Inactivation of a Defined Active Site in the Mouse 20S Proteasome Complex Enhances Major Histocompatibility Complex Class I Antigen Presentation of a Murine Cytomegalovirus Protein

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Summary

Proteasomes generate peptides bound by major histocompatibility complex (MHC) class I molecules. Avoiding proteasome inhibitors, which in most cases do not distinguish between individual active sites within the cell, we used a molecular genetic approach that allowed for the first time the in vivo analysis of defined proteasomal active sites with regard to their significance for antigen processing. Functional elimination of the δ/low molecular weight protein (LM P) 2 sites by substitution with a mutated inactive LM P2 T1A subunit results in reduced cell surface expression of the MHC class I H -2Lδ and H -2Dδ molecules. Surface levels of H -2Lδ and H -2Dδ molecules were restored by external loading with peptides. However, as a result of the active site mutation, MHC class I presentation of a 9-mer peptide derived from a protein of murine cytomegalovirus was enhanced about three- to fivefold. Our experiments provide evidence that the δ/ LM P2 active site elimination limits the processing and presentation of several peptides, but may be, nonetheless, beneficial for the generation and presentation of others.

Key words: proteasome • antigen processing • mutation • active site • low molecular weight protein 2 T1A

Using proteasome-specific inhibitors, the proteasome system has been shown to be involved in antigen processing and to represent the major source for the generation of MHC class I peptides (1–4). The 20S proteasome is an NH₂-terminal nucleophile hydrolase containing an active site threonine residue (5). It is a cylinder-shaped particle composed of four stacked rings of seven subunits each. In eukaryotes, the seven different α type subunits occupy positions in the two outer rings, whereas the two inner rings are formed by seven different β type subunits (6). The proteolytic activity is restricted to the lumen of the cylinder and is mediated by three of the seven β type subunits, i.e., subunits δ (β1), M B1 (β5), and Z (β2) (parentheses, new nomenclature according to Groll et al., reference 7). Therefore, in total, the 20S proteasome complex possesses six active sites within the two inner β rings. By induction with the cytokine IFN-γ, the active site bearing constitutive β subunits are replaced by their IFN-γ-inducible counterparts low molecular weight protein 2 (LM P2)† (β1), LM P7 (β5), and M ECL-1 (β2) during proteasome assembly (3, 8, 9).

†Abbreviations used in this paper: LM P, low molecular weight protein; M CA, amidoo-4-methylcoumarin; M CM V, murine CM V.

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inhibitors, it has been shown that the inhibition of some of the proteasomal peptidase activities affects the processing of MHC class I antigens (1, 18). However, there exists little active site specificity of the available proteasome aldehyde inhibitors. Even the active site specificity of lactacytin demonstrated in vitro is difficult to control in cell experiments since, depending on the experimental condition, lactacytin affects more than one type of active site (19, 20). Therefore, experimental setups using proteasome inhibitors in most cases do not allow one to draw any conclusions on the functional importance of a specific active site for the generation of a defined MHC class I antigen. Such knowledge is, however, important to better understand the basic rules of antigen processing and to develop strategies that may allow either up- or downregulation of the generation of a defined antigenic peptide.

To overcome these problems, we made use of a recently described mutation in the nonconstitutive LM P2 (iβ1) subunit in which the NH₂-terminal active site threonine was replaced by alanine (21). This T1A mutation resulted in the impairment of correct maturation by autocatalytic processing of the subunit and rendered an proteolytically inactive LM P2 subunit. In this study, we used the inactive mutant to study the functional importance of the δ/LM P2 (iβ1/ iβ1) active sites with regard to MHC class I antigen presentation. Overexpression of the mutant LM P2 T1A subunit in mouse fibroblast cells resulted in an effective replacement of the proteolytically active δ (iβ1) subunit. As a consequence, the mutant LM P2 T1A cells contain proteasomes in which two of the six active sites of the 20S proteasome complex are eliminated. Our experiments demonstrate that the deletion of these active sites generally limits the production of peptides bound to H-2Ld and H-2Dd molecules. At the same time, the mutation enhances the generation and presentation of an H-2Ld epitope derived from the cytomegalovirus pp89 protein.

