Mutational analysis of subunit iβ2 (MECL-1) demonstrates conservation of cleavage specificity between yeast and mammalian proteasomes

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Abstract Proteasomes are the major protein-degrading complexes in the cytosol and regulate many cellular processes. To examine the functional importance of the MC14/MECL-1 proteasome active site subunits, cell lines expressing a catalytically inactive form of MECL-1 were established. Whereas mutant MECL-1 was readily incorporated into cytosolic proteasomes, replacing the constitutive MC14 subunit, removal of the prosequence was incomplete indicating that its processing required autocatalytic cleavage. Functional analyses showed that the absence of the MC14/MECL-1 active sites abrogated proteasomal trypsin-like activity, but did not affect other catalytic activities. Our data demonstrate a conservation of cleavage specificity between mammalian and yeast proteasomes.

Key words: Interferon γ-inducible subunit; 20S proteasome; MECL-1 T1A; Autocatalytic processing; Peptide hydrolysis

1. Introduction

Proteasomes are enzyme complexes abundant in the cytosol and probably also the nucleus of all eukaryotic cells (for review see [1,2]). They play a central role in the cytosolic proteolysis of misfolded proteins and of many short-lived and long-lived proteins. Moreover, proteasomes regulate multiple cellular processes such as cell cycle control, transcription factor activation and also MHC class I antigen processing in higher eukaryotes.

Proteasomes consist of a 20S catalytic core which associates with regulatory particles, e.g. PA28 or 11S and the 19S complex or PA700 [1,2]. Binding to the 19S complex confers protein degrading activity and substrate specificity on the 20S core which by itself lacks the ability to degrade proteins, probably due to limited access to the proteolytic channel [3]. One well characterized specificity of 19S is that for polyubiquitin-conjugated substrates, which are bound by at least one but probably several of the 19S subunits [4]. The PA28 regulator is selectively expressed in higher eukaryotes and enhances the capacity of proteasomes to degrade antigens into peptides that bind to MHC class I molecules [5,6]. In PA28-containing cells, both 19S and PA28 can bind to the 20S core, solely or simultaneously [7], likely resulting in a different tuning of 20S activity.

The 20S catalytic core consists of four heptameric rings. The outer rings contain the α-subunits α1–α7, the two inner rings the β-subunits β1–β7 of which three, β1 or δ, β2 or MC14 (mouse) or Z (human), and β5 or MB1, exert catalytic activity [1,8]. Remarkably, in interferon γ (IFNγ)-induced cells of higher eukaryotes three additional catalytically active subunits, LMP2 (iβ1), MECL-1 (iβ2) and LMP7 (iβ5), are transcribed which replace the constitutive subunits δ, MC14 or Z and MB1 in the proteasome complex, resulting in the formation of so called immunoproteasomes [9,10].

Since proteasome substrate choice, kinetics of protein degradation and the precise cleavages mediated are of extreme importance to many cellular processes, e.g. MHC class I antigen processing, knowledge of the proteolytic mechanisms of proteasomes is essential. Using tri- and tetrapeptide substrates, the substrate specificity of the active site subunits has been determined, though with inconsistent results. While several groups showed that replacement of constitutive for IFNγ-induced subunits enhanced the trypsin-like (T-L) and chymotrypsin-like (ChT-L) activity of proteasomes, opposite results were obtained by others [11–15]. Recently, mutant subunits with an inactivated catalytic site were used to further elucidate substrate specificities. In mouse cells, substitution of δ by inactive LMP2 T1A was shown to abrogate cleavages after acidic residues [16], confirming earlier studies ascribing the proteasomal peptidyl glutamyl hydrolyzing (PGPH) activity to the δ subunit [9]. In yeast, a detailed analysis of proteasomes with different inactive catalytic sites led to the assignment of PGPH activity to β1, T-L activity to β2 and ChT-L activity to each of the three catalytic subunits [17,18]. The cleavage site preferences determined with tri- and tetrapeptides appeared to correspond with proteasomal site usage in the yeast enolase 1 protein, though the much broader specificities of the β1 and β2 active centers did not in every case correspond to the preferences deduced from tri- and tetrapeptides [19].

