targeting sequence to be functional. Our studies suggest an additional level of interaction between Sos and Sevenless that is independent of Drk, and may involve the PH domain contained within the NH_2-terminus. In agreement with our results, *Drosophila* NCat and a construct including Cat and the PH domain activate Ras in mammalian cell lines whereas Cat and CatC are inactive (*18*). Furthermore, our studies are consistent with, and provide further evidence for, an inhibitory role for the COOH-terminus proposed for mammalian Sos (*21*). Our results are consistent with a model in which a signal transfer particle (*25*) forms apically in the eye disc within which proteins interact with each other through multiple domains, as is seen in transcription complexes. In this model, upon activation of the receptor, the inhibition of the catalytic domain of Sos by its COOH-terminus might be alleviated by Drk through its bipartite binding to Sos and to the tyrosine-phosphorylated Sevenless protein.

REFERENCES AND NOTES

1. S. L. Zipursky and G. M. Rubin, *Annu. Rev. Neurosci.* **17**, 373 (1994) and references cited therein; P. Sternberg, *Annu. Rev. Genet.* **27**, 497 (1993); N. Perrimon, *Curr. Op. Cell Biol.* **6**, 260 (1994).
2. R. D. Rogge, C. A. Karlovich, U. Banerjee, *Cell* **64**, 39 (1991).
3. M. A. Simon, D. D. Bowtell, G. S. Dodson, T. R. Laverty, G. M. Rubin, *ibid.* **67**, 701 (1991).
4. L. Bonfini, C. A. Karlovich, C. Dasgupta, U. Banerjee, *Science* **255**, 603 (1992).
5. M. A. Simon, G. S. Dodson, G. M. Rubin, *Cell* **73**, 169 (1993).
6. J. P. Olivier *et al.*, *ibid.*, p. 179.
7. S. G. Clark, M. J. Stern, H. R. Horvitz, *Nature* **356**, 340 (1992).
8. E. J. Lowenstein *et al.*, *Cell* **70**, 431 (1992).
9. T. Pawson and J. Schlessinger, *Curr. Biol.* **3**, 434 (1993) and references cited therein.
10. S. Jones, M.-L. Vignais, J. R. Broach, *Mol. Cell. Biol.* **11**, 2641 (1991); D. Broek *et al.*, *Cell* **48**, 789 (1987).
11. K. Touhara, J. Inglese, J. A. Pitcher, G. Shaw, R. J. Lefkowitz, *J. Biol. Chem.* **269**, 10217 (1994); J. E. Harlan, P. J. Hajduk, H. S. Yoon, S. W. Fesik, *Nature* **371**, 168 (1994).
12. The nucleotide positions for the constructs are as follows: NCat: 1 to 3721; Cat: 2584 to 3721; CatC: 2584 to 5533; C: 3465 to 5533; and PH: 1702 to 2178. Details of the construction of each clone are available on request. For each deletion construct, a minimum of 10 independent transformant lines were isolated. Qualitatively, the transformant lines from a given construct had similar effects. For simplicity, only the results from the transformants with the strongest genetic effects are shown.
13. K. Basler, B. Christen, E. Hafen, *Cell* **64**, 1069 (1991).
14. Protein immunoblot analysis demonstrated that the amount of expression of Sos in Sos^{JC2} is not different from that seen in wild-type flies, suggesting that Sos^{JC2} encodes an overactive rather than an overexpressed product.
15. R. Rogge, R. Cagan, A. Majumdar, T. Dulaney, U. Banerjee, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5271 (1992).
16. C. A. Karlovich, L. Bonfini, and U. Banerjee, unpublished observations.
17. Because the sE elements in the constructs are followed by an hsp70 promoter, excessive amounts of Sos fragments can be obtained if transformant flies are heat shocked. However, the resulting phenotypes then become independent of the receptor. We have therefore not used these expression conditions for the results reported here. Instead, in these experiments, the sE elements utilize the weak basal activity of the hsp70 promoter.
18. L. McCollam *et al.*, in preparation.
19. The GST-PH fusion construct was made by subcloning a Pvu II *Sos* complementary DNA fragment encoding amino acid positions 453 to 681 into the Sma I site of the pGEX3X vector (Pharmacia). The preparation of fusion protein and fly lysates was as described (*6*) except that the lysates were clarified by centrifugation at 50,000*g* for 2.5 hours. The binding assay was done essentially as described (*6*).
20. U. Banerjee, P. J. Renfranz, D. R. Hinton, B. A. Rabin, S. Benzer, *Cell* **51**, 151 (1987).
21. A. Aronheim *et al.*, *ibid.* **78**, 949 (1994).
22. The rabbit polyclonal antibody to Sos, provided by S. L. Zipursky, was generated against the COOH-terminal 416 amino acids of the Sos protein.
23. H. Krämer, R. L. Cagan, S. L. Zipursky, *Nature* **352**, 207 (1991).
24. L. Buday and J. Downward, *Cell* **73**, 611 (1993).
25. A. Ullrich and J. Schlessinger, *ibid.* **61**, 203 (1990).
26. N. Franceschini and K. Kirshfeld, *Kybernetik* **9**, 159 (1971).

