Most antigenic peptides presented on major histocompatibility complex class I molecules are generated by proteasomes. Interferon-$\gamma$ (IFN-$\gamma$), which stimulates antigen presentation, induces new proteasome $\beta$-subunits LMP2 and LMP7, which replace the homologous $\beta$-subunits Y ($\delta$) and X ($\epsilon$). As a result, the capacity of the proteasome to cleave model peptides increases after hydrophobic and basic residues and falls after acidic residues. To clarify the function of these subunits, we examined the effects of overexpressing subunits X ($\delta$) and Y ($\epsilon$). Transfection of the Y gene into HeLa cells stimulated the proteasomal cleavage after acidic residues without altering other peptidase activities. This effect was proportional to the amount of the Y subunits and opposite to the effect of its homolog, LMP2. Y appears to promote cleavages after acidic residues. Furthermore, in mutants lacking the LMP genes (in contrast to wild-type cells), interferon-$\gamma$ treatment increased the proteasome content of Y subunits and enhanced postacidic cleavages. Transfection with CDNA for the X subunit reduced hydrolysis after hydrophobic and basic residues, an effect opposite to transfection of LMP2 and LMP7. Surprisingly, transfection of X increased the amounts not only of X, but also of Y, while decreasing LMP2 content. Thus, the loss of the Y subunit upon interferon-$\gamma$ treatment or LMP2 transfection accounts for the suppression of postacidic cleavages, and the loss of X contributes to the increased hydrolysis after hydrophobic and basic residues. These adaptations should favor the production of the kinds of peptides that are presented on major histocompatibility complex class I molecules.

Antigenic peptides derived from intracellular proteins are continuously being presented to the immune system in association with MHC class I molecules on the surface of most mammalian cells. If a peptide is displayed to which the immune system is not tolerant (e.g. a viral peptide), the cell is destroyed by cytolytic CD$^8^+$ lymphocytes (1, 2). Antigen presentation thus requires the continuous proteolytic digestion of intracellular proteins to oligopeptides, some of which are transported by the TAP transporter into the endoplasmic reticulum. Peptides of 8–9 residues then bind to appropriate MHC class I molecules and are transported to the cell surface (1, 2).

Recent studies have demonstrated that proteasomes degrade the bulk of cytosolic proteins and thus generate the great majority of antigenic peptides (3). The 20 S particle functions as the proteolytic core of the 26 S (2000 kDa) proteasome complex, which catalyzes the ATP-dependent degradation of ubiquitin-conjugated proteins (4–6). The 20 S particle contains 28 distinct but related subunits arranged in four superimposed rings (7, 8). The two internal rings are composed of seven related $\beta$-subunits, which contain the proteolytic active sites (4–6, 9–11). The mammalian proteasome exhibits at least five distinct peptidase activities, including ones which preferentially cleave model peptides on the carboxyl side of large hydrophobic residues (the "chymotrypsin-like" activity), basic residues (the "trypsin-like" activity), and acidic amino acids (the postglutamyl cleavages) (12). It is still unclear which peptidase activities are associated with which $\beta$-subunit(s).

These endopeptidase activities are differentially regulated by the cytokine interferon-$\gamma$ (IFN-$\gamma$) as part of its mechanism for stimulation of antigen presentation (13, 14). Although two groups have reported discrepant results (15, 16), it is now well established that proteasomes isolated from IFN-$\gamma$-treated cells cleave peptide substrates with hydrophobic or basic carboxyl termini faster than those of control cells (17–20). By contrast, the postglutamyl peptidase activity was reduced after IFN-$\gamma$ treatment (17–20). These IFN-$\gamma$-induced modifications are of particular interest, since they should enhance the production from proteins of peptides whose carboxyl termini are hydrophobic or basic residues. Such peptides are preferentially transported into the endoplasmic reticulum by the TAP transporters (2) and bind selectively to MHC class I molecules (21).