We and others [20–22] recently described the identification of MC14 or Z (β2) as the third subunit which is exchanged for an induced homologue, MECL-1 (iβ2), in IFNγ-induced cells. To start to elucidate the function of the MC14/MECL-1 subunits, we used a catalytically inactive (T1A) form of MECL-1 to replace MC14 in mouse fibroblast cells. Testing the peptide hydrolyzing activity of MECL1 T1A proteasomes we noticed a complete abrogation of T-L activity, indicating that MC14 is solely responsible for the T-L activity of mouse proteasomes. On the basis of these and previous data, we conclude that despite the evolutionary distance between yeast and murines the cleavage specificity of proteasomes, a major regulatory cellular complex, is conserved.

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2. Materials and methods

2.1. DNA constructs

To generate mMECL-1 T1A, the base triplet ACT encoding Thr-1 in mMECL-1 wild type (wt) cDNA was exchanged for a codon encoding alanine (GCT) by polymerase chain reaction (PCR)-based mutagenesis. The following synthetic oligonucleotides were used: primer 1, 5′-GCAGGCGAATTCATGCTGAAGCAGGCAGTG-3′ containing an *Eco*RI restriction site and the ATG translation initiation site; primer 2, 5′-GCACGCAAGACCGGGGCTACCATCGCGGGGCT-T-3′ (forward) and primer 3, 5′-AAGCCCCGCGATGGTAGCCCC-GGTCTTGCGTGC-3′ (reverse), introducing the A to G point mutation in the codon encoding Thr-1; primer 4, 5′-CTGTTTAGA-TCTTCATTCCACCTCCATGGC-3′ containing the translation stop site and a *Bgl*II restriction site. Primer combinations 1 and 3 and 2 and 4 were used to generate an MECL-1 T1A 5′ and an MECL-1 T1A 3′ fragment from mMECL-1 wt cDNA. The two fragments were annealed in a consecutive PCR, the product was amplified with primers 1 and 4 and cloned into the eukaryotic expression vector pSG5.

2.2. Cell lines and transfections

Mouse fibroblast B8 cells, B8 LMP2+LMP7 (BC27H7) [12] and B8 LMP2+LMP7+MECL-1 (B27M.2) transfectant cells [23] were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin, streptomycin, 0.02% 2-mercaptoethanol, 250 μg/ml G418, and 400 μg/ml hygromycin for LMP transfectant cells. T2 LMP2+LMP7 transfectant cells (T27.10) [24] were grown in RPMI with 10% FCS, L-glutamine, penicillin, streptomycin and 1 mg/ml G418. The pSG5 mMECL-1 T1A expression construct was transfected into T27.10 cells using electroporation [26] and in BC27H7 cells using calcium phosphate precipitation. Transfected cells were plated under cloning conditions and selected with 200 μg/ml hygromycin and 2.5 μg/ml puromycin for T2 and B8 transfectant cells respectively.

2.3. Western blot analysis

Expression of mMECL-1 T1A was tested by Western blot analysis as described [23].

2.4. Proteasome purification and NEPHGE-, IEF-PAGE

Purification of 20S proteasomes from cell lines was performed as described [12,25]. Purified TCA-precipitated proteasomes (60 μg) were separated by two-dimensional NEPHGE-PAGE or IEF-PAGE [20]. Gels were stained with 0.2% Coomassie brilliant blue and the proteasome subunits were assigned as described [20] or identified by microsequencing [26]. Alternatively, the separated proteins were transferred to Immobilon P membrane and probed with mMECL-1-specific rabbit antiserum.

2.5. Peptide digestion assays

The cleavage properties of the 20S proteasomes were tested against the fluorogenic peptides Bz-VGR-AMC, Suc-LLVY-AMC, and Z-LLE.βNA (Bachem, Heidelberg, Germany). Peptide substrates were diluted in assay buffer (50 mM Tris-HCl pH 7.5, 25 mM KCl, 10 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA) and incubated with 200 ng 20S proteasomes at the concentrations specified in the figure legends, for 1 h at 37°C. Fluorescence emission was measured at 460 nm for free AMC groups (excitation 390 nm) and at 405 nm for free βNa groups (excitation 355 nm).

