Specific Interactions between ATPase Subunits of the 26 S Protease*

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The regulatory complex of the 26 S protease contains at least 15 distinct subunits. Six of these subunits (S4, S6, S6', S7, S8, and S10b) belong to a novel subfamily of presumptive nucleotidases that we call subunit 4 (S4)-like ATPases. Each of these putative ATPases was synthesized in reticulocyte lysate containing [35S]methionine, and the radiolabeled proteins were used in binding studies. S4, S6, S10b, and S6' displayed specific binding to components of the regulatory complex separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional PAGE. S4 bound to S7, and S6 bound two proteins: S8 and centrinactin, a component of the dynactin complex. S10b bound to S6' and bound much more weakly to S1 and p50, another component of the dynactin complex. S6' bound to S10b. Two subunits, S7 and S8, did not bind any components present on nitrocellulose membranes, presumably because S7 and S8 are already oligomeric following synthesis. Co-translation and sucrose gradient sedimentation of 35S-labeled ATPases demonstrated the formation of S6'-S10b dimers in solution but revealed more complex associations, namely the formation of trimers and tetramers, among S4, S6, S7, and S8. Progressive COOH-terminal deletions that removed as much as 300 amino acids from S4 had no effect on the binding of S4 to S7. In striking contrast, truncation of 85 NH2-terminal amino acids from S4 abrogated binding, clearly implicating the NH2 terminus of S4 in its specific interaction with S7. Since S4-like ATPases contain putative coiled-coils within the first 150 NH2-terminal amino acids, we propose that coiled-coils are responsible for the specificity of the observed subunit associations and that these associations are important for self-assembly of the regulatory complex.

The ATP/ubiquitin (Ub)3-dependent proteolytic pathway is involved in a diverse set of cellular processes including cell cycle regulation (1–5), antigen presentation by major histocompatibility complex class I molecules (6–9), and the selective degradation of short lived and abnormal intracellular proteins (10–13). ATP-dependent conjugation of Ub to e-amino groups of lysine residues in protein substrates often marks them for degradation by the 26 S protease complex (14, 15), which at present is the only cytosolic enzyme known to degrade Ub-conjugated proteins. The 26 S protease is composed of at least 25 protein subunits with molecular masses ranging from 20 to 110 kDa. Considerable evidence shows that the multicatalytic protease (MCP) or proteasome (16) constitutes the proteolytic core of the 26 S enzyme. In the presence of ATP, the multicatalytic protease associates with a 19 S regulatory complex (RC) that confers ATP dependence and Ub recognition to the 26 S protease (17–21). Electron micrographs show that the MCP cylinder is capped at one or both ends by RCs to produce mushroom- or dumbbell-shaped structures (22–24).

Among the polypeptides constituting the human RC, subunit 4 (S4) was the first component to be cloned and sequenced (25). The deduced amino acid sequence of S4 predicts a 440-residue protein bearing a putative nucleotide binding site near the center of the sequence (26). Comparison of the predicted amino acid sequence of S4 with sequences in the GenBank™ data base reveals significant similarity to a number of proteins originally thought to be components of transcription complexes but found recently to be subunits of the 26 S protease (14, 25). These S4-like ATPases include the human immunodeficiency virus tat-binding proteins 1 and 7 (TPB-1 and TPB-7, respectively) (21, 27–29); MSS1 (30, 31), and the thyroid receptor-interacting protein 1 (Tripl)2 (21, 32, 33) or Sug1p (34, 35), all of which are reasonably well conserved in the central ATPase module and at the COOH terminus but diverge considerably over an NH2-terminal region encompassing −150 amino acids.

In contrast to the substantial amount of information available on the structure of MCP, including the x-ray structure of the enzyme from Thermoplasma acidophilum (36–40), little is known about the organization of the regulatory complex. In an attempt to determine specific interactions between ATPases of the human RC, we have employed a nitrocellulose filter binding assay. In this report, we show that S4, S6, S10b, and S6' bind specific subunits within the regulatory complex. The specific interactions observed on filters were confirmed by the co-sedimentation of in vitro-translated ATPases on sucrose gradients. Truncation experiments demonstrate that the NH2 terminus of S4 is responsible for its interaction with S7. Based on the presence of putative coiled-coils in the amino termini of the ATPases, we propose that coiled-coil interactions are involved in assembly of the regulatory complex.

EXPERIMENTAL PROCEDURES

Preparation of Regulatory Complex—Human 26 S protease was partially purified from 20 units of outdated blood as described by Dubiel et al. (25). Briefly, packed and washed human erythrocytes were lysed

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†† These abbreviations are: Ub, ubiquitin; MCP, multicatalytic protease; RC, regulatory complex; TBP-1, tat-binding protein 1; TBP-7, tat-binding protein 7; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; pBS, pBluescript SK+; TBST, Tris-buffered saline-Tween 20.

