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Mapping Subunit Contacts in the Regulatory Complex of the 26 S Proteasome

S2 AND S5b FORM A TETRAMER WITH ATPase SUBUNITS S4 and S7*

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The 19 S regulatory complex (RC) of the 26 S proteasome is composed of at least 18 different subunits, including six ATPases that form specific pairs S4-S7, S6-S8, and S6′-S10b *in vitro*. One of the largest regulatory complex subunits, S2, was translated in reticulocyte lysate containing [^{35}S]methionine and used to probe membranes containing SDS-polyacrylamide gel electrophoresis separated RC subunits. S2 bound to two ATPases, S4 and S7. Association of S2 with regulatory complex subunits was also assayed by co-translation and sedimentation. S2 formed an immunoprecipitable heterotrimer upon co-translation with S4 and S7. The non-ATPase S5b also formed a ternary complex with S4 and S7 and the three proteins assembled into a tetramer with S2. Neither S2 nor S5b formed complexes with S6′-S10b dimers or with S6-S8 oligomers. The use of chimeric ATPases demonstrated that S2 binds the NH_2-terminal region of S4 and the COOH-terminal two-thirds of S7. Conversely, S5b binds the COOH-terminal two-thirds of S4 and to S7's NH_2-terminal region. The demonstrated association of S2 with ATPases in the mammalian 19 S regulatory complex is consistent with and extends the recent finding that the yeast RC is composed of two subcomplexes, the lid and the base (Glickman, M. H., Rubin, D. M., Coux, O., Wefes, I., Pfeifer, G., Cejka, Z., Baumeister, W., Fried, V. A., and Finley, D. (1998) *Cell 94*, 615–623).

The 26 S proteasome is the major intracellular protease in eukaryotes and the only protease known to degrade proteins modified by polyubiquitin (polyUb)[1] chains (2–5). Because of its central role in the ubiquitin-mediated proteolytic pathway, this large ATP-dependent enzyme is involved in a wide variety of cellular processes, including cell cycle regulation (6–13), antigen presentation (14–18), inflammation (19), and the selective degradation of short-lived and abnormal intracellular proteins (5, 20). The 26 S proteasome is assembled from two multisubunit complexes: the 20 S proteasome and the 19 S regulatory complex (21–27). The 20 S proteasome is composed of 28 subunits arranged into four stacked heptameric rings. These rings form a cylinder with the proteolytic active sites isolated from the external solvent within a central chamber (28, 29). The 28 subunits can be grouped into two families according to their evolutionary relationship to the α and β subunits present in the *Thermoplasma acidophilum* proteasome. The α subunits form two identical end rings, whereas the two inner rings are made of β subunits that contain the catalytic NH_2-terminal Thr nucleophiles (30, 31). The fact that the ends of *Saccharomyces cerevisiae* proteasomes are sealed (29) suggests that in eukaryotes a mechanism must exist to deliver protein substrates to the catalytic sites. The 19 S regulatory complex is an obvious candidate for providing this function.

In the presence of ATP, the 20 S proteasome associates with the 19 S regulatory complex (RC), which confers polyUb chain recognition, ATP dependence, and the ability to degrade proteins (23, 25, 32). The human and *S. cerevisiae* regulatory complexes are composed of at least 18 different subunits (33, 34). Six of the subunits (S4, S6, S6′, S7, S8, and S10b) constitute the S4-like ATPase subfamily within the "AAA" family of ATPases (35). Sequence identity in the nucleotide binding modules of these proteins is ~60%, and overall, the S4-like ATPases are the RC subunits most conserved throughout evolution (36, 37). S4-like ATPases are essential for growth in yeast and are thought to catalyze the unfolding and translocation of substrates down the proteasome's axis (4, 38). Some of the remaining 12 non-ATPase subunits have limited homology to the p40 and p47 components of the eukaryotic initiation factor 3 complex and the Sgn3, Sgn5, and Sgn6 subunits of the signalosome (34, 39, 40). One of the non-ATPase subunits, S5a, binds ubiquitin (Ub) conjugates and polyUb chains *in vitro* (41). It also inhibits Ub-lysozyme and Ub-cyclin B conjugate degradation when added in excess to reticulocyte lysates and *Xenopus* egg extracts, respectively (42). However, because S5a is not essential in yeast there must be other RC components that recognize polyUb chains (43).

