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Molecular cloning and expression of subunit 9 of the 26S proteasome

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Abstract Seven peptides from subunit 9 (S9) of the human 26S proteasome were sequenced and this information was used to clone a HeLa cDNA that encodes the 46 kDa subunit. Rabbit polyclonal antisera were made against a ubiquitin fusion protein containing 12 amino acids from S9 and against a full-length S9 expressed in *E. coli*. Western blot analysis showed that the S9-specific antibodies bound the 26S proteasome and its regulatory complex separated on non-denaturing gels. In SDS-PAGE samples of the two complexes, the S9-specific antibodies bound a single 46 kDa subunit. Thus, a cDNA encoding a novel 26S protease subunit has been isolated, sequenced, and expressed.

Key words: Subunit 9; 26S proteasome; Sequencing

1. Introduction

The 26S proteasome is a multisubunit complex that is responsible for the degradation of a variety of cellular proteins [1,2]. The enzyme is composed of at least 30 different subunits ranging in size from 20 kDa to 110 kDa.

Three factors (CF-1, CF-2, CF-3) can be combined to assemble the 26S proteasome in vitro [3]. CF-3 is the multicatalytic protease (MCP) or 20S proteasome [4,5], which contributes the small (20–30 kDa) subunits and peptide cleavage activity to the 26S proteasome. A large particle, which may be CF-1 alone or CF-1 and CF-2 together, has been purified from several sources and it has been shown to combine with MCP to form the 26S proteasome. This particle, first purified from rabbit reticulocytes and called the 'ball' [6], has also been named the μ particle in *Drosophila* [7], PA700 in bovine red blood cells [8], and the 19S cap complex from *Xenopus* [9]. The terms ATPase complex [10] and regulatory complex or RC [11] have also been used. We prefer the last term because this particle is composed of subunits with several different activities, including substrate selection [11,12] and nucleotide binding and hydrolysis [13]. There is also in vivo evidence that these multisubunit complexes (MCP and RC) exist as preformed complexes and assemble into the 26S proteasome in the cell [14].

The regulatory complex provides all of the larger subunits (40–110 kDa) and two subunits with molecular weights less than 30 kDa to the 26S proteasome. The subunits unique to the RC are designated S1–S15 based on their SDS-PAGE mobilities, with the largest subunit (110 kDa) designated S1 and the smallest subunit (25 kDa) designated S15 [13,15].

At least six 26S proteasome subunits probably have nucleotide binding and ATPase activity [16], based on their sequence similarities to the AAA family of proteins (for ATPases Associated with Various Cellular Activities) [17]. Interactions between these ATPase subunits (S4, S6, S6′, S7, S8, and S10b) of the 26S proteasome have recently been described [18]. One of the ATPase subunits (S8) has been shown to bind and presumably select c-Fos for degradation by the 26S proteasome [12]. A subunit (S5a) that binds ubiquitin-conjugated substrates has also been identified, sequenced, and expressed [19,20]. However, the primary structures of subunits 9, 11, and 15 of the 26S proteasome remain to be determined. Here we report the cloning and expression of a cDNA that encodes subunit 9 of the 26S proteasome.

2. Materials and methods

The human 26S proteasome and its regulatory complex were purified from whole blood [15]. The rabbit 26S proteasome, regulatory complex, and 20S proteasome were isolated from reticulocytes [6]. The proteins were electrophoresed through denaturing gels and non-denaturing gels as described [10]. Rabbit polyclonal antisera were produced using Titermax research adjuvant as recommended by the manufacturer (CytRx Corp., Norcross, GA). Western blots on polyvinylidene fluoride (PVDF) membranes (Millipore Immobilon-P, Bedford, MA) were developed using peroxidase-conjugated goat anti-rabbit IgG as secondary antibody (Organon Teknika Corp., West Chester, PA) and enhanced chemiluminescence (ECL) (DuPont NEN, Boston, MA) for antibody detection.

Subunits of the human 26S proteasome were separated by SDS-PAGE, then electrotransferred to PVDF membranes, stained with Ponceau S and excised from the membrane. Direct sequencing of S9 indicated that its N-terminus is blocked so peptides were generated that were digested with endoproteinase Lys-C (Boehringer Mannheim Corp., Indianapolis, IN) [21]. The peptides were separated by HPLC and sequenced directly by automated Edman degradation [15].

