Two new proteases in the MHC class I processing pathway

Lars Stoltze[1], Markus Schirle[1], Gerold Schwarz[2], Christian Schröter[2], Michael W. Thompson[3], Louis B. Hersh[3], Hubert Kalbacher[2], Stefan Stevanovic[1], Hans-Georg Rammensee[1] and Hansjörg Schild[1]

The proteasome generates exact major histocompatibility complex (MHC) class I ligands as well as NH_2-terminal-extended precursor peptides. The proteases responsible for the final NH_2-terminal trimming of the precursor peptides had, until now, not been determined. By using specific selective criteria we purified two cytosolic proteolytic activities, puromycin-sensitive aminopeptidase and bleomycin hydrolase. These proteases could remove NH_2-terminal amino acids from the vesicular stomatitis virus nucleoprotein cytotoxic T cell epitope 52–59 (RGYVYQGL) resulting, in combination with proteasomes, in the generation of the correct epitope. Our data provide evidence for the existence of redundant systems acting downstream of the proteasome in the antigen-processing pathway for MHC class I molecules.

The major site of cytotoxic T cell (CTL) epitope production is the proteasome[1], a multicatalytic protease complex located in the cytosol that degrades proteins in a ubiquitin- dependent manner. Some of the resulting peptides have the correct size for binding to major histocompatibility complex (MHC) class I molecules, can be transferred by transporter associated with antigen processing (TAP) into the lumen of the endoplasmic reticulum (ER) and once there, associate directly with MHC molecules. Others depend on the removal of amino acids to fit into the binding groove of MHC class I molecules[2] and are therefore termed "precursor peptides". However, until now, none of the proteases involved in the trimming of CTL epitope precursors had been identified. Indirect evidence implies aminopeptidase involvement, but it is not known whether these enzymes reside in the cytosol or the ER[3–5]. It is likely that proteases involved in processing exist in both of these cellular compartments. It has been suggested that cytosolic leucine amino peptidase (LAP), which can be induced by interferon γ (IFN-γ), plays a role in antigen processing[6]. It has also been hypothesized that another cytosolic protease, tri-peptidyl-peptidase II (TPP-II), replaces even more relevant functions of the proteasome not related to antigen processing[7]. In the ER no protease has been proposed for the removal of amino acids flanking CTL epitopes.

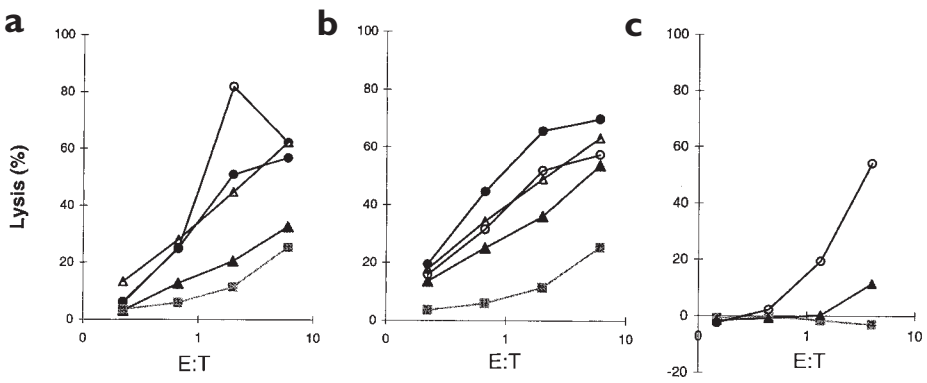


Figure 1. AAF-CMK–mediated inhibition of CTL recognition for VSV minigene–transfected and VSV–infected cells. (**a**) Recognition of MC-5v by LS-Vn in a ^{51}Cr-release assay after treatment with acid wash and AAF-CMK. Cells were incubated, for 2 h at 37 °C, with 50 μM AAF-CMK (▲), followed by an acid wash and a further 2 h incubation with AAF-CMK. Targets were MC-5v cells treated with AAF-CMK (50 μM) and RGYVYQGL (100 nM) added during ^{51}Cr labeling (△), acid wash only (●), untreated MC-5v (○) or MC-57 (□). CTLs were used at the given effector:target ratios (E:T). (**b**) As **a** but using MC-v instead of Mc-5v transfectants. (**c**) MC-57 cells were preincubated with AAF-CMK (50 μM) for 2 h and infected with VSV. A standard 4-h ^{51}Cr-release assay with LS-Vn cells, specific for RGYVYQGL, as effector cells followed.

