Harlan Sprague-Dawley (Indianapolis, IN). All mice used in these experiments were females; 4 to 6 weeks of age and were housed in specific pathogen-free conditions and handled in accordance with institutional guidelines. OVA-specific T_H1 and T_H2 cells were generated in vitro from DO11.10 splenocytes as described [C. S. Hsieh, S. E. Macatonia, A. O'Garra, K. M. Murphy, *J. Exp. Med.* **181**, 713 (1995); D. A. Randolph, C. J. Carruthers, S. J. Szabo, K. M. Murphy, D. D. Chaplin, *J. Immunol.* **162**, 2375 (1999)]. On day 7 after culture with antigen under polarizing conditions, cells were frozen in fetal calf serum containing 10% dimethyl sulfoxide. Before transfer into recipient mice, cells were thawed and restimulated with 0.3 μM OVA (323–339) peptide and irradiated BALB/c splenocytes. Three days later, the cells were diluted 1:4 in medium supplemented with IL-2 (40 U/ml), and used for passive transfer 7 days after thawing. The polarized cytokine profiles of each batch of cells were confirmed by intracellular cytokine staining or by enzyme-linked immunosorbent assay (ELISA) with Quantikine ELISA kits (R&D Systems, Minneapolis, MN). Naïve $CD4^+$ T cells were prepared by generating single-cell suspensions from the LNs and spleens of RAG-2–deficient DO11.10 TCR transgenic mice and centrifuging the cells over a Histopaque-1119 gradient to eliminate erythrocytes and dead cells. The resulting cells were >70% $CD4^+$, and of these >85% were L-selectin$^+$ naïve cells. For passive transfer, 2×10^7 T cells were washed, resuspended in 0.25 ml of phosphate-buffered saline (PBS) and then injected intravenously (iv) into mice that had been anesthetized with Metofane. As indicated, some mice were also injected with OVA (100 μg) in 50 μl of incomplete Freund's adjuvant administered intraperitoneally or subcutaneously in the footpad. In some experiments, T cells were incubated with pertussis toxin (100 ng/ml) for 2 hours at 37°C and then washed five times before transfer.

