Integration of the ubiquitin-proteasome pathway with a cytosolic oligopeptidase activity

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Cytosolic proteolysis is carried out predominantly by the proteasome. We show that a large oligopeptidase, tripeptidylpeptidase II (TPPII), can compensate for compromised proteasome activity. Overexpression of TPPII is sufficient to prevent accumulation of polyubiquitinated proteins and allows survival of EL-4 cells at otherwise lethal concentrations of the covalent proteasome inhibitor NLVS (NIP-leu-leu-leu-vinylsulfone). Elevated TPPII activity also partially restores peptide loading of MHC molecules. Purified proteasomes from adapted cells lack the chymotryptic-like activity, but still degrade longer peptide substrates via residual activity of their Z subunits. However, growth of adapted cells depends on induction of other proteolytic activities. Therefore, cytosolic oligopeptidases such as TPPII normalize rates of intracellular protein breakdown required for normal cellular function and viability.

The proteasome is commonly placed at the core of cytosolic proteolysis, a process essential for normal cellular physiology (1–3). Modulation of proteasome-dependent proteolysis interferes with many cell biological processes, such as cell cycle regulation (4), activation of transcription factors (5), intracellular protein breakdown (1), and antigen presentation (6). In mammalian cells, this proteolytic complex has at least three distinct catalytically active β subunits: X, Y, and Z, held responsible for the chymotrypsin-, caspase- and trypsin-like activities, respectively (7). X, Y, and Z can be replaced with their IFN-γ-inducible counterparts, LMP-2, LMP-7, and MECL-1 (2).

Although much is known about this large protease in structural terms (8, 9), less clear are aspects of its control and connections with other intracellular proteases. Genetic evidence is consistent with an essential role for the ubiquitin (Ub)-proteasome system to maintain cell viability, and yeast whose proteasome activity is ablated are not viable (10, 11). Exposure of mammalian cells to pharmacological agents that interfere with proteasome function generally leads to cell cycle arrest (12) and death (13). Proteasome inhibitors include the natural compounds lactacystin and epoxomycin (14, 15) as well as C-terminally modified peptide derivatives (1, 16), which all bind to the N-terminal threonine in the catalytic site of active β subunits (17).

We explored the essential nature of proteasomal proteolysis by culturing mouse thymic tumor cells in the continuous presence of high concentrations of a proteasome inhibitor, NLVS (NIP-leu-leu-leu-vinylsulfone) (16, 17). Upon initial exposure to NLVS, a significant proportion of cells die, leaving only a small population that continues to survive and grow. Such ''adapted'' EL-4 cells withstand otherwise lethal concentrations of this inhibitor and grow at normal rates (18). We observed an increase in a hydrolyzing activity capable of cleaving the peptide substrate, Ala-Ala-Phe (AAF)-7-amido-4-methylcoumarin (AMC), and showed that inhibition of this activity impedes growth of NLVS-adapted cells (18). In EL-4 cells adapted to 6 μM of the proteasome inhibitor lactacystin, a modest increase in AAF-AMC hydrolyzing activity was likewise observed, and tripeptidylpeptidase II (TPPII) was identified as one protease responsible for this activity (19). TPPII is a large cytosolic oligopeptidase that sequentially removes tripeptides from the free N terminus of short polypeptides, but other than its ability to inhibit cholecystokinin in the brain, its precise role in cellular physiology is largely unknown (20, 21). Although at least some of the AAF-AMC activity is attributable to TPPII, it is not known whether TPPII is required for resistance to proteasomal inhibition. We now show that TPPII can compensate for impaired proteasome activity to permit continued Ub-dependent proteolysis and maintain cell viability in the presence of high concentrations of NLVS, as well as compensate for the loss of other proteasome-dependent functions.

Methods

Preparation of Lysates, Purification of 20S Proteasomes from EL-4 and Adapted Cells, and Labeling Experiments. EL-4 and adapted EL-4 cells were maintained as described (18), and lysates from EL-4 and adapted cells were prepared by published methods (16). Subcellular fractionation was achieved by centrifugation at 100,000 *g* for 1 h, and the supernatants were spun at 100,000 *g* for 5 h. 20S proteasomes were purified from 5×10^9 EL-4 and adapted cells according to a previously published protocol (22). Synthesis and Na[^{125}I] labeling of NLVS and tyr-leu-leu-leu-vinyl-sulfone (YL_3VS) have been described (23) as was the labeling of cell extracts and purified proteasome fractions (16). In brief, 10^6 cpm of ^{125}I-NLVS or ^{125}I-YL_3VS were incubated with 20 μg of extract or 0.5 μg of purified 20S proteasome for 2 h at 37°C before visualization by autoradiography of silver-stained SDS/PAGE gels. A total of 1×10^6 cells were incubated with different concentrations of lactacystin (E.J. Corey, Harvard University, Boston) for 12 h at 37°C before cell extract preparation and incubation with ^{125}I-YL_3VS for 2 h at 37°C.

