MHC Class I Antigen Processing of an Adenovirus CTL Epitope Is Linked to the Levels of Immunoproteasomes in Infected Cells

Alice J. A. M. Sijts, Sybille Standera, René E. M. Toes, Thomas Ruppert, Nico J. C. M. Beekman, Peter A. van Veelen, Ferry A. Ossendorp, Cornelis J. M. Melief and Peter M. Kloetzel

_J Immunol_ 2000; 164:4500-4506; doi: 10.4049/jimmunol.164.9.4500
http://www.jimmunol.org/content/164/9/4500

References
This article cites 43 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/164/9/4500.full#ref-list-1

Why _The JI_? Submit online.
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

Subscription
Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
MHC Class I Antigen Processing of an Adenovirus CTL Epitope Is Linked to the Levels of Immunoproteasomes in Infected Cells

Alice J. A. M. Sijts,* Sybille Standera,* René E. M. Toes,† Thomas Ruppert,* Nico J. C. M. Beekman,‡ Peter A. van Veelen,‡ Ferry A. Ossendorp,‡ Cornelis J. M. Melief,† and Peter M. Kloetzel²*

Proteasomes are the major source for the generation of peptides bound by MHC class I molecules. To study the functional relevance of the IFN-γ-inducible proteasome subunits low molecular mass protein 2 (LMP2), LMP7, and mouse embryonal cell (MEC) ligand 1 in Ag processing and concomitantly that of immunoproteasomes, we established the tetracycline-regulated mouse cell line MEC217, allowing the titrable formation of immunoproteasomes. Infection of MEC217 cells with Adenovirus type 5 (Ad5) and analysis of Ag presentation with Ad5-specific CTL showed that cells containing immunoproteasomes processed the viral early 1B protein (E1B)-derived epitope E1B192–200 with increased efficiency, thus allowing a faster detection of viral entry in induced cells. Importantly, optimal CTL activation was already achieved at submaximal immunosubunit expression. In contrast, digestion of E1B-polypeptide with purified proteasomes in vitro yielded E1B192–200 at quantities that were proportional to the relative contents of immunosubunits. Our data provide evidence that the IFN-γ-inducible proteasome subunits, when present at relatively low levels as at initial stages of infection, already increase the efficiency of antigenic peptide generation and thereby enhance MHC class I Ag processing in infected cells. The Journal of Immunology, 2000, 164: 4500–4506.

The MHC class I Ag processing pathway allows CTL to react fast and efficiently to viral infections. Most MHC class I-presented CTL epitopes derive from proteasome-mediated degradation of Ags in the cytosol (for review see Ref. 1). Peptide fragments released by the proteasome are translocated into the endoplasmic reticulum (ER) by TAP and, if conforming to the binding motif, associate with MHC class I molecules for transport to the cell surface.

To detect viruses shortly after cellular infection when viral loads are still low, the MHC class I Ag processing pathway should function with high efficiency. Although ER translocation of antigenic peptides, MHC class I binding, and cell surface transport seem not to curtail CTL epitope presentation (2, 3), proteolytic peptide generation has been identified as a rate-liming step in Ag processing (4, 5).

Proteasomes are multicatalytic enzyme complexes consisting of a catalytic 20S core that associates with regulatory complexes (19S or PA700 and 11S or PA28), which tune proteasome function (for review, see Refs. 6 and 7). The induction of cells with IFN-γ results in enhanced expression of most components of the MHC class I Ag processing pathway, including that of the proteasome activator PA28, which has been demonstrated to enhance MHC class I Ag presentation (8, 9). IFN-γ also induces the expression of three proteasome subunits, low molecular weight protein 2 (LMP2), LMP7, and multicatalytic endopeptidase complex-like 1 (MECL-1) (7), which are called the immunosubunits. These subunits replace the constitutive catalytic subunits in the proteasome complex, resulting in the formation of so-called immunoproteasomes.

