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Research Paper

Substrate specificity of the human proteasome

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Abstract

Background: Regulated proteolysis by the proteasome is crucial for a broad array of cellular processes, from control of the cell cycle to production of antigens.

Results: The rules governing the N-terminal primary and extended substrate specificity of the human 20S proteasome in the presence or absence of 11S proteasome activators (REGα/β and REGγ) have been elaborated using activity-based proteomic library tools.

Conclusions: The 11S proteasome activators are shown to be important for both increasing the activity of the 20S proteasome and for altering its cleavage pattern and substrate specificity. These data also establish that the extended substrate specificity is an important factor for proteasomal cleavage. The specificities observed have features in common with major histocompatibility complex (MHC) class I ligands and can be used to improve the prediction of MHC class I restricted cytotoxic T-cell responses.

Keywords: Proteasome; Substrate specificity; Combinatorial libraries; Antigen presentation

1. Introduction

The 20S proteasome is an intracellular protease found in almost all living cells from prokaryotes to eukaryotes and is responsible for the majority of cytosolic and nuclear protein degradation [1,2]. The enzyme forms the proteolytic core of the larger ATP-dependent 26S proteasome that is essential for maintaining the integrity of the cell by degrading abnormal proteins and short-lived proteins that are involved in the regulation of cell cycle, gene transcription, and metabolism. A specialized function of the proteasome in mammals is the production of epitopes for presentation on the major histocompatibility complex (MHC) class I molecules. The cleavage specificity of proteasomes is believed to be an important factor in antigen presentation because MHC class I epitopes must conform to stringent structural requirements of both length and composition for efficient presentation.

The 20S proteasome consists of four heptameric rings stacked upon one another to form a cylindrical particle. Two inner β-rings contain catalytic subunits whose active sites face a central proteolytic chamber and two flanking α-rings that separate the proteolytic chamber from the external solvent. Early examination of the 20S proteasome substrate specificity suggested that it contained multiple distinct activities [3]. Several mutational, biochemical and structural studies on the yeast enzyme led to the conclusion that three of the seven β-subunits in eukaryotic proteasomes harbor proteolytic activity [4–6]. The β1-subunit (PGPH or Y) is associated with cleavage after acidic amino acids and the β2-subunit (trypsin-like or Z) is associated with cleavage after basic amino acids, and the β5-subunit (chymotrypsin-like or X) is associated with cleavage after hydrophobic amino acids. Additional activities for cleavage after branched-chain amino acids (BrAAP) and small neutral amino acids have also been reported but whether these activities are due to additional active subunits remains controversial [7].

The 20S proteolytic core can exist in several forms depending on its association with cap complexes. Association with the 19S cap (PA700) yields the 26S proteasome, a complex responsible for ubiquitin-mediated degradation of proteins [8]. Association with the interferon γ (IFNγ)-

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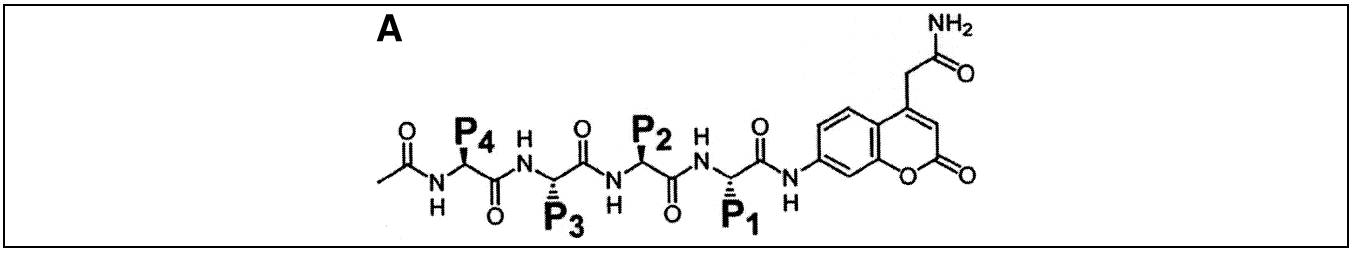


Fig. 1. (A) Structure of fluorogenic peptide libraries. (B) Results from the one-position fixed positional scanning library where the *y*-axis represents the rate of substrate cleavage (in relative fluorescence units) over time per nanomolar of 20S proteasome (RFU/s/nM) and the *x*-axis represents the P1-amino acid. The three non-fixed positions contain an equimolar mixture of 19 amino acids (Cys and Met excluded, Nle included and indicated with 'n') for a total of 6859 substrates/well. (C) Ratio of the activity of the 11S proteasome activator (REGα/β and REGγ) complexes over the activity of the uncomplexed 20S in the P1-library (changes in P1-Gly and P1-Pro are not shown due to minimal activity of the uncomplexed 20S toward these amino acids). Data from these figures show that the 11S proteasome activators (REGα/β and REGγ) selectively enhance specific proteasomal cleavage.

