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# Pathways Accessory to Proteasomal Proteolysis Are Less Efficient in Major Histocompatibility Complex Class I Antigen Production\*

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Benedikt Kessler<sup>‡§¶</sup>, Xu Hong<sup>§¶</sup>, Jelena Petrovic<sup>¶</sup>, Anna Borodovsky<sup>‡\*\*</sup>, Nico P. Dantuma<sup>¶‡‡</sup>,  
Matthew Bogyo<sup>§§</sup>, Herman S. Overkleef<sup>¶¶</sup>, Hidde Ploegh<sup>‡</sup>, and Rickard Glas<sup>¶¶¶</sup>

From the <sup>‡</sup>Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, <sup>¶</sup>Mikrobiologiskt och Tumör Biologiskt Centrum, Karolinska Institutet, Stockholm S-171 77, Sweden, and the <sup>§§</sup>Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

Degradation of cytosolic proteins depends largely on the proteasome, and a fraction of the cleavage products are presented as major histocompatibility complex (MHC) class I-bound ligands at the cell surface of antigen presenting cells. Proteolytic pathways accessory to the proteasome contribute to protein turnover, and their up-regulation may complement the proteasome when proteasomal proteolysis is impaired. Here we show that reduced reliance on proteasomal proteolysis allowed a reduced efficiency of MHC class I ligand production, whereas protein turnover and cellular proliferation were maintained. Using the proteasomal inhibitor adamantane-acetyl-(6-aminohexanoyl)3-(leuciny)3-vinyl-(methyl)-sulphone, we show that covalent inhibition of all three types of proteasomal  $\beta$ -subunits ( $\beta_1$ ,  $\beta_2$ , and  $\beta_5$ ) was compatible with continued growth in cells that up-regulate accessory proteolytic pathways, which include cytosolic proteases as well as deubiquitinating enzymes. However, under these conditions, we observed poor assembly of H-2D<sup>b</sup> molecules and inhibited presentation of endogenous tumor antigens. Thus, the tight link between protein turnover and production of MHC class I ligands can be broken by enforcing the substitution of the proteasome with alternative proteolytic pathways.

Several cytosolic proteases, including the 26 S proteasome, bleomycin hydrolase, puromycin-sensitive amino peptidase and leucine-aminopeptidase, contribute to the generation of MHC<sup>1</sup> class I ligands (1–3). However, the 26 S proteasome, a

large multicatalytic proteinase complex, carries out the bulk of both cytosolic protein degradation and MHC class I ligand production (1, 2). This protease has a multisubunit 20 S core structure containing two sets of three distinct catalytic sites, X ( $\beta_5$ ), Y ( $\beta_1$ ), and Z ( $\beta_2$ ), associated with one or two 19 S regulatory accessory complexes (1, 2). The proteasome generates a wide range of peptide cleavage products (3–24 amino acids in length) that are ultimately degraded into free amino acids (4–6). In mammalian cells, a minor subset of peptides is rescued from further degradation and is translocated from the cytosol into the endoplasmic reticulum for assembly with MHC class I molecules. The MHC class I pathway is thereby assured constitutive production of ligands through cytosolic proteolysis.

In the case of an immunological challenge, mammalian cells express IFN- $\gamma$ -inducible proteasomal  $\beta$ -subunits (LMP7/ $\beta_{5i}$ , LMP2/ $\beta_{1i}$ , and MECL-1/ $\beta_{2i}$ ) that replace the constitutively expressed subunits in newly synthesized proteasomes (1). Such replacement leads to increased proteasomal production of peptides with hydrophobic C termini, usually preferred for both TAP transport and MHC class I binding (7). However, the majority of potential MHC class I ligands, as deduced from their primary structure, are not efficiently processed, although the correct motifs for TAP transport and MHC class I binding are contained in the protein sequence (8, 9). Such failure may depend on proteolysis by cytosolic proteases inefficient at generating the requisite cleavage products. Thus, it is possible that MHC class I processing may be regulated by differential participation of non-proteasomal peptidases in cytosolic protein degradation. Impaired proteasomal activity can be functionally compensated, at least in part, by another large cytosolic peptidase, tripeptidyl-peptidase II (10–13). Despite covalent inhibition by NLVS (14) or lactacystin (15), EL-4 lymphoma cells adapted to growth in the presence of this inhibitor (denoted EL-4ad) maintain cytosolic proteolysis and cell viability by a mechanism that includes compensatory up-regulation of tripeptidyl-peptidase II (10–12). However, it is unknown whether the adapted state has functional consequences at the level of MHC class I ligand generation and antigen presentation to CTLs.

We show that lymphoma cells with reduced reliance on proteasomal activity no longer efficiently produced MHC class I ligands, although cytosolic proteolysis continued, and proliferation was not altered compared with control cells. Assembly of H-2D<sup>b</sup> molecules was dramatically reduced, and endogenous tumor antigens were not presented efficiently under these conditions. This phenotype contributed to escape from tumor re-

sulphone; AMC, 7-amino-4-methylcoumarin; FACS, fluorescence-activated cell sorter; CMK, chloromethyl ketone; APC, antigen presenting cell.

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¶¶ Present address: Leiden Inst. of Chemistry, Gorlaeus Laboratory, 2300 RA Leiden, The Netherlands.

¶¶¶ To whom correspondence should be addressed: MTC, Karolinska Institutet, Theorells Väg 3, Stockholm S-171 77, Sweden. Tel.: 46-8-58589687; Fax: 46-8-7467637; E-mail: rickard.glas@mtc.ki.se.

<sup>1</sup> The abbreviations used are: MHC, major histocompatibility complex; IFN- $\gamma$ , interferon- $\gamma$ ; NLVS, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-vinyl sulfone; CTL, cytotoxic T lymphocyte; GFP, green fluorescent protein; Ub, ubiquitin; AAF-VS, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Ala-Ala-Phe-vinyl sulfone; LLG(*cis*)-VS, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Gly(*cis*)-vinyl sulfone; Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS, adamantane-acetyl-(6-aminohexanoyl)3-(leuciny)3-vinyl-(methyl)-

