The proteasome inhibitor PI31 competes with PA28 for binding to 20S proteasomes

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Received 25 July 1999; received in revised form 3 August 1999

Abstract PI31 is a previously described inhibitor of 20S proteasomes. Using recombinant PI31 we have analyzed its effect on proteasomal hydrolyzing activity of short fluorogenic substrates and of a synthetic 40-mer polypeptide. In addition, we investigated its influence on the activation of 20S proteasome by the proteasome activator PA28. PI31 inhibits polypeptide degradation already at concentrations which only partially inhibit fluorogenic substrate turnover and immunosubunits do not influence the PI31 binding affinity. Furthermore our data demonstrate that PI31 is a potent competitor of PA28-mediated activation.

Key words: Proteasome; Proteasome inhibitor 31(PI31); Proteasome activator (PA28)

1. Introduction

The proteasome is considered to be the central protease complex and major mediator of controlled proteolysis in the cytosol of eukaryotic and prokaryotic cells and in archaebacteria. The proteasome consists of a catalytically active core complex, the 20S, which is controlled by a variety of specific regulatory proteins including activators, inhibitors, and modulators (for review see [1]). While the function of the proteasome is increasingly well understood – for example its role in cell cycle progression, degradation of transcription factors or in the selective proteolysis of antigens for presentation by MHC class I molecules (for review see [2]) – the influence of regulatory complexes on proteasomal activity is not yet clear.

The 20S core particle consists of four rings each containing seven subunits. The outer terminal rings are built of alpha subunits, whereas the inner two rings contain the catalytically active beta subunits. While in archeae both the alpha subunits and the beta subunits are encoded by one single gene each [3], in eukaryotes each of the seven subunits is encoded by its own specific gene. Of the seven beta subunits only three (delta/β2, MC14/β1 and MB1/β5) display catalytic activity.

DeMartino and coworkers performed a biochemical screen to identify complexes which could modify the activity of the 20S proteasome. They found two activators, termed PA28 [4] or 11S regulator [5] and PA700 [6] or 19S regulator [7]. These activators bind to the terminal rings of the 20S core particle to form proteasome-regulator complexes, and greatly increase proteolytic activity towards short fluorogenic substrates.

PA700 is a complex of 700 kDa composed of at least 16 subunits, which are organized in two distinguishable subcomplexes, the base and the lid. The base consists of six ATPases which bind to the proteasome via an ATP-dependent mechanism, and displays unfoldase activity, as shown recently by our group [8]. The lid is connected to the base most likely by one single subunit and has the ability to bind poly-ubiquitin conjugates. The complex formed by the 20S proteasomal core complex and PA700 is called the 26S proteasome. Regulation of proteolysis by 26S proteasomes is considered to occur predominantly at the level of ubiquitin conjugation onto substrates, which thereby are targeted towards the lid and for degradation (for review see [9]).

The proteasomal function is modulated by IFNγ through the induction of homologues of the three catalytically active 20S proteasome beta subunits, the so-called immunosubunits. These homologues are preferentially incorporated and change the enzymatic activity of the 20S proteasome, resulting in altered cleavage site usage in viral and self proteins that encompass MHC class I binding peptides [10].

The other activating complex, PA28, is a high-affinity activator of the proteasome's multiple peptidase activities. PA28 is found in mammalian cells, at relatively low levels, however, it is strongly inducible by IFNγ. It binds to the 20S proteasome and has been shown to modulate the catalytic activity of the 20S proteasome complex in vitro to favour antigenic peptide production [11,12]. Furthermore, the overexpression of PA28 in mouse cells enhances the cell surface presentation of specific viral CTL epitopes [13].

In the original screen for complexes that modulate 20S proteasome activity, the group of DeMartino identified another regulatory protein, the proteasome inhibitor PI31 [14]. While the physiological role of this protein is not yet understood, important functions in for example modulating the role of PA28 could be hypothesized. Therefore, we produced recombinant PI31 and tested the influence of this protein on the enzymatic activity of different proteasome populations and on PA28-effected proteasome activation.

