LMP2⁺ proteasomes are required for the presentation of specific antigens to cytotoxic T lymphocytes

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Background: Major histocompatibility complex (MHC) class I molecules present short peptides generated by intracellular protein degradation to cytotoxic T lymphocytes (CTL). The multisubunit, non-lysosomal proteinases known as proteasomes have been implicated in the generation of these peptides. Two interferon-γ (IFN-γ)-inducible proteasome subunits, LMP2 and LMP7, are encoded within the MHC gene cluster in a region associated with antigen presentation. The incorporation of these LMP subunits into proteasomes may alter their activity so as to favour the generation of peptides able to bind to MHC class I molecules. It has been difficult, however, to demonstrate a specific requirement for LMP2 or LMP7 in the presentation of peptide epitopes to CTL.

Results: We describe a T-cell lymphoma, termed SP3, that displays a novel selective defect in MHC class I-restricted presentation of influenza virus antigens. Of the MHC-encoded genes implicated in the class I pathway, only LMP2 is underexpressed in SP3 cells. Expression of IFN-γ in transfected SP3 cells simultaneously restores LMP2 expression and antigen presentation to CTL. Expression of antisense-LMP2 mRNA in these IFN-γ-transfected cells selectively represses antigen recognition and the induction of surface class I MHC expression. Moreover, the expression of this antisense-LMP2 mRNA in L929 fibroblast cells, which constitutively express LMP2 and have no presentation defect, blocks the presentation of the same influenza virus antigens that SP3 cells are defective in presenting.

Conclusions: Our results show that the LMP2 proteasome subunit can directly influence both MHC class I-restricted antigen presentation and class I surface expression.

Background

The expression of major histocompatibility complex (MHC) class I molecules is frequently downregulated on the surface of malignant tumor cells [1]. Because these molecules present tumor-specific peptides to cytotoxic T lymphocytes (CTL), their downregulation allows the tumour cell variants to escape lysis and detection by CTL, a natural process of immune selection [2]. Crystallographic studies have shown clearly how peptides bind to class I molecules [3]. In addition, the characterization of self-peptides eluted from purified class I molecules has shown that they are usually 8–10 amino acids long, and that the peptides presented by a particular class I molecule have certain amino-acid sequence features in common — these features are known as an allele-specific motif [4,5].

The peptides presented by class I molecules can be derived from intracellular pathogens, endogenous self proteins or tumor-specific proteins, and are transported from the cytosol into the endoplasmic reticulum, where they associate with the MHC class I heavy chain and light chain (β₂ microglobulin) components [6–8]. Studies of mutant cell lines with defects in intracellular antigen presentation associated with low levels of cell-surface class I expression have demonstrated the crucial roles of the peptide itself, as well as of the transporters associated with antigen processing (TAPs), in class I assembly and cell-surface expression [9–12]. Moreover, analysis of a functional polymorphism of TAP2 in the rat, and the development of TAP- and ATP-dependent *in vitro* peptide transport assays, have shown that the TAP1–TAP2 heterodimeric complex is a peptide transporter involved in the delivery of peptides to class I molecules [13–16].

Less is known about how peptides suitable for binding to class I molecules are derived from intracellular proteins. Recently, evidence has accumulated that proteasomes
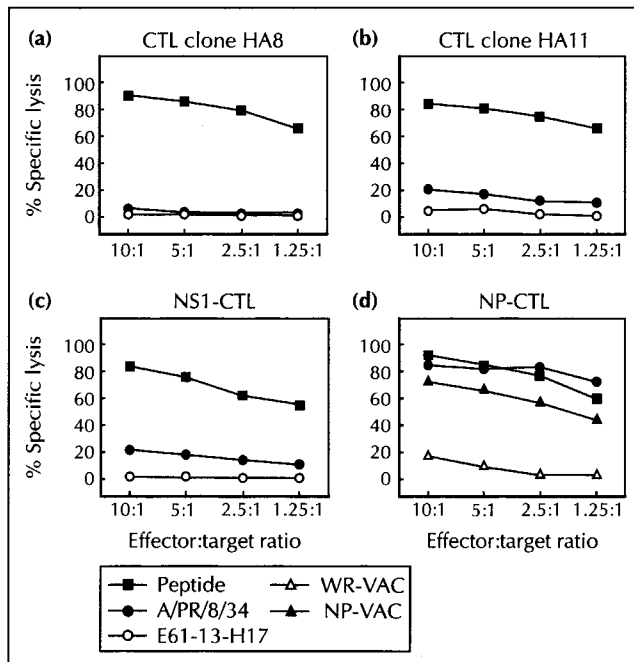
Fig. 1. Selective recognition of influenza-infected SP3 target cells by CTL specific for different influenza viral antigens. **(a)** CTL clone HA8, specific for residues 354–362 of influenza A/PR/8/34 HA. **(b)** CTL clone HA11, specific for residues 259–266 of A/PR/8/34 HA. **(c)** NS1-CTL, specific for residues 152–160 of A/PR/8/34 NS1. **(d)** NP-CTL, specific for residues 50–57 of A/PR/8/34 NP. SP3 cells were infected with A/PR/8/34 (●) or control influenza virus strain E61-13-H17 (○), or incubated with 1 mM of the appropriate synthetic peptide epitope (■) and used as targets for CTL (at the indicated effector:target ratios) in chromium-release assays. NP-CTL were also incubated with SP3 cells infected with 10 pfu per cell of the recombinant vaccinia viruses NP-VAC (▲) or control WR-VAC (△).