Materials and Methods

Cell Lines. The BALB/c-derived mouse fibroblast cell lines C4 and B8 were used. The B8 cell line, which is derived from the C4 cells, constitutively expresses the IE1 pp89 of the murine cytomegalovirus (22). The B8 cell line was subcloned by limiting dilution and one clone was chosen as recipient for the transfection experiments. The generation of the cDNA constructs of LM P2 and LM P2 T1A, transfection by conventional calcium phosphate precipitation and selection are described in detail in reference 21.

Purification of 20S Proteasomes and Assay of Proteolytic Activity. Purification of 20S proteasomes and assay of proteolytic activity is described in detail elsewhere (16, 17). The method is based on a modification of the method of in vivo labeling and the use of the proteasomal peptidase activities. The technique described here is a modification of the method used for the assay of proteasomal peptidase activities (16, 17). The technique described here is a modification of the method used for the assay of proteasomal peptidase activities (16, 17). The technique described here is a modification of the method used for the assay of proteasomal peptidase activities (16, 17).

Results and Discussion

Efficient Incorporation of the LM P2 T1A Subunit into the 20S Proteasomes. To investigate the consequence of a de-
fined active site elimination in the mouse 20S proteasome complex, the murine fibroblast cell line B8 was stably transfected with cDNAs either encoding a wild-type LM P2 or a mutated LM P2 subunit in which the active site threonine 1 residue was exchanged against alanine by site-directed mutagenesis (21). Overexpression of the LM P2 or LM P2 T1 A subunits results in efficient incorporation of these subunits into the 20S proteasome complex (Fig. 1). The incorporation of the LM P2 proteins is associated with an almost complete exchange against subunit δ. Accordingly, by immunoblotting with anti-δ antibody only after overexposure of the enhanced chemiluminescence blot, negligible amounts of residual δ subunit can be identified in 20S proteasomes of B8-LM P2 and B8-LM P2 T1 A cells. Arrows, the position of a 21-kD marker protein.

Table 1. Effects of Transfection of Wild-type B8 Cells with LM P2 and LM P2 T1 A cDNAs on the Peptidase Activities of Proteasomes

| Substrate       | B8         | B8-LM P2    | B8-LM P2 T1 A |
|-----------------|------------|-------------|---------------|
|                 | V<sub>max</sub> | K<sub>m</sub> | V<sub>max</sub> | K<sub>m</sub> | V<sub>max</sub> | K<sub>m</sub> |
|                 | μmol/h mg | μM          | μmol/h mg   | μM          | μmol/h mg   | μM          |
| VGR-MCA         | 0.31       | 57          | 0.31         | 76          | 0.35        | 100         |
| GGL-MCA         | 0.85       | 25          | 0.79         | 33          | 0.75        | 52          |
| LLVY-MCA        | 1.11       | 29          | 1.20         | 41          | 1.33        | 70          |
| GLF-MCA         | 0.18       | 35          | 0.14         | 35          | 0.05        | 83          |

V<sub>max</sub> and K<sub>m</sub> values were determined with 20S proteasomes and the substrates Bz-Val-Gly-Arg-MCA for the trypsin-like activity, Z-Gly-Gly-Leu-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and MeO-suc-Gly-Leu-Phe-MCA for the chymotrypsin-like activity. The peptide-glutamyl-peptide hydrolyzing activity measured with the substrate Cbz-Leu-Leu-Glu-bn was undetectable in both B8-LM P2 and B8-LM P2 T1 A transfectants and is therefore not shown.

in the B8-LM P2 T1 A mutant. Also, the K<sub>m</sub> value, the measure for the binding affinity of substrates, was only moderately influenced by subunit substitution or active site mutation. For all substrates, we monitored an approximately twofold increase in the K<sub>m</sub> for the LM P2 T1 A proteasome. One possible reason for the observed increase in K<sub>m</sub> values in the LM P2 T1 A mutant could be that the NH<sub>2</sub>-terminal extension of 8–10 amino acids of the mutant subunit influences the accessibility of the other active sites and hence the substrate binding affinity. Apart from this, the data suggest that the active site under investigation has little effect upon the trypsin and chymotrypsin-like peptide substrates, which is in agreement with the previous finding that the δ/LM P2 site affects the peptide glutamyl peptide hydrolyzing activity (PDGH activity) of the proteasome complex. This activity is completely eliminated in these cells (data not shown). On the other hand, these data show that the different hydrolyzing activities, as monitored with unphysiologically short substrates, are in fact overlapping and that the attractive model of three different proteolytic specificities each mediated by one of three pairs of active sites is perhaps too simple. In a recent investigation, Elever and coworkers (27) came to a similar conclusion by showing that short peptide hydrolyzing activities are overlapping and that different active sites cleave more than one type of short fluorogenic substrate. Interestingly, the incorporation of the LM P2 subunit into 20S proteasomes as such and not its activity seems to affect the enzymatic characteristics of the active sites of the neighboring subunit Z (p2) and the more distant subunit M B1(p5). This suggests once more (14) that the incorporation of this IFN-γ-inducible subunit may also influence the structure function relationship within the proteasome complex.