13. Spleens and LNs were embedded in OCT (Tissue Tek, Elkhart, IN), frozen on dry ice, and then stored at −70°C. Cryosections were cut, air-dried, and fixed in acetone for 10 min. Slides were incubated for 15 min in blocking solution (PBS with 0.5% Tween-20 and 3% normal goat serum) containing avidin (four drops per milliliter; Vector Labs, Burlingame, CA) followed by 15 min in blocking solution containing biotin (four drops per milliliter; Vector Labs). Antibody staining was by incubation for 1 hour in blocking solution containing biotinylated KJ1-26 antibody and fluorescein isothiocyanate (FITC)–B220 (Pharmingen, San Diego, CA). The slides were washed in PBS with 0.5% Tween-20 and then incubated for 20 min in 0.5% H_2O_2 in methanol to quench endogenous peroxidase activity. Color development was for 1 hour with the AP-ABC reagent (Vector Labs) together with horseradish peroxidase–anti-FITC (Sigma). Alkaline phosphatase activity was detected with the Vector Blue Substrate Kit (Vector), and peroxidase activity was detected with Sigma Fast diaminobenzidine substrate (Sigma).
14. RNA was prepared from freshly isolated naïve $CD4^+$ T cells or from T_H1 and T_H2 cells 7 days after initiation of culture under polarizing conditions with guanidine isothiocyanate–CsCl [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)]. Ribonuclease protection assays were performed with 10 μg of RNA (RiboQuant kit, Pharmingen) with the mCR-5 and mCR-6 template sets. For Northern (RNA) blot analysis, 40-μg samples of RNA were fractionated with 1% agarose gel in formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham Life Science). Gene expression was detected with radiolabeled probes (Multiprime system, Amersham Life Science). For CXCR3, the entire coding region was used as a probe. For CCR7, a 415–base pair Nco I–Hind III fragment was used. Hybridization was at 42°C in 50% formamide and washing was in 0.2× standard saline citrate at 42°C.
15. D. Randolph and D. Chaplin, unpublished data.
16. Cells (2×10^7 per milliliter) were incubated in 3 μM Fura-2 AM (Molecular Probes, Eugene, OR) for 20 min in T cell medium at 37°C in the dark. The suspensions were diluted 10-fold in medium and incubated an additional 20 min, then washed three times in fluorimetry buffer [25 mM Hepes, 1 mM $CaCl_2$, 125 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.5 $MgCl_2$, 0.1% glucose, 0.1% BSA (pH 7.4)] and stored on ice until use. The cells were equilibrated briefly at 37°C and then either SLC or Mig (Peprotech, Rocky Hill, NJ) was added. Fluorescence was measured and the average bulk intracellular Ca^{2+} concentrations were calculated with a Hitachi F2000 fluorimeter as described [G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440 (1985)]. After the intracellular Ca^{2+} concentration had returned to a stable baseline, 10 μM ionomycin, 0.1% Triton-X 100, and 4 mM EGTA were added sequentially as controls.
17. Chemokine receptor cDNAs were amplified from T_H1 and T_H2 RNA with the Titan One Tube RT-PCR System (Boehringer Mannheim). Forward and reverse primers for CCR7 were GAGACTCGAGAGAGCACCATGGACCCAGG and GAGAGAATTCCTACGGGGAGAAGGTTGTGG and for CXCR3 were GAGACTCGAGATGTACCTTGAGGTTAGTGAACG and GAGAGAATTCGAATTACAAGCCCAGGTAGG (underlined nucleotides designate Xho I or Eco RI sites). Primer sequences for other chemokine receptors tested are available on request. Polymerase chain reaction products were purified by gel electrophoresis, digested with Xho I and Eco RI, and cloned into the Xho I and Eco RI sites of the retroviral plasmid hCD4-RV (provided by T. Murphy, Washington University School of Medicine). hCD4-RV is similar to green fluorescent protein (GFP)–RV as described [S. Ranganath *et al.*, *J. Immunol.* **161**, 3822 (1998)], but with truncated human CD4 as a selectable marker, rather than GFP. Retroviral stocks were prepared with the Phoenix cell packaging line (provided by K. M. Murphy, Washington University School of Medicine) according to the protocol of G. Nolan (Stanford University). Primary T cells from DO11.10/RAG-$2^{-/-}$ mice were activated under polarizing conditions with OVA (323–339) peptide and infected 24 hours later with retroviral supernatants and polybrene (6 μg/ml, Sigma). Six days after activation, transduced cells were purified with FITC–anti-huCD4 followed by anti-FITC MicroBeads on a MiniMACS column (Miltenyi Biotec, Auburn, CA). Purified huCD4-expressing cells were restimulated with OVA peptide and irradiated BALB/c splenocytes and either used directly 7 days later or frozen for subsequent use.
18. Mig has previously been reported to be preferred over SLC as a ligand for CXCR3 [H. Soto *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8205 (1998)].
19. D. Randolph, G. Huang, D. Chaplin, unpublished data.
20. For measurement of T cell help in vivo, primed B cells were prepared in BALB/c mice by immunization with NP-BSA (100 μg) in complete Freund's adjuvant. Ten days later, splenic B cells were purified with anti-CD43 magnetic beads on a MidiMACS column (Miltenyi Biotec). Purified B cells (1×10^7) were transferred to naïve, sublethally irradiated (400 rads) BALB/c mice either alone or with 10^5 OVA-specific control T_H2 cells or with 10^5 CCR7-transduced T_H2 cells. The recipient mice were immunized ip with NP-OVA (100 μg) adsorbed to 2 mg of alum in 0.5 ml of PBS. Seven days later, anti-NP IgG1 titers were measured in sera by ELISA. For evaluation of helper function in vitro, 10^6 primed B cells were incubated at 37°C in wells on a 96-well plate with 1×10^5 control transduced or CCR7-transduced T_H2 cells in T cell medium containing either NP-OVA (10 μg/ml) or NP–keyhole limpet hemocyanin (10 μg/ml). After 2 days, the medium containing the antigen was removed and replaced with fresh medium. Five days later, culture supernatants were collected and anti-NP IgG1 titers were measured.
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25. We thank T. Murphy and K. Murphy for the retroviral vectors and valuable advice, and O. Kanagawa for the DO11.10/RAG-$2^{-/-}$ mouse strain. Supported by NIH grants T32 GM07200 (to D.A.R.) and AI34580 (to D.D.C.). D.D.C. is an investigator of the Howard Hughes Medical Institute.