may generate such peptides. The 20S proteasome [17–19] is an abundant, non-lysosomal protease that catalyzes the cleavage of peptide bonds on the carboxy-terminal side of basic, hydrophobic or acidic amino-acid residues. It is a cylindrical particle composed of fourteen different subunits arranged in four stacked rings [20–22], and is differentially distributed in the cytoplasm, nucleus and other cellular compartments, depending on the cell type studied [23–25]. The 20S proteasome forms the core of a larger 26S protease, which catalyzes the ATP-dependent degradation of ubiquitinated proteins [26,27], and there is some evidence that ubiquitin-dependent proteolysis is involved in the processing of some class I-restricted antigens [28,29]. In addition, two IFN-γ-inducible genes, designated *LMP2* and *LMP7*, which exhibit homology to genes encoding proteasome subunits, have been found adjacent to the *TAP* genes within the MHC gene cluster [30–33].

In vitro proteolysis assays using proteasome fractions have demonstrated that LMP2 and LMP7 may alter the preferred cleavage specificity of the proteasome [34, 35]. In addition, purified proteasomes containing these two proteins are able to generate epitopes from synthetic peptides [36] and intact proteins [37]. However,

functional experiments with mutant cell lines lacking both LMP2 and LMP7 have provided little evidence for the involvement of LMP2 and LMP7 in class I-restricted antigen presentation [38–42], and studies of mutant mice lacking expression of LMP2 or LMP7 [43,44] have failed to define precisely the respective functions of these proteasome subunits in the class I antigen-presentation pathway.

Here, we describe a T-cell lymphoma, known as SP3, that has a selective, epitope-specific antigen-presentation defect. The defect is associated with a lack of LMP2 expression but a normal level of cell-surface MHC class I molecules. Antigen presentation and LMP2 expression are restored by IFN-γ, but the expression of an anti-sense-LMP2 mRNA prevents this restoration. Moreover, the expression of this antisense mRNA in L929 fibroblast cells, which constitutively express LMP2 and have no presentation defect, reproduces the presentation defect of SP3 cells. LMP2-containing (LMP2+) proteasomes are therefore required for the presentation of specific antigens to CTL.

Results and discussion

An antigen-presentation defect in SP3 cells

The stable metastatic cell line termed SP3 was generated by syngeneic *in vivo* passage of the AKR-mouse-derived BW5147 T-cell lymphoma (BW-O) [45]. SP3 cells do not have a significant defect in their cell-surface levels of the class I molecules H-2K^k and H-2D^k (see Fig. 4). After infection with the influenza virus strain A/PR/8/34, however, SP3 cells are inefficiently recognized by the K^k-restricted influenza hemagglutinin-specific CTL clones HA8 and HA11 (Fig. 1a,b). This antigen-presentation defect is not limited to HA, because a K^k-restricted epitope of the influenza non-structural protein NS1 is also poorly recognized by specific polyclonal CTL (NS1-CTL) (Fig. 1c). SP3 cells can, however, be efficiently lysed by CTL clones HA8 and HA11, and the polyclonal NS1-CTL, in the presence of the appropriate peptide (Fig. 1). It is unlikely, therefore, that a mutation has occurred affecting the K^k heavy chain of SP3 cells. The lack of recognition is not due to an aborted infection by the influenza virus, because the same SP3 cells are efficiently lysed by K^k-restricted, influenza nucleoprotein (NP)-specific CTL (NP-CTL; Fig. 1d). The presentation defect is also observed in SP3 cells infected with a recombinant vaccinia virus expressing either a full-length, cytoplasmic form of HA (L⁻H1-VAC) or a 70-residue HA fragment (KG34-VAC) (Table 1).

To identify the step in the class I antigen-presentation pathway in which SP3 cells are defective, we generated recombinant vaccinia viruses expressing only the HA8 or the HA11 peptide epitopes. In contrast to SP3 cells expressing full-length HA, SP3 cells infected with vaccinia viruses expressing these HA peptide epitopes were

Table 1. Presentation of full-length or pre-processed hemagglutinin by SP3 cells to K^k-restricted CTL clones.