2. Materials and methods

2.1. Generation of mouse PI31 expression construct

Murine PI31 was cloned by RT-PCR (reverse transcriptase-polymerase chain reaction) using mouse fibroblast mRNA as a template and oligonucleotide primers from the 5′ UTR and 3′ UTR of PI31, with the sequences GCTTTCTGCATAGTCCCCTCC and CAAGAATACTGACGCTGATG, respectively. The PCR was performed with PFU-polymerase (Promega, Madison, WI, USA) in 30 cycles using an annealing temperature of 55°C.

The product was cloned into the Topo-cloning vector (Invitrogen, Groningen, The Netherlands) and sequenced with an ABI-PRISM sequencer. The obtained cDNA was cloned into the 4T3pGex gluta-

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PII: S0014-5793(99)01072-8

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murine  MAGLEVLFASAAPSMSCPQDALVCFLHWEVVTNGYYALGTGDQQGPSDKKSELLPAKWNSNKELYALRYESKDGARKLLL  80
        :::::::::::::    : :::::::::::::: ::  :: ::: :: ::::::::: :: :: :: :::: ::: ::::
human   MAGLEVLFASAAPAITCRQDALVCFLHWEVVTHGYCGLGVGDQPGPNDKKSELLPAGWNNNKDLYVLRYEYKDGSRKLLV  80

murine  KAVSVENGMIINVLELGTQQVADLTLNLDDYIDAEDLSDFHRTYKNSEELRSQIRSGIITPIHEQWEKARANSSPREFPP 160
        ::  ::  :: :::: : :::::::::::::::::: : :::::::::::::: : ::::::::::::::   :  :::::
human   KAITVESSMILNVLEYGSQQVADLTLNLDDYIDAEHLGDFHRTYKNSEELRSRIVSGIITPIHEQWEKANVSSPHREFPP 160

murine  ATAREVDPLQISSNRPHTSRQPCWGDSLSPIAVGGNYLNPFGCQRGGMIVDPLRSGFPRVLIDPSSGLPNRLPPGAVPPG 240
        ::::::::: :    ::::::: : : : :  :::  : :::  :::::::::::::::: ::::::::::::::::::::
human   ATAREVDPLRIPPHHPHTSRQPPWCDPLGPFVVGGEDLDPFGPRRGGMIVDPLRSGFPRALIDPSSGLPNRLPPGAVPPG 240

murine  ARFDPFGPIGTSPSGPNPDHLPPPGYDDMYL 271
        :::::::::::::: :::::::::::::::::
human   ARFDPFGPIGTSPPGPNPDHLPPPGYDDMYL 271
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Fig. 1. Alignment of the human and the murine PI31 protein sequences.

thione S-transferase fusion vector (CLONTECH, Palo Alto, CA, USA) using the oligonucleotide primers CCCGAATTCCATGG-CGGGTCTCGAGGTTCTC at the 5′ and GATGCGGCCGCTCA-CAGGTACATGTCATCGTAG at the 3′ end. The resulting recombinant protein has three additional amino acids (i.e. GlySerPro) after the thrombin cleavage at the N-terminus.

2.2. Expression of the recombinant PI31

The recombinant mPI31/glutathione S-transferase fusion protein was expressed in *Escherichia coli* strain DH5α according to standard procedures. Briefly, bacteria were grown in the presence of 2% glucose at 37°C until logarithmic phase (OD_{600} 0.6) and then shifted to 30°C. IPTG was added to an end concentration of 100 mM. Cultures were harvested, resuspended in ice cold lysis buffer (50 mM Tris (pH 7.5) and 150 mM NaCl) with PMSF and lysed with the French Press.

Lysates were cleared and loaded on a gluthatione column (Sigma, St. Louis, MO, USA) at 4°C, which was extensively washed with PBS and then incubated with 10 U biotinylated thrombin (Novagen, Madison, WI, USA) in thrombin cleavage buffer (20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) at 4°C overnight. recPI31 was recovered from the column with the streptavidin agarose bound thrombin (Novagen) and pelleted. The supernatant was loaded onto a Resource Q FPLC column (Pharmacia, Upsala, Sweden) in 100 mM Tris (pH 7.2) which was then eluted with a linear gradient from 100 mM to 500 mM NaCl. Recombinant mPI31 eluted at a salt concentration of 160 mM NaCl.

2.3. 20S proteasome preparations

20S proteasomes were purified according to standard procedures [10].

2.4. Fluorogenic peptide digestion assays

20S proteasome activity assays with short fluorogenic oligopeptides and the in vitro polypeptide digestions were performed as described [10].