27. We thank G. Rubin, M. Simon, E. Hafen, and B. Dickson for fly stocks and helpful discussions, L. Zipursky for the antibody to Sos, and J. Colicelli, D. Broek, and O. Witte for clones and helpful advice. Supported by a NIH grant (RO1EY08152-06), a Faculty Research Award (FRA426) from American Cancer Society, a McKnight Award and a Sloan Award to U.B., and a genetics training grant (USPHS NRSA GM-07104) and a biotechnology training grant (USPHS NRSA GM-08375) for C.A.K.

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Proteasome from *Thermoplasma acidophilum*: A Threonine Protease

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The catalytic mechanism of the 20*S* proteasome from the archaebacterium *Thermoplasma acidophilum* has been analyzed by site-directed mutagenesis of the β subunit and by inhibitor studies. Deletion of the amino-terminal threonine or its mutation to alanine led to inactivation of the enzyme. Mutation of the residue to serine led to a fully active enzyme, which was over ten times more sensitive to the serine protease inhibitor 3,4-dichloroisocoumarin. In combination with the crystal structure of a proteasome-inhibitor complex, the data show that the nucleophilic attack is mediated by the amino-terminal threonine of processed β subunits. The conservation pattern of this residue in eukaryotic sequences suggests that at least three of the seven eukaryotic β-type subunit branches should be proteolytically inactive.

The 26*S* proteasome (*1*) is the central protease of the ubiquitin-dependent pathway of protein degradation (*2*). The catalytic core of the complex is formed by the 20*S* proteasome, a barrel-shaped particle of four stacked, seven-membered rings (*3*). The rings are formed by 14 different but related subunits, which fall into two families (*4*), with the α-type subunits forming the outer rings and the β-type subunits forming the inner rings of the complex (*5*). Some (possibly all) β-type subunits contain a prosequence, which is cleaved autocatalytically during the assembly of the complex (*6*, *7*). The 20*S* proteasome has also been detected in the archaebacterium *Thermoplasma acidophilum* where it is of simpler composition, being formed by only two related subunits, α and β, which have given their names to the eukaryotic subunit families (*8*). The structure of the *Thermoplasma* proteasome has been determined to 3.4 Å by x-ray crystallography, both unliganded and in complex with *N*-acetyl-Leu-Leu-norleucinal (*9*). Although proteasomes have not yet been described in other prokaryotes, genomic sequencing has revealed the existence of proteins in eubacteria that are significantly related to eukaryotic β-type subunits (*10*). One of these proteins, from the nocardioform actinomycete *Rhodococcus* sp., is part of a complex, high-molecular weight protease with a specificity similar to that of the *Thermoplasma* proteasome (*11*).

The 20*S* proteasome of eukaryotes was initially characterized as a multicatalytic protease with chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase activities (*12*) and, on the basis of inhibitor studies, has more recently been proposed to contain up to five different proteolytic components (*13*). The lack of sequence similarity to other proteases (*14*) and the inconclusive nature of inhibitor studies (*13*, *15*) have prevented the assignment of the proteasome to one of the known protease families, but its sensitivity toward 3,4-dichloroisocoumarin and toward peptide aldehydes has indicated that it may be an unusual serine or cysteine protease.

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The proteasome of *Thermoplasma* has only a single, chymotrypsin-like activity, although experiments with denatured substrates show that the enzyme hydrolyzes virtually any peptide bond (*16*). Assembly of the complex is guided by the α subunits, which can also form rings in the absence of β subunits but remain entirely inactive. The β subunits are monomeric in the absence of α subunits, cannot catalyze the cleavage of the pro-peptide, and remain inactive; but if the pro-peptide is removed by genetic deletion, the β subunits form disordered aggregates and regain low but significant proteolytic activity (*7*). This result supported the assignment of the active site to the β subunits (*17*). On the basis of the hypothesis that the proteasome is an unusual kind of serine or cysteine protease, all serine and histidine residues in the *Thermoplasma* β subunit have been mutated individually without significantly impairing proteolytic activity (the subunit contains no cysteine) (*18*). In combination with previous studies (*14*, *15*), this result showed that the proteasome does not belong to any of the four known classes of proteases.