Targets	Effector:target ratio	L⁻H1-VAC (full-length HA)	KG34-VAC (70 residue HA)	HA354-362-VAC (peptide HA8)	HA259-266-VAC (peptide HA11)
			HA8 CTL effector		
SP3	10:1	7 %	1 %	84 %	2 %
	5:1	6 %	3 %	76 %	1 %
	2.5:1	6 %	2 %	64 %	0 %
SP3+IFN-γ	10:1	45 %	40 %	80 %	1 %
	5:1	36 %	38 %	74 %	0 %
	2.5:1	30 %	33 %	70 %	0 %
			HA11 CTL effector		
SP3	10:1	11 %	6 %	7 %	78 %
	5:1	9 %	1 %	2 %	67 %
	2.5:1	6 %	0 %	1 %	52 %
SP3+IFN-γ	10:1	45 %	4 %	5 %	81 %
	5:1	36 %	1 %	3 %	73 %
	2.5:1	30 %	1 %	1 %	56 %

SP3 cells, untreated or incubated with 500 U ml⁻¹ of IFN-γ for 48 h, were infected with 10 pfu of recombinant vaccinia virus per cell. Cytotoxicity assays were performed using a standard 6 h [^{51}Cr]-release assay [61]. Percent-specific lysis is shown for cell lines with a spontaneous release of less than 15 %. Similar results were obtained from at least three separate experiments.

efficiently lysed by HA8 or HA11 CTLs (Table 1). These results indicate that SP3 cells have a processing defect which prevents the efficient presentation of some intracellular antigens, but that their ability to transport antigenic peptides and peptide–class I complexes is not impaired. This latter point was confirmed by the finding, based on northern and western blotting experiments (data not shown), that SP3 cells express normal levels of TAP1 and TAP2.

IFN-γ restores HA antigen presentation by SP3 cells
IFN-γ has been shown to correct class I antigen-presentation defects in other tumor cell lines [46,47]. We therefore tested the ability of SP3 cells to present antigen after pre-incubation with IFN-γ. Treatment with IFN-γ restored the ability of HA8 and HA11 CTLs to lyse SP3 cells that were infected with a vaccinia virus expressing full-length HA (Table 1). Similarly, IFN-γ-treated SP3 cells infected with KG34-VAC were lysed by HA8 CTLs (Table 1). SP3-IFN-γ cells — stable transfectants expressing a murine recombinant IFN-γ gene [48] — were also able to process HA (data not shown). Thus, SP3 cells have a selective defect in the processing of long polypeptide sequences of HA that is fully reversed by expressing IFN-γ.

SP3 cells are regulatory mutants for LMP2 expression
LMP2 and LMP7 are IFN-γ-inducible proteasome subunits proposed to be involved in antigen presentation. Their expression might, therefore, be altered in SP3 cells. Immunoprecipitation of SP3 cell lysates with an anti-rat proteasome antiserum [49] followed by two-dimensional gel electrophoresis showed that these cells lack the LMP2 subunit, but constitutively express the LMP7 subunit (Fig. 2a). This phenotype is cell-type specific and not

generally observed in the thymocytes from which SP3 cells are originally derived [25].

SP3 cells are regulatory mutants, because LMP2 mRNA and protein could both be induced in them by IFN-γ (Fig. 2b,d and Fig. 3). In addition to inducing LMP2, IFN-γ treatment also led to a dramatic decrease in the expression of a constitutively expressed proteasome subunit (Fig. 2a,b) — probably the δ subunit [50]. Thus, as reported previously [51–53], incorporation of LMP2 and the presumed δ subunit into proteosomes was found to be mutually exclusive and IFN-γ-regulated. In contrast, the level of LMP7 in the proteasome remained constant relative to the levels of the constitutive subunits and was unaffected by IFN-γ treatment. A parallel analysis of precipitates obtained using an anti-LMP antiserum (anti-H-2^d allo-antiserum) [54] showed that IFN-γ generated a specific LMP2⁺ proteasome subset in SP3 cells that could not be precipitated in the absence of LMP2 (Fig. 2c,d; [55]).

