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Chromosomal Localization and Immunological Analysis of a Family of Human 26S Proteasomal ATPases

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The 26S proteasome is a eukaryotic ATP-dependent protease functioning as a protein death machine. It is a large multisubunit complex, consisting of a catalytic 20S proteasome and two regulatory modules, named PA700. The PA700 complex is composed of multiple subunits of 25-110 kDa, which are classified into two subgroups, a subgroup of at least 6 ATPases that constitute a unique multi-gene family encoding homologous polypeptides conserved during evolution and a subgroup of approximately 15 non-ATPase subunits, most of which are structurally unrelated to each other. In the present study, we report the chromosomal localization and immunological properties of six members of the human 26S proteasomal ATPase family. By use of the fluorescence *in situ* hybridization method, the S4 (*PSMC1*), MSS1 (*PSMC2*), TBP1 (*PSMC3*), TBP7 (*PSMC4*), p45 (*PSMC5*), and p42 (*PSMC6*) genes were mapped to human chromosomes 19p13.3, 7q22.1-q22.3, 11p11.2, 19q13.11-q13.13, 17q23.1-q23.3, and 12q15, respectively, indicating that the genes for multiple ATPases of the 26S proteasome are located on different chromosomes. Immunoblot analysis revealed that all these ATPases were associated with the purified 26S proteasome and that some of them showed striking heterogeneity in their electrical charges.

There is accumulating evidence that the 26S proteasome is involved in the destruction of bulk proteins with rapid and slow turnover rates in a wide variety of biological processes such as cell cycle progression, apoptosis, metabolic regulation, signal transduction, and antigen processing (1). It also plays a critical role in selective removal of abnormal/harmful proteins generated in cells, including rapid breakdown of unassembled or misfolded proteins in the endoplasmic reticulum, which constitutes a form of a protein quality control, and implies that it functions as a self-surveillance system for maintaining cellular homeostasis (2). The 26S proteasome is a very large protein complex with a molecular mass of approximately 2000 kDa. It consists of a central 20S proteasome and two large V-like terminal modules named PA700 (also called 19S ATPase complex; see review 3) attached to the central part in opposite orientations, such that it appears as a dumbbell-shaped particle. The 20S proteasome, functioning as a catalytic machine, consists of multiple homologous subunits with molecular masses of 21-32 kDa arranged in a stack of four rings that comprise a cylindrical particle (4). Two identical rectangular modules, PA700, with possible regulatory roles are composed of a set of heterogeneous proteins of sizes of 25-110 kDa (1). The components of the latter regulatory complex can be classified into two subgroups, one of multiple ATPase subunits and the other of a set of non-ATPase subunits (5).

The 26S proteasome has intrinsic ATPase activity that seems to play an essential role in its proteolytic function (6). So far, at least 6 different species of ATPase belonging to the same family have been suggested to be associated with the human 26S proteasome (5). Genes homologous to all 6 human ATPase genes have been cloned from yeast, and recent analysis of the entire genomic DNA sequence of *Saccaromyces cerevisiae* revealed that no more their 6 members of this family of ATPases are present in this yeast. The most highly conserved region between these proteins is a central one of ~220 amino acids, called the conserved AAA module (ATPases associated with a variety of cellular activities), which is present in all members of the protein family and contains a consensus motif for the putative ATP binding site (7). The AAA protein family has over 100 recognized members so far. In addition to pro-

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teasomal ATPases, the family contains proteins involved in very diverse cellular functions, such as vesicle fusion, peroxisome biogenesis, protein sorting in the mitochondrion, and transcription. Thus the AAA-ATPase is suggested to serve as a common module catalyzing energy-requiring biological reactions.

In this report, we verified the chromosomal localization of six members of the human 26S proteasomal ATPase family by the fluorescence *in situ* hybridization method (FISH). We also examined their immunological properties, and found that all these ATPases are associated with the 26S proteasome.

MATERIALS AND METHODS

Chromosomal mapping. Direct R-banding FISH based on FISH combined with the replicated prometaphase R-band was used. For suppression of repetitive sequences contained in this clone, we used human Cot-1 DNA (BRL) as described by Lichter *et al.* (8) with slight modifications (9). Labeling, hybridization, rinsing, and detection were carried out by routine procedures. Provia 100 (Fuji, ISO100) was used for microphotography (filter combination, Nikon B-2A). We isolated independent genomic clones of the 6 proteasomal ATPases and used each clone as a probe. These clones contained an insert of about 40 kb in a pWE15 cosmid vector. The library was purchased from Clontech Laboratories.