2 C. Gorben, L. Hoffman, and M. Rechsteiner, unpublished results.
with 4 volumes of 1 mM diethiothreitol (DTT) in water. The mixture was stirred for 30 min at 4 °C and centrifuged for 1 h at 10,000 rpm. Glycerol was added to the supernatant fraction to a final concentration of 20%, and the lysate was then mixed overnight with 1.7 liters of Fractogel TSK HW 55 equilibrated in 10 mM Tris- HCl, pH 7.0, containing 1 mM DTT at 4 °C. The DDE/DEAE column was eluted with a 2-liter column and washed with 2 column volumes of 75 mM KCl in TSDG (10 mM Tris- HCl (pH 7.0), 10 mM NaCl, 25 mM KCl, 1.1 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 20% glycerol) at a flow rate of 2.5 ml/min. Proteins were eluted with a linear gradient of 75–400 mM KCl in TSDG and assayed for ATP-dependent peptidase activity using succinylphenethylaminothiocyanuric (Spectrapath, Eastman Kodak Co.) as substrate. Under these conditions, the regulatory complex is eluted last from the column (above 240 mM KCl) relative to MCP and the 26 S protease (20). Active fractions containing 26 S protease or regulatory complexes were pooled and concentrated to 70 ml using an XM300 membrane in a large stirred vessel (above 240 mM KCl) relative to MCP and the 26 S protease (20). Active fractions containing 26 S protease or regulatory complexes were pooled and concentrated to 70 ml using an XM300 membrane in a large stirred vessel and concentrated to 70 ml using an XM300 membrane in a large stirred vessel and concentrated to 70 ml using an XM300 membrane in a large stirred vessel.

Preparation of Antibodies and Immunoprecipitation—Polyclonal anti-serum against recombinant S4 from Schizosaccharomyces pombe was prepared in a New Zealand White rabbit injected intramuscularly with 63 μg of protein emulsified in TiterMax™ adjuvant (Vectax). Immune serum was obtained from blood collected 31 days after injection and used for immunoprecipitation of in vitro translated S4 proteins as described by Harlow and Lane (45). Briefly, aliquots (5 μl) of 35S-labeled translation products were diluted to 500 μl with radioimmune precipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% SDS, 0.5% Nonidet P-40, 1 mM EDTA, 50 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A). S4-specific antiserum (1 μl) was then added to each sample and incubated overnight at 4 °C with continuous mixing. Immune complexes were precipitated with 75 μl of protein A-Sepharose for 1 h at 4 °C followed by centrifugation. The sedimented beads were washed 5 times with radioimmune precipitation buffer at 4 °C and applied to SDS-PAGE. Proteins were visualized on a PhosphorImager screen or x-ray film.

Antibody binding was visualized by enhanced chemiluminescence (Amersham or EN DuPont) using secondary antibodies labeled with horseradish peroxidase (46).

Sucrose Gradient Sedimentation of in Vitro Translated ATPases—35S-Labeled ATPase preparations in rabbit reticulocyte lysate were sedimented through sucrose gradients as described (46). Samples (1–2 x 10⁶ cpm in a volume of 100 μl) were layered atop 4.9 ml 5–20% sucrose gradients and centrifuged at 39,000 rpm in a Beckman SW 50.1 rotor for 18 h at 4 °C. Fractions (0.25 ml) were collected from the bottom using a peristaltic pump, and 30-μl aliquots were applied to SDS-PAGE. The gels were fixed in 40% methanol, 10% acetic acid in water, dried under vacuum, and exposed to a PhosphorImager screen. The distribution of 35S-labeled ATPases in the gradients was compared with protein standards (catalase, aldolase, and bovine serum albumin (BSA); Pharmacia) to estimate their size.

Sequence Analyses—Prediction of coiled-coil regions in S4-like ATPases was carried out as described by Carr and Kim (47) using the computer program described by Lupas et al. (48) (version 2.1) using a window size of 28 residues.

RESULTS

Composition of the Human Red Blood Cell Regulatory Complex—The human red blood cell RC is composed of at least 15 distinct subunits with molecular masses between 25 and 110 kDa. A typical two-dimensional map of purified RCs is shown in Fig. 1. Using the simple nomenclature introduced by Dubiel et al. (49), the regulatory complex subunits are designated S1–S13. The subunits can be classified into ATPases and non-ATPases. Five ATPases are clearly present in our preparations: S4, S6, S7, S8, and S9 (Table I). A sixth nucleotidease, TBP-1, has been indirectly identified in the course of these studies (see below). Since the migration of TBP-1 and S6 is identical on two-dimensional gels, it has been designated TBP-1 as S6. The non-ATPase subunits include S1–S3, S5a, S5b, S9, S10a, and S11–S13 (Table I). One of these proteins, S5a, binds Ub-lysylase conjugates (50) and inhibits Ub-mediated proteolysis by the 26 S protease (51). The remaining non-ATPase subunits have unique sequences with limited homology to each other or other classes of proteins (49).