LMP2⁺ proteasomes are required for presentation of HA to CTL
In order to test whether the HA presentation defect was due solely to a lack of LMP2, we used different vectors to express LMP2 in SP3 cells. However, transfection of these cells with the *LMP2* gene did not allow them to produce LMP2⁺ proteasomes, even though *LMP2* mRNA and unprocessed LMP2 protein could be detected in the transfected cells. A similar failure of *LMP2* mutant cells to incorporate expressed LMP2 into proteasomes has been reported previously [42]. It is possible, therefore, that additional components required for post-translational processing events that are essential in proteasome assembly are absent in SP3 cells but are induced by IFN-γ.
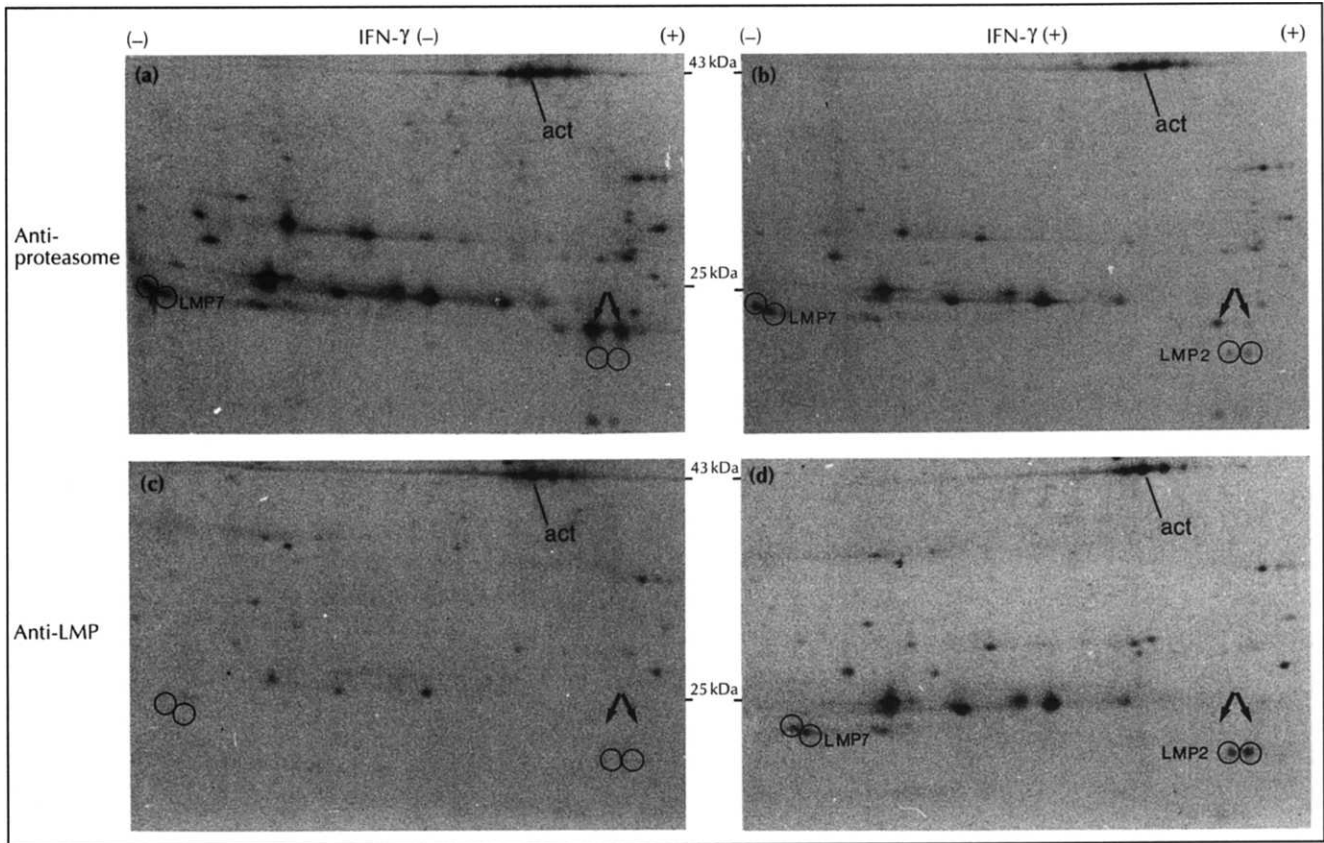
Fig. 2. Modulation by IFN-γ of the proteasome subunit composition in SP3 cells. Two-dimensional gel electrophoresis of proteasomes from untreated **(a,c)** or IFN-γ-treated **(b,d)** SP3 cells. Lysates were immunoprecipitated with an anti-rat-proteasome antiserum (a,b) or an anti-H-2^d allo-antiserum (anti-LMP) (c,d). The position of actin (act) is indicated for reference. Circles indicate the location of processed LMP2 (present in (b) and (d); molecular weight 21 kDa, isoelectric point of 4.7) and LMP7 (present in (a),(b) and (d), molecular weight 24 kDa, isoelectric point of 7.5). The long arrows indicate the presumed δ proteasome subunit.