3. Results and discussion

To examine the functional importance of the MC14 (or Z)/MECL-1 pair of proteasome subunits, the mouse MECL-1 (mMECL-1) sequence was modified to encode an alanine instead of the active site threonine at position 1 and resulting construct was transfected into LMP2+7 containing human lymphoblast T2 cells (T27.10). T27.10 cells were chosen because the presence of LMP2 in particular and to a lesser extent of LMP7 should support the incorporation of newly transfected mMECL-1 into the proteasome complex [10,23,26]. Positive transfectant clones were identified by immunoblot analysis with a mMECL-1-specific polyclonal rabbit antiserum. Surprisingly, this procedure revealed three bands for MECL-1 in transfectant cells, as shown for a representative clone in Fig. 1A (lane 1). Based on migrational behavior, we identified the upper band as the unprocessed mMECL-1 T1A precursor protein [23]. The second prominent band migrating slightly more slowly than the MECL-1 wt form (lane 2) most likely represents a partially processed form of mMECL-1 T1A (see below), whereas the faint 29 kDa band corresponding in molecular weight (MW) to the mMECL-1 wt subunit (lane 2) must result from cross-reaction of the antiserum with the human MECL-1 (hMECL-1) subunit which is endogenously expressed in T2 cells (see below).

As reported for LMP2 T1A [25], the inactivation of the Thr-1 catalytic center prevented autocatalytic cleavage of the LMP2 prosequence at the Gly −1 Thr +1 site. A second processing event within the prosequence was not prevented suggesting that the cleavage of proteasomal propeptides might be achieved by a two-step mechanism. To further characterize the influence of the T1A mutation in mMECL1, 20S proteasomes were purified of T27.10 mMECL-1T1A transfectant cells and of T27.10 cells for comparison, and separated by two-dimensional IEF-PAGE (Fig. 2A,B). The position of mMECL-1 T1A in the Coomassie-stained gel of T27.10 mMECL-1 T1A proteasomes (Fig. 2B) was established by immunoblotting with mMECL1-specific antibodies (Fig. 2C). N-terminal sequencing of the respective spots revealed the

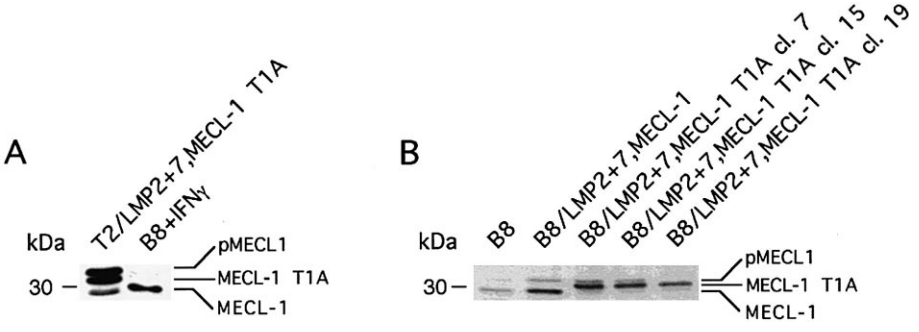

Fig. 1. Incorporation of catalytically inactive mMECL-1 T1A into 20S proteasomes of T2 (A) and B8 (B) transfectant cells. T2 and B8 cells were transfected with an mMECL-1 T1A expression vector as described in Section 2. Cellular lysates of transfectant clones were separated by SDS-PAGE and probed with a mMECL-1-specific polyclonal rabbit antiserum. Controls included lysates of B8 cells that were treated with IFNγ for 3 days (A) and of B8 and B8 LMP2+7+mMECL-1 wt cells (B). MECL-1, MECL-1 T1A and the precursor form (pMECL-1) are indicated. Molecular weight markers in kDa are shown on the left.