Activity Assays and *in Vitro* Digestions with Purified Proteasome. Assays for chymotryptic-, tryptic-, and caspase-like activities were performed by using the fluorogenic substrates Suc-LLVY-AMC, Boc-LLR-AMC, and Z-YVAD-AMC (Bachem), respectively, as described (16). *In vitro* proteasome digestion reactions containing 0.5 μg of purified proteasome and 20 μg of peptide DPVHGEFAPGNYPALWSYAMG (Sendai $NP_{317–338}$) were performed as described (24). Digestion reactions were analyzed

Abbreviations: NLVS, NIP-leu-leu-leu-vinylsulfone; YL_3VS, Tyr-leu-leu-leu-vinylsufone; TPPII, tripeptidylpeptidase II; Ub, ubiquitin; AMC, 7-amido-4-methylcoumarin; AAF, Ala-Ala-Phe; TIC, total ionic current; FP, fluorophosphonate.

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by C18 (Waters Delta Pak 3.9 mm × 150 mm) reverse phase-HPLC (Hewlett–Packard HP-1100). Gradient of solvent A (H_2O, 0.1% CH_3COOH) and solvent B (CH_3CN, 0.1% CH_3COOH) was 0 to 5 min at 5 to 30% B, 5 to 15 min at 30 to 40% B, 15 to 20 min at 40 to 60% B at 1 ml/min. Digestion products were analyzed by online electrospray MS (LCZ, Micromass, Manchester, U.K.) linked to the HPLC system (LC-MS). The molecular mass of degradation products were calculated from the m/z peaks in total ionic current (TIC). UV peaks of each degradation product and starting substrate were integrated and compared by measuring the appearance of fragments and degradation of initial substrate over time.

Transfections, Western Blots, Pulse–Chase, and Flow Cytometry. A total of 15×10^6 EL-4 cells were electroporated (960 μFa, 0.25 kV) with 15 μg of linearized murine TPPII cDNA (a generous gift from B. Tomkinson, Karolinska Institut, Stockholm, Sweden), and mock transfections used pcDNA3 vector DNA (Invitrogen). Cells were selected at 675 μg/ml of G418 (geneticin) in RPMI plus 10% calf serum, 10% glutamine, and penicillin/streptomycin. At 1–2 weeks, 24 single-cell clones were assayed for AAF-AMC and Suc-LLVY-AMC hydrolysis as described above. An AAF/LLVY activity ratio was determined and values that exceed those of the mock-transfected clones provided selection criteria for positive TPPII clones (6 of 24). TPPII activity was assessed by Western blot using fluorophosphonate (FP)-biotin as described below, and by size exclusion fractionation of lysates on a superose 6 column. Western blot analysis (25) using FP-biotin, a derivative of diisopropylfluorophosphate-biotin, was used as described (26). Pulse–chase experiments were performed as described (18) after 4-h incubation of cells with 50 μM NLVS before 1-h starvation and chase points up to 4 h. Lanes were quantitated on an Alphaimager 2000 (Alpha Innotech, San Leandro, CA). FACS analysis on 2×10^5 EL-4, adapted and TPPII cells incubated with 0, 5, 10, 50 and 100 μM of NLVS over 4 days was performed by FACSCalibur (Becton Dickinson) for determining the percentage of living cells.

Characterization of ^{3}H-Labeled MHC Class I-Associated Peptides. EL-4 and TPPII transfectants were incubated with 50 μM NLVS for 16 h at 37°C. Metabolic labeling with L-[3,4,5-^{3}H]leucine and L-[4,5-^{3}H]tyrosine (Amersham Pharmacia), K^b immunoprecipitation using P8 antiserum, and peptide elution was performed as described (27). Peptides were resolved on a Hewlett–Packard HPLC system (HP 1100) using reverse-phase chromatography (C18, 0.21 × 25 cm, Vydac) with a gradient of H_2O + 0.1% CH_3COOH (A) and acetonitrile + 0.1% CH_3COOH (B) at 0.4 ml/min: 0 to 10 min at 0–10% B, 10 to 15 min at 10% B, 15 to 60 min at 10 to 40% B, 60 to 65 min at 40–95% B. One-minute fractions were collected, mixed with Aquasol (NEN), and counted in a liquid scintillation counter (LKB).

Results

Proteasome Cleavage Activity Is Compromised in EL-4 Cells Adapted to Toxic Concentrations of NLVS. In adapted EL-4 cells, hydrolysis of the proteasome substrate Suc-LLVY-AMC is reduced essentially to background levels, with a concomitant decrease in ^{125}I-NLVS labeling of proteasomal β subunits (Fig. 1*A*) (18). Vinylsulfone probes can be directed to distinct proteasome β subunits depending on the peptide scaffold used (23). We exposed adapted cell extracts the more recently developed tetrapeptide vinylsulfone ^{125}I-YL$_3$-VS, which readily modifies the Z polypeptide, a β subunit less efficiently labeled by ^{125}I-NLVS (16, 17, 23). Strong residual labeling of the Z subunit with ^{125}I-YL$_3$VS is seen in extracts from adapted EL-4 cells, whereas ^{125}I-NLVS shows little if any labeling, consistent with our earlier observations (Fig. 1*A*). We conclude that in NLVS-adapted cells, the Z subunit, and to a lesser extent Mecl-1, remain