In contrast to the substantial information on the mechanism (30, 31) and structure of the 20 S proteasome, which includes crystal structures for the *T. acidophilum* and *S. cerevisiae* enzymes (28, 29), little is known about the arrangement and function of subunits in the regulatory complex. We previously showed that the six ATPases associate in pairs and proposed that their NH_2-terminal regions are involved in assembly of the regulatory complex (33). In this report we demonstrate that one of the ATPase pairs, S4-S7, interacts directly with subunits 2 and 5b. Experiments using radiolabeled S2 fragments indicate that a central portion of the S2 sequence specifically binds S4,

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[1] The abbreviations used are: polyUb, polyubiquitin; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; NPAGE, nondenaturing gel electrophoresis; PCR, polymerase chain reaction; RC, regulatory complex; RIPA, radioimmunoprecipitation assay buffer; TBST, Tris-buffered saline with Tween 20.

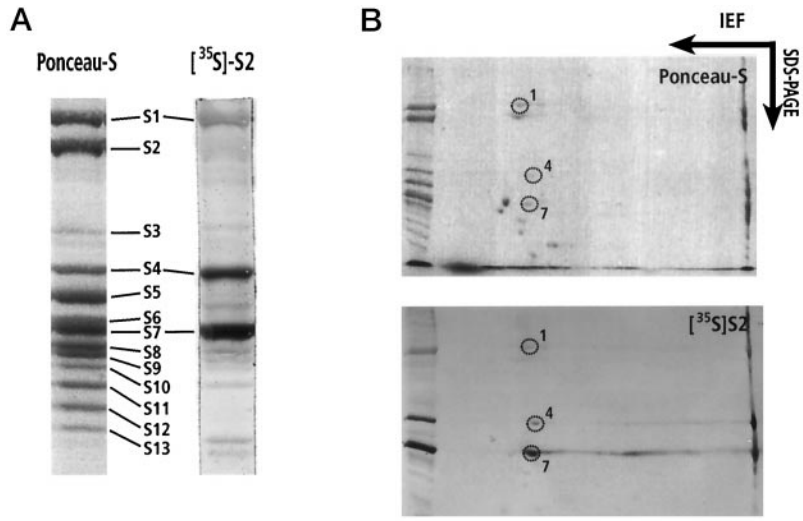

FIG. 1. **Binding of ^{35}S-labeled S2 to separated regulatory complex subunits.** *A*, purified regulatory complexes (50 μg) of the 26 S protease were separated on 10% SDS gels and transferred to nitrocellulose. The position of migration of individual subunits was determined by Ponceau S staining (*left*). The bound proteins were visualized by exposure to a PhosphorImager screen and autoradiography (*right*). Subunits of the RC are labeled from *top* to *bottom* according to Dubiel *et al.* (36). *B*, samples of purified regulatory complexes (25 μg) were subjected to two-dimensional gel electrophoresis as described under "Materials and Methods." Proteins were transferred to nitrocellulose, stained with Ponceau S (*top*), and incubated with ^{35}S-labeled S2 (*bottom*). The membrane was exposed to a PhosphorImager screen and x-ray film to visualized the bound ^{35}S-labeled S2. The position of migration of individual RC subunits in the two-dimensional gel was determined as described previously (33). Significant streaking of S4 and S7 occurred during two-dimensional PAGE as shown by the binding of ^{35}S-labeled S2 to "basic" forms of these two subunits.

and using chimeras between the S6′ ATPase and S4 or S7, we show that the NH_2-terminal portion of S4 and the COOH-terminal region of S7 bind to S2. By contrast, the COOH-terminal region of S4 and the NH_2-terminal portion of S7 are required for their association with S5b. The results presented here are consistent with the recent demonstration by Glickman *et al.* (1) that the yeast RC is composed of two subcomplexes, the lid and the base. We extend their findings by demonstrating specific contacts between subunits within the base subcomplex of the human RC. This information may provide insights regarding catalytic mechanisms of the regulatory complex.

