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Identification, molecular cloning, and characterization of subunit 11 of the human 26S proteasome

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Abstract We sequenced five peptides from subunit 11 (S11), a 43 kDa protein of the human 26S proteasome, and used this information to clone its cDNA. The S11 cDNA encodes a 376 amino acid protein with a p*I* of 5.6 and a molecular mass of 42.9 kDa. Translation of S11 RNA in the presence of [^{35}S]methionine produces a radiolabeled protein that co-migrates with S11 of the human 26S proteasome on SDS-PAGE. Polyclonal antiserum made against recombinant S11 recognizes a protein of the same size in extracts of bacteria expressing S11 and in purified 26S proteasomes from human red blood cells or rabbit reticulocytes. The S11 sequence does not contain motifs that suggest a biological function. S11 is, however, the human homolog of Rpn9, a recently identified subunit of the yeast 26S proteasome.

Key words: 26S proteasome; 19S regulatory complex; S11; Human chromosome 11; Rpn9; Ubiquitin

1. Introduction

A large number of proteins in eukaryotic cells are degraded by the ubiquitin (Ub)-proteasome pathway [1,2]. Over the past several years the list of substrates that are ubiquitinylated and degraded has grown dramatically and it now includes a large number of important regulatory molecules such as cyclins, cdk inhibitors, transcription factors, etc. [3]. The enzyme responsible for degrading Ub-conjugated substrates is the 26S proteasome. This large energy-dependent protease is formed from the 20S proteasome and a 19S regulatory complex (RC) [4–7]. The 20S proteasome or multicatalytic protease is the core proteolytic component, and its three or more active sites cleave a wide range of peptide bonds. The 19S regulatory complex, or PA700, contributes 18 regulatory subunits [8,9], which provide several biochemical functions. Six are members of the AAA family of ATPases [10] and confer nucleotidase activity with the expended energy being thought to move substrates to the central proteolytic chamber within the 20S proteasome. One RC subunit, S5a, has been shown to bind poly-Ub chains [11,12] and another component, presumably S13, is a ubiquitin isopeptidase [13]. The remaining subunits have not been assigned definite biochemical functions, but they are probably involved in substrate selection and/or intracellular localization of the 26S proteasome. The sequences for all but one of the RC subunits in the mammalian 26S proteasome have now been identified. We report here the sequence and some biochemical properties of that last subunit, S11, a 43 kDa protein in the human 26S proteasome.

2. Materials and methods

2.1. Protein purification and electrophoresis

The 26S proteasome and its regulatory complex were purified from outdated human blood [14,15]. The rabbit 26S proteasome, regulatory complex, and 20S proteasome were isolated from phenylhydrazine-induced reticulocytes as described [16]. Proteins were separated on denaturing and non-denaturing gels in a Bio-Rad mini-gel apparatus (6 cm resolving gel, 1 cm stacking gel, 8 cm wide, 1 mm thick) [16,17]. Complexes separated by non-denaturing gel electrophoresis were assayed for peptidase activity by overlaying gels with 200 μM sLLVY-MCA, and the complexes were subsequently stained with Coomassie brilliant blue. For Western blots of denatured and non-denatured proteins, the proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Immobilon-P, Bedford, MA) in 20% methanol/25 mM Tris-base/200 mM glycine buffer pH 8, stained with Ponceau S (Sigma, St. Louis, MO), then blocked with 5% non-fat milk in Tris-buffered saline (TBS). The membranes were incubated with antisera in 5% milk/TBS overnight at 4°C. Antibody binding was detected using peroxidase-conjugated goat anti-rabbit IgG as secondary antibody (Organon Teknika Corp., West Chester, PA) and enhanced chemiluminescence (ECL) (Dupont NEN, Boston, MA).

2.2. Protein sequence

Subunits of the human 26S proteasome were separated by SDS-PAGE [8] and electroblotted onto PVDF membranes for amino acid sequencing. Subunit 11 could not be directly sequenced, so peptides were generated by endoproteinase Lys-C digestion of the protein on PVDF membranes [18]. The peptides were separated by HPLC and their amino acid sequence was determined by automated Edman degradation.