A dive Site Dletion Leads to Reduced Surface Levels of MHC Class I Alleles. To investigate the effect of the δ/LM P2 active site mutation on the generation of antigenic peptides
in vivo, we analyzed the cell surface expression of MHC class I molecules whose assembly and efficient transport to the cell surface is dependent on the loading with suitable peptides. Flow cytometric analysis of several independent B8-LMP2 T1A cell clones with allele-specific antibodies revealed an ~40% reduction in the cell surface expression of the H-2D\(d\) and H-2L\(d\) molecules when compared with B8, B-LMP2, or B8 mock-transfected control cells (Fig. 2). No difference in cell surface expression was found for the H-2K\(d\) molecules (data not shown). That this is not a clonal effect is demonstrated by the finding that identical data were obtained with different B8-LMP2 T1A cell lines. These results suggest that the elimination of the two active sites restricts the overall quality of peptide generated, thus possibly limiting the supply of peptide and, in consequence, negatively affecting MHC class I molecule assembly and cell surface expression.

To test this hypothesis we took advantage of the observation that MHC class I molecules can reach the cell's surface without prior peptide binding when cells are incubated at 27°C and that empty MHC class I molecules can be stabilized by binding of externally added peptides (24, 25). B8-LMP2 T1A cells were therefore loaded either with a synthetic 9-mer peptide that binds to H-2L\(d\) or peptides extracted from B8 cells. As expected from its binding characteristics, the synthetic 9-mer peptide restores the level of H-2L\(d\), but not that of H-2D\(d\) on B8-LMP2 T1A cells (Fig. 3 C). Furthermore, peptides extracted from nontransfected B8 cells were able to stabilize the levels of both MHC alleles on the surface of B8-LMP2 T1A cells (Fig. 3 D). These data demonstrate that it is indeed the lack of peptides that is responsible for reduced MHC class I expression on the surface of B8-LMP2 T1A cells. In support of this, pulse chase experiments and immunoprecipitation of H-2D\(d\) and
H-2Dd and H-2Ld molecules. In contrast, the level of H-2Kd amount of peptides available for binding to MHC class I proteins therefore decreases the overall efficiency of loading. The elimination of active sites in the 20S proteasome does not increase at the cell surface at higher temperatures, but under the same experimental conditions, the number of H-2Kd molecules does not increase at the cell surface. MHC expression independent of peptide supply. How-ever, under the same experimental conditions, the number of Kd molecules does not increase at the cell surface at 27°C, even when peptides extracted from B8 cells are externally loaded. The elimination of active sites in the 20S proteasome complex therefore decreases the overall amount of peptides available for binding to MHC class I H-2Dd and H-2Ld molecules. In contrast, the level of H-2Kd molecules is not reduced. Interestingly, all three haplotypes possess similar preferences for the COOH-terminal anchor residue but differ with regard to the residue preference at position 2 of the epitope. This indicates that the functional importance of the activity of the β/LMP2 varies depending on the type of peptide products that have to be generated for binding to a given MHC class I haplotype. In addition, despite the fact that the peptide hydrolyzing activities of the different active sites of the 20S proteasome are overlapping as deduced from in vitro data obtained with short fluorogenic peptide substrates (27), the β/LMP2 active sites exert a specific cleavage property that is responsible for the in vivo generation of a specific peptide quality from natural protein substrates.