These IFN-$\gamma$-induced changes in peptidase activity result from the expression and incorporation into proteasomes of the MHC-encoded $\beta$-subunits LMP2 and LMP7 (22–24). These effects do not occur in mutant lymphoblasts (721.174) that lack LMP2 and LMP7 due to a deletion in the MHC region (17). In addition, cells transfected with the LMP7 gene, proteasomes exhibit increased chymotrypsin- and trypsin-like activities (20). Moreover, the degree of enhancement of the chymotrypsin-like activity is proportional to the amount of LMP7 subunits actually incorporated in the proteasome fraction (20). Transfection of cells with the LMP2 gene increases the basic activity and suppresses the postglutamyl activity (20). LMP2 and LMP7 are not obligatory for class I antigen presentation (25–27) but are important in this process. Macrophages and spleen cells from mutant mice with a deletion of LMP2 (28) or LMP7 (29) exhibit defects in antigen-specific T-cell responses.

The total cellular content of proteasomes does not change...
upon IFN-γ treatment but their subunit composition is altered. The LMPs expressed after IFN-γ stimulation are incorporated into β-rings in places generally occupied by homologous subunits. Moreover, in proteasomes from IFN-γ-treated cells, two subunits Y and X (30) are missing or greatly reduced in amount. Subunit Y (19, 30), which was named by others as the β-subunit (31) or subunit 10 (32) or MB1 (33), has about 69% identity with LMP7 (30). In addition, upon IFN-γ treatment, one other β-subunit, Z, is replaced by its homolog, MECL-1 (19), which is not encoded in the MHC region.

To clarify further the basis for the functional changes induced by IFN-γ, we have attempted to define the catalytic functions associated with the subunits lost upon IFN-γ treatment. Since incorporation of LMP2 in place of Y reduces cleavage of acidic substrates and for Western blotting. Hydrolysis of substrates (see below). Cells were routinely grown in the presence of 5 units/ml hygromycin to maintain expression of the transfected gene. In Western blotting (see below). As expected, this subunit was present in the proteasomes isolated from transfected HeLa cells, but proteasomes from the transfected cells (two independent clones) contained from 31% to 93% more Y subunits than controls, as assessed by quantitative Western blot of the proteasome fraction. However, the total cellular content of proteasomes did not differ significantly between the control cells and transfectants. Therefore, some particles from transfected cells must have contained more Y subunits than proteasomes from controls. We then compared the peptidase activities of preparations from many cultures of control and transfected cells. The maximal postglutamyl peptidase activity (Vₘₐₓ) of proteasomes from Y-transfected cells as measured with the substrate, Cbz-LLE-βNA, was significantly higher (34%, p < 0.001) than that of proteasomes from control cells (Table I). In contrast, the "chymotrypsin-like" activity (assayed with Suc-LLVY-MCA) and "trypsin-like" activity (assayed with Boc-LRR-MCA) were not affected by the transfection of Y (Table I). Thus, after IFN-γ treatment, the disappearance of the Y (β) subunit from proteasomes (19, 30) may account for the reduction in the postglutamyl peptidase activity (17, 19).

The vector used for transfection in these experiments was carried as a low-copy episome and was maintained in cells by selection for hygromycin resistance (i.e., expression of hygromycin phosphotransferase). When the transfectant cell lines were grown without the drug, the levels of subunit Y in their proteasomes fell in some cells to even lower levels than in control cells. The proteasomes that contained less Y than controls cleaved the acidic substrate at a significantly (p < 0.01) lower rate (Vₘₐₓ = 0.71 ± 0.05 mmol×mg proteasome⁻¹×h⁻¹; n = 8) than controls (Vₘₐₓ = 0.95 ± 0.06; n = 8). These variations in subunit Y content of proteasomes in different clones allowed us to examine in the same cells the effects of different amounts of Y on proteasome function. A strong correlation was found between the minimal capacities of proteasomes to cleave the postglutamyl bond and the amounts of subunit Y incorporated into these particles (r = 0.95; Fig. 1). As the amount of Y in proteasomes from transfected cells increased from 70 to 150% of the level in control cells, there was a proportional rise in the capacity to catalyze postglutamyl cleavages. No further increase in this activity was seen in preparations that had even higher levels of subunit Y; the reason for this plateau in activity is unclear at present.
Interestingly, supranormal levels of Y were not associated with any alterations in the chymotrypsin-like and trypsin-like activities. The maximal capacity of the preparations with subnormal amounts of Y to hydrolyze the other model substrates was also reduced. Cleavage of the hydrophobic substrate was 29% lower than in control cells (Table II). Proteasomes from control cultures (quantified by Western blotting and phosphorimaging; see under “Materials and Methods”) after transfection, the amount of Y in control cells was taken as 100%. The regression coefficient for the least square line going through first n = 8 points, r = 0.95.