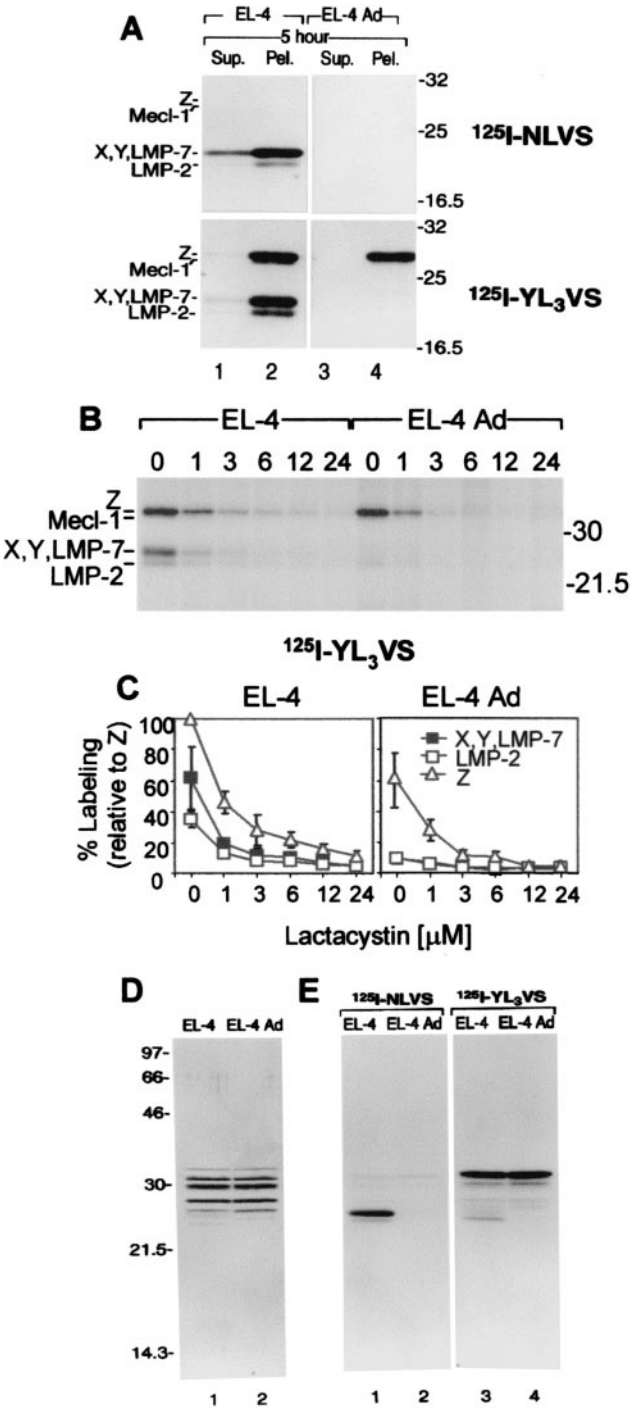

Fig. 1. Labeling of subcellular fractions and purified proteasomes from EL-4 and adapted cells with covalent active site-directed affinity probes. (*A*) Twenty micrograms of each subcellular fraction was incubated with ^{125}I-NLVS or ^{125}I-YL$_3$VS for 2 h at 37°C, resolved by 12.5% SDS/PAGE, and visualized by autoradiography. The affinity probe labeling of proteasomal β subunits labeled is described elsewhere (23). (*B*) Lactacystin was incubated with intact EL-4 and adapted EL-4 cells for 12 h at 37°C, lysed, labeled with ^{125}I-YL$_3$VS, and analyzed by 12.5% SDS/PAGE and autoradiography. (*C*) Quantitative analysis of results in Fig. 3*B*. Bands representing the Z, X/Y/LMP-7, or LMP-2 subunits were quantified by using PhosphorImager analysis (IMAGEQUANT, Molecular Dynamics). Mean values and standard deviations of three separate experiments are shown. (*D*) Purified 20S from EL-4 and adapted cells were incubated with ^{125}I-NLVS or ^{125}I-YL$_3$VS for 2 h at 37°C and visualized by silver staining the 12.5% SDS/PAGE and by autoradiography (*E*). Note: in proteasomes from adapted cells, LMP-7 is modified by NLVS and comigrates with LMP-8 (*A*, lane 2) (18).

