Growth Retardation in Mice Lacking the Proteasome Activator PA28γ

(Received for publication, September 28, 1999)

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The proteasome activator PA28 binds to both ends of the central catalytic machine, known as the 20 S proteasome, in opposite orientations to form the enzymatically active proteasome. The PA28 family is composed of three members designated α, β, and γ. PA28α and PA28β form the heteropolymer mainly located in the cytoplasm, whereas PA28γ forms a homopolymer that predominantly occurs in the nucleus. Available evidence indicates that the heteropolymer of PA28α and PA28β is involved in the processing of intracellular antigens, but the function of PA28γ remains elusive. To investigate the role of PA28γ in vivo, we generated mice deficient in the PA28γ gene. The PA28γ-deficient mice were born without appreciable abnormalities in all tissues examined, but their growth after birth was retarded compared with that of PA28γ+/− or PA28γ+/+ mice. We also investigated the effects of the PA28γ deficiency using cultured embryonic fibroblasts; cells lacking PA28γ were larger and displayed a lower saturation density than their wild-type counterparts. Neither the expression of PA28αβ nor the subcellular localization of PA28αα was affected in PA28γ−/− cells. These results indicate that PA28γ functions as a regulator of cell proliferation and body growth in mice and suggest that neither PA28α nor PA28β compensates for the PA28γ deficiency.

The proteasome with an apparent sedimentation coefficient of 20 S is a protein-killing machine equipped with a variety of catalytic centers that presumably contribute to the hydrolysis of multiple peptide bonds in single polypeptide substrates by a coordinated mechanism (see Ref. 1 and references therein). The 20 S proteasome is a barrel-like particle with a molecular mass of ~750 kDa, appearing as a stack of four rings made up of two outer α rings and two inner β rings. Tertiary structural analysis indicates that the center of the α ring is almost closed, thus preventing penetration of substrates into the interior of the β ring on which the proteolytically active sites are located (2). Presumably because of this structural feature, the 20 S proteasome exists as a latent form in cells. The latent proteasome is activated fully by a recently identified endogenous protein, the proteasome activator PA28 (also known as the 11 S regulator) (3, 4). PA28 differs from another well known proteasome regulator designated PA700. The latter is made up of multiple subunits of 25–110 kDa and associates with the 20 S proteasome to form the 26 S proteasome, a eukaryotic ATP-dependent protease complex with a molecular mass of ~2 MDa (reviewed in Refs. 5–8). The 26 S proteasome is responsible for the degradation of a wide variety of cellular proteins tagged with a polyubiquitin chain that functions as a degradation signal. Extensive studies conducted during the past decade have established that the 26 S proteasome plays a critical role in various biological processes by regulating the levels of cellular proteins rapidly, timely, and/or irreversibly (9, 10).

The native PA28 is a protein complex with a molecular mass of ~200 kDa that binds directly to both ends of the 20 S proteasome to form a football-like structure (5, 11). Binding of PA28 greatly stimulates multiple peptidase activities of the 20 S proteasome. However, the football-like proteasome lacks the ability to degrade large protein substrates, suggesting that PA28 may cooperate with the 26 S proteasome in a sequential proteolytic pathway (2, 3). Indeed, our recent work indicates the existence of a “hybrid-type” proteasome (12), with PA28 attached to one end and PA700 to another end of the 20 S proteasome. Similar to the 26 S proteasome, this type of proteasome appears to function as an ATP-dependent protease. 1 PA28 is composed of two subunits, named PA28α and PA28β, that share ~50% amino acid identity (13). These subunits assemble into a heterohexamer (α3β3) with alternating α and β subunits (14) or a heterohexamer (α3β4) or a mixture of α3β4 and α4β3 (15). Cloning of PA28α and PA28β cDNAs revealed that the α and β subunits are structurally similar to a nuclear protein, termed Ki antigen, which was initially identified with autoantibodies found in sera of patients with systemic lupus erythematosus (13). Ki antigen associates reversibly with the 20 S proteasome, indicating that it is a genuine component of the PA28 protein family (16). Therefore, Ki antigen was renamed PA28γ (16). Intriguingly, PA28γ appears to form a homopolymer, presumably PA28γ/γ or PA28γ/+. Upon

* This work was supported in part by grants-in-aid for Scientific Research from The Ministry of Education, Science, Sports, and Culture of Japan, The Human Frontier Science Promotion Organization, and The Naito Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡‡ This paper is available on line at http://www.jbc.org

1 N. Tanahashi, Y. Murakami, Y. Minami, N. Shimbara, B. H. Hendil, and K. Tanaka, manuscript in preparation.
stimulation with IFN-γ, the protein levels of both PA28α and PA28β increase markedly in a variety of cells, whereas that of PA28γ remains unchanged or is slightly decreased (reviewed in Refs. 17 and 18). Furthermore, PA28α and PA28β are located primarily in the cytoplasm, whereas PA28γ occurs in the nucleus without appreciable localization in the cytoplasm (19, 20). Thus, the two forms of the PA28 complex differ in terms of the responsiveness to IFN-γ and subcellular localization.

