Production of a Specific Major Histocompatibility Complex Class I-restricted Epitope by Ubiquitin-dependent Degradation of Modified Ovalbumin in Lymphocyte Lysate*

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Peptide epitopes presented through class I major histocompatibility complex (MHC class I) on the cell surface, are generated by proteolytic processing of protein-antigens in the cytoplasm. The length and amino acid sequence determine whether a given peptide can fit into the peptide binding groove of class I heavy chain molecules and subsequently be presented to the immune system. The mode of action of the processing pathway is therefore of great interest. To study the processing mechanism of MHC class I-restricted intracellular antigens, we reconstituted the proteolytic processing of a model antigen in a cell-free system. Incubation of oxidized and urea-treated OVA in lymphocyte lysate resulted in partial degradation of the antigen. Degradation of the antigen depended on the presence of ATP. Addition of methylated ubiquitin abolished the reaction which was then restored by addition of an excess of native ubiquitin, indicating that the breakdown of the antigen in lymphocyte lysate is mediated by the ubiquitin proteolytic system. Upon incubation of modified OVA in lymphocyte lysate, a specific antigenic peptide was generated. The peptide was recognized by cytotoxic T lymphocytes directed against OVA-derived, H-2Kb-restricted peptide (SIINFEKL), and by a monoclonal antibody that recognizes cell-bound R9-SIINFEKL complexes. Formation of the peptide epitope depended on the presence of ATP and ubiquitin. These results indicate that proteolytic processing of modified OVA is carried out by the ubiquitin-mediated degradation system. The experimental system described provides a tool to analyze the molecular mechanisms underlying the generation of specific, MHC class I-restricted peptide epitopes.

Cells display foreign and altered intracellular protein-antigens to cytotoxic T-lymphocytes through MHC1 class I molecules. Intracellular antigens are not presented directly. The intracellular antigen is first proteolyzed in the cytoplasm of the antigen presenting cell to yield short peptides. Suitable peptides are then translocated through specialized peptide transporters (termed TAP) into the lumen of the endoplasmic reticulum where they bind to newly synthesized MHC class I molecules. The entire complex is then stabilized and transported to the plasma membrane where it is presented to the immune system (1).

Proteolysis and generation of antigenic peptides is carried out by the multicatalytic protease (26 S proteasome). Direct evidence for the involvement of the proteasome in the processing of MHC class I-restricted intracellular antigens came from experiments in which membrane-permeable inhibitors of proteasomes were added to cells and the capacity of these cells to present antigens was monitored. These inhibitors, which block proteasome activity in vitro, inhibited the cellular turnover of short-lived and long-lived proteins, assembly of class I molecules as well as presentation of OVA introduced into the cytoplasm (2).

The proteasome constitutes the major proteolytic activity in the cytosol and nucleus of all eukaryotes (3). It is composed of two multisubunit complexes: a proteolytic core particle (20 S proteasome) and a regulatory component termed proteasome activator 700 (PA700 or 19 S particle) (4). ATP-dependent assembly of 20 S proteasomes with PA700 cap complexes forms 26 S proteasomes (5).

The 20 S proteasome is an ATP-independent protease that in vitro only cleaves peptides (6). The 20 S proteasome can proteolyze several completely unfolded proteins, but, only when activated by treatment with SDS (7). The physiological function of this particle is therefore unknown. The 20 S proteasome can also associate with the PA28 activator complex (11 S regulator) that enhances in vitro cleavage of short peptides but not of proteins (8, 9). The two homologous subunits of PA28 (α and β) are inducible by interferon-γ (10, 11), suggesting a role for this activator in the processing pathway. PA28 stably expressed in a mouse fibroblasts line significantly enhanced the class I-mediated presentation of two viral epitopes, pointing to a role for the 20 S-PAPA28 complex in vivo (12). The reason for the augmenting effect seems to be favorable modulation of proteasomal cleavage activity (13). Interferon-γ also induces the expression of the 20 S proteasome subunits LMP2 and LMP7, which replace constitutive β-type subunits (14). Through use of precursor peptides as substrates it has been demonstrated that incorporation of LMP2 and LMP7 may alter the cleavage specificity of the 20 S proteasome in a manner that favors the generation of peptide epitopes (15). In vivo it has been shown that presentation of most antigens is unaffected by LMP2 and LMP7. However, expression of LMP2 and LMP7 can restore defects in surface presentation of certain viral antigens.
through specific MHC class I molecules in LMP-deficient cell lines (16). These observations indicate that proteolytic processing is selectively influenced by the subunit composition of the 20 S proteasome and by the association of specific regulators such as PA28. The underlying mechanism for the selective effect of LMP2 and LMP7 on the processing of protein-antigens is obscure.