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Impaired Immunoproteasome Assembly and Immune Responses in *PA28*$^{-/-}$ Mice

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In vitro PA28 binds and activates proteasomes. It is shown here that mice with a disrupted *PA28b* gene lack PA28a and PA28b polypeptides, demonstrating that PA28 functions as a hetero-oligomer in vivo. Processing of antigenic epitopes derived from exogenous or endogenous antigens is altered in *PA28*$^{-/-}$ mice. Cytotoxic T lymphocyte responses are impaired, and assembly of immunoproteasomes is greatly inhibited in mice lacking PA28. These results show that PA28 is necessary for immunoproteasome assembly and is required for efficient antigen processing, thus demonstrating the importance of PA28-mediated proteasome function in immune responses.

Cytotoxic T cells eliminate infected cells by recognizing foreign antigens processed in a proteasome-dependent manner and presented by class I molecules of the major histocompatibility complex (MHC) (*1*). The peptidase activities of the proteasome can be activated in vitro by the interferon-inducible proteasome regulators PA28a and PA28b (*2, 3*). A role for PA28 in MHC class I antigen presentation has been suggested (*3, 4*). However, the underlying mechanism by which PA28 influences antigen processing via a protea-

some-mediated pathway remains elusive.

In mice, there are at least two functional copies of *PA28a*, whereas *PA28b* has only one functional copy (*5*). We therefore decided to generate mice with a disrupted *PA28b* gene [Web figure 1 (*6*)]. The availability of $PA28b^{-/-}$ mice also allowed us to address the question of whether PA28a by itself plays a role in vivo. We found that while PA28a and PA28b remain associated with the proteasomes in wild-type cells, under identical conditions no proteasome-associated PA28a and PA28b polypeptides were detected in $PA28b^{-/-}$ cells (Fig. 1A). Immunoblotting with PA28b-specific polyclonal antibodies confirmed that PA28b is not expressed in $PA28b^{-/-}$ mice (Fig. 1C). Surprisingly, even upon interferon induction, the expression of the PA28a polypeptide was not detected, or was at very low levels, under either native (Fig. 1B) or denaturing (Fig. 1C) conditions in $PA28b^{-/-}$ mice. Thus, PA28 functions as a hetero-oligomer in vivo, ruling out an in vivo role for PA28a as a homo-heptamer (*7*). These findings led us to conclude that $PA28b^{-/-}$ mice have a functional phenotype equivalent to mice defective in both *PA28a* and *PA28b* loci.

We next examined the effect of PA28 deficiency on the expression of immune molecules with focus on MHC class I molecules [Web figure 2 (*6*)]. To determine the quality of peptides bound to class I molecules we examined the stability of class I heterodimers by a class I thermostability assay (*8*). We found that class I molecules expressed in $PA28^{-/-}$ cells were less stable than in wild-type cells (*9*), suggesting the acquisition of peptides with low affinity. Indeed, a major difference in the spectrum of peptides presented by class I molecules between interferon-treated wild-type and $PA28^{-/-}$ cells was observed, revealing a 70% reduction in the production of hydrophobic peptides (eluants at 30 to 35% of acetonitrile) in $PA28^{-/-}$ cells (Fig. 2A). The finding that interferon treatment does not diminish the PA28 deficiency strongly suggests that PA28 is required for MHC antigen presentation.