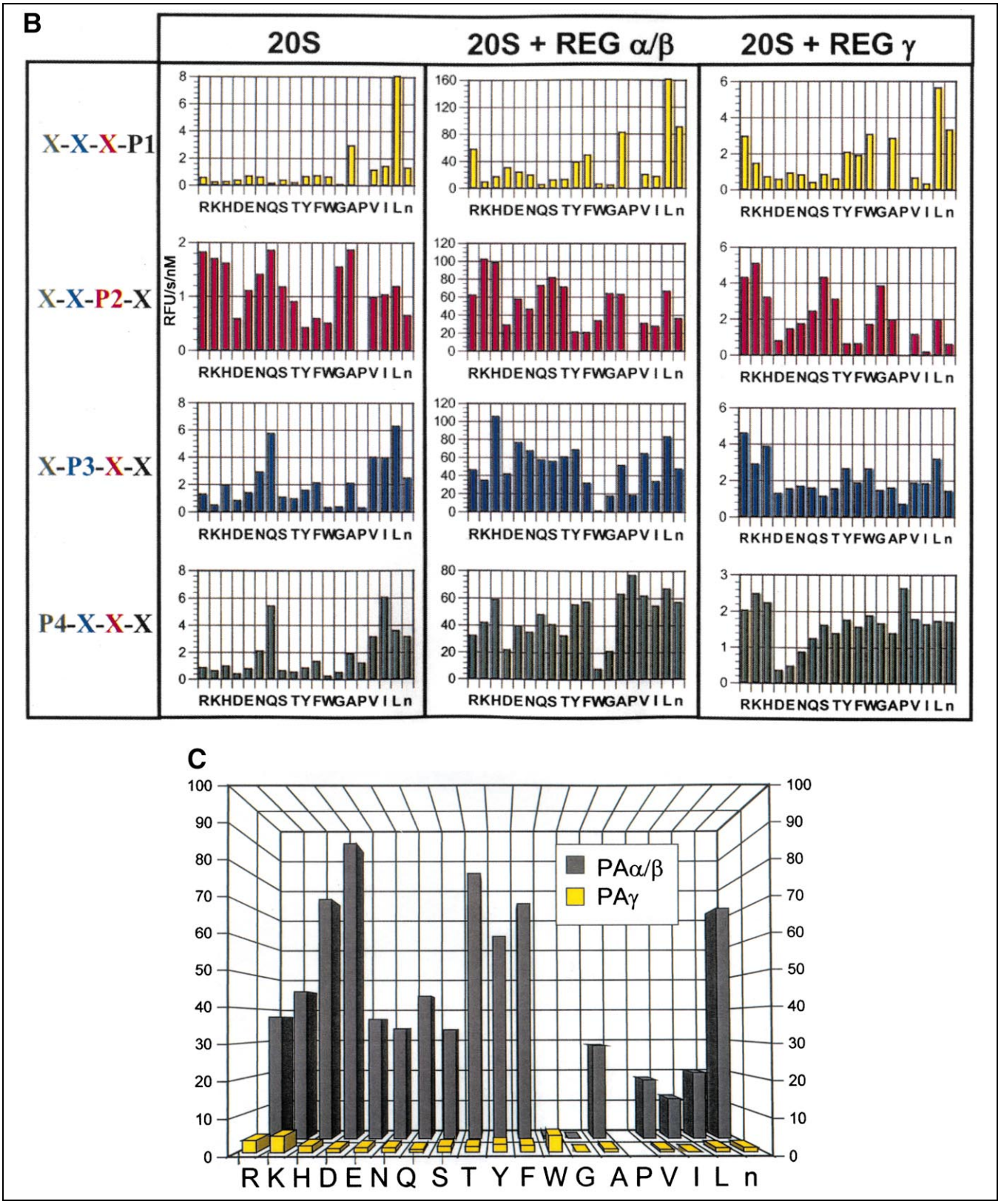
inducible REGα/β cap (11S or PA28) increases hydrolysis of most peptides indicating an activation of all three catalytic β-subunits [9]. Association of the proteasome with another 11S regulatory cap, REGγ [10], causes selective activation of the trypsin-like(Z) subunit to yield a proteasome complex with enhanced activity to cleave after basic amino acids. In addition, asymmetrical hybrid proteasomes have been identified that consist of one REGα/β cap and one 19S cap [11]. The importance of the 20S proteasome and REGα/β in generating the C-termini of some MHC class I ligands has been demonstrated [9,12–14].

Defining the substrate specificity of the proteasome is necessary for understanding its role in CTL epitope generation and other degradation functions. Through the use of peptide and selected protein substrates, several groups have studied the cleavage specificity of the 20S proteasome [5,15–17]. However, the guiding principles of proteasome cleavage specificity are still largely unknown because methods to efficiently sample large numbers of substrates have been lacking. Recently collections of covalent inhibitors were designed to evaluate the specificity of the proteasome [18]. The approach provided some important information on the extended substrate specificity of proteasome subunits and produced a subunit selective inhibitor, however, their studies do not necessarily depict a complete picture of substrate specificity since the compounds employed all contained asparagine at the P1-position. Furthermore, irreversible inhibitors provide information on the initial steps of peptide bond hydrolysis, substrate binding and acylation, but they do not take into account subsequent essential steps, deacylation and product release. Indeed, the lack of a correlation between covalent modification by peptide vinyl sulfones and substrate hydrolysis has been recently demonstrated [19]. Defining the primary and extended specificity of the 20S proteasome in the presence or absence of various activators will increase our understanding of proteasomal activity, provide tools for probing function through the design of substrates and inhibitors, and will aid in the prediction of epitope production and destruction. Here we describe in detail, the P4, P3, P2 and P1 substrate specificity of the human 20S proteasome and proteasome–REG complexes.

2. Results and discussion

To reveal the N-terminal P1 to P4 substrate specificity of the human 20S proteasome and its 11S REG complexes, exhaustive peptide library configurations of the P4–P1 sites were synthesized containing the C-terminal fluorogenic leaving group 7-amino-4-carbamoylmethylcoumarin (acc) (Fig. 1A) [20]. Proteolytic release of the coumarin leaving group results in increased fluorescence and allows for the sensitive and continuous determination of cleavage rates. The substrates were synthesized in two positional scanning formats and provide the facile determination of a protease's P4–P1 substrate specificity. In the first format, the 'one-position fixed' library, the substrates are pooled in four sets of 19 sublibraries (76 wells of 6859 substrates/well) where in each sublibrary one position in the tetrapeptide is known and the other three positions contain an equimolar mixture of the 19 amino acids (cysteine is excluded and methionine is replaced with the isosteric amino acid, norleucine). In the second library, the 'two-position fixed' library, the format is more complex in that two positions in the tetrapeptide are known and the other two positions contain an equimolar mixture of the 19 amino acids (2166 wells of 361 substrates/well). Screening of the one-position fixed library allows for a gross overview of the substrate specificity of the proteasome at each site, while screening of the two-position fixed library allows for a more detailed dissection of the dependencies between sites.

The substrate cleavage preferences of the 20S proteasome are dramatically altered depending on the 11S REG present (Fig. 1B). While activity from all three subunits is present in the 20S proteasome, the major activity is cleavage after the P1-aliphatic amino acids leucine and alanine with an absence of cleavage after P1-proline (Fig. 1B). Cleavage after aliphatic amino acids constitutes