The 26 S proteasome is an ATP-dependent protease that degrades mostly ubiquitin-conjugated proteins (17). The ubiquitin proteolytic system is a major pathway for degradation of proteins in the cytoplasm and nucleus of all eukaryotes. In this system, proteins are targeted for degradation by their prior ligation to ubiquitin, a 76-amino acid polypeptide which was highly conserved during evolution (18). Covalent attachment of ubiquitin to proteins requires ATP and is carried out by the ubiquitin ligase system (19). Ligation of ubiquitin is initiated by formation of an isopeptide bond between the carboxyl terminus of a single ubiquitin molecule and an e-amino group of a lysine residue of the target protein (20). A polyubiquitin chain is then elongated by the successive formation of isopeptide bonds between the e-amino group of lysine 48 of the previously incorporated ubiquitin molecule and the carboxyl terminus of the incoming ubiquitin molecule (21). The serial addition of ubiquitin molecules results in the synthesis of a long polyubiquitin chain (high molecular weight ubiquitin-protein conjugate). Polyubiquitinated proteins are targeted to proteolysis by the 26 S proteasome. The protein moiety of the conjugate is degraded to short peptides and free ubiquitin is released for reutilization (22).

It has been shown that increased susceptibility to ubiquitination can facilitate the class I-restricted presentation of influenza virus nucleoprotein (23), HIV-1 nef (24), and β-galactosidase (25). However, the general role of ubiquitination in the targeting of intracellular antigens is still unknown. Furthermore, in-depth study of the proteolytic processing of foreign antigens to yield antigenic MHC class I-restricted epitopes has been impeded due to the absence of a suitable system in which the process can be reconstituted in vitro. Here we report the development of such an in vitro system based on the processing of a model protein-antigen, oxidized and urea-treated OVA (uOVA) in lymphocyte lysate. Using it we show that the model antigen is degraded by the ubiquitin-proteasome dependent pathway, rather than by the 26 S proteasome. This system will facilitate biochemical characterization of all cytoplasmic reactions leading to the generation of the antigenic epitopes.

**EXPERIMENTAL PROCEDURES**

**Materials**

Albumin from chicken egg (type VII) and ubiquitin from bovine erythrocytes were from Sigma. Synthetic H-2Kb-restricted OVA-peptide (SIINFEKL) was prepared by AnaSpec (San Jose, CA). Reductively methylated ubiquitin was prepared according to a previously published procedure (26). In the preparations used, >99% of the amino groups were blocked, as determined by reaction with fluorescamine (26).

**Cell Lines, Recombinant Viruses, and Monoclonal Antibodies**

The T lymphoma cell lines RMA and its mutant counterpart, RMA/S (27), were kindly provided by Professor Lea Eisenbach (The Weizmann Institute, Rehovot, Israel). Recombinant vaccinia virus expressing the H-2Kb-restricted OVA epitope was a kind gift from Dr. J. W. Wedell (NIH, Bethesda, MD). MAB Y-3 was from American Type Culture Collection; mAb 25-D1.16 was generated by Dr. Angel Forgard (28).

**Mice**

Nine- to 10-week-old female C57Bl mice were purchased from Harlan Sprague-Dawley and kept under laminar flow in the animal facility of The Sackler Faculty of Medicine, Tel Aviv University.

**Preparation of urOVA**

The urOVA was prepared by a two-step procedure.

**Step 1: Oxidation**—The reaction mixture contained the following components in a final volume of 100 µl: OVA (2 mg), 100 mM sodium phosphate buffer (pH 7.4), 1 mM sodium iodine. The reaction at room temperature was initiated by addition of 50 µg of chloramine-T and was terminated after 2 min by addition of 100 µg of sodium metabisulfite. The mixture was then loaded onto a PD10 column (Pharmacia Biotech Inc.) equilibrated in buffer A: Tris-HCl (pH 7.6), sodium chloride, 50 mM. The column was then washed by successive addition of 0.5-ml portions of buffer A. Fractions containing oxidized OVA (eluting between 2.5 and 3.5 ml) were pooled. Aliquots were then subjected to treatment with urea.

