/β on antigen presentation is dependent on a change of proteasome subunit composition. We have analyzed the assembly and subunit composition of proteasomes in fibroblast transfectants overexpressing both, α and β subunits of PA28. In these transfectants we found a marked enhancement in the presentation of the immunodominant H-2L^d-restricted pp89 epitope of murine cytomegalovirus, although the 20S proteasome composition was the same as in recipient cells. We, therefore, conclude that PA28α/β can enhance antigen processing independently of changes in 20S proteasome subunit composition or assembly.

Key words: MHC class I / Antigen processing / CTL / Cytomegalovirus / Immunodominant epitope

1 Introduction

The proteasome is the main protease in the cytoplasm and the nucleus which is responsible for the generation of most peptide ligands of MHC class I molecules [1]. The proteolytic core complex of the proteasome system is the 20S proteasome which is constructed like a cylinder of four stacked rings [2]. The outer two rings consist of seven different α-type subunits that bind to regulatory complexes of the 20S core particle, whereas the two inner rings are made up of seven different subunits of the β-type. Three of the β-subunits designated δ, MB1, and MC14 (Z) bear the active centers of the 20S proteasome. Upon stimulation of cells with IFN-γ, these constitutively expressed subunits are replaced by inducible subunits named LMP2, LMP7, and MECL-1 during the *de novo* assembly of 20S proteasomes. This subunit exchange alters the cleavage pattern of the proteasome [3] which leads to an enhancement in the generation of some epitopes, although the bulk of MHC class I ligands can still be generated in cell lines or mice which are deficient for LMP2 or LMP7 [4–7].

The proteasome regulator PA28α/β (also named '11S regulator') was discovered by virtue of its ability to stimulate the different peptidolytic activities of the 20S proteasome [8, 9]. PA28 consist of two subunits, α and β, which are both transcriptionally induced by IFN-γ stimulation. They contribute about equally to the formation of hexa- or heptameric rings that associate with the α-endplate of the 20S proteasome [10–12]. Overexpression of the α subunit of PA28 resulted in a marked enhancement in the presentation of two virus-derived epitopes on MHC class I molecules, suggesting a function for PA28 in antigen

Abbreviations: MCMV: Murine cytomegalovirus **NEPHGE:** Non equilibrium pH gradient electrophoresis

processing [13]. Although the *in vivo* mechanism of how PA28 enhances antigen presentation remains to be determined, we found that during the *in vitro* processing of polypeptides by the 20S proteasome the addition of PA28 accelerated the production of MHC class I ligands by inducing concerted dual cleavages, leading to the direct excision of class I ligands [14, 15]. The finding that PA28 plays an important role in antigen processing was recently corroborated by the phenotypic analysis of PA28β gene targeted mice [16]. LPS blasts or macrophages from these mice were barely able to present several MHC class I-restricted epitopes and the *in vivo* generation of CTL to lymphocytic choriomeningitis virus and the expansion of $CD8^+$ T cells after MCMV infection were dramatically reduced. Interestingly, the incorporation of the inducible subunits LMP2, LMP7, and MECL-1 into proteasomes was greatly diminished in PA28$\beta^{-/-}$ mice which led to the proposal that the lack of immunoproteasomes may account for the observed defect in antigen presentation [16].

In this study we tested this hypothesis by analyzing the proteasome composition of fibroblast transfectants which overexpress α and β subunits of PA28. These cells presented the immunodominant H-2L^d-restricted epitope encompassing residues 168–176 of the MCMV immediate early protein pp89 much better as compared to wild-type recipient cells, although the subunit composition of 20S proteasomes of PA28 transfectants and recipient cells were indistinguishable. This data indicate that PA28 has an effect on antigen processing which does not necessarily rely on changes in 20S proteasome subunit composition.