Since Y (i) is believed to be replaced by LMP2 upon treatment with IFN-γ (30, 32, 33), we measured the levels of LMP2 incorporated into proteasomes containing different amounts of subunit Y. Only small amounts of LMP2 were detectable by Western blot analysis in preparations from control HeLa cells. Nevertheless, the levels of LMP2 appeared to be inversely related to the amounts of Y in the proteasome fractions of HeLa cells (Fig. 2). In contrast, the amounts of LMP7 did not differ significantly in proteasomes from control and Y-transfected cells. Transfection of the Y gene thus had the following effects on proteasomes that were opposite to those of IFN-γ: 1) increasing amounts of the subunit Y, 2) lower amounts of LMP2, and 3) higher postglutamyl activity. These effects were also opposite to those seen upon transfection of HeLa cells with cDNA for LMP2 (20), which resulted in two independent preparations in an approximately 60% lower level of Y in proteasomes and a reduction in postglutamyl depeptidase.

Effects of Transfection of CDNA Encoding the X Subunit—We next analyzed how alterations in the level of expression of subunit X affected proteasome composition and activity. In initial studies, we failed to generate stable transfecants of HeLa cells with cDNA for X, but were successful with the 721 lymphoblast cell line. Subunit X, as well as Y, LMP2, and LMP7 are all constitutively present in human lymphoblasts, as assessed by Western blotting. After transfection of these cells with the X subunit cDNA (in two independent transfectant lines), the proteasomes contained 52% more X-polypeptide than in controls (Table II). Proteasomes from the transfected cells degraded the hydrophobic peptide, Suc-LLVY-MCA, at a significantly lower rate than those from control cells (Table III). The amount of LMP7 in the transfected cells gradually decreased after many passages of the cells. Therefore, it was possible to analyze how clonal variations in the levels of this subunit affects the chymotrypsin-like activity of proteasomes. As shown in Fig. 3, as the level of X increases in different clonal variants, the maximal capacity to cleave the hydrophobic substrate decreases. This inverse correlation is highly significant (r = 0.89). Thus, the loss of X that occurs upon IFN-γ treatment of X can contribute to the increase in the chymotrypsin-like activity of proteasomes in cytokine-stimulated cells.

Elevated expression of X also caused a significant decrease in the trypsin-like activity but had no effect on hydrolysis of the acidic substrate (Table III). These effects on peptidase activity of X are opposite to those induced by overexpressing LMP7. This finding is consistent with the earlier proposals, based largely on sequence similarities, that LMP7 replaces the X subunit when it is incorporated into proteasomes (30, 32, 33). However, increasing the levels of X in the transfected cells was not accompanied by a significant fall in the levels of LMP7 (Table II). In addition, the amounts of subunit X did not fall significantly after transfection of LMP7 cDNA into HeLa cells (in three different preparations) or into mutant lymphoblasts with a deletion in the MHC region covering the LMP2 and LMP7 loci (in six preparations).

Interpretation of these effects of transfection of X cDNA is complicated by the surprising finding that proteasomes from the transfected wild-type lymphoblasts (721 cells) expressing elevated amounts of X subunit cDNA also had higher levels of Y than control and lower levels of LMP2 (Table II). Moreover, comparison of different clones indicates that when more X was incorporated into the proteasomes of the different transfecants, more Y was also found in them and less LMP2 was present (Table II, Fig. 4). Similarly, in Y-transfected HeLa cells, not only did the amount of Y subunit increase (55 ± 8% greater than control, n = 4), but also the amount of X increased significantly (by 58 ± 9%: p < 0.05). Consistent with these findings, proteasomes from LMP2-transfected HeLa cells have significantly decreased amounts of both Y (as mentioned above) and X (52 ± 12%; n = 3; p < 0.05) subunits. Thus, the two constitutive subunits appear to influence the incorporation of each other; possibly, they interact directly with each other in
the particle, or they are incorporated in a linked manner.