In general, active site residues of enzymes can be identified by the conservation patterns of aligned sequences. This is because related proteins retain the same basic three-dimensional fold and functionally important residues, long after evolutionary divergence may have led to a randomization of the amino acid sequence at nonessential positions (*19*). Proteasome sequences are significantly related by statistical criteria (*10*), indicating that all proteasome subunits belong to one protein family and will therefore assume the same basic fold. This extrapolation from sequence conservation to structural similarity is borne out by the crystal structures of the *Thermoplasma* α and β subunits, which are similar (root-mean-square deviation 1.33 Å) despite only 26% sequence identity (*9*). The structural conservation implies that those subunits that have retained proteolytic activity should all function by the same catalytic mechanism because evolution of a different mechanism would have caused large changes in the active-site geometry and thus also in the three-dimensional scaffold. This implication does not contradict the observation that eukaryotic proteasomes have multiple specificities because substrate specificity appears not to be determined by the catalytic mechanism but by the structure of the substrate binding pocket.

An alignment of β-type sequences from *Thermoplasma* and eukaryotes (Fig. 1) shows that, apart from glycines, the only completely conserved residue is Asp^{51}, which is also conserved in all α-type subunits. This

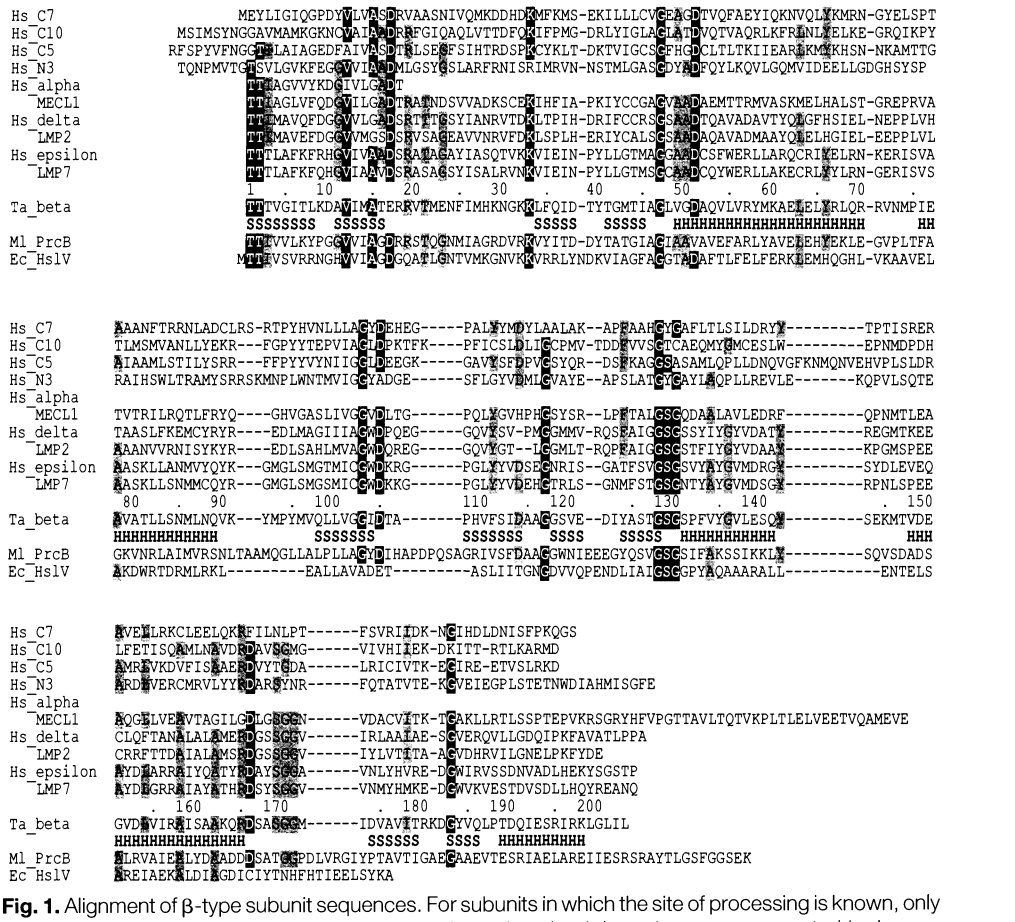

Fig. 1. Alignment of β-type subunit sequences. For subunits in which the site of processing is known, only the mature sequences are shown. The seven eukaryotic subunit branches are represented by human sequences (Hs). The constitutively expressed subunits δ and ε can be replaced after interferon γ stimulation by the closely related MHC-encoded subunits LMP2 and LMP7 (*22*). A similar relationship connects the closely related α and MECL1 subunits (*23*); for α, only the NH_2-terminal sequence is known. The numbering and secondary structure (H, α-helix; S, β strand) refer to the *Thermoplasma* sequence (Ta). For *Mycobacterium leprae* (Ml) PrcB, the presumed mature form is shown (*11*). The HslV-related proteins are represented by the *Escherichia coli* sequence (Ec). Residues highlighted in reverse type are conserved in at least eight of ten branches of β-type subunits, as defined by the dendrogram in Fig. 2; Ml PrcB and Ec HslV are considered different branches. Shaded residues are conserved in five of ten branches. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