2 Results

2.1 Characterization of PA28α/β double transfectants

Previously, we have generated transfectants of the BALB/c-derived fibroblast line B8 which overexpressed murine and human PA28α proteins [13]. The B8 line was chosen as a recipient because it expressed the transfected immediate early gene 1 of MCMV encoding the pp89 protein constitutively at low levels. The processing and presentation of the H-2L^d-restricted immunodominant epitope YPHFMPTNL encompassing residues 168–176 of pp89 could thus be monitored in these transfectants, yielding evidence that PA28α overexpression enhanced pp89 presentation. As it is now established that PA28 exists *in vivo* as a heterohexa- or heptamer of the two subunits α and β we have recently generated stable PA28α/β double transfectants [17]. Two clones designated BPαβ2 and BPαβ13, were selected for this study, which, according to Northern and Western blots, expressed the highest amounts of PA28 α and β [17].

To further characterize the PA28 transfectants, we compared the stoichiometry of PA28α and β in B8 recipient cells, in a PA28α single transfectant (BPαm.4), and a PA28α/β double transfectant (BPαβ13). Using an antibody raised against a peptide of PA28α, we performed metabolic labeling and immunoprecipitation from total lysates of these clones as well as from the 11S fraction of sucrose gradients containing the ringform of PA28 (Fig. 1). In total lysates we found that in the PA28α/β double transfectant both subunits were overexpressed and present in about equal amounts whereas in the PA28α single transfectant the α subunit was more abundant than the β subunit. However, when we performed the immunoprecipitation from 11S fractions of glycerol gradients on which we separated the total lysates (Fig. 1, right panel), we found that in single and double transfectants PA28α and β appeared in about equal stoichiometry and were in both cases more abundant than in B8 recipient cells. This result indicates that the overexpression of PA28α does not change the stoichiometry of α and β in the PA28 ring and further suggests that overexpression of PA28α can increase the amount of the 11S regulator by recruiting more endogenously expressed PA28β monomers into the PA28α/β ring. These results indicate that an up-regulation of pp89 presentation in PA28α transfectants is not due to a change in the composition

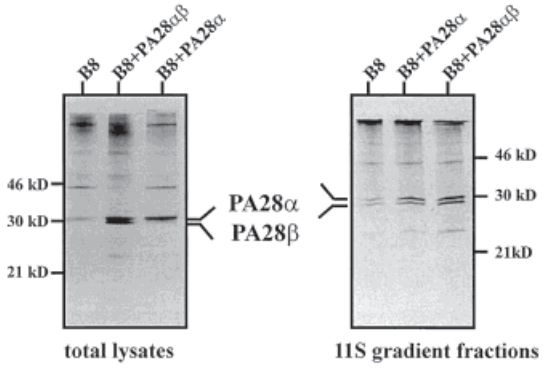

Fig. 1. Immunoprecipitation of PA28α/β from total lysates (left panel) or the 11S gradient fraction of sucrose gradients (right panel) from metabolically labeled B8 recipient cells (B8), the PA28α single transfectant BPαm4 (B8+PA28α), and the PA28α/β double transfectant BPαβ13 (B8+PA28αβ). Cells were labeled with [^{35}S]methionine for 45 min and immunoprecipitation with a PA28α-specific antibody was performed directly from lysates or from the 11S fraction after separation over sucrose gradients. The precipitates were separated by electrophoresis according to Schaegger and Jagow [26] and exposed for autoradiography. The positions of PA28α and PA28β are indicated.

of the 11S regulator but that an increase in the amount of the ring form of PA28α/β is responsible for this effect. A prediction from this comparison of single and double transfectants would be that PA28α/β double transfectants may likewise present the pp89 epitope at a higher level.

