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We have compared the subunit composition and enzymatic activity of purified 26S proteasomes from Burkitt’s lymphoma (BL) cells and in vitro EBV-transformed lymphoblastoid cell lines (LCLs) of normal B cell origin. Low expression of the IFN-γ-regulated β low molecular mass polypeptide (Lmp)2, Lmp7, and MECL-1 was demonstrated in a panel of seven BL lines that express the germinall center cell phenotype of the original tumor. Coexpression of Lmp2 and Lmp7 with the constitutively expressed subunits δ and MB1 was demonstrated in the BL lines by immunoprecipitation and two-dimensional gel fractionation of the 20S proteasomes. Coexpression of these subunits correlated with reduced levels of chymotrypsin- and trypsin-like activities detected by the cleavage of fluorogenic substrates. Down-regulation of Lmp2 and Lmp7 and decreased chymotrypsin- and trypsin-like activities were also observed in purified proteasomes from a c-myc-transfected subline of the ER/EB2–5 LCL that has adopted a BL-like phenotype. A synthetic peptide analogue of the immunodominant epitope from the EBV nuclear Ag 4 (E4416 – 424Y) was cleaved by proteasomes from BLs and A1, while proteasomes from LCLs were inactive. Cleavage of the E4416 – 424Y peptide was not affected by treatment of the BL cells with IFN-γ despite both significant up-regulation of Lmp2 and Lmp7 and reconstitution of chymotrypsin and trypsin-like activities against fluorogenic substrates to LCL-like levels. The results demonstrate that B cell lines representing different stages of B cell activation and differentiation express proteasomes with different subunit compositions and enzymatic activity. This may result in the generation of a distinct set of endogenous peptides and influence the immunogenicity of these cells. The Journal of Immunology, 1998, 160: 3281–3289.

The presentation of endogenously expressed protein Ags to CTLs requires degradation in the cytosol and transport of the generated peptides into the lumen of the endoplasmic reticulum (ER) by a heterodimeric complex known as TAP. In the ER, the peptides associate with newly synthesized class I heavy chains and β2-microglobulin (β2m) to form stable trimolecular complexes that are subsequently transported to the cell surface (reviewed in Ref. 1). Recent evidence suggests that the multicatalytic protease complex known as the proteasome participates in the generation of antigenic peptides for MHC class I-restricted presentation (2, 3).

The proteasome is composed of a 20S cylindrical proteolytic core that binds activator complexes designated 19S regulator and 11S regulator (or PA28) at its ends. The 19S regulator associates with the 20S proteasome to form the 26S protease, which is involved in the targeted degradation of ubiquitin-conjugated proteins (reviewed in Ref. 4). The 20S core consists of 14 subunits ranging in molecular mass from 21 to 32 kDa (reviewed in Ref. 5). The subunits are classified as α and β type based on their homology to the subunits of an ancestral proteasome found in the archaeabacterium Thermoplasma acidophilum (6). The active proteolytic sites are contained in the β subunits that constitute the two inner rings of the catalytic core (7). The genes encoding for two β subunits, designated Lmp2 and Lmp7, have been mapped to the MHC class II locus (8–10). Lmp2 and Lmp7 are induced by treatment with IFN-γ and replace the two constitutively expressed subunits, δ and MB1 (11). A third subunit exchange has been detected following IFN-γ treatment of mouse fibroblasts; the non-MHC-encoded MECL-1 subunit was shown to replace the thus far poorly characterized β subunit MC14 (12, 13).

The proteasome cleaves peptide bonds after hydrophobic (chymotrypsin-like activity), basic (trypsin-like activity), and acid (peptidylglycine) peptide-hydrolyzing residues (reviewed in Ref. 14), but very little is known about the contribution of the various β subunits to this enzymatic activity. Recent evidence suggests that incorporation of low molecular mass polypeptide (Lmp)2 and Lmp7 in the 20S proteasome core following IFN-γ treatment may alter the peptidase activity, favoring cleavage at the carboxyl side of hydrophobic and basic residues (15, 16). It is not known whether additional differences in the expression of catalytic or regulatory subunits may occur as a result of cell type and/or state of activation.