We previously observed that minigene transfectants expressing NH_2- as well as COOH-terminal extended versions of the VSV nucleoprotein cytolytic epitope, amino acids 52–59 (RGYVYQGL), or VSV NP(52–57), require proteasomal activity to remove COOH-terminal but not NH_2-terminal amino acid extensions. This concurs with the finding that only NH_2-terminally extended but not the correct VSV NP CTL epitope can be generated by purified 20*S* proteasomes *in vitro*[8]. We decided to characterize the proteases involved in NH_2-terminal trimming and focused on identification of the cytosolic proteases that are capable of removing amino acids at the NH_2 terminus of CTL epitope precursors. The existence of

[1]Institute for Cell Biology, Department of Immunology, University of Tübingen, Auf der Morgenstelle 15, D-72076 Tübingen, Germany. [2]Institute of Physiological Chemistry, Hoppe-Seyler Str. 4, D-72076 Tübingen, Germany. [3]Department of Biochemistry, 800 Rose Lexington, University of Kentucky, Lexington KY 40536, USA Correspondence should be addressed to H. S. (hansjoerg.schild@uni-tuebingen.de)

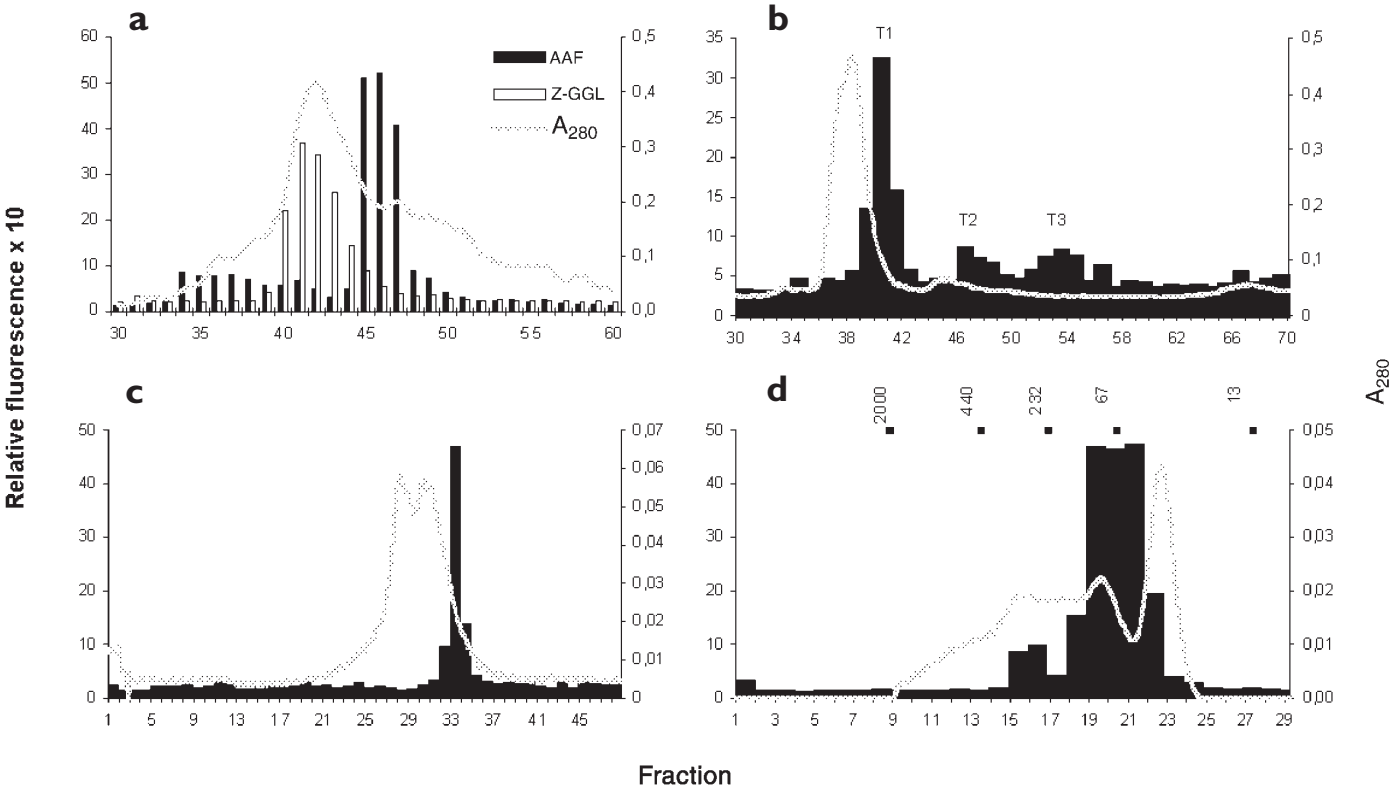


Figure 2. Purification of proteolytic activities of interest. (**a**) Free flow electrophoresis profile: samples of each fraction were incubated with either Z-GGL-AMC for endopeptidase activity or AAF-AMC for aminopeptidase activity. (**b**) Pooled fractions 45 and 46 from **a** were applied to a DEAE-toyopearl-ion exchange column. Each fraction was tested for AAF-AMC hydrolyzing activity. (**c**) Pooled fractions 40 and 41 from **b** were applied to a MonoQ ion exchange column and treated as before. (**d**) Pooled fractions 33 and 34 from **c** were applied to a Superdex gel filtration column and treated as before. (Filled squares denote peak elution of markers: dextran blue, 2000 kD; ferritin, 440 kD; thyroglobin, 232 kD; BSA, 67 kD; and RNAse, 13 kD. RF, open and filled bars; A_{280}, histograms.)

different cytosolic precursor peptides, and therefore indirect evidence of these proteases, has been reported for the CTL epitope SIINFEKL[9]. We identified two proteases, puromycin-sensitive aminopeptidase and bleomycin hydrolase, representing the dominant cytosolic activities involved in removal of the five NH_2-terminally extending amino acids of RGYVYQGL. These are, therefore, promising candidates for assisting proteasomes in the generation of MHC class I ligands.

Results

AAF-CMK inhibits NH_2-terminal trimming