2.2 Overexpression of PA28 α and β leads to an enhancement of MCMV pp89 presentation

We compared the H-2L^d-restricted presentation of the immunodominant MCMV pp89 epitope by the two clones BPαβ2 and BPαβ13 with that of B8 recipient cells. Cytolytic assays were performed using B8, BPαβ2, and BPαβ13 as targets and pp89/H-2L^d-specific CTL as effectors (Fig. 2A). Both PA28α/β transfectants were lysed by pp89-specific CTL two to three times more efficiently compared to B8 wild-type cells in three independent experiments. Enhanced presentation of the pp89 epitope in PA28α/β double transfectants was also found when B8, BPαβ2, and BPαβ13 cells were used as stimulators of a pp89/H-2L^d-specific T cell hybridoma which we have recently generated (Fig. 2B). The TCR-mediated stimulation of this hybridoma can be quantified in lacZ assays because it expresses a lacZ reporter gene under the control of the IL-2 promotor/enhancer [18, 19]. In spite of the better presentation of the pp89 epitope, the cell surface expression of the H-2 molecules D^d, K^d, and L^d was not altered in B8 cells transfected with PA28α alone or PA28 α and β together as previously determined by flow cytometry [13, 17].

To exclude that the higher pp89 epitope presentation in PA28α/β double transfectants was due to a higher expression level of the pp89 protein in these cells, we performed pp89 Western analysis (Fig. 3). As a negative control we used the C4 fibroblast line from which the B8 clone had been obtained by transfection of the pp89 gene. No differences in pp89 expression were detected between B8 cells and the PA28α/β transfectants. This indicates that the better presentation of pp89 in BPαβ2 and BPαβ13 cells can not be attributed to higher pp89 protein levels in these clones and that PA28 overexpression is unlikely to affect the degradation rate of the pp89 protein.

2.3 The PA28α/β-mediated enhancement of pp89 presentation does not rely on changes in 20S proteasome subunit composition

Cells obtained from PA28β gene targeted mice were recently reported to lack the IFN-γ inducible proteasome subunits LMP2, LMP7, and MECL-1 in their 20S proteasomes and it was suggested that this may be the reason for the observed defects in antigen presentation [16]. Hence, we decided to compare the subunit composition of 20S proteasomes from B8 cells with that of BPαβ2 and BPαβ13 transfectants in order to investigate whether changes in the subunit composition of their 20S proteasomes may account for the increase in pp89 presentation on H-2L^d. As controls for the expression of LMP2, LMP7, and MECL-1 we used IFN-γ treated B8

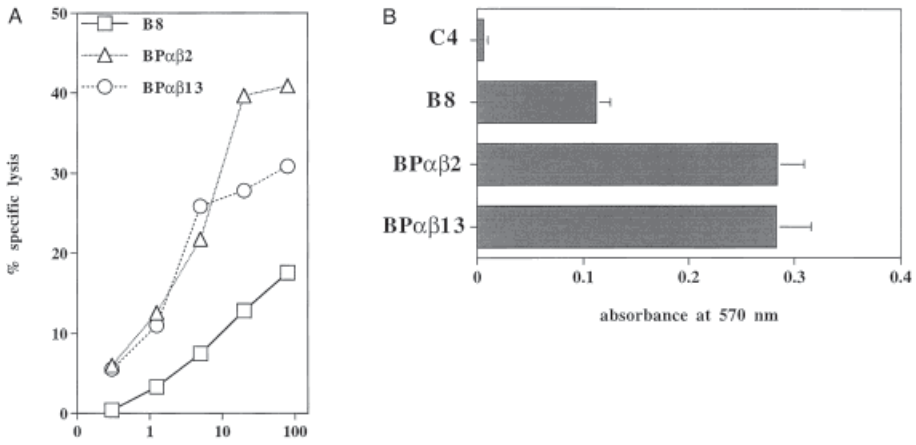

Fig. 2. Comparison of the H-2L^d restricted presentation of the pp89 epitope by B8 cells and the PA28α/β transfectants BPαβ2 and BPαβ13. (A) Cytolytic chromium-release assay using H-2L^d/pp89-specific CTL as effectors. The effector to target ratio (E:T) is plotted versus per cent specific lysis. Data represent the means of six replicate cultures. (B) Stimulation of a H-2L^d/pp89-specific T cell hybridoma by C4, B8, BPαβ2, and BPαβ13 cells. C4 are the parental cells from which the clone B8 has been obtained by transfection of the pp89 gene. The absorbance of the generated dye at 570 nm is plotted for each stimulator cell. The values are the means of three replicate cultures.