residue has been mutated to asparagine in *Thermoplasma* β with the surprising result that the proteolytic activity of the mutant proteasome was three times greater (*18*). Because Asp^{51} is not required for catalytic activity and no other potential nucleophile is entirely conserved, we conclude that not all eukaryotic β-type subunits are proteolytically active.

Potential nucleophiles that are highly but not entirely conserved are found in two regions of high sequence similarity between eukaryotic and eubacterial β-type sequences (*10*); these regions are (i) the NH_2-terminal 30 residues of mature subunits and (ii) a GlySerGly motif (Fig. 1). The potential nucleophile in region (ii), Ser^{129}, could be mutated without impairment of the proteolytic activity in *Thermoplasma* β (*18*). Of the potential nucleophiles in region (i), the most conspicuous are two threonines present at the NH_2-terminus of several processed eukaryotic subunits as well as in all bacterial subunits. Deletion of the two threonines in *Thermoplasma* β or their mutation to alanine yielded proteasomes that were correctly folded and assembled but that were entirely inactive (*20*). Deletion or mutation of the NH_2-terminal threonine alone was sufficient to yield this phenotype. Of 16 mutations introduced into the β subunit (*18*), partly in highly conserved positions such as Asp^{51}, Ser^{129}, and Ser^{169}, this mutation was the first to result in a loss of proteolytic activity.

These results indicated that the NH_2-terminal threonine contributes to the activity of the proteasome, and we therefore tested whether serine could be substituted at this position. Surprisingly, the $Thr^1 \rightarrow Ser$

Table 1. Kinetic parameters of Suc-LLVY-AMC (succinyl-leucyl-leucyl-valyl-tyrosyl-7-amido-4-methylcoumarin) hydrolysis. Activity was assayed by incubating 0.5 μg of purified proteasomes with ten different concentrations of substrate (0.01 to 0.7 mM) for 15 or 30 min at 60°C (*18*). Values for V_{max} and K_m were determined by direct linear plotting (*24*). For calculation of k_{cat}, we assumed 14 active sites per particle. Values for K_m and k_{cat} are averages of triplicate experiments in which the standard errors were 15 and 10%, respectively. For inactivation kinetics, purified protein (0.02 μM) was first incubated with eight different inhibitor concentrations [3,4-dichloroisocoumarin (DCI) 0.01 to 0.1 mM; *N*-acetyl-leucyl-leucyl-norleucinal, (AcLLnL), 0.001 to 0.05 μM] for 60 min at 20°C, and proteolytic activity was measured with 100 μM Suc-LLVY-AMC. The dissociation constant for the enzyme-inhibitor complex (K_i) and the first-order rate constant for formation of inactive enzyme (k_2) were determined by application of the direct linear plot method (*24*). $K_{obs}/[I]$ is given by k_2/K_i (*25*). Values are the averages of triplicate experiments in which the standard error was about 10%.