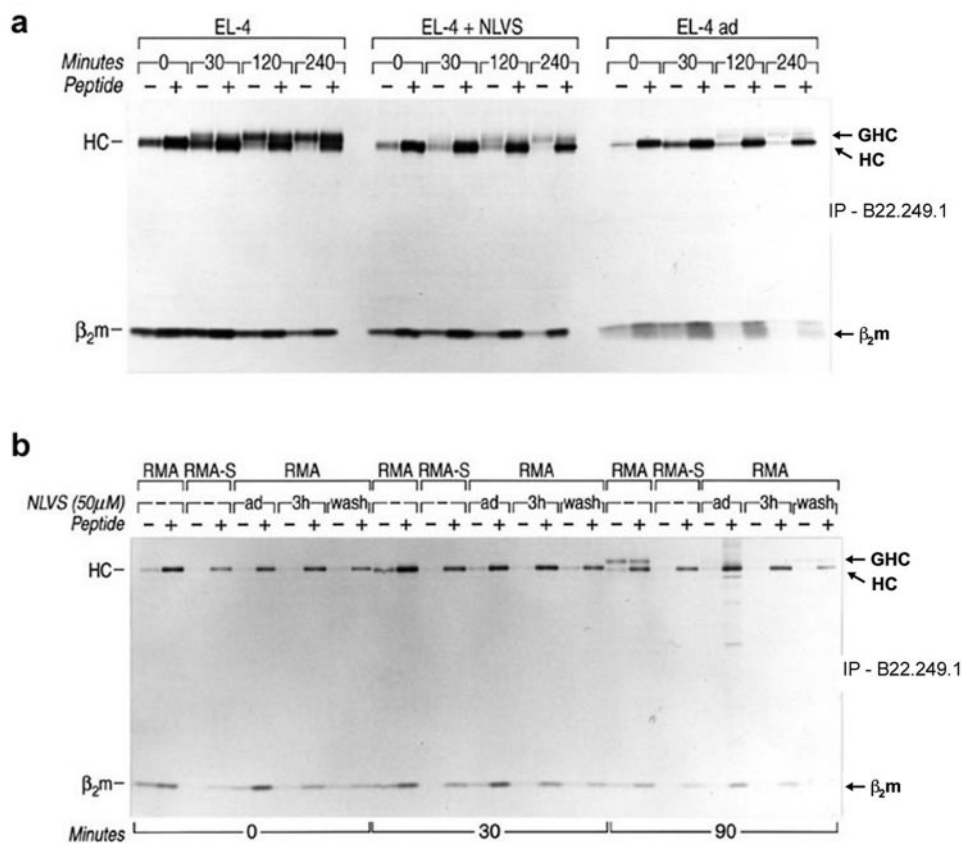


FIG. 1. Impaired assembly of MHC class I molecules in cells with reduced reliance on proteasomal activity. *a*, pulse-chase experiments were performed on control EL-4 cells, EL-4 cells treated with 50  $\mu M$  NLVS for 16 h, or EL-4ad cells. H-2D<sup>b</sup> molecules were immunoprecipitated (IP) with anti-H-2D<sup>b</sup> antibody B22.249.1 in the presence or absence of an H-2D<sup>b</sup>-binding peptide, followed by SDS-PAGE analysis and autoradiography. *b*, RMA cells left untreated or treated with 50  $\mu M$  NLVS for 3 h (3h), adapted (ad) to 50  $\mu M$  NLVS (RMAad), or adapted to NLVS and washed (wash) and RMA-S cells were subjected to pulse-chase experiments. H-2D<sup>b</sup> molecules were immunoprecipitated in the presence or absence of an H-2D<sup>b</sup>-binding peptide, followed by SDS-PAGE analysis and autoradiography. Glycosylated heavy chains (GHC) that were transported from the endoplasmic reticulum, heavy chains (HC), and  $\beta_2$ -microglobulin ( $\beta_2m$ ) are indicated with arrows.

jection in tumor graft experiments in syngeneic C57Bl/6 mice. Using a GFP reporter to measure proteolysis, we show in live cells that non-proteasomal serine peptidase activity participated in protein degradation, but inhibition of these enzymes failed to have a significant effect on the assembly of H-2K<sup>b</sup> molecules. Continued proteolysis in proteasome-impaired cells afforded the cell the requisite housekeeping functions while preventing the full display of the usual set of MHC class I-restricted epitopes.

#### MATERIALS AND METHODS

**Cells and Transfections**—EL-4 is a benzopyrene-induced thymoma cell line of the H-2<sup>b</sup> haplotype, derived from C57Bl/6 mice. RMA is a Rauscher's virus-induced T cell lymphoma cell line and is also derived from C57Bl/6. Adaptation to the proteasomal inhibitor NLVS was obtained by incubation of these cells in RPMI 1640 medium containing 5% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine, and 10  $\mu M$  NLVS. Gradually outgrowing cells were selected and cultured in 50  $\mu M$  NLVS over a period of several weeks as described previously (10). EL-4.Ub-R-GFP and EL-4.Ub-M-GFP cells were obtained by electroporation of EL-4 cells with constructs Ub-R-GFP and Ub-M-GFP (16), respectively, and stable clones were selected with 0.5 mg/ml G418. Electroporation was performed in a Bio-Rad Gene-Pulser at 250 V and 960 microfarads.

**Proteasomal Inhibitors**—NLVS (14) covalently modifies all catalytically active subunits of the proteasome, but with preference for the  $\beta$ -subunits with chymotryptic specificity. Several derivatives of NLVS were obtained by variations in the peptide scaffold: 4-hydroxy-5-iodo-3-nitrophenylacetyl-Ala-Ala-Phe-vinyl sulfone (AAF-VS) and 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Gly(*cis*)-vinyl sulfone (LLG(*cis*)-VS). LLG-VS was obtained in the *cis*- and *trans*-isomers due to the absence of a side chain on the P1 glycine. Whereas the *trans*-form of

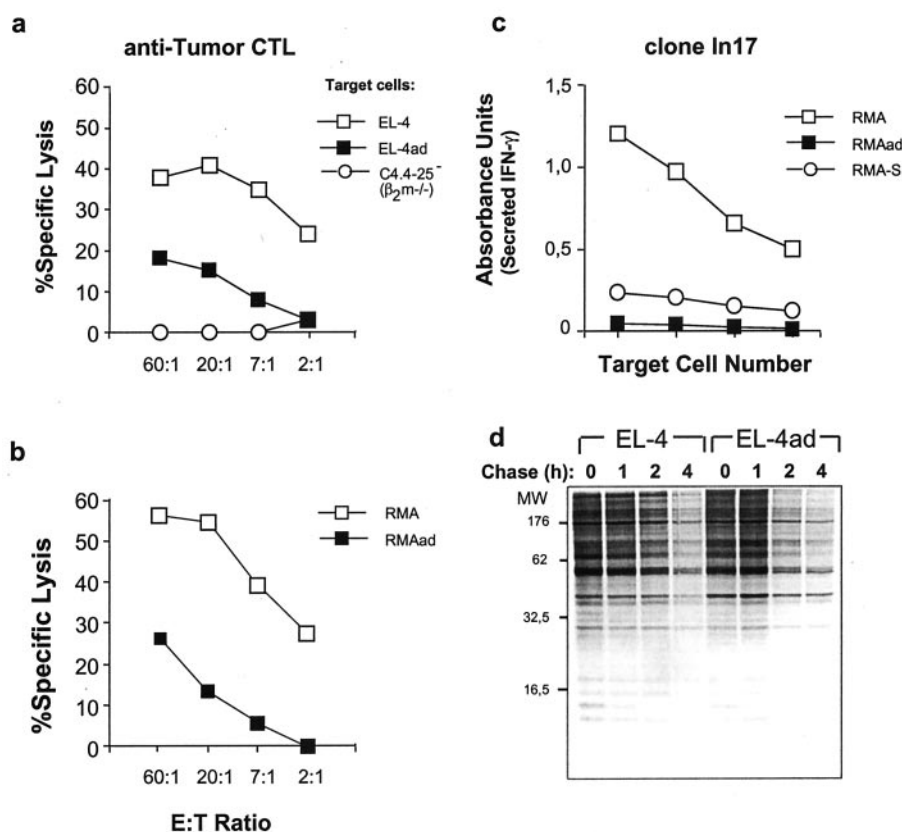
LLG-VS modifies proteasomal  $\beta$ -subunits, the *cis*-form modifies yet uncharacterized targets in the cytosol distinct from the proteasome. Adamantane-acetyl-(6-aminohexanoyl)3-(leucyl)3-vinyl-(methyl)-sulphone (Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS) is an N-terminally extended vinyl sulfone inhibitor that blocks all proteasomal  $\beta$ -subunits in a covalent manner (17).