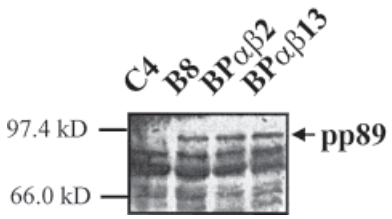

Fig. 3. Western analysis of MCMV pp89 expression in transfectants of C4 fibroblasts. B8 has been derived from C4 cells by transfection of the immediate early gene 1 of MCMV. BPαβ2 and BPαβ13 are PA28α/β double transfectants of B8. The pp89 protein is indicated and migrates at about 90 kDa; the identity of additional bands migrating below pp89 is unknown.

cells as well as the B8-derived LMP2/LMP7/MECL-1 triple transfectant B27 M6 [20]. The cells were metabolically labeled for 4 h and the 20S proteasomes were allowed to mature in a chase period of 5 h. The proteasomes were immunoprecipitated and the subunit composition analyzed on 2D IEF/PAGE gels (Fig. 4). The subunit composition of B8, BPαβ2, and BPαβ13 was identical and in particular no enhancement in the incorporation of LMP2 and MECL-1 was observed. The subunit LMP7 has a basic isoelectric point and hence migrates out of our IEF/PAGE gels, but a previous Western analysis did not provide any evidence for LMP7 up-regulation in our PA28α/β transfectants [17]. We therefore conclude that the enhancement of H-2L^d-restricted pp89 presentation in our transfectants can not be attributed to changes in the composition of 20S proteasomes but must rely on a different effect mediated directly or indirectly by PA28α/β.

2.4 Overexpression of PA28α/β does not shift proteasome assembly towards the assembly of immunoproteasomes in IFN-γ-treated cells

The analysis of PA28β$^{-/-}$ mice suggested that PA28 is required for the assembly of immunoproteasomes. We tested in the PA28α/β double transfectant BPαβ13 whether the overexpression of PA28α/β would shift proteasome assembly from the production of constitutive 20S proteasomes towards the assembly of immunoproteasomes shortly after the initiation of IFN-γ treatment. First, we performed a kinetic experiment to determine whether a time period of IFN-γ stimulation could be determined during which the synthesis of LMP2, LMP7, and MECL-1 precursors was initiated but which was insufficient to bring the endogenous PA28α/β expression in B8 cells to the same level as in the BPαβ13 transfectant. After 12 h of IFN-γ stimulation, the synthesis of LMP2, LMP7, and MECL-1 was fully initiated in B8 and