Proteasomes are the central enzymes responsible for the generation of major histocompatibility complex (MHC) class I ligands (reviewed in Refs. 17, 18, and 21). Recently, Groettrup et al. (22) found that, together with the MHC-encoded LMP genes (23), PA28α plays an important role in the generation of class I binding peptide by altering the cleavage pattern of the proteasome. More recently, this form of PA28 was found to enhance the induction of virus-specifickiller T cells in vivo (23) and to favor proliferation of dominant class I ligands in vitro (24, 25). These findings suggest that the IFN-γ-inducible PA28 plays an important role in the generation of class I ligands, and no information is available concerning the biological or biochemical function of the PA28γ homopolymer. To gain insights into its biological function, we created mice lacking the PA28γ gene (Pmec3). PA28γ was not essential in mouse development, but its absence resulted in retardation of cell proliferation and body growth.

EXPERIMENTAL PROCEDURES

Construction of the PA28γ Targeting Vector—A 12-kb NcoI fragment containing the entire coding region of the PA28γ gene was obtained from a 129/SvJ mouse genomic library (Stratagene) as previously reported (26, 27). The targeting vector consisted of the 6.2-kb genomic DNA containing the 1.2-kb neomycin resistance gene derived from pMC1neo/poly(A) (see Fig. 1A). A 1.1-kb diphtheria toxin gene (DT-A) derived from pMC1D7-3 was attached to the 5’ end of the PA28γ-neomycin construct.

Production of PA28γ Null Mice—The targeting vector was linearized with ScaI and transfected by electroperoration into T7E embryonic stem (ES) cells (courtesy of Dr. S. Aizawa, Kumamoto University). ES cells were selected in 200 μg/ml G418 (Life Technologies, Inc.). Colonies of ES cells with homologous recombination events were identified by polymerase chain reaction (PCR) amplification of a 2.4-kb fragment using the primer pair derived from the 5’-upstream region of the PA28γ gene (5’-CAGCTGGATTGAAAGAGGAGATGCTGTC-3’) and from the neomycin gene (5’-GTCTTTTTTGCAGAGCCCTCAAGAACGAC-TC-3’). To verify the results of PCR screening, genomic DNAs prepared from the PCR-positive ES clones were digested with EcoRI and BamHI. cDNA (26) was 32P-labeled using random primers and used as a probe. D, Western blot analysis of extracts prepared from PA28γ-/-, PA28γ+/−, and PA28γ+/- mice. The blot was probed with the anti-PA28γ antibody.

Table I

| Sex | Genotype | Number | Body weight Mean ± S.D. (grams) |
|-----|----------|--------|----------------------------------|
| Male | +/-      | 16     | 32.9 ± 3.8                      |
|     | +/+      | 44     | 31.0 ± 3.1                      |
|     | +/-      | 23     | 29.5 ± 3.2                      |
| Female | +/-     | 22     | 23.9 ± 3.4                      |
|       | +/+      | 46     | 23.2 ± 2.8                      |
|       | +/-      | 26     | 21.7 ± 2.9                      |

(26, 27) were described elsewhere. PA28γ-null ES cells were obtained from mouse embryonic fibroblasts (MEF) (see Ref. 26). Western blot analysis was performed using anti-mouse PA28γ antibody and a mouse antiserum to IFN-γ. MEFs were transfected with a plasmid encoding a human PA28γ gene (26). The expression of PA28γ in MEFs was determined by immunoblotting. The expression of PA28γ in MEFs was determined by Western blot analysis. Full-length mouse PA28γ cDNA (26) was 32P-labeled using random primers and used as a probe. D, Western blot analysis of extracts prepared from PA28γ-/-, PA28γ+/−, and PA28γ+/- mice. The blot was probed with the anti-PA28γ antibody.
FIG. 2. Growth retardation in PA28γ-deficient mice. A, growth curves in two pairs of male littermates are shown. Each pair is represented by a square or circle. Open and filled symbols indicate wild-type and PA28γ-deficient mice, respectively. Mice were weighed at indicated intervals, and their weight was plotted against age in weeks. B, analysis of body weight in a total of 175 mice obtained by intercross-breeding of PA28γ+/− heterozygotes is shown. Body weight in 85 male mice (left) and 90 female mice (right) at 24 weeks of age is shown. There are statistically significant differences between the PA28γ−/− and PA28γ+/- groups (p < 0.01) and between the PA28γ−/− and PA28γ+/- groups (p < 0.05) in both male and female cohorts. Statistical analysis was performed by one-way analysis of variance and by using Sheffe’s method as a post hoc test (male: analysis of variance, F(2, 80) = 4.995, p = 0.009; female: analysis of variance, F(2, 91) = 3.747, p = 0.0273). n.s., not significant.