The association of the IFN-γ-inducible subunits alters the catalytic specificity and structural effects imposed on the proteasome (16-18).4

To clarify the contribution of immunoproteasomes to Ag processing, 25- to 40-mer polypeptides harboring CTL epitopes have been digested with purified proteasomes. Indeed, certain antigenic peptides were liberated with greater efficiency in the presence of the LMPs and MECL-1, but the generation of other peptides was not affected (Refs. 14 and 19 and our unpublished observations). Although generation of a murine CMV pp89 CTL epitope was enhanced in the presence of LMP2 in vitro, overexpression of this subunit in pp89-transfected fibroblast cells did not enhance recognition by specific CTL (20). Other investigators showed that the absence of the LMPs hampered the processing of a subset of MHC class I-presented CTL epitopes in the human 721.174 and T2 mutant cell lines (21-24). In one study, the adverse effect of lacking LMP expression was reversed by the introduction of LMP7 (24). Mice lacking either LMP2 or LMP7 expression show only partial deficits in antigenic peptide presentation (25, 26), making a major impact of the LMP subunits on MHC class I Ag processing questionable.

Copyright © 2000 by The American Association of Immunologists 0022-1767/00/$02.00

4 Abbreviations used in this paper: ER, endoplasmic reticulum; Ad5, Adenovirus type 5; E1B, early 1B protein; LMP, low molecular weight protein; MECL, multicatalytic endopeptidase complex-like 1; TET, tetracycline; NEPHGE, nonequilibrium pH gradient gel electrophoresis; RP-HPLC, reversed phase HPLC.
So far, all studies using intact cellular systems only examined the influence of LMP2 and LMP7 on antigenic peptide generation, neglecting the potential contribution of the third IFN-γ-induced subunit MECL-1. Recently, the IFN-γ-inducible subunits were shown to incorporate interdependently into proteasomes, implicating that under physiological conditions the assembly of proteasomes containing LMP2, LMP7, and MECL-1 is favored (27, 28). Thus, because of potential functional cooperation, these three subunits should be regarded in combination to further elucidate their role in MHC class I Ag processing.

To this aim, transfected cells were generated expressing LMP2, LMP7, and MECL-1 under control of an inducible promoter. Uninduced cells containing constitutive proteasomes and induced cells containing immunoproteasomes were infected with adenovirus type 5 (Ad5), and MHC class I presentation of a viral CTL epitope derived from the early 1B protein (E1B) (29) was studied. Compared with uninduced cells, induced cells processed the E1B CTL epitope with greater efficiency, resulting in a better recognition by E1B-specific CTL already at early time points after infection. Consistent with this, the presence of the IFN-γ-inducible subunits enhanced the proteasome-mediated liberation of the antigenic nonamer from E1B polypeptide in vitro. We discuss the possible mechanisms by which immunoproteasomes may improve MHC class I Ag processing efficiency.

Materials and Methods

DNA constructs

The expression vectors pTET-Splice and pTET-tTAk, containing the tetacycline (TET)-regulated tTAk transcription activator, were described by Shokett et al. (30). cDNAs encoding LMP2 (H-2b haplotype), LMP7, and MECL-1 were cloned into pTET-Splice using standard methods.

Cell lines and transfections

The MEC-18 cell line was derived from a primary culture of embryonal cells (MEC) of a C57BL/6 p53−/− mouse and cultured in IMEM (Biochrom, Berlin, Germany) with 10% FCS, 2 mM L-glutamine, 20 μM 2-ME, penicillin, and streptomycin. Human WEHI-164 clone 13 cells were maintained as described (31). The CTL clones 100B6 (28) and 100 PKOB4 (31) are specific for the Ad5 CTL epitope E1B192–200 in the context of H-2Db MHC class I molecules.