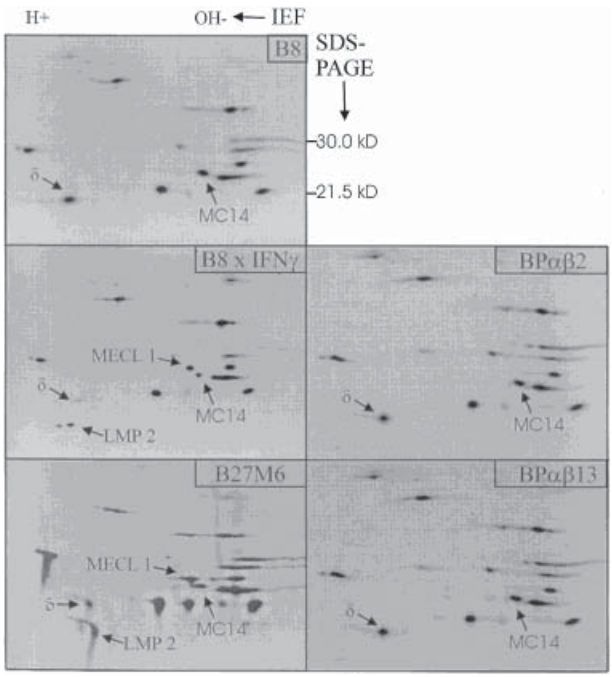

*Fig. 4.*Two-dimensional IEF/PAGE analysis of proteasomes immunoprecipitated from two different PA28α/β double transfectants (BPαβ2, BPαβ13), one LMP2/LMP7/MECL-1 triple transfectant (B27 M6) as well as the parental B8 line grown in the presence and absence of IFN-γ. The cells were labeled with [^{35}S]methionine/cystein for 4 h and then grown for 5 h in chase medium to allow full maturation of 20S proteasomes. The position of the house-keeping subunits δ and MC14 and the IFN-γ inducible subunits LMP2 and MECL-1 are indicated on the autoradiographies.

BPαβ13 cells but as shown in the Western analysis presented in Fig. 5A, the steady state amount of PA28 in IFN-γ induced B8 cells was still far lower than in IFN-γ – stimulated or unstimulated BPαβ13 cells. Next, we performed a pulse chase experiment with B8 and BPαβ13 cells that had been treated for 12 h with IFN-γ. The cells were labeled for 1 h and chased for 1 and 4 h since proteasome assembly should occur within this time period. The proteasome was immunoprecipitated and the subunit composition was analyzed by NEPHGE/PAGE (Fig. 5B). After 12 h of IFN-γ stimulation, the incorporation of LMP2 and LMP7 was clearly initiated, but also the constitutively expressed subunits were still visible after 1 h (data not shown) and 4 h of chase (Fig. 5B). Clearly, we did not observe a shift towards the assembly of immunoproteasomes containing LMP2 and LMP7 in BPαβ13 cells as compared to B8 recipient cells, although both cell lines were equally stimulated with IFN-γ according to flow cytometric quantification of the IFN-γ -mediated enhancement in MHC class I cell surface expression (data not shown). Taken together, we

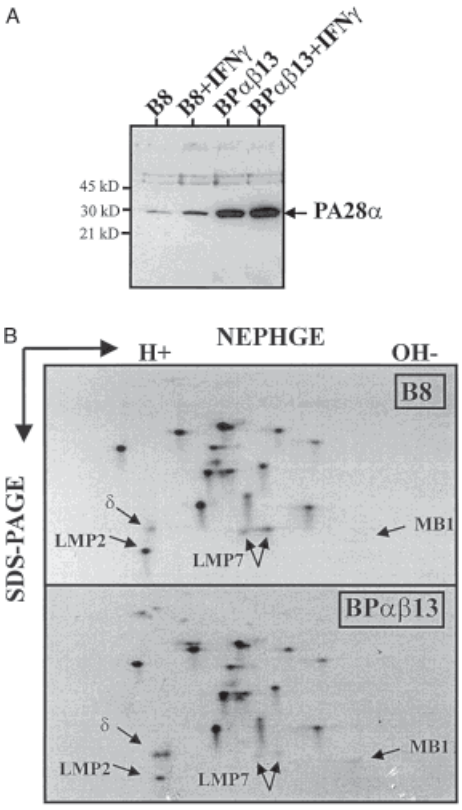

Fig. 5. Overexpression of PA28α/β does not shift proteasome assembly towards the preferential production of immunoproteasomes. (A) Western analysis for PA28α expression in unstimulated B8 cells and the PA28α/β transfectant BPαβ13 and in the same cells after 12 h of stimulation with IFN-γ. (B) NEPHGE/SDS-PAGE of 20S proteasome subunits from IFN-γ treated B8 and BPαβ13 cells. The cells were pretreated for 12 h with IFN-γ and labeled for 1 h with [^{35}S]methionine/cysteine. After a chase period of 4 h 20S proteasomes were immunoprecipitated and analyzed for subunit composition on 2D gels by autoradiography. The IFN-γ-inducible subunits LMP2 and LMP7 are labeled as well as their respective constitutive homologues δ and MB1.

conclude that – at least in this system – a constitutive overexpression of PA28α and β can not direct the assembly of 20S proteasomes towards the production of immunoproteasomes.