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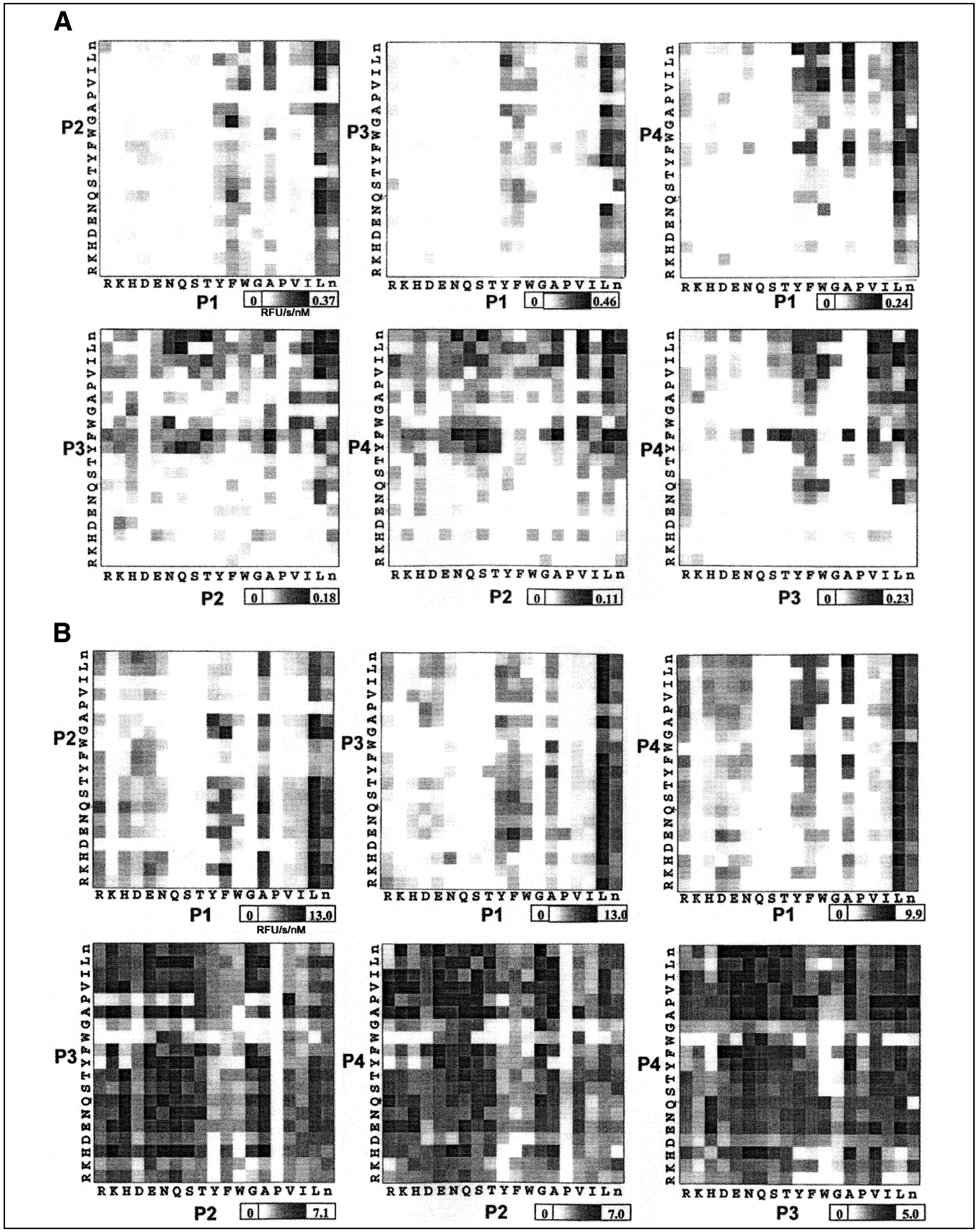
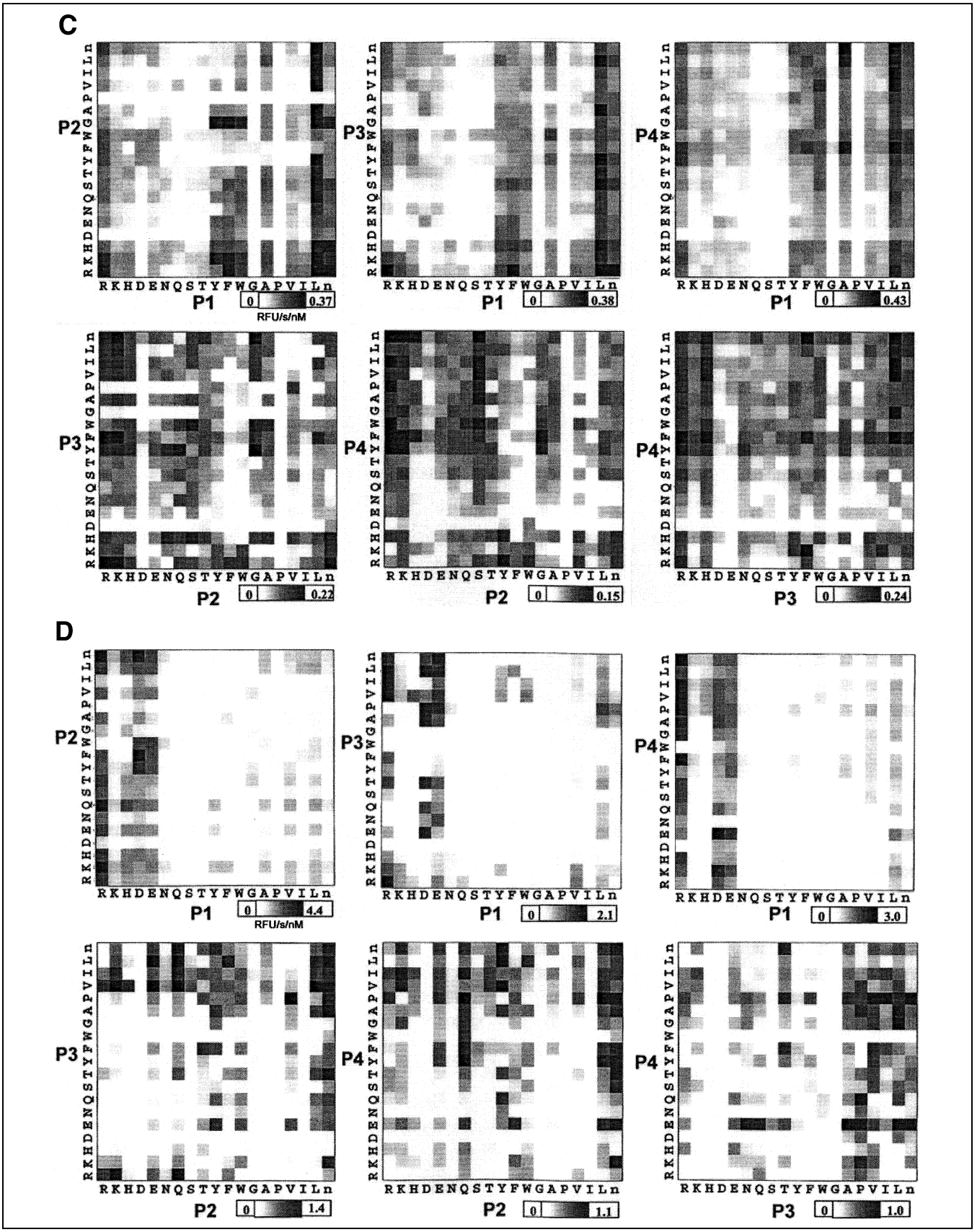


Fig. 2. Results from the two-position fixed libraries where the shade of the square represents the rate of substrate cleavage (in relative fluorescence units) over time per nanomolar of 20S proteasome (RFU/s/nM) and the *x*- and *y*-axes represent the positions P1-, P2-, P3- or P4-amino acid. The two positions in the substrate that are not held constant contain an equimolar mixture of 19 amino acids (Cys and Met excluded, Nle included and indicated with 'n') for a total of 361 substrates/well. (A) 20S proteasome alone, (B) 20S proteasome plus PAα/β, (C) 20S proteasome plus PAγ, (D) 20S proteasome plus PAα/β and 5 μM lactacystin.