MATERIALS AND METHODS

Preparation of 26 S Proteasome and Regulatory Complex—Human and bovine 26 S proteasomes and regulatory complexes were purified from red blood cells as described (33). The purified protein complexes were assayed for ATP-dependent peptidase activity (44) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of Full-length Subunit 2—A DNA fragment comprising nucleotides 1–93 (fragment A) was generated by overlapping extension PCR (45), using the S2 cDNA sequence published by Tsurumi *et al.* (46). A G27A point mutation, which did not change the S2 amino acid sequence, was introduced in the cDNA to remove an *Hae*II restriction site. A second fragment (fragment B) comprising nucleotides 53–233 was obtained using a partial pBluescript-S2 clone (nucleotides 63–2882) as a template. Fragments A and B were then annealed and amplified (fragment C). The original clone and fragment C were digested with *Bam*HI and *Hae*II, ligated, and transformed into Novablue competent cells. The full-length S2 was then subcloned into pAED4 between the *Bam*HI and *Eco*RI sites. The resulting S2 clone was sequenced to verify that mutations were not present.

Analysis of the S2 Sequence—The human S2 clone used in these studies differs at four residues (E32G, A60V, A415G, and V793M) from the sequence published by Tsurumi *et al.* (46). The cDNA was sequenced twice in both directions, and ambiguities were not found at these positions. Both Val^{60} and Gly^{415} in our S2 sequence are conserved in the budding yeast Rpn1 and the fission yeast Mts4 proteins, whereas Glu^{32} and Val^{793} are not (12, 46). Thus, Gly^{32} and Met^{793} in the S2 clone used here may represent polymorphisms.

Antibodies—An S2 fragment (nucleotides 1249–2013) was produced by PCR using pAED4-S2 as template and cloned into pAED4 using the *Xho*I-*Bam*HI restriction sites. This fragment was subcloned into pET-16b (Novagen) and introduced into BL21(DE3) competent cells. Recombinant protein was produced by induction with 0.25 mM isopropyl-β-D-thiogalactopyranoside and used to immunize New Zealand White rabbits as described by Harlow and Lane (47). Anti-S4 and anti-S5b polyclonal antibodies were prepared as described (33, 48). Anti-S10b polyclonal antiserum was a gift from Robert Benezra of the Memorial-Sloan Kettering Cancer Center (New York).

Preparation of ATPase Chimeras—Unique *Nde*I and *Kpn*I sites were introduced in the cDNA sequences of the six regulatory complex ATPases by overlapping extension PCR (45). The resulting products were subcloned into pAED4 under the control of the T7 promoter and sequenced to verify that no other mutations were present. The constructs were digested with *Nde*I and *Kpn*I, and the purified DNA fragments were used to replace the first 330, 327, and 294 base pairs of the S4, S6′, and S7 sequences, respectively.

In Vitro Transcription and Translation—S2, S5b, and ATPase chimeras were cloned into pAED4 and transcribed from the T7 promoter. Coupled transcription and translation of ATPase subunits, S5b, S2, and ATPase chimeras was performed as described previously (33), except that the reactions contained 80 μCi of [^{35}S]methionine (1000 Ci/mmol, NEN Life Science Products). Unincorporated methionine was removed on 1 or 5 ml Sephadex-G25 columns equilibrated in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl. The radiolabeled proteins were used without further purification in binding or sucrose gradient sedimentation assays.

Gel Electrophoresis—SDS-PAGE was performed using 10% separating gels and 4.5% stacking gels according to Laemmli (49). Two-dimensional gel electrophoresis (two-dimensional PAGE) of regulatory complexes was performed in a Bio-Rad Mini-PROTEAN®II 2-D cell using 2.4% (w/v) ampholines, pH 3.5–9.5, and 0.3% (w/v) ampholines, pH 9–11, and a Mini-PROTEAN®II slab gel system (50). Nondenaturing polyacrylamide gel electrophoresis (NPAGE) was performed as described previously (23).