**Step 2: Urea Treatment**—Urea (96 mg) was dissolved in a 150-µl aliquot containing approximately 600 µg of oxidized OVA. Incubation was for 2 h at room temperature. The mixture was then subjected to filtration on a PD10 column (equilibrated in buffer A) as described above. The protein containing fractions were pooled and concentrated in Centricon 30 (Amicon). The concentrated protein was further dialyzed for 2 h against cold buffer A. The final protein preparation termed urOVA was stored in aliquotes at −70 °C.

**Preparation of 125I-ur-OVA**

OVA (1 mg) was radioiodinated as described above for the oxidation (“cold iodination”) of OVA, except that 1 mCi of Na125I (Amersham) was included in the reaction mixture. The radioiodinated protein was then treated with urea as described above.

**Large Scale Cultures of RMA Cells**

RMA cells were grown in spinner flask bottles (Bellco Glass Inc.) in RPMI 1640 containing 10% fetal calf serum to 1.5 × 10⁶ cell/ml. The cells were then pelleted and washed twice with phosphate-buffered saline. Cell pellets were either processed immediately or stored at −70 °C.

**Preparation of RMA Lysates**

RMA cell pellets were resuspended in 5 ml/10⁶ cells of buffer containing 20 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, and 1 mM EDTA. Lysis was under nitrogen pressure in a cell disruption chamber (Parr Instruments). The crude cell extract was subjected to fractional centrifugation at 1,000 × g and then at 10,000 × g. The 10,000 × g supernatant was subjected to ultracentrifugation for 1 h at 100,000 × g. The resulting supernatant (lysate) was stored in aliquotes at −70 °C.

**Preparation of OVA-specific CTL**

CTL specific for the H-2Kb-restricted OVA epitope derived from amino acid 257–264 of OVA were obtained from splenocytes of C57Bl/6 mice immunized with recombinant vaccinia virus expressing the octapeptide epitope as a minigene as described previously (29).

**Degradation Assays**

Degradation reaction mixtures contained the following components in a final volume of 25 µl: 40 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol, 5 mM MgCl₂, 1 mM ATP, 1 mM creatine phosphate, 1.25 units of creatine phosphokinase, RMA lysate (70 µg of total protein), 0.5 µg of 125I-urOVA, and additional compounds as indicated in the figure legends. Incubation was for the indicated periods of time at 37 °C. In reactions without ATP, 2-deoxyglucose (20 mM) and hexokinase (1.5 µg) (Boehringer Mannheim) were added instead of ATP and the ATP re-generating system. Reactions were stopped by addition of trichloroacetic acid. Degradation was determined by measuring the amount of soluble radioactivity after addition of trichloroacetic acid. ATP-dependent degradation was calculated in reactions performed in the presence of ATP after subtraction of trichloroacetic acid soluble radioactivity released in a parallel incubation carried out in the absence of ATP. ATP-independent degradation was determined in reactions performed in the absence of ATP after subtraction of acid-soluble radioactivity present in parallel reaction mixtures to which trichloroacetic acid was added at time 0.

**Conjugation Assays**

Conjugation assays were carried out in a reaction mixture containing the following components in a final volume of 25 µl: 40 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol, 5 mM MgCl₂, 0.5 µg of 125I-ur-OVA (approximately 100,000 cpm), RMA lysate (70 µg), 1 mM ATP, 80 µM ubiquitin aldehyde (30), and additional compounds as indicated. Reactions with-
out ATP contained deoxyglucose (20 mM) and hexokinase (1.5 μg) instead of ATP-S. Incubation was for 1 h at 37 °C. Reactions were then separated by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gels. Gels were dried and exposed to XAR-5 film (Kodak).

Proteolytic Processing Assays

Step 1: Degradation of urOVA—Reactions were carried out as described above in a final volume of 2 ml containing the following components: 40 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol, 5 mM MgCl$_2$, 1 mM ATP, 10 mM creatine phosphate, 100 units of creatine phosphokinase, RMA lysate (5.6 mg of total protein), 150 μg of urOVA, and additional compounds as indicated in the figure legends. Reactions were incubated at 37 °C for various time periods as indicated in the text and then immediately subjected to acid extraction.