Two of the peptide sequences were homologous to a 437 bp pig EST (GenBank accession number L20453), so non-degenerate oligonucleotide primers were designed from this sequence. Using these primers and a HeLa cDNA template, a probe DNA was generated by the polymerase chain reaction (PCR) (Perkin Elmer Corp., Branchburg, NJ). An appropriately sized DNA product was subcloned (Gibco BRL CloneAMP, Grand Island, NY), sequenced to confirm its identity, then random prime-labeled with ^{32}P and used as a probe to screen a HeLa lambda ZAPII cDNA library (Stratagene Cloning Systems, La Jolla, CA). DNAs from three positive plaques were sequenced, and all three had sequences in common. The cDNA described here had the most 5′ sequence.

In vitro transcription and translation (TNT from Promega Biotec Corp., Madison, WI) of the S9 cDNA produced a protein of ~47.5 kDa, as predicted by the cDNA sequence. DNA encoding the open reading frame (with introduced restriction endonuclease sites *Nde*I and *Xho*I) was amplified by PCR, subcloned into prokaryotic expression vectors pAED4 and pET16b, and sequenced to confirm that no mutations were introduced. In vivo expression with IPTG induction (0.1 mM, 2 h, 37°C) of *E. coli* BL21-DE3 cells produced N-terminus histidine-tagged (2896 Da) S9 (from vector pET16b, Novagen Inc., Madison, WI) of the expected ~50 kDa size. The his tag was cleaved off by factor Xa protease digestion as recommended by the manufacturer (New England Biolabs, Inc., Beverly, MA).

Two rabbit polyclonal antisera against S9 were prepared. A ubiquitin-S9 fusion protein (VKRDIQENDEEA) was constructed [22], purified, and injected into a New Zealand white rabbit (105 μg pro-

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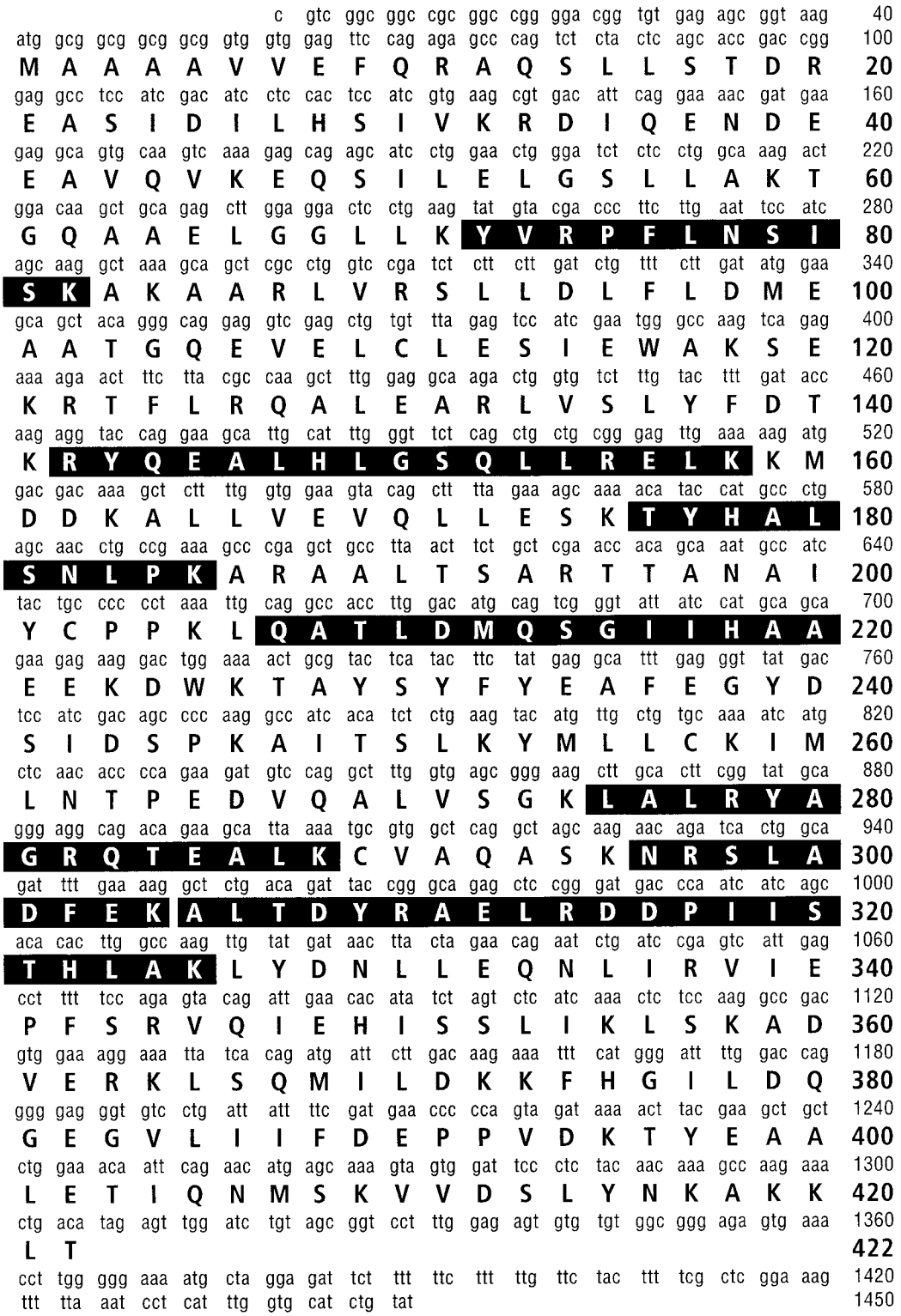