Immunological analyses. PA700 and the 26S proteasome were purified, as described before (10). Samples (10 μg) of PA700 or samples (50 μg) of the 26S proteasome were subjected to SDS-PAGE or two-dimensional PAGE, respectively, and were electrophoretically transferred to an Immobilon PVDF (polyvinylidene fluoride) membrane filter (Millipore) with a Sartoblot (Sartorius). Then the filter was processed for Western blotting as recommended by the manufacturer. Polyclonal antibodies against human MSS1, TBP1, and p42 were raised in rabbits by immunization with the corresponding proteins of known sequence synthesized in *E. coli* by use of the pET system. Anti-peptide antibodies were raised in rabbits by immunization with synthetic peptides based on the previously reported sequences for human S4, TBP7, and p45. Proteins were dye-stained with Coomassie brilliant blue R.

RESULTS AND DISCUSSION

We determined the localization of the genes for 6 proteasomal ATPases in human chromosomes by the FISH method. For this, we first isolated independent genomic clones of the 6 proteasomal ATPases and used each clone as a probe. To determine the chromosomal location of the genes encoding the 6 ATPases, we examined 100 typical R-band metaphase plates with each probe for the S4 (*PSMC1*), MSS1 (*PSMC2*), TBP1 (*PSMC3*), TBP7 (*PSMC4*), p45 (*PSMC5*), and p42

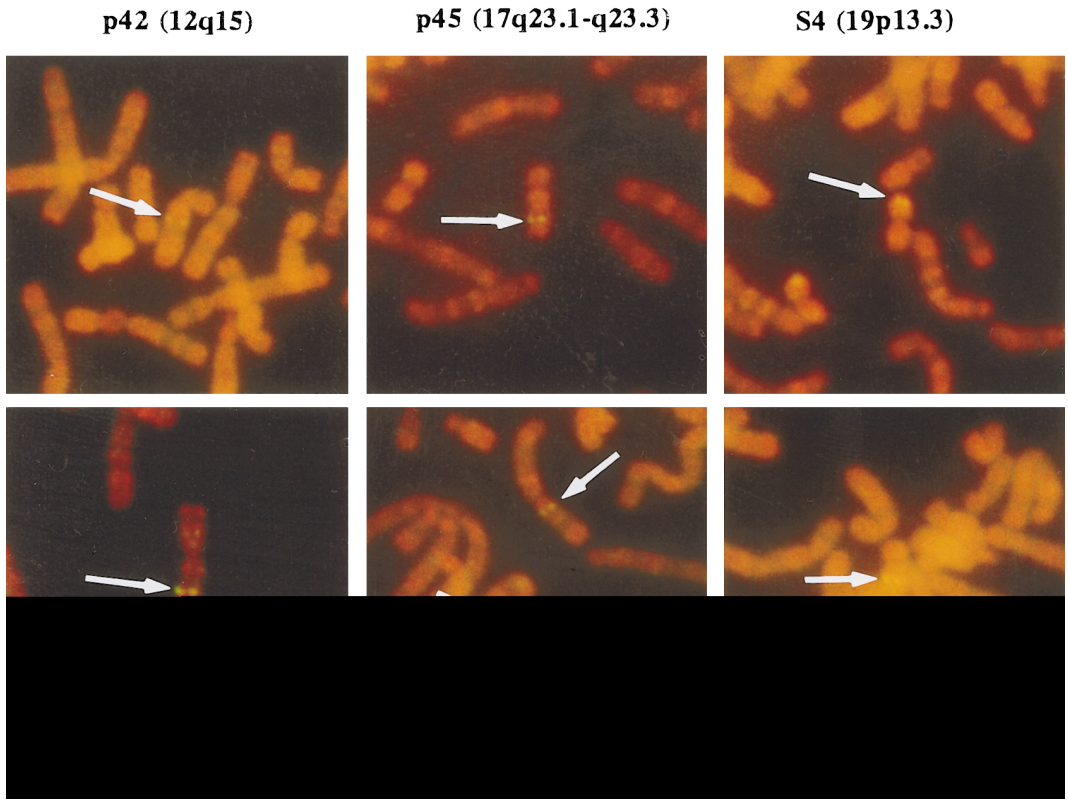

FIG. 1. Partial R-banded metaphase plates after *in situ* hybridization with 6 fluorescent ATPase DNA probes. For details of analysis, see the text. Arrows indicate the signals on 19p13.3, 7q22.1-q22.3, 11p11.2, 19q13.11-q13.13, 17q23.1-q23.3, and 12q15, showing the chromosomal localization of the human 26S proteasomal S4 (*PSMC1*), MSS1 (*PSMC2*), TBP1 (*PSMC3*), TBP7 (*PSMC4*), p45 (*PSMC5*), p42 (*PSMC6*) genes, respectively.