Transfected cell lines were established using calcium-phosphate precipitation. MEC/tTAk cells were transfected with pTET-tTAk and pLXSP, and positive clones were selected with hygromycin B (200 μg/ml) in medium containing 400 ng/ml TET. The presence of tTAk was confirmed by Northern analyses. MEC/tTAk clone 29 (MEC-29) was further transfected with pTET-Splice. TET-inducibility of tTAk expression was verified by Western blot analysis of cell lysates using a polyclonal 20S-proteasome-specific rabbit antiserum for 1 h at 4°C. Sepharose beads were washed with NET buffer (50 mM Tris.Cl (pH 8), 150 mM NaCl, 5 mM EDTTA) with 0.5% Nonidet P-40, resuspended in nonequilibrium gradient gel electrophoresis (NEPHGE) sample buffer, and subjected to NEPHGE two-dimensional gel electrophoresis. Gels were exposed for autoradiography. Radioactive signals were quantified using a Molecular Dynamics (Sunnyvale, CA) phosphor imager.

Proteasome isolations

20S proteasomes used in this study were freshly prepared as described (14) and kept on ice. The purity of the proteasome preparations, checked by Coomassie-stained SDS-PAGE, was >95%.

NEPHGE two-dimensional gel electrophoresis

Purified proteasomes were subjected to NEPHGE-PAGE as described (14). Gels were Coomassie-stained according to standard procedures.

Ad5 and infections

Ad5 dl7001 (33) lacking the E3 region was propagated in 911 cells as described (34). Viral stocks were aliquoted and stored at −80°C in 10% glycerol. Adhered MEC217 cells were infected at a multiplicity of infection of 100 in PBS with 0.5% BSA for 35 min at room temperature. This procedure results in infection of 100% of cells. Infected cells were cultured in Iscove’s medium at 37°C until use in a CTL assay.

Eμ′-release assay

MEC217 cells were cultured for 3 days in the absence or presence of TET and then infected with Ad5 dl7001 or left uninfected. After 24 h of infection, the cells were harvested and used as targets in a 16-h Eμ′-release assay with CTL clone 100B6 as described (35).

TNF-α production assay

MEC217 cells cultured for 2 days without or with 10 ng/ml or 400 ng/ml TET were infected with Ad5 dl7001 or left uninfected. After 2 h of infection, the cells were harvested and used as targets in a TNF-α production assay. Target cells were placed in the wells of 96-well plates (1 × 104 cells/well) in a volume of 50 μl. CTL clone 100 PKOB4 cells (5000 cells/well) were added in a volume of 50 μl, and the plates were incubated at 37°C for 2.5, 10, and 22 h. Sixty microliters of supernatant of independent triplicate wells were transferred into the wells of fresh 96-well plates and were further incubated for 1 h at 37°C. The concentration of TNF-α was measured by sandwich ELISA. The OD570s of wells with WEHI cells cultured in the presence of Ad5 infected cells was determined by measuring the OD570s of the microcultures with a Dynatech (Chantilly, VA) MR5000 ELISA reader. The OD570s of wells with WEHI cells cultured in the presence of recombinant TNF-α (35 pg/ml) were used as a reference for total WEHI cell death.

Peptides

Z-LLE-βNA was purchased from Bachem (Heidelberg, Germany). Synthetic AKVLRDPCYKISKLVRNRCVYISNGAEVEIDRERV (Ad5 E1B E1B192–213) and VNRNCVY1 (E1B192–206) were kindly provided by Dr. P. Henklein (Institute of Biochemistry).

Peptide digestion assays and quantitation of CTL epitope generation

Digestion assays using Z-LLE-βNA as a substrate were performed as described (14). To determine proteasome-mediated cleavage of Ad5 E1B 40-mer, 15 μg of polypeptide and 1 μg of purified 20S proteasomes were incubated in 100 μl assay buffer (20 mM HEPES/KOH (pH 7.8), 2 mM MgAc2, 5 mM DTT) at 37°C for the time periods specified in the figure legends, and then they were frozen. Samples were analyzed by reversed phase HPLC (RP-HPLC) (HPLC system HP1100 (Hewlett-Packard, Waldbronn, Germany) system equipped with a μRPC C2/C18 SC 2.1/10 column (Pharmacia, Freiburg, Germany); eluent A, 0.05% trifluoroacetic acid; eluent B in 30 min, 63–95% eluent B in 4 min; flow rate, 50 μl/min). Analysis was performed on line with an ion trap mass spectrometer (ThermoQuest, Egselsbach, Germany) equipped with an electrospray ion source. Each scan was acquired over the range m/z = 300–1300 in 3 s. The peptides