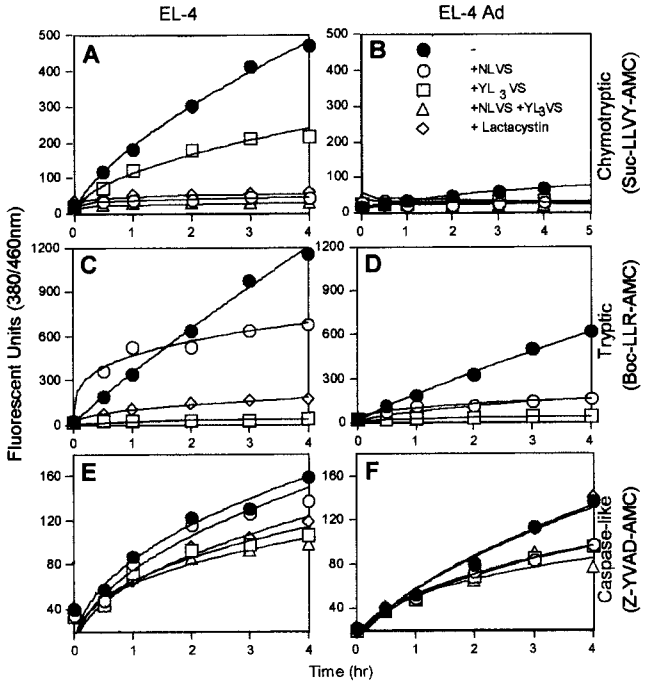

Fig. 2. Residual tryptic- and caspase-like activities in purified 20S from EL-4-adapted cells. Purified EL-4 20S incubated with (*A*) 20 μM LLVY-AMC, (*C*) 20 μM LLR-AMC, or (*E*) 100 μM YVAD-AMC. Purified EL-4 adapted 20S incubated with (*B*) 20 μM LLVY-AMC, (*D*) 20 μM LLR-AMC, or (*F*) 100 μM YVAD-AMC. Reactions were incubated with no inhibitor (●), 50 μM NLVS (○), 50 μM YL_3VS (□), 50 μM NLVS + 50 μM YL_3VS (△), or 50 μM lactacystin (◇). Note scale differences for panels.

partially unmodified and two of six catalytically active proteasomal β subunits present in each complex are thus expected to retain proteolytic activity. This proteasomal activity is similar to that found in EL-4 cells acutely treated with toxic concentrations of NLVS, where YL_3VS also labels residual Z and MECL-1 subunits (data not shown).

EL-4 cells adapted to lactacystin were reported (19) although it is not clear whether these cells grow at rates and to densities comparable to normal EL-4 cells, as do NLVS-adapted EL-4 cells (18). Does lactacystin inactivate the Z subunits? Lactacystin incompletely modifies the Z subunit at concentrations up to 6 μM, as indicated by residual labeling of the Z subunit with ^{125}I-YL_3VS in cells treated with lactacystin for 12 h, whereas the other catalytic β subunits are more completely inhibited (Fig. 1 *B* and *C* and data not shown). Lactacystin (6 μM) has a greater inhibitory effect on the Z subunit (80%, Fig. 1*C Left*) than did NLVS (40%, Fig. 1*C Right*). From this result we infer that in lactacystin-adapted EL-4 cells (19), the Z subunit may not be completely modified and hence some must remain catalytically active.

To study the extent of residual proteasomal activity in NLVS-adapted cells, we purified 20S proteasomes from EL-4 and adapted EL-4 cells and assayed them for purity by SDS/PAGE and silver staining (Fig. 1 *D* and *E*). We obtained labeling profiles for these purified preparations by using ^{125}I-NLVS and ^{125}I-YL_3VS to detect residual unmodified β subunits. As observed in adapted EL-4 cell lysates, ^{125}I-NLVS labeling of the adapted cell 20S proteasomes is starkly reduced, whereas labeling of the Z subunit with ^{125}I-YL_3VS is comparable to that seen in control EL-4 cells (Fig. 1*D*). Fluorescently labeled peptide substrates and a synthetic peptide of 21 residues were used to assess proteolysis *in vitro*. The chymotrypsin-like activity is essentially eliminated (Fig. 2 *A* and *B*), consistent with our previous observation (18). These data further support the observation that NLVS mediates its *in vivo* effect on the proteasome (18, 28) without blocking the Z and MECL-1 subunits completely. Surprisingly, when assayed by using substrates for the trypsin-like and caspase-like activities, the proteasomes isolated from adapted EL-4 cells show only modest inhibition of peptide bond hydrolysis compared with control cells (Fig. 2 *C–F*). We examined the hydrolysis of fluorescently labeled substrates in the presence of NLVS, YL_3VS, NLVS + YL_3VS, and lactacystin. NLVS preferentially inhibits Suc-LLVY-AMC hydrolysis (Fig. 2 *A* and *B*), whereas YL_3VS displays the greatest inhibition on the Boc-LLR-AMC hydrolyzing activity (Fig. 2 *C* and *D*, see also Fig. 1*A*). The combination of YL_3VS and NLVS eliminates most proteolysis with minor residual caspase-like activity (Fig. 2 *E* and *F*).