VSV NP(52–59) is not generated directly by 20*S* proteasomes, instead the NH_2-terminal flanking amino acids of RGYVYQGL (SLSDL) are removed by lactacystin-independent proteolytic activity[8]. We tested the ability of several protease inhibitors to interfere with generation of the VSV CTL epitope from NH_2-terminal-extended minigene transfectants. Alanine-alanine-phenylalanine-chloromethylketon (AAF-CMK) fulfilled these requirements. AAF-CMK inhibited RGYVYQGL processing in the minigene transfectant MC-5v (**Fig. 1a**) but not in MC-v cells, the transfectants expressing the correct CTL epitope (**Fig. 1b**). The presentation of RGYVYQGL in MC-v cells in the presence of AAF-CMK showed that the inhibitor had no general inhibitory effect on the MHC class I antigen presentation pathway. CTL activation was normal when inhibitor-treated cells incubated with the VSV CTL epitope peptide were used (**Fig. 1a**). The effect of AAF-CMK was not limited to the processing of CTL epitopes from minigenes; cells infected with VSV and incubated with AAF-CMK could not present the CTL epitope RGYVYQGL to T cells (**Fig. 1c**). AAF-CMK was also able to inhibit CTL epitope processing from vaccinia virus–encoded proteins and generation of the ovalbumin (OVA)-derived CTL epitope SIINFEKL in EG-7 transfectants that expressed the full-length OVA (see Web Figure 1 on the supplementary information page of *Nature Immunology* on line). Generation of this epitope was dependent on NH_2-terminal trimming[6,9].

A lack of CTL epitope generation can be attributed to inhibition of nonproteasomal proteolytic activities because AAF-CMK does not inhibit the proteasome in any of its proteolytic activities[10] (L. Stoltze, unpublished data).

Purification of NH_2-terminal trimming activities

We characterized the proteasome-independent activities required to generate VSV NP(52–59) from the NH_2-terminal-extended minigene construct in MC-5v cells, and chose three selection criteria that had to be fulfilled by the respective protease(s). AAF-CMK had to be capable of inhibiting the protease; removal of the five NH_2-terminal amino acids (SLSDL) flanking the VSV NP epitope RGYVYQGL had to occur efficiently; and it was imperative that the protease was insensitive to lactacystin, which had no effect on NH_2-terminal trimming. Because various CTL epitope precursors of OVA had been identified in the cytosol[9], we started our search for proteases in this compartment. In addition, LLnL (which also inhibits ER resident proteases[11]), like lactacystin, had no inhibitory effect on the presentation of VSV NP(52–59) in MC-5v cells (data not shown).