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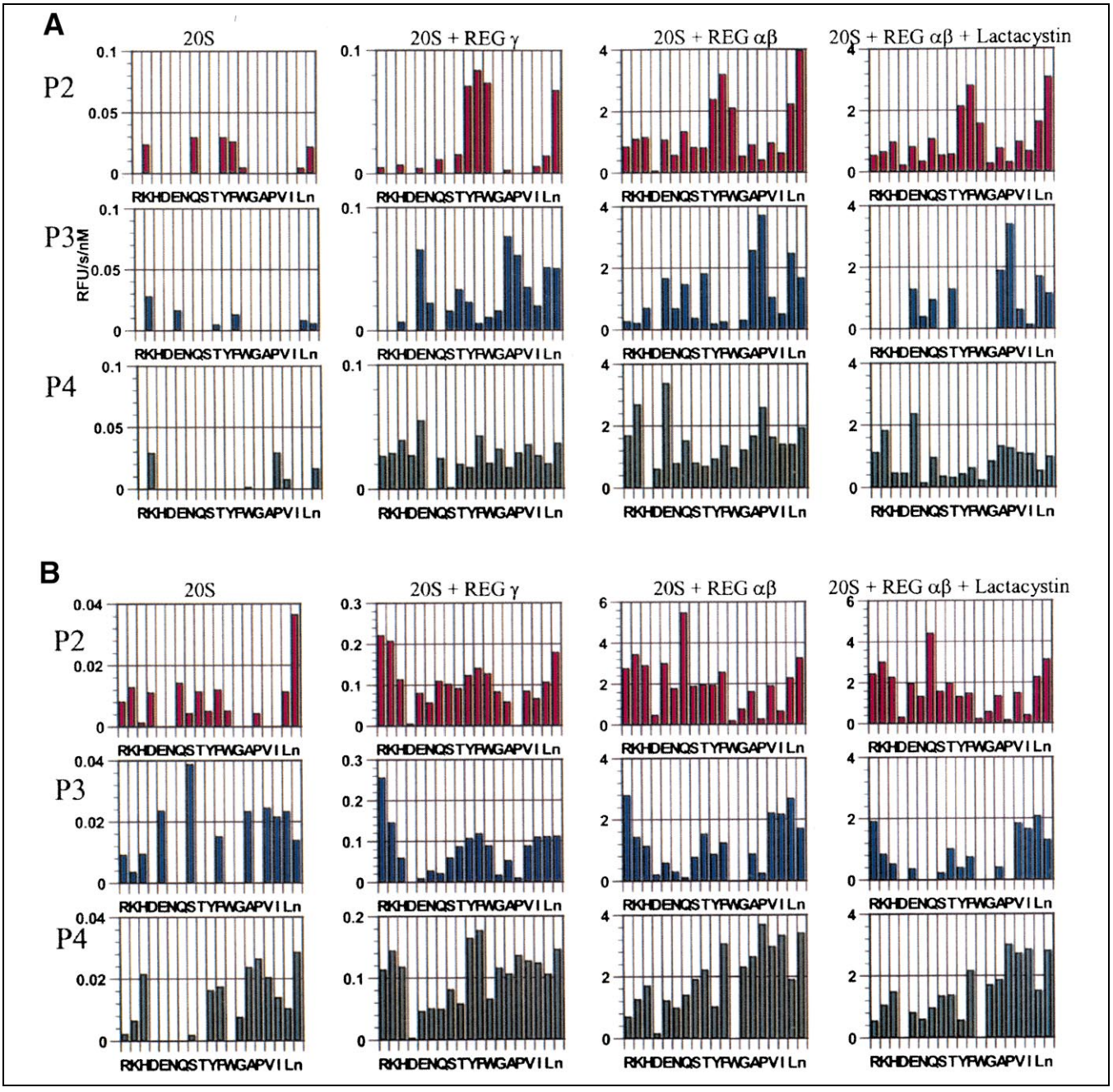

Fig. 3. Histograms of the two-position fixed library showing the extended substrate specificity when the P1-position is held constant as (A) arginine, (B) aspartate, (C) phenylalanine and (D) leucine. The *x*-axis represents a fixed amino acid and the *y*-axis represents the rate of substrate cleavage (in relative fluorescence units) over time per nanomolar of 20S proteasome (RFU/s/nM).

the major activity in all forms of the proteasomes tested, however, in the case of REGα/β there is an additional increase in cleavage after basic, acidic, polar and aromatic amino acids (Fig. 1C). A more narrow proteasome activation profile is observed in the presence of REGγ (Fig. 1B). The selective activation for cleavage after basic amino acids by the REGγ complexed proteasome was expected from previously published results [10], however a dramatic increase in cleavage after tryptophan residues was also observed (Fig. 1C).

Extended substrate specificity of the proteasome and its complexes is also revealed from the activity profiles. The P2-substrate specificity profile is similar for all complexes, however, the profiles from the P3 and P4 positions show striking differences between the proteasome complexes; broad for REGα/β and biased towards basic amino acids for the REGγ complex. The preference for basic amino acids in the primary and extended cleavage sites by the REGγ complex may reflect its localization to the nucleus [21] and its potential role in degrading nuclear proteins that have a higher composition of basic amino acids [22]. An additional intriguing difference between the two