2.3. DNA sequence

Since the five S11 peptide sequences were not encoded by any known protein, they were used to BLAST search for partial cDNA sequences in the expressed sequence tag (est) database. Several human ests matched the S11 sequences and they were aligned to construct a predicted human S11 sequence (AF400658, AA340701, AA249403, N40401, AA345552, AA642032, AA252642, AA215613, AA633271). Oligonucleotides designed for both ends of the predicted S11 cDNA sequence were used to amplify a DNA by the polymerase chain reaction (PCR) with rTth polymerase (GeneAmp XL-PCR, Perkin-Elmer, Foster City, CA) using cDNA synthesized (5 PRIME→3 PRIME Inc., Boulder, CO) from human myeloblast KG-1 RNA prepared by guanidinium isothiocyanate (5 PRIME→3 PRIME Inc., Boulder, CO). The PCR product was cloned into the pAMP plasmid (CloneAMP, Gibco BRL, Gaithersburg, MD), sequenced on both strands, and subcloned into the expression vector pAED4 (a gift from Don S. Doering, Whitehead Institute, Cambridge, MA) at the *Nde*I-*Xho*I sites. Although there were ambiguities between est sequences, the entire S11 cDNA sequence matches at least one est sequence with a single exception: a C in the est sequence is a T at position 69 of

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Abbreviations: est, expressed sequence tag DNA; IPTG, isopropyl-β-D-thiogalactopyranoside; PCI, proteasome-COP9-initiation factor helical motif; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; rS11, recombinant S11; RC, regulatory complex; S11, subunit 11 of the 26S proteasome; TBS, Tris-buffered saline; Ub, ubiquitin; 2-D gel electrophoresis, two-dimensional gel electrophoresis

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the S11 sequence. However, the predicted amino acid sequence is identical between the two sequences. The putative cDNA sequence for mouse S11 (used in Fig. 4) was constructed from est DNAs available in the database (AA646226, AA437964, AA208986). The plant *Arabidopsis thaliana* has an S11 sequence on chromosome 4 (ATF13C5). Sequence analysis by PROSITE does not identify any functional motifs. The human 26S proteasome subunit 11 sequence has been assigned the GenBank accession number AF086708.

2.4. S11 synthesis, protein binding and antiserum production

Radiolabeled S11 was synthesized by transcription and translation in a rabbit reticulocyte extract (TNT-T7 Coupled Reticulocyte Lysate System, Promega Corp., Madison, WI). For protein binding studies (data not shown), 10^6 cpm of [^{35}S]S11 protein was incubated with proteins bound to membranes [11,19], and the radioactive bands were visualized by autoradiography (Kodak X-OMAT AR film, Rochester, New York). A 43 kDa S11 protein was expressed from pAED4 in BL21 (DE3) cells (Novagen Inc., Madison, WI) by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h at 37°C. The S11 protein was found mainly in the insoluble fraction, prepared as described (Harlow and Lane) and emulsified with Titermax Gold research adjuvant (CytRx Corp., Norcross, GA) for immunization of a New Zealand White rabbit. The S11 antiserum was used at 1:1000 dilution for Western blotting.

3. Results

Most of the subunits of the 19S regulatory complex of the human 26S proteasome have now been identified, revealing that six members belong to an ATPase family (S4, S6, S6′, S7, S8, S10b). Of the remaining non-ATPase subunits, S5a is a polyUb binding protein [11,12], but functions for the other 10 subunits are not apparent from their amino acid sequences. We previously identified the non-ATPase S9 by using peptide sequence information to generate probes for screening a