In order to investigate whether the LMP2 subunit was directly involved in HA presentation, we suppressed LMP2 expression in the SP3–IFN-γ cells by use of an antisense-LMP2 mRNA. A full-length cDNA encoding H-2^k LMP2 was cloned in reverse orientation into the expression plasmid pREP4, generating pREP4αLMP2;

after electroporation into SP3–IFN-γ cells, stable clones were isolated. As a control, the empty pREP4 vector was introduced into SP3 and SP3–IFN-γ cells. Unexpectedly, the double-transfected clones (SP3–IFN-γ-αLMP2 cells) exhibited a significant decrease in the levels of cell-surface class I molecules compared to SP3–IFN-γ cells (Fig. 4),
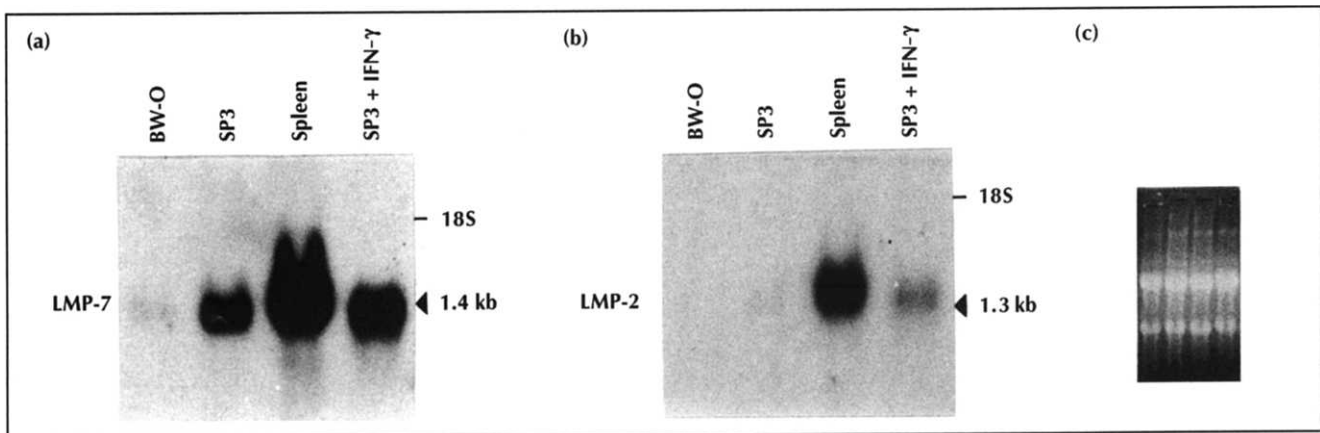

Fig. 3. Effects of IFN-γ on LMP mRNA expression in SP3 cells. Northern hybridization analysis of **(a)** LMP7 expression and **(b)** LMP2 expression. Total RNA was isolated from BW-O cells, SP3 cells, AKR mouse splenocytes and SP3 cells pre-incubated with IFN-γ. **(c)** The blotted gel after staining with ethidium bromide.
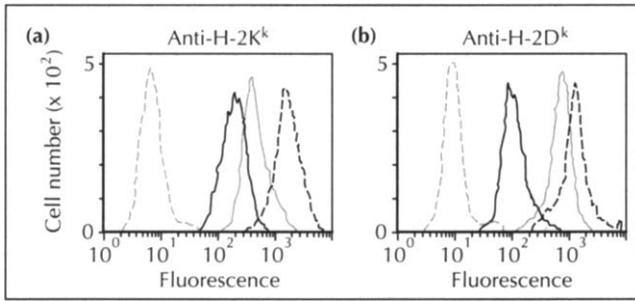
Fig. 4. Reduction of surface class I MHC molecules by antisense-LMP2 mRNA in SP3-IFN-γ cells. Indirect immunostaining of SP3-pREP4 cells (black solid line), SP3-IFN-γ-pREP4 cells (black broken line) and SP3-IFN-γ-αLMP2 cells (grey solid line) with (a) antibody 16-3-1N (ATCC), specific for H-2K^k, and (b) antibody 15-5-5S (ATCC), specific for H-2D^k, followed by staining with fluorescein isothiocyanate-conjugated goat-anti-mouse immuno-globulin G. Background staining is represented by the grey broken line.

whereas the level of class I molecules was unaffected in cells transfected with the empty pREP4 vector (SP3-IFN-γ-pREP4 cells). The reduction of class I MHC expression was not due to the loss of IFN-γ expression in SP3-IFN-γ-αLMP2 cells, because the addition of exogenous IFN-γ did not restore the expression of surface class I molecules to normal levels (data not shown).