In Vitro Translation and Sucrose Gradient Sedimentation of...
Subunit Interactions in the 26 S Protease

35S-Labeled ATPases—To examine protein-protein interactions between ATPases and other components in the regulatory complex, we translated S4, S6, S6′, S7, S8, and S10b in the presence of [35S]methionine and used the resulting radiolabeled proteins in solid phase binding assays. Fig. 2A shows a 10% SDS-polyacrylamide gel of the translation products used in this study. Each full-length radiolabeled protein migrated on SDS-PAGE with a mobility expected for its molecular mass. The smaller radioactive products are presumably generated by initiation from internal methionines or by proteolysis. Translation of the S6′ sequence consistently produced two bands of approximately 48 and 46 kDa; both products were found to be active in the binding assays (see below).

The radiolabeled translation products were also characterized by centrifugation on 5–20% sucrose gradients (Fig. 2B).

About 90% of full-length S6′ and S6, and about 70% of S4 and S10b sedimented slower than BSA, consistent with their being monomers (Table II). By contrast, S7 and S8 sedimented as larger species (Fig. 2B). S7 partitioned between a fraction that co-sedimented with BSA and one that co-sedimented with aldolase, indicating that all radiolabeled S7 molecules in the lysate were oligomeric. Similarly, full-length S8 sedimented faster than BSA and slower than aldolase, suggesting that it is present as a trimer in the lysate (compare with the sedimentation of BSA–S10b dimers in Fig. 6).

Binding of 35S-Labeled ATPases to Subunits of the Regulatory Complex—To determine whether each ATPase interacted with subunits in the RC, human erythrocyte regulatory complexes were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated in the presence of each 35S-ATPase. The results presented in Fig. 3B demonstrate that each 35S-ATPase binds different subunits of the regulatory complex. S4 binds to components of approximately 51 and 50 kDa, and S6 binds subunits of 46–50 kDa whereas S6′ binds a 44-kDa species. Regions on the nitrocellulose filter with bound 35S-protein were excised, boiled in SDS-PAGE sample buffer, and analyzed on 10% SDS-polyacrylamide gels; all bound radioactivity was present as full-length 35S-ATPases (Fig. 3C). No binding was observed when membranes were incubated with radiolabeled S7 and S8 (not shown). We attribute the lack of binding by these members of the S4-like subfamily to the fact that S7 and S8 are already oligomeric following translation (Table II).

To identify more precisely the regulatory complex components recognized by S4-like ATPases, we performed two-dimensional gel electrophoresis of the RC, transferred the proteins to nitrocellulose, and incubated the membranes with the 35S-labeled translation products as described above. The autoradiograms in Fig. 4 demonstrate that each ATPase bound a different RC subunit. S4 bound S7 (panel labeled S4), which has three or more isoforms with isoelectric points in the range 5.5–6.0. S6 bound several proteins with isoelectric points ranging from pH 6.5 to 7.0. These are shown below to be isoforms of subunit 8 and the actin homolog, centrin. S6′ bound isoforms of S10b (panels labeled S6 and S6′, respectively). We also tested [35S]S10b for binding to RC components. This subunit bound to S6, which migrates as a single species with a pI of −5.0 (panel labeled S10b) and, to a lesser extent, to S1 and a

![Fig. 1. Two-dimensional profile of the 26 S protease regulatory complex from human red blood cells.](image)

**Table I**

Subunits of human red blood cell regulatory complex

| Component | S4-like ATPase | Identification | References |
|-----------|----------------|----------------|------------|
| S1        | No             | Peptide sequencing | 51         |
| S2        | No             | Peptide sequencing | 51         |
| S3        | No             | Peptide sequencing | 51         |
| S4        | Yes            | Peptide sequencing, antibody binding, in vitro translation | 25, this study |
| S5        | No             | Peptide sequencing, antibody binding | 52, 53, 54 |
| S5b       | No             | Peptide sequencing, antibody binding | 52         |
| S8 + S6'  | Yes            | Peptide sequencing, antibody binding, in vitro translation, ATPase binding | 29, this study |
| S7        | Yes            | Peptide sequencing, in vitro translation | 31, this study |
| Centrin   | No             | Peptide sequencing | 55, this study |
| S8        | Yes            | Peptide sequencing, antibody binding, in vitro translation | This study |
| S9        | No             | Peptide sequencing | 51         |
| S10a      | No             | Peptide sequencing | 51         |
| S10b      | Yes            | Antibody binding | This study |
| S11       | No             | Peptide sequencing | 56, this study |
| S12       | No             | Peptide sequencing | 51         |

*a* Refer to legend for Fig. 1.