Step 2: Acid Extraction—The reaction mixture was adjusted to pH 2 by addition of trifluoroacetic acid to a final concentration of 0.6% (v/v), then sonicated at full power for 30 s in a bath sonicator and rotated for an additional 30 min at 4 °C. The mixture was then microcentrifuged and the clear supernatant was subjected to size exclusion chromatography to remove proteins.

Step 3: Isolation of Low Molecular Weight Material—The acid extract was loaded onto a 1.6 × 50-cm G-25 Sephadex (medium) column equilibrated to 0.1% trifluoroacetic acid, 90% acetonitrile; gradient, 4–60% B in 56 min; flow rate, 0.2 ml/min). All the material eluting between 20 and 36 min (24–45% B) was loaded onto a 1.6 × 25-cm C18 column (Vydac) (eluent A, 0.1% trifluoroacetic acid, 4% acetonitrile; eluent B, 0.085% trifluoroacetic acid, 90% acetonitrile; gradient, 4–60% B in 56 min; flow rate, 0.2 ml/min). All the material eluting between 20 and 36 min (24–45% B) was harvested by the continuous manual collection of individual peaks and the clear supernatant was subjected to ultrafiltration in Centricon 3 (Amicon). The filtrate was collected, dried, and subjected to reverse-phase HPLC.

Step 4: Reverse-phase HPLC—Low molecular weight material from step 3 was separated on a 2.1 × 150-mm C18 column (Vydac) (eluent A, 0.1% trifluoroacetic acid, 4% acetonitrile; eluent B, 0.085% trifluoroacetic acid, 90% acetonitrile; gradient, 4–60% B in 56 min; flow rate, 0.2 ml/min). All the material eluting between 20 and 36 min (24–45% B) was harvested by the continuous manual collection of individual peaks absorbing at 214 nm. The organic solvent was then removed from each fraction by three freeze-dry wash cycles. OVA-peptide eluted consistently between 32 and 35 min (36–39% solvent B).

Step 5: Cytotoxicity Assays—Each fraction from step 4 was dissolved in 160 μl of serum-free ISCOV medium, divided evenly into three separate wells of a 96-well plate each containing 5 × 10$^5$ [%35S]methionine-labeled RMA/S cells (in 50 μl of ISCOV). Peptides were allowed to bind to the cells for 3 h at 37 °C. OVA-specific CTL (in 100 μl of RPMI 1640 medium containing 20% fetal calf serum) were then added at an effector:target ratio of 50:1 for further incubation for 5 h at 37 °C. Following the second incubation period, cells were pelleted. CTL-mediated killing of RMA/S cells was then determined by measuring the amount of [%35S]methionine released to the culture medium in each well. The result for each peptide is the mean value of the triplicate incubation. The proportion of target cells lysed by CTL is expressed as percent specific lysis and is computed according to the equation,

\[
\text{% Specific lysis} = \frac{\text{experimental release} - \text{media release (no effectors)}}{\text{total release} - \text{media release}} \times 100
\]

Flow Cytometry

Cells were incubated with primary antibodies for 30 min at 4 °C, washed with phosphate-buffered saline, 5% fetal calf serum, 0.1% sodium azide, then incubated with fluorescein isothiocyanate-F(ab')$_2$, goat anti-mouse IgG for 30 min at 4 °C, washed, and resuspended in the same medium plus propidium iodide to exclude dead cells during analysis. Stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mansfield, MA).

RESULTS

Degradation of urOVA in RMA Lysate Is Mediated by the Ubiquitin-dependent Proteolytic System—To investigate the mechanism of the cytoplasmic reactions involved in the proteolytic processing of MHC class I-restricted intracellular antigens it was necessary to reconstitute the degradation of a foreign antigen in a cellular lysate. urOVA served as a model antigen since modification by oxidation followed by treatment with urea substantially increases the susceptibility of the an-

FIG. 1. Degradation of [%35S]urOVA in RMA lysate. Radiolabeled urOVA was incubated in RMA lysate for the indicated time periods without ATP, ●; with ATP, □; with ATP in the presence of: ubiquitin (20 μM), ■; MeUb (20 μM), □; MeUb (20 μM) and ubiquitin (40 μM), ○. Degradation was determined as described under “Experimental Procedures.”

tigen to degradation in rabbit reticuloocyte lysate.\(^2\) We then tested whether proteolysis of the antigen results in the formation of an OVA-specific antigenic epitope by comparing the conditions required for degradation with those required for generation of the antigenic peptide.