Fig. 1. Nucleotide sequence and deduced amino acid sequence for subunit 9 of the human 26S proteasome. The nucleotide sequence is shown in lower case, and the amino acid sequence is shown in upper case. The boxed amino acid sequences were identified by direct peptide sequencing.

tein, initial and two boosting immunizations in Titermax). This antiserum was used here at a 1:2000 dilution. Also, histidine-tagged S9 recombinant protein was gel-purified and injected into a second rabbit (150 μg his-S9, initial and two booster injections). Both antisera bind to the recombinant his-S9 and to S9 separated from the 26S proteasome.

3. Results and discussion

The 26S proteasome was purified from human red blood cells and its subunits were separated by SDS-PAGE and electroblotted onto PVDF membranes. Subunit 9 (~46 kDa) was

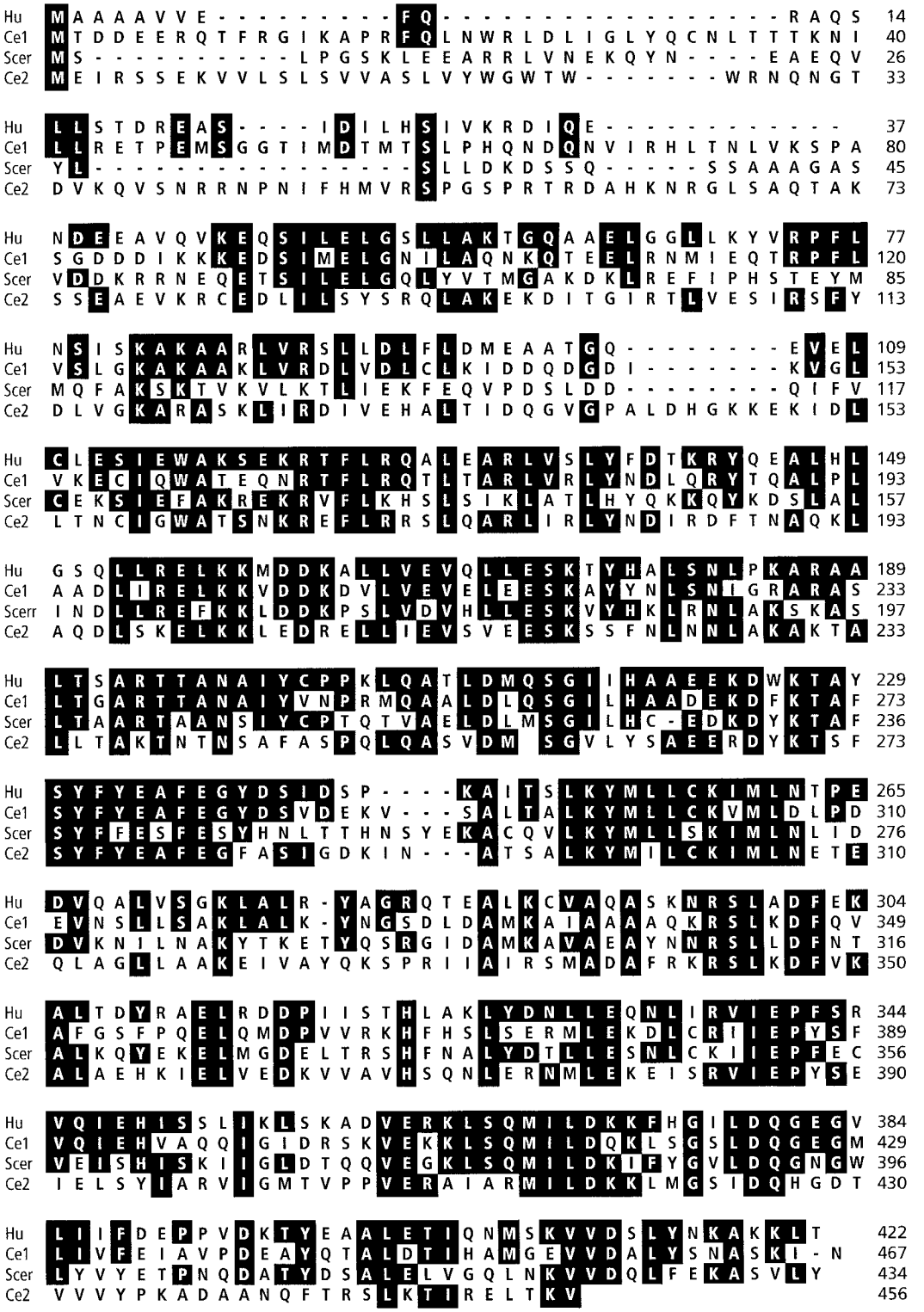

Fig. 2. Putative S9 homologs from *C. elegans* and *S. cerevisiae*. The open reading frame of the human S9 cDNA was aligned with the sequences of open reading frames from *C. elegans 1* (GenBank accession number U13876), *S. cerevisiae* (GenBank accession number X95644), and *C. elegans 2* (GenBank accession number Z11505). Sequences that are identical to human S9 are boxed. The percentage of amino acids identical to human S9 is 52% Ce1, 42% Scer, and 35% Ce2. The alignments were made by the clustal method of the DNASTAR software package (Madison, WI).

excised and subjected unsuccessfully to amino acid sequencing. Because the full-length protein apparently has a blocked N-terminus, peptides were generated by endoproteinase lys-C digestion [21]. Seven peptides, encompassing 96 amino acids, were sequenced. Two of the peptide sequences were identical to an open reading frame in a pig EST. Non-degenerate oligonucleotide primers designed from these sequences were used to prepare a DNA probe by PCR. This DNA was used to

