In Vivo Functional Analysis of the Ras Exchange Factor Son of Sevenless

Chris A. Karlovich,* Laura Bonfini,* Linda McCollam, Ronald D. Rogge, Andrea Daga, Michael P. Czech, Utpal Banerjee†

The Son of sevenless (Sos) protein functions as a guanine nucleotide transfer factor for Ras and interacts with the receptor tyrosine kinase Sevenless through the protein Drk, a homolog of mammalian Grb2. In vivo structure-function analysis revealed that the amino terminus of Sos was essential for its function in flies. A molecule lacking the amino terminus was a potent dominant negative. In contrast, a Sos fragment lacking the Drk binding sites was functional and its activity was dependent on the presence of the Sevenless receptor. Furthermore, membrane localization of Sos was independent of Drk. A possible role for Drk as an activator of Sos is discussed and a Drk-independent interaction between Sos and Sevenless is proposed that is likely mediated by the pleckstrin homology domain within the amino terminus.

Intercellular signaling mediated by the guanosine triphosphate (GTP)–binding protein Ras has been implicated in the specification of cell fate in a number of developmental processes (*1*). In the *Drosophila* eye, signaling through the Sevenless receptor tyrosine kinase in the presumptive R7 cell leads to the activation of Ras and, consequently, the proper specification of that cell as a photoreceptor neuron (*1*). Two members of this signaling system have been demonstrated to function between Sevenless and Ras. One is Sos (*2*–*4*), a guanine nucleotide transfer factor that converts Ras from an inactive guanosine diphosphate (GDP)–bound form to the active GTP-bound form. The second is Drk (*5*, *6*), a gene product with similarity to *Caenorhabditis elegans* Sem-5 (*7*) and mammalian Grb2 (*8*) that contains one SH2 (Src homology 2) domain and two SH3 domains. The SH3 domains of Drk or Grb2 bind to specific proline-rich sequences in the COOH-terminal end of Sos (*9*), and the SH2 domain binds to phosphotyrosines on activated tyrosine kinase receptors (*5*–*9*). Together, these data suggest that Drk serves as an adaptor, linking activated tyrosine kinase receptors to Sos.

The role of Drk may be a purely passive one, in that it may only serve to place Sos in spatial proximity to Ras within the cell membrane. Alternatively, the binding of Drk could serve to activate Sos. To investigate these issues, and to gain further insight into the mechanistic role of Sos in signal transduction, we initiated an in vivo structure-function analysis of the Sos gene product. We divided the *Drosophila* Sos molecule into three domains (Fig. 1A). The catalytic domain, Cat, is defined as the region of similarity to the *Saccharomyces cerevisiae* CDC25 protein, which activates Ras in yeast (*10*). The region COOH-terminal to Cat, defined as C, includes two proline-rich motifs that bind to the SH3 domains of Drk (*5*, *6*). The NH_2-terminal domain, N, contains the rest of the protein, including a segment with similarity to pleckstrin (the pleckstrin homology or PH domain). In mammalian systems, PH domains bind to both the βγ subunits of heterotrimeric GTP-binding proteins and to phosphatidylinositol-4,5-bisphosphate (*11*). We generated deletion constructs (Fig. 1A) containing different combinations of these domains and transformed them into flies (*12*) with a vector that includes two tandem repeats of the *sevenless* enhancer (sE) (*13*). Use of the sE element allows increased levels of expression of the constructs in cells that normally express Sevenless.

To assess the effects of the constructs on R7 development, transformants were studied in the genetic background sev^{E4}; Sos^{JC2}/Sos^{+}, in which the Sevenless signal is attenuated (*2*). In this background, only 17% of the ommatidia develop an R7 cell (Fig. 1B). In *sevenless*^{E4} (sev^{E4}) flies, no R7 cells develop, but in the sev^{E4}; Sos^{JC2}/Sos^{+} combination, the loss of signal due to the sev^{E4} mutation is partially compensated for by the Sos^{JC2} allele which encodes an overactive Sos protein product (*14*). When placed in the sev^{E4}; Sos^{JC2}/Sos^{+} genetic background, a Sos fragment that enhances the Sevenless signal is expected to cause an increased number of R7 cells to develop. In contrast, a fragment that interferes with the Sevenless signal will reduce the number of R7 cells that appear.