Degradation of Long Peptide Substrates by Purified Adapted Cell 20S Proteasome Exhibit Delayed Kinetics. What are the differences between fully active proteasomes and proteasomes with only active Z/Mecl-1 sites when assayed on larger substrates? We analyzed the fragmentation pattern of a 21-mer peptide from Sendai $NP_{317–358}$ by combined liquid chromatography and MS. Remarkably, we observed no alteration in the most abundant fragments generated by proteasomal proteolysis; qualitative differences may occur in the generation of less abundant fragments. Cleavage by the 20S proteasome purified from adapted cells is delayed, most visibly after 4–8 h (Fig. 3). Importantly, the preferred cleavage immediately C terminal to the Leu residue at the C terminus of the Sendai NP cytotoxic T lymphocyte epitope is still executed by adapted cell proteasomes (Fig. 3*B*). Notwithstanding selection of adapted cells on an inhibitor that completely blocks chymotrypsin-like activity (Fig. 2*B*), the remaining active Z subunits must be capable of cleaving C terminal of leucine, although less efficiently than the X/LMP7 subunits (24, 29). Thus, adapted cells possess partially functional proteasomes that can cleave polypeptides with surprisingly similar specificity but with reduced efficiency. Although these observations suggest that proteasome function continues in adapted cells, the residual activity itself is insufficient for cell survival, as growth of adapted cells depends on induction of the nonproteasomal AAF-AMC hydrolyzing activity (18).

Elevated TPPII Activity Permits Survival and Continued Ub-Dependent Protein Degradation in EL-4 Cells Exposed to Toxic Concentrations of NLVS. What events regulate cell adaptation? If TPPII can account for the adapted state, then an increase in expression of TPPII might suffice for acquired resistance to proteasome inhibitors such as NLVS. We transfected a TPPII cDNA plasmid (30) into EL-4 cells. Lysates from 24 individual neomycin-resistant colonies were screened for an increase in the ratio of AAF-AMC/Suc-LLVY-AMC substrate hydrolysis, preferred by TPPII and the proteasome, respectively. Six TPPII-transfectant clones exhibited a 1.5-fold or higher induction of this ratio relative to mock transfectants, and we subsequently used a FP-biotin active site probe to detect catalytically active TPPII protein (26). We found a 3-fold increase in FP-biotin labeling of lysates from TPPII transfectant clones compared with control cells, and a slight increase in TPPII-labeling was observed in NLVS-adapted cells (Fig. 4*A*). Comparison of AAF-AMC hydrolysis profiles obtained from size exclusion chromatography fractions of EL-4 and TPPII lysates also show that the high molecular weight TPPII complex is more abundant in TPPII transfected cells (data not shown).

We next tested the TPPII-transfected EL-4 clones and control cells for their response to proteasomal inhibition imposed by NLVS (Fig. 4*B*). Cultures of EL-4 cells undergo massive death when exposed to 50 or 100 μM NLVS, yet TPPII-transfected

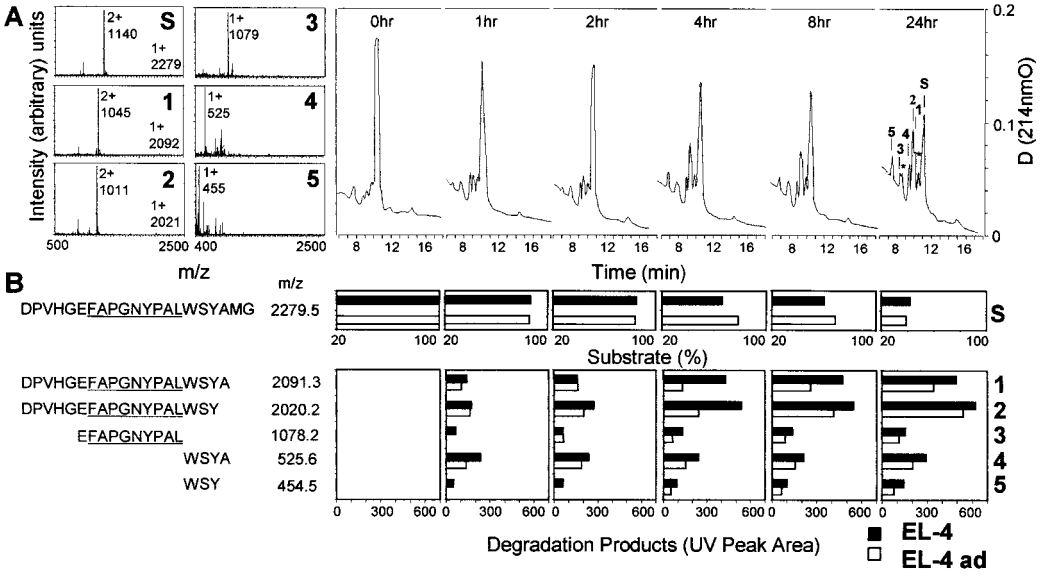

Fig. 3. Purified EL-4-adapted 20S have a reduced degradation rate compared with EL-4 20S. Degradation products of Sendai $NP_{317–338}$ peptide, incubated with purified EL-4 or EL-4-adapted 20S for specified times, were analyzed by reverse-phase HPLC and on-line MS (liquid chromatography-MS). (*A*) Reverse-phase HPLC chromatograms illustrate the formation of degradation products (boxes 1–5) generated by the EL-4 20S over indicated time intervals. The initial 21 residue peptide (S) and irrelevant peaks (*) are indicated. Mass spectra of each fragment are shown on the left. (*B*) The fragmentation products generated by the 20S from EL-4 (filled bars) and adapted cells (open bars) were identified by molecular mass in TIC chromatograms (data not shown) and quantified by their UV peak integration area.