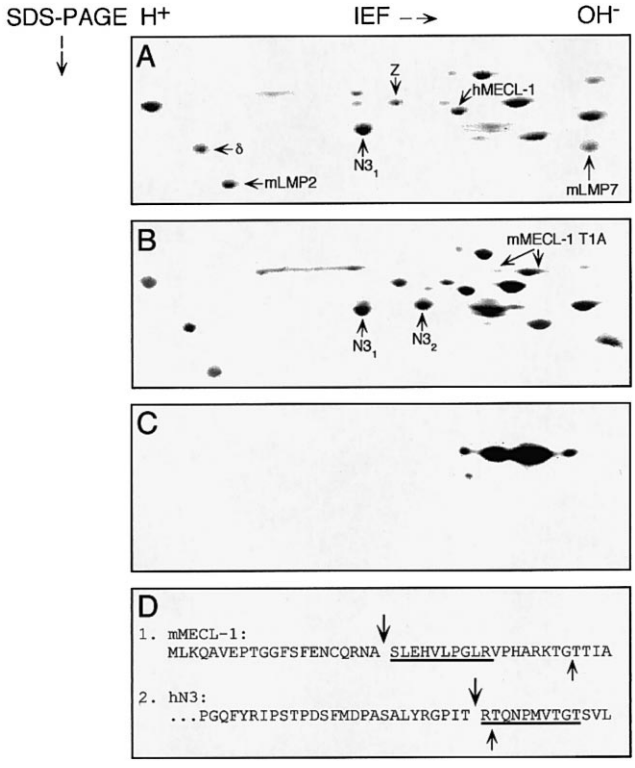

Fig. 2. Incorporation of mMECL-1 T1A into 20S proteasomes of T2 transfectant cells. T27.10 cells expressing mMECL-1 T1A were expanded and 20S proteasomes were purified as described [25]. Proteasome aliquots from T27.10 (A) and from T27.10-mMECL-1 T1A (B) of 60 μg were separated by two-dimensional IEF/SDS-PAGE and the subunits were visualized with Coomassie brilliant blue (A, B). The positions of relevant β-subunits are indicated in A. Spots containing misprocessed mMECL-1 and $N3_{(2)}$ present in mutant proteasomes are indicated in B. mMECL-1 T1A-containing proteasomes were additionally blotted to Immobilon P and probed with an anti-mMECL-1 antiserum (C). The N-termini of mMECL-1 T1A and of N3 were identified by Edman degradation as described [25]. The sequences obtained are underlined in the mMECL1 and N3 prosequences, given in D. Large arrows indicate the actual cleavage sites of these proteasome subunits in the mutant cell line, while small arrows designate the expected cleavage site in wild type subunits.

presence of mMECL-1 T1A and identical N-termini in the two most prominent spots, thus, MECL-1 T1A exists in at least two isoforms which have different charges. Both isoforms migrated with an MW corresponding to that of the middle MECL-1 signal in Fig. 1A. Similarly to the partially processed product of the LMP2 T1A protein [25], we found that autocatalytic cleavage of the mMECL1 propeptide was prevented at the conserved Gly Thr site and that an additional processing event had occurred, at position −18 within the prosequence (Fig. 2D). Apart from transfected mMECL-1 T1A also the endogenous hMECL-1 wt form is visible in the Coomassie gel (Fig. 2A,B). Thus, due to the presence of LMP2 in the cell line used, exogenous mMECL1 T1A and the endogenous hMECL1 are incorporated efficiently into the mature proteasome complex. Of note, the unprocessed precursor mMECL-1 T1A protein is not incorporated into the proteasome complex and is therefore not present in the two-dimensional gel.

Interestingly, an additional protein spot was seen in proteasomes containing mMECL1 T1A, not present in proteasomes from T27.10 cells (Fig. 2A,B). Microsequencing identified this spot as subunit N3 containing an additional amino acid, Arg, at the N-terminus (Fig. 2D), explaining the charge shift of this spot with respect to correctly processed N3 (Fig. 2A). These observations suggest that the inactivation of MECL-1 influences correct processing of its neighbor across the two contacting β-rings, N3. Our data are consistent with results obtained upon inactivation of Pup1 in yeast proteasomes [17]. Thus, the processing mechanism of eukaryotic proteasomes appears to be a conserved process.