Electroblotting and Incubation with Radioactive Subunits and ATPase Chimeras—Human or bovine regulatory complexes separated by SDS-PAGE were transferred to nitrocellulose overnight at 100 mA according to the method of Towbin *et al.* (51). Proteins were stained with Ponceau S, and the position of individual subunits was marked with waterproof ink. The membranes were blocked overnight at 4 °C in 10% nonfat dried milk in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Subsequently, the membranes were incubated with *in vitro* translated ^{35}S-labeled S2 or ^{35}S-labeled ATPase chimeras ($1–2 \times 10^6$ cpm/ml) overnight at 4 °C in 5% milk in TBST. The membranes were washed five times for 5 min and once for 10 min in TBST, air-dried, and exposed to a PhosphorImager screen or x-ray film (X-Omat AR (Eastman Kodak Co.) or Hyperfilm™-βmax (Amersham Pharmacia Biotech)) for autoradiography.

Sucrose Gradient Sedimentation—^{35}S-Labeled subunits were sedimented through 5–20% or 10–30% sucrose gradients as described pre-

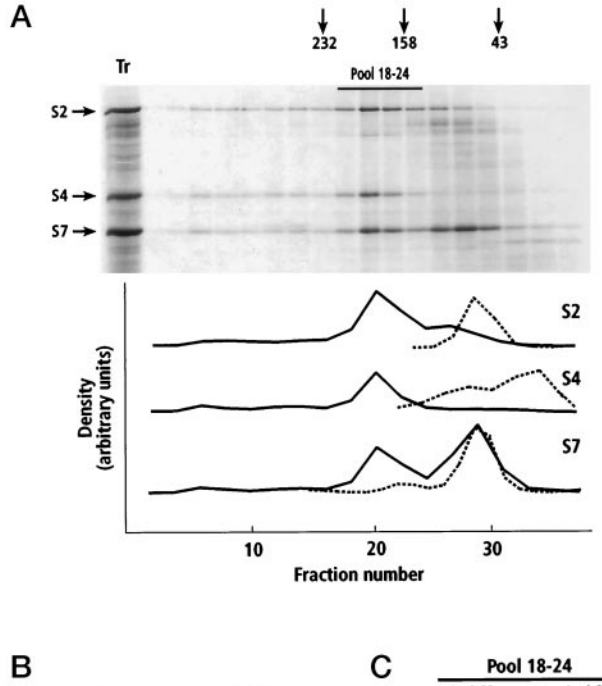

FIG. 2. **Sucrose gradient sedimentation of co-translated ^{35}S-labeled S2, S4, and S7.** *A*, *top*: S2, S4, and S7 were co-transcribed and co-translated in rabbit reticulocyte lysate containing [^{35}S]methionine. The translation mixture was layered atop a 10–30% sucrose gradient and centrifuged at 39,000 rpm in a Beckman SW 50.1 rotor for 18 h at 4 °C. Fractions (125 μl) were collected from the bottom, and 60-μl aliquots were analyzed on a 10% polyacrylamide gel. Proteins were visualized by exposure to a PhosphorImager screen. Sizing standards included catalase (232 kDa), aldolase (158 kDa), and ovalbumin (43 kDa). *Tr* designates a sample of the translation mixture. *Bottom*, the relative intensity of bands corresponding to full-length ^{35}S-labeled S2, S4, or S7 was determined by PhosphorImager analysis. The values (*density*) were then plotted *versus* fraction number to determine the distribution of S2 and the two ATPases across the gradient. The *dashed lines* show the distribution of S2, S4, and S7 translated alone. The *solid lines* show the distribution of each subunit upon co-translation. *B*, S2 was transcribed and translated in reticulocyte lysate containing [^{35}S]methionine and immunoprecipitated with either preimmune IgG (*P*) or anti-S2 and anti-S4 (*I*) polyclonal antibodies as described under "Materials and Methods." The *asterisks* designate smaller translation products of S2 presumably generated by proteolysis or initiation from internal methionine residues. *C*, based upon the distribution of S2 across the gradient shown in *A*, fractions were pooled as indicated and immunoprecipitated with either anti-S2 or anti-S4 antibodies. The relative abundance of S2, S4, and S7 in the precipitates was determined by densitometry using the NIH Image version 1.61 software. Upon correction of the density values (arbitrary units) for the number of Met residues in each protein, the S2:S4:S7 ratio was 0.6:1.0:0.8 in the anti-S2 precipitate and 0.3:1.0:0.9 in the anti-S4 precipitate. The difference in S2 abundance in both samples may result from the fact that S4 and S7 form tetramers upon synthesis (33) which, like the trimer, sediment near the aldolase marker. Thus, S4-S7 tetramers are presumably precipitated along with S2-S4-S7 ternary complexes using anti-S4 antibodies. It also appears that S2 proteolysis occurred during immunoprecipitation because S2 fragments can be detected in anti-S2 and anti-S4 precipitates (*asterisks*).