(*PSMC6*) genes. As shown in Fig. 1, the signals for FISH employed were localized to chromosomes 19p13.3, 7q22.1-q22.3, 11p11.2, 19q13.11-q13.13, 17q23.1-q23.3, and 12q15, respectively. No doublet signals were observed on other chromosomes. During the preparation of this manuscript, Hoyle et al. (11) reported that the *PSMC3* and *PSMC5* genes were localized to chromosomes 11p12-p13 and 17q24-q25, respectively, in which results are fairly consistent with those obtained by us presently. Our results indicate that the genes for multiple ATPases of the 26S proteasome are located on different chromosomes. The localization of these ATPase subunit genes differs from that observed for not only approximately 10 of the 20S proteasome genes (for a review, see ref. 1), but also two non-ATPase subunit genes of the 26S proteasome, p40 (equivalent to Mov-34) and p112, which are localized on chromosomes 16q23-24 and 2q37.1-37.2, respectively (12, 13). The high homologies among these 6 ATPases suggest that these genes might have evolved from a common ancestral gene, but it is unknown why they became located on different chromosomes rather than formed a cluster of related genes.

The cDNAs encoding some ATPases, such as S4 (14), p45 (15), and p42 (16), were cloned directly by screening with oligonucleotide probes synthesized corresponding to their protein sequences, but the other three cDNAs for MSS1, TBP1, and TBP7, were identified as proteins with high homology to S4 (for reviews, see refs. 3 and 5). Therefore, as it is uncertain whether all these proteins are actual components of the PA700 complex, we examined PA700 immunologically to determine whether it contains all 6 members of this family of ATPase subunits. As shown in Fig. 2, the respective antibodies against the 6 members of the ATP-binding protein family described above, i.e., S4, MSS1, TBP1, TBP7, p45 and p42, all gave strong signals at positions corresponding to their sizes deduced from their cDNA structures, although some non-specific signals also were observed. To confirm this finding, we carried out immunoblotting for the purified 26S proteasome separated by two-dimensional electrophoresis. As shown in Fig. 3, these antibodies specifically reacted with their respective subunits ranging from 40 kDa to 55 kDa, providing strong evidence that the 6 isolated putative ATPase cDNAs encode genuine components of the 26S proteasome. Intriguingly, some antibodies, particularly anti-p45/p42 antibodies, gave multiple spots, which were also detected by protein staining. This heterogeneity with similar sizes, but distinct charges, suggests their post-translational modifications, but the mechanism and significance are unknown. Further study is necessary to clarify this issue.

The proteolysis system, consuming metabolic energy, is thought to play an essential role in regulation of cellular functions by catalyzing rapid and irreversible reactions for various important biological processes (1, 2). In eukaryotic cells, ubiquitin and the proteasome appear to be organized into a major ATP-dependent, multi-enzymatic proteolytic cascade system (2). One role of the ATPase is to supply energy continuously for the selective degradation of target proteins by the active 26S proteasome. The energy produced by the PA700 ATPases is presumed to be used for unfolding of protein substrates, mostly ubiquitinated, to allow them for their entry into the central cavity of the 20S proteasome where the active sites are located (3). It is unclear, however, why so many ATPases are associated with the 26S proteasome. As a single ATPase subunit may be sufficient to supply energy for proteolysis, perhaps these ATPases have other functions besides the supply of energy for proteolysis. Interestingly, p45 was also identified as the thyroid hormone receptor-interacting protein, TRIP1 (17). The yeast homologues of p45 and p42 were initially found as SUG1 and SUG2, respectively that were originally identified in a genetic search for transcription factors that interact with the GAL4 regulatory protein of yeast (18, 19). Recently, the mammalian SUG1 homologue was found to have a DNA helicase activity (20). These findings suggest strongly that these ATPases have dual or multiple roles in not only proteolysis but also transcription. Moreover, each of the 6 ATPase is an essential for cell viability in the budding yeast, because the chromosomal disrup-