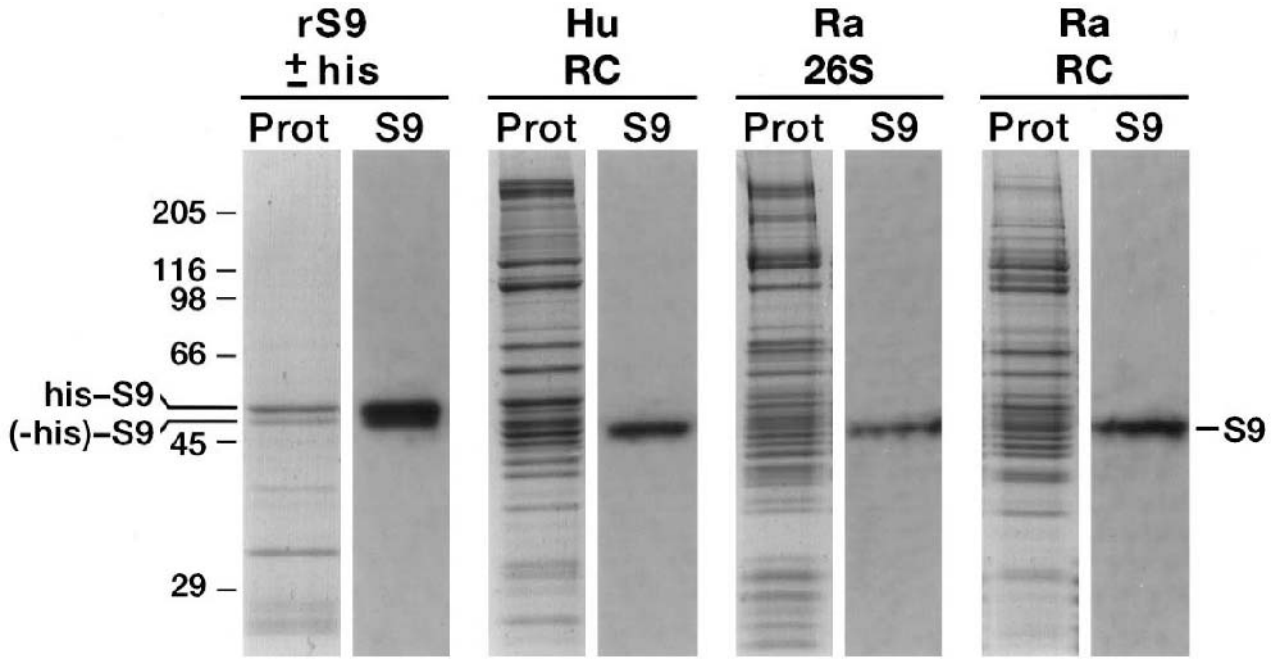

Fig. 3. Immunodetection of S9 protein. Recombinant histidine-tagged S9 expressed in *E. coli* (rS9, digested with factor Xa protease to remove the his tag), human regulatory complex from red blood cells (Hu RC), and rabbit reticulocyte 26S proteasome (Ra 26S) and regulatory complex (Ra RC) were electrophoresed through denaturing 12% acrylamide gels and Coomassie stained. Proteins in duplicate gels were electroblotted onto PVDF membranes and probed with S9-specific antibodies (1:2000 of Ub-S9 serum). The Coomassie-stained protein gel is on the left (Prot) of each pair, and the protein bound by S9-specific antibodies is on the right (S9).

screen a HeLa cDNA library. Eight positive clones were identified. The three clones with the strongest hybridization signals were sequenced, and the cDNA with the longest 5′ sequence is described here.

The nucleotide sequence of the S9 cDNA and its deduced amino acid sequence are shown in Fig. 1. This 1494 bp cDNA encodes a previously unreported protein of 422 amino acids. The predicted molecular weight (47 469 Da) is slightly larger than expected from the mobility of subunit 9 on SDS-PAGE gels (~46 kDa), but the predicted p*I* of 6.3 matches that of