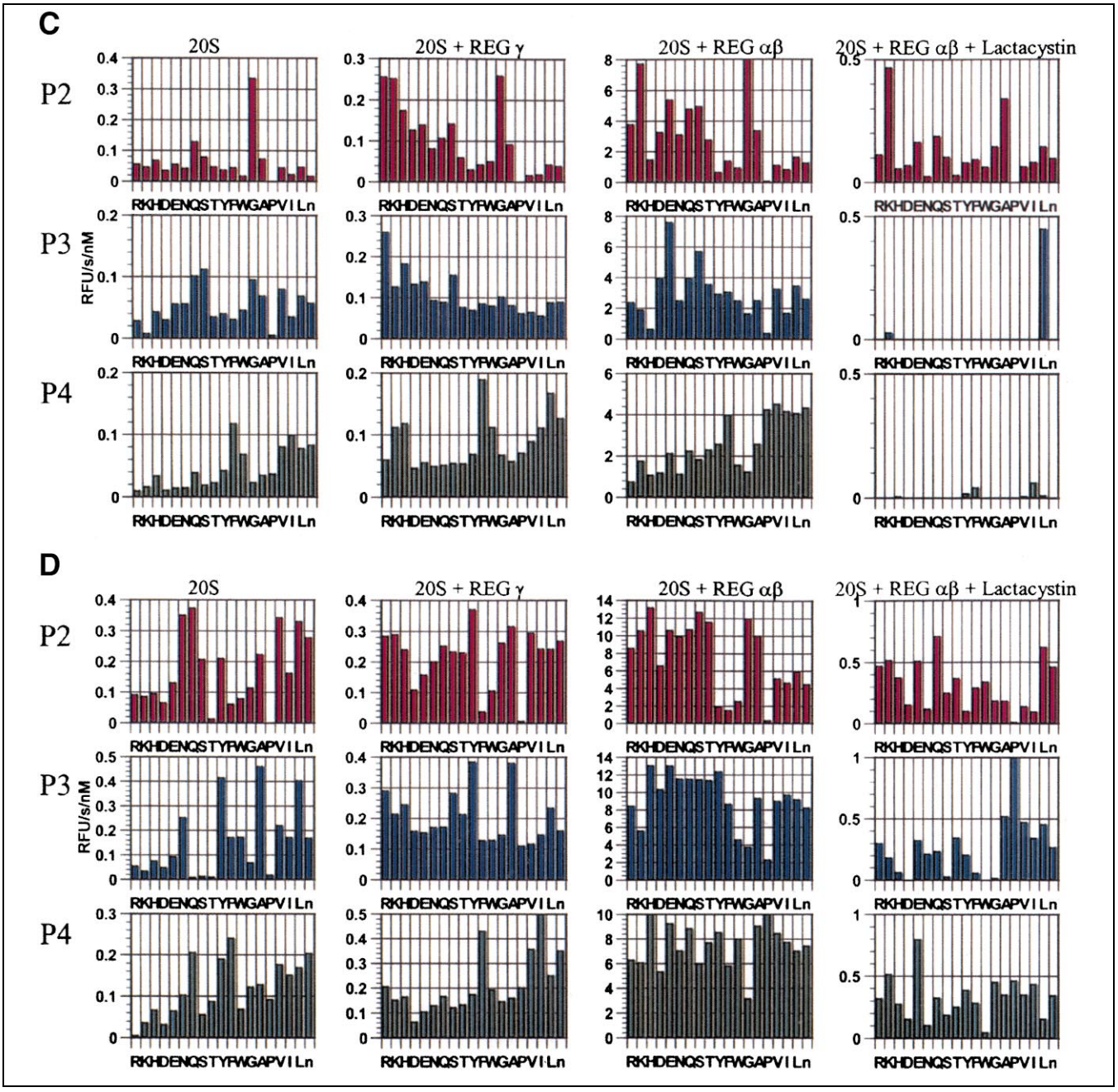

Fig. 3 (*Continued*).

complexes is seen in the selectivity for tryptophan. While the REGα/β complex disfavors tryptophans in the primary and extended sites, the REGγ complex favors tryptophan in these positions. Another obvious proteasomal sequence preference uncovered from these profiles is the presence or absence of proline at the primary and extended sites. Proline at the P1 and P2 positions is not tolerated for all forms of the proteasome tested. Proline is accepted at the P3 position and is the most favored amino acid in the P4 position for the two REG–proteasome complexes. Proline is postulated to be an important factor in the efficient production of class I epitopes through the prevention of proteasomal cleavage within the epitope [23]. With the majority of proteasomal products yielding sequence lengths smaller than the 8–9 amino acids, as required for class I presentation [24], protection of epitopes from internal cleavage is an important issue to address.

A comprehensive and exhaustive view of all interdependencies for the P4 to P1 diversity space was assessed employing the two-position fixed library (Fig. 2). The resulting activity profiles mirror those seen in the one-position fixed library: the activity of the 20S proteasome clusters in the hydrophobic regions of the profiles; the activity of the REGα/β–proteasome complex broadens at all substrate

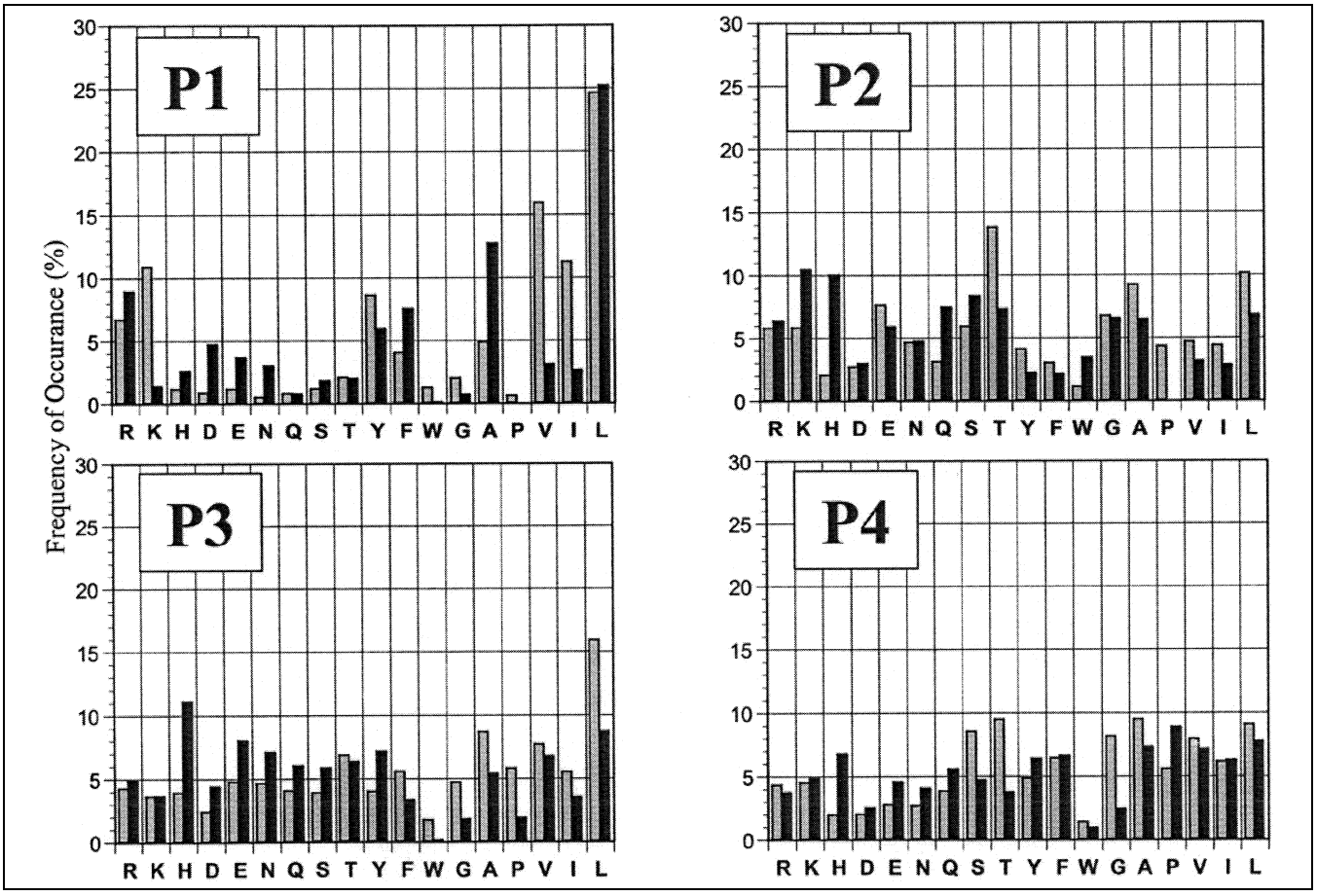

Fig. 4. Histogram relating the frequency of amino acids at the C-termini of 5640 MHC class I epitopes (gray bars) from the MHCPEP database [30] to the activity of the 20S proteasome-PAα/β complex in the one-position fixed library (black bars).

positions; the activity of the REGγ–proteasome complex also broadens but clusters in the basic region of the profiles.

While each of the three active β-subunits displays substrate specificity determinants that distinguish it from the others, the β1 (or PGPH)-subunit, with preference for cleavage after acidic amino acids, displays the most restrictive extended substrate specificity (Figs. 2 and 3A). In the presence of REGα/β or REGγ the β1-subunit displays a preference for aromatic or large aliphatic amino acids at the P2-position, proline, alanine or large aliphatic amino acids at the P3-position and broad selectivity at the P4-position (Fig. 3A).