cells survive (Fig. 4*B*) and continue to proliferate, as do NLVS-adapted cells. Mock transfectants behave similarly to EL-4 cells (data not shown). Is overexpression of TPPII sufficient to prevent the accumulation of Ub conjugates when the proteasome is inhibited? Ub conjugates do not accumulate in adapted EL-4 cells beyond levels observed in untreated EL-4 cells (16,

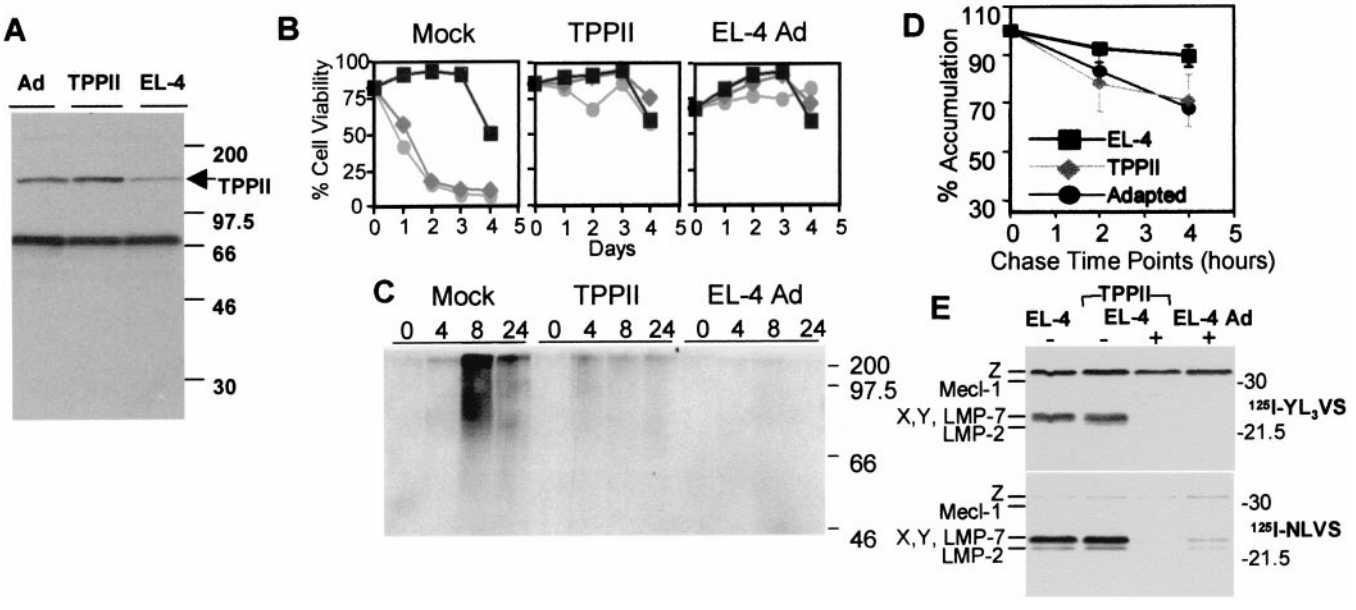

Fig. 4. Elevated levels of TPPII enable EL-4 cells to survive toxic concentrations of NLVS and process Ub-conjugated proteins. (*A*) Relative TPPII activity. FP-biotin binds TPPII at its active site in EL-4, TPPII transfectants, and adapted cells. The 60-kDa band is an unidentified polypeptide detected by Streptavidin-horseradish peroxidase. (*B*) Viability of EL-4, adapted, and TPPII transfectant cells treated with 0 (squares), 50 (rectangles), and 100 μM (circles) NLVS over 4 days as monitored by flow cytometry. (*C*) Western blot of accumulated poly-Ub-conjugated proteins using lysates from EL-4, TPPII transfectant, and adapted cells at 0, 4, 8, and 24 h after treatment with 50 μM NLVS assayed for an accumulation of polyubiquitinated proteins as described in *Materials and Methods*. Less polyubiquinated material is observed after 24 h, the result of partial cell death. One of 10 experiments is shown. (*D*) Pulse–chase analysis of polyubiquitinated material: control EL-4 (squares), adapted (circles), and TPPII transfectant cells (rectangles) were pulsed with [^{35}S]methionine and 50 μM NLVS and chased over time. Ub conjugates were analyzed by immunoprecipitation, SDS/PAGE, and autoradiography. Lanes were quantified by densitometric scan, and standard error mean was calculated. (*E*) NLVS is not a substrate for TPPII. EL-4 cells transfected with TPPII were either untreated or incubated with 50 μM NLVS for 24 h at 37°C. Lysates prepared from EL-4, TPPII transfectants, and adapted EL-4 cells were incubated with ^{125}I-NLVS as described above.