Evason from CTL-mediated rejection is likely to be a critical step in tumor development. The EBV-associated Burkitt’s lymphoma (BL) is a classical example of a human tumor that evades CTL surveillance. The BL tumor cells are phenotypically similar to germinal center centroblasts/centrocytes with high expression of CD10 and CD77 and a lack of adhesion molecules and activation markers such as CD23 and CD39 (17). BL-derived cell lines are classified into three major groups that differ in cell phenotype and pattern of growth in vitro. Group I BL lines grow as a single-cell suspension and have retained the surface marker expression of the
original tumor. Group II cell lines are characterized by co-expression of CD10 and activation markers, up-regulation of adhesion molecules, and growth in large clumps. BL lines expressing a group III phenotype closely resemble EBV-transformed lymphoblastoid cell lines (LCLs) and have lost expression of CD10 and BLA (reviewed in Ref. 18). The viral gene expression of EBV-positive group I BL lines is restricted to the EBV nuclear Ag (EBNA), while drift to a group III phenotype is accompanied by expression of six nuclear Ags (EBNA1 to EBNA6) and three latent membrane proteins (LMP1, -2A, and -2B) (19). EBV-positive BL lines that express a group I/II phenotype are not lysed by EBV-specific CTLs (20). Recent evidence suggests that several steps in the Ag-processing and -presentation pathway may be impaired in these cells. Down-regulation of the TAP-1 and TAP-2 transporters was demonstrated in BL lines that failed to present CTL epitopes derived from EBNA3 (21). Lysis was partially restored by transfection of a mimicking gene encoding the epitope linked to an ER localization sequence (21) or by transactivation of the TAP-1 gene following LMP1 up-regulation/transfection (22). However, the demonstration that full reconstitution of TAP activity did not restore presentation of an HLA A11-restricted epitope derived from EBNA4 (23) suggests the presence of an additional defect(s) located upstream of peptide transport and MHC class I assembly and maturation.

We have now compared the subunit composition and cleavage specificity of purified proteasomes derived from BL lines expressing a group I/II phenotype, in vitro EBV-transformed LCLs that constitutively express a group III phenotype, and an LCL that has acquired BL-like characteristics due to constitutive expression of the c-myc oncogene and down-regulation of the EBV-encoded EBNA2 and LMP1. We demonstrate that the BL cell phenotype is associated with lower expression of three IFN-γ-inducible β subunits and different cleavage specificity of the proteasome. Thus, a characteristic set of antigenic peptides may be produced in these cells.

Materials and Methods

Cell lines

BL28 was established from an EBV-negative tumor biopsy (24), while Akata (25), BL72 (24), and WW1-BL (26) were established from EBV-positive tumor biopsies. The BL28 and Akata cell lines have been classified as group I BLs on the basis of surface marker and EBV Ag expression, while BL72 and WW1-BL express a group II phenotype. The DH-LCL was established by spontaneous outgrowth from the lymphocytes of a normal EBV-seropositive donor cultured in the presence of 0.1 μM cyclosporin A. This cell line carries an EBV strain that lacks the immunodominant HLA A11-restricted epitope E4416–424 (27). The QJZ-LCL, JAC-LCL and EA-LCL were obtained by in vitro infection of normal B lymphocytes with EBV of a EBV strain that lacks the immunodominant HLA A11-restricted epitope E4416–424 (28) (EBNA2 and LMP1-deficient T2 line (28) was used as a control in the analysis of Lmp7 expression). The A1 subline was obtained by stable transfection of a cDNA of the EBNA4 epitope E4416–424 (IVTDFSVIK) with a synthetic analogue of the EBNA4 epitope E4416–424 (IVTDFSVIK) with a

Phε-Tyr substitution in position 5 (E4416–424V) was synthesized by the Merrifield solid-phase method (31) (Alta Bioscience, Birmingham, U.K.). HPLC-purified peptide was dissolved in PBS at a concentration of 0.5 μg/mL and stored at −20°C.