The Journal of Immunology

4501
were identified by their molecular masses calculated from the m/z peaks of the single or multiple charged ions and were confirmed by mass spectrometric sequencing analyses. To measure E1B 192–200 generation, the digests were diluted in PBS with 0.5% BSA and 80 μM 2-ME and were titrated on IFN-γ-induced uninfected MEC217 cells in 96-well plates (2 × 10^4 cells/well). After 30 min at 37°C, cells were washed with PBS used as targets in a TNF-α production assay. A standard curve generated in the same experiment by diluting synthetic E1B 192–200 on MEC217 cells was used to quantify E1B 192–200 in the digests.

Results

TET-regulated expression of proteasome immunosubunits

To examine the effect of LMP2, LMP7, and MECL-1 on proteasome-mediated Ag degradation and CTL epitope generation, transfectant mouse embryonal cell (MEC) lines expressing these subunits under a TET-regulatable inducible promoter were generated as described in Materials and Methods. A total of 54% of the established MEC lines showed inducible expression of the three introduced subunits in immunoblot analyses, as demonstrated for a representative clone (MEC217) in Fig. 1. This clone was selected for complete processing of the transfected subunits into the mature form, lacking the presequence, which indicates efficient incorporation into 20S proteasomes. MEC217 cells cultured in the presence of TET, which inactivates tTAk, did not express the LMPs or MECL-1 at detectable levels, and thus lack any significant expression of the inducible proteasome subunits. In the absence of TET, both LMPs and MECL-1 were clearly detectable (Fig. 1). Kinetic experiments established that the levels of induced LMP2 increased from the first up to the third day of TET removal and then stabilized. In contrast, TET-mediated repression of gene transcription resulted in a substantial drop of LMP2 levels within 2 days, probably due to cell division (not shown). The expression of the transfected subunits did not influence the levels of endogenous PA28α or β (not shown), implicating the absence of stabilizing effects of immunoproteasomes on PA28, which could serve as a mechanism to optimize MHC class I Ag processing. Thus, in MEC217 cells the levels of immunosubunits are tightly regulated by TET, and immunoproteasomes are expressed in the absence of elevated levels of PA28.

Dose-dependent exchange of constitutive for induced proteasome subunits in MEC217 cells

To further study the efficiencies of subunit exchange, MEC217 cells were cultured in the presence of graded concentrations of TET and then subjected to pulse-chase analysis (Fig. 2). As expected, high concentrations of TET (400 ng/ml) fully suppressed expression of the transfected subunits, resulting in a 100% constitutive proteasome population. In contrast, culture in 10 ng/ml TET effected a roughly 55% replacement of constitutive for induced subunits among newly assembled proteasomes, as measured by quantifying the radioactive signals for δ and LMP2 (Fig. 2). In the absence of TET, a nearly complete exchange of δ for LMP2 and MB1 for LMP7 was reached (Fig. 2). Thus, LMP2, LMP7, and MECL-1 efficiently replace their constitutive homologues δ, MB1, and MC14 in induced MEC217 cells.

To estimate the effect of induced immunosubunit expression on the total cellular proteasome population, MEC217 cells were grown in bulk culture in the absence and in the presence of 400 ng/ml and 10 ng/ml TET for 3 days, and 20S proteasomes were isolated. The exchange rates of constitutive for induced subunits
LMP2 and LMP7, estimated from Coomassie-stained two-dimensional gels, were 0, 40, and 60% for cultures with 400 ng/ml TET, 10 ng/ml TET, and without TET, respectively (not shown). Because replacement of δ reduces the proteasomal peptidyl glutamyl hydrolyzing activity (14), the subunit exchange efficiencies were further established by measuring the enzymatic activity of the isolated proteasomes against Z-LLE-βNA. Whereas constitutive proteasomes cleaved this substrate efficiently, immunoproteasomes of cells cultured with 10 ng/ml TET or without TET hydrolyzed Z-LLE-βNA to relative levels of roughly 50 and 20% only (Fig. 3), reflecting the gradual substitution of δ for LMP2 in the proteasome preparations. Thus, these experiments (Figs. 2 and 3) reveal an efficient assembly of immunoproteasomes in induced MEC217 cells that can be leveled by titration of the TET concentration in the medium.