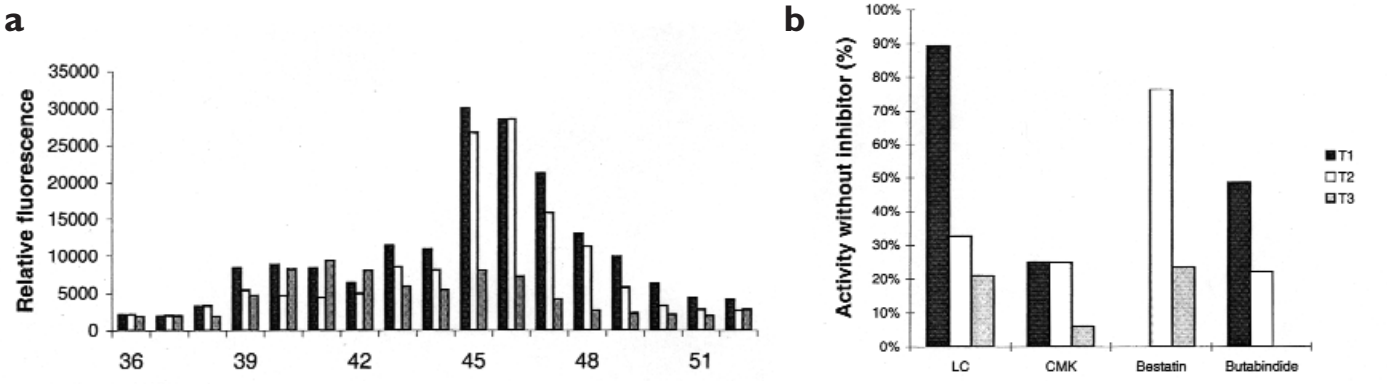


Figure 3. Fulfillment of selection criteria for purification steps. (**a**) Each fraction of the free flow electrophoresis was tested for SLSDL-AMC hydrolyzing activity (filled bars) and susceptibility to inhibition by AAF-CMK (open bars) and lactacystin (shaded bars) at concentrations of 50 μM each. (**b**) The three major activity peaks of the DEAE column, T1–T3, were characterized by their inhibitor profile using AAF-AMC as substrate. Lactacystin (LC), AAF-CMK (CMK) and bestatin were applied at 50 μM, butabindide at 5 μM. (**c**) T1–T3 were characterized by their ability to cleave SLSDL-AMC.

We purified cytosol from Cox cells (a human EBV-transformed B cell line) and applied it to free flow electrophoresis, which allows the careful separation of large protease complexes. The proteasome, in particular, could be easily distinguished from other dominant cytosolic aminopeptidase activities. Endopeptidase activity against benzoxycarbonyl-glycine-glycine-alanine-aminomethylcoumarine (Z-GGL-AMC) was found only in fractions 40–44, whereas the main aminopeptidase activities against alanine-alanine-phenylalanine-aminomethylcoumarine (AAF-AMC) eluted in fractions 45–47 (**Fig. 2a**). Immunoblot analysis detected proteasomes in fractions 40–44 only (data not shown). Initially AAF-AMC cleavage was chosen as the marker for aminopeptidase activity because a protease inhibited by AAF-CMK should also cleave the AAF-AMC substrate.

The main aminopeptidase activity in fractions 45–47 fulfilled the selection criteria. SLSDL-AMC, a fluorogenic substrate consisting of the natural five amino acids flanking VSV NP(52–59), was cleaved efficiently and the activity could be inhibited by AAF-CMK (**Fig. 3a**). However, this SLSDL-AMC cleavage activity was not inhibited by lactacystin. The minor aminopeptidase activity in fractions 34–38 did not fulfill the selection criteria and was therefore excluded from further analysis.

To purify the relevant enzymatic activities further, fractions 45 and 46 were pooled and applied to a diethylaminoethyl (DEAE)-toyopearl-ion exchange column. Three AAF-AMC–hydrolyzing activities, T1–T3, were resolved (**Fig. 2b**). T1 was insensitive to lactacystin but not to AAF-CMK and could hydrolyze SLSDL-AMC (**Fig. 3b**,**c**). No specific aminopeptidase could be assigned to T2 but, because of its inhibition by lactacystin and the lack of SLSDL-AMC hydrolysis, it was excluded from further investigations. T3 was TPP-II, as identified by immunoblotting (western blotting) and by its complete inhibition by butabindide, the most specific TPP-II inhibitor known[12] (data not shown and **Fig. 3b**). The TPP-II–containing activity T3 was characterized no further because it lacked SLSDL-AMC–hydrolyzing activity and was lactacystin-sensitive.

The fractions with T1 activity fulfilled the selection criteria and were applied to a MonoQ ion-exchange column. This resulted in further separation of the proteases present in the fraction but again only one type of AAF-AMC–hydrolyzing activity was observed (**Fig 2c**). By applying the pooled fractions to a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel, two major bands at ~100 kD and ~50 kD were revealed. To investigate whether these proteins resulted from one protease complex they were applied to a Superdex gel filtration column as the final purification step (**Fig 2d**). Two activity peaks were observed. The major activity was in fractions 19–22, corresponding to a size of 100 kD. The minor activity was in fractions 15 and 16 with a size of 300 kD.