Western blotting

The equivalents of 2 × 10^6 cells were fractionated by SDSPAGE in denaturing 12% acrylamide gels and blotted onto nitrocellulose filters according to standard procedures (11). The blots were probed with LMP2, LMP7, MECL-1, and 5-specific rabbit sera (obtained from Drs. J. Trowsdale and I. Correa) Division of Immunology; Department of Pathology University of Cambridge, Cambridge, U.K. (33) at a 1:1000 dilution or with a 1:50 dilution of the mouse mAb MCP21 (a kind gift of Dr. K. B. Hendri) August Krogh Institute; University of Copenhagen, Copenhagen, Denmark. Expression of the EBV Ags EBNA2 and LMP1 was assessed using a 1:20 dilution of a previously characterized human serum HR containing high Ab titers to all EBV As (viral capsid Ag, 1:640; EBNA1, 1:80; EBNA2A, 1:160; EBNA2B, 1:80; EBNA6A, 1:320) and a 1:10,000 dilution of the LMP1-specific mAb S-12 (34). The c-Myc protein was detected using the mAb 9E10 (a kind gift of Dr. G. Evan) Imperial Cancer Research Fund (ICRF), London, U.K. at 1:500 dilution. The blots were reacted with horseradish peroxidase-labeled anti-rabbit, anti-human, or anti-mouse Abs, and the immunocomplexes were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham, Little Chalfont, U.K.).

2D Gel electrophoresis

Five × 10^6 cells were metabolically labelled by culture for 5 h at 37°C in 2 ml of methionine-free RPMI 1640 medium supplemented with 10% FCS (Life Technologies) and 100 μCi of [35S]methionine (Amersham). The cells were lysed for 30 min at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 5 mM EDTA, 0.5% Nonidet P-40, and 1 mM PMSF, and the nuclei were pelleted by centrifugation at 15,000 g. The supernatants were transferred to a clean Eppendorf tube and preclaried by incubation with 2 μl mouse serum for 1 h at 4°C on a rocking platform, followed by 2× 105 suspension with 50 μl of a 10% suspension fixed Staphylococcus aureus (Sigma) in NET buffer and a final wash in 50 mM ammonium acetate (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% v/v Nonidet P-40. Immunoprecipitation with the mAb MCP21 (35) was performed from equal amounts of TCA-precipitable material for 3 h at 4°C, and the immunocomplexes were recovered with 50 μl of S. aureus for 2 h at 4°C. After three washes in NET buffer and a final wash in 50 mM ammonium acetate (pH 7.4), the pellet was resuspended in 1.5 ml of distilled water and lyophilized. The immunoprecipitates were dissolved in 50 μl of nonequilibrium pH gel electrophoresis (NPHPGE) buffer (9 M urea, 5% mercaptoethanol, 2% Triton X-100, 2.5% Phosphate 3–10, 10,000 μCi of [35S]methionine (Amersham). The A1 subline was obtained by stable transfection of the plasmid encoding the EBNA4 epitope E4416–424 (IVTDFSVIK) with a

Purification of the 26S proteasome complex

26S proteasomes were purified from 2 × 10^6 cells according to the protocol of Driscoll et al. (16). Briefly, frozen cell pellets were resuspended in a buffer containing 20 mM Tris-Cl (pH 7.5), 2 mM MgCl2, 0.1 mM EDTA, 2 mM ATP, and 1 mM DTT, and membranes were broken by 50 strokes in a Dounce homogenizer. The nuclei were removed by centrifugation at 15,000 g for 15 min, and the supernatant was further centrifuged at 100,000 g for 60 min. The 100,000-g supernatant was applied to a Mono-Q (HR 5/5) column and fractionation by fast protein liquid chromatography (FPLC) (Pharmacia). Bound proteins were eluted with an NaCl gradient of 0.5 M from the column (0.02 M NaCl). The second dimension was run on denaturing 12.5% acrylamide gels. The gels were dried, exposed to a PhosphorImager screen for 24 h (LCLs) or 48 h (BLs), and then densitometric analysis was performed using the Image Quant software (Molecular Dynamics, Sunnyvale, CA).
Expression of Lmp2, Lmp7, MECL-1, and MC6 subunits in BL cells. Lysates from $2 \times 10^6$ LCL and BL cells were separated by SDS-PAGE in a 12% resolving gel and transferred to nitrocellulose filters. The blots were probed with rabbit sera specific for human Lmp2, Lmp7 and MECL-1 and were also probed with the mAb MCP21 that recognizes the $\alpha$ subunit MC6.

Enzyme assays

The fluorogenic peptides N-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (SucLLVY-MCA) and N-tert-butoxycarbonyl-Leu-Arg-Arg-7-amino-4-methylcoumarin (BocLRR-MCA) were used to assess proteasome activity. The rates of cleavage by purified proteasomes were analyzed with different substrate concentrations at 37°C for 1 h. The reactions were quenched with cold ethanol, and fluorescence was measured in a luminescence spectrometer (Perkin-Elmer, Beaconsfield, U.K.) using an excitation of 380 nm and emission of 440 nm. The $V_{\text{max}}$ for each proteasome preparation was calculated according to the Lineweaver-Burk equation (37) and expressed as nmol of substrate degraded per $\mu$g of enzyme per h.