The sE(NCatC) and sE(NCat) constructs increased transmission of the Sevenless signal, raising the fraction of ommatidia with R7 cells to 33% and 40%, respectively (Fig. 1B). This effect is comparable to that obtained if one copy of $Gap1^{sxt}$, a negative regulator of Ras, is removed from the system (*15*) or if a second copy of Sos^{JC2} is added (*2*). In contrast, sE(CatC), sE(Cat), sE(C), sE(N), and sE(PH) decreased the fraction of R7-containing ommatidia to less than 3%, comparable to that seen when one copy of either *boss*, which encodes the ligand for Sevenless, or Ras1, or Sos is removed from the system (*4*). In this assay, it is critical that the sE elements are present, because the endogenous *Sos* promoter is too weak to elicit these phenotypic responses (*16*).

To assay for dominant negative effects,

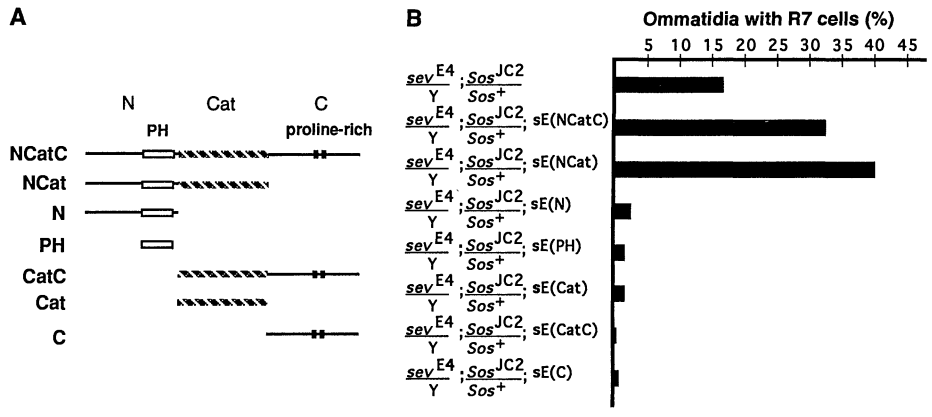

Fig. 1. *Sos* constructs and their effects on Sevenless signaling in a sensitized genetic background. (**A**) Predicted protein products of the *Sos* constructs. (**B**) Effects of *Sos* constructs on R7 development in a sev^{E4}; Sos^{JC2}/Sos^{+} genetic background. For each genotype, one copy of a given construct was used, except for sE(PH) which had very low amounts of expression and the results shown were obtained from flies with two copies of the construct. The eyes were scored by the pseudopupil technique (*26*) for the presence of R7 cells. For each genotype, at least 2000 ommatidia were counted.

C. A. Karlovich, L. Bonfini, R. D. Rogge, A. Daga, U. Banerjee, Department of Biology and Molecular Biology Institute, University of California, Los Angeles, CA 90024, USA.
L. McCollam and M. P. Czech, Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, MA 01605, USA.
*These two authors contributed equally to this work.
†To whom all correspondence should be addressed.