FIG. 3. Growth properties and single cell size of MEFs. A, growth kinetics and saturation densities of primary MEFs at passage 4 are shown. Cells (4 × 10^5) from wild-type (open symbols) and PA28γ−/− (filled symbols) were plated in 60-mm culture dishes and counted daily. Two independent pairs of MEFs (represented by circles and squares) were used. B, single cell size of MEF with a wild-type or PA28γ-deficient genotype is shown. MEFs were harvested at the logarithmic growth phase. Forward scatter intensity was measured by flow cytometry. Open and filled histograms indicate wild-type and PA28γ−/− MEFs, respectively.

13.5-day-old embryos. Cells were cultured at 37 °C (5% CO₂) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with penicillin, streptomycin, l-glutamate, sodium pyruvate, and nonessential amino acids. For G₁ synchronization by serum starvation, asynchronous cultures at confluence were placed in DMEM containing 0.1% FBS for 72 h. For continuous labeling, G₁ synchronized cells were trypsinized, reseeded at a density of 1.5 × 10^5 cells per 10-cm dish in DMEM with 10% FBS in a series of 6-cm culture dishes. Cells were counted daily with a hemocytometer.

Flow Cytometric Analysis—For cell size measurement, MEFs were trypsinized, washed with ice-cold PBS, and their forward scatter was measured by FACScan (Becton Dickinson) as soon as possible. For cell cycle analysis, MEFs at logarithmic growth were pulse-labeled with 10 μM BrdUrd (Sigma) for 30 min, trypsinized, and fixed in 70% ethanol. For continuous labeling, G₁ synchronized cells were trypsinized, replated at a density of 1.5 × 10^5 cells per 10-cm dish in DMEM with 10% FBS containing 65 μM BrdUrd, trypsinized after 3, 6, 9, 12, and 24 h, and fixed in 70% ethanol. Fixed cells were kept at −20 °C until analysis. Cells were treated with 2 N HCl to denature the DNA, followed by neutralization by sodium borate. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd antibodies (PharMingen) and counterstained with propidium iodide containing RNase (20 μg/ml). The stained cells were analyzed by FACScan. Cell debris and fixation artifacts were gated out, and G₁, S, and G₂/M populations were quantitated using CellQuest software. In each experiment, a similar number of events was analyzed.

Immunohistochemistry—PA28γ−/− and PA28γ−/− MEFs were cultured on a slide glass, fixed with 4% paraformaldehyde/PBS, quenched with 50 mM NH₄Cl, permeabilized with 0.2% Triton X-100/PBS for 30 min at room temperature, and blocked with BlockAce™ (Yukijirushi) overnight at 4 °C. Cells were then incubated with a primary antibody solution containing 0.1% Tween 20 for 1 h, washed in Triton X-100/PBS, and incubated with a secondary FITC-conjugated anti-rabbit antibody. As a control, nonimmune rabbit sera were used. Cells were embedded in SlowFade (Molecular Probes) mounting medium.

RESULTS

Targeted Disruption of the PA28γ Gene Creates a Null Mutation—The targeting construct was designed to delete the genomic fragment extending from exon 2 to exon 8 of the PA28γ gene (Fig. 1A). PCR screening of 600 colonies resistant to G418 revealed that three ES cell clones underwent homologous recombination. Southern blot analysis with the probe external to...
Weight was intermediate in PA28

Gates are indicated by

Stained with propidium iodide for DNA content and with an FITC-conjugated anti-BrdUrd antibody for measuring replicative DNA synthesis.

Approximately 10^4 cells were examined in each analysis, and experiments were repeated four times.

The percentage of cells that entered the S phase was significantly smaller in PA28^+/+ mice than in wild-type mice (data not shown).