Peptide degradation assays

The E4416–424Y peptide was $^{125}$I-labeled by chloramine T-catalyzed iodination (38). Aliquots (100 $\mu$L) of the proteasome preparation were desalted by ultrafiltration (Microcon-10, Amicon, Beverly, MA), and 1 $\mu$L of enzyme was incubated with 30 ng of iodinated peptide at 37°C for the indicated time in 30 $\mu$L of assay buffer (20 mM Tris-HCl (pH 7.5), 2 mM MgCl$_2$, 0.1 mM EDTA and 1 mM DTT). The degradation products were resolved in 7 $\times$ 8 cm $\times$ 1 mm tricine gels (39). Dried gels were exposed to a PhosphorImager screen and analyzed with the Image Quant software (Molecular Dynamics). Where indicated, the proteasomes were preincubated with lactacystin for 20 min at 37°C before addition of the substrate.

Results

Expression of the IFN-$\gamma$-regulated $\beta$ subunits

The levels of expression of the IFN-$\gamma$-regulated $\beta$ subunits Lmp2, Lmp7, and MECL-1 were compared by Western blot analysis in both a panel of BL lines that have retained the phenotypic characteristics of the original tumor and in regular in vitro EBV-transformed LCLs of normal B cell origin. A representative experiment, including two group I lines (Akata and BL28), two group II lines (WW1-BL and BL72), and two reference LCLs, is shown in Figure 1. A significant down-regulation of Lmp2 and a less marked down-regulation of Lmp7 and MECL-1 was detected in all four BL lines compared with LCLs. Similar results were obtained in three additional group I BLs: DG75, BL41, and Rael (data not shown). In repeated experiments, the expression of Lmp2 appeared to be somewhat lower in group I compared with group II BLs. All the cell lines contained similar amounts of proteasomes as judged by the detection of comparable levels of the constitutively expressed $\alpha$ subunit MC6.

Proteasome subunit composition

The proteasome subunit composition was further investigated by immunoprecipitation from metabolically labeled cells and by 2D gel fractionation. At least two independent experiments were performed for each cell line tested. The various proteasome subunits were identified by comparison with published data (12, 35) and, when specific Abs were available, by Western blot analysis when specific Abs were available. The position of the Lmp2, $\delta$, Lmp7, and MB1 subunits is indicated.

Proteasome purification and enzymatic activity

To investigate whether the differences in subunit composition detected by Western blot and 2D gel analysis correlated with differences in enzymatic activity, we have analyzed the cleavage specificity of 26S proteasomes isolated from LCL and BL cells. A representative experiment illustrating the FPLC purification procedure and the enzymatic activity of proteasomes from the DH-LCL is shown in Figure 3. The chromatographic profile at 280 nm
Methods.
In each experiment, equal amounts of TCA-precipitable radioactivity was loaded. One of two independent experiments performed with each LCL is shown.

while group III BLs and LCLs have the phenotype of activated B original tumor resemble germinal center centrocytes/centroblasts, while group III BLs and LCLs have the phenotype of activated B

Correlation between cell phenotype and proteasome activity
BL lines that have maintained the phenotypic characteristic of the original tumor resemble germinal center centrocytes/centroblasts, while group III BLs and LCLs have the phenotype of activated B

Table I. Relative expression of constitutive and IFN-γ-inducible β subunits in LCLs and BLs