*b* Hoffman, L., and Rechsteiner, M. (1997) *FEBS Lett.* **404**, 179–184.

*c* An S10 protein different from S10b was identified by peptide sequencing (51). Hence, the more *geodic* protein described by Dubiel et al. (51) is referred to as S10a, and the more *basic* S4-like ATPase identified in this study is called S10b.

*d* L. Hoffman and M. Rechsteiner, unpublished data.
Regulatory complex ATPases were transcribed and translated in rabbit reticulocyte lysate in the presence of [35S]methionine as described under “Experimental Procedures.” The radiolabeled proteins were then layered atop 5–20% sucrose gradients (5 ml) and centrifuged at 39,000 rpm in a Beckman SW50.1 rotor for 18 h at 4 °C. The gradients were fractionated (0.25 ml) from the bottom using a peristaltic pump, and aliquots (30 μl) of the fractions were subjected to SDS-PAGE and PhosphorImager analysis. Abbreviations are as follows: S4, subunit 4; S6, subunit 6 or tat-binding protein; S7, subunit 7 or MSS1; S8, subunit 8 or Trip-1; S10b, subunit 10b; S6’, subunit 6’ or tat-binding protein 1.

### TABLE II

Association state of translated ATPases

| Monomer | % isotope as | Oligomer |
|---------|-------------|----------|
| S4      | >70.0       | >20.0    |
| S6      | 90.0        | 10.0     |
| S7      | >90.0       | >90.0    |
| S10b    | 75.0        | 25.0     |
| S6’     | >90.0       |          |

*Except where indicated, the numbers represent the means of triplicate experiments.

The amount of radioactivity sedimenting at or deeper than the BSA position is referred to as the “oligomeric” fraction, since it is not possible to define the specific association of [35S]ATPases with unlabeled components of the lysate.

50-kDa component that migrates on two-dimensional gels just above S6. Since it might seem that S6’ and S6 bind the same subunit(s) on two-dimensional gels, a membrane containing bound RC subunits was incubated in the presence of both [35S]labeled S6 and S6’. The presence of two distinct labeled stripes in the panel labeled S6’ + S6’ demonstrates that these ATPases bind separate components of the regulatory complex.

Identification of the Binding Partners of S4, S6, S10b, and S6’—We used several approaches to clearly identify the bound target(s) of each radiolabeled ATPase. Based on the migration of in vitro translated ATPases on two-dimensional gels, we determined that the major subunits bound by [35S]S4, [35S]S10b, and [35S]S6 were S7, S6, and S8, respectively (see Fig. 5). Although the presence of S6’ in human erythrocyte regulatory complexes has yet to be demonstrated by direct chemical methods, we assume it is present in our preparations based upon the reported subunit composition of the bovine red blood cell 19 S complex (21). Since the predicted molecular masses and isoelectric points of S6’ (TBP-7) and S6’ (TBP-1) are virtually identical, we could not determine which of these components was bound by radiolabeled S10b. Therefore, His6-tagged recombinant S6’ and S6 were expressed in Escherichia coli, affinity-purified, applied to SDS-PAGE, and transferred to nitrocellulose. The immobilized proteins were then incubated with [35S]labeled S10b as described above. Although equivalent amounts of S6’ and S6 proteins were present on the membrane, radiolabeled S10b bound only to S6’ (Fig. 5, bottom panel), indicating that the binding partner of S10b is S6’ (TBP-1) and not S6 (TBP-7). Moreover, polyclonal antibodies demonstrated that the major subunit bound by S6’ was S10b (panel labeled anti-aCA + anti-S10b). These experiments demonstrate that the major binding partner of each ATPase is another ATPase: S4–S7; S10b–S6’; and S6–S8.

Antibody reactivity demonstrated that S6 also bound centrin (panel labeled anti-aCA), a 45-kDa component of the dynactin complex (57). Interestingly, yet another subunit of the dynactin complex, a 50-kDa species, was bound by [35S]S10b as shown upon Western blotting with a monoclonal antibody against the novel protein p50 (54) (panel labeled “anti-p50”). Thus, two of the three non-ATPase proteins bound by [35S]-ATPases are members of the dynactin complex; subunit 1 is the other non-ATPase to which an [35S]-labeled ATPase binds.