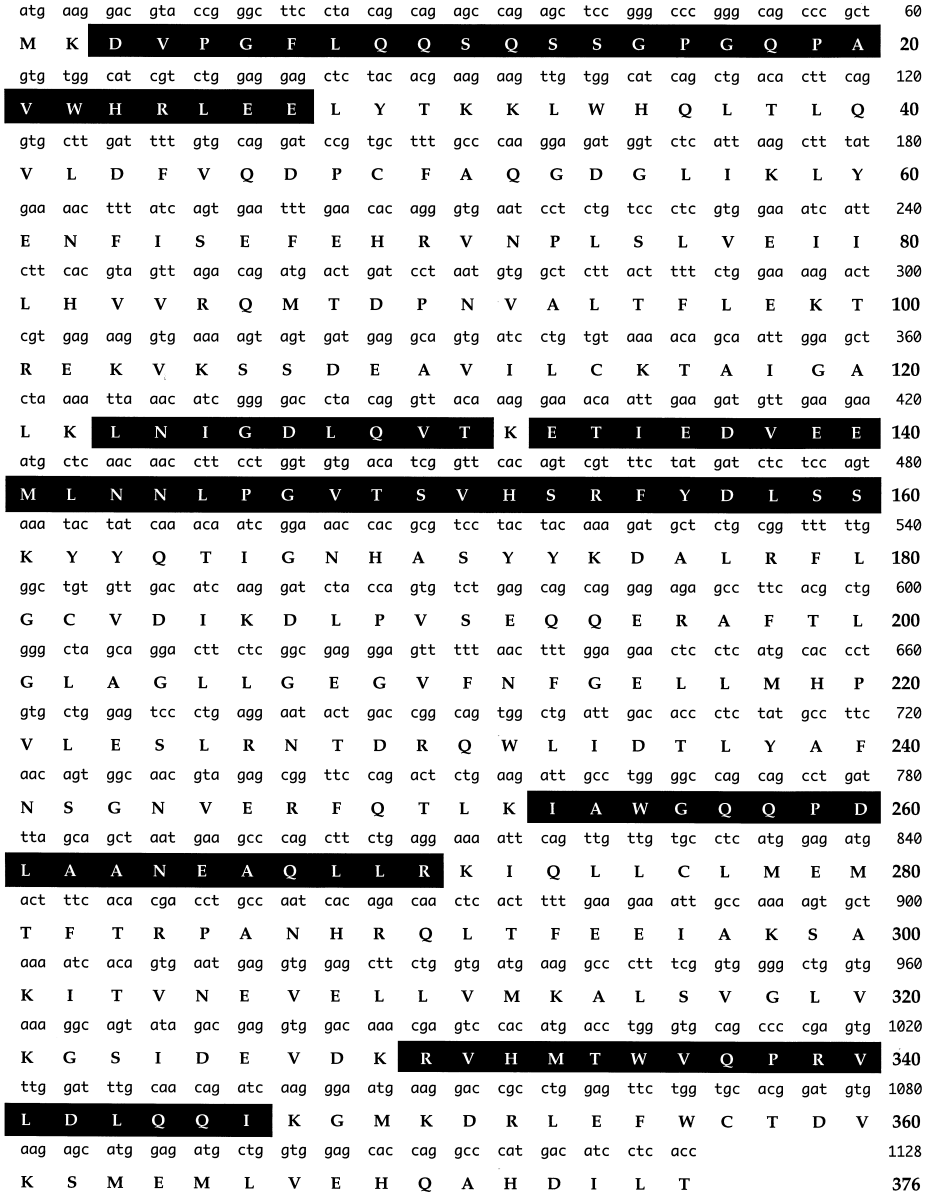

Fig. 1. Nucleotide and amino acid sequence of human 26S proteasome subunit 11. Five peptides (boxed sequence) from the 43 kDa S11 of the human proteasome were directly sequenced. Using sequences from est projects available in the database, specific oligonucleotide primers were designed and the corresponding cDNA was synthesized by PCR. An apparent genomic S11 sequence is encoded on human chromosome 11 (11p15.5; AF015416). The DNA sequence for human 26S proteasome subunit 11 has been given the GenBank accession number AF086708.

cDNA library [20]. We have employed a similar approach to isolate a cDNA encoding the prominent, but previously unidentified, S11.

Five peptide sequences were generated from human 26S proteasome S11. A search of the sequence database did not reveal a match to any known proteins. However, several mammalian DNAs that encoded the peptides were available from est projects. These ests were aligned to produce a candidate full-length cDNA (see Section 2). Primers were synthesized for the 5′ and 3′ ends of the putative cDNA, RNA was prepared from KG-1 human myeloblast cells, and dT-primed and S11-specific-primed cDNAs were synthesized from the RNA. Finally, the S11 cDNA was amplified by PCR. The 1128 bp DNA sequence and the corresponding protein sequence is shown in Fig. 1, with boxes around the directly sequenced peptides. The cDNA encodes a 376 amino acid protein with a predicted molecular mass of 42 934 Da and a p*I* of 5.63.

To test whether the cloned cDNA encodes a protein which co-migrates with the 26S proteasome subunit 11 on SDS-PAGE, the cDNA was transcribed and translated in a rabbit reticulocyte lysate containing [^{35}S]methionine. For the purpose of comparing the relative migration, S9 and S10b of the regulatory complex were also synthesized. The resulting radiolabeled proteins were then separated by SDS-PAGE (Fig. 2, left panel), which confirmed that the 422 amino acid S9 migrated the slowest, the 389 amino acid S10b migrated a little faster, and the 376 amino acid S11 migrated the fastest of the three. [^{35}S]S11 also co-migrated precisely on SDS-PAGE gels with subunit 11 from purified 19S regulatory complexes (Fig. 2, right panel). Thus, the cloned S11 cDNA encodes the full-length protein. Using far Western blot analyses [11,19], [^{35}S]S11 was found to bind the 26S proteasome and the 19S regulatory complex, but not the 20S proteasome, indicating specific protein-protein interactions between S11 and RC subunits (data not shown). These in vitro translation experiments show that the putative S11 cDNA encodes a protein of the expected molecular weight for the 26S proteasome subunit and indicate that the ^{35}S-labeled S11 protein binds to other components of the regulatory complex.