Growth Properties of PA28^γ−/− MEFs in Vitro—MEFs were prepared from PA28^+/+ and PA28^γ−/− embryos 13.5 days postcoitum, and their growth properties were examined in vitro. At passage 4, PA28^γ−/− MEFs showed a significantly slower growth rate and a lower saturation density than their wild-type counterparts (Fig. 3A). Single MEF size was measured using flow cytometry as forward scatter intensity. PA28^γ−/− MEFs were slightly larger than wild-type ones (Fig. 3B), consistent with the observation that they showed lower saturation densities.

Entry into the S Phase Is Impeded in PA28^γ−/− MEFs—To investigate the cause of growth retardation, proliferating MEFs at the logarithmic phase were pulse-labeled with BrdUrd and analyzed by flow cytometry. As indicated in Fig. 4A, PA28^γ−/− cells showed an increase in the number of G_1 phase cells and a decrease in the number of S phase cells relative to wild-type cells. To confirm this observation, MEFs synchronized at the G_1 phase were continuously labeled with BrdUrd. The percentage of cells that entered the S phase was significantly smaller in PA28^γ−/− cells than in wild-type cells (Fig. 4B). These results indicate that entry into the S phase is slightly impeded in PA28^γ−/− MEFs.
Expression of PA28α and PA28β in PA28γ-deficient Cells—We next examined whether the absence of PA28γ influences the expression of PA28α or PA28β. Western blot analysis using MEF extracts showed that the levels of neither PA28α nor PA28β are altered by the deficiency of PA28γ (Fig. 5A). Under normal conditions, PA28α and PA28β are co-localized predominantly in the cytoplasm, whereas PA28γ occurs mainly in the nucleus (19, 20). However, PA28α has two possible nuclear localization signals (27). Therefore, it was of interest to examine whether the PA28α/β complex can enter the nucleus in the absence of PA28γ and compensate for the deficiency of PA28γ. As shown in Fig. 5B, immunohistochemical analysis of PA28γ−/− MEFs indicated that PA28γ is located predominantly in the nuclei excluding the nucleoli (upper right panel) as reported previously (19, 20). No significant staining was observed in PA28γ−/− cells (lower right panel). On the other hand, the subcellular distribution of PA28α was essentially the same between PA28γ−/− and PA28γ−/+ MEFs (upper and lower left panels). Thus, the PA28α/β heteroplymer is unlikely to take over the function of PA28γ in PA28γ-deficient cells.

DISCUSSION

In this report, we generated PA28γ-deficient mice by gene targeting (Fig. 1) and found that PA28γ affects body size, cell growth, and cell proliferation (Figs. 2 and 3). Flow cytometric analysis of MEFs (Fig. 4) revealed that the proportion of cells that entered the S phase was significantly lower in PA28γ−/− cells than in wild-type cells. The PA28γ homopolymer can associate with the 20 S proteasome (16) and stimulate the latent proteasome activity strongly (28). Thus, the phenotype of PA28γ-deficient mice raises the possibility that PA28γ might be involved in the degradation of nuclear proteins regulating cell cycle progression. For example, loss of PA28γ may delay the degradation of cyclin-dependent kinase inhibitors such as p21cip1 and p27kip1, the degradation of which is required for G1 to S transition in the cell cycle (29). Therefore, we examined the protein levels of p27 in PA28γ−/− and PA28γ−/+ MEFs by Western blot analysis but could not find any obvious difference (data not shown).

A series of recent studies has established that the PA28αβ complex is involved in the generation of MHC class II ligands (reviewed in Refs. 17, 18, and 22). Therefore, we investigated whether PA28γ-deficient mice show any abnormalities in their immune system. The cellularity and size of the spleen and thymus did not show any obvious difference between PA28γ−/− and wild-type mice (data not shown). Furthermore, disruption of the PA28γ gene did not alter the CD4/CD8 ratios in lymphocytes, the T/B cell ratio in peripheral lymphocytes, or the expression level of cell surface MHC class I molecules (data not shown). These results indicate that PA28γ is unlikely to play a pivotal role in the MHC class I antigen processing/presentation system. This is consistent with the observation that PA28γ has been found in invertebrates that do not have the adaptive immune system (26). However, the present study does not rule out the possibility that PA28γ plays a specialized role in antigen presentation such as the processing of antigens in the nucleus.

Because PA28γ localizes almost exclusively in the nucleus and is highly conserved between vertebrates and invertebrates, we supposed that PA28γ might have an essential role in maintaining cellular activities. Contrary to this initial expectation, PA28γ turned out to be dispensable. Subcellular localization of PA28γ, and hence, that of the PA28αβ complex did not change significantly between PA28γ-deficient and wild-type cells (Fig. 5). Thus, it appears unlike that dispensability of PA28γ ensues from the compensation provided by the PA28αβ complex. A better understanding of the biological functions of the PA28γ family proteins might be gained by creating mutant mice lacking all three members of this family. Such work is in progress in our laboratories.