Effect of IFN-γ and MHC Gene Deletion on Subunit Y: As reported previously (17), proteasomes from the mutant lymphoblasts, 721.174, which lack the LMP2 and LMP7 genes, have higher postglutamyl cleaving activity than proteasomes from wild-type 721 cells. Moreover, treatment of these mutant cells with IFN-γ resulted in an anomalous further increase in the hydrolysis of the acidic substrate (17), in contrast to wild-type cells, where IFN-γ caused a suppression of this activity (17). To determine whether these changes correlate with changes in the expression of Y or X, we analyzed proteasome preparations from the mutant lymphoblastoid cells by Western blotting. Both LMP2 and Y subunits are present in proteasomes isolated from the wild-type lymphoblast line. In contrast, proteasomes from mutant cells have no LMP2, but contain significantly more Y and X than did the wild-type preparations (Table IV), which can account for their greater postglutamyl cleaving activity (17). Furthermore, after stimulation with IFN-γ, proteasomes from wild-type cells had a 47% decrease in the amount of Y subunits and a 52% decrease in X subunits (Table IV), in accord with prior reports (30, 32, 33), and a decreased maximal capacity to cleave the acidic peptide (by 51 ± 3% n = 3). It seems likely that the reduced ability to hydrolyze the acidic substrate is due to the decreased content of Y subunits. By contrast, in proteasomes from the mutant 721.174 cells treated with IFN-γ, both the amount of Y and the peptidylglutamyl peptide activity increased (by 56 ± 6%, n = 3). These findings are further evidence that content of Y correlates closely with the postglutamyl activity. It is noteworthy that IFN-γ increased not only the amount of Y but also the amount of X (Table IV). Thus, incorporation of Y and X seems to influence each other’s incorporation.

**DISCUSSION**

The Y and X subunits of the proteasome are normally expressed in most cells but disappear upon stimulation by IFN-γ, concomitant with the appearance of the new β-subunits LMP2, LMP7, and MECL-1 (19). The major finding of the present report is that altering the expression of the X (α) and Y (β) subunits by transfection alters the composition and peptidase activities of proteasomes. Thus, the loss of these components could contribute to the functional changes induced by IFN-γ and possibly other cytokines (17, 19). When IFN-γ stimulates transcription of these new subunits, it does not suppress the transcription of Y or X (30). Therefore a post-translational event appears to prevent either the processing of X and Y or their incorporation into proteasomes. Possibly, X and Y are unable to compete successfully with the LMPs for incorporation into proteasomes; alternatively, IFN-γ might activate some complex mechanism that favors incorporation of LMPs. It seems likely that LMP2 and LMP7 play some role in the exclusion of X and Y, since IFN-γ stimulation of mutant cells that
lack the LMP genes actually causes an increase in the levels of X and Y in proteasomes, in contrast to the decrease seen in wild-type cells. Presumably, in this deletion mutant, due to the lack of LMP subunits, there is no mechanism to exclude the incorporation of X and Y into new proteasomes. Moreover, we have found unexpectedly that enhanced expression of X or Y subunits in the transfected cells increased the extent of incorporation of both subunits into proteasomes. This result suggests that an interaction between these two subunits is important in the assembly of the particle, since the β-rings of the proteasome contain seven tightly packed subunits, and incorporation of a single β-subunit should exclude one of the seven others.

A variety of findings on the archaebacterial proteasome (4-6, 10) have indicated that β-subunits participate in the active sites of the proteasome (4-6, 13, 14), as was also suggested by studies involving LMP2 and LMP7 transfection (20) and mutation analysis in yeast (9). The resolution of the x-ray crystal structure of Thermoplasma proteasomes (10) and inhibitor modifications of β-subunits (11) indicate that the amino-terminal threonine of each β-subunit serves as the active site nucleophile in peptide hydrolysis. The more complex mammalian particle contains several different β-subunits, although only some of these, including LMP2, LMP7, Y (d), X (e), and MECL-1, have an amino-terminal threonine (4-6, 11).