Identification of proteases

The fractions of the Superdex column containing the proteolytic activities were separated on an 8% SDS gel. Fractions 19–21 contained a strong band of approximately 100 kD and fractions 15 and 16 contained a dominant band at 50 kD (**Fig. 4**). We analyzed the prominent protein bands by in-gel trypsin digestion and tandem mass spectrometry. The results identified the 100-kD protein as puromycin-sensitive aminopeptidase (PSA) and the 50-kD protein as bleomycin hydrolase (BH). These sizes fitted the calculated molecular weights obtained from gel filtration of 300 kD for human BH, which has been reported to exist as a homohexamer of 300 kD[13], and of 100 kD for PSA. Minor bands were iden-

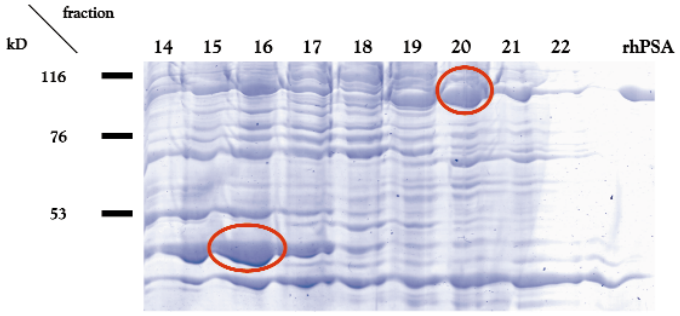
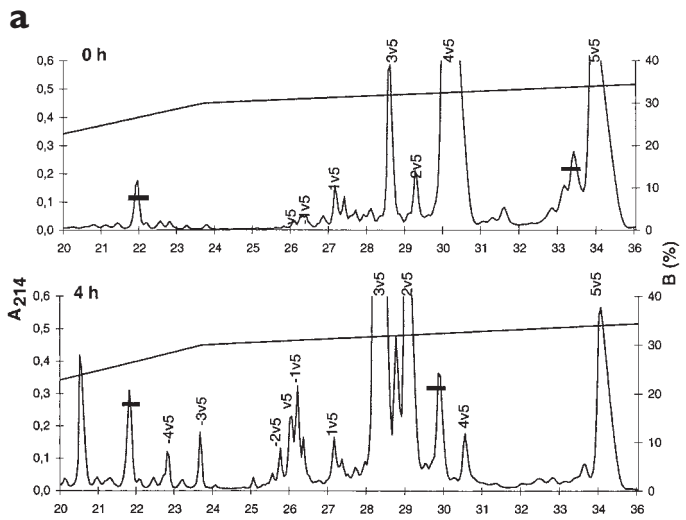


Figure 4. Identification of proteolytic activities of interest. The active fractions of the Superdex column were loaded onto an 8% SDS gel and stained with Coomassie blue. Recombinant human PSA (rhPSA) was loaded into the last lane. The most prominent protein bands were identified as 50-kD BH and 100-kD PSA (circled). The minor bands were identified as proteins with no known proteolytic activity.



b

PSA digest of:	SLSDLRGYVYQGL 5v	SLSDLRGYVYQGLKSGNV 5v5
fragment found:	LSDLRGYVYQGL 4v	LSDLRGYVYQGLKSGNV 4v5
	SDLRGYVYQGL 3v	SDLRGYVYQGLKSGNV 3v5
	DLRGYVYQGL 2v	DLRGYVYQGLKSGNV 2v5
	RGYVYQGL v	LRGYVYQGLKSGNV 1v5
	GYVYQGL -1v	RGYVYQGLKSGNV v5
	YVYQGL -2v	GYVYQGLKSGNV -1v5
	VYQGL -3v	YVYQGLKSGNV -2v5
	YQGL -4v	VYQGLKSGNV -3v5
	QGL -5v	YQGLKSGNV -4v5
		QGLKSGNV -5v5

c

BH digest of:	SLSDLRGYVYQGL 5v	SLSDLRGYVYQGLKSGNV 5v5
fragment found:	LSDLRGYVYQGL 4v	LSDLRGYVYQGLKSGNV 4v5
	SDLRGYVYQGL 3v	SDLRGYVYQGLKSGNV 3v5
	DLRGYVYQGL 2v	DLRGYVYQGLKSGNV 2v5
	RGYVYQGL v	LRGYVYQGLKSGNV 1v5
	YVYQGL -2v	RGYVYQGLKSGNV v5
	VYQGL -3v	GYVYQGLKSGNV -1v5
		YVYQGLKSGNV -2v5
		VYQGLKSGNV -3v5

Figure 5. Characterization of the aminopeptidase activity of PSA and BH. Recombinant human PSA was incubated with 5v and 5v5. (**a**) HPLC elusion profiles (min) of time point 0 h and 4 h. Peaks are marked with the identified peptides, **–** mark irrelevant peaks. (**b**) Summary of identified peptides in PSA digests. (**c**) Summary of 5v and 5v5 digest by purified human BH. When the correct COOH terminus exists the CTL epitope is generated by both proteases. (B%, straight lines; A_{214}, histograms.)

tified by the same procedure but revealed proteins with no known protease activity (data not shown).