**Peptide Substrates and Peptidase Assays**—To assay the activity of the proteasome, we used the fluorogenic substrates succinyl-LLVY-AMC, benzyloxycarbonyl-GGL-AMC, *t*-butyloxycarbonyl-LRR-AMC, and benzyloxycarbonyl-YVAD-AMC (Sigma). To assay tripeptidyl-peptidase II activity, we used AAF-AMC (Sigma). Cell extracts or proteasome-enriched fractions and substrate (100  $\mu M$ ) were mixed in 50 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 2 mM ATP in a final volume of 100  $\mu l$ . Peptide hydrolysis was monitored by fluorescence spectroscopy (PerSeptive Biosystems, Framingham, CT) with excitation at 380 nm and fluorescence reading at 460 nm.

**Proteasome Purifications**—Preparation of proteasome-enriched fractions was performed using 0.5–1  $\times 10^9$  control or adapted EL-4 cells and C57Bl/6 livers. Cells were washed with phosphate-buffered saline and lysed by vortexing with glass beads in 50 mM Tris base (pH 7.5), 250 mM sucrose, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 2 mM ATP. Glass beads and cell debris were removed by sequential centrifugations at 3000 and 14,000 rpm, respectively. Microsomes were removed by centrifugation for 1 h at 100,000  $\times g$ , and large cytosolic proteins or protein complexes containing proteasomes and tripeptidyl-peptidase II were then sedimented at 100,000  $\times g$  for 5 h. The resulting pellet was dissolved in 50 mM Tris base (pH 7.5), 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 2 mM ATP, and 30% glycerol.

**Pulse-Chase Experiments**—Cells were starved in methionine/cysteine-deficient medium for 45–60 min, pulsed with [<sup>35</sup>S]methionine for 15 min, and chased for the indicated times. Cells were collected by centrifugation and lysed in 0.5% Nonidet P-40 lysis buffer, and MHC class I molecules were immunoprecipitated with rabbit anti-p8 serum

**FIG. 2. Decreased tumor antigen presentation due to reduced reliance on proteasomal activity.**  $^{51}\text{Cr}$  release assays were performed using antitumor-specific CTLs tested against  $^{51}\text{Cr}$ -labeled EL-4 (a) and RMA (b) target cells. RMA-S and C4.4-25<sup>-</sup> are MHC class I mutant cell lines used to control for nonspecific target cell killing. Tumor antigen (NK-GENAQAI<sup>30</sup>)-specific CTL clone In17 was used to test antigen presentation by RMA, RMA-S, and RMAad cells (c). Antigen recognition was quantified by detection of IFN- $\gamma$  secretion by effector cells, as described previously (31). Bulk protein turnover was assessed by metabolic labeling of control EL-4 and EL-4ad cells with  $^{35}\text{S}$  for 15 min, and the fate of radiolabeled proteins was followed by a chase for the indicated times, SDS-PAGE separation, and autoradiography (d).  $\beta_2\text{m}^{-/-}$ ,  $\beta_2$ -microglobulin-deficient; *E/T*, effector to target ratio.



(H-2K<sup>b</sup> cytoplasmic tail), monoclonal antibody Y3 (H-2K<sup>b</sup>  $\alpha_1\alpha_2$ ), or antibody B22.249.1 (H-2D<sup>b</sup>  $\alpha_1\alpha_2$ ) as described previously (18). Viral peptide epitopes are known to stabilize the MHC class I complex at 4 °C. We added D<sup>b</sup>-binding peptide ASNENMDAM (influenza NT60, amino acids 366–374) at 10  $\mu\text{M}$  to detect the presence of unfolded heavy chains. Immune complexes were removed by adsorption to staphylococcus A and analyzed by SDS-PAGE as previously described (10).

**Generation of CTLs and  $^{51}\text{Cr}$  Release Assays**—For the generation of CTLs specific for tumor antigens expressed by EL-4 cells, we primed B6 mice two to three times with control or adapted EL-4 cells transfected with B7.1. Priming of responses with EL-4 cells not expressing B7.1 led to very low CTL responses or no response at all. To generate RMA cell-specific CTLs, we primed C57BL/6 mice with irradiated RMA cells two to three times; and for the subsequent generation of *in vitro* effector cells,  $25 \times 10^6$  splenocytes were restimulated *in vitro* with  $1-2 \times 10^6$  irradiated tumor cells for 5 days. The H-2D<sup>b</sup> restricted CTL clone In17 was acquired from Elisabeth Wolpert and Vanooi Fredriksson (MTC, Karolinska Institutet). The conditions for generation and analysis of this CTL have been described previously (23).

**Tumor Growth Experiments**—Control EL-4 and EL-4ad cells (cultured in RPMI medium 1640 supplemented with 5% fetal calf serum) were washed with phosphate-buffered saline and resuspended in 200  $\mu\text{l}$ /inoculate. The cells were inoculated into the right flanks of syngeneic C57BL/6 mice at  $10^4$  or  $10^6$  cells/animal. Some of the mice were irradiated with 400 rads prior to tumor inoculation to inhibit antitumor immune responses. Outgrowth of the tumors was monitored by palpations weekly.