Enhanced Specific Cytolysis of Cells Expressing the LMP2 T1A Protein. So far the experiments showed that the functional elimination of two defined active sites in the 20S proteasome complex affects the quality of peptide generation and results in the downregulation of certain, but not all, MHC class I molecules. To determine how far the active site mutation influences the presentation of a specific peptide, we analyzed the different transfectant B8 cell lines with regard to their ability to present an immunodominant 9-mer peptide of the murine CMV (MCMV) pp89 to a H-2Ld-specific T cell line in a cytotoxicity assay. As shown in Fig. 4, B8 cells and B8 control-transfectant cells were lysed to the same extent, whereas B8-LMP2 cells were slightly less susceptible to lysis. In contrast, three- to five-fold less pp89/H-2Ld-specific cytotoxic T cells were required to lyse the B8-LMP2 T1A cells.

To exclude the possibility that increased pp89 expression in B8-LMP2 T1A cells was responsible for the observed effect, we investigated the expression of pp89 by Northern blot analysis since pp89 is quite stable at the posttranslational level (22). As shown in Fig. 4 B, no significant differences in the expression of pp89 can be detected in the investigated cell lines. Thus, despite the elimination of two active sites, sufficient pp89 antigen is generated to allow an increase in peptide-specific MHC class I presentation. Although in vitro experiments do not necessarily reflect the in vivo situation, it is interesting to note that in vitro digests experiments of the pp89 25-mer synthetic polyepitope harboring the 9-mer epitope (15) show that the improved MHC class I presentation may be due to altered proteasomal processing properties. Although β/LMP2 proteasomes have the tendency to destroy the epitope, mutant LMP2 T1A proteasomes do not use the internal cleavage site and thus seem to preserve the epitope (Ruppert, T., unpublished observations). In consequence, the increased maximum of lysis observed may be due to an increase in specific peptide supply. Considering that the overall H-2Ld surface expression is reduced in B8-LMP2 T1A cells, these experiments represent the first example that the specific elimination of a proteasomal active site, in this case, β/LMP2, may be beneficial for presentation of certain class I epitopes, despite reduced MHC class I expression.
References

1. Rock, K.L., G. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A.L. Goldberg. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on M HC class I molecules. Cell. 78:761–771.

2. Coux, O., K. Tanaka, and A.L. Goldberg. 1996. Structure and functions of the 20S and 26S proteasomes. Annu. Rev. Biochem. 65:801–847.

3. Groettrup, M., A. Soza, U. Kuckelkorn, and P.-M. Kloetzel. 1996. Peptide antigen production by the proteasome: complexity provides efficiency. Immunol. Today. 17:429–435.

4. Lehner, P.J., and P. Cresswell. 1996. Processing and delivery of peptides presented by the M HC class I molecules. Curr. Op. Immunol. 8:59–67.

5. Seemüller, E., A. Lupas, D. Stock, J. Löwe, R. Huber, and W. Baumeister. 1995. Proteasome from Thermoplasma adophilum: a threonine protease. Science. 268:579–582.

6. Grziwa, A., W. Baumeister, B. Dahllmnn, and F. Kopp. 1991. Localization of subunits in proteasomes from Thermoplasma adophilum by immunoelectron microscopy. FEBS Lett. 290:186–190.

7. Groll, M., L. Ditzel, J. Löwe, D. Stock, M. Bochtler, H.D. Bartunik, and R. Huber. 1997. Structure of the 20S proteasome from yeast at 2.4 Å resolution. Nat. 386:463–473.

8. Früh, K., M. Gossen, K. Wang, H. Bujard, P.A. Peterson, and Y. Yang. 1994. Displacement of the housekeeping proteasome subunits by M HC-encoded LS Ps. PNAS. 91:8611–8615.

9. Groll, M., R. Kraft, S. Kostka, S. Stander, R. Stohwasser, and P.-M. Kloetzel. 1996. A third interferon-gamma induced subunit exchange in the 20S proteasome. Eur. J. Immunol. 26:863–869.

10. Kelly, A., S.H. Powis, R. Glynn, E. R adley, S. Beck, and J. Trowsdale. 1991. Second proteasome-related gene in the human M HC class II region. Nat. 353:667–668.

11. Ortiz-Navarrete, V., A. Seelig, M. Gernold, S. Frentzel, P.-M. Kloetzel, and G.J. Hammerling. 1991. Subunit of the 20S proteasome (multicatalytic proteinase) encoded by the major histocompatibility complex. Nature. 353:662–664.