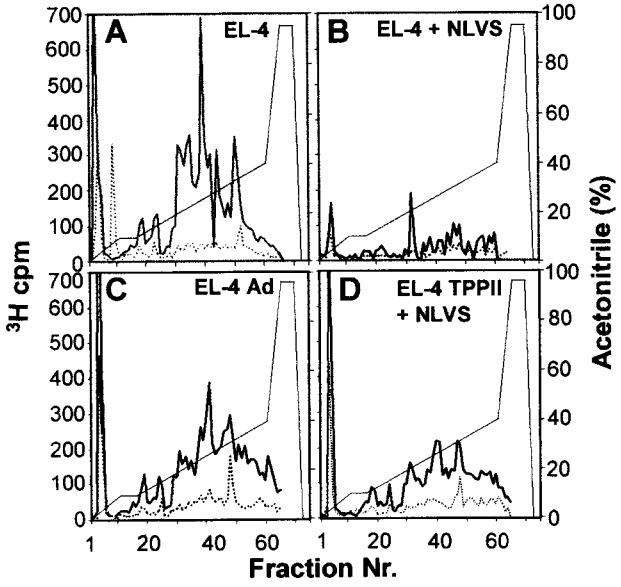

Fig. 5. HPLC analysis and comparison of K^b-eluted radiolabeled peptides from control NLVS-treated EL-4 cells, TPPII transfectant, and adapted cells. Control EL-4 cells (*A*), cells treated with NLVS for 16 h (*B*), adapted cells (*C*), and TPPII transfectants treated with NLVS (*D*) were labeled with ^{3}H-leucine and ^{3}H-tyrosine for 6 h. Class I molecules (H-$2K^b$) were immunoprecipitated and bound peptides (solid lines) were eluted and separated by HPLC. Peptides loaded on HPLC were normalized for total cell incorporation of radiolabeled amino acids. Mock-precipitated material (dotted lines) refers to nonspecific background. Peptide standards included with each run showed elution times between runs to differ by less than a minute.

18), but do accumulate in 4 h in EL-4 cells treated with NLVS (Fig. 4*C*). Remarkably, Ub conjugates do not accumulate in NLVS-treated TPPII-transfected EL-4, and pulse–chase experiments confirmed the ability of TPPII transfectants to clear Ub conjugates (Fig. 4*D*). We conclude that overexpression of TPPII is sufficient to normalize the flux of proteins that transit the Ub-dependent degradation machinery.

One obvious possibility for the resistance of TPPII transfectants to NLVS treatment is that NLVS can be degraded by TPPII. However, we found that proteasomal β subunits of NLVS-treated EL-4 cells were similarly modified in both control and TPPII transfectants, indicating that TPPII does not prevent NLVS from reaching the proteasome's active sites (Fig. 4*E*). Furthermore, ^{125}I-NLVS is not measurably degraded during incubation with lysates prepared from EL-4, TPPII transfectants, and EL-4 adapted cells, as assayed by TLC (data not shown). We therefore conclude that NLVS, unlike lactacystin, which inhibits TPPII to 50% at 10 μM (19), does not directly inhibit nor is a substrate for TPPII.

Increased TPPII Activity Partially Restores Peptide Supply for MHC Class I Assembly During Proteasomal Inhibition. A fraction of cleavage products generated by the proteasome are antigenic peptides presented at the cell surface by MHC class I molecules for T cell recognition (6). We assessed the impact of the proteasome inhibitor NLVS on the generation of antigenic peptides by pulsing EL-4 cells, NLVS-treated EL-4 cells, adapted EL-4 cells, and TPPII overexpressing EL-4 cells with ^{3}H-Leu and ^{3}H-Tyr (the major anchor residues for K^b binding peptides). Radiolabeled peptides were eluted from isolated K^b molecules and analyzed by reversed-phase HPLC (Fig. 5). Exposure to NLVS dramatically decreases the amount of peptides presented (Fig. 5*B*) in accordance with inhibition of class I assembly and maturation (18) (data not shown). In NLVS-exposed TPPII transfectants, we observed partial restoration of each set of K^b-binding peptides in a similar manner as observed in EL-4 adapted cells (Fig. 5 *C* and *D*). This identifies TPPII as a proteolytic enzyme with an impact on MHC class I antigen production in EL-4 cells. We also found that EL-4-adapted cells maintained peptide loading of H-$2K^b$ molecules, although some differences in peptide species may arise between control and adapted cells (Fig. 5 *A* and *C*).

Discussion

Blocked proteasome function impairs cell viability in eukaryotes (2), in contrast to prokaryotic cells (31). Redundancy of the prokaryotic proteolytic machinery may derive from other proteases present in the cytosol, such as ATP-dependent Lon or downstream proteases such as the Tricorn protease TRI (32, 33). In eukaryotic cells, proteasome-mediated proteolysis is considered mostly responsible for protein turnover, but the contribution of other cytosolic oligopeptidases is less clear.