To study the influence of PA28 on the generation of cytotoxic T lymphocyte (CTL) epitopes, we monitored the generation of two dominant epitopes, Ova257-264 and NP366, derived from the antigens ovalbumin and influenza nuclear protein, respectively. Ovalbumin was introduced into the cytoplasm of LPS blasts made from wild-type and $PA28^{-/-}$ mice and the presentation of the class I K^b-restricted Ova257-264 CTL epitope was assayed. Flow cytometry analysis of the ovalbumin-loaded LPS blasts with a monoclonal antibody (25D1.16) recognizing the class I K^b-β2m-Ova257-264 trimer (*10*) showed that, contrary to wild-type cells, $PA28^{-/-}$ cells fail to present the Ova257-264 epitope (*10*). The lack of Ova257-264 epitope presentation in $PA28^{-/-}$ mice was confirmed by CTL assays, demonstrating that the Ova257-264–specific T cell clone B3 (*11*) lysed the ovalbumin-loaded LPS blasts from wild-type but not $PA28^{-/-}$ mice (Fig. 2B). We also found that the subdominant ovalbumin epitope Ova55-72 was not presented by $PA28^{-/-}$ LPS blasts (*12*). Because difference in CTL responses was not observed when cells were exogenously loaded with synthetic Ova257-264 peptide (*12*), the possibility that $PA28^{-/-}$ cells are inefficient CTL targets was eliminated. Furthermore, when wild-type and

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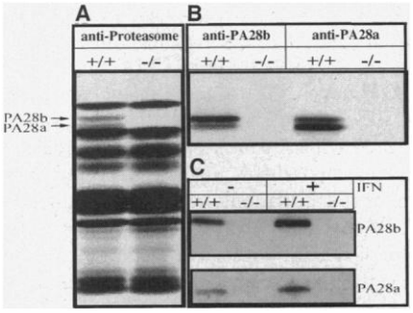

Fig. 1. Expression of PA28a and PA28b. (**A**) Physical association of PA28 and the proteasome. Splenocytes from wild-type (+/+) and $PA28^{-/-}$ (–/–) mice were metabolically labeled for 2 hours followed by a 4-hour chase in the presence of the proteasome inhibitor lactacystin at a concentration of 10 μmol/ml and lysed with a buffer containing 1% digitonin. Co-immunoprecipitations were performed using an antiserum specific for proteasome subunit C9 (*3*). (**B**) Immunoprecipitation analysis of PA28 expression. Splenocytes were metabolically labeled for 60 min and lysed with a buffer containing 1% NP-40. Immunoprecipitations were carried out with PA28b- or PA28a-specific antisera (*3*). (**C**) Immunoblot analysis of PA28 expression. After a 24-hour incubation in the presence (+) or absence (–) of interferon at 500 U/ml, splenocytes were lysed in SDS sample buffer. The lysates were separated by SDS-PAGE, electro-blotted, and probed with PA28a- (*18*) or PA28b-specific antisera (*3*).