3 Discussion

Several lines of evidence have indicated that the proteasome regulator PA28α/β functions in the processing of antigens for presentation on MHC class I molecules. Like other proteins along the class I pathway both subunits of PA28 are inducible with IFN-γ [21]. PA28α/β has not been found in species which lack a cellular immune system and it affects the activity of the proteasome, the main protease involved in antigen processing. The overexpression of PA28α enhanced the presentation of two viral antigens [13] and PA28β gene targeted mice showed a severe deficiency in class I-restricted antigen presentation [16]. The first *in vivo* evidence how PA28 may accomplish an elevation in class I-restricted antigen presentation was recently obtained by Preckel et al. [16] who found that the incorporation of the inducible subunits LMP2, LMP7, and MECL-1 into 20S proteasomes was severely reduced in cells from PA28β-deficient mice. Because LMP2 and LMP7 have been shown to improve the antigen presentation of several class I epitopes it has been suggested that PA28 improves antigen processing by promoting the assembly of immunoproteasomes. The principal finding of the present study is that the PA28-dependent elevation of MCMV pp89 presentation did not rely on changes in the subunit composition of 20S proteasomes. Therefore, we can conclude that other *in vivo* functions of PA28 must exist in antigen processing which are independent of effects on the subunit composition and assembly of 20S proteasomes.

A second result of this study is that we did not observe an enhancement in the incorporation of LMP2, LMP7, and MECL-1 in 20S proteasomes from fibroblasts overexpressing PA28α and β (Fig. 4). Moreover, the assembly of immunoproteasomes was not favored in PA28α/β double transfectants when these transfectants as well as B8 parental cells were stimulated with IFN-γ (Fig. 5). Therefore, we were unable to obtain experimental support for a function of PA28 in the assembly of immunoproteasomes as proposed by Preckel et al. [16] in our experimental system. However, our experiments were based on PA28 overexpression rather than deletion and we can not exclude that PA28 is required in low amounts to maintain immunoproteasome assembly. In B8 fibroblasts, the basal expression of LMP2, LMP7, and MECL-1 on mRNA and protein level is very low which could prevent a higher level of incorporation of these subunits into 20S proteasomes. Moreover, the small induction of PA28α/β in B8 cells observed 12 h after IFN-γ stimulation may be sufficient to allow the assembly of immunoproteasomes at a maximal rate. Nevertheless, we can conclude that if there is a role for PA28α/β in the promotion of immunoproteasome assembly, the higher steady state levels obtained after prolonged IFN-γ stimulation in cells are not required for this function. This result suggests that the up-regulation of PA28α/β expression must serve different functions which are unrelated to the assembly of LMP2, LMP7, and MECL-1 into the proteasome. The latter conclusion is again consistent with our finding that PA28 could enhance MCMV pp89 presentation without elevating the level of LMP2, LMP7, and MECL-1 in BPαβ2 and BPαβ13 cells.

The notion that the effects of PA28 are not exclusively mediated via the subunits LMP2, LMP7, and MECL-1 is also supported by the phenotype of PA28$\beta^{-/-}$ mice which seem to have a more fundamental defect in antigen presentation than LMP2 or LMP7-deficient mice [7, 6]. It is noteworthy, however, that cells from lymph nodes of LMP7$^{-/-}$ mice show a significant reduction in H-2D^b and H-2K^b cell surface expression which was not observed in the same cell population from PA28$\beta^{-/-}$ mice. This may suggest that the residual incorporation of LMP7 in the latter mice is sufficient to provide the full quantity of class I ligands needed for a maximal class I surface expression in lymphocytes. Interestingly, neither the overexpression of PA28α/β in fibroblasts nor the lack of PA28α/β in PA28$\beta^{-/-}$ mice altered the MHC class I cell surface expression although the presentation of individual antigens was enhanced or reduced, respectively. It, therefore, seems that the impact of PA28 is epitope specific, a characteristic, which has also been reported for LMP2 and LMP7. The H-2L^d-restricted presentation of MCMV pp89 in B8 cells overexpressing LMP2 and LMP7 did not lead to an elevation of epitope presentation [13] whereas PA28α/β did. In contrast, the overexpression of LMP2, LMP7, and MECL-1 enhanced the H-2L^d-restricted presentation of the NP118 epitope of lymphocytic choriomeningitis virus whereas the overexpression of PA28 α and β had no effect [17]. These results suggest that PA28α/β and the incorporation of LMP2, LMP7, and MECL-1 in proteasomes affect antigen presentation in a different way which argues against the idea that PA28α/β exerts its effect exclusively via promoting the assembly of immunoproteasomes.