Parameter	Inhibitor	Wild type	Thr¹-Ser mutant
K_m (μM)		85	68
K_{cat} (10^{-3} sec^{-1})		30	30
V_{max} (μM sec^{-1} μg^{-1})		22	23
$K_{obs}/[I]$	AcLLnL	117,000	120,000
(M^{-1} sec^{-1})	DCI	166	2,300

mutant was fully active, and its kinetic parameters were essentially indistinguishable from those of the wild-type enzyme (Table 1). Nevertheless, an effect of the mutation could be seen on the sensitivity toward the serine protease inhibitor 3,4-dichloroisocoumarin; in particular, the serine mutant was almost 15 times more sensitive to this compound than the wild type was. From this result we conclude that the side chain hydroxyl group of the NH_2-terminal threonine provides the initial nucleophilic attack in the wild-type enzyme. No effect of the serine mutant could be observed on the sensitivity toward the inhibitor *N*-acetyl-Leu-Leu-norleucinal. In the proteasome crystal structure (*9*), this inhibitor is found in close proximity to the side chain of the NH_2-terminal threonine, providing additional evidence for the role of the threonine hydroxyl group as the attacking nucleophile. The identification of Thr¹ as the active-site nucleophile allowed us to deduce from its conservation pattern (Fig. 1) that members of the C5-, C7-, and C10-type β subunits were unlikely to carry proteolytic activity, whereas all other β subunits, including all bacterial proteins, were potentially active.

As has been discussed (*18*), proteolysis requires a second conserved residue that carries an unprotonated nucleophilic group, frequently with a p*K* in the physiological range. Its function is to strip the proton from the active-site nucleophile, thus initiating the attack, and then to donate the proton back to the leaving NH_2-terminal group of the cleaved substrate. Two possible proton acceptors-donors are highly conserved in the potentially active β subunits: Lys³³ and the amino group of Thr¹. Their involvement in catalysis is supported by the crystal structure of the *Thermoplasma* β subunit (*9*), which shows the Lys³³ side chain in close proximity to Thr¹. We have explored the role of Lys³³ in catalysis by mutating it to alanine and arginine. The mutant proteasomes folded and assembled correctly but remained entirely inactive. This result showed that Lys³³ is essential for functional reasons, rather than structural reasons but did not clarify whether Lys³³ is involved directly in proton transfer or whether it serves to polarize the Thr¹ amino group. Both roles appear possible on the basis of the crystal structure (*9*), but the more favorable p*K* of the Thr¹ amino group makes this the more likely primary acceptor-donor. The geometry of its interaction with the side chain hydroxyl group, either directly or mediated by water, is potentially quite favorable (*9*). The Thr¹ NH_2-terminus is freed by cleavage of the pro-peptide in *Thermoplasma* and in all eukaryotic subunits that contain this residue, with the exception of N3-type subunits where processing occurs eight residues prior to Thr¹. In eubacterial subunits, the NH_2-terminal residue of the mature form has only been determined for a subunit from *Rhodococcus* sp. (*11*), which is closely related in sequence (64% identity) to *Mycobacterium* PrcB (Fig. 1); this subunit also starts with Thr¹.

The involvement of the Thr¹ amino group in the catalytic mechanism of proteasomes is supported by the enzymatic mechanism of penicillin acylase, which was reported after this report was submitted (*21*). In this enzyme, the amino group of an internal serine residue is freed by proteolytic cleavage and forms a single-residue catalytic center with the hydroxyl group of the serine side chain. The interaction between the amino and hydroxyl groups is mediated by a water molecule.

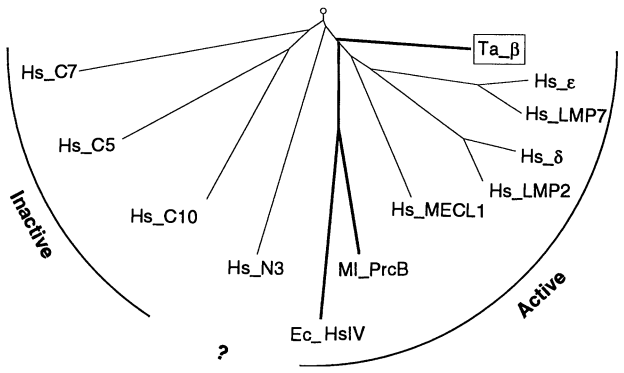

Fig. 2. Dendrogram of β-type subunits. Bacterial branches are shown bold. The dendrogram was calculated by distance-based methods from the alignment in Fig. 1 as described (*10*). The labels "active" and "inactive" refer to the proteolytic activity deduced from the conservation pattern of Thr¹. The question mark for the N3 branch refers to the fact that subunits in this family contain Thr¹ but lack potential proton acceptors-donors (or both) and may therefore also be inactive.