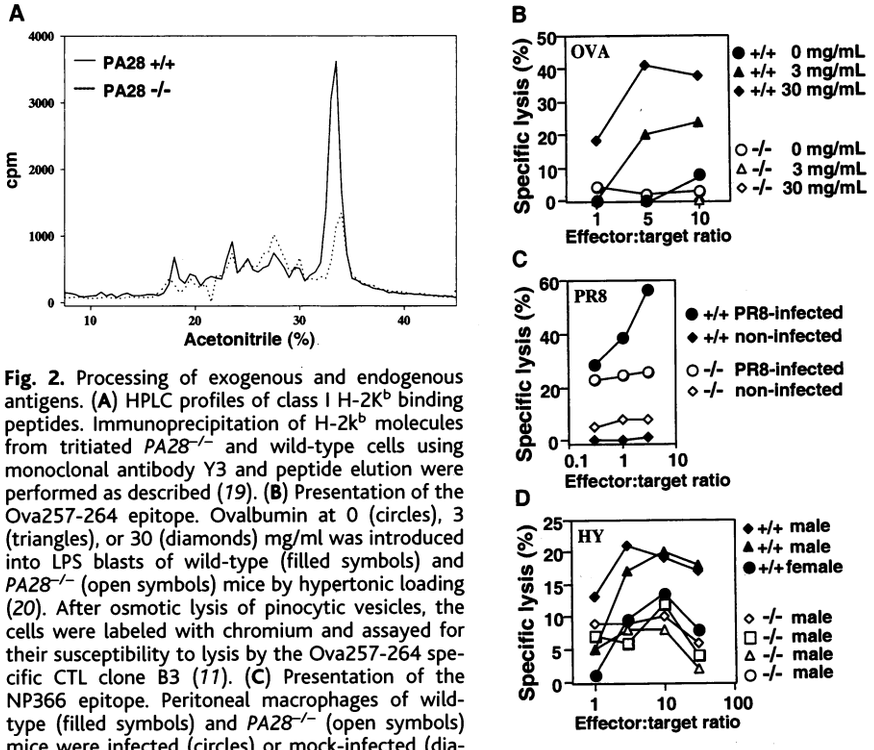

Fig. 2. Processing of exogenous and endogenous antigens. (**A**) HPLC profiles of class I H-$2K^b$ binding peptides. Immunoprecipitation of H-$2k^b$ molecules from tritiated $PA28^{-/-}$ and wild-type cells using monoclonal antibody Y3 and peptide elution were performed as described (*19*). (**B**) Presentation of the Ova257-264 epitope. Ovalbumin at 0 (circles), 3 (triangles), or 30 (diamonds) mg/ml was introduced into LPS blasts of wild-type (filled symbols) and $PA28^{-/-}$ (open symbols) mice by hypertonic loading (*20*). After osmotic lysis of pinocytic vesicles, the cells were labeled with chromium and assayed for their susceptibility to lysis by the Ova257-264 specific CTL clone B3 (*11*). (**C**) Presentation of the NP366 epitope. Peritoneal macrophages of wild-type (filled symbols) and $PA28^{-/-}$ (open symbols) mice were infected (circles) or mock-infected (diamonds) with influenza virus PR8 during a 1-hour labeling with chromium. Susceptibility of macrophages to lysis by an NP366-specific CTL clone (*21*) at the indicated effector:target ratios was assayed. (**D**) Presentation of the male self-antigen HY. HY-specific CTLs as effectors were prepared from female HY transgenic splenocytes and restimulated as described (*22*). LPS blasts from male wild-type mice (filled diamond and filled triangle) or male $PA28^{-/-}$ mice (open symbols) were used as targets. As a negative control, LPS blasts from a female wild-type mouse (filled circle) were used.

PA28$^{-/-}$ cells were infected with influenza virus, *PA28*$^{-/-}$ cells were again significantly less sensitive to lysis by NP366-specific CTLs than wild-type cells (Fig. 2C). These findings further demonstrate that, without PA28, the presentation of these CTL epitopes is severely impaired.

The processing of endogenous antigens was further investigated by comparing the ability of LPS blasts from wild-type and *PA28*$^{-/-}$ mice to process and present the male self-antigen HY (Fig. 2D). The CD8-enriched splenocytes from female mice bearing a transgenic T cell receptor specific for the D^b-restricted HY antigen were used to generate CTLs. HY-specific CTLs lysed male wild-type LPS blasts but they failed to lyse male *PA28*$^{-/-}$ and female wild-type cells, demonstrating that processing of endogenous HY self-antigen is severely impaired in *PA28*$^{-/-}$ cells. Thus, PA28 is important for both the processing of exogenous and endogenous antigens.