Characterization of PSA activity

We performed *in vitro* peptide digests using recombinant PSA incubated with NH_2- and COOH-terminally extended VSV NP(52–59). As previously described[23], PSA behaved exclusively as an aminopeptidase (**Fig. 5**). A 19-mer containing five amino acids added to the NH_2 and COOH terminals, called 5v5, was digested by continuous removal of NH_2-terminal amino acids only. PSA acted on the substrates with fast kinetics so that from the time the substrate was added to the time the reaction was quenched, smaller fragments were generated and could be detected by HPLC (**Fig. 5a**, 0 h). PSA generated the correct CTL epitope from the CTL epitope precursor 5v (**Fig. 5b**). This was observed in a single digest of 5v by PSA only, as well as in a combination digest of 5v5 by proteasome and PSA. It was thus shown that PSA is responsible for the trimming activity because the proteasome alone was unable to generate the epitope[8]. However, unlike LAP, PSA does not stop at the start of VSV NP(52–59)[6]. Based on the activity profiles obtained during the purification procedure (**Fig. 3**), PSA activity was inhibited by AAF-CMK and puromycin but not lactacystin[14] (and data not shown).

Hydrolyzing activity of BH

The inhibitor profile for BH met the requirements of our purification profile (**Fig. 3**) because it was AAF-CMK–sensitive, but lactacystin-insensitive. In addition, BH was more sensitive to E64 (a typical cysteine protease inhibitor) than it was to AAF-AMC[15]. (Web Figure 2 on the supplementary information page of *Nature Immunology* on line). Digests of peptides corresponding to VSV NP(52–59) minigenes resulted in a similar pattern to that observed for PSA (**Fig. 5c**). BH trimmed peptides generated by the proteasome in double digests of 5v5, which lead to VSV NP(52–59) generation. Like PSA, BH did not stop trimming at the beginning of the CTL epitope. This implied that both BH and PSA could generate the CTL epitope from precursor peptides containing the correct COOH terminus.

Discussion

We investigated the contribution of cytosolic proteases other than proteasomes to the generation of the H2-K^b ligand of VSV NP(52–59), RGYVYQGL. We found that the protease inhibitor AAF-CMK, which does not inhibit proteasome function, interfered with the generation of the VSV NP CTL epitope from NH_2-terminally extended minigenes (5v), but not from minigenes encoding the CTL epitope (v) itself. CTL epitope generation was also AAF-CMK–sensitive after infection of target cells with VSV or vaccinia viruses encoding the entire protein but not the CTL epitope itself. We also found that the generation of the OVA CTL epitope SIINFEKL in cells expressing the entire OVA protein was inhibited by AAF-CMK. Thus, AAF-CMK inhibition of 5v-minigene processing is not a minigene artifact, and there seem to be similarities in the processing requirements of SIINFEKL and RGYVYQGL CTL epitopes. Generation of the SIINFEKL from NH_2-terminally extended minigenes was also shown to be proteasome-independent but dependent on additional aminopeptidases[16].

Cytosolic proteases were analyzed for AAF-CMK inhibition, resistance to lactacystin and the ability to remove the NH_2-terminal amino acids of the 5v minigene, and two proteases were identified. Their inhibitor profiles and substrate specificities easily distinguished them from proteasomes and TPP-II. Both enzymes acted as aminopeptidases. Because these data were obtained *in vitro* and BH, at least, contains some endopeptidase activity, and taking into consideration that interacting or regulating factors of these proteases light still await identification, it cannot be ruled out that *in vivo* those enzymes also reveal other activities. For this reason, we suggest grouping the additional proteases involved in the class I processing pathway under the general term “trimpeptidases”.