we introduced the Sos fragments into a sev^{+}/sev^{E4} genetic background. Because sev^{E4} is a fully recessive mutation, sev^{+}/sev^{E4} flies have a functional Sevenless signaling system that results in an eye containing R7 cells in all ommatidia. An introduced protein fragment that functions as a dominant negative would compete with the endogenous components and disrupt the wild-type signal, thereby giving rise to ommatidia that lack R7 cells. The use of the sev^{+}/sev^{E4} heterozygote rather than a sev^{+}/sev^{+} fly increases the sensitivity of this assay. The sE(CatC) construct had a strong dominant negative effect, causing 70% of the ommatidia to lack R7 cells (Table 1). The constructs sE(C), sE(Cat), sE(N), and sE(PH) also disrupted the wild-type signaling system, but with lower efficiency. The differences in magnitudes of the effects brought about by sE(CatC) and the rest of the constructs could either result from differences in their amounts of expression or stability in flies, or to inherent differences in their ability to disrupt wild-type Sevenless function. The inhibitory effects on signaling seen in these assays likely result from competition by the introduced fragments of Sos with endogenous components of the Ras pathway. For example, CatC and C could be titrating out Drk and Sev from the system, whereas Cat could be titrating Ras away from the Sevenless signaling complex.

Like Sos^{JC2}, the sE(NCatC) construct promoted the development of R7 cells in sev^{E4} flies (Fig. 2, A and B). Thus, the construct sE(NCatC) is a suppressor of the sev^{E4} allele of *sevenless*. This allele of *sevenless* encodes a full-length protein product whose presumed residual receptor activity is essential for the suppression by sE(NCatC). A null mutation, sev^{d2}, expressing no Sevenless protein, was not rescued by sE(NCatC) (Fig. 2, A and C). Thus, even when expressed in increased amounts, Sos required the presence of the Sevenless receptor to produce its biological effects (*17*).

The sE(NCat) construct promoted R7 development in a sev^{E4} background just as effectively as did sE(NCatC) (Fig. 2, A and D). Thus sE(NCat) is a suppressor of sev^{E4} despite lacking 469 amino acids from the COOH-terminus, including the Drk binding sites. If Drk was the only linker between Sevenless and Sos, we would have expected Sevenless not to interact with NCat at all. It could be argued that when overexpressed, NCat is active without being targeted to Sevenless. However, sE(NCat) did not suppress sev^{d2}, which makes no Sevenless protein (Fig. 2, A and E). Thus, the Sevenless molecule is critical for NCat function, suggesting an interaction between NCat and Sevenless. Because NCat does not bind Drk (*18*), this interaction is different from the one mediated by Drk. In a sev^{E4}; Sos^{JC2}/drk^{-} background, R7 cells fail to develop. Introducing sE(NCat) partially rescues this phenotype (*16*).

In the above assay, the receptor dependence described for NCat was not a reflection of low amounts of signaling by sev^{E4} through the endogenous Sos molecule. To demonstrate this, we removed one copy of endogenous Sos^{+} from flies that were genetically sev^{E4}; sE(NCat)/sE(NCat) and found that the frequency at which R7 cells appeared was not affected. Furthermore, introducing as many as two extra copies of Sos^{+} did not cause the formation of R7 cells in a sev^{E4} genetic background.

The NH_2-terminus of *Drosophila* Sos contains the pleckstrin homology domain between amino acids 474 and 591. This domain is found in more than 25 signaling molecules. Because the PH domain functions in protein-substrate interactions (*11*), it could conceivably contribute to the interaction between Sos and Sevenless. We therefore generated transformant flies expressing sE(PH), a construct in which the PH domain is transcribed under the control of the *sevenless* enhancer. Introduction of sE(PH) into sev^{E4}; Sos^{JC2}/Sos^{+} flies reduced the number of R7 cells that developed (Fig. 1B). Furthermore, sE(PH) caused the appearance of some ommatidia with no R7 cells in a sev^{+}/sev^{E4} background (Table 1). This suggests that the PH domain expressed on its own can compete with components of the endogenous *sevenless* signaling pathway as a weak dominant negative.