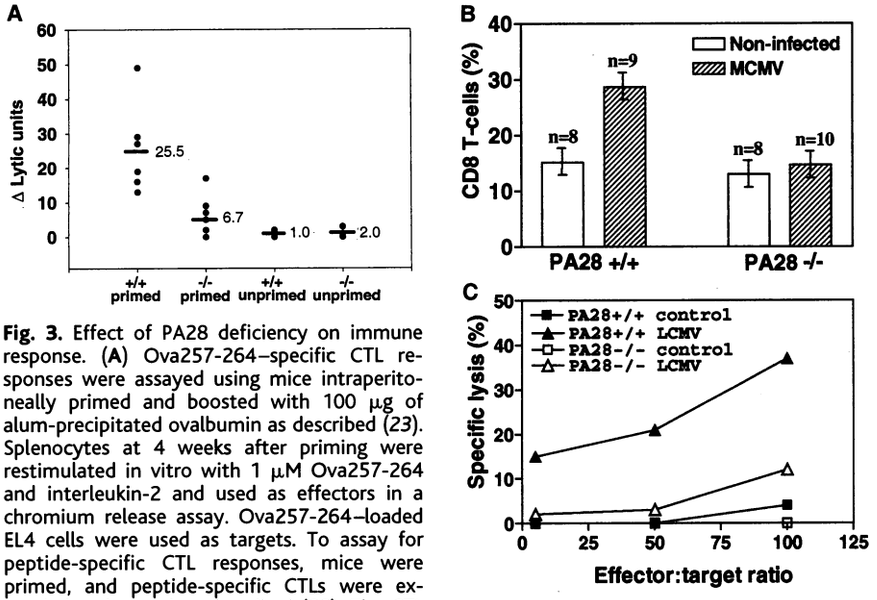

Fig. 3. Effect of PA28 deficiency on immune response. (**A**) Ova257-264–specific CTL responses were assayed using mice intraperitoneally primed and boosted with 100 μg of alum-precipitated ovalbumin as described (*23*). Splenocytes at 4 weeks after priming were restimulated in vitro with 1 μM Ova257-264 and interleukin-2 and used as effectors in a chromium release assay. Ova257-264–loaded EL4 cells were used as targets. To assay for peptide-specific CTL responses, mice were primed, and peptide-specific CTLs were expanded and tested as described (*22*). The CTL responses of nonprimed mice were analyzed in the same fashion. To facilitate comparison of responses among experiments, the percent release values were transformed into net lytic units as indicated. (**B**) MCMV-induced CD8 T cell response. Splenic T cells from wild type and *PA28*$^{-/-}$ mice that were infected or mock-infected with MCMV (Smith strain VR-1399 from American Type Culture Collection) for 7 days were stained with anti-CD8 antibodies (*18*) and analyzed by flow cytometry. Error bars indicate SEM. (**C**) LCMV-induced CTL response. At 6 days after infection with LCMV [Armstrong strain (*14*)], splenocytes from wild-type (filled symbols; $n = 5$) and *PA28*$^{-/-}$ (open symbols; $n = 6$) mice were used as effector cells. Susceptibility of LCMV-infected (triangles; LCMV) or noninfected (squares; control) target cells [MC57 fibroblasts (*14*)] to lysis by the effector cells at the indicated effector:target ratios was assayed.

To determine the consequence of these antigen processing defects in the generation of an immune response, wild-type and *PA28*$^{-/-}$ mice were immunized with ovalbumin or Ova257-264 peptide and assayed for the Ova257-264–specific CTL response (Fig. 3A). Almost identical CTL responses were observed when *PA28*$^{-/-}$ mice and wild-type littermates were primed with the Ova257-264 peptide (*12*). In contrast, the CTL response in ovalbumin-primed *PA28*$^{-/-}$ mice was significantly lower than in ovalbumin-primed wild-type littermates, demonstrating that PA28 deficiency results in an impaired priming of CTL in vivo.

Two well-characterized infectious disease models, murine cytomegalovirus (MCMV) (*13*) and lymphocytic choriomeningitis virus (LCMV) (*14*), were also used to examine how in vivo immune responses to pathogens are affected by a lack of PA28. Splenocytes from wild-type and *PA28*$^{-/-}$ mice that were either uninfected or infected with MCMV for 7 days were stained with CD8 antibodies (Fig. 3B). Without MCMV infection, CD8-positive T cells in wild-type and *PA28*$^{-/-}$ mice were 15% and 14% of total splenic T cells, respectively. After MCMV infection, CD8-positive T cells increased to 28% in wild-type mice, whereas CD8-positive T cells in *PA28*$^{-/-}$ mice remained almost unchanged at 15%. In the LCMV infectious disease model (Fig. 3C), the results showed that 37% and 12% of specific lysis were achieved with T cells from LCMV-infected wild-type and *PA28*$^{-/-}$ mice, respectively, demonstrating that only a third of wild-type LCMV-specific CD8 T cell activity was generated in LCMV-infected *PA28*$^{-/-}$ mice. These data demonstrate that the antigen processing defects in *PA28*$^{-/-}$ mice result in impaired CTL responses and that PA28 is required for proper immune function during pathogen infection.