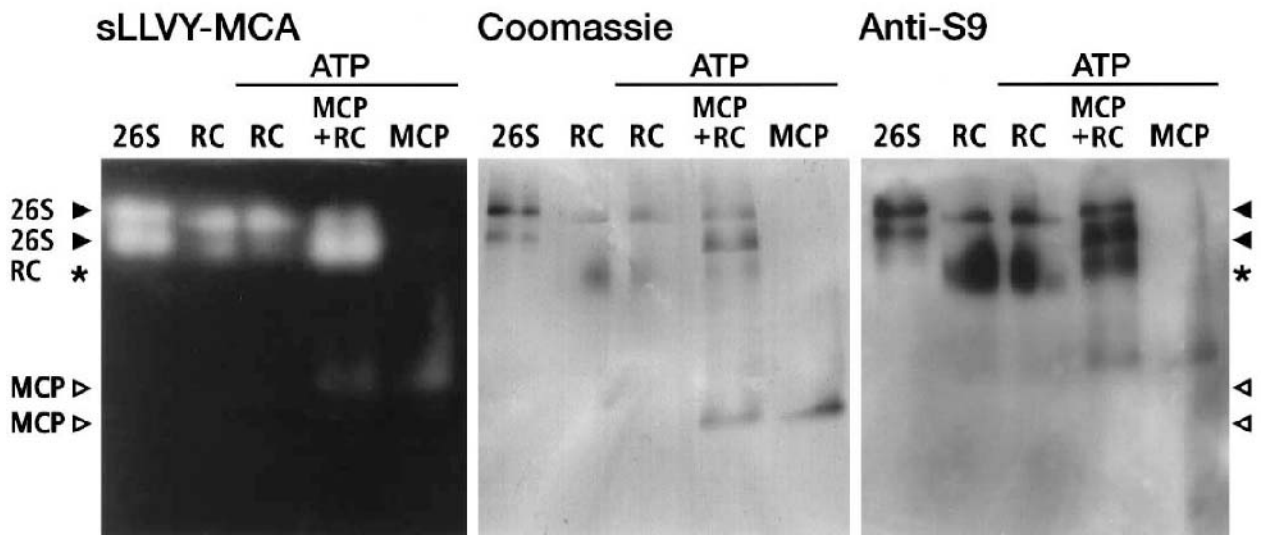

Fig. 4. Immunodetection of S9 in the 26S proteasome and its regulatory complex. Rabbit reticulocyte 26S proteasome, the regulatory complex (RC), and the multicatalytic protease (MCP), plus the RC and MCP preincubated alone or together with 2 mM ATP to assemble the 26S proteasome, were electrophoresed through non-denaturing gels. The peptidase activity of these complexes was assayed by fluorescent substrate overlays (sLLVY-MCA), and the protein complexes were stained (Coomassie). Duplicate gels were electroblotted onto PVDF membranes and probed with S9-specific antiserum (Anti-S9). The peptidase activities of the slow and fast forms of the 26S proteasome are identified by the closed arrowheads. Fast and slow forms of MCP are identified by open arrowheads. The faster migrating MCP complex stains well with Coomassie but has little peptidase acitivity, and the slightly slower migrating complex is not visible by Coomassie staining, but it has significant peptidase activity. These fast/slow pairs of both the 26S proteasome and MCP were first described in [6]. RC, which does not hydrolyze peptides, is seen by Coomassie staining to migrate just below the 26S doublet and is identified by an asterisk. A small amount of S9-containing complex which copurified with MCP was detectable with the S9-specific antibodies, but this complex did not comigrate with MCP on non-denaturing gels.

the 26S subunit identified as S9 on two-dimensional gels [18]. All seven of the peptides directly sequenced from S9 are present in the deduced amino acid sequence (boxed). The amino acid sequence of the open reading frame is not homologous to members of the S4-ATPase family, but it does have 9 dileucine repeats, a motif that was previously noted in subunit 5b [23]. However, there is no other significant homology to S5b (or to any other 26S subunit), and S9-specific antibodies did not bind to any 26S subunit other than S9 (see below). The Coils 2.1 algorithm [24] suggests that the protein can form coiled coils, and possible myristylation and phosphorylation sites were identified by a computer search of the Prosite database.

Although the human S9 cDNA does not show significant homology to any mammalian proteins in the GenBank database, there is significant similarity to a 467 residue open reading frame in *Caenorhabditis elegans* ('Ce1', Fig. 2). With 52% identity, this sequence probably represents the S9 homolog in *C. elegans*. On chromosome IV of *Saccharomyces cerevisiae*, an apparent S9 homolog of 434 amino acids is present ('Scer', 42% identity). Another *C. elegans* sequence ('Ce2', 456 amino acids) has less, but still significant, homology to human S9 (35% identity), suggesting that it may be a family member. As shown in Fig. 2, significant similarities between human, worm, and yeast sequences are scattered throughout the entire sequence of S9.