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>δ</th>
<th>Lmp2</th>
<th>Ratio</th>
<th>MB1</th>
<th>Lmp7</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIZ-LCL</td>
<td>1,113</td>
<td>2,432</td>
<td>0.45</td>
<td>547</td>
<td>11,858</td>
<td>0.05</td>
</tr>
<tr>
<td>JAC-LCL</td>
<td>1,418</td>
<td>5,223</td>
<td>0.27</td>
<td>0</td>
<td>14,388</td>
<td>0</td>
</tr>
<tr>
<td>EA-LCL</td>
<td>0</td>
<td>2,026</td>
<td>0</td>
<td>1,100</td>
<td>11,938</td>
<td>0.09</td>
</tr>
<tr>
<td>DH-LCL</td>
<td>0</td>
<td>1,491</td>
<td>0</td>
<td>0</td>
<td>4,668</td>
<td>0</td>
</tr>
<tr>
<td>BL28</td>
<td>11,642</td>
<td>2,369</td>
<td>4.9</td>
<td>4,324</td>
<td>23,393</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>3,912</td>
<td>793</td>
<td>4.9</td>
<td>1,211</td>
<td>9,734</td>
<td>0.12</td>
</tr>
<tr>
<td>DG75</td>
<td>3,049</td>
<td>266</td>
<td>11</td>
<td>1,145</td>
<td>4,618</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>1,759</td>
<td>593</td>
<td>3</td>
<td>1,739</td>
<td>5,983</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>902</td>
<td>0</td>
<td></td>
<td>2,317</td>
<td>5,305</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>1,128</td>
<td>0</td>
<td></td>
<td>2,071</td>
<td>2,227</td>
<td>0.9</td>
</tr>
<tr>
<td>BL72</td>
<td>15,720</td>
<td>11,710</td>
<td>1.3</td>
<td>8,379</td>
<td>86,815</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>2,741</td>
<td>2,604</td>
<td>1</td>
<td>883</td>
<td>10,479</td>
<td>0.09</td>
</tr>
</tbody>
</table>

The OD of the indicated subunits was measured from a PhosphorImager scan of the 2D gels developed for 24 h (LCLs) and 48 h (BL2) as described in Materials and Methods. In each experiment, equal amounts of TCA-precipitable radioactivity was loaded. One of two independent experiments performed with each LCL is shown.

FIGURE 3. Purification and functional characterization of the 26S proteasome complex. 26S proteasomes were purified from cytosolic cell extracts by FPLC fractionation as described in Materials and Methods. A, Chromatogram profile of fractions 36 to 57 in one representative experiment performed with the DH-LCL. Bound proteins were eluted from the column with NaCl gradient (−). The eluted proteins were detected by monitoring UV absorbance (A280), and the peptide-hydrolyzing activity (●) was assayed as described using 10 µl of each fraction. B, Effect of the proteasome inhibitor lactacystin on the degradation of the SucLLVY-MCA substrate. One microgram of enzyme was incubated for 20 min at 37°C with the indicated concentration of the inhibitor. The substrate was then added to the reaction mixture and fluorescence was measured after an incubation for 1 h at 37°C. C, Enzyme kinetics analysis of proteasome activity on the SucLLVY-MCA and BocLRR-MCA substrates. One microgram of enzyme was incubated with an increasing substrate concentration (25–200 µM). The cleavage activity was evaluated as fluorescence units and is expressed as nmol of substrate degraded per µg of enzyme per h.

The V max values are 111 nmol/µg/h for SucLLVY-MCA and 100 nmol/µg/h for BocLRR-MCA.

of fractions 36 to 57 is presented in panel 3A. Each fraction was assessed for proteolytic activity against the fluorogenic substrate SucLLVY-MCA. One single activity peak was identified between fractions 48 and 52 corresponding to a single A280 peak eluted at 42% buffer B. The cleavage of the SucLLVY-MCA substrate was inhibited in a dose-dependent manner by the specific proteasome inhibitor lactacystin (Fig. 3B) and by other proteasome inhibitors such as z-LLL and LnLL but not by the aminopeptidase specific inhibitor bestatin (data not shown), confirming that the enzymatic activity was associated with the proteasome complex. Furthermore, Lmp2 and Lmp7 were detected in these fractions by Western blot analysis (data not shown). The purity of the proteasome preparations was confirmed by SDS-PAGE fractionation and silver staining that revealed the presence of closely migrating polypeptides with a molecular mass ranging from 20 to 32 kDa and additional bands with a molecular mass of between 30 and 110 kDa (data not shown). A similar migration pattern was reported for purified 26S proteasome from rabbit reticulocytes (40).