**Sedimentation Properties of Co-translated [35S]-ATPases—** The oligomeric state of translated S7 and S8 (Table II) could explain their lack of interaction with RC subunits bound to nitrocellulose. For this reason, we turned to sucrose gradient analysis of co-translated ATPases in an attempt to confirm that the specific binding partners of S7 and S8 are S4 and S6,
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Little is known about the arrangement of subunits in the regulatory complex or the assembly of this part of the 26 S protease. Because a protein-binding assay identified subunit 5a (S5a) as a Ub-conjugate recognition component (49) of the 26 S protease, we were encouraged to attempt similar experiments with the S4-like ATPases. The experiments presented in Figs. 3 and 4 demonstrate that four of the six S4-like ATPases bind specific subunits within the regulatory complex. Interestingly, each ATPase binds another ATPase. S4 binds to S7, S6 binds to S8, and S10b and S6’ bind each other. Subunit 7 is identical to Mss1, an ATPase identified as a suppressor of tat-mediated transactivation (31). S8, the binding partner of S6, was isolated as a protein that binds the thyroid hormone receptor in vitro (60). Mammanlian S8 is the homolog of Saccharomyces cerevisiae Sug1p, an ATPase subunit of the yeast 26 S protease (35).

The binding of [35S]S6’ to nitrocellulose-bound S10b and of [35S]S10b to filter-bound S6’ are consistent with the presence of these two ATPases within the bovine modulator complex (58). The modulator is a 250-kDa particle that enhances the ATP-dependent activation of MCP by the bovine regulatory complex or PA700. Thus, high affinity contacts between S4-like ATPases and S6’ and S10b, behaved as expected from the filter assay. When translated alone, these ATPases sedimented slower than BSA consistent, with each being a monomer. However, when they were co-translated, the S6’ and S10b proteins sedimented to the same position between the BSA and aldolase markers (Fig. 6A). No interactions were observed between S6’ or S10b and the remaining four ATPases (data not shown). Sedimentation analysis revealed interactions between S4 and other ATPases that were not apparent from the nitrocellulose assay. More than 70% of S6 co-sedimented with S4, and co-translation of S4 and S8 resulted in a significant shift of S4 to a multimeric species sedimenting near the aldolase marker (Fig. 6, B and C, respectively). In these cases, the radiolabeled ATPases sedi-
mented deeper than expected for heterodimers, suggesting that they formed trimers or tetramers. Thus, in solution, S4 was found to associate with three ATPases: S6, S8, and (as expected) S7 (see Fig. 6D). The sedimentation behavior of S6 was considerably influenced by co-translation of S8 (see Fig. 6E). Both ATPases sedimented near the aldolase marker, consistent with their presence in a trimer. The position of sedimentation of S8 was not changed upon co-translation with S6 (panel E) possibly because S8 already formed trimers upon synthesis (Fig. 2B); however, the amount of radiolabeled S8 increased in the presence of S6 (not shown).

The NH2 Terminus of S4 Confers Binding Specificity—Because it is unlikely that refolding generates native proteins on the nitrocellulose filters, the specific binding observed in Figs. 4 and 5 suggest that smaller regions present in S4-like ATPases might mediate their reciprocal binding. To investigate this possibility, we generated progressive COOH-terminal deletions of S4 and used the in vitro translated products to probe RC subunits separated on SDS gels. A schematic representation of the various S4 deletions is shown in Fig. 7A. Translation products generated from each deletion migrated on SDS-PAGE at the predicted molecular mass, and the authenticity of the translation products was confirmed by immunoprecipitation (Fig. 7B). As shown in Fig. 7C, progressive COOH-terminal deletions of S4 up to Thr167 had no effect on its binding to S7. By contrast, deletion of the first 228 base pairs of the S4 cDNA resulted in a truncated protein (S4-(85–440)) unable to bind S7. This finding clearly implicates the NH2-terminal region of S4 in binding. Because of low sequence similarity in this region between members of the S4 family, we propose that the NH2 termini of S4-like ATPases confer binding specificity.