The β2 (or trypsin-like)-subunit displays the greatest difference between the REGα/β and REGγ proteasome complexes (Figs. 2 and 3B). In the presence of REGγ, the β2-subunit's preference for cleavage after basic amino acids expands to the extended binding sites, with a striking preference for basic amino acids in the P2 and P3 positions. In the presence of REGα/β the β2-subunit's preference for basic amino acids at P3 is retained; however, the bias for basic amino acids in P2 and P4 is significantly diminished in comparison to REGγ. Further differences between the β2-subunit of the REGα/β and REGγ proteasome complexes are manifested in what amino acids are not acceptable at the extended positions. REG-associated proteasomes do not accept proline, aspartic acid or tryptophan at P2, and tryptophan is disfavored at the P3 and P4 sites for the β2-subunit. This is in stark contrast to the extended specificity of the β2-subunit in the REG γ-complexed proteasome that has significant, if not preferential, activity for tryptophan in the P3 and P4 positions of the substrate sequence. Unlike the preference for proline in the P3 position of the β1-subunit, the β2-subunit disfavors proline in this position. Moreover, aspartic acid is disfavored at each of the extended sites of the β2-subunit for all forms of the 20S proteasome tested.

Cleavage after the aromatic amino acids is increased for REGγ-proteasomes while remaining approximately the same or somewhat lower for cleavage after aliphatic amino acids (Fig. 1). In contrast, the REGα/β-proteasome shows increased cleavage after aliphatic amino acids and the aromatic amino acids, tyrosine and phenylalanine, but not tryptophan. The extended substrate specificity profile has similar features for all forms of the 20S proteasome when P1 is held constant as phenylalanine (Figs. 2 and 3C). These features include preference of glycine and absence of proline at P2 and preference for phenylalanine at P4.

Table 1
Aldehyde inhibitor kinetics (20S+α/β)

Substrate	Ac-EPFD-al K_i (nM)	Ac-PRQR-al K_i (nM)	Ac-PEGF-al K_i (nM)	Ac-HHSL-al K_i (nM)	Z-LLL-al K_i (nM)
Ac-nRnR-ACC	NI[a]	210 ± 30	NI	NI	1900 ± 570
Ac-EPFD-ACC	40 ± 4	NI	NI	NI	860 ± 170
Ac-EPFE-ACC	32 ± 11	NI	NI	11 800 ± 3900	49 ± 15
Ac-EPFL-ACC	45 ± 10	NI	NI	6730 ± 2900	400 ± 60
Ac-PEGF-ACC	NI	NI	38 700 ± 4700	840 ± 130	1.0 ± 0.2
Ac-HHSL-ACC	NI	NI	31 400 ± 5200	720 ± 90	0.7 ± 0.1
Ac-VRGW-ACC	NI	NI	NI	8462 ± 5200	2.4 ± 1.3

[a] NI = no inhibition (less than 20% inhibition observed at 50 μM inhibitor).

One notable difference with the REGγ complex is an increase in basic amino acids at the extended sites.

Substrate preferences in the extended sites for cleavage after P1 aliphatic amino acids are extremely broad for all forms of the proteasome. The P2 site displays the most selectivity with a lack of large bulky amino acids at this position, in addition to the pervasive absence of proline (Figs. 2 and 3C). The acceptability of most amino acids in the extended sites may account for the large rates of cleavage after aliphatic amino acids seen in the libraries. A possible reason for the seemingly broad sequence-independent activity is that several subunits cleave after aliphatic amino acids, a property that has been qualitatively demonstrated with active site mutants of the yeast 20S proteasome [15]. Screening the substrate library with the REGα/β complex in the presence of the β5-specific proteasome inhibitor lactacystin [25], resulted in abolition of most of the cleavage after hydrophobic and aliphatic amino acids (Fig. 2D). Examination clearly shows that the extended substrate profile for P1-Leu now approximates that of P1-Asp, indicating that both the β1- and β5-subunits cleave after aliphatic amino acids. This is consistent with the observation by Cardozo et al. that a proline residue at P3 directs substrates to the BrAAP activity of the proteasome [26].

Several studies suggest that catalytic activation of the 20S proteasome by 11S activators is achieved by opening a pore through the α-rings. The recent structure of the yeast 20S proteasome and the REGα homolog from *Trypanosoma brucei* (PA26) shows that upon complex formation, conformational changes occurring in the α-subunits result in a dramatic increase in pore size [27]. While the substrate profiles generally support this model, the model does not completely account for the selective changes in primary and extended substrate specificity observed in the results presented here. One must propose that binding of REGs to the proteasome results in both increased substrate access to the central proteolytic chamber and altered conformations in the substrate binding pockets of the catalytically active β-subunits.

Subunit-specific substrate sequences often translate into selective inhibitors, but not as a rule. By examining the substrate preferences observed for the β1-, β2-, and β5-subunits, we designed four peptide aldehyde inhibitors. C-terminal aldehydes are capable of forming a stable hemi-acetal with the hydroxyl of the nucleophilic N-terminal threonine residue. As predicted, the aldehyde inhibitor designed against the β1-subunit Ac-Glu-Pro-Phe-Asp-H selectively inhibited the cleavage of substrates designed to monitor the β1-subunit activity, all with a similar K_i of approximately 40 nM (Table 1). These data further demonstrate that the extended substrate specificity is an important determinant in the partitioning of tetrapeptides with aliphatic amino acids at P1 to specific active sites. While both Ac-Glu-Pro-Phe-Leu-acc and Ac-His-His-Ser-Leu-acc contain leucine at P1, only the former substrate with the β1-like extended sequence is inhibited by Ac-Glu-Pro-Phe-Asp-H. Even at a concentration of 50 μM this peptide aldehyde inhibited less than 20% of the hydrolysis of substrate designed to monitor the other subunits. The peptide aldehyde inhibitor designed against the β2-subunit, Ac-Pro-Arg-Gln-Arg-H, inhibits only the substrate used to monitor β2 activity, Ac-Nle-Arg-Nle-Arg-acc with a K_i of 210 nM (Table 1). The aldehyde inhibitors designed against the optimal substrate specificity of the β5-subunit, Ac-Pro-Glu-Gly-Phe-H and Ac-His-His-Ser-Leu-H were not very potent inhibitors, with K_is of approximately 30 μM and 800 nM, respectively (Table 1). This is in contrast to the very tight inhibition ($K_i \sim 1$ nM) observed with carbobenzoxy-Leu-Leu-Leu-H (MG-132). This result is consistent with the observation by Dick et al. [28] that the β5-subunit is prone to substrate inhibition by a substrate that contains large bulky P2-amino acid, whereas, substrate inhibition is not observed with substrates that contain small or polar amino acids in the P2-position (data not shown). Caution must be exercized when drawing conclusions about the substrate specificity of an enzyme through the use of inhibitors since the same features that produce an efficiently hydrolyzed substrate will not necessarily produce a potent inhibitor.