We now provide direct evidence that increased TPPII activity is sufficient for cell viability and the maintenance of Ub-dependent proteolysis in EL-4 lymphoma cells when proteasome function is compromised. Earlier work describes the resistance of EL-4 cells to otherwise toxic concentrations of NLVS, where no proteasome activity was detected (18). Surprisingly, NLVS-adapted cell proteasomes retain partial activity, but this activity alone is insufficient to maintain cell viability (Fig. 4*B*); auxiliary protease activity(s) are required.

Our recent analysis of multiple independent harvests of adapted cells shows a variable increase in AAF-AMC hydrolyzing activity in the 2- to 5-fold range (unpublished data). Elevated AAF-AMC hydrolysis also occurs in EL-4 cells adapted to 6 μM lactacystin and has been attributed to TPPII (19). However, whether TPPII alone was sufficient for cellular adaptation has thus far not been addressed. Our results suggest that TPPII, as one of perhaps several oligopeptidases, can act in concert with the proteasome in the degradation of cytosolic proteins and peptides. Moreover, TPPII has broad tissue distribution, which also suggests that in addition to its specific function as a cholecystokinin-inactivating peptidase in neurons (19), it may have a more universal role in proteolysis.

Lactacystin can be used in lieu of NLVS to obtain adapted EL-4 cells (19), but substantial differences exist between these adapted cell populations. The Z subunit (bearing trypsin-like activity) is modified by lactacystin more effectively at lower concentrations than by NLVS. Moreover, lactacystin partially inhibits TPPII (19), whereas NLVS does not (Fig. 4*E* and unpublished data).

Proteasome activity in EL-4 cells is not blocked completely by NLVS at 50 μM or by lactacystin at 6 μM, the range of inhibitor concentration used for adaptation (Fig. 1 *A* and *B*; ref. 19). The use of ^{125}I-YL_3VS reveals the basis for the continued partial activity of purified proteasomes from adapted cells, which retain trypsin and caspase-like activities. Although residual "caspase-like" activity persisted, we did not detect active Y subunits in adapted proteasomes, a subunit that also is modified by ^{125}I-YL_3VS (23) (Fig. 1 *A* and *D*). In yeast, deletion mutants that lack all proteasomal activity are not viable (10, 11). We suspect that mammalian cells likewise will not survive such a challenge. Reagents that block Z subunit activity such as YL_3VS either lack membrane permeability because of their free amino terminus or lose functional activity as observed for N-acetylated YL_3VS. As long as adequate Z subunit-specific reagents are not available, the specific role of the Z subunit *in vivo* will remain elusive. Purified 20S proteasomes from EL-4 and adapted cells process a 21-mer peptide and produce identical predominant cleavage products, albeit at a reduced rate for proteasomes from adapted cells. In yeast mutants defective in the β5/pre2 subunits (cor-

responding to mammalian X/LMP7), cleavage after hydrophobic and small aliphatic residues continues (24, 29). Therefore, the Z subunit alone can carry out cleavages usually attributed to the chymotryptic-like activity. Nevertheless, the residual Z subunit activity alone does not suffice to maintain cell viability: adapted cells treated with the covalent inhibitor AAF-CMK die (18).

Neither EL-4 cells with elevated TPPII activity nor NLVS-adapted cells accumulate Ub-conjugated proteins (Fig. 4*C*). If Ub isopeptidase activities continue unabated in adapted cells—and there is no evidence that proteasome inhibitors block isopeptidase activity—TPPII could degrade previously Ub-conjugated proteins. Indeed, TPPII possesses endopeptidase activity *in vitro* (19). For NLVS- or lactacystin-inhibited proteasomes, the release of product may be impaired and cause inhibition of proteolytic activity by a negative feedback mechanism. Feedback inhibition may occur at each point in the degradation pathway from accumulation of ubiquitinated, deubiquitinated, or partially processed proteins to low abundance of peptide products, leading to the induction of stress-inducible genes involved in heat-shock responses (1, 25, 34). To restore *in vivo* rates of proteolysis to acceptable levels, partially degraded protein substrates may be redirected to other proteases such as TPPII as a means for continued cytosolic proteolysis. A direct interaction between Ub conjugation machinery, Ub isopeptidases, and the 26S proteasome is a matter of record (35, 36). A similar linkage may occur to couple TPPII activity to the Ub-proteasome machinery to optimize protein degradation.

Large cytosolic proteases other than TPPII also may contribute to proteolysis. Bleomycin hydrolase is a cysteine protease that shares structural similarity with the proteasome and has broad tissue distribution, but its major physiological role remains to be determined (37). Dipeptidyl peptidase III (DPPIII) (monomer: 80 kDa) participates in the terminal stages of protein degradation, favoring oligopeptides of four or more residues as substrates (38). Therefore, TPPII and DPPIII as well as other proteases may help increase the efficiency of proteolysis in adapted cells. A striking observation was the partial restoration in TPPII transfectants of the peptide pool presented by MHC class I molecules (Fig. 5). The interdependence of proteasomal proteolysis and TPPII or similar activities suggests a level of integration of the different components for cytosolic proteolysis. Not only is there coupling between Ub conjugation, deubiquitination, and proteasomal activity, oligopeptidases that act downstream of the proteasome should be viewed as an essential element of the proteolytic cascade.

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