On the basis of the sequence conservation of Thr¹, we have divided the eukaryotic β-type subunits into an inactive and a potentially active group with regard to proteolytic activity. The two groups segregate on different branches of a 20S subunit dendrogram (Fig. 2), with N3-type subunits occupying a middle position. N3-type subunits contain Thr¹ but lack both potential proton acceptors-donors, Lys³³, and the free amino group at Thr¹ and are therefore most likely also proteolytically inactive. This assignment is supported by the conservation pattern of two glycine-rich sequences, $Gly^{128}SerGly$ and $Ser^{169}GlyGly$, that are in direct proximity to the active-site residues (*9*) and are conserved in active subunits but not in N3 or in inactive subunits (Fig. 1). The prediction that three, maybe four, of the eubacterial β-type subunits are proteolytically inactive is entirely compatible with the available data. Thus, LMP2, LMP7, and their constitutive homologs δ and ε, belong to the active group, in concordance with their established role in proteasome activity (*22*). All three eukaryotic β-subunit branches that are predicted to be active contain a constitutive member and a interferon γ inducible homolog; LMP2 is the inducible homolog of δ (*22*), LMP7 of ε (*22*), and MECL1 of α (*23*).

REFERENCES AND NOTES

1. J.-M. Peters, Z. Cejka, J. R. Harris, J. A. Kleinschmidt, W. Baumeister, *J. Mol. Biol.* **234**, 932 (1993); T. Yoshimura *et al.*, *J. Struct. Biol.* **111**, 200 (1993).
2. A. L. Goldberg, *Eur. J. Biochem.* **203**, 9 (1992); M. Rechsteiner, *Cell* **66**, 615 (1991); A. Hershko and A. Cienchanover, *Annu. Rev. Biochem.* **61**, 761 (1992).
3. J. Hase *et al.*, *Biochim. Biophys. Acta* **611**, 205 (1980); W. Baumeister *et al.*, *FEBS Lett.* **241**, 239 (1988); R. Hegerl, G. Pfeifer, G. Pühler, B. Dahlmann, W. Baumeister, *ibid.* **283**, 117 (1991); G. Pühler *et al.*, *EMBO J.* **11**, 1607 (1992).
4. G. Pühler, F. Pitzer, P. Zwickl, W. Baumeister, *Syst. Appl. Microbiol.* **16**, 734 (1994); A. Lupas, A. J. Kos-