One of the initial steps in antigen processing is the degradation of a protein by the proteasome. The generated peptide fragments are then transported to the endoplasmic reticulum by the transporter associated with antigen processing (TAP) where they are loaded onto MHC class I molecules for surface display. Peptides with C-terminal hydrophobic and charged amino acids are preferences shared by the proteasome, TAP [29] and MHC class I

molecules. The power of the profiling method presented here is that all possible non-prime tetrapeptide sequences are simultaneously and continuously monitored. Because the proteasome functions in generating the C-termini of MHC class I epitopes, knowledge of not only the optimal cleavage sites but also the sub-optimal and disfavored cleavage sites should permit construction of reliable algorithms to determine what degradation sequences are likely to be generated from a protein sequence. Indeed, even though proteasome processing is an early step in antigen presentation, a correlation exists between the results presented here and amino acid sequences found in the C-terminal portions of MHC class I ligands (Fig. 4) [30,31]. In practical terms, analysis of the proteasome substrate specificity data presented here could allow for the design of more immunogenic antigens in vaccines, namely, epitopes that not only contain optimal cleavage sites but also are resistant to internal degradation [32]. Furthermore, the development of the function-based proteomics tools described in this manuscript allows for monitoring of the precise changes in substrate specificity of proteasomes from various tissues, cells or even other organisms [33,34]. This is of considerable interest since the β1-, β2-, and β5-subunits can be replaced by the IFNγ-induced subunits, lmp2, MECL1, and lmp7. While these IFNγ-induced proteasomal subunits have been postulated to play a role in the processing of certain antigens, their role is still somewhat controversial [16,35,36]. The proteomics tools presented here may not only aid in dissecting the function of specific proteasome populations, but may also aid in validating them as targets for therapeutic intervention.

3. Significance

The proteasome plays a crucial function in maintaining the fidelity of an organism through the degradation of abnormal and transient proteins and through the production of the epitopes for MHC class I presentation. Exhaustive and complex configurations of fluorogenic substrates have proven to be useful in defining the N-terminal primary and extended substrate specificity of the human 20S proteasome in the presence of 11S proteasome activators (REGα/β and REGγ). The resultant profiles enable the characterization of global proteasome activity and the dissection of the individual proteasome subunit activities. Traditionally, the cleavage preference for the amino acid N-terminal to the scissile bond (P1) of the substrate has been relied upon to define the activity of the proteasome subunits. However, the profiling data presented here suggests that the extended specificity is a critical feature of substrate hydrolysis. The wealth of information generated enables the design of more specific substrates and inhibitors for targeting proteasome complexes and defines the pool of peptides generated from the proteasome for MHC class I presentation.

4. Materials and methods

4.1. Enzyme and REG preparation

20S proteasome, REGα/β and REGγ preparation: human 20S proteasome was prepared from outdated blood by previously described methods [37]. 11S activator caps, REGα/β and REGγ, were prepared as previously described [10].

4.2. Substrate and inhibitor synthesis

Substrates and substrate libraries were synthesized using methods described previously [20]. Aldehyde inhibitor Ac-Pro-Arg-Gln-Arg-H was prepared using semicarbazone methodology [38]. Inhibitors, Ac-Glu-Pro-Phe-Asp-H, Ac-Pro-Glu-Gly-Phe-H, and Ac-His-His-Ser-Leu-H, were prepared using Weinreb amide methodology [39].

4.3. Substrate library kinetic screening

Purified 20S proteasome (25 nM for assays without REG, 1 nM for assays with 70 nM REGα/β, and 10 nM for assays with 700 nM REGγ) in a buffer containing 60 mM Tris pH 7.5, 10 mM KCl, and 5 mM $MgCl_2$, was pre-incubated at 37°C for 10 min to allow for association of 20S with REG caps. The pre-incubated proteasome was then added to the substrate libraries and fluorescence was monitored for 30 min at λ_{ex} of 380 nm and λ_{em} of 450 nm in a Molecular Devices Gemini XS microtiter plate reader. Inhibitors were assayed in the same manner with pre-incubation of 1 nM 20S proteasome, 70 nM REGα/β and $4\times K_i$ concentration of inhibitor.

4.4. Inhibitor analysis

Multiple concentrations of inhibitors (0.0001 mM to 50 μM) were pre-incubated at 37°C for 10 min with 20S proteasome and REGα/β. The inhibited proteasome was then added to 5 μM of purified substrate and the K_i was determined using the Henderson equation for tight-binding inhibitors (Table 1).

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References

[1] O. Coux, K. Tanaka, A.L. Goldberg, Structure and functions of the 20S and 26S proteasomes, Annu. Rev. Biochem. 65 (1996) 801–847.
[2] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev. Biochem. 67 (1998) 425–479.
[3] S. Wilk, M. Orlowski, Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex, J. Neurochem. 40 (1983) 842–849.
[4] M. Groll, L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H.D. Bartunik, R. Huber, Structure of 20S proteasome from yeast at 2.4 Å resolution, Nature 386 (1997) 463–471.

[5] A.K. Nussbaum, T.P. Dick, W. Keilholz, M. Schirle, S. Stevanovic, K. Dietz, W. Heinemeyer, M. Groll, D.H. Wolf, R. Huber, H.G. Rammensee, H. Schild, Cleavage motifs of the yeast 20S proteasome β subunits deduced from digests of enolase 1, Proc. Natl. Acad. Sci. USA 95 (1998) 12504–12509.

[6] M. Groll, W. Heinemeyer, S. Jager, T. Ullrich, M. Bochtler, D.H. Wolf, R. Huber, The catalytic sites of 20S proteasomes and their role in subunit maturation: a mutational and crystallographic study, Proc. Natl. Acad. Sci. USA 96 (1999) 10976–10983.

[7] M. Orlowski, S. Wilk, Catalytic activities of the 20 S proteasome, a multicatalytic proteinase complex, Arch. Biochem. Biophys. 383 (2000) 1–16.

[8] D. Voges, P. Zwickl, W. Baumeister, The 26S proteasome: a molecular machine designed for controlled proteolysis, Annu. Rev. Biochem. 68 (1999) 1015–1068.

[9] M. Rechsteiner, C. Realini, V. Ustrell, The proteasome activator 11 S REG (PA28) and class I antigen presentation, Biochem. J. 345 (2000) 1–15.

[10] C. Realini, C.C. Jensen, Z. Zhang, S.C. Johnston, J.R. Knowlton, C.P. Hill, M. Rechsteiner, Characterization of recombinant REGα, REGβ, and REGγ proteasome activators, J. Biol. Chem. 272 (1997) 25483–25492.

[11] K.B. Hendil, S. Khan, K. Tanaka, Simultaneous binding of PA28 and PA700 activators to 20 S proteasomes, Biochem. J. 332 (1998) 749–754.

[12] T. Preckel, W.P. Fung-Leung, Z. Cai, A. Vitiello, L. Salter-Cid, O. Winqvist, T.G. Wolfe, M. Von Herrath, A. Angulo, P. Ghazal, J.D. Lee, A.M. Fourie, Y. Wu, J. Pang, K. Ngo, P.A. Peterson, K. Fruh, Y. Yang, Impaired immunoproteasome assembly and immune responses in PA28−/− mice, Science 286 (1999) 2162–2165.