It may seem unexpected that the overexpression of PA28α alone and the co-expression of PA28α and β enhance pp89 presentation to a similar extent. To investigate this phenomenon we have analyzed the composition of PA28 in the 11S sucrose gradient fraction of B8 cells either overexpressing PA28α alone or PA28α and β together. Interestingly, in both cell lines the stoichiometry of PA28α and β was about equimolar and markedly elevated when compared to B8 recipient cells (Fig. 1). This indicates that although recombinant PA28α protein forms stable homoheptameric rings *in vitro* which activate peptide hydrolysis by 20S proteasomes [11] these do not prevail *in vivo*. It seems that overexpression of PA28α by itself can elevate the formation of PA28α/β heterohexa- or heptamers by recruiting more of the endogenously expressed β monomers into the 11S regulator which would explain why the effect on pp89 presentation was similar in single or double transfected cells. In accordance with these findings the elimination of PA28β in gene targeted mice led to a simultaneous disappearance of PA28α proteins which most likely are instable as monomers and and homo-oligomers *in vivo*.

Our experiments indicate that PA28 can elevate class I-restricted antigen presentation independent of its effects on 20S proteasome composition. How PA28 achieves this elevation is still a matter of debate. The careful kinetic analysis of the fragmentation of two polypeptides by 20S proteasomes in the presence and absence of PA28 *in vitro* yielded evidence that PA28 induced coordinated dual cleavages leading to the accelerated appearance of fragments of appropriate length for binding to MHC class I molecules [15]. It was suggested that PA28 may coordinate the cleavage of two juxtaposed active centers of the 20S proteasome to achieve this aim. However, as the site-directed inactivation of 20S proteasome subunits did not affect the length distribution of proteasomal peptide products [22] additional mechanisms may exist which determine the length of peptide products. Recently, Baumeister and colleagues provided evidence that it is the capacity to retain peptides of a certain length in the lumen of the 20S proteasome which will determine the size distribution of peptide products [23]. An attractive hypothesis based on this finding would be that PA28 may control the size of the openings in the 20S proteasome through which finite peptide products leave the complex. As a further alternative, it was recently suggested that PA28α/β may adopt chaperone-like functions by supporting the refolding of denatured proteins [24]. These and other hypotheses will now need to be addressed experimentally to define the role of PA28 in antigen processing at the molecular level.

4 Materials and methods

4.1 Cell lines

The murine fibroblast clone C4 was derived from embryonic BALB/c mice by SV40 infection *in vitro*. The clone B8 was derived from C4 cells by cotransfection of the immediate early gene 1 of MCMV encoding the pp89 protein and a neomycin resistance gene (Hartmut Hengel, U.H.K., unpublished). The PA28α transfectant BPαm4 had been obtained by cotransfection of B8 cells with a mouse PA28α expression construct and the puromycin resistance construct pLXSP as previously described [13]. The PA28α/β transfectants BP$\alpha\beta$.2 and BP$\alpha\beta$13 were obtained by cotransfection of the BPαm4 clone with a PA28β expression construct and the hygromycin resistance construct pLXSH [17, 25]. All cells were maintained in IMDM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 250 μg/ml G418 (for B8, BPαm.4, BP$\alpha\beta$.2/13), 2.5 μg/ml puromycin (for BPαm4, BP$\alpha\beta$2/13), 400 μg/ml hygromycin B (for BP$\alpha\beta$2/13).