The first proteolytic activity, PSA, was identified 20 years ago[17] and was named after its inhibition by puromycin. PSA, as a Zn protease, is conserved throughout animal species[18], shows broad tissue distribution[19] and its gene has housekeeping structure[20]. In MC57 cells PSA expression cannot be induced by IFN-γ, as tested by reverse transcription–polymerase chain reaction and immunoblot analysis (data not shown). So far, neither its function nor any physiological substrates have been determined although PSA contains two motifs that are 72%

and 40% similar to proteasome motifs[14]. Its broad distribution, as well as the fact that a putative precursor peptide such as 5v is a substrate *in vitro* for this enzyme, renders it a promising trimpeptidase candidate. In addition, it has been shown that metalloproteases in general may play a role in antigen processing[21]. The Zn protease PSA could be the first known candidate in this group of proteases to function as a trimpeptidase.

The second protease to be identified is BH. This cysteine protease is also conserved throughout evolution, demonstrates a housekeeping gene structure[22] and is widely distributed in many tissues[23]. Consequently, we were also able to purify it from MC57 and EL-4 cells (data not shown). BH hydrolyses the anticancer drug bleomycin[24] but as with PSA, no physiological function or substrates are known even though it has already been determined that its structure resembles that of the proteasome[13]. In addition to its aminopeptidase activity, BH also displays endopeptidase activity[25]. We extended these studies by using various NH_2-terminal-blocked substrates and observed a slow but reproducible endopeptidase activity especially after R. This endopeptidase activity was comparable to that described for TPP-II. It remains to be seen whether this endopeptidase activity has any relevance *in vivo* and indeed represents the postulated proteolytic activity for nonproteasomal CTL epitope generation[7,26,27], which is still unidentified. Additional evidence supporting the role of BH as a possible trimpeptidase is that BH, as a cysteine protease, is completely inhibited by the cystein protease inhibitor E64[15,23]. E64 was described as having an inhibitory effect on the processing of a class I ligand[28]. A further link to the proteasome pathway is the observation that, in yeast, human BH binds to the homolog of the ubiquitin-conjugating enzyme 9[29]. E64 did not, however, inhibit VSV NP(52–59) generation in MC-5v cells. This observation may be explained easily by the compensatory effect of PSA, which is not inhibited by E64. CTL epitopes may therefore be generated by a variable set of proteases with complementary and redundant specificities.

From our data we suggest the following model for the generation of RGYVYQGL. The proteasome generates the correct COOH terminus and a distant cut in the NH_2-terminal region. BH and PSA act as complementary and redundant systems responsible for the final trimming of the correct NH_2 terminus. (BH and PSA may be the constitutive trimpeptidases in this model because they are broadly distributed and are not induced by IFN-γ in MC57 cells, data not shown.) LAP, never detacted in our experimental setup, could instead contribute (as an IFN-γ–inducible peptidase) to higher diversity and efficiency of CTL epitope generation like the IFN-γ–inducible β-subunites of the proteasome[30,31] or PA28[32]. Considering all these possibilities, the diversity of peptide generation in the class I processing pathway appears more complex than was anticipated. The apparent complementarity and redundancy of this system implies a more efficient, as well as more reliable, production of class I ligands, reducing the chance for immune escape through interference with antigen processing by viruses or tumor cells.

Methods

Cell lines. All cell lines were maintained in RPMI with 10% fetal calf serum (FCS). The mouse fibroblast cell line, MC57, and resulting transfectants of this line as well as the CTL line LS-Vn specific for VSV NP(52–59) are described elsewhere[8]. Cox cells, a human EBV-transformed B cell line, were provided by C. A. Müller, Tübingen and grown in roller bottles.

Acid wash, AAF-CMK assay. The acid wash protocol was performed as previously described[8]. Briefly, cells were preincubated for 2 h in 50 μM of AAF-CMK (Sigma, St. Louis, MO) at 37 °C, treated for 1.5 min with "acid" (a 1:1 mix of 0.263 M citric acid and 0.132 M NaH_2PO_4 at pH 3.0), neutralized to pH 7.5 by adding 1 ml of 0.15 M NaH_2PO_4 and washed twice with PBS. This was followed by a further 2-h incubation in medium containing inhibitor and a standard 4 h ^{51}Cr-release assay. Spontaneous release values were 14–22%. VSV-infected cells were preincubated for 2 h at 37 °C in AAF-CMK (50 μM), infected for 1.5 h with VSV (1×10^7 plaque-forming units) in 200 μl of RPMI without FCS but containing 50 μM of AAF-CMK. Subsequently the cells were incubated for 2 h at 37 °C and the ^{51}Cr-release assay followed as for the acid wash.