12. Groettrup, M., S. Stander, R. Stohwasser, and P.-M. Kloetzel. 1997. The subunits M ECL1 and LM P2 are mutually required for incorporation into the 20S proteasome. Proc. Natl. Acad. Sci. 94:8970–8975.

13. Gaczynska, M., K.L. Rock, and A.L. Goldberg. 1993. Gamma-interferon expression of M HC genes regulates peptide hydrolysis by proteasomes. Nature. 365:264–267.

14. Kuckelkorn, U., S. Frentzel, R. Kraft, S. Kostka, M. Groettrup, and P.-M. Kloetzel. 1995. Incorporation of major histocompatibility complex encoded subunits LM P2 and LM P7 changes the quality of the 20S proteasome polyprotein processing products independent of interferon-gamma. Eur. J. Immunol. 25:2605–2611.

15. Boes, B., H. Engel, T. R uppert, G. Multhaup, U.H. Koszinowski, and P.-M. Kloetzel. 1994. Interferon-gamma modulates the proteolytic activity and the cleavage site preference of 20S mouse proteasomes. J. Exp. Med. 179:901–909.

16. Fehling, H.J., W. Swat, C. Laplace, R. Kühn, K. Rajewsky, U. Müller, and H. Von Boehmer. 1994. M HC class I expression in mice lacking the proteasome subunit LM P-7. Science. 265:1234–1237.

17. Van Kaer, L., P.G. Ashton-Rickardt, M. Eichberger, M. Gaczynska, K. Nagashima, K.L. Rock, A.L. Goldberg, P.C. Doherty, and S. Tonegawa. 1994. Altered peptidase and viral specific T cell response in LM P2 mutant mice. Immunity. 1:533–541.

18. Cernando, V., A. Benham, V. Braud, S. Mukherjee, K. Gould, B. McCino, J. Neefjes, and A. Townsend. 1997. The proteasome specific inhibitor lactacytin blocks presentation of cytotoxic T lymphocyte epitopes in human and murine cells. J. Immunol. 27:336–341.

19. Fenteany, G., R.F. Standaert, W.S. Lane, S. Choi, E.J. Correy, and S.L. Schreiber. 1993. Inhibition of proteasome activities and subunit-specific amino- terminal threonine modification by lactacytin. Science. 268:726–731.

20. Dick, L.R., A.A. Cruikshank, L. Grenier, F.D. Melandi, S.L. Nunes, and R.L. Stein. 1996. MHC class Ii molecules. Proc. Natl. Acad. Sci. 93:5533–5538.

21. Schmidtke, G., R. Kraft, S. Kostka, P. Henklein, C. Frömmel, J. Löwe, R. Huber, P.-M. Kloetzel, and M. Schmidt. 1996. Analysis of mammalian 20S proteasome biogenesis: the maturation of β subunits is an ordered two step mechanism involving autocatalysis. EMBO J. 15:6887–6890.

22. Hengel, H., P. Lucin, S. Jonjic, T. Ruppert, and U.H. Koszinowski. 1994. Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. J. Virol. 68:289–297.

23. Maniatis, T., J. Sambrook, and E.F. Fritsch. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

24. Ljunggren, H.G., N.J. Stam, C. Olen, J.J. Neefjes, P. Hoglund, M.T. Heemels, J. Bastin, T.N. Schumacher, A. Townsend, K. Karre, et al. 1990. Empty MHC class I molecules come out in the cold. Nature. 346:476–480.

25. Schumacher, T.N., M.T. Heemels, J.J. Neefjes, W.M. Kast, C.J. Melief, and H.-L. Ploegh. 1990. Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. Cell. 62:563–567.

26. Del Val, M., H.J. Volkmann, J. R othbard, S. Jonjic, M. Messere, J. Schickedanz, M. Ederhaye, and U.H. Koszinowski. 1988. Molecular basis for cytolytic T lymphocyte recognition of the murine cytomegalovirus immediate early protein pp89. J. Virol. 62:3965–3972.

27. Eleuteri, A.M., R.A. Koansk, C. Cardzo, and M. Orlowski. 1997. Bovine spleen multicatalytic proteinase (proteasome): replacement of X, Y, and Z subunits by LM P2, LM P7, and MECL1 and changes in properties and specificity. J. Biol. Chem. 272:11824–11831.