To address whether the PH domain interacts with Sevenless, a fusion protein was made with glutathione S-transferase (GST) fused to the PH domain (*19*). The fusion protein was bound to glutathione-Sepharose beads, and the beads were incubated with homogenates made from flies expressing the constitutively active *sevenless* allele, sev^{S11}. Protein immunoblot analysis using MAb150-C3 raised against Sevenless (*20*) demonstrated that the GST-PH fusion protein did bind Sevenless (Fig. 3). Neither GST alone nor a control fragment from the c-Abl protein bound Sevenless. We were unable to show binding between either NCatC or NCat and Sevenless by coimmunoprecipitation. The interaction between NCatC or NCat and Sevenless may therefore be weak or transient. The binding appeared not to be dependent on receptor activation because a catalytically inactive mutant, $Sev^{S11\text{-}lysmet}$ (*13*), also bound to GST-PH. However, genetic analysis suggests that catalytic activity of Sevenless is essential for the development of R7. For example, sE(NCat), which includes the PH domain, cannot rescue the catalytically defective $sev^{S11\text{-}lysmet}$ (*16*). As with other tyrosine kinase receptors, the kinase activity could be important for the transmission of more than one signal. Although it is not clear if the interaction between the PH domain and Sevenless is direct, it is likely that

Table 1. Effects of Sos constructs on R7 development in a sev^{+}/sev^{E4} genetic background. In each case, at least 2000 ommatidia were scored. Two copies of each construct were used in this assay except for sE(PH), for which expression was low and four copies were introduced.

Genotype	Ommatidia lacking R7 cells (%)
sev^{+}/sev^{E4}	0
sev^{+}/sev^{E4}; sE(CatC)	70
sev^{+}/sev^{E4}; sE(C)	20
sev^{+}/sev^{E4}; sE(N)	10
sev^{+}/sev^{E4}; sE(Cat)	5
sev^{+}/sev^{E4}; sE(PH)	7

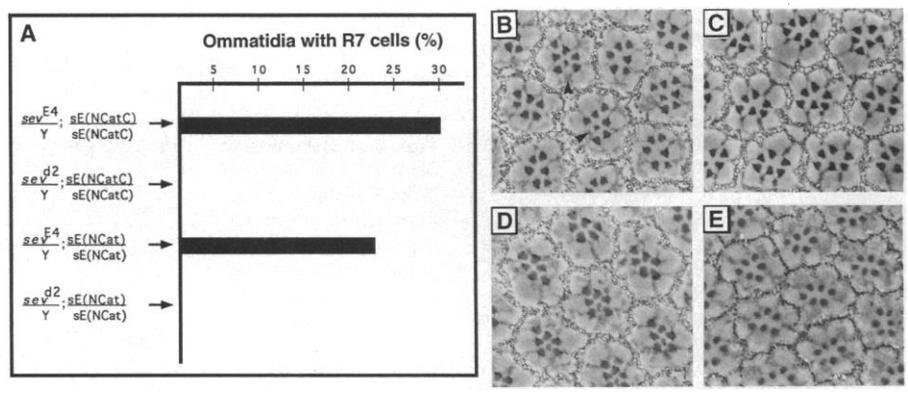

Fig. 2. Suppression of sev^{E4}, but not the null allele sev^{d2} by sE(NCat) and sE(NCatC). (**A**) Fraction of ommatidia in which R7 cells appear in sev^{E4} and sev^{d2} backgrounds in the presence of sE(NCatC) and sE(NCat). For each genotype, two copies of either sE(NCatC) or sE(NCat) were used and at least 2000 ommatidia were scored. (B through E) Distal tangential sections from adult eyes. The dark structures in each facet or ommatidium are light-gathering organelles called rhabdomeres. The centrally positioned R7 rhabdomere is marked with an arrow. (**B**) sev^{E4}; sE(NCatC). (**C**) sev^{d2}; sE(NCatC). (**D**) sev^{E4}; sE(NCat). (**E**) sev^{d2}; sE(NCat).

the binding between Sevenless and the PH domain contributes at least in part to the observed Drk-independent genetic interaction between NCat and Sevenless.