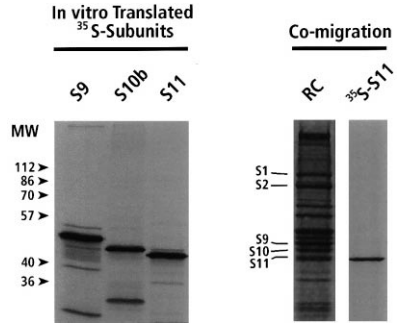

Fig. 2. Co-migration of recombinant S11 with human 26S proteasome S11. S11 DNA was subcloned into the pAED4 plasmid and the S11 RNA was transcribed by T7 polymerase then translated into ^{35}S-labeled protein in a rabbit reticulocyte lysate system. The DNAs encoding 26S proteasome subunits 9 and 10b were also transcribed and translated into ^{35}S-proteins. 1×10^6 cpm of these proteins were separated in 10% SDS gels, stained with Coomassie blue, dried onto filter paper, and exposed to X-OMAT AR film. The ^{35}S-proteins for S9, S10b, and S11 are shown on the left. The two lanes on the right show that when the radiolabeled S11 protein is mixed with the human regulatory complex (10 μg), then resolved by SDS-PAGE, [^{35}S]S11 overlies precisely with subunit 11, suggesting that the S11 cDNA represents the full-length protein.

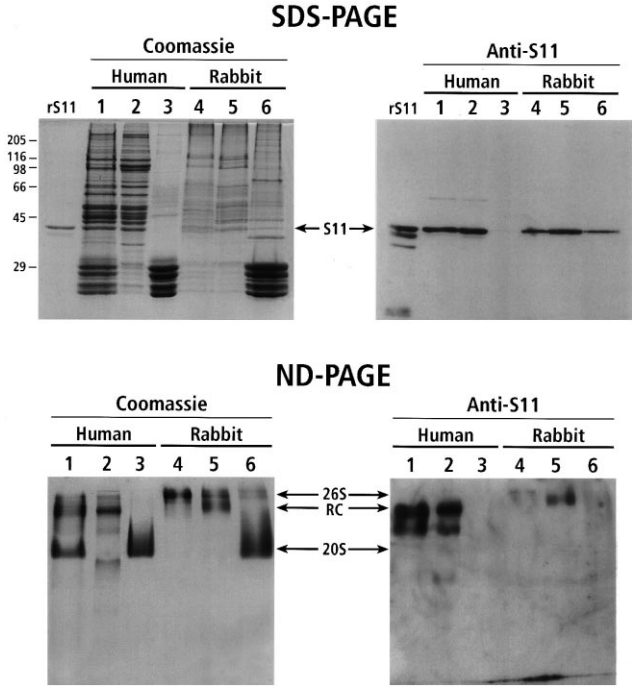

Fig. 3. Recognition of S11 in human and rabbit 26S proteasomes by antibodies to recombinant S11. Recombinant S11 (rS11), human 26S proteasomes (lane 1), human 19S regulatory complex (lane 2), human 20S proteasomes (lane 3), rabbit 26S proteasome (lane 4), rabbit 19S regulatory complex (lane 5), and rabbit 20S proteasomes (lane 6) were separated by denaturing (top) or non-denaturing gel electrophoresis (bottom) and either Coomassie blue stained in the gel (left), or transferred onto PVDF membranes for immunoblotting with anti-S11 polyclonal antiserum (right). Antibody detection by enhanced chemiluminescence identifies recombinant S11 and S11 purified with the 26S proteasome and the RC. The low molecular weight proteins detected in the rS11 preparation (top, right) are presumably S11 degradation products.