2.5. PA28 complex isolation

PA28 was isolated from mouse embryonal cells which express PA28α and β at high levels from a Tet-regulated promoter (van Hall and Sijts, in preparation). 6×10^6 cells cultured for 3 days in the absence of Tet, to induce PA28 expression, were harvested, lysed with 0.1% Triton lysis buffer and three times freeze/thawed. Lysates were applied to a 10–40% glycerol gradient, which was centrifuged for 15.49 h at 40 000 rpm at 4°C. Fractions of 600 μl were collected and tested for their capacity to activate isolated 20S proteasome as described [10]. Fraction 9 was chosen for further experiments, since it

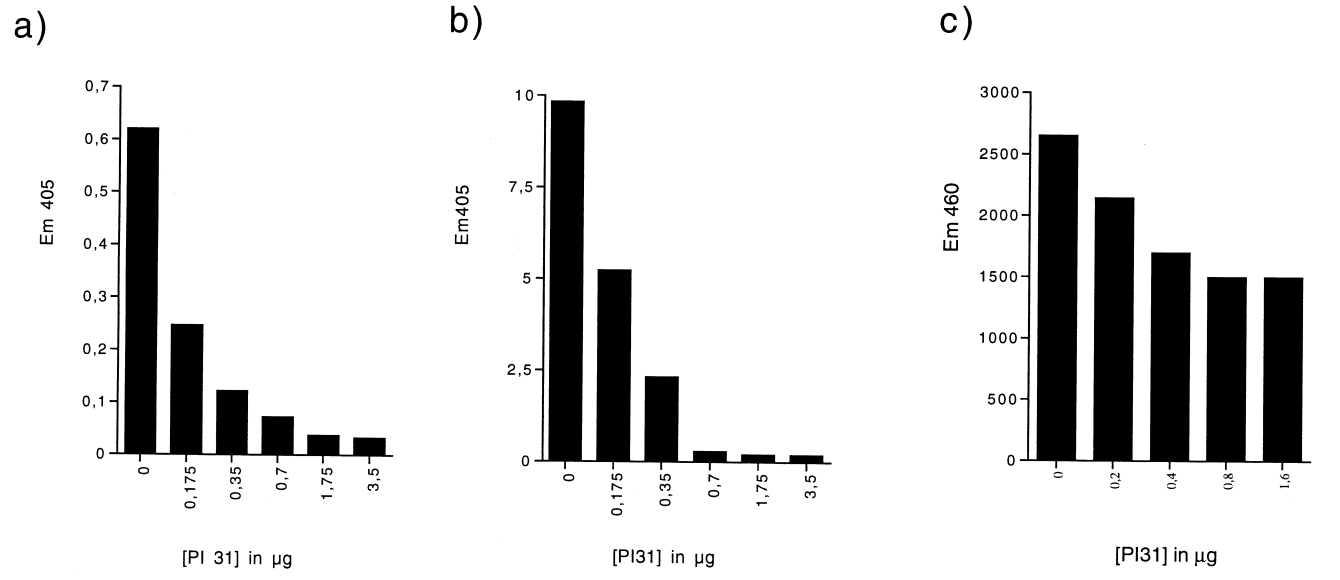

Fig. 2. PI31 is an efficient inhibitor of 20S proteasome activity. Peptide cleavage activity was assayed by incubating specific substrates with 200 ng purified 20S proteasome in the presence of various concentrations of PI31 in a volume of 100 μl for 60 min at 37°C. Release of the fluorescent groups was measured with a spectrofluorometer at 390 nm excitation/460 nm emission for Suc-LLVY-AMC (a) and Bz-VGR-AMC (c) or at 355 nm excitation/405 nm emission for Z-LLE-βNa (b), and plotted on the *y* axis. Mean values of duplicate wells are plotted.

strongly activated the purified 20S proteasome and did not exhibit proteolytic activity in the absence of 20S proteasome. The PA28 concentration in this fraction was estimated by Western blot analysis with titrated standard concentrations of recombinant PA28β protein.

3. Results

3.1. Cloning of mouse PI31

To investigate the influence of the proteasome inhibitor PI31 on proteasome function we cloned the protein coding region of the mouse PI31 cDNA to express PI31 as a GST fusion protein.