Purification procedure of protease activities. Unless stated otherwise, all steps were performed at 4 °C. Up to 20 ml of cell pellet washed with free-flow buffer (250 mM sucrose, 10 mM tri(hydroxymethyl)aminomethane–acetic acid, pH 6.9, at room temperature) was lysed with 10–20 strokes in a 5 ml Potter-Elvehjem-homogenizor with a 60 μm gap at 1000 rpm. The pellet was centrifuged for 3 min at 20000*g* and the supernatant a second time for 12 min at 400000*g* (Beckman TL 100 ultracentrifuge, Beckman, Palo Alto, CA). The resulting cytosol was separated on a free-flow electrophoresis machine (Dr. Weber, GmbH, München) at 800 V at 10 °C. Active fractions were pooled and applied to a DEAE-toyopearl-ion exchange column (TosoHass, Stuttgart, Germany). A linear gradient of 62–200 mM KCl over 147 min was used in a buffer containing 10% glycerol, $MgCl_2$ (1.1 mM), DTT (1 mM), NaCl (10 mM) and Tris HCl (10 mM), pH 7.6 (at room temperature). Resulting active fractions were concentrated on a MonoQ ion exchange column (Pharmacia Biotech, Uppsala). A buffer similar to that of DEAE was used with a 20 min linear gradient from KCl (25–250 mM). The remaining active fractions were pooled and applied to a Superdex 200 gel filtration column (Pharmacia Biotech). The buffer used was similar as for the other columns but contained NaCl (200 mM). The active fractions obtained were concentrated by speed vac and loaded onto 10% SDS-PAGE gels.

Activity assay. All assays containing fluorogenic substrates were incubated at 37 °C for 0.5–2 h in the corresponding column buffer. Conditions for the various assays after each purification step were as follows. Free Flow: fraction (200 μl), substrate (50 μM), AAF-CMK or lactacystin (50 μM, provided by E. J. Corey, Harvard University). DEAE: fraction (50 μl), substrate (200 μM). For inhibitor studies: fraction (10 μl), substrate (100 μM), lactacystin, AAF-CMK, bestatin (Sigma) (100 μM) or butabindide (10 μM, provided by J. C. Schwartz, Paris). MonoQ: fraction (5 μl), substrate (200 μM). Superdex 200: fraction (30 μl), substrate (200 μM).

In-gel digest and mass spectrometry. These were stained for 5 min with Coomassie blue R-250 (Sigma), destained for 10 min and the relevant protein bands excised and sliced into small pieces. After destaining and drying, gel slices were reduced with 2 mM DTT and alkylated with 20 mM iodacetamide at 55 °C for 1 h each. After washing and drying, trypsin digests were done at 37 °C overnight (1 μg of trypsin per gel slice). The resulting peptides were analyzed by tandem mass spectrometrical analysis on a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF, Micromass, Manchester, UK) equipped with an nanoflow electrospray ionization source. Metal-coated glass capillary nanoflow needles were from Protana (Type normal, Odense, Denmark). The needles were filled with approximately 3 μl of the sample diluted in 50:49:1 MeOH:water:formic acid and then opened by breaking the tapered end of the tip under a microscope. A stable spray was observed by applying a needle voltage of 800–1200 V, a backpressure of 2 psi and a source temperature of 45 °C. The estimated flow rate was 20–50 nl/min. Fragmentation of the parent ion was achieved by collision with argon atoms. Q1 was set to the mass of interest ± 0.5 Daltons and an optimized collision energy applied. The integration time for the TOF analyzer was 1 s with an interscan delay of 0.1 s.

Peptide digests by recombinant PSA and purified BH. Peptide (20 μg) was incubated with recombinant human PSA expressed by the bacculovirus system (0.4 μg) or purified human BH (4 μg). The reaction buffer was the same used for the DEAE column, which contained 25 mM KCl at final volume of 300 μl. Digests lasted 4 h and 60 μl aliquots were taken at various time points. Reactions were stopped by adding 10% acetic acid and then storing the sample at -20 °C. Samples were desalted on a SMART system equipped with a μRPC C2/C18 SC 2.1/10 column (Pharmacia). Eluent A, 0.1% TFA; eluent B,80% acetonitrile containing 0.081% TFA. Step gradient: 0–30% B in 23.5 min, 30–36% B in 16.5 min; flow rate: 150 μl/min, gradient started 5 min after sample injection. Eluted peptides were identified by Maldi-TOF-MS (Hewlett Packard, Palo Alto, CA).

Acknowledgements

We thank L. Yakes for reading the manuscript. Supported by the Deutsche Forschungsgemeinschaft (Leibnizprogram Ra369/4-1, to H. G. R.); Sonderforschungsbereich 510 (to H. S.); the European Union (Biomed 95-0263), Merck KGaA (Darmstadt, Germany), CTL Immunotherapies Corporation (Chatsworth, CA, USA) and a grant from the NIH/NIDA, DA 02243 (to L. B. H.).

Received 16 August 2000; accepted 28 September 2000.

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