Our genetic assays indicated that the NH_2-terminal domain of Sos is critical for its function. To determine if any sequence outside of the Cat fragment is essential for the catalytic function of *Drosophila* Sos in activating Ras, we transformed the Sos constructs into the temperature-sensitive yeast mutant, *cdc25*-5. The Cat fragment functioned as a guanine nucleotide releasing protein for Ras in vivo, rescuing *cdc25*-5 and allowing it to grow at a nonpermissive temperature (Fig. 4). NCatC also rescued *cdc25*-5 but was degraded (*16*), so we are uncertain whether the rescue was caused by the full-length product. NCat was also fully functional as an activator of Ras in yeast. Thus NCat was active in both yeast and flies whereas Cat was active in yeast, but not in flies. Because *Drosophila* Ras is virtually identical to yeast Ras, we conclude that in flies the NH_2-terminus probably does not have a catalytic function in the activation of Ras, but rather it may be essential for the proper presentation of Sos to Ras within the Sevenless signaling complex.

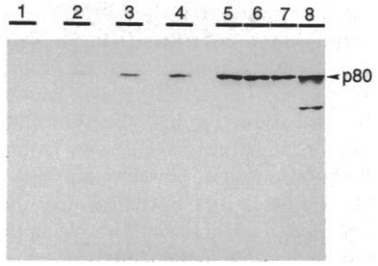

Fig. 3. Interaction of a GST-PH fusion protein with Sevenless. Lanes 1 to 3, fusion proteins incubated with lysate from sev^{S11}. Lane 1, GST-Abl(C); lane 2, GST; lane 3, GST-PH; lane 4, GST-PH incubated with lysate from $sev^{S11\text{-}lysmet}$; lanes 5 through 8, supernatants from the corresponding GST-Abl(C), GST, and GST-PH incubations. The 80-kD protein is the predicted primary translation product of the sev^{S11} allele; the lower band corresponds to the 60-kD β subunit of Sevenless formed by cleavage of the Sevenless precursor.

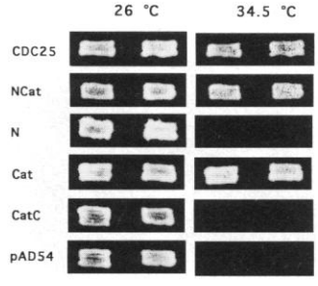

Fig. 4. Complementation of a *S. cerevisiae* $cdc25^{ts}$ mutation by *Sos* constructs. Growth of the cdc25-5 temperature-sensitive mutation at restrictive (34.5°C) and permissive (26°C) temperatures was examined after transformation with the pAD54 plasmid containing a complementing fragment of the *S. cerevisiae CDC25* gene or the *Sos* complementary DNA fragments NCat, N, Cat, or CatC. The *CDC25* construct includes the complementing fragment of the *CDC25* gene in the multicopy plasmid pAD5 (*10*). The construct N lacking the catalytic domain and the yeast shuttle vector pAD54 were used as negative controls. For each construct, two independent transformants are shown.

CatC failed to rescue the *cdc25*-5 mutant (Fig. 4). Thus, the ability of Cat to activate Ras was lost when the COOH-terminus was added onto it. Protein immunoblot analysis confirmed that Cat and CatC were expressed in similar amounts (*16*). The fact that Cat activated Ras whereas CatC did not is consistent with the observation (*21*) that the COOH-terminus may have an inhibitory influence on the catalytic domain.

There appears to be a close link between membrane localization and functional activity of mammalian Sos (*21*). Thus, the function of Grb2 could be to translocate Sos from the cytosol to the cell membrane. We tested for membrane localization by staining third instar eye imaginal discs with an antibody to the COOH-terminal region of Sos (*22*). When sE(NCatC) discs were stained with the antibody, the full-length Sos protein was primarily localized to the apical microvilli above the adherens junctions (Fig. 5A). This apical localization is similar to that of Boss (*23*), Sevenless (*20*), and Drk (*6*).