To identify the mouse homologue of the human PI31 the mouse EST databases of the GenBank were screened with sequence fragments of the published human PI31 protein (accession number: D88378). Received EST sequences were used to select oligonucleotide primers located in the predicted 3′ UTR and the 5′ UTR of the mouse PI31 cDNA. PI31 cDNA was cloned by RT-PCR using polyT-RNA of BALB/c mouse fibroblast cells (see Section 2). The deduced mPI31 amino acid sequence has the same length as hPI31 and displays 83% identity (Fig. 1). The C-terminus of PI31, which is unusually rich in proline residues can be separated into a strongly conserved region (residues 204 to 271; two conservative changes in 67 amino acids) and a less well conserved region (residues 150 to 203; 17 out of 54 amino acids are non-conservatively exchanged). The remaining protein regions are relatively well conserved (28 amino acid exchanges in a total of 149 residues) and, according to structure prediction programs (Preditor EMBL, Heidelberg, Germany), maintains a dominant helical structure, which is also predicted for the human PI31.

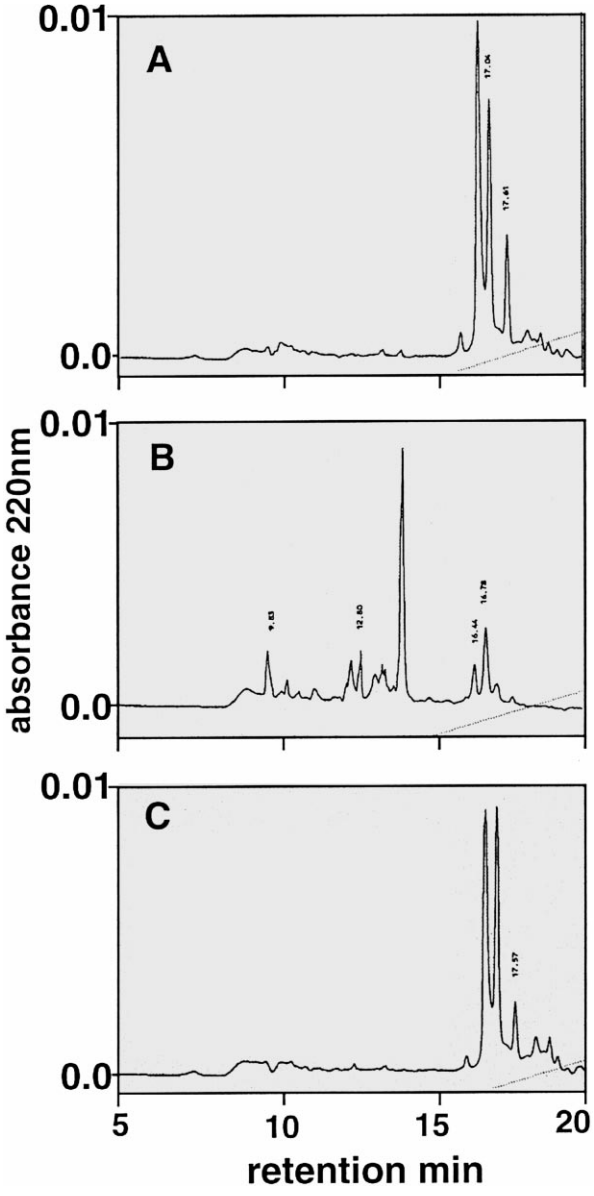

Fig. 3. PI31 inhibits the digestion of a synthetic hepatitis B virus 40-mer polypeptide. 660 ng peptide was incubated in a volume of 100 μl for 8 h at 37°C without proteasomes (A) and with 1 μg purified 20S proteasome in the absence (B) and in the presence (C) of 8 μg PI31. Digestion products were separated by HPLC.