DISCUSSION

FIG. 3. Binding of [35S]labeled ATPases to 26 S protease subunits separated by SDS-PAGE. Purified regulatory complexes of the 26 S protease were separated on 10% SDS-gels. Proteins were either stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose and incubated with [35S]labeled ATPase subunits (B). Subunits of the RC are labeled by subunit number from top to bottom between panels A and B. The position of migration of molecular weight markers is indicated by the numbers to the left of panel A. M in panel B refers to subunits of the RC identified by Ponceau S staining and marked with radioactivity ink. The [35S]-labeled ATPases used as probes are indicated at the top of each lane in panel B. Luciferase (LUC.) was used as a negative control for binding. Two-dimensional gel electrophoresis indicated that S8 was not bound by [35S]-S4 in the filter assay (see Figs. 5 and 6). Thus, the S8-like component bound in panel B is likely to constitute a breakdown product of subunit 7. Binding of S4 to a subunit that migrates on SDS-gels at the position of S5 (B) was inconsistent; thus, it is considered nonspecific. The asterisk denotes an unidentified target of S6’. C, the areas containing [35S]-labeled proteins shown in B were excised from the membrane, boiled in SDS sample buffer to release the bound [35S]-ATPases, and separated by SDS-PAGE (lanes 4–6). For comparison, the total [35S]-labeled translation products of S4, S6, and S6’ are shown in lanes 1–3. Full-length [35S]-ATPases are marked by open arrows. The two [35S]-S6’ translation products are designated by closed arrows (see Fig. 2A). respectively. Pairs of ATPases were co-translated in the presence of [35S]methionine and sedimented through 5–20% sucrose gradients (Fig. 6). The position of each ATPase was determined by PhosphorImager analyses of SDS-PAGE gels on individual fractions from the sucrose gradient. The relative intensities of each ATPase band in the fractions were used to plot their distribution across the gradient. Two of the ATPases, S6’ and S10b, behaved as expected from the filter assay. When translated alone, these ATPases sedimented slower than BSA consistent, with each being a monomer. However, when they were co-translated, the S6’ and S10b proteins sedimented to the same position between the BSA and aldolase markers (Fig. 6A). No interactions were observed between S6’ or S10b and the remaining four ATPases (data not shown). Sedimentation analysis revealed interactions between S4 and other ATPases that were not apparent from the nitrocellulose assay. More than 70% of S6 co-sedimented with S4, and co-translation of S4 and S8 resulted in a significant shift of S4 to a multimeric species sedimenting near the aldolase marker (Fig. 6, B and C, respectively). In these cases, the radiolabeled ATPases sedimented deeper than expected for heterodimers, suggesting that they formed trimers or tetramers. Thus, in solution, S4 was found to associate with three ATPases: S6, S8, and (as expected) S7 (see Fig. 6D). The sedimentation behavior of S6 was considerably influenced by co-translation of S8 (see Fig. 6E). Both ATPases sedimented near the aldolase marker, consistent with their presence in a trimer. The position of sedimentation of S8 was not changed upon co-translation with S6 (panel E) possibly because S8 already formed trimers upon synthesis (Fig. 2B); however, the amount of radiolabeled S8 increased in the presence of S6 (not shown).

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ATPases are likely to occur in the regulatory complex of the 26 S protease and in additional protein complexes (e.g. the modulator).

In the experiments shown in Fig. 3B, equal cpn of radiolabeled full-length \(^{35}S\)-ATPases were incubated with each membrane. Although the relative amounts of bound isotope suggest that S6' and S6 may bind their target subunits with higher affinity than does S4, it is clear that filter binding is a qualitative and not a quantitative assay. For example, the apparent binding of a specific ATPase could easily be influenced by the presence of endogenous competitors in the lysate. Moreover, the regions mediating contact between membrane-bound subunits and soluble ATPases may renature to different extents after electrophoresis and electroblotting of the bound target. Finally, binding could well depend upon the association state of the probe \(^{35}S\)-ATPase. In this regard, we have attributed the lack of binding of S7 and S8 to nitrocellulose-bound S4 and S6 to the fact that these ATPases formed trimmers in the translation reactions (Fig. 2B and Table II). Whether newly translated S7 or S8 molecules are bound to themselves, to other ATPases, to chaperonins, or to other lysate components is an open question. If free S4 and S6 subunits are abundant in reticulocyte lysate, this could explain the oligomeric state of S7 and S8, respectively. In this regard, we have found by immunoblotting of sucrose gradient fractions that free unlabeled S4 is present in the commercial reticulocyte lysate (not shown).

The sedimentation behavior of S7 (e.g. the presence of two peaks centered at positions characteristic of 80- and 150-kDa proteins) and S8 (a peak centered around 120 kDa) suggests the formation of trimers and tetramers that complicate the solution binding assay. Genetic studies in the fission yeast *Schizosaccharomyces pombe* have suggested interactions between S4 and S7 (4) and S4 and the product of the *let1* gene,\(^4\) presumably S8, *in vivo*. The sedimentation analyses provide biochemical evidence for association between S4, S6, S7, and S8 (Fig. 6) and suggest that these ATPases are in direct contact or in close proximity to one another within the regulatory complex of the human 26 S protease. By contrast, S10b and S6' associated tightly with one another but did not interact with the other ATPases in either the filter or sedimentation assays. These two subunits may, in fact, be localized elsewhere in the regulatory complex (see Ref. 58 and below).

The six ATPase subunits of the regulatory complex belong to a protein subfamily characterized by the conserved nucleotide-binding sequence, GPPGXXGK, a conserved DEID sequence about 50 residues toward the COOH terminus, and by a conserved cysteine residue close to the COOH terminus (25). These putative ATPases are members of a larger family of proteins referred to as "AAA" (ATPases associated with a variety of cellular activities) widely distributed in nature (61). Sequence identity within the ATPase module of S4 homologs is above 60% and spans over 180 amino acid residues of the six ATPases (25, 28, 59). Sequence identity decreases substantially at the NH\(_2\) terminus (<20% identity), which in S4 contains the domain found to mediate its interaction with S7 (Fig. 7C). Regions within the first 100 amino acids of S4-like ATPases are predicted to form coiled-coils (Table III), which are known to mediate protein-protein interactions (62). Based on this observation, we proposed (14) that the NH\(_2\)-terminal domains of regulatory complex ATPases are involved in the selection of substrates for destruction by the 26 S protease. Recent work by Wang et al. (63) supports this hypothesis by showing that c-Fos and S8 bind each other specifically through their coiled-coil regions.