**Immunosubunits enhance MHC class I Ag processing in infected cells**

To examine the effects of LMP2, LMP7, and MECL-1 on MHC class I Ag processing, we used Ad5, against which specific CTL clones exist as a model system. MEC217 cells were cultured in the presence or absence of TET and infected with Ad5 mutant dl7001. Dl7001 lacks the viral E3 region and thus the E3-19K protein, which retains MHC class I molecules in the ER. Viral infection by itself did not affect proteasome subunit composition in MEC217 cells (data not shown). Presentation of the viral E1B 192–200 epitope by H-2Db MHC class I molecules was tested in an Eu31-release assay with CTL clone 100B6 (Fig. 4A). Whereas uninduced infected MEC 217 cells (+TET) were not lysed above background levels, induced infected cells (−TET) expressing the proteasome immunosubunits appeared highly susceptible to lysis by E1B192–200-specific CTL. Infected MEC-29 cells containing tTAk only and cultured with and without TET
were not lysed (not shown), indicating that tTAk expression or the presence of TET did not influence the results. Observed effects were reproduced in a second independent experiment (not shown).

To confirm and expand these results, we performed a more sensitive assay that measures TNF-α production by activated CTL. MEC217 cells cultured for 2 days with different concentrations of TET (400 ng/ml and 10 ng/ml) or without TET were infected with Ad5 d7001 and then cocultured for different time periods with E1B-specific CTL clone 100 PKOB4. TNF-α secretion was measured (Fig. 4B). Because 100 PKOB4 CTL lack perforin expression and thus do not lyse their target cells efficiently and because Ad5 infection is not lytic in mouse cells, decreases in target cell numbers during the assay were only minimal. Both uninduced and induced infected MEC217 cells were recognized by E1B192–200-specific CTL (Fig. 4B). However, cells expressing LMP2, LMP7, and MECL-1 induced significantly higher levels of TNF-α release than did cells cultured with 400 ng/ml TET, already at early time points after infection. Thus, the presence of immunoproteasomes enhanced the processing efficiency of E1B192–200, resulting in a faster recognition of infected cells by virus-specific CTL. Moreover, the kinetics of CTL activation by cells cultured in the presence of 10 ng/ml TET and in the absence of TET appeared identical (Fig. 4B), indicating that the lower levels of immunosubunits sufficed to effect efficient E1B192–200 presentation.

Enhanced antigenic peptide generation by immunoproteasomes

To explain the observed enhancement of MHC class I Ag processing in induced MEC217 cells, the effects of the inducible proteasome subunits on E1B degradation and E1B192–200 generation were further analyzed in vitro. A 40-mer E1B polypeptide (E1B176–215) encompassing the E1B CTL epitope was synthesized and incubated with purified 20S proteasomes from induced and uninduced MEC217 cells. Generated digestion products were analyzed by RP-HPLC and on-line analyzed with a mass spectrometer. The identity of detected peptide fragments was confirmed by ms/ms analysis. Remarkably and consistent with earlier findings with murine CMV pp89 25-mer (U. Kuckelhorn and P. M. Kloetzel, manuscript in preparation), we found that over the course of digestion (as measured at 3 and 6 h), immunoproteasomes degraded the E1B 40-mer faster than constitutive proteasomes did (not shown). After 3 h of digestion, when turnover of the E1B 40-mer was still incomplete, the E1B192–200, flanking fragments E1B176–191 and E1B201–215 were among the predominant peptide products (data not shown). The E1B epitope VNIRNCCYTI was just discernable from background in the HPLC-mass spectrometry of digests of constitutive proteasomes (Fig. 5, upper left panel; at 21.63 min). In contrast, immunoproteasomes produced this peptide efficiently, as is evidenced by a clearly distinguishable peak at 21.74 min (Fig. 5, upper right panel). The same relative differences in E1B192–200 abundancies were found in the 6-h-digestion products (Fig. 5, lower panels). Because not only E1B192–200 but also the N- and C-terminal flanking fragments (176–191 and 201–215, respectively) were most prevalent in immunoproteasome digests, we infer that the observed better liberation the E1B epitope is caused by a more efficient usage of the Leu191-Val192 and Ile215, Ser216 cleavage sites. Peptides with the correct COOH terminus of the epitope but prolonged at the N terminus were not detected, implying that both types of proteasomes generated the E1B CTL epitope at exact size.