The activity of the purified proteasome preparations was estimated in enzyme kinetics assays (Fig. 3C). One microgram of the enzyme was incubated with an increasing concentration (25–200 µM) of the SucLLVY-MCA (chymotrypsin-like) and BocLRR-MCA (trypsin-like) substrates for 1 h at 37°C, and cleavage was evaluated as fluorescence units. The V max was calculated according to the Lineweaver-Burk equation (37) and is expressed as nmol of substrate degraded per µg of enzyme per h. Proteasomes purified from BL cells were tested for chymotrypsin- and trypsin-like activities in parallel with proteasome preparations derived from LCLs and from the Lmp2/7-deficient cell line T2. The results of four to eight experiments performed with two proteasome preparations from BL28, BL72, three LCLs, and T2 are summarized in Fig. 4. The maximal activity of the BL-derived proteasomes was ~20 nmol/µg/h for SucLLVY-MCA and between 20 and 30 nmol/µg/h for BocLRR-MCA. Comparable values were obtained in a more restricted set of experiments performed with purified proteasomes from BL41 and DG75 (data not shown). This activity was similar to that of T2-derived proteasomes (23 nmol/µg/h and 13 nmol/µg/h, respectively) and was four- to sixfold lower than the activity of proteasomes derived from LCLs.

Correlation between cell phenotype and proteasome activity
BL lines that have maintained the phenotypic characteristic of the original tumor resemble germinal center centrocytes/centroblasts, while group III BLs and LCLs have the phenotype of activated B
blasts. To assess whether different stages of B cell activation/differentiation are associated with changes in subunit composition and enzymatic activity of the proteasome, we have used the ER/EB2–5 LCL and its c-myc transfected subline A1 as a model. ER/EB2–5 has been obtained by in vitro transformation of B lymphocytes with a recombinant EBV that carries an EBNA2-estrogen receptor fusion gene (29). Withdrawal of estrogen from the culture medium results in down-regulation of EBNA2 and LMP1, loss of the activated phenotype, and growth arrest. Expression of a constitutively activated c-myc gene drives cell proliferation in the A1 transfectant in the absence of EBNA2 and LMP1 (Fig. 5A) and allows the expression of a stable BL-like phenotype (30). Lmp2 and Lmp7 were strongly down-regulated in the A1 subline compared with the parental ER/EB5–2 LCL (Fig. 5A). Proteasomes purified from these cells exhibited decreased chymotrypsin- and trypsin-like activities and resembled those derived from authentic BL cells and the T2 cell line (Fig. 5B).

Cleavage of the E4416–424Y peptide

We have previously shown that HLA A11-positive BL cells fail to present the A11-restricted epitope E4416–424. The defect could not be overcome by expression of a preformed epitope even after full reconstitution of TAP-1/TAP-2 activity, suggesting that the exogenous peptide may be degraded before entering the ER lumen (23). To investigate whether the proteasome may be involved in this degradation, an analogue of the E4416–424 peptide that carries a Phe7Tyr substitution in position 5 to allow iodination (E4416–424Y) was used as substrate for degradation by proteasomes from LCLs, BLs, and the ER/EB2–5-A1 pair. As illustrated by the representative experiment shown in Figure 6, proteasomes purified from QJZ-LCL (Fig. 6B) and ER/EB2–5 (Fig. 6D) either did not cleave or only marginally cleaved the E4416–424Y peptide. Similar results were obtained in repeated experiments performed with proteasome preparations derived from two additional LCLs. In contrast, the E4416–424Y peptide was cleaved by proteasome derived from BL28 (Fig. 6A), A1 (Fig. 6C), and BL72 (data not shown). Two major degradation products are indicated in Figure 6 as a and b. The a product migrated slightly faster in tricine gels compared with the uncleaved peptide, while the electrophoretic mobility of the b product was considerably higher. The appearance of these degradation products was inhibited in a dose-dependent manner by the specific proteasome inhibitors lactacystin (Fig. 6, A–D) and z-LLL (data not shown), but not by other protease inhibitors such as aprotinin, leupeptin, bestatin, and pepstatin (data not shown).

Effect of IFN-γ

IFN-γ up-regulates the expression of several β subunits (11–13, 41) and modulates the proteolytic activity of human and mouse 20S proteasomes (15, 16, 41). Therefore, we have investigated the effects of IFN-γ on Lmp2/7 expression and on the enzymatic activity of the BL proteasomes. Representative experiments performed with the BL28 and BL72 cell lines are shown in Figure 7. Treatment of the BL lines for 48 h in medium containing 500 U/ml.
of IFN-γ resulted in a strong up-regulation of Lmp2 (a 14-fold increase in BL28 and a 5-fold increase in BL72), and a two- to threefold increase was observed for Lmp7 (Fig. 7, A and B). A concomitant down-regulation of the δ subunit was also observed (Fig. 7, A and B). This change in subunit composition correlated with enhanced cleavage of the SucLLVY-MCA and BocLRR-MCA fluorogenic substrates (Fig. 7C) corresponding to chymotrypsin- and trypsin-like activities similar to those observed in LCLs (cf Fig. 4 and Fig. 7C). As illustrated by the representative experiment shown in Figure 8, treatment with IFN-γ did not influence the ability of the BL proteasomes to cleave the E4416–424Y peptide. This is in line with the inability of BL cells treated with IFN-γ to present the EBNA4 Ag (23).