Immunoprecipitation and western immunoblotting experiments confirmed that the expression of LMP2 and LMP2$^+$ proteasomes was suppressed in these clones, but the total proteasome pool size was unaffected (Fig. 5). Cytotoxicity assays showed that, in contrast to SP3-IFN-γ cells, SP3-IFN-γ-αLMP2 cells were completely unable to present HA to HA8 CTL, whereas NP recognition was preserved. The phenotype of these cells therefore mimicked that of the parental SP3 cells (Fig. 6). These results demonstrate that SP3 cells require LMP2$^+$ proteasomes in order to present HA to CTL efficiently.

There are several possible explanations for the epitope-selectivity of the antigen-presentation defect in SP3 cells.
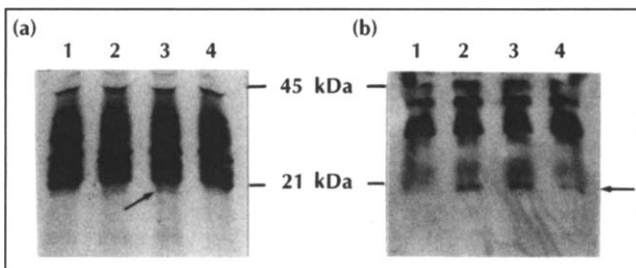

Fig. 5. Suppression of LMP2$^+$ proteasomes by antisense-LMP2 mRNA expression. (a) Immunoprecipitation of total proteasome pools by rabbit anti-rat-proteasome antiserum analyzed by one-dimensional SDS–PAGE on a 10 % gel. (b) Western immunoblot analyzing LMP2 expression. Lane 1, SP3 cells; lane 2, SP3 cells treated with IFN-γ; lane 3, SP3-IFN-γ-pREP4 cells; lane 4, SP3-IFN-γ-αLMP2 cells. The arrows indicate the location of the processed form of LMP2. Antisense-LMP2 mRNA does not alter the overall content of proteasomes, but there is a dramatic decrease in the LMP2$^+$ proteasome subset in the same precipitates.
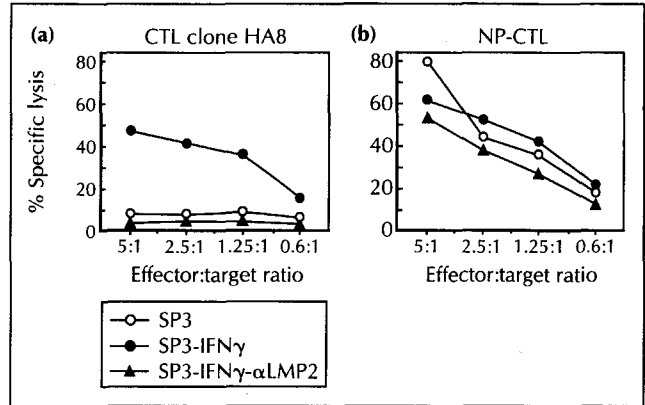

Fig. 6. Cytotoxicity assay showing that antisense-LMP2 mRNA specifically suppresses HA8 epitope recognition by CTL. Control SP3-pREP4 cells (O), SP3-IFN-γ-pREP4 transfectants (●) and SP3-IFN-γ-αLMP2 cells (▲) were infected with A/PR/8/34 and used as targets for (a) HA8 CTL or (b) NP-CTL, at the indicated effector:target ratios, in a chromium-release assay.

CTL with distinct specificities may have different affinities for antigen, or may be triggered by different levels of antigen. Different affinities of different epitopes for the K^k class I molecule is unlikely to be the explanation, as the efficiently presented NP epitope has a lower binding affinity than the poorly presented HA epitopes [56]. It is possible that the NP epitope is much more efficiently processed from the intact protein than are HA epitopes, because the NP epitope is immunodominant in the polyclonal CTL response to influenza virus. Variations in the intracellular locations of protein antigens might also affect their processing.

In order to confirm the critical role of LMP2 in the generation of HA epitopes, H-2^k L929 fibroblast cells, which efficiently present HA to CTL [57], were transfected with pREP4αLMP2. Stable transfectants were isolated as a polyclonal population and used as targets in cytotoxicity assays; HA8 and NP CTLs were used as effectors. Constitutive levels of cell-surface class I
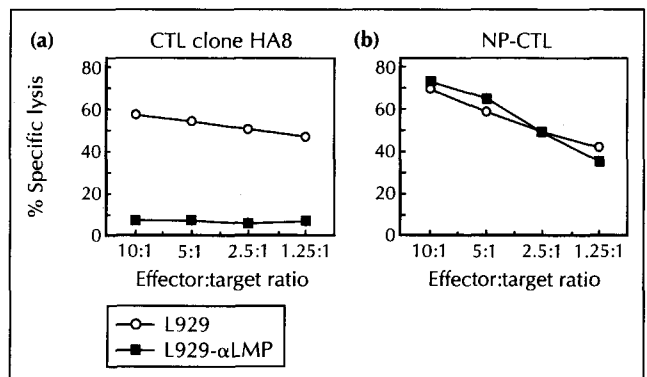