The relative efficiencies of E1B192–200 production by different proteasome populations were further analyzed using E1B192–200-specific CTL as read out. First, peptide fragments generated during a 90-min digestion of E1B 40-mer with constitutive and with immunoproteasomes were separated by RP-HPLC, and targeting activity of the individual fractions was assayed with E1B192–200-specific T cells. As expected, only one single fraction which corresponded to the fraction in which synthetic E1B192–200 eluted sensitized uninfected target cells for T cell recognition (data not shown), supporting our notion that both constitutive and immunoproteasomes generate E1B192–200 precisely.

To minimize potential losses during HPLC purification, E1B192–200 generation was quantified from the unfractionated peptide digests. Dilutions of digests generated with 20S proteasomes of MEC217 cultured in the presence of 400 or 10 ng/ml TET or in the absence of TET were loaded on uninfected MEC217 cells and cocultured with CTL clone 100 PKOB4, and TNF-α release into the culture supernatant was determined using WEHI cells. Relative antigenic peptide quantities (Fig. 6) were calculated from a standard curve generated with synthetic E1B192–200 in the same experiment. Two independent measurements showed that proteasomes of cells grown without TET produced the E1B epitope 3-fold more efficiently than proteasomes of cells grown with 10 ng/ml TET and 10-fold more efficiently than proteasomes of uninduced cells. Thus, the incorporation of LMP2, LMP7, and MECL-1 into the proteasome enhanced E1B192–200 generation (Fig. 6) in a dose-responsive fashion.

Discussion

The IFN-γ-inducible subunits LMP2, LMP7, and MECL-1 are believed to convert proteasomes into particles with specialized Ag processing function. However, the actual effects of these subunits on proteasome-mediated Ag degradation and CTL epitope generation are still largely unclear. In this paper, we demonstrate for the first time that the three immunosubunits, simultaneously expressed, enhance MHC class I presentation of an Ad5-derived
epitope by infected cells, resulting in improved detection of cellular infection by CTL.

The TET-based inducible gene expression system allowed us to mimic the conditions of IFN-γ induction and to examine the influences of the LMPs and MECL-1 on Ag processing detached from other IFN-γ-mediated effects. By titrating the TET concentration, the effects of the immunosubunits could be studied in correlation to their expression levels. We found that immunoproteasome-containing MEC217 cells processed and presented the Ad5 E1B192–200 epitope more efficiently than uninduced cells did, resulting in a higher susceptibility to CTL-mediated lysis (Fig. 4A) and an increased ability to activate CTL at earlier time points after infection (Fig. 4B). Thus, the presence of the LMPs and MECL-1 even at relatively low levels (Fig. 4B) allows the efficient detection of intracellular viruses already at early stages of infection, resulting in a rapid elimination of infected cells. Interestingly, neither the Eu³⁺-release assay nor the TNF-α production assay showed a clear enhancement of MHC class I presentation of an E1A-derived CTL epitope by induced cells (not shown), indicating that immunoproteasomes may not significantly influence the processing of this epitope. Thus, the immunosubunits might alter the generation efficiency of different epitopes differentially.