Due to the presence of hMECL-1 in T27.10 mMECL-1T1A cells mMECL-1 T1A competes with endogenous hMECL-1 for incorporation. Therefore, the resulting proteasome population did not contain sufficient amounts of mMECL-1T1A, which would allow the analysis of enzymatic activities of the MC14/MECL-1 active sites. To allow such an analysis, mMECL-1 T1A cDNA was transfected into B8 LMP2+7 (BC27H7) mouse fibroblast cells cells which have low levels of endogenous mMECL-1 [11]. Like observed in T2 cells, mMECL-1 T1A expressed in BC27H7 cells migrated as a 31 kDa protein (Fig. 1B, lanes 3–5), indicating that this subunit was incorporated into the 20S proteasomes of the transfectant clones but was processed incompletely. Endogenous mMECL-1 wt was barely detectable, like in the B8 parental cell line (Fig. 1B, lane 1, Fig. 3E). Thus, the established clones express mMECL-1 T1A at high levels and lack any significant amounts of endogenous mMECL-1.

To examine the efficiency of MC14 for mMECL-1 T1A exchange, proteasomes were purified of B8 LMP2+7+ mMECL-1 T1A clone 7 (B8.27.MT1A) cells and of B8+ LMP2+LMP7+mMECL-1 (B27M.2) cells as a control. Coomassie-stained two-dimensional IEF-PAGE gels (Fig. 3) showed the presence of MC14 and mMECL-1 in B27M.2 proteasomes (Fig. 3B). While a spot representing MC14 was visible in proteasomes of B8.27.MT1A cells, mMECL-1 wt was not detected (Fig. 3A). Instead, an additional spot was observed which on the basis of its MW and isoelectric point (IP) was identified as mMECL-1 T1A). Further immunoblot analyses (not shown) revealed the presence of only minor quantities of mMECL-1 T1A, precluding N-terminal sequencing, at more than one position in the B8.27.MT1A Coomassie gel as observed for T2 proteasomes (Fig. 2). The intensities of the visible mMECL-1 T1A spot and MC14 spot in Fig. 3A suggest roughly equal incorporation rates for the two subunits in B8.27.MT1A proteasomes. Therefore, the exchange efficiency of MC14 for mMECL-1 T1A is similar to that observed for mMECL-1 wt and MC14 in B27M.2 cells (Fig. 3B).

The subunit composition of proteasomes of B8.27.MT1A, B27M.2 and of B8 cells which influences enzymatic activity (see below) was further characterized by two-dimensional NEPHGE-PAGE (Fig. 3D–F). Proteasomes purified from B8 cells did not contain any substantial amounts of LMP2 and LMP7 nor of mMECL-1 (Fig. 3E) as was already previously shown by Boes et al. and Groettrup et al. [11,23] and is demonstrated in Fig. 1. Both proteasomes of B27M.2 and of B8.27.MT1A cells showed an efficient replacement of δ and MB1 for LMP2 and LMP7. In the context of these experiments in particular the exchange of δ versus LMP2 is of importance since LMP2 also determines the incorporation efficiency of MECL-1 [23]

To examine the function of the MECL-1 catalytic site, we compared the peptide hydrolyzing activities of the three pro-