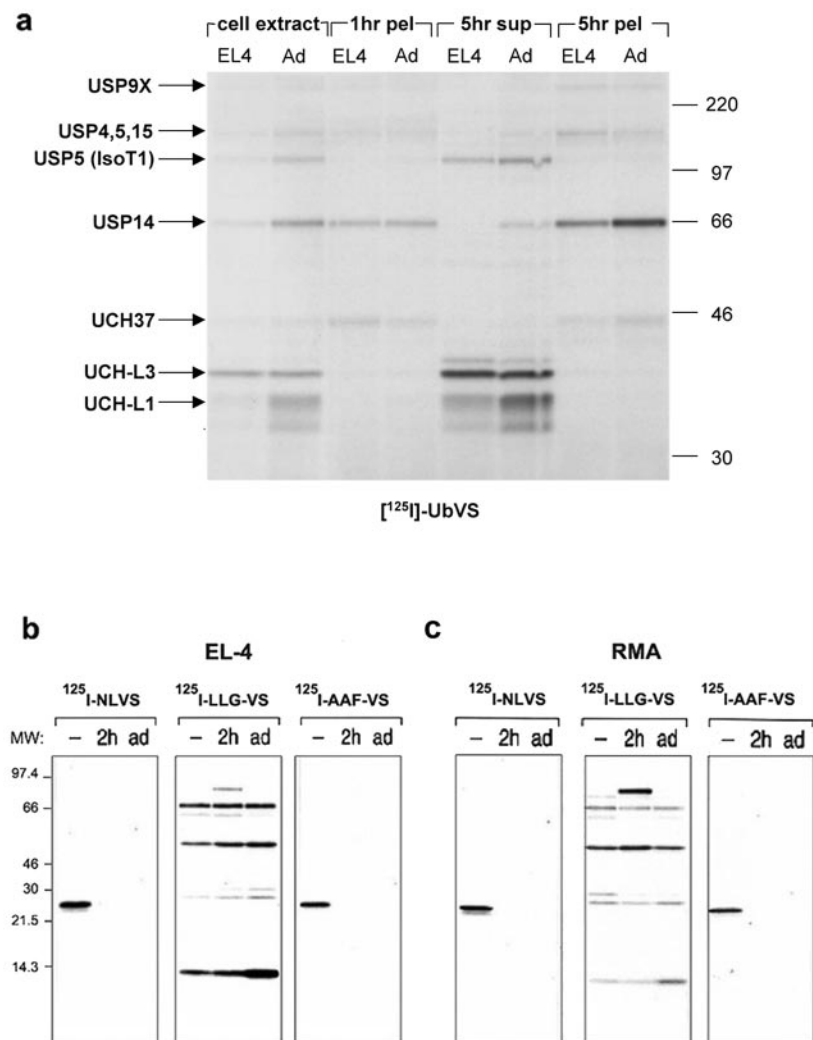
## RESULTS

**Tumor Cells with Reduced Reliance on Proteasomal Proteolysis Fail to Efficiently Produce MHC Class I Ligands**—EL-4 cells can adapt to proliferate in the presence of high concentrations of NLVS, a covalent proteasomal inhibitor (denoted EL-4ad cells) (10). MHC class I molecules show allelic variation in their ability to undergo assembly and transport during proteasomal inhibition, and H-2D<sup>b</sup> is one allele that fails to assemble in cells that are treated with proteasomal inhibitors for short-term periods (19–21). We therefore investigated H-2D<sup>b</sup> ligand production in EL-4ad cells, which proliferate with low proteasomal activity. In control EL-4 cells, almost all folded H-2D<sup>b</sup>

molecules were transported from the endoplasmic reticulum within 120 min after onset of the chase, as judged from their acquisition of Golgi-specific glycan modifications (Fig. 1a, left panel). In EL-4 cells treated with NLVS (50  $\mu\text{M}$ , 3 h), only a minimal fraction of H-2D<sup>b</sup> heavy chains were transported even after long chase times (Fig. 1a, middle panel), as reported previously (21). Stabilization of H-2D<sup>b</sup> molecules in cell lysates of NLVS-treated EL-4 cells by addition of the influenza nucleoprotein-(366–374) peptide confirmed that most of these H-2D<sup>b</sup> heavy chains were devoid of peptide ligand (+ lanes) (22). In EL-4ad cells, a fraction of H-2D<sup>b</sup> resumed folding, although at much lower levels compared with control EL-4 cells. The majority of H-2D<sup>b</sup> heavy chains in EL-4ad cells remained unassembled in the endoplasmic reticulum devoid of peptide, as indicated by the stabilizing effect of nucleoprotein-(366–374) added to lysates of these cells (Fig. 1a, right panel). In RMAad cells, which were similarly adapted to NLVS, maturation of H-2D<sup>b</sup> molecules was comparable to that observed in EL-4ad cell (Fig. 1b). Despite normal proliferation, tumor cells can therefore avoid production of most H-2D<sup>b</sup> ligands by reduced reliance on proteasomal activity.

Tumor cells often acquire deficiencies in MHC class I antigen presentation to escape from host immune detection. To test whether reduced reliance on proteasomal activity has functional consequences, we tested presentation of endogenous tumor antigens to CTLs by EL-4ad and RMAad cells. Both EL-4 and RMA cells express H-2K<sup>b</sup>-restricted (gagL<sup>75–83</sup>) as well as H-2D<sup>b</sup>-restricted (env<sup>189–196</sup>) murine leukemia virus-derived peptides and, in addition, an endogenous H-2D<sup>b</sup>-restricted tumor epitope (23). We generated antitumor CTLs by priming C57BL/6 mice and subsequent *in vitro* restimulation of splenocytes with B7.1-transfected EL-4 cells. We found that tumor antigen-specific CTLs performed efficient killing of control EL-4 cells, whereas EL-4ad cells were not efficiently recognized, although the latter were killed significantly better than

**FIG. 3. Proteolysis accessory to the proteasome analyzed by covalent vinyl sulfone probes.** *a*, isopeptidase activities were analyzed using ubiquitin-vinyl sulfone (*UbVS*) in cellular fractions from control EL-4 and EL-4ad (*Ad*) cells. *1hr pel* and *5hr pel*, protein pellets created by centrifugation at  $100,000 \times g$  for 1 and 5 h, respectively. This procedure sediments high molecular mass proteins or protein complexes. *b* and *c*, different radiolabeled peptide vinyl sulfones ( $[^{125}\text{I}]\text{NLVS}$ ,  $[^{125}\text{I}]\text{LLG-VS}$ , and  $[^{125}\text{I}]\text{AAF-VS}$ ) exhibiting different specificities were incubated with cell lysates prepared from untreated (–), NLVS-treated (2 h; *2h*), or NLVS-adapted (*ad*) lymphoma cell lines. Lysates prepared from EL-4 (*b*) and RMA (*c*) cells were separated by SDS-PAGE and analyzed by autoradiography. Whereas the first and third compounds efficiently labeled the  $\beta_5$ - and  $\beta_{5i}$ -subunits of the proteasome,  $[^{125}\text{I}]\text{LLG-VS}$  labeled other yet uncharacterized cellular proteases.



C4.4-25<sup>–</sup>, a  $\beta_2$ -microglobulin-deficient variant of EL-4 (Fig. 2*a* and data not shown). We also found that RMAad cells likewise had a reduced ability to present endogenous tumor antigens compared with control RMA cells. Although RMAad target cells were killed at higher levels than TAP-deficient RMA-S cells, 5–10 times more CTLs were required to obtain the same degree of killing as seen on RMA target cells (Fig. 2*b*). Even more pronounced differences were obtained using CTL clone In17, specific for tumor antigen-specific peptide NKGNAQAI restricted by H-2D<sup>b</sup> (20). In line with previous data, we found that In17 detected the presence of the tumor-specific epitope on RMA cells, but failed to recognize RMA-S cells (Fig. 2*c*). Furthermore, no recognition of RMAad cells was observed. MHC class I-restricted presentation of a tumor antigen-specific peptide can thereby be inhibited when proteasomal proteolysis is inhibited in a suitable manner. Because we used IFN- $\gamma$  secretion as readout for antigen detection by In17, these data also exclude that the differences in CTL killing were due merely to differences in target cell apoptosis when comparing control and NLVS-adapted target cells. These data support the conclusion that EL-4ad cells fail to display the full repertoire of MHC class I-associated antigens at the cell surface.

The chymotrypsin-like activity of the proteasome is required for the production of most MHC class I ligands and is normally rate-limiting for intracellular proteolysis (1, 4, 7). To visualize protein turnover in EL-4ad cells, we performed pulse-chase experiments with  $[^{35}\text{S}]\text{methionine}$  and displayed labeled pro-

tein by SDS-PAGE. As expected from the proliferation rates of these cell lines (10), we observed a similar rate of decay of labeled proteins when comparing control EL-4 and EL-4ad cells (Fig. 2*d*). We conclude that NLVS-adapted cells have a severely inhibited chymotryptic proteasomal activity, as deduced from experiments employing active site-directed covalent probes. When complemented by the induction of other cytosolic proteases (10–12), the remaining proteasomal activity is adequate for normal protein turnover, but not for production of all class I ligands.