To make antigen for production of S11-specific antibodies, the S11 cDNA was subcloned into pAED4 and overexpressed in bacteria by IPTG induction. The recombinant S11 (rS11) protein was found primarily in the insoluble fraction, which was used to immunize a rabbit. The recombinant S11 protein and 26S proteasomes, 19S regulatory complexes, and 20S proteasomes isolated from human red blood cells and rabbit reticulocytes were separated by denaturing (SDS-PAGE) and non-denaturing (ND-PAGE) gel electrophoresis (Fig. 3). Duplicate sets of proteins were stained with Coomassie blue (left panels) or transferred onto PVDF membranes and probed with anti-S11 serum (right panels). The S11-specific antibodies recognize the 43 kDa subunit and native complexes of the 26S proteasome and 19S regulatory complex from both human and rabbit preparations. Furthermore, anti-S11 reacted with a component of the predicted p*I* after two-dimensional (2-D) gel electrophoresis of purified human RC (data not shown). The anti-rS11 antibodies show that the protein expressed from the cDNA is the same protein present in the 26S proteasome and the 19S regulatory complex.

Alignment of S11 sequences from human, mouse, worm, and yeast shows that amino acid conservations are scattered throughout the protein (Fig. 4). The human and mouse proteins are 97% identical (363/376) to each other, with 90%

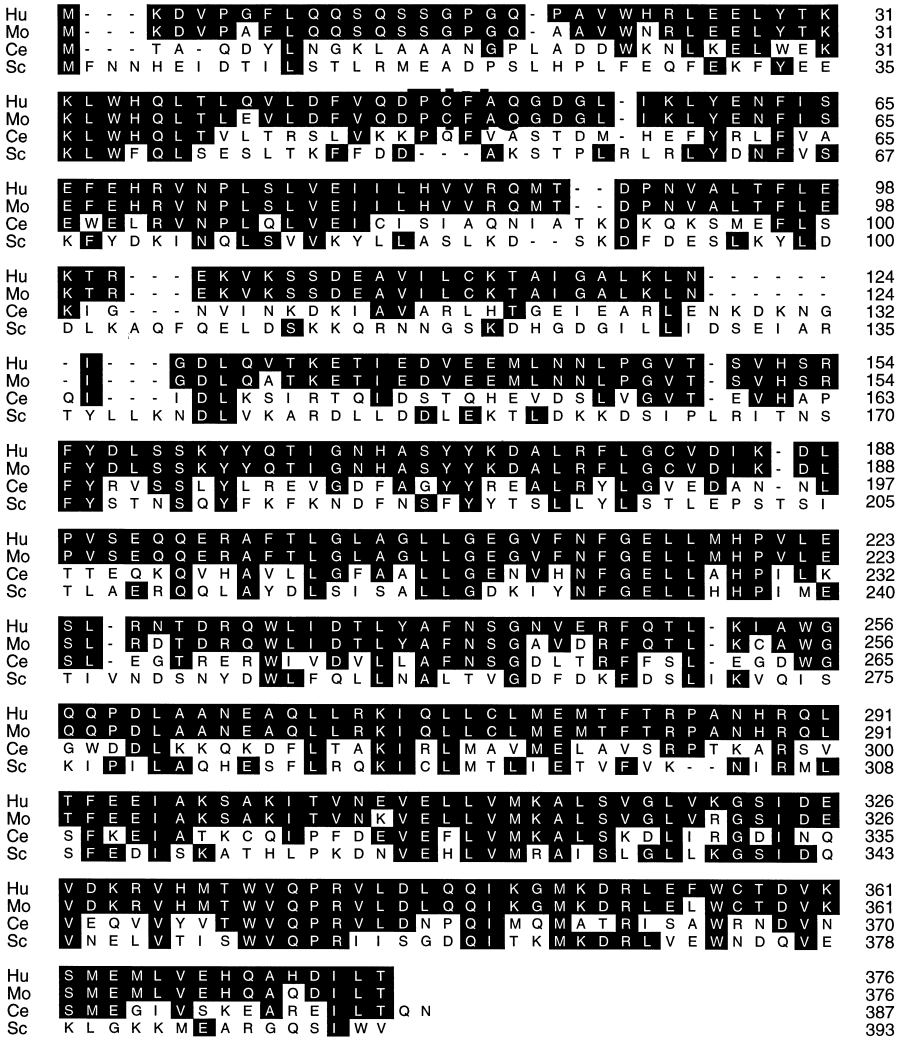

Fig. 4. Alignment of S11 sequences from human, mouse, worm and yeast. The S11 sequences for human, mouse, worm and yeast (Hu, Mo, Ce, Sc, respectively) are aligned here with amino acids identical to human S11 enclosed in boxes. The putative cDNA sequence for mouse S11 was constructed from est DNAs available in the database (AA646226, AA437964, AA208986). Human S11 is encoded on chromosome 11 (AF015416). The *Saccharomyces cerevisiae* S11 is encoded on yeast chromosome IV (U33007/SCD9461), and *Caenorhabditis elegans* S11 is encoded on worm chromosome III (Z49130/CET06D8).

nucleotide identity. The worm sequence shares 41% amino acid identity with human S11, and the yeast amino acid sequence is 31% identical to human S11. From yeast to humans, three stretches of conserved amino acids are striking (KLWF/HQL, NFGELL, WVQPR), but functional motifs are not obvious after a search of the PROSITE database.