Fig. 7. Cytotoxicity assay testing the effect of antisense-LMP2 mRNA on L929 cells. L929 cells (O) or L929 cells transfected with pREP4αLMP2 (■) were infected with A/PR/8/34 and used as targets for (a) HA8 CTL or (b) NP-CTL, at the indicated effector:target ratios, in a chromium-release assay.

molecules were not decreased in cells expressing anti-sense-LMP2 mRNA (data not shown). When these cells were infected with influenza virus, however, they showed a clear block in presentation of the HA8 epitope, in comparison with the parental L929 cells; in contrast, presentation of the NP epitope was unaffected (Fig. 7). Similar results were obtained in three experiments, and have also been confirmed in assays using clones derived by limiting dilution from the polyclonal population of L929 transfectants expressing antisense-LMP2 mRNA. Moreover, infection of these L929-αLMP2 cells with the recombinant vaccinia virus expressing the minimal HA8 epitope permitted their efficient lysis by HA8 CTL (data not shown). Therefore, the phenotype of L929 cells expressing antisense-LMP2 mRNA mimics that of SP3 cells. Our results are consistent with the hypothesis that the LMP2 proteasome subunit has proteolytic activity [58], and that this activity is required for the generation of some epitopes recognized by CTL.

Conclusions

Our results provide clear evidence for the involvement of LMP2, not only in antigen presentation, but also in the upregulation of class I expression. Because neither the absence of LMP2 nor the presence of antisense-LMP2 mRNA alter the constitutive level of class I molecules on the surface of SP3 cells, it appears that, upon IFN-γ induction, LMP2 specifically controls the supply of peptide by forming novel 'immune' LMP2$^+$ proteasomes that also contain LMP7 and other constitutively expressed subunits. Thus, although the SP3 (LMP2$^-$7$^+$) proteasomes supply sufficient peptides to maintain the basal level of class I molecules, the repertoire of peptides generated is deficient for the presentation of some viral antigens and for inducing tumor rejection [48].

Our results may therefore explain why LMP2 mutant animals have normal constitutive levels of MHC class I molecule expression, but nevertheless have a significant deficiency in their CD8$^+$ T-cell repertoire [43]. If the repertoire is dependent on maximal peptide diversity, associated with a completely efficient class I antigen-presentation capacity, then the absence of LMP2 would result in reduced CD8$^+$ T-cell diversity. Our results further suggest that the reduction in the basal level of expression of surface class I molecules observed in LMP7 knock-out mice [44] is due to the fact that LMP7 is a component of both constitutive and 'immune' LMP2$^+$ proteasomes.

The antisense approach we have employed has proved to be a useful tool in dissociating the specific functions of LMP2 from the pleiotropic effects mediated by IFN-γ. It should be possible to use this approach to study other proteasome subunits, for which natural mutants are not yet available, and to provide a source of various types of proteasomes for use in in vitro assays.

Materials and methods

Cell lines
The original BW-O cell line was derived from the non-metastatic BW5147 T-cell lymphoma (H-2^k, AKR/J mouse origin, Salk Institute, California). It gave rise to the SP3 metastatic variant upon successive syngeneic in vivo passages [45]. The H-2^k L929 fibroblast cell line has been well documented. Cells were cultured in RPMI 1640 or DMEM medium supplemented with 10 % fetal calf serum (FCS; Gibco). For treatment with IFN-γ, cells were incubated for 48 h with 500 U ml^{-1} mouse recombinant IFN-γ (Holland Biotechnology).

CTL and synthetic peptides
The CTL used in these experiments are K^k-restricted, and have been described previously. The polyclonal NP-specific CTL recognize amino-acid residues 50–57 of A/PR/8/34 NP [57,59]. The polyclonal NS1-specific CTL recognize amino-acid residues 152–160 of A/PR/8/34 NS1 [60]. CTL clones HA11 and HA8 recognize amino-acid residues 259–266 and 354–362, respectively, of A/PR/8/34 HA [57]. The CTL were restimulated weekly with peptide-treated syngeneic spleen cells and human recombinant IL-2 (20 Cetus U ml^{-1}). Peptides were synthesized and purified by standard methods. Cytotoxicity assays were performed using a standard [^{51}Cr]-release assay [61].