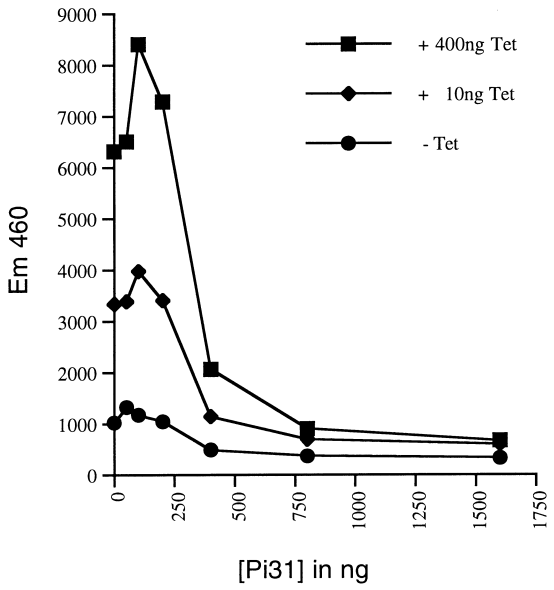

Fig. 4. PI31 inhibits 20S proteasome with the same kinetics regardless of their subunit composition. 20S proteasomes were purified from MEC217 cells grown in the presence or absence of 10 ng/ml or 400 ng/ml tetracycline. The purified proteasomes had either an almost complete, a 55% or 0%, exchange of constitutive for immunosubunits, respectively. Peptide cleavage activity of these proteasomes in the presence of various concentrations of PI31 were assayed by incubating Suc-LLVY-AMC with 200 ng purified 20S proteasome as described in Fig. 2a.

3.2. Expression of PI31 as a GST fusion protein

mPI31 cDNA was cloned into a GST expression vector and expressed in *E. coli*. The secreted recombinant protein was bound to a glutathione column, PI31 was cleaved off with thrombin (see Section 2) and purified by FPLC using a Resource Q column. The protein was tested for its capacity to inhibit 20S activity by monitoring the hydrolysis of short fluorogenic peptide substrates. As shown in Fig. 2, cleavage after tyrosine (chymotrypsin-like or ChT-l activity) and after glutamic acid (peptidyl-glutamyl hydrolyzing or PGPH activity) were inhibited by PI31 whereas cleavage after arginine (trypsin-like or T-l activity) was less sensitive to inhibition. While a substantial overall reduction in activity was achieved, PI31, even when added at relatively high concentrations, failed to

abrogate 20S activity completely (Fig. 2). In contrast, already concentrations of recombinant PI31, which had minor effects on cleavage of short fluorogenic peptides, in particular on such as Bz-VGR-AMC, inhibited the digestion of polypeptides by the 20S proteasome completely (Fig. 3). Altogether, we conclude that the activity of recombinant mPI31 is comparable to that described for the purified bovine PI31 [14].

3.3. PI31 inhibits activity of both constitutive and immunoproteasomes

To test whether inhibition by PI31 is influenced by changes in the subunit composition of the 20S proteasome complex, purified 20S proteasomes containing either the constitutive or the IFNγ-inducible immunosubunits were tested for their specific inhibition kinetics. Proteasomes used were purified from mouse fibroblast B8 cells which show a roughly 50% exchange of constitutive for immunosubunits upon culture in the presence of INFγ, and from mouse embryonal MEC217 cells which express the immunosubunits in an inducible and titratable fashion from a tetracycline (Tet)-regulated promoter (Sijts and Kloetzel, in preparation). Testing the activity of the purified proteasome populations against different short fluorogenic peptide substrates, we found that the incorporation of the immunosubunits reduced cleavage of the Suc-LLVY-AMC peptide as described previously [10,11]. Titrating PI31 into the experiments showed that the kinetics of inhibition were identical for the different proteasome populations tested (Fig. 4). Therefore, we conclude that PI31 acts as a general inhibitor of 20S activity and that its function is not affected by proteasomal subunit composition.

3.4. PI31 alters 20S activity but not specificity

We previously reported that the INFγ-induced proteasome activator PA28 modifies the cleavage mechanism of proteasomes such as to promote coordinated double cleavage events in certain polypeptide substrates [12]. To determine whether PI31 alters the quality of proteasome-mediated cleavages, we digested synthetic polypeptides with purified 20S proteasome of uninduced MEC217 cells in the absence or presence of PI31 at a concentration that reduces cleavage activity to 15%, as measured with fluorogenic Suc-LLVY-AMC substrate. While PI31 diminished polypeptide substrate turnover drastically, we did not detect any changes in the quality of the digestion products generated (Fig. 5). Higher concentrations of PI31 abolished polypeptide degradation completely (Fig. 3). These data indicate that PI31 inhibits 20S activity without influencing cleavage specificity.