The present work provides evidence that the NH\(_2\) termini may also be required for assembly of the S4-like ATPases during formation of the regulatory complex. Deletion of the first 85 amino acid residues of S4 results in total loss of binding to subunit 7 (Fig. 7C). Although the NH\(_2\) terminus of S4 does not conform to the principles set in the computer algorithm of

\(^4\) G. McGurk and C. Gordon, unpublished results.
Lupas et al. (48, 64), our binding studies indicate that a coiled-coil region is likely to exist in S4. Notably, the Lupas algorithm predicts robust coiled-coils at the NH2 termini of the remaining five regulatory complex ATPases. Further experimentation is required to determine whether the coiled-coil regions in S4-like ATPases are involved in assembly of the regulatory complex, selection of substrates for degradation, or both. The NH2 termini in the S4 subfamily of ATPases could fill both roles.

Fig. 5. Identification of the target subunits of 35S-labeled ATPases. Top three panels, co-migration of in vitro translated ATPases with regulatory complex subunits is shown. Purified regulatory complexes (50 μg) were mixed with in vitro translated 35S-ATPases and subjected to two-dimensional gel electrophoresis as described under “Experimental Procedures.” The gels were stained with Coomassie Brilliant Blue, dried under vacuum, and exposed to x-ray film for autoradiography. The positions of migration of stained RC subunits are indicated by the dotted areas. Middle three panels, RC subunits were transferred to nitrocellulose following two-dimensional gel electrophoresis, stained with Ponceau S, and subjected to immunoblotting with anti-centractin, anti-S10b, or anti-p50. The open arrow indicates the position of migration of anti-centractin, which is identical to the position of the more acidic component bound by 35S6 (see Fig. 4). Weak cross-reactivity with S7 and S8 was observed with anti-S10b antibodies (panel labeled anti-S10b). The asterisk denotes protein precipitated at the top of the first dimension gel. Bottom panel, binding of 35S-S10b to purified S6 and S6′ is shown. Purified recombinant His6-tagged TBP-1 (S6′) and TBP-7 (S6) were separated on a 10% polyacrylamide gel, transferred to nitrocellulose, and stained with Ponceau S (Protein). The membrane was subsequently incubated with 35S10b as described under “Experimental Procedures.” This experiment demonstrated that two distinct ATPases, S6 and S6′, co-migrate to the same position on SDS-PAGE and two-dimensional gels.

Fig. 6. Sucrose gradient sedimentation of co-translated 35S-ATPases. Pairs of ATPases were transcribed and translated in rabbit reticulocyte lysate containing 35S-methionine. The translation mixtures were sedimented on 5–20% sucrose gradients as described under “Experimental Procedures.” Conditions for sedimentation and analysis of the gradient fractions are given in the legend for Table II, except that in panels B and C, the gradients were centrifuged for 20 h. Arbitrary units represent density values of full-length 35S-ATPases obtained by PhosphorImager analysis but corrected for differences in incorporation of isotope between translation reactions (ATPases translated alone versus co-translated ATPases). The dashed lines represent the distribution of 35S-ATPases translated alone. The solid lines show the distribution of 35S-ATPases upon co-translation with other S4 family members. The co-translated ATPases are indicated in parentheses. Sedimentation markers are catalase (232 kDa) (C), aldolase (158 kDa) (A), and BSA (67 kDa) (B).

Lupas et al. (48, 64), our binding studies indicate that a coiled-coil region is likely to exist in S4. Notably, the Lupas algorithm predicts robust coiled-coils at the NH2 termini of the remaining five regulatory complex ATPases. Further experimentation is required to determine whether the coiled-coil regions in S4-like ATPases are involved in assembly of the regulatory complex, selection of substrates for degradation, or both. The NH2 termini in the S4 subfamily of ATPases could fill both roles. After
Subunit Interactions in the 26 S Protease

Predicted NH\(_2\)-terminal coiled-coil regions in S4-like ATPases

| S4-like ATPase | Localization in protein sequence | Probability (no weights) |
|----------------|----------------------------------|-------------------------|
| S4             | 74–102                           | 0.94*                   |
| S6             | 37–71                            | 0.98                    |
| S7             | 41–68                            | 0.99                    |
| S8             | 42–69                            | 1.00                    |
| S10b           | 25–52                            | 1.00                    |
| S6'            | 48–75                            | 0.99                    |

* Numbers represent the position in the sequence relative to the first methionine in the mature protein.