When urOVA was incubated in lymphocyte lysate (prepared from the lymphoma cell line RMA) it was degraded in a time-dependent fashion, in the presence but not in the absence of ATP (Fig. 1). The requirement for ATP for urOVA degradation suggested that proteolysis is carried out by the ubiquitin-proteasome proteolytic pathway. To determine that polyubiquitination is indeed required for urOVA degradation, the effect of the ubiquitin derivative, MeUb, on the degradation and ligation of urOVA to ubiquitin was tested. This derivative was used in the past to demonstrate ubiquitin requirement for degradation of mitotic cyclins in clasm extracts (31). In MeUb, all lysine e-amino side groups are chemically blocked by reductive methylation (20). Conjugation of MeUb to the proteolytic substrate is carried out by the ubiquitin ligase system. However, whenever MeUb is incorporated into a growing ubiquitin chain, it terminates the chain extension, because its lysine 48 residue is blocked. Thus, in the presence of MeUb, low (short) rather than high, molecular weight conjugates are formed. Low molecular weight ubiquitin-protein conjugates (in contrast to HMW ubiquitin-protein adducts) are poorly degraded by the 26 S proteasome (22). Thus, MeUb acts as an effective inhibitor of the ubiquitin system because it competes with native ubiquitin for the conjugating enzymes but produces futile ubiquitin-protein conjugates. As seen in Fig. 1, addition of MeUb to the reaction mixture almost completely inhibited degradation of urOVA in RMA lysate. This inhibition could be overcome by addition of excess native ubiquitin.

We next tested the effect of MeUb on the ligation of ubiquitin to urOVA (Fig. 2). In the absence of ATP or ubiquitin, HMW ubiquitin-OVA conjugates were not visible (lanes 1 and 2). Addition of ubiquitin stimulated the formation of HMW conjugates (lane 3). Addition of MeUb resulted only in the formation of low molecular weight ub-OVA conjugates (lane 4). Native ubiquitin relieved inhibition by MeUb and restored generation of HMW ub-OVA conjugates (lane 5). These results and the inhibitory effect of MeUb on urOVA degradation (Fig. 1) indicate that formation of polyubiquitin chains is required for the degradation of urOVA in RMA lysate. The absence of detecta-

\(^2\) A. Ciechanover, unpublished data.
ble ubiquitin-OVA conjugates in the presence of ATP, and the fact that addition of ubiquitin greatly stimulated the formation of HMW ubiquitin-OVA conjugates indicate that the concentration of free ubiquitin in RMA lysate is very low. Nonetheless, the comparable rates of degradation of urOVA with and without added ubiquitin (Fig. 1) suggest that upon addition of excess ubiquitin, proteolysis becomes rate-limiting.

Degradation of urOVA in RMA Lysate in the Presence of ATP Produces an H-2K\(^b\)-restricted Peptide Epitope—Proteolytic processing of OVA in vivo produces a specific H-2K\(^b\)-restricted epitope (32), henceforth the OVA-peptide. We therefore tested if degradation of urOVA in lymphocyte lysate also produces the expected antigenic epitope. RMA lysate was incubated either in the presence or absence of urOVA. Peptides were then extracted and purified by reverse phase chromatography on HPLC (Fig. 3). Individual HPLC-purified peptide fractions were preincubated with target RMA/S cells and then tested for recognition by OVA-specific H-2K\(^b\)-restricted CTL in a cytotoxicity assay. Lysis of target RMA/S cells was elicited by a single peptide fraction isolated from a reaction carried out in the presence of urOVA (Fig. 4B). OVA-peptide eluted consistently at the position marked by the arrow in Fig. 3 (between 36 and 39% of solvent B). No significant reactivity was observed by peptides isolated from the incubation without urOVA (Fig. 4A).

It is an open question whether an individual antigen can be processed by the 20 S as well as by the 26 S proteasome. We therefore tested if appearance of the antigenic peptide resulting from the processing of urOVA required ATP. As depicted in Fig. 5, OVA-peptide was generated in the presence but not in the absence of ATP. This result indicates that the primary protease that processes urOVA is the 26 S proteasome. However, the possibility that the OVA-peptide is generated initially as a longer precursor by the 26 S proteasome complex and is subsequently edited by the 20 S proteasome, cannot be excluded.