3.5. Competition between PI31 and PA28

PA28 activates proteasomes in vitro and facilitates proteasome-mediated liberation of MHC class I binding peptides from viral substrates [11]. We analyzed whether PI31 can compete PA28 function. PA28 was isolated from mouse embryonal cells which express both PA28α and PA28β at high levels, from a Tet-regulated promoter (van Hall and Sijts, in preparation). Cell lysates of induced cells were applied to a glycerol gradient and fractions 6 to 11 were identified as 20S activating fractions containing PA28 complexes in peptide digestion assays and by Western analysis using PA28-specific antisera (see Section 2). The same fractions of uninduced MEC cells that did barely express PA28 did not activate 20S activity, excluding the possibility that the observed enhanced substrate cleavage was mediated by putative other proteases present in the selected fractions.

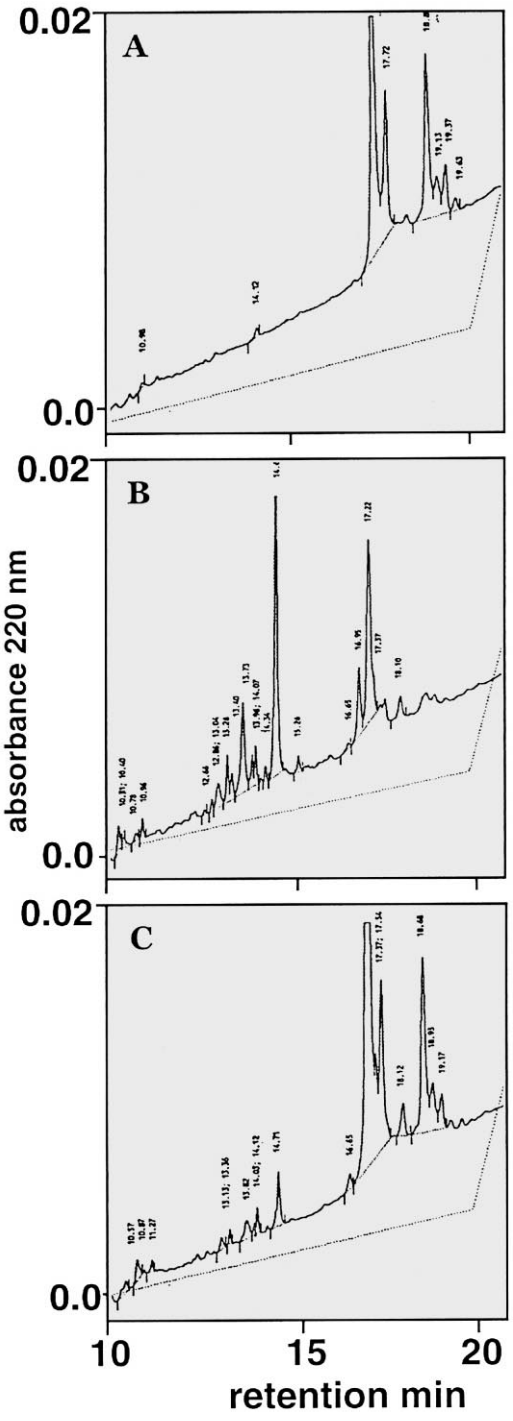

Fig. 5. PI31 does not influence the quality of the proteasomal digestion products. A synthetic HepB virus 40-mer polypeptide was digested for 24 h as described in Fig. 3 without proteasome (A) and with 1 μg purified 20S proteasome in the absence (B) and in the presence (C) of 1.1 μg PI31.

To test the influence of PI31 on PA28-mediated proteasome activation, we assayed the effect of PI31 on activated 20S using Suc-LLVY-AMC as substrate. Increasing amounts of PI31 were added to serial dilutions of PA28. The kinetics of activation and inhibition were measured and plotted double reciprocally in a Lineweaver-Burk plot, as shown in Fig. 6A.