*Up-regulation of Deubiquitinating Enzymes and Non-proteasomal Peptidases in EL-4ad Cells*—The activity of the ubiquitin-specific protease USP14 is associated with the 19 S cap proteasome and is up-regulated when the proteasome is inhibited (24). More generally, inhibition of proteasomal proteolysis should lead to accumulation of ubiquitin-conjugated substrates. Adaptation to proteasomal inhibitors might well include increased activity of deubiquitinating enzymes to deal with such accumulation. We therefore examined whether this was the case also in EL-4ad cells using  $^{125}\text{I}$ -labeled ubiquitin-vinyl sulfone (24). Cellular fractions of control EL-4 and EL-4ad cells (cytosolic as well as proteasome-enriched fractions) were incubated with ubiquitin-vinyl sulfone, and covalently modified polypeptides were separated by SDS-PAGE. We found increased labeling of IsoT1, USP14, and UCH-L1 in EL-4ad cells compared with control EL-4 cells (Fig. 3*a*), in line with what was observed in acutely treated EL-4 cells (24). More

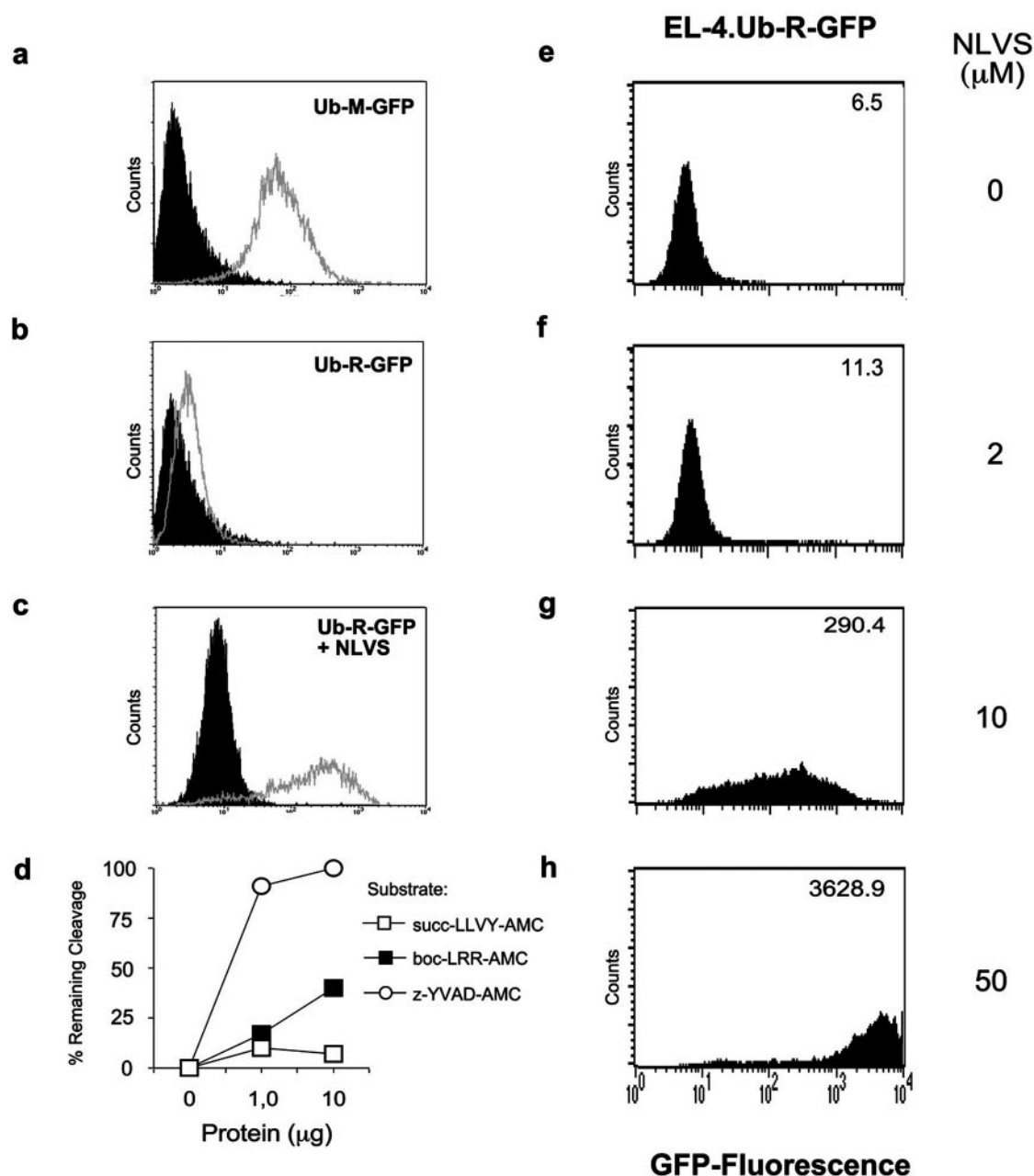


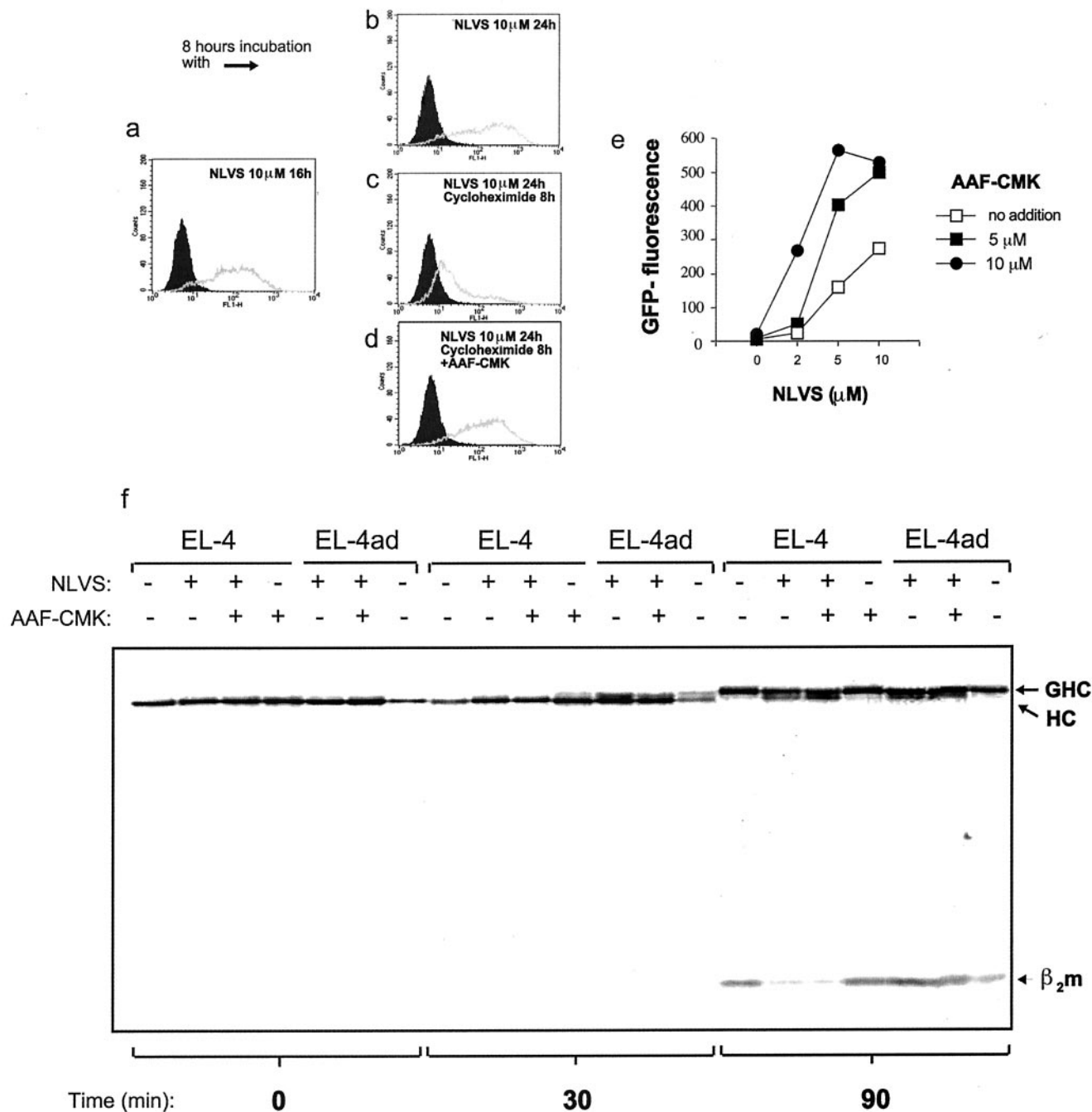
FIG. 4. **Measuring proteasomal degradation by fluorescent ubiquitinated substrates in live cells.** Shown are the results from FACS analysis of EL-4 cells stably transfected with Ub-M-GFP (*a*) or Ub-R-GFP (*b* and *c*). EL-4.Ub-R-GFP cells were left untreated (*b*) or were treated with  $10 \mu\text{M}$  NLVS (*c*). The level of proteasomal activity in EL-4.Ub-R-GFP cells treated with  $10 \mu\text{M}$  NLVS was measured by cytosolic high molecular mass protein ( $100,000 \times g$ , 5 h) cleavage of the fluorogenic peptide substrates succinyl (*succ*)-LLVY-AMC, *t*-butyloxycarbonyl (*boc*)-LRR-AMC, and benzyloxycarbonyl (*z*)-YVAD-AMC (*d*). Also shown are the results from FACS analysis of EL-4.Ub-R-GFP cells treated with up to  $50 \mu\text{M}$  NLVS (*e-h*).