Influenza and recombinant vaccinia viruses
Influenza virus strains A/PR/8/34 (Mount Sinai strain) and E61-13-H17 [62] were used for infection in the cytotoxicity assays. Recombinant vaccinia viruses were generated by standard methods, and were expressed from the P7.5 vaccinia promoter. L$^-$H1-VAC [63], KG34-VAC [57] and NP-VAC [64] have been described previously. HA259-266-VAC and HA354-362-VAC express the peptide epitopes recognized by CTL clones HA11 and HA8, respectively, and were generated as described [65].

Northern blots
Total RNA extraction, electrophoresis, blotting on to Hybond-N nylon membrane and hybridization were carried out according to standard protocols [66]. Each lane was loaded with 30 μg RNA. DNA probes were labeled by random priming (Boehringer kit) using [α^{32}P]dATP (Amersham). The TAP1 probe consisted of the 1.45 kb and 1.25 kb EcoRI fragments isolated from the plasmid pHbApr-1-neo-510-15 (containing the full-length rat TAP1 cDNA) [39,67]. The TAP2 probe comprized the 0.9 kb EcoRI fragment from plasmid pEII-4 (containing the mouse TAP2 genomic sequence) [68]. For LMP2 mRNA detection, we used a 0.8 kb EcoRI–SalI fragment isolated from the plasmid pcDNAneo1-W10 (containing the full length cDNA of H-2^d LMP2) [30], and for LMP7 mRNA detection, we used a 0.9 kb EcoRI fragment isolated from plasmid W14-4 (containing H-2^d LMP7 cDNA sequences (J. Monaco, unpublished data)).

Western blots
Total cell lysate preparation, electrophoresis and transfer onto nitrocellulose were carried out according to standard procedures [66]. TAP1 and TAP2 were detected using rabbit antisera raised against synthetic peptides corresponding to the carboxyl terminus of the respective rat TAP polypeptides, as described [39,69]. LMP2 was detected using a rabbit polyclonal antiserum directed against recombinant rat LMP2 (S.T. and A.J.R., unpublished data). Following incubation with 500-fold

diluted horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Amersham), blots were developed using an enhanced chemiluminescence kit (Amersham).

Metabolic radiolabeling and immunoprecipitation

Cells (10^7) were incubated for 1 h in RPMI 1640 medium lacking cysteine and methionine (Gibco) and containing 10 % FCS at 37°C in an atmosphere of 7 % CO_2 in air. Following incubation, a mixture of [^{35}S]methionine and [^{35}S]cysteine (Translabel, ICN Radiochemicals, California) was added to a final specific activity of 200 μCi ml^{-1} and the cells were incubated for a further 5 h under the same conditions. Subsequently, cell lysates were prepared as described previously [70], except that no peptide was added to the extracts. For analysis by two-dimensional gel electrophoresis, 300 μl of each lysate, prepared from untreated or IFN-γ-treated SP3 cells, was divided into two aliquots and incubated on ice for 1 h with either 15 μl of a rabbit anti-proteasome antiserum [49] or 30 μl of an anti-H-2^d (BALB/b anti-BALB/c) allo-antiserum [54]. Following incubation, 100 μl of a 10 % suspension of protein A–Sepharose CL-4B (Pharmacia) was added to each sample. After a 1 h incubation at 4 °C, the beads were washed and resuspended in sample buffer (pH 9.5) containing 9 M urea, 4 % Nonidet-P40, 2 % 2-mercaptoethanol and 2 % 9–11 ampholytes (Ampholines, LKB Instruments, Maryland) [71].

Two-dimensional gel electrophoresis

The samples prepared as described above were analysed by high resolution two-dimensional electrophoresis [71]. The first-dimension isoelectric focusing gels contained isoelectric point 3.5–10 resolyte (BDH), and the second-dimension slab gels were 8–18 % linear polyacrylamide gradients. Carbamylated rabbit muscle creatine phosphokinase (EC 2.7.3.2, Sigma) was used as an internal standard for each isoelectric focusing gel, and Rainbow protein molecular weight markers (Amersham) were used as standards for the second-dimension gels. Gels were fixed, dried and subjected to autoradiography for 2–3 weeks.

LMP2 antisense mRNA expression plasmid

A full-length LMP2 cDNA was isolated by RT–PCR using mRNA purified from L929 cells and the oligonucleotides 5'-CCGAGCCCCGCTCTGCTGAGA-3' and 5'-GGAAG-GGACTTCTGGGGATCAG-3'. After verification of its sequence, the cDNA was inserted in reverse orientation into the expression vector pREP4 (Invitrogen) to give plasmid pREP4αLMP2. This plasmid was introduced into SP3 cells by electroporation, and into L929 cells as a calcium phosphate precipitate. Transfectants were selected with 2 mg ml^{-1} and 0.75 mg ml^{-1} hygromycin B, respectively.

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