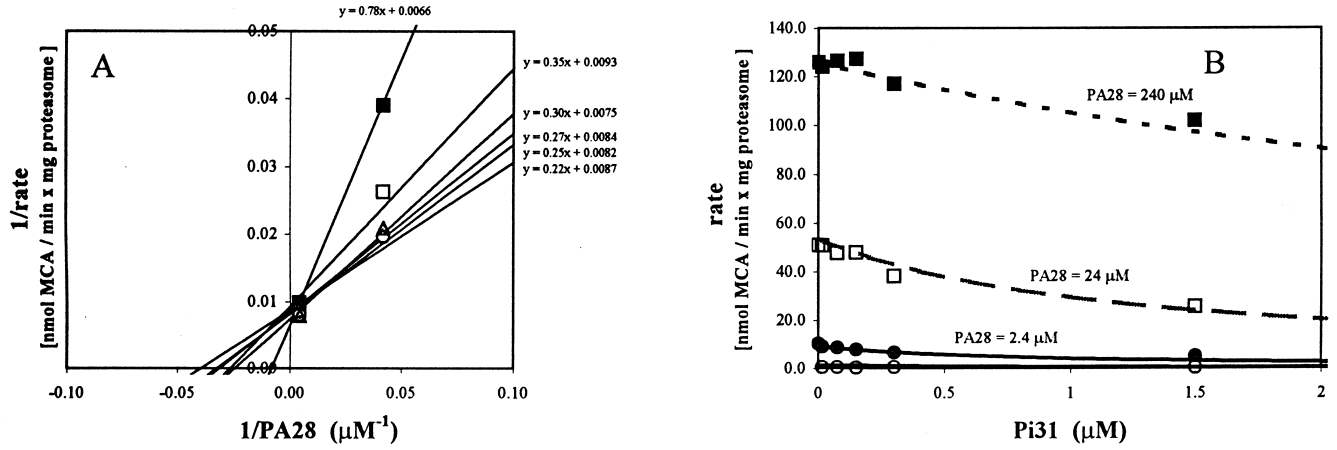

Fig. 6. Kinetic analysis of the interaction of PA28 and PI31 with the 20S proteasome. A: Activation kinetics: Double reciprocal plot of the cleavage rate versus concentration of PA28 at increasing concentrations of PI31 (○, 0.0 μM; ●, 0.015 μM; △, 0.075 μM; ▲, 0.15 μM; □, 0.3μM; ■, 1.5 μM). The regression lines reveal increasing slopes but constant intercept indicative for competitive inhibition. For a better visual inspection of slopes and intercepts the plot is only shown in the range of small 1/*S* values. B: Inhibition kinetics: Cleavage rate versus concentration of PI31 at four different concentrations of PA28 was analyzed by using the rate equation

$$v = \frac{v_o + v_a \frac{(PA28)}{K_a}}{1 + \frac{(PA28)}{K_a} + \frac{(PI31)}{K_i}}$$

which describes competitive binding of PA28 (with dissociation constant K_a) and PI31 (with dissociation constant K_i) to the proteasome. The parameters v_o and v_a refer to the maximal cleavage rates of the core 20S proteasome and the PA28-activated proteasome. Fitting this rate equation to the data (see bold lines) provided the following numerical estimates:

v_o	1.49 nmol MCA/min mg proteasome
v_a	148.4 nmol MCA/min mg proteasome
K_a	44.4 μM
K_i	0.79 μM

Increasing concentrations of PI31 resulted in progressively ascending slopes. However, there was no systematic change of the intercept. This is a hallmark of a competitive inhibitor.

In order to assess the binding constants for PI31 and PA28, we fitted the rate equation given in the legend of Fig. 6 to the measured dependency between cleavage rate and inhibitor concentration at various concentrations of PA28, as is shown in Fig. 6B. The affinity of PI31 to the 20S proteasome was estimated to be at least 50 times higher than that of PA28. In support of this, preincubation of 20S proteasome with PI31 or with PA28 at room temperature or at 37°C did not affect the kinetics of PI31-mediated inhibition of the 20S proteasome (data not shown). These data indicate that PI31 and PA28 compete with each other for the binding to the 20S proteasome and that the inhibition of 20S proteasomes by PI31 is reversible only at high concentrations of PA28.

4. Discussion

Here we used recombinant mouse PI31 to investigate the influence of PI31 on proteasome activity and on PA28-mediated proteasome activation.

Our experiments show that PI31 inhibits the activity of 20S proteasomes isolated from IFNγ-induced cells and from uninduced cells with the same kinetics, indicating that immunosubunits do not influence the PI31 binding affinity.