The S9 cDNA was subcloned into pET16b, and expression of the recombinant histidine-tagged S9 (rS9) in *Escherichia coli* generated a protein of the expected ~50 kDa size. Rabbits were immunized with the recombinant histidine-tagged S9 and with a ubiquitin-S9 fusion protein [22] containing the S9 sequence VKRDIQENDEEA, and the polyclonal sera were used in Western blot analyses. The recombinant his-S9 (rS9), human regulatory complex (Hu RC), rabbit 26S proteasome (Ra 26S) and rabbit RC (Ra RC) were electrophoresed through denaturing gels and stained with Coomassie brilliant blue; proteins in duplicate gels were transferred to PVDF membranes and probed with anti-S9 sera. The antibodies recognized the recombinant S9 protein and a ~46 kDa protein in preparations of the human regulatory complex, the rabbit 26S proteasome and the rabbit regulatory complex. S9 from human and rabbit 26S proteasome preparations migrated identically on SDS-PAGE (Fig. 3).

For reasons not yet understood, the S9 cDNA encodes a recombinant protein with slightly slower mobility on SDS-PAGE than S9 purified from the 26S proteasome. In vitro transcription and translation of the S9 cDNA in rabbit reticulocyte lysate resulted in a protein that migrated on SDS-PAGE like the recombinant protein (47.5 kDa), that is, slightly slower than subunit 9 in the 26S proteasome (46 kDa). Since the S9 protein in HeLa extracts migrated the same as the S9s in both the rabbit reticulocyte and human red blood cell 26S proteasomes (data not shown), the difference in mobility between the protein encoded by the HeLa cDNA and the protein purified with rabbit or human 26S proteasomes is not tissue- or species-specific. The slightly more rapid migration of S9 from intact 26S proteasomes may be due to post-translational cleavages or other modifications.

To show that S9 is a component of the 26S proteasome and its regulatory complex, the native complexes were separated by non-denaturing gel electrophoresis, electroblotted onto PVDF membranes, and probed with antibodies to S9 (Fig. 4). The antibodies bound to the 26S proteasome and its regulatory complex, but not to the multicatalytic protease. Incubation of RC and MCP with ATP to form the 26S proteasome caused S9 to shift its position on non-denaturing gels, from the location of the RC to that of the 26S proteasome. The comigration of S9 with free RC and with the 26S proteasome after assembly of RC and MCP provides further evidence that S9 is an integral subunit of the 26S proteasome's regulatory complex.

4. Conclusion

Several 26S proteasome subunits have suspected activities such as nucleotide binding or hydrolysis (S4, S6, S6′, S7, S8, S10b), and Ub-conjugate binding (S5a). S9 can be added to the equally long list of 26S proteasome subunits that have been identified, cloned, and sequenced, but whose role in the multisubunit complex remains unknown (S1, S2, S3, S5b, S10a, S12, S13). For example, yeast with mutations in S1 (Sen3) accumulate Ub-conjugates [25]. The S2 cDNA was obtained by a two-hybrid screen for proteins that bind the tumor necrosis factor (TNF) receptor [26]. A tumor transplantation antigen in mice, called P91A, has been identified as subunit 3 of the 26S proteasome [27]. A protein enriched in dileucine repeats has been identified as subunit 5b [23]. S10a and S12 are other fully sequenced subunits of the 26S proteasome with unknown activities [13,28]. Nin1p is a protein from yeast that represents subunit 13 [29,30] and it is needed for transition through the cell cycle. Interestingly, nin1-1 mutants can be suppressed by overexpression of S3 or S5a [31]. The common phenotypes for mutations in these regulatory complex subunits are cell cycle arrest and temperature-sensitive growth defects. Still, the biochemical activity provided by each subunit remains unknown. All of these regulatory complex subunits, including S9, presumably function in maintaining the structural integrity of the multisubunit complex, localizing the proteasome within the cell, or selecting substrates for degradation.

In summary, subunit 9 of the 26S proteasome has been identified and its cDNA cloned and expressed. No higher eukaryotic homologues were identified by searching the GenBank database, but there are apparent S9 homologues in *S. cerevisiae* and in *C. elegans*. The S9 cDNA encodes a novel protein whose function is presently unknown, but it is not a member of the S4 ATPase family, nor is it related to previously identified 26S proteasome subunits. Both the recombinant his-S9 protein and the S9-specific antibodies reported here should be useful tools for studying the expression, activity, and role of this protein in the 26S proteasome.

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