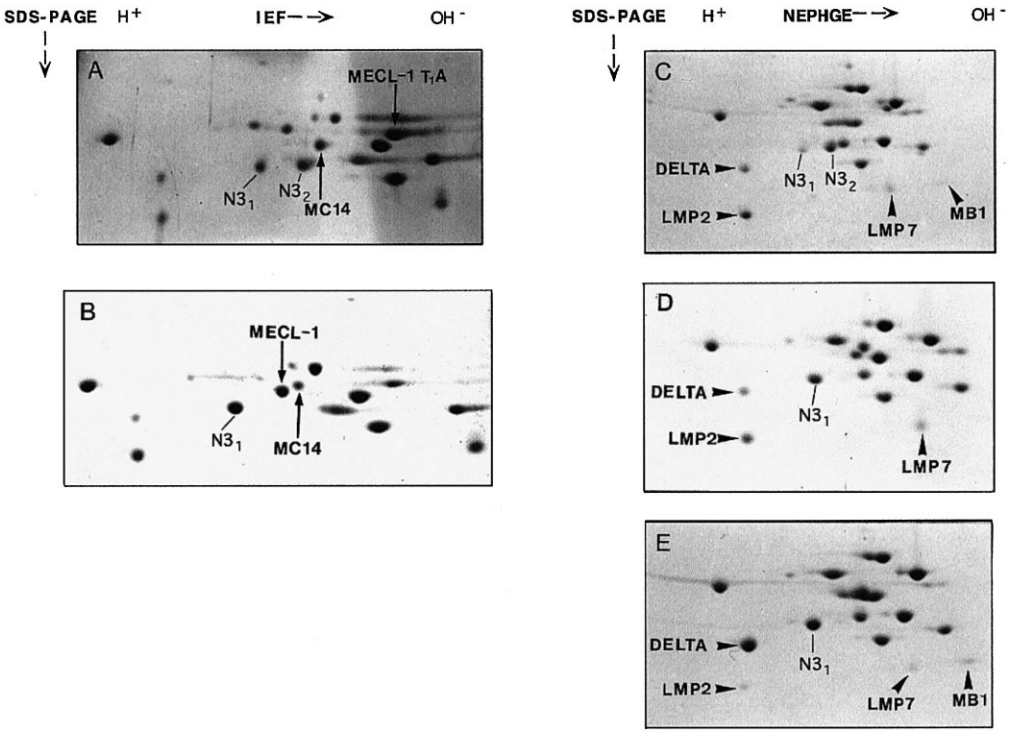

Fig. 3. Subunit composition of 20S proteasomes of B8.27.MT1A (A, C), B27M.2 (B, D) and of B8 (E) cells. 20S proteasomes were purified of B8.27.MT1A (A, C), B27M.2 (B, D) and of B8 (E) cells and subjected to two-dimensional IEF-PAGE (A, B) to analyze the exchange of MC14 for MECL-1 or mMECL-1 T1A or NEPHGE-PAGE to analyze the δ/LMP2 or MB1/LMP7 exchange (C–E). Separated proteins were stained with Coomassie blue. Proteasome subunits were assigned on the basis of their migrational behavior as indicated [20].

teasome populations shown in Fig. 3. Consistent with our earlier observations [11,12,24], we found that the incorporation of the IFNγ-inducible subunits reduced the T-L, PGPH and ChT-L activities of B8 proteasomes as measured using the Bz-VGR-AMC, Z-LLE-βNA, and SUC-LLVY-AMC substrates respectively (Fig. 4). It may be worth mentioning that in particular the PGPH activity is most drastically reduced due to the presence of LMP2. Remarkably, while proteasomes containing mMECL-1 T1A instead of mMECL-1 converted the SUC-LLVY-AMC and Z-LLE-βNA peptides to about the same extent as wild type immunoproteasomes, mMECL-1 T1A proteasomes failed to hydrolyze the Bz-VGR-AMC tripeptide. Therefore, we infer that the T-L activity of mammalian proteasomes, as defined with short peptide substrates, is largely mediated by the MC14/MECL-1 catalytic sites. Also, the MC14/MECL-1 subunits do not play any significant role in the hydrolysis of hydrophobic or acidic substrates. Interestingly, these data corroborate previous findings by the groups of Wolf and Rammensee [17,18], showing that the β2 catalytic site of yeast proteasomes is to a large extent responsible for T-L activity. Likewise, we previously showed that mammalian δ exerts the proteasomal PGPH activity, consistent with the recent data of cited investigators who assigned this activity to the yeast β1 subunit [16]. From these