active ubiquitin removal could prepare these substrates for degradation by other proteases.

Two additional active-site probes with different peptide scaffolds were used (25),  $[^{125}\text{I}]\text{AAAF-VS}$  and  $[^{125}\text{I}]\text{LLG-VS}$ , to examine whether residual proteasomal activity is mediated by the  $\beta_5/\beta_{5i}$ -subunits (X/LMP7). None of these probes labeled  $\beta_5/\beta_{5i}$ -subunits (X/LMP7) in lysates of EL-4ad cells, whereas strong labeling was detected in lysates of control EL-4 cells (Fig. 3*b*). This confirms that virtually no catalytic activity remains for the  $\beta_5/\beta_{5i}$ -subunits (X/LMP7) in EL-4ad cells, which is important in view of the fact that small amounts of peptide ligand suffice to load MHC class I molecules with peptide (26). Interestingly, using  $[^{125}\text{I}]\text{LLG}(cis)\text{-VS}$ , we detected a series of modified polypeptides distinct from proteasomal  $\beta$ -subunits. Be-

cause vinyl sulfones are mechanism-based probes (14, 27), we conclude that these polypeptides correspond to additional, yet to be identified, proteases. This activity is not inhibited by NLVS, further supporting the alteration in proteolytic specificity in EL-4ad cells (Fig. 3*b*, middle panel). Labeling of the  $\beta_5/\beta_{5i}$ -subunits (X/LMP7) with  $[^{125}\text{I}]\text{NLVS}$  was likewise inhibited in RMAad cells when tested with these peptide vinyl sulfones (Fig. 3*c*).

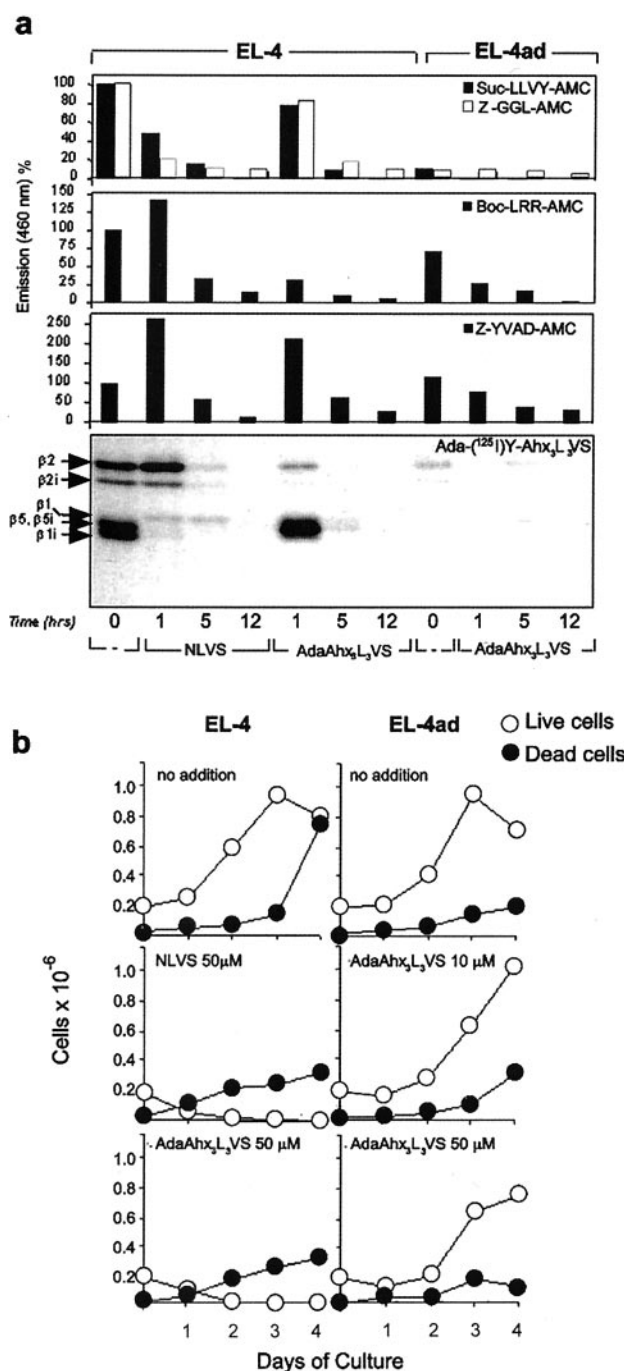
*Proteolysis Accessory to the Proteasome Supports Protein Degradation, but Is Relatively Ineffective in MHC Class I Ligand Production*—We next made stable EL-4 transfectants expressing Ub-R-GFP to monitor proteasomal degradation of a protein substrate in live cells (16). GFP was converted into an N-end rule substrate and was degraded in EL-4 cells due to its