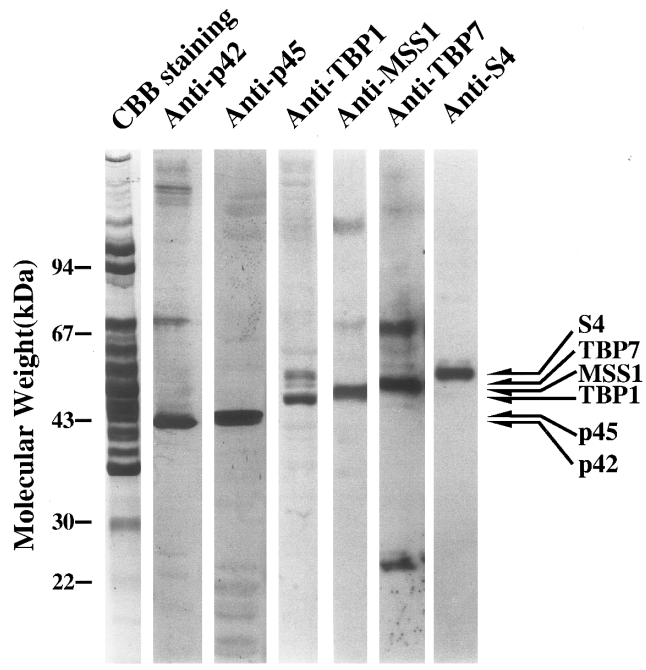

FIG. 2. Immunoblotting of the PA700 complex with antibodies against the 6 ATPases. The antibodies used are described in "MATERIALS AND METHODS". Samples (10μg) of purified PA700 were subjected to SDS-PAGE and then used for immunoblot analysis. Proteins were stained with Coomassie brilliant blue R (left lane), or immunostained (other lanes). Arrows on the right indicate the positions of the various ATPase subunits.

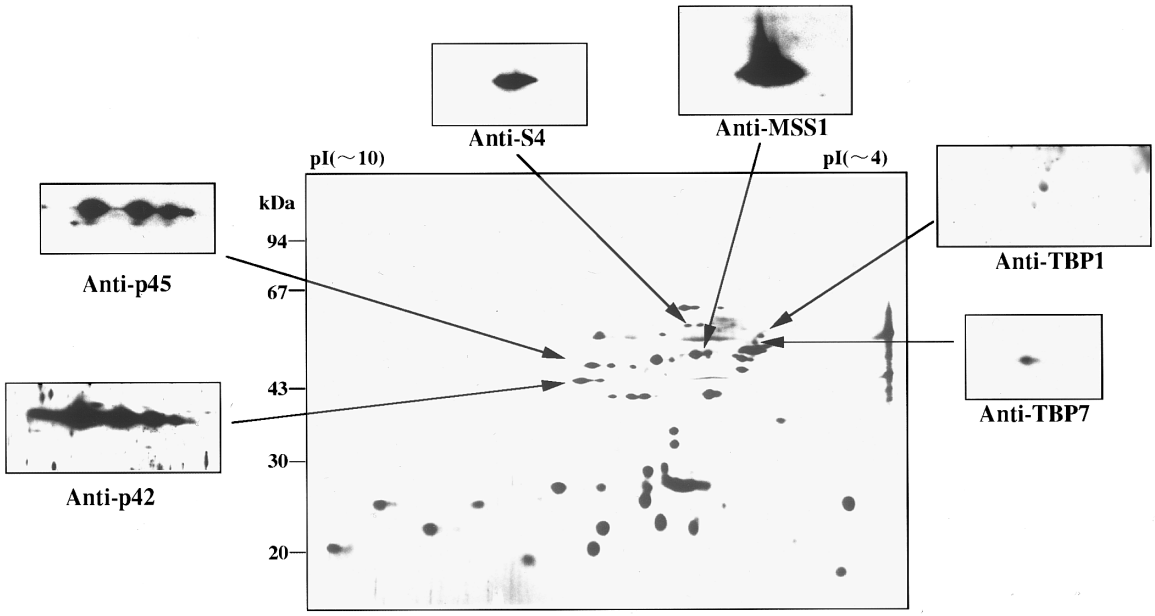

FIG. 3. Immunoblotting with 6 anti-ATPase antibodies of the human 26S proteasome complex separated by two-dimensional polyacrylamide gel electrophoresis. Samples (50 μg) of the purified 26S proteasome were subjected to two-dimensional PAGE. Protein staining and immunoblotting were carried out, as for FIG. 2. Data for immunoblotting show only the portions of the blot reactive with the respective antibodies.

tion of individual ATPase genes resulted in lethality (for a review, see ref. 2), indicating that their roles are not redundant. Although the sequences of ATP-binding domains among these 6 ATPases are highly homologous, there is little sequence similarity among these proteins at their amino and carboxyl termini, which may exert their unique functions. The structure-functional relationship of individual ATPase subunits remains to be solved.

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