Although the calculated score for the potential coiled-coil region of S4 is above the value of 1.3 designated by Lupas et al. (48) as diagnostic, some of its properties are atypical. In contrast to the remaining five regulatory complex ATPases, the Lupas algorithm fails to predict a coiled-coil region in the S4 sequence when increased weights for the core residues (a, d) are used. Thus, further studies will be required to establish firmly that residues 74–102 in S4 actually form a coiled-coil with S7.

One of these, S1, is the largest subunit of the regulatory complex and has been implicated in the degradation of a protein involved in tRNA splicing (66). The amount of \([^{[35]}S]\)S10b bound to S1 is considerably lower than the amount bound to its ATPase partner S6' (Fig. 4). However, the binding to S1 may well be significant, since the ATPases must associate with other subunits in the regulatory complex. For this reason, we believe that the observed association is an example of an interaction between ATPases and non-ATPase components.

At least two components of the dynactin complex are present in our regulatory complex preparations: p50 (54), which bound \([^{35]}S]\)S10b, and centriatin (55), bound by radiolabeled S6. Dynactin is a 20 S complex that interacts with cytoplasmic dynein and activates dynein-mediated vesicular transport (57, 67).

There are at least three possibilities to explain the presence of centriatin and p50 in our preparations of regulatory complex and their binding to ATPases: (a) these proteins are common subunits of both the 26 S protease and dynactin complex; (b) both centriatin and p50 are proteolytic substrates of the 26 S protease; or (c) the regulatory complex/26 S protease and dynactin interact with each other in vivo. The last possibility is attractive, since association of the 26 S protease with the cytoskeleton would allow targeting of the 26 S enzyme to different cellular locations (e.g. centromeres at anaphase); the dynactin/dynein complex could provide the motive force required for its movement. We are currently characterizing the binding of regulatory complex subunits to centriatin and p50.*

In summary, we have shown that the S4-like ATPases associate with each other in highly specific pairs. Moreover, this association is mediated by their NH\(_2\) termini, which contain putative coiled-coil regions likely to be important in heterotypic interactions that promote the formation of the regulatory complex. The high degree of specificity in the observed binding supports the biological relevance of such interactions. Assigning functions to the different regions in these S4-like ATPases should lead to a better understanding of the structure and function of the 26 S protease.

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FIG. 7. Binding of truncated forms of S4 to S7. A, schematic representation of full-length S4 and four truncated derivatives used to probe RC subunits bound to nitrocellulose filters. Regions in S4 were deleted by restriction endonuclease digestion as described under "Experimental Procedures." B, the \([^{35]}S\)-labeled translation products of S4 and its truncated derivatives (A) were analyzed on a 12% SDS-gel either directly (–) or following immunoprecipitation (IP) with S4-specific rabbit antiserum. Under the conditions used for immunoprecipitation, none of the translation products of S6, S6', and luciferase was precipitated by this antiserum (not shown). C, human RC subunits separated by SDS-PAGE (10% polyacrylamide) were transferred to nitrocellulose and incubated with \([^{35]}S\)-labeled S4 or truncated S4 derivatives (A and B). Binding of the radiolabeled products was detected by PhosphorImager analysis or autoradiography.

incorporation into the regulatory complex, these regions might become free to bind potential substrates.

The fact that six distinct ATPases are found in the regulatory complex raises the question of whether they form a hexameric ring like p97, another member of the AAA family (65). If the ATPases sit in direct contact with the proteasome α-subunits, this model is difficult to reconcile with published images of the 26 S protease (22, 23). A hexameric ring of roughly 45-kDa proteins would have a diameter larger than the underlying proteasome and would extend beyond the edges of the cylinder. By contrast, a tetramer of ATPases would be expected to have a diameter less than the proteasome, more consistent with the images of Peters et al. (22) and Yoshimura et al. (23). In this regard, we found that S4 bound three ATPases in solution, S6, S7, and S8, in contrast to S10b and S6', which only bound each other both on nitrocellulose filters and on sucrose gradients (Figs. 4 and 6). These results raise the possibility that a tetramer of ATPases (S4, S6, S7, S8) contacts the proteasome and that two ATPases, S10b and S6', are located more peripherally.

Three non-ATPases were bound by radiolabeled subunit 10b.

TABLE III

Predicted NH\(_2\)-terminal coiled-coil regions in S4-like ATPases

| S4-like ATPase | Probability (no weights) |
|----------------|-------------------------|
| S4             | 0.94*                   |
| S6             | 0.98                    |
| S7             | 0.99                    |
| S8             | 1.00                    |
| S10b           | 1.00                    |
| S6'            | 0.99                    |

* Numbers represent the position in the sequence relative to the first methionine in the mature protein.