The incorporation of Y and X subunits was found to alter the peptidase activities of the proteasome in an opposite fashion to LMP2 and LMP7. The simplest interpretation of our data is that the Y (d) subunit is primarily responsible for the postglutamyl hydrolyzing activity. For example, in different transfectants, this activity was directly proportional to the content of Y, and it fell under conditions where Y decreased, e.g., IFN-γ treatment or transfection with LMP2 (20, 36, 37). It is noteworthy that the sequence of Y resembles very closely that of the PRE3 subunit in yeast, and mutations in this subunit can prevent cleavage of the acidic substrate (30, 38). The presence of the X (e) subunit somehow reduces the chymotrypsin- and trypsin-like activities. Interestingly, the yeast proteasome subunit, PRE1, is very closely related to X in sequence, and mutations in PRE1 reduce cleavages after both hydrophobic and basic residues by yeast proteasomes (30, 39). The simplest model to explain these observations would be that the X subunit stabilizes hydrolysis of both types of peptides, but more slowly than LMP7. In any case, these findings argue that these specific peptidase activities are associated with these sequences and suggest remarkable conservation of the catalytic functions of specific subunits. Although the transfected polypeptides themselves may possess higher or lower catalytic activity, they may also influence the activities on other β-subunits through allosteric interactions. In addition, since the overexpression of a single β-subunit can alter the composition of proteasomes in complex ways, caution is needed in assigning specific catalytic functions to particular subunits.

Since both the Y (d) subunit is homologous to LMP2, and the X (e) is closest in sequence to LMP7, it was predicted that upon IFN-γ stimulation, LMP2 replaces Y (d), while LMP7 replaces X (e) (30, 32, 33). While some of our results support this model, they suggest that other replacements are also possible. Increased incorporation of X in wild-type cells did not decrease the incorporation of LMP7. Thus, X appears to have replaced some other subunit. In lymphoblasts where LMP2 and LMP7 expression is constitutive, the X transfectant did contain lower levels of LMP2 protein, possibly some other subunit(s) are also reduced. In addition, increased expression of LMP7 did not suppress the content of X in proteasomes although such a suppression has been observed by others (41). Presumably, LMP7 can also replace some other β-subunit(s). Although transfection studies are excellent means to dissect the roles of an individual gene product, the resulting cells may give a distorted picture of the changes seen with IFN-γ treatment, which usually leads to a coordinate induction of three new subunits (LMP2, LMP7, and MECL-1). Also, the levels of LMP7 present in our transfectants were not as high as are observed after IFN-γ stimulation. Perhaps when more LMP7 is produced it is more effective in replacing X (41).

Clear evidence for subunit competition was obtained when LMP2 and Y were transfected. In proteasomes from Y (d) transfectants, the amounts of LMP2 were reduced, and in LMP2 transfectants, lower amounts of Y were present. However, the alterations in subunit composition in these transfected cells are probably more complex than a simple replacement of LMP2 by Y. Because our analysis is limited to those subunits for which we have antibodies suitable for quantitative Western blotting, we cannot rule out the possibility that there are additional alterations in other subunits.

A surprising result was that the levels of X and Y in proteasomes could be increased by IFN-γ stimulation or gene transfection in the mutant strain that lacked LMP2 and LMP7. Therefore, either proteasomes can be generated that have more than one Y or X subunit or some proteasomes lack these subunits under basal conditions. In either case, this finding indicates that even in the absence of cytokine stimulation cell may contain heterogenous populations of proteasomes that differ in subunit composition and functional capacity.

The degradation of proteins by the proteasome appears to be the major pathway for generating MHC class I-presented peptides (3, 13, 14). The deletion of LMP2 (28, 40) or LMP7 (29) decreases the efficiency with which at least some antigens are presented. Therefore, changes in the proteasome’s catalytic activities after incorporation of LMPs clearly promote antigen presentation. The loss of X and Y lead to enhanced capacity to cleave peptides after hydrophobic and basic residues and reduced cleavage after acidic residues. Therefore, during the course of protein breakdown, these immunoproteasomes should generate more peptides that have basic or hydrophobic amino acid-carboxyl termini, a property required for transport into the endoplasmic reticulum and for binding to MHC class I molecules (2, 21). However, studies of how proteasomes from the various transfectants degrade protein substrates or ubiquitin-conjugated proteins should clarify exactly how these β-subunits affect the frequency with which such peptides are generated.

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