Because PA28 has been shown to interact with proteasomes in vivo (*3*), we examined whether PA28 deficiency results in an alteration of proteasome subunit composition. We found that, compared to wild-type proteasomes, the interferon-inducible catalytic subunits LMP2/7 and MECL1 (*1*) were almost completely absent in the *PA28*$^{-/-}$ proteasomes, whereas the constitutively expressed catalytic subunits X, Y, and Z remained present (Fig. 4, A and C). Whereas X, Y, and Z subunits were rapidly displaced by LMP2/7 and MECL in wild-type proteasomes (Fig. 4A), only a small fraction of X subunit was displaced by LMP7 in *PA28*$^{-/-}$ proteasomes after a 4-hour chase (Fig. 4, A and C). Fur-

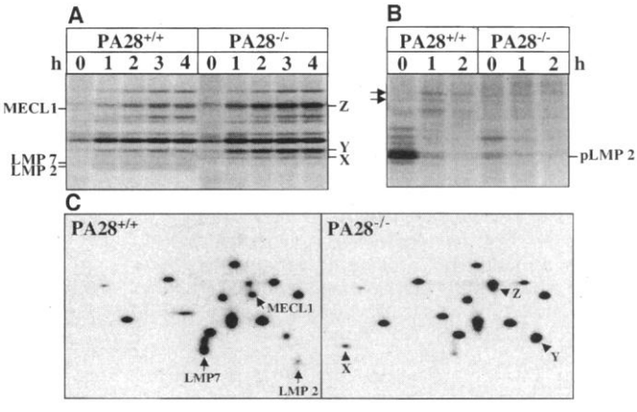

Fig. 4. PA28 promotes assembly of immunoproteasomes. (**A** and **B**) At 24 hours after interferon induction, *PA28*$^{-/-}$ and wild-type cells were metabolically labeled for 30 min and chased in the presence of lactacystin for the indicated times (in hours). Immunoprecipitations were performed with (A) C9- or (B) LMP2-specific antiserum (*3*). Anti-LMP2 antiserum has been shown to recognize 15S immunosubunit precursors containing proteasome assembly intermediates via LMP2 precursor (*3*). PA28a and PA28b are indicated with arrows. LMP2 precursor is labeled as pLMP2. (**C**) As in (A), the anti-C9 immunoprecipitates from cells that were subjected to a 4-hour chase were analyzed by two-dimensional gel electrophoresis as described (*3*). It was noted that lactacystin treatment not only stabilizes the association of proteasome and PA28 but also results in increased and decreased electrophoretic mobilities of LMP7 and LMP2, respectively [Web figure 3 (*6*)]. Proteasome catalytic subunits, which were identified by two-dimensional gel electrophoresis and immunoblotting with catalytic subunit-specific antisera (*3*), are indicated.

thermore, while immunosubunit precursors-containing 15*S* proteasome assembly intermediates were detected in wild-type cells, under identical conditions 15*S* complexes were hardly detectable in *PA28*$^{-/-}$ cells (Fig. 4B). Notably, PA28 was found to be associated with the immunosubunit precursor-containing 15*S* complexes, suggesting that PA28 is required for the incorporation of immunosubunits into proteasomes.