To confirm that the active peptide fraction contains H-2K\(^b\)-restricted OVA-peptide, we tested if it is recognized by the monoclonal antibody 25-D1.16 that exclusively recognizes cell-bound K\(^\alpha\)-SIINFEKL complexes (28) (Fig. 6). Three peptide fractions (11–13) were tested for recognition by 25-D1.16 and Y-3, a monoclonal antibody that recognizes K\(^\alpha\)-peptide complexes (33). The mAb 25-D1.16 recognized only fraction 12, whereas Y-3 recognized all three peptide fractions. These results indicate that fraction 12 contained the peptide SIINFEKL. CTL activity (Fig. 6f) coincided with 25-D1.16 activity. The only difference between the two peptide-detection methods was that OVA-specific CTL recognized fraction 11 as well. This is likely due to the greater sensitivity of CTL that are able to detect the minute amounts of OVA-peptide eluting in this fraction.

Quantitation of K\(^{\alpha}\)-SIINFEKL complexes by mAb 25-D1.16 revealed that only 2 fmol of OVA-peptide were recovered following incubation of 3 nmol of urOVA for 30 min in RMA lysate. During this incubation period, approximately 2% (60 pmol) of urOVA was degraded (Fig. 1). The yield of OVA-peptide is therefore only 3 \(\times\) 10\(^{-5}\) of the maximal theoretical yield expected if every urOVA molecule degraded would result in the formation of one molecule of OVA-peptide. The possible reasons for the low abundance of the antigenic peptide are discussed below.
Generation of OVA-peptide Is Ubiquitin-dependent—We demonstrated that degradation of urOVA in RMA lysate requires polyubiquitination. If peptides are generated directly by proteolysis, then the generation of OVA-peptide is also expected to require formation of high molecular weight urOVA-ubiquitin conjugates. As illustrated in Fig. 7B, production of OVA-peptide in the presence of ATP was almost completely abrogated in the presence of MeUb. Inhibition in the presence of MeUb was overcome by addition of excess native ubiquitin. The magnitude of inhibition by MeUb and recovery of activity by native ubiquitin were in correlation with the degree of urOVA degradation in each reaction (Fig. 7A). These results confirm that proteolytic processing of urOVA in RMA lysate is largely if not exclusively dependent upon polyubiquitination and is carried out by the 26 S and not the 20 S proteasome.

**DISCUSSION**

We have developed an experimental system to study the mechanistic and regulatory aspects of the processing of MHC class I-restricted intracellular antigens. The foregoing results demonstrate that it is possible to correlate intermediate steps in the processing pathway with the formation of the end product, namely the specific MHC class I-restricted peptide epitope. Although urOVA, the protein we used as a model antigen is in fact an artificial one, it was proteolyzed by the ubiquitin-proteasome pathway previously shown to be a physiological route by which MHC class I-restricted antigens are processed (34). It is worth mentioning in this context that the basic molecular mechanism of the ubiquitin proteolytic system has also been resolved through use of non-physiological protein substrates such as the hen egg lysozyme, bovine serum albumin, and oxidized ribonuclease A (35).

The question of whether ubiquitination is obligatory for the generation of immunogenic peptides was addressed in this study by directly comparing the requirements for degradation and production of a specific immunogenic peptide. We found that, in the presence of ATP but in the absence of ubiquitination urOVA is not degraded (Figs. 1 and 2) and OVA-peptide is not produced (Figs. 5 and 7), indicating that ubiquitination of the antigen is essential for the generation of the antigenic peptide. The results presented in this work strongly imply that targeting of antigens to degradation by the 26 S proteasome is both necessary and sufficient for production of antigenic peptides. Since most intracellular proteins are marked for degradation by prior conjugation to ubiquitin, it is conceivable that the ubiquitin-mediated proteolytic pathway is the major physiological route for the processing of MHC class I-restricted antigens. However, it is possible that, similar to ornithine decarboxylase that is degraded by the 26 S proteasome in a ubiquitin-independent manner (36), specific antigens may also be processed by this protease without being ubiquitinated. The possibility of a ubiquitin-independent but proteasome-dependent pathway for class I presentation is demonstrated by Michalek and colleagues (37). The investigators show that na-

**FIG. 5. Effect of ATP on generation of OVA-peptide.** urOVA was incubated in RMA lysate for 2 h at 37 °C either in the absence (A) or presence (B) of ATP. Peptides were then isolated and tested for recognition by OVA-specific CTL.