**FIG. 5. Degradation of an N-end rule substrate is influenced by non-proteasomal oligopeptidases.** Shown are the results from FACS analysis of EL-4.Ub-R-GFP cells incubated with 10  $\mu$ M NLVS for 16 h (a) and further incubated for 8 h in the presence of no addition (b), cycloheximide (c), or 10  $\mu$ M AAF-CMK (d). EL-4.Ub-R-GFP cells were incubated in the presence of NLVS (0, 2, 5, or 10  $\mu$ M) in combination with AAF-CMK (0, 5, or 10  $\mu$ M) (e). Control EL-4 and EL-4ad cells were pulsed with [<sup>35</sup>S]methionine, and transport of H-2K<sup>b</sup> molecules was followed by immunoprecipitation and SDS-PAGE in the presence (+) or absence (-) of 50  $\mu$ M NLVS and 10  $\mu$ M AAF-CMK (f). The cells were incubated with the protease inhibitors for 3 h prior to metabolic labeling. *GHC*, glycosylated heavy chains; *HC*, heavy chains;  $\beta_2$ m,  $\beta_2$ -microglobulin.

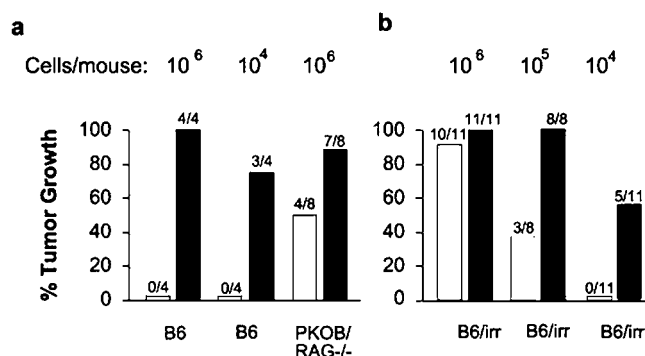
N-terminal arginine, whereas Ub-M-GFP was comparatively stable (Fig. 4, a–c) because of the presence of a methionine residue. NLVS treatment of EL-4.Ub-R-GFP cells led to accumulation of fluorescence, as detected by FACS. In line with data from yeast mutants (28, 29), efficient inhibition of primarily the chymotryptic proteasomal activity was sufficient for accumulation of fluorescence (Fig. 4, c and d). In addition, the accumulation of R-GFP fluorescence observed in cells exposed to 10  $\mu$ M NLVS also correlated with the induction of cellular toxicity and subsequent cell death (data not shown). These data further confirm that NLVS is indeed an efficient inhibitor of proteasomal protein degradation in live cells.

We next tested whether inhibition of accessory pathways has any effect on cytosolic proteolysis. To do this, we accumulated high levels of the R-GFP substrate in live EL-4.Ub-R-GFP cells and then blocked protein synthesis to study changes in the steady state of the substrate (Fig. 5, a–d). This revealed residual substrate degradation in the continued presence of 10  $\mu$ M NLVS because a substantial fraction of the substrate was removed after 8 h. However, this was inhibited by treatment with AAF-CMK, an efficient inhibitor of tripeptidyl-peptidase II and other serine oligopeptidases (Fig. 5, c and d). Although inhibition of oligopeptidases by AAF-CMK had minor effects on untreated EL-4.Ub-R-GFP cells, we observed a significant effect



**FIG. 6. EL-4ad cell proliferation despite inhibition of all catalytic sites of the proteasome by Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS.** *a*, control EL-4 or EL-4ad cells were incubated with either 50 μM NLVS or 50 μM Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS for the indicated times, and cell lysates were submitted to differential centrifugation for partial purification of proteasomes. The samples were either tested for cleavage of the peptide reporter substrates succinyl (Suc)-LLVY-AMC, benzyloxy-carbonyl-GGL-AMC, *t*-butyloxycarbonyl (Boc)-LRR-AMC, and benzyloxy-carbonyl (Z)-YVAD-AMC (upper three panels) or labeled with Ada-<sup>[125]</sup>I-Tyr-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS, followed by SDS-PAGE and autoradiography (lower panel). *b*, EL-4ad cell viability was mostly independent of proteasomal proteolysis. EL-4 (left panels) or EL-4ad (right panels) cells were left untreated (upper panels) or were incubated with 50 μM NLVS (middle left panel) or 10 μM (middle right panel) or 50 μM (lower panels) Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS for the indicated times. Live (○) and dead (●) cells were counted by trypan blue exclusion.

on R-GFP accumulation upon treating EL-4.Ub-R-GFP cells with both NLVS and AAF-CMK in combination (Fig. 5e). These data show that non-proteasomal oligopeptidase activity indeed



**FIG. 7. Increased *in vivo* tumorigenicity of cells with reduced reliance on proteasomal proteolysis.** Control EL-4 (open bars) and EL-4ad (closed bars) cells were grafted at 10<sup>4</sup> to 10<sup>6</sup> cells into the right flanks of syngeneic C57Bl/6 mice (*a*) or perforin/RAG-1<sup>-/-</sup> mice (PKOB/RAG-/-) (*b*). Frequency of tumor growth is displayed.

contributes to cytosolic proteolysis, especially during situations of limiting or insufficient proteasomal activity.

To further study whether oligopeptidases inhibitable by AAF-CMK are important in generating MHC class I ligands, we performed a pulse-chase experiment with [<sup>35</sup>S]methionine metabolic labeling and precipitation of H-2K<sup>b</sup> molecules. A substantial fraction of H-2K<sup>b</sup> molecules continue to assemble in EL-4ad cells (10). We examined whether this may be due to ligands produced by oligopeptidases inhibitable by AAF-CMK. We found that this treatment had minor effects on the assembly and transport of H-2K<sup>b</sup> molecules in EL-4ad cells and also in control EL-4 cells with active proteasomes (Fig. 5f). We conclude that pathways accessory to proteasomal proteolysis that are inhibited by AAF-CMK support protein degradation, but reveal poor yields of MHC class I ligands.