Sos localization was not dependent on the Sevenless protein. Sos remained apical in both sev^{E4} and sev^{d2} genetic backgrounds (Fig. 5, B and C). To establish if Drk functions in the apical localization of Sos, strains expressing NCatC but mutant for the Drk gene product were constructed. The *drk* allele used, $drk^{E(sev)2B}$, is pupal lethal and has a point mutation in its SH2 domain (*5*) that prevents receptor binding and results in delocalization from the apical membrane (*6*). In the $drk^{E(sev)2B}/drk^{E(sev)2B}$ genetic background, NCatC remained localized to the apical membranes (Fig. 5D). Thus in this system, membrane localization of Sos occurred independently of Drk. This result was also confirmed when discs expressing C were stained with antibody to Sos. The C fragment contains Drk binding sites and sE(C) transformant flies contained the same amount of Drk as wild-type flies. Yet the C protein product was found in a diffuse and cytoplasmic pattern (Fig. 5E). The C fragment was enriched in the apical half of the disc, but Drk did not fully localize this fragment to the apical membranes. This is unlikely to be a result of expression of large amounts of sE(C), because protein immunoblot analysis (*16*) revealed that the amount of expression of C was less than that for NCatC or CatC.

The CatC fragment was localized to the apical membranes (Fig. 5F). This localization was also independent of Drk (*16*). Because CatC is apically localized and C is not, we conclude that the presence of the Cat domain is essential for the localization of Sos to its proper subcellular compartment. The above results suggest that binding of Sos to Sevenless is a two-step process. The first step involves the translocation of Sos to apical membranes, and this can take place independently of Drk. The second step is mediated by Drk and results in Sos binding to Sevenless. This contrasts with the finding in transfected mammalian cell lines in which Sos is cytoplasmic and is translocated to the membrane through the mediation of Drk (*21*, *24*).

Taken together, the results from our experiments allow us to make several conclusions about Sos function in the developing eye imaginal disc. The NH_2-terminal region of Sos was essential for its function in *Drosophila*; a molecule lacking the NH_2-terminus, CatC, had a dominant negative effect. In contrast, the *Drosophila* Sos fragment lacking the COOH-terminus, NCat, was fully active and, unlike its mammalian counterpart (*21*), did not require a membrane

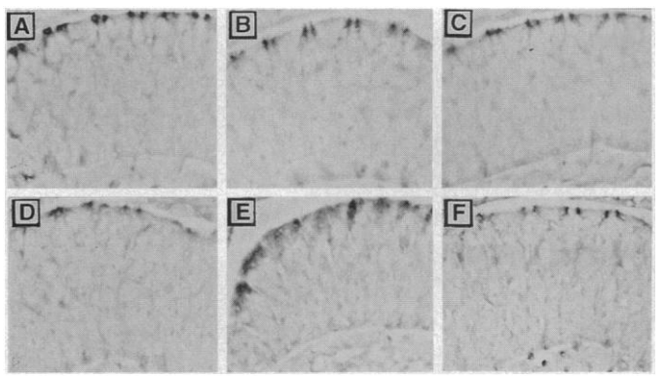

Fig. 5. Localization of Sos in eye imaginal discs. Eye imaginal discs from larvae expressing full-length and fragments of Sos were stained with an antibody to the COOH-terminus of Sos (*22*) in the presence of NP-40 (0.4%), embedded in resin, and sectioned perpendicular to the surface of the disc. In each case, two copies of the construct were used. Anterior is to the right and the apical surface is at the top. (**A**) sE(NCatC). (**B**) sev^{E4}/sev^{E4}; sE(NCatC). (**C**) sev^{d2}/sev^{d2}; sE(NCatC). (**D**) $drk^{E(sev)2B}/drk^{E(sev)2B}$; sE(NCatC). (**E**) sE(C). (**F**) sE(CatC).

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.

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Proteasome from *Thermoplasma acidophilum*: A Threonine Protease

Erika Seemüller, Andrei Lupas, Daniela Stock, Jan Löwe, Robert Huber, Wolfgang Baumeister*

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.

E. Seemüller, A. Lupas, W. Baumeister, Abteilung für Strukturbiologie Max-Planck Institut für Biochemie, D-82152 Martinsried Germany.
D. Stock, J. Löwe, R. Huber, Abteilung für Strukturforschung, Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany.

*To whom correspondence should be addressed.