4. Discussion

Two pieces of evidence indicate that the human cDNA we have cloned represents full-length S11 of the 26S proteasome. First, in vitro synthesis produces a protein that co-migrates with human RC subunit 11 (from which the original peptide sequences were derived). Second, expression of the protein in a prokaryotic expression system also results in a protein that co-migrates with human S11. This result was confirmed when the S11-specific serum detected proteins migrating identically in bacterial extracts of expressed S11 and in the human regulatory complex. Since migration of the S11 protein on SDS-PAGE is indistinguishable whether the protein was made in a eukaryotic system (rabbit reticulocytes), a prokaryotic system (*Escherichia coli*), or purified with the human 26S proteasome, S11 within the regulatory complex is presumably not post-translationally modified, at least in a way that affects migration on SDS-PAGE or 2-D gels. However, our initial sequencing attempts did indicate that the N-terminus of the protein is blocked.

There is also considerable evidence that the S11 cDNA encodes a bona fide subunit of the 26S proteasome. All five of the peptide sequences originally derived from the human RC are encoded by this cDNA. The size of the protein translated from this cDNA (in both a eukaryotic system and a prokaryotic system) is identical to the 26S proteasome subunit on SDS-PAGE. The [^{35}S]S11 protein synthesized in a eukaryotic extract binds to the 26S proteasome and to the 19S regulatory complex, as has been observed for other components of these multisubunit complexes (i.e. the ATPases). Antiserum made against recombinant S11 recognizes the 26S proteasome

complex and a 43 kDa subunit of the purified complex. In addition, individual subunits of the yeast regulatory complex have recently been identified [9]. Most of the 17 yeast RC subunits had previously been identified in the higher eukaryotic complex, but a few novel proteins were found. One of these, Rpn9, is the yeast homolog of the subunit that we have identified here. Interestingly, deletion of the yeast Rpn9 gene results in a slow-growth phenotype [9].

A loosely defined α-helical sequence motif that is apparently common among subunits of the 26S proteasome, COP9 proteins, and initiation factors ('the PCI domain') has recently been identified [21]. In addition to S11 and Rpn9 [9], the other 26S proteasome subunits with PCI domains are S3, S9, S10a, S12, S13, and p55, which are all non-ATPase subunits with roles in the 26S proteasome yet to be established.

The sequences of human S11 and 3 homologs reveal two pairs of highly conserved Trp residues in the C-terminal half of each protein (W232, W255, W335, W356 in the human sequence). The pairs have the proper spacing for being WW motifs [22,23], but the cluster of aromatic residues typically found near the center of WW motifs is not present in human S11 or its homologs. Thus, the functional significance of the two regions flanked by tryptophan residues is not yet clear.

The sequence of S11 is almost identical to the sequence of human p40.5 reported by Hori et al. [24] with the exception of two residues. N13 and T253 in the p40.5 sequence are S and I, respectively, in the sequence of S11 reported here. These discrepancies could represent sequencing errors in the p40.5 sequence since S13 is conserved in the bovine protein [24]. However, the difference at position 253 cannot be resolved since four out of five N-terminal residues (T253–Q257) sequenced chemically in a bovine peptide [24] differ from the cDNA-deduced human sequence and I253 in S11 is not conserved in the homologs of mouse, *Caenorhabditis elegans*, or *Saccharomyces cerevisiae* (Fig. 4). Furthermore, the evidence presented herein proves that the cloned and sequenced cDNA encodes a true subunit of the 26S proteasome, which was only inferred in the report of Hori et al. [24].

In conclusion, we have determined the cDNA sequence of subunit 11, a 43 kDa component of the human 26S proteasome, specifically a subunit of its 19S regulatory complex. It is a non-ATPase subunit with no obvious homologies to proteins with known biochemical activities. Its identification, its ability to bind the 26S proteasome and the 19S regulatory complex, and the availability of antiserum specific for the protein, should provide avenues for further investigation into its role in 26S proteasome function.

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