4.2 Western blot analysis

Western blotting and chemiluminescence detection were performed exactly as previously described [17]. Primary antibodies used were rabbit polyclonal antibodies recognizing PA28α [25] and MCMV pp89 (U.H.K., unpublished).

4.3 Metabolic labeling, immunoprecipitation, and electrophoresis

The metabolic labeling of cells with [^{35}S]methionine/cysteine and immunoprecipitation with protein A Sepharose was performed essentially as previously outlined [17] with minor modifications. For the experiments shown in Fig. 1, 1×10^7 cells were starved for 30 min and labeled for 45 min prior to lysis by threefold freezing and thawing in 400 μl of buffer A (50 mM Tris/HCl pH 8.0, 5 mM $MgCl_2$, 0.2% Triton-X100, 0.75 μM aprotinin, 10 μM leupeptin, 2.8 μM pepstatin, 0.85 mM phenylmethylsulfonyl fluoride). The postnuclear supernatant was either used directly for immunoprecipitation or was loaded onto a 10-40% (w/v) sucrose gradient in 50 mM Tris/HCl pH 8.0 and centrifuged in a Sorvall TH641 rotor at 40,000 rpm for 16 h. Gradient fractions of 600 μl were drawn and fractions corresponding to 11S which stimulated 20S proteasome activity were used for immunoprecipitation with a polyclonal rabbit antibody raised against a PA28α peptide [25]. The immunoprecipitates were electrophoresed according to Schaegger and Jagow [26] prior to autoradiography.

For the experiments shown in Fig. 4, 5×10^6 cells were starved for 1 h, labeled for 4 h, and chased for 5 h to allow maturation of proteasome precursors. Lysis was performed for 30 min at 4°C in buffer B (50 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% NP40 and 0.75 μM aprotinin, 10 μM leupeptin, 2.8 μM pepstatin, 0.85 mM phenylmethylsulfonyl fluoride) and immunoprecipitation was performed with a rabbit polyclonal antiserum raised against purified 20S proteasomes from mouse liver. IEF/SDS-PAGE and autoradiography were performed exactly as described [27].

For the experiment shown in Fig. 5B, 3×10^6 cells were pretreated for 12 h with 100 U/ml mouse rIFN-γ (Roche, Rotkreuz) and were starved for 30 min, labeled for 1 h, and chased for 1 h or 4 h in the presence of IFN-γ. Lysis and immunoprecipitation of mouse 20S proteasomes were performed as for the experiment in Fig. 4 but the precipitates were analyzed by non-equilibrium pH gradient gel electrophoresis (NEPHGE)/SDS-PAGE as previously described [27].

4.4 Cytolytic assays

Generation of MCMV-specific polyclonal CTL was achieved by *in vitro* restimulation of spleen cells from mice that were latently infected with MCMV [28]. The CTL were propagated by weekly restimulation and addition of 100 U/ml recombinant human IL-2 and 10^{-8} M synthetic MCMV pp89 nonapeptide ^{168}YPHFMPTNL^{176}. For the production of target cells, transfectants were trypsinized and labeled with $Na_2{}^{51}CrO_4$ for 90 min and a 4–6 h standard cytolytic assay was performed with 1,000 target cells and graded numbers of effector cells in fourfold dilution steps. Data represent the mean of a specific lysis of six replicates. Specific lysis was calculated as (experimental release – spontaneous release) / (total release – spontaneous release) × 100%.

4.5 LacZ assay

For lacZ assays, MCMVpp89-specific T cell hybridomas pp89Hyb [17] were cocultured overnight with 5×10^4 stimulator cells in 96-well plates at an effector to target ratio of 1:1. The cultures were washed once with PBS and lysed by addition of 100 μl of Z buffer [0.15 mM chlorophenolred β-galactoside (CPRG, Roche, Rotkreuz), 100 mM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP40 in PBS]. After 4 h incubation at 37°C the absorbance at 570 nm (reference wavelength at 620 nm) was read using a SpectraFluor Plus plate reader (Tecan, Gröding, Austria).

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