REFERENCES

1. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
2. Gross, M., DitzeL, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) Nature 386, 463–469
3. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) J. Mol. Biol. 267, 10515–10525
4. Duhel, W., Pratt, G., Ferrell, K., and Rechsteiner, M. (1992) J. Biol. Chem. 267, 22369–22377
5. Baumeister, W., Walz, J., Zuhl, F., and Seemüller, E. (1998) Cell 92, 367–380
6. Rechsteiner, M. (1998) in Ubiquitin and the Biology of the Cell (Peters, J.-M., Harris, J. R., and Finley, D., eds) pp. 141–189, Plenum Publishing Corp., New York
7. DeMartino, G. N., and Slaughter, C. A. (1999) J. Biol. Chem. 274, 22123–22128
8. Tanaka, K. (1998) Biochem. Biophys. Res. Commun. 247, 537–541
9. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
10. Ciechanover, A. (1998) EMBO J. 17, 7151–7169
11. Gray, C. W., Slaughter, C. A., and DeMartino, G. N. (1994) J. Mol. Biol. 236, 7–15
12. Hendil, K. B., Khan, S., and Tanaka, K. (1998) Biochem. J. 332, 749–754
13. Ahn, J. Y., Tanahashi, N., Akiyama, K., Hisamatsu, H., Noda, C., Tanaka, K., Chung, C. H., Shihmara, N., Willy, P. J., Mott, J. D., Slaughter, C. A., and DeMartino, G. N. (1995) FEBS Lett. 366, 37–42
14. Song, X., Mott, J. D., von Kampen, J., Pramanik, B., Tanaka, K., Slaughter, C. A., and DeMartino, G. N. (1996) J. Biol. Chem. 271, 26410–26417
15. Zhang, Z., Krutchinsky, A., Endicott, S., Realiini, C., Rechsteiner, M., and Standing, K. G. (1999) Biochemistry 38, 5651–5658
16. Tanahashi, N., Yokota, K., Ahn, J. Y., Chung, C. H., Fujimura, T., Takahashi, E., DeMartino, G. N., Slaughter, C. A., Toyonoa, T., Yamamura, K., Shimbara, N., and Tanaka, K. (1997) Genes Cells 2, 195–211
17. Tanaka, K., and Kasahara, M. (1998) Immunol. Rev. 163, 161–176
18. Fruh, K., and Yang, Y. (1999) Curr. Opin. Immunol. 11, 76–81
19. Soza, A., Kneule, C., Groettrup, M., Henklein, P., Tanaka, K., and Kloeetz, P.-M. (1997) FEBS Lett. 413, 27–34
20. Wojcik, C., Pawelz, T., Tanaka, K., and Wilk, S. (1998) Eur. J. Cell Biol. 77, 151–160
21. Rock, K. L., and Goldberg, A. L. (1999) Annu. Rev. Immunol. 17, 739–779
22. Groettrup, M., Ruppert, T., Kuehn, L., Seeger, M., Stander, S., Koszinowski, U., and Kloeetz, P.-M. (1995) J. Biol. Chem. 270, 25688–25695
23. Groettrup, M., Soza, A., Eggars, M., Kuehn, L., Dick, T. P., Schild, H., Rammensee, H. G., Koszinowski, U. H., and Kloeetz, P. M. (1996) Nature 381, 166–168
24. Dick, T. P., Ruppert, T., Groettrup, M., Kloeetz, P. M., Kuehn, L., Koszinowski, U. H., Stevanovic, S., Schild, H., and Rammensee, H.-G. (1996) Cell 86, 253–262
25. Shimbara, N., Ogawa, K., Nakajima, H., Yasunaka, N., Hishita, Y., Nawa, S., Tanahashi, N., and Tanaka, K. (1998) J. Biol. Chem. 273, 23962–23971
26. Kandil, E., Kohda, K., Ishibashi, T., Tanaka, K., and Kasahara, M. (1997) Immunogenetics 46, 337–344
27. Kohda, K., Ishibashi, T., Shimbara, N., Tanaka, K., Matsuda, Y., and Kasahara, M. (1998) J. Immunol. 160, 4923–4935
28. Realiini, C., Jensen, C. C., Zhang, Z., Johnston, S. C., Knozowln, J. R., Hill, C. P., and Rechsteiner, M. (1997) J. Biol. Chem. 272, 25483–25492
29. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512