It has been demonstrated that immunosubunits are required for efficient antigen processing (*1*) and that, in vitro, PA28 does not preferentially activate immunoproteasomes and does not alter proteasome substrate specificity (*15*). Thus, our findings strongly suggest that the inability to generate immune responses in *PA28*$^{-/-}$ mice was a consequence of a decreased cellular level of immunoproteasomes. Taken together with the fact that, in PA28-expressing cells, immunoproteasomes have greater stability than proteasomes not containing immunosubunits (*3, 12*) and that PA28 interacts with the α-subunit ring of the proteasome (*1*), we hypothesize that, by inducing conformational changes of the proteasome α-subunit ring (*3, 16*), PA28 promotes immunoproteasome assembly. PA28 might facilitate recruitment and assembly of the immunosubunit-containing proteasome β-subunit ring onto the α-subunit ring, which serves as a scaffold for the proteasome β-subunit ring formation and Ump1p-mediated maturation (*17*). As a consequence of increased cellular level of immunoproteasomes, MHC class I antigen processing and presentation are greatly enhanced by PA28.

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9. Splenocytes were metabolically labeled for 60 min followed by a 60-min chase. NP-40 cell lysates were incubated at 31°, 37°, and 43°C for 45 min before being subjected to immunoprecipitation with anti-K^b or D^b. The amounts of class I molecules were quantified. It was found that while an equivalent amount of class I molecules was present in wild-type and *PA28*$^{-/-}$ splenocytes before temperature challenge, the amount of class I molecules in *PA28*$^{-/-}$ cells after the temperature challenge decreased to ~80% of the wild-type levels.
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18. Antisera specific to the proteasome subunit C9, LMP2, PA28a, and PA28b have been described (*3*). Antibodies specific to CD4, CD8, CD3-e, TCR-ab, CD23, CD25, CD28, CD45, CD69, class I, and class II molecules were purchased from PharMingen (San Diego, CA). 25D1.16 (*10*) and a PA28a-specific antiserum (*2*) were kindly provided by A. Porgador and M. Rechsteiner, respectively. Metabolic labeling, immunoprecipitation, immunoblotting, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described (*3*).
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Global Transposon Mutagenesis and a Minimal Mycoplasma Genome

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Mycoplasma genitalium with 517 genes has the smallest gene complement of any independently replicating cell so far identified. Global transposon mutagenesis was used to identify nonessential genes in an effort to learn whether the naturally occurring gene complement is a true minimal genome under laboratory growth conditions. The positions of 2209 transposon insertions in the completely sequenced genomes of *M. genitalium* and its close relative *M. pneumoniae* were determined by sequencing across the junction of the transposon and the genomic DNA. These junctions defined 1354 distinct sites of insertion that were not lethal. The analysis suggests that 265 to 350 of the 480 protein-coding genes of *M. genitalium* are essential under laboratory growth conditions, including about 100 genes of unknown function.

One important question posed by the availability of complete genomic sequences (*1–3*) is how many genes are essential for cellular life. We are now in a position to approach this problem by rephrasing the question "What is life?" in genomic terms: "What is a minimal set of essential cellular genes?"

Interest in the minimal cellular genome predates genome sequencing [for a review, see (*4*)]. The smallest known cellular genome (*5*) is that of *Mycoplasma genitalium*, which is only 580 kb. This genome has been completely sequenced, and analysis of the sequence revealed 480 protein-coding genes plus 37 genes for RNA species (*2*).

The fraction of nonminimal genomes that is essential for cell growth and division has been experimentally measured in yeast (12%) and in the bacterium *Bacillus subtilis* (9%) (*6*). The indispensable portion of the *B. subtilis* genome was estimated to be 562 kb, close to the size of the *M. genitalium* genome. Theoretical approaches to defining a minimal gene set have also been attempted. With the availability of the first two complete genome sequences (*Haemophilus influenzae* and *M. genitalium*) and the assumption that genes conserved across large phylogenetic distances are likely to be essential, a minimal gene set of 256 genes was proposed (*7*).

Mycoplasma pneumoniae is the closest known relative of *M. genitalium,* with a genome size of 816 kb, 236 kb larger than that of *M. genitalium* (*3*). Comparison of the two genomes indicates that *M. pneumoniae* in-

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References and Notes

[8] **Empty and Peptide-Containing Conformers of Class I Major Histocompatibility Complex Molecules Expressed in Drosophila melanogaster Cells**

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