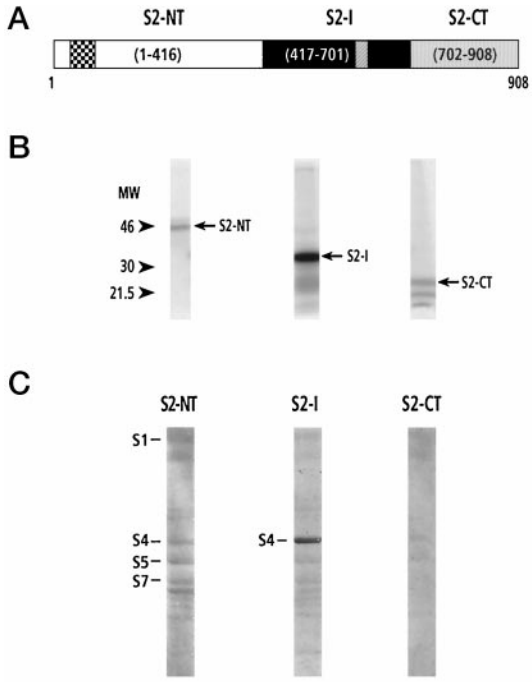

FIG. 3. **Binding of ^{35}S-labeled S2 fragments to regulatory complex subunits separated by SDS-PAGE.** *A*, schematic representation of three fragments of S2, S2-NT (residues 1–416, *white*), S2-I (residues 417–701, *black*), and S2-CT (residues 702–908, *shaded*), generated by PCR and transcribed and translated in rabbit reticulocyte lysate containing [^{35}S]methionine. *B*, SDS-PAGE (10% polyacrylamide) analysis of the resulting ^{35}S-labeled S2 fragments used for the binding experiments in *C*. *C*, the radiolabeled fragments (equal amounts of counts/min/ml) were incubated with nitrocellulose membranes containing regulatory complex subunits separated by SDS-PAGE. Bound ^{35}S-labeled proteins were visualized by autoradiography and by exposure to a PhosphorImager screen. The position of migration of individual RC subunits was determined by Ponceau S staining of the filters after SDS-PAGE and electroblotting and marked with waterproof ink. Because binding of the S2-NT fragment to RC components was significantly less than binding of the whole S2 molecule (see Fig. 1), we consider the observed binding to be nonspecific. *MW*, molecular weight markers. The (▨) denotes a putative coiled coil in the S2 sequence (52, 53). The (▨) represents a KEKE motif (55).

viously (33). The gradients were fractionated from the bottom (125 μl fractions), and 60-μl aliquots were analyzed by SDS-PAGE. The gels were fixed in 40% methanol, 10% acetic acid in water, dried under vacuum, and exposed to a PhosphorImager screen or Hyperfilm™-βmax for autoradiography. The sedimentation of protein standards (catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (BSA, 67 kDa), and ovalbumin (43 