Discussion

Previous results have suggested that defects in the production of antigenic peptides for MHC class I assembly and presentation may contribute to the immunoescape of BL cells (21–23). We have investigated this phenomenon by comparing the subunit composition and enzymatic activity of proteasomes derived from BL cells and from EBV-transformed LCLs of normal B cell origin.

A significant down-regulation of the β subunit Lmp2 and a less marked down-regulation of Lmp7 and MECL-1 were demonstrated in a panel of seven BL cell lines expressing a group I or group II BL phenotype (Fig. 1 and data not shown). The different subunit composition of proteasomes from BLs and LCLs was further confirmed by immunoprecipitation and 2D gel fractionation of the 20S proteasome complexes (Fig. 2). The β subunits δ and MB1 were clearly coexpressed with the IFN-γ-inducible Lmp2 and Lmp7 in BL cells but were expressed at very low levels or were not detected in LCLs. Since the presence of Lmp2/δ and Lmp7/MB1 appears to be mutually exclusive (11), the coexpression of these subunits in BLs is likely to reflect the presence of structurally different subsets of proteasomes. This possibility is in line with the findings of Brown et al. that demonstrate the existence of at least four types of proteasomes in a mouse macrophage cell line (42); only one subset of the proteasomes contained Lmp2. The significance of our findings is further confirmed by the strong down-regulation of Lmp2 and Lmp7 in the A1 transfectant that has shifted toward a BL-like phenotype due to constitutive expression of c-myc and down-regulation of EBNA2 and LMP1 (30) (Fig. 5A). The difference between ER/EB2–5 and A1 suggests that the
distinct pattern of proteasome β subunit composition of BLs may be a property of the particular stages of B cell activation/differentiation represented by the tumor rather than the result of in vivo selection for a nonimmunogenic phenotype.

Controversial data exist concerning the influence of Lmp2 and Lmp7 on the degradation of fluorogenic substrates and the relevance of this activity for the production of antigenic peptides in vivo. Gaczynska et al. (15) and Driscoll et al. (16) have shown that incorporation of these subunits in the proteasome complex enhances the chymotrypsin- and trypsin-like activities, while the peptidylglutamyl hydrolyzing activity was slightly diminished (15) or unaffected (16). Contrasting results were reported by Kloetzel and coworkers who showed that transfection of Lmp2 and/or Lmp7 in both T2 and the mouse B8 fibroblasts correlated with decreased chymotrypsin-like and peptidylglutamyl hydrolyzing activities (43–45). The discrepancy may be explained by differences in the protocols used for proteasome purification, which could result in variable degrees of contamination by other cellular proteases. The significance of these findings for the production of antigenic peptides is also unclear. While the presence of Lmp2 and Lmp7 appears to be important for the generation of certain immunogenic peptides, such as the immunodominant epitope from the murine CMV immediate early Ag pp89 (41, 44), these β subunits are clearly not essential for the production of other CTL epitopes derived from the influenza nucleoprotein and matrix protein (46–48).