Our in vitro digestion experiments with E1B polypeptide showed that immunoproteasomes yielded E1B192–200 more efficiently than constitutive proteasomes did (Figs. 5 and 6), indicating that the presence of the LMPs and MECL-1 supported the production of this antigenic peptide. Because digestion with immunoproteasomes instead of with constitutive proteasomes led to a faster degradation of E1B polypeptide (not shown) and a more efficient generation of the E1B176–191 and E1B201–215 single cleavage products (Fig. 5), we infer that the observed enhanced antigenic peptide production in the presence of immunoproteasomes is likely to follow from more frequent cleavage at the Leu¹⁹¹-Val¹⁹² and Ile²⁰⁰-Ser²⁰¹ peptide bonds. Although our mass spectrometry analyses revealed the presence of at least one cleavage site within the E1B192–200 sequence (between Cys¹⁹⁷ and Cys¹⁹⁸; not shown), truncated E1B epitopes containing either the correct N or COOH terminus were not detected (not shown). Thus, although cleavage at internal sites may influence the usage of the Leu¹⁹¹-Val¹⁹² and Ile²⁰⁰-Ser²⁰¹ peptide bonds and thereby the efficiency of antigenic peptide liberation, it is unlikely that E1B192–200 peptide is degraded after previous generation.

Although the magnitude of antigenic peptide production in vitro appeared to be determined by the absolute levels of IFN-γ-inducible subunits (Fig. 6), we found that in intact cells relatively low levels of immunoproteasomes were sufficient to reach maximal Ag presentation (Fig. 4B). Therefore, already the smaller enhancement of E1B192–200 production in infected cells under conditions of submaximal immunosubunit expression, reflecting initial IFN-γ induction, results in enough MHC class I peptide complexes to achieve maximal CTL activation. Taken together, our observations underscore the physiological relevance of in vitro peptide digests with 20S proteasomes, which is in agreement with previous studies that showed a correlation between polypeptide digestion analyses in vitro and MHC class I presentation of the studied antigenic peptides by intact cells (8, 9, 37–39).

Nevertheless, the intriguing question of how to translate the data obtained with polypeptide digests to the situation in intact cells remains. Cytosolic (19S-containing) proteasomes are believed to degrade full-length proteins, mainly targeted by linkage to ubiquitin moieties which are recognized by subunits of the 19S component. Indeed, studies examining MHC class I Ag processing of Ags that were introduced by electroporation or that were intracellularly secreted by bacteria demonstrated that the substrate-targeting mechanisms limited the rate of protein degradation and antigenic peptide generation (4, 40). Alternative theories concerning cytosolic protein degradation propose that nonproteosomal enzymes mediate the first cleavages in Ags, after which the generated fragments are further cleaved by proteasomes, thereby circumventing the need for 19S. Furthermore, in case of endogenously synthesized Ags, so-called defective ribosomal translation products may serve as an additional source of antigenic peptides (41). Compatible with either theory, the incorporation of the IFN-γ-inducible subunits may change the degradative mechanism of proteasomes, resulting in altered preferences (11, 14, 15) and frequencies (this study) of cleavage site usage and consequently in improved antigenic peptide generation. Preliminary data (not shown) indicate that PA28, which increases MHC class I Ag presentation of certain viral Ags (8, 9), does not influence epitope generation from the Ad5 E1A and E1B proteins.

Immunofluorescence studies using LMP2-specific mAbs reveal an asymmetrical localization of LMP2 proteasomes in induced cells (C. Knuehl and P. M. Kloetzel, unpublished observations). Whereas an antiserum specific for 20S complexes shows an equal division of proteasomes over cytoplasm and nucleus, LMP2-containing proteasomes mainly seem to colocalize with TAP around the ER. This finding would corroborate previous biochemical data demonstrating that 20S proteasomes copurify with microsomal fractions (42) and with ER fractions (43). Thus, immunoproteasomes, unlike constitutive proteasomes, may reside in the vicinity of TAP and may deliver the generated peptides for immediate transportation into the ER, which may contribute to an improved MHC class I Ag presentation.

Thus, although not essential for the generation of many CTL epitopes, immunoproteasomes may tune the Ag processing machinery by multiple ways to improve the efficiency of MHC class I Ag presentation, allowing an optimal immunosurveillance by CTL.
In antigen presentation...a...