ter, W. Baumeister, *Enzyme Protein* **47**, 252 (1993).
5. A. Grziwa, B. Dahlmann, Z. Cejka, U. Santarius, W. Baumeister, *J. Struct. Biol.* **109**, 168 (1992); T. M. Schauer *et al.*, *ibid.* **111**, 135 (1993).
6. K. Früh *et al.*, *J. Biol. Chem.* **267**, 22131 (1992); S. Frentzel, B. Pesold-Hurt, A. Seelig, P.-M. Kloetzel, *J. Mol. Biol.* **236**, 975 (1994).
7. P. Zwickl, J. Kleinz, W. Baumeister, *Nature Struct. Biol.* **1**, 765 (1994).
8. B. Dahlmann *et al.*, *FEBS Lett.* **251**, 125 (1989); P. Zwickl, F. Lottspeich, B. Dahlmann, W. Baumeister, *ibid.* **278**, 217 (1991); P. Zwickl *et al.*, *Biochemistry* **31**, 964 (1992).
9. J. Löwe *et al.*, *Science* **268**, 533 (1995).
10. A. Lupas, P. Zwickl, W. Baumeister, *Trends Biochem. Sci.* **19**, 533 (1994).
11. T. Tamura, I. Nagy, A. Lupas, F. Lottspeich, Z. Cejka, R. De Mot, W. Baumeister, unpublished data.
12. S. Wilk and M. Orlowski, *J. Neurochem.* **35**, 1172 (1980); M. Orlowski and S. Wilk, *Biochem. Biophys. Res. Commun.* **101**, 814 (1981).
13. M. Orlowski, C. Cardozo, C. Michaud, *Biochemistry* **32**, 1563 (1993).
14. G. N. DeMartino *et al.*, *Biochim. Biophys. Acta* **1079**, 29 (1991); T. Tamura *et al.*, *ibid.* **1089**, 95 (1991).
15. W. M. Mason, *Biochem. J.* **265**, 479 (1990); H. Djaballah *et al.*, *Eur. J. Biochem.* **209**, 629 (1992); B. Dahlmann *et al.*, *ibid.* **208**, 789 (1992); C. Cardozo, A. Vinitsky, M. C. Hidalgo, C. Michaud, M. Orlowski, *Biochemistry* **31**, 7373 (1992); P. J. Savory, H. K. Djaballah, H. Angliker, E. Shaw, A. J. Rivett, *Biochem. J.* **296**, 601 (1993); K. L. Rock *et al.*, *Cell* **78**, 761 (1994).
16. T. Wenzel and W. Baumeister, *FEBS Lett.* **326**, 215 (1993); *Nature Struct. Biol.* **2**, 199 (1995).
17. The location of the active site in the β subunits had been proposed previously because the MHC-encoded subunits that alter the enzymatic activity of the proteasome in response to immune stimulation are β-type subunits (*22*), as are all known eubacterial subunits (*10*) and all yeast subunits whose mutation abolishes a specific proteolytic activity [W. Heinemeyer, J. A. Kleinschmidt, J. Saidowsky, C. Escher, D. H. Wolf, *EMBO J.* **10**, 555 (1991); W. Hilt, C. Enenkel, A. Gruhler, T. Singer, D. H. Wolf, *J. Biol. Chem.* **268**, 3479 (1993); W. Heinemeyer, A. Gruhler, V. Möhrle, Y. Mahé, D. H. Wolf, *ibid.*, p. 5115; C. Enenkel *et al.*, *FEBS Lett.* **341**, 193 (1994)].
18. E. Seemüller, A. Lupas, F. Zühl, P. Zwickl, W. Baumeister, *FEBS Lett.* **359**, 173 (1995).
19. M. O. Dayhoff, R. M. Schwartz, B. C. Orcutt, in *Atlas of Protein Sequence and Structure*, M. O. Dayhoff, Ed. (National Biomedical Research Foundation, Washington, DC, 1978), vol. 5, suppl. 3; C. Chothia and A. Lesk, *EMBO J.* **5**, 823 (1986); N. L. Summers, W. D. Carlson, M. Karplus, *J. Mol. Biol.* **196**, 175 (1987).
20. Mutations were introduced by the inverse polymerase chain reaction (PCR) [T. Clackson, D. Guessow, P. T. Jones, *PCR: A Practical Approach* (Oxford Univ. Press, Oxford, 1991)] into vector pRSET5a [R. Schoepfer, *Gene* **124**, 83 (1993)] containing the α and β genes of the *Thermoplasma* proteasome [P. Zwickl, F. Lottspeich, W. Baumeister, *FEBS Lett.* **312**, 157 (1992)] and confirmed by DNA sequencing. The β-gene was deleted for the 21 5′ nucleotides that code for the pro-sequence and elongated at the 3′ end by an affinity tag that codes for six consecutive histidine residues. Wild-type and mutant proteasomes were overexpressed in *E. coli* BL21 (DE3) [F. W. Studier *et al.*, *Methods Enzymol.* **185**, 60 (1990)], purified on a Ni-NTA resin (Diagen GmbH, Hilden, Germany) and diluted to a final concentration of 10 μg/ml in 20 mM tris-HCl, 1 mM EDTA, 1 mM NaN_3, pH 7.5. Correct processing of mutant β subunits was confirmed by Edmann degradation, and the correct assembly of mutant proteasomes was confirmed by electron microscopy. Because all mutants were made in a construct in which the β pro-sequence had been genetically replaced by an initiator methionine, we are unable to assess the effect of the mutations on the autocatalytic processing of the pro-sequence. The initiator methionine was cleaved in all mutants, presumably by the *E. coli* methionine aminopeptidase. This indicates that similar processing should occur in the *E. coli* proteasome homolog HslV, where the conserved threonines are also only preceded by an initiator methionine.
21. H. J. Duggleby *et al.*, *Nature* **373**, 264 (1995).
22. R. Glynne *et al.*, *ibid.* **353**, 357; V. Ortiz-Navarette *et al.*, *ibid.*, p. 662; C. K. Martinez and J. J. Monaco, *ibid.*, p. 664; A. Kelly *et al.*, *ibid.*, p. 667; Y. Yang, J. B. Waters, K. Früh, P. A. Peterson, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4928 (1992); A. L. Goldberg and K. L. Rock, *Nature* **357**, 375 (1992); M. T. Michalek *et al.*, *ibid.*, p. 552; M. G. Brown, J. Driscoll, J. J. Monaco, *J. Immunol.* **151**, 1193 (1993); J. Driscoll, M. G. Brown, D. Finley, J. J. Monaco, *Nature* **365**, 262 (1993); M. Gaczynska, K. L. Rock, A. L. Goldberg, *ibid.*, p. 264; M. Gaozynska, K. L. Rock, T. Spies, A. L. Goldberg, *Proc. Natl. Acad. U.S.A.* **90**, 9213 (1994); B. Boes *et al.*, *J. Exp. Med* **179**, 901 (1994); M. Aki *et al.*, *J. Biochem.* **115**, 257 (1994).
23. K. Tanaka, personal communication.
24. R. Eisenthal and A. Cornish-Bowden, *Biochem. J.* **139**, 715 (1974).
25. C. G. Knight, *Proteinase Inhibitors* (Elsevier, New York, 1986).
26. We thank M. Boicu for DNA sequencing, F. Lottspeich for protein sequencing, U. Santarius for electron microscopy of mutant proteasomes, and P. Zwickl for many helpful discussions.