**Evidence for Continued Cell Survival and Growth without Significant Proteasomal Activity**—EL-4ad cells continue to depend on proteasomal β-subunit activity, at least to some extent (30). NLVS fails to block β<sub>2</sub>- and β<sub>2i</sub>-subunits (Z/MECL-1) *in vivo*, a pattern of inhibition that is shared between NLVS and other covalent proteasomal inhibitors such as lactacystin (15) and epoxomicin (31). To examine if residual proteasomal activity influences the viability of EL-4ad cells, we used Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS, a cell-permeable tripeptide vinyl sulfone that covalently modifies all proteasomal β-subunits with comparable efficiency (17). We found that proteasome-enriched fractions from control EL-4 or EL-4ad cells treated with either NLVS or Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS had almost completely blocked chymotryptic and trypsin-like proteasomal activities, whereas the caspase-like activity was 70% inhibited (Fig. 6a). Initially, at early time points, we observed an induction of the trypsin- and caspase-like specificities during inhibitor treatment, possibly due to allosteric effects on the proteasome upon binding of the inhibitor to the XLMP7 site (32). Consistent with the enzyme assays using fluorogenic peptide substrates, labeling of β-subunits with Ada-<sup>[125]</sup>I-Tyr-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS in cell lysates followed by separation of the β-subunits by SDS-PAGE confirmed that all proteasomal active sites were covalently modified during treatment of live cells with the Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS inhibitor (Fig. 6a, lower panel). Furthermore, EL-4ad cells proliferated regardless of the presence of Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS, whereas control EL-4 cells died within 48 h (Fig. 6b). To confirm that proteasomes of proliferating EL-4ad cells were indeed modified, we prepared proteasome-enriched fractions from cells treated for several days with Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS. This analysis revealed results similar to those observed in acutely treated cells. Essentially no residual tryptic and chymotryptic activities and inhibited caspase-like activity were detected (data not



shown). Thus, tumor cells may adapt and proliferate normally even when proteasomal  $\beta$ -subunit activity is almost absent.

**Escape from *in Vivo* Immune Detection by Tumor Cells with Reduced Reliance on Proteasomal Activity**—*In vivo* tumors frequently acquire mutations in genes encoding proteins of the MHC class I-processing pathway, and these are believed to be the result of immune selection (33). Therefore, we explored whether an adapted phenotype similar to that of EL-4ad cells was selected during growth *in vivo*. We inoculated control EL-4 and EL-4ad cells into syngeneic C57Bl/6 mice and observed tumors in most mice inoculated with EL-4ad cells at both  $10^6$  and  $10^4$  cells/animal, whereas control EL-4 cells failed to grow and produce tumors (Fig. 7a). The tumor-forming ability of EL-4ad cells was dependent, at least in part, on escape from immune recognition because both EL-4ad and control EL-4 cells formed tumors in mice with a deficiency of perforin and RAG-1 (PKOB/RAG<sup>-/-</sup>) (Fig. 7a). Furthermore, after low dose irradiation (400 rads) of the C57Bl/6 mice, commonly used to reduce *in vivo* transplantation barriers (34), both control EL-4 and EL-4ad cells were able to grow after inoculation of  $10^6$  cells/animal, whereas only EL-4ad cells grew at  $10^4$  cells/animal (Fig. 7b). In conclusion, we have shown that the ability of NLVS-adapted cells to proliferate independently of proteasomal  $\beta$ -subunit activities leads to reduced immune recognition *in vivo*.

#### DISCUSSION

This study shows that tumor cells may avoid efficient production of MHC class I ligands and hence immune recognition by modulation of proteasomal activity. Pathways accessory to proteasomal proteolysis can reduce the extent to which cells depend on proteasomal activity. In our case, cells adapted to growth in the presence of proteasomal inhibitors were unable to maintain normal levels of MHC class I ligand production. In addition, using the vinyl sulfone inhibitor Ada-Ahx<sub>3</sub>-Leu<sub>3</sub>-VS, we showed that inhibition of all catalytic  $\beta$ -subunit activities of the proteasome (more efficiently than achieved with NLVS) was compatible with continued cell growth of EL-4ad cells. These results indicate that it is possible for mammalian cells to partly escape from production of MHC class I ligands by aversion to pathways of protein degradation involving proteases other than the proteasome.

Although MHC class I processing is a rather inefficient process overall, in which most (>99%) of the cleaved peptides are never displayed at the cell surface, it is an adequate method for screening of the bulk of cellular protein content for the presence of foreign antigens (26). The steady-state level of MHC class I at the surface of cells depends on both its transport and removal from the cell surface (35), and transport of H-2D<sup>b</sup> is substantially inhibited in EL-4ad cells. Despite this, the cell-surface H-2D<sup>b</sup> (and also H-2K<sup>b</sup>) levels detected by FACS are almost normal, suggesting that the rate of decay at the cell surface may be reduced when transport is slow (data not shown). Earlier data on MHC class II transport in cathepsin S<sup>-/-</sup> mice have revealed a similar feature; and also in this case, an absence of T cell detection is observed for certain antigens (36, 37). In the course of an immune response, the proteolytic specificity in antigen processing has profound influence on the generation of MHC class I ligands, as illustrated by the IFN- $\gamma$ -dependent substitution of proteasomal  $\beta$ -subunits (7). The fact that pathways accessory to proteasomal proteolysis, such as tripeptidyl-peptidase II, can contribute to maintaining proteolysis when the proteasome is inhibited allows for mammalian cells to alter the spectrum of cleavage fragments in the cytosol more dramatically (10–13). This notion is supported by the up-regulation of several deubiquitinating enzymes in EL-4ad cells, observed otherwise in cells suffering from acute pro-

teasomal inhibition (24). USP14 is associated with the 19 S regulatory complex, and its precise role in proteasomal proteolysis remains to be established. Other deubiquitinating enzymes are also up-regulated in EL-4ad cells, such as IsoT1 and UCH-L1, which participate in the disassembly of free polyubiquitin chains (38, 39). When the proteasome is blocked, the removal of ubiquitin from ubiquitin-conjugated substrates may be a crucial step to engage alternative proteolytic pathways.

Reduced expression of several components of the MHC class I antigen-processing pathway is often observed in human tumors. This includes down-regulation of the IFN- $\gamma$ -inducible proteasomal  $\beta$ -subunits ( $\beta_{1i}$ ,  $\beta_{2i}$ , and  $\beta_{5i}$ ) (40), important for production of MHC class I ligands, as well as down-regulation of other gene products involved in antigen processing (41–43). Tumors may fail to produce certain immunodominant ligands due to altered proteasomal specificity (40, 44). Our data show that reduced reliance on proteasomal proteolysis biases cytosolic proteolysis to produce peptides that are less fit for MHC class I assembly, thereby down-regulating the pool of potential MHC class I-restricted epitopes. Such down-regulation is stably retained in rapidly proliferating cells and can be induced at fairly high frequency (10). Furthermore, EL-4ad cells appear to use this phenotype to avoid immunological rejection during the formation of tumors *in vivo*. An EL-4ad-like phenotype may be preferentially selected in tumors that are poorly antigenic, a trait observed in many types of tumors. Interestingly, in Burkitt's lymphomas, the oncogene *c-myc* is known to induce down-regulation of a number of components of the MHC class I-processing pathway, including down-regulation of proteasomal chymotryptic activity and up-regulation of tripeptidyl-peptidase II, thus linking the deficiency in antigen processing directly to oncogene expression (45, 46). This study reveals a new strategy for regulation of MHC class I processing: reduced reliance on proteasomal activity to down-regulate generation of MHC class I ligands

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**Pathways Accessory to Proteasomal Proteolysis Are Less Efficient in Major Histocompatibility Complex Class I Antigen Production**

Benedikt Kessler, Xu Hong, Jelena Petrovic, Anna Borodovsky, Nico P. Dantuma, Matthew Bogyo, Herman S. Overkleeft, Hidde Ploegh and Rickard Glas

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