Degradation experiments performed with the fluorogenic substrates SucLLVY-MCA and BocLRR-MCA (Fig. 4) and the E4416–424Y analogue (Fig. 6) indicate that proteasomes derived from both BL cells and the A1 transfectant have a different cleavage specificity compared with proteasomes derived from LCLs. The low expression of Lmp2 and Lmp7 correlated with less efficient cleavage of hydrophobic and basic fluorogenic substrates (cf Fig. 1 and Fig. 4, Fig. 5A and 5B). This was not an artifact due to contamination by other proteases, such as aminopeptidases, since the cleavage of fluorogenic substrates was not influenced by a wide panel of protease inhibitors. The E4416–424Y analogue was cleaved by proteasomes from BLs but was completely resistant to cleavage by proteasomes from LCLs (Fig. 6). Treatment of BL cells with IFN-γ resulted in up-regulation of Lmp2 and Lmp7, down-regulation of δ, and enhanced chymotrypsin- and trypsin-like activities against fluorogenic substrates (Fig. 7, A–C) but did not affect the cleavage of the E4416–424Y peptide (Fig. 8, A and B). This indicates that the enzymatic activity involved in the degradation of E4416–424Y is not directly modulated by Lmp2 and Lmp7. Conceivably, other catalytic subunits may be responsible for cleavage of this substrate. At least five enzymatic activities have been associated with the proteasome including chymotrypsin-like activity, trypsin-like activity, peptidylglutamyl hydrolyzing activity, branch chain amino acid preferring activity, and cleavage between small neutral amino acids (reviewed in 14). The E4416–424Y peptide contains at least three sites for branch chain amino acid preferring activity (P2, P7, and P8) and one site for the peptidylglutamyl hydrolyzing activity (P4). It is not known which β subunits confer these specificities. The constitutively expressed δ, MB1, and MC14 subunits may be involved. If this is the case, our failure to modulate the cleavage specificity by IFN-γ treatment may be due to the relatively long half-life of the proteasome (49), which may result in the persistence of significant levels of these subunits. Alternatively, the proteasome specificity detected in both BL cells and the A1 subline may be due to differential expression of regulatory components of the proteasome complex. Recent reports have focused the attention on the PA28 regulatory subunit that is known to associate with the 20S core (50, 51). Addition of PA28 to purified 20S proteasomes was shown to enhance the production of immunogenic nonamer peptides from longer precursors (50). This correlated with a more efficient presentation of the immunodominant epitope from murine CMV pp89 in mouse fibroblasts transfected with human or mouse PA28 (51). So far, we have been unable to test the expression of PA28 in BL cells due to a lack of specific reagents, and we cannot exclude its involvement. However, PA28 was shown to be induced by IFN-γ treatment (52), and it is therefore unlikely that differences in the expression of this regulatory subunit would be sufficient to explain the degradation of E4416–424Y substrate by proteasomes from untreated and IFN-γ-treated BL cells. Finally, although we have clearly shown that the cleavage of the E4416–424Y peptide was inhibited by the proteasome-specific inhibitor lactacystin and by the peptide aldehyde z-LLL (Fig. 6 and data not shown), we cannot formally exclude the possibility that other as yet unidentified proteases may be sensitive to these inhibitors and may therefore be responsible for the enzymatic activity observed in BL cells. It is noteworthy that higher concentrations of lactacystin were required to inhibit the degradation of the synthetic peptide compared with the fluorogenic substrates (cf Fig. 3B and Fig. 6). This is likely due to the different enzymatic activities involved in the cleavage, since lactacystin was shown to inhibit chymotrypsin-like, trypsin-like, and peptidylglutamyl hydrolyzing activities with different kinetics (53).

Taken together, our results strongly indicate that the different peptidase activities of the BL proteasomes may result in the production of characteristic sets of peptides that are different from those produced in other cell types. We have previously shown that BL cells cannot present the E4416–424 epitope even after full reconstitution of TAP function (23). Our present observations may offer an explanation for this phenomenon, since degradation of this peptide may prevent its accumulation in the cytosol. While the
significance of this finding for the immunescape of BL is unclear because the tumor does not express viral Ags that are recognized by EBV-specific CTLs, differences in proteasome cleavage specificity could provide an efficient route of immunescape in other EBV-associated malignancies, such as nasopharyngeal carcinoma and Hodgkin’s disease, which express at least some of the highly immunogenic viral Ags serving as targets for the efficient rejection of LCL cells (reviewed in Ref. 54). Indeed, down-regulation of the proteasome β subunits Lmp2 and Lmp7 has been shown to favor immunescape in other tumor models such as renal cell carcinoma and small lung carcinoma (55, 56).

We have shown that differences in proteasome subunit composition and cleavage specificity are associated with the particular stage of B cell activation/differentiation represented by the BL tumor. This finding has interesting implications for understanding the life-long persistence of EBV-infected cells in healthy virus carriers in the face of strong CTL responses. The virus has been detected in circulating “resting” B cells (57) that, similar to BL, express only LMP2A and EBNA1 (58–60). EBNA1 may not act on the life-long persistence of EBV-infected cells in healthy virus carriers with a Burkitt’s lymphoma (BL)-like phenotype. J. Immunol. 159:353.