[13] T.P. Dick, T. Ruppert, M. Groettrup, P.M. Kloetzel, L. Kuehn, U.H. Koszinowski, S. Stevanovic, H. Schild, H.G. Rammensee, Coordinated dual cleavages induced by the proteasome regulator PA28 lead to dominant MHC ligands, Cell 86 (1996) 253–262.

[14] M. Groettrup, A. Soza, M. Eggers, L. Kuehn, T.P. Dick, H. Schild, H.G. Rammensee, U.H. Koszinowski, P.M. Kloetzel, A role for the proteasome regulator PA28α in antigen presentation, Nature 381 (1996) 166–168.

[15] T.P. Dick, A.K. Nussbaum, M. Deeg, W. Heinemeyer, M. Groll, M. Schirle, W. Keilholz, S. Stevanovic, D.H. Wolf, R. Huber, H.G. Rammensee, H. Schild, Contribution of proteasomal β-subunits to the cleavage of peptide substrates analyzed with yeast mutants, J. Biol. Chem. 273 (1998) 25637–25646.

[16] C. Cardozo, R.A. Kohanski, Altered properties of the branched chain amino acid-preferring activity contribute to increased cleavages after branched chain residues by the 'immunoproteasome', J. Biol. Chem. 273 (1998) 16764–16770.

[17] N.P. Emmerich, A.K. Nussbaum, S. Stevanovic, M. Priemer, R.E. Toes, H.G. Rammensee, H. Schild, The human 26 S and 20 S proteasomes generate overlapping but different sets of peptide fragments from a model protein substrate, J. Biol. Chem. 275 (2000) 21140–21148.

[18] T. Nazif, M. Bogyo, Global analysis of proteasomal substrate specificity using positional-scanning libraries of covalent inhibitors, Proc. Natl. Acad. Sci. USA 98 (2001) 2967–2972.

[19] J. Li, X. Gao, J. Ortega, T. Nazif, L. Joss, M. Bogyo, A. Steven, M. Rechsteiner, Proteasome activation by 11S REG (PA28) homologs: lysine 188 substitutions convert the pattern of proteasome activation by REGγ to that of REGs α and β, EMBO J. 20 (2001) 3359–3369.

[20] J.L. Harris, B.J. Backes, F. Leonetti, S. Mahrus, J.A. Ellman, C.S. Craik, Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries, Proc. Natl. Acad. Sci. USA 97 (2000) 7754–7759.

[21] P. Brooks, G. Fuertes, R.Z. Murray, S. Bose, E. Knecht, M.C. Rechsteiner, K.B. Hendil, K. Tanaka, J. Dyson, J. Rivett, Subcellular localization of proteasomes and their regulatory complexes in mammalian cells, Biochem. J. 346 (2000) 155–161.

[22] A. Reinhardt, T. Hubbard, Using neural networks for prediction of the subcellular location of proteins, Nucleic Acids Res. 26 (1998) 2230–2236.

[23] N. Shimbara, K. Ogawa, Y. Hidaka, H. Nakajima, N. Yamasaki, S. Niwa, N. Tanahashi, K. Tanaka, Contribution of proline residue for efficient production of MHC class I ligands by proteasomes, J. Biol. Chem. 273 (1998) 23062–23071.

[24] A.F. Kisselev, T.N. Akopian, K.M. Woo, A.L. Goldberg, The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation, J. Biol. Chem. 274 (1999) 3363–3371.

[25] G. Fenteany, R.F. Standaert, W.S. Lane, S. Choi, E.J. Corey, S.L. Schreiber, Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin, Science 268 (1995) 726–731.

[26] C. Cardozo, A. Vinitsky, C. Michaud, M. Orlowski, Evidence that the nature of amino acid residues in the P3 position directs substrates to distinct catalytic sites of the pituitary multicatalytic proteinase complex (proteasome), Biochemistry 33 (1994) 6483–6489.

[27] F.G. Whitby, E.I. Masters, L. Kramer, J.R. Knowlton, Y. Yao, C.C. Wang, C.P. Hill, Structural basis for the activation of 20S proteasomes by 11S regulators, Nature 408 (2000) 115–120.

[28] R.L. Stein, F. Melandri, L. Dick, Kinetic characterization of the chymotryptic activity of the 20S proteasome, Biochemistry 35 (1996) 3899–3908.

[29] S. Uebel, W. Kraas, S. Kienle, K.H. Wiesmuller, G. Jung, R. Tampe, Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries, Proc. Natl. Acad. Sci. USA 94 (1997) 8976–8981.

[30] V. Brusic, G. Rudy, A.P. Kyne, L.C. Harrison, MHCPEP, a database of MHC-binding peptides: update 1997, Nucleic Acid Res. 26 (1998) 368–371.

[31] H.G. Rammensee, T. Friede, S. Stevanoviic, MHC ligands and peptide motifs: first listing, Immunogenetics 41 (1995) 178–228.

[32] C.J. Luckey, G.M. King, J.A. Marto, S. Venketeswaran, B.F. Maier, V.L. Crotzer, T.A. Colella, J. Shabanowitz, D.F. Hunt, V.H. Engelhard, Proteasomes can either generate or destroy MHC class I epitopes: evidence for nonproteasomal epitope generation in the cytosol, J. Immunol. 161 (1998) 112–121.

[33] T. Frisan, V. Levitsky, A. Polack, M.G. Masucci, Phenotype-dependent differences in proteasome subunit composition and cleavage specificity in B cell lines, J. Immunol. 160 (1998) 3281–3289.

[34] M.C. Mutomba, W.Y. To, W.C. Hyun, C.C. Wang, Inhibition of proteasome activity blocks cell cycle progression at specific phase boundaries in African trypanosomes, Mol. Biochem. Parasitol. 90 (1997) 491–504.

[35] F. Momburg, V. Ortiz-Navarrete, J. Neefjes, E. Goulmy, Y. van de Wal, H. Spits, S.J. Powis, G.W. Butcher, J.C. Howard, P. Walden, G.J. Hammerling, Proteasome subunits encoded by the major histocompatibility complex are not essential for antigen presentation, Nature 360 (1992) 174–177.

[36] D. Arnold, J. Driscoll, M. Androlewicz, E. Hughes, P. Cresswell, T. Spies, Proteasome subunits encoded in the MHC are not generally required for the processing of peptides bound by MHC class I molecules, Nature 360 (1992) 171–174.

[37] W. Dubiel, G. Pratt, K. Ferrell, M. Rechsteiner, Purification of an 11 S regulator of the multicatalytic protease, J. Biol. Chem. 267 (1992) 22369–22377.

[38] R. Dagnino, T.R. Webb, Improved synthesis of arginine peptide aldehydes, Tetrahedron Lett. 35 (1994) 2125–2128.

[39] J.-A. Fehrentz, B. Castro, An efficient synthesis of optically active α-(*t*-butoxycarbonylamino)-aldehydes from α-amino acids, Synthesis 1983 (1983) 676–678.