Competition experiments with PA28 and PI31 clearly show that PI31-mediated inhibition of 20S proteasomes is directly competing with PA28-mediated activation. Kinetic analyses show that this competition is, due to the clearly distinct affinities of the two molecules for the 20S proteasome, strongly biased towards PI31. Only at a concentration that exceeds the concentration of PI31 at least 50-fold, PA28 would be able to overrule PI31-mediated inhibition of 20S proteasomes.

Our experiments demonstrate that at high concentrations the PA28 complex competitively interferes with PI31-mediated inhibition of 20S proteasomes. Thus, PA28 is in principle able to replace PI31 from its binding site on the 20S complex. Since it has been well documented that the PA28 complex binds the two outer alpha rings of the 20S complex, it is tempting to speculate that this is also the binding site of PI31. Structural analysis of PI31 predicts that the N-terminal region adopts a predominantly helical structure (amino acids 1–149) which is followed by an extended C-terminal region (amino acids 150–271) which is rich in proline residues. Therefore, we propose that the N-terminal portion of PI31 binds the 20S alpha rings, allowing the proline rich region to protrude into the inner cavity of the 20S proteasome. As such, the C-terminal sequences of the proline rich region (amino acids 204–271) would reach the inner cavity of the 20S proteasome and thereby either inhibit access to the proteasomal channel or inhibit the activity of the active centers directly. In support of this, we find that the C-terminus of PI31 which would be of functional importance is highly conserved between the human and the murine homologue, whereas the N-terminal part of the proline rich portion (amino acids

150–203), which would have the role of a spacer in the proposed model, is not as well conserved. Guided by this model, the differential effects of PI31 on the degradation of the different short fluorogenic peptides become more conceivable. One possibility would be that these peptides bypass PI31 with different probabilities, resulting in the measurement of different inhibition kinetics. On the other hand, one could propose that the C-terminus of PI31 preferentially interferes with specific active centers. For example inhibition of β2, which mediates trypsin-like activity, might be weaker. This would explain the residual activities for the Z-LLE-βNa and the Suc-LLVY-AMC substrate and the less strong inhibition kinetics for the Bz-VGR-AMC substrate.

Taken together, our results show that PI31 inhibits 20S activity independent of subunit composition, and overrules the effect of PA28 on proteasome activation.

One could speculate that competitive PA28 levels could be achieved at a very local level in specific compartments or by specific protein modifications. However, this remains to be shown in further studies using cellular systems.

Acknowledgements: This work was funded by the Deutsche Forschungsgemeinschaft Kl 427/10-1 and EU-Grant B104-CT97-0505 to P.-M.K.

References

[1] Ciechanover, A. (1998) EMBO J. 17, 7151–7160.
[2] Pamer, E. and Cresswell, P. (1998) Annu. Rev. Immunol. 16, 323–358.
[3] Zwickl, P., Grziwa, A., Puhler, G., Dahlmann, B., Lottspeich, F. and Baumeister, W. (1992) Biochemistry 31, 964–972.
[4] Ma, C.P., Slaughter, C.A. and DeMartino, G.N. (1992) J. Biol. Chem. 267, 10515–10523.
[5] Dubiel, W., Pratt, G., Ferrell, K. and Rechsteiner, M. (1992) J. Biol. Chem. 267, 22369–22377.
[6] Ma, C.-P., Vu, J.H., Proske, R.J., Slaughter, C.A. and DeMartino, G.N. (1994) J. Biol. Chem. 269, 3539–3547.
[7] Dubiel, W., Ferrel, K. and Rechsteiner, M. (1995) Mol. Biol. Rep. 21, 27–34.
[8] Braun, B.C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P.-M., Finley, D. and Schmidt, M. Nat. Cell Biol., in press.
[9] Hershko, A. and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479.
[10] Kuckelkorn, U., Frentzel, S., Kraft, R., Kostka, S., Groettrup, M. and Kloetzel, P.-M. (1995) Eur. J. Immunol. 25, 2605–2611.
[11] Groettrup, M., Ruppert, T., Kuehn, L., Seeger, M., Standera, S., Koszinowski, U. and Kloetzel, P.M. (1995) J. Biol. Chem. 270, 23808–23815.
[12] Dick, T.P. et al. (1996) Cell 86, 253–262.
[13] Groettrup, M. et al. (1996) Nature 381, 166–168.
[14] Ma, C.-P., Slaughter, C.A. and DeMartino, G.N. (1992) Biochim. Biophys. Acta 1119, 303–311.