**FIG. 6. Detection of urOVA-derived peptide by anti-Kb-SIINFEKL mAb.** Peptide fractions were isolated following degradation of urOVA in RMA lysate with ATP and ubiquitin. Three fractions (11–13) eluting at the position of elution of the OVA-derived antigenic epitope (Fig. 3) were tested for recognition by mAb 25-D1.16 by flow cytometry and OVA-specific CTL in a cytotoxicity assay. For flow cytometry, RMA/S cells, preincubated at 28 °C for 15 h, were pulsed in Iscove’s modified Dulbecco’s medium for 4 h at 37 °C with 25 mM SIINFEKL (OVA 257–264) (b) or with HPLC-purified peptide fractions 11–13 (c-e). The cells were then washed and stained with 25-D1.16 mAb (anti-Kb-SIINFEKL) complex; mlgG1, k isotype; thick lines), isotype control (dotted lines), or with Y3 mAb (anti-Kb; thin lines). Non-pulsed RMA/S (a), CTL activity (f).
tive OVA microinjected into the cytosol of antigen presenting cells requires ubiquitin ligation for MHC class I presentation, whereas presentation of microinjected, reductively methylated OVA was ubiquitin-independent but sensitive to proteasome inhibitors.

The absolute requirement of ATP and ubiquitin for the proteolytic processing of urOVA suggests that the 26 S proteasome degrades the antigen and that the 20 S particle has no independent role. In agreement with the observation that the in vitro activated 20 S proteasome can proteolyze completely unfolded proteins, Dick and co-workers (38) demonstrated that the SDS-activated 20 S proteasome can produce specific class I epitopes from completely denatured OVA and β-galactosidase. Unassembled 20 S proteasomes do exist in the cytoplasm and it is possible that they can be activated by association with specific regulators such as PA28 (39). Yet 20 S proteasomes cannot degrade native antigens. Therefore, it has been proposed that the function of the 20 S proteasome may be to edit precursor peptides that are initially produced by partial proteolysis of intact antigens by the 26 S proteasome (13). The ability of PA28 to associate with 20 S proteasomes and to stimulate their peptide cleavage (but not proteolytic) activity in vitro and the fact that PA28 stimulates the presentation of viral epitopes in vivo, strongly support this hypothesis.

The remarkably low recovery of OVA-peptide from degradation of urOVA (Fig. 6) may be attributed to several factors apart from the slim possibility that most of the peptide was lost during isolation. One probable reason may be low frequency of cleavage within urOVA at sites just flanking the epitope (OVA 257–264). Low recovery of OVA-peptide may also be caused by instability of the antigenic peptide in the absence of microsomes. It has been demonstrated in vivo that peptide epitopes derived from cytoplasmic proteins are either undetectable or present at very low amounts in the absence of class I molecules that specifically bind these peptides (40). This suggests that peptides finding no class I receptors are fully degraded by cytoplasmic peptidases in a very short time.

The question of how peptides that are released from proteasomes reach the TAP peptide transporter in the endoplasmic reticulum membrane remains open. Based on the findings that cytosolic hsp70 and hsp90 bind cytosolic peptides and elicit tumor and viral-specific immunity (41), Srivastava (42) expressed the hypothesis that hsp species may generally serve to carry antigenic peptides from the proteasome to the peptide transporters. This hypothesis remains to be challenged. In contrast, Frisan et al. (43) have recently suggested that binding of antigenic peptides to hsp70 may be detrimental for class I presentation. These investigators have demonstrated that presentation of an immunodominant HLA-A11 epitope derived from EBNA4 is impaired in two Burkitt’s lymphoma cell lines. The defect cannot be corrected by expression of the preformed epitope in the cytoplasm or by treatment with interferon-γ, which up-regulates the expression of TAP. Maturation of class I molecules is unaffected in these cells, implying that the defect most likely occurs after proteolysis and before transport of the peptides to the endoplasmic reticulum. Understanding the interaction of putative peptide chaperones with the 26 S proteasome and TAP is an unresolved problem. The underlying molecular mechanism that may cause the defect in presentation of EBNA4 may be correlated to an impaired function of a putative peptide chaperone that either cannot bind peptides or cannot discharge them. This and other questions about the mechanism and regulation of the pathway for the proteolytic processing of MHC class I-restricted antigens, are now experimentally approachable.

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