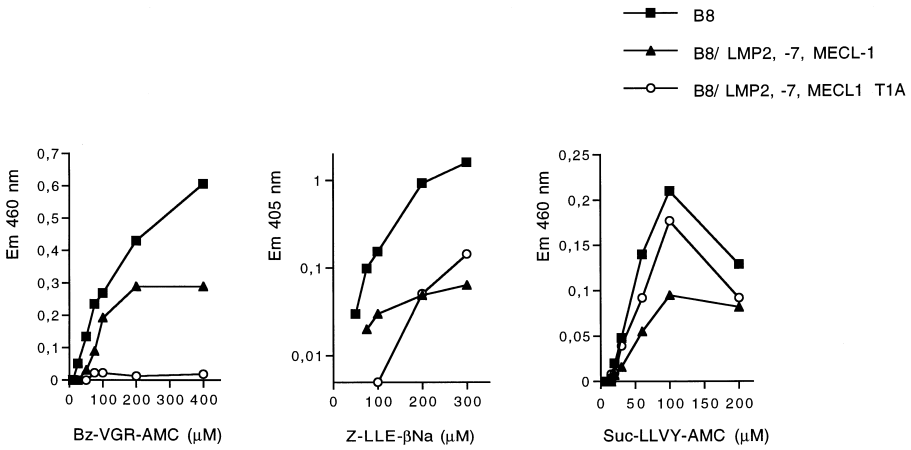

Fig. 4. Replacement of MC14 by mMECL T1A inhibits the T-L activity of 20S proteasomes. Peptide cleavage activities were assayed by incubating specified substrates with 200 ng purified 20S proteasomes in a volume of 100 μl for 60 min at 37°C. Release of the fluorescent groups was measured with a spectrofluorometer at 355 nm excitation/405 nm emission (Z-LLE-βNA) or 390 nm excitation/460 nm emission (SUC-LLVY-AMC, Bz-VGR-AMC) and plotted on the *y*-axis. Mean values of duplicate wells are plotted.

biochemical experiments we infer that the proteolytic activities of the yeast and mammalian proteasomal catalytic centers are conserved, a conclusion which is in agreement with the structural data obtained from the yeast proteasome X-ray structure [3].

Since the ratio between MC14 and mMECL-1 T1A in the isolated proteasome population of B8.27.MT1A cells was estimated to be roughly 1:1 (Fig. 3A), the observed complete abolition of one of the proteasome-hydrolyzing activities was unexpected. These data can be interpreted such that proteasome mixed populations with mMECL-1 T1A in one and MC14 in the other β-ring are formed, whereby we propose that the inactive mutant subunit may affect the activity of the second homologous site. Such an interpretation is supported by the almost complete elimination of the T-L activity in the B8.27.MT1A mutant which would not be expected if considerable amounts of proteasomes containing only MC14 were present in our preparation. Indeed, other investigators using intact cellular systems have demonstrated the formation of proteasome mixed populations containing both constitutive proteasome subunits and their induced homologues [21,27]. Further experiments using tagged proteasome subunits to purify proteasome subpopulations will be needed to examine the hydrolyzing activities and thus the functional importance of such proteasomes.

Rammensee and coworkers [19] showed that the hydrolyzing activities of yeast proteasome subunits as determined with short substrates corresponded to a large extent with the cleavage site preferences in polypeptide substrates. This finding was unprecedented considering previous studies by Groettrup et al. [12] demonstrating that the usage of specific cleavage sites in the pp89 polypeptide could not be assigned to single catalytic subunits in the case of mammalian proteasomes. Further studies using a larger set of polypeptides and different mutant mammalian proteasomes will be needed to fully understand the contribution of individual catalytic sites and of proteasome structure to substrate cleavage. These investigations are of functional importance in particular for the design of drugs to interfere with specific proteasome activities as desired, for example, for therapy of $CD8^+$ T cell-mediated autoimmune disease. In addition, they may help to predict proteasome cleavage site usage, relevant for the development of vaccines to induce T cell-mediated immunity.

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