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TECHNICAL COMMENTS

Herpes-Like Sequences in HIV-Infected and Uninfected Kaposi's Sarcoma Patients

Recently, Yuan Chang *et al.* (*1*) detected the presence of unique DNA sequences in 90% of Kaposi's sarcomas (KS) and 15% of non-KS tissues in HIV-infected individuals. These sequences, amplified by polymerase chain reaction (PCR) procedures and sequenced by Chang *et al.*, share DNA homology with genes of the capsid and tegument proteins of *Herpes saimiri* and Epstein-Barr viruses (*1*). In evaluating the presence of this herpesvirus-like sequence, we have synthesized primers for one of the reported sequences, designated the KS330Bam fragment, and used the PCR procedure to examine 13 KS biopsies, 12 corresponding normal tissues, 7 KS-derived cell lines, and peripheral blood mononuclear cells (PBMC) from 30 subjects (*2*).

Our studies confirm those of Chang *et al.* (*1*) and indicate that the herpesvirus-like sequence can be found in all 13 KS biopsies studied, including one from an individual not infected with human immunodeficiency virus (HIV) (Table 1). All 13 biopsy donors were homosexual men living in the United States. Corresponding control skin and other nearby tissues from eight of these men were negative for the sequence. In the four exceptions, the presence of KS cells in the control biopsies could not be excluded by histologic examination. In one patient whose KS tumors were positive for the KS330Bam sequence, subsequent biopsies of three resolved KS skin lesions were negative. These results were obtained after the patient had received chemotherapy and the biopsies revealed no histologic evidence of KS.

The SLK cell line, derived from an HIV-negative KS patient (*3*), was negative for the KS330Bam sequence (Fig. 1), as were six cell lines derived in our laboratory from KS tissue (*4*). This herpesvirus-like sequence was also not detected in EBV-carrying cell lines (for example, Raji) nor cells infected with HHV-6 (*5*).

We have detected the KS330Bam sequence as well in the PBMC of 10 KS patients and not in the PBMC of 20 non-KS subjects studied (*6*). Six of the non-KS individuals were HIV-infected, while the others were healthy, HIV-seronegative subjects. Of the 10 KS patients, three were not infected with HIV (Table 1).

With the use of immunomagnetic bead selection (*7*), we have isolated cell subsets from the PBMC and localized the KS330Bam sequence primarily to the $CD19^+$ B cell population (Table 1). Notably, the $CD8^+$ cells were negative. These findings are consistent with the presence of this sequence in some B cell lymphomas (*1*). Finally, in attempts to examine the possible route of transmission of this KS-associated virus, we examined cells and cell-free fluid from saliva and

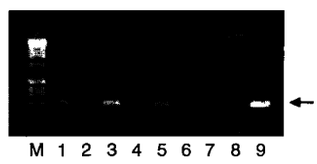

Fig. 1. Analysis of PCR amplified herpesvirus-like sequence. Total DNA from tissue or cells was amplified, and electrophoresis was performed on an agarose gel (*2*). M, DNA molecular weight marker. Lanes 1 and 2, tumor and control tissue from KS+ HIV+ patient; lanes 3 and 4, tumor and control tissue from KS+ HIV− patient; lane 5, KS+ HIV+ PBMC; lane 6, KS+ HIV-PBMC; lane 7, KS-HIV-PBMC; lane 8, SLK line; and lane 9, positive control KS tumor tissue. Arrow indicates the mobility of the 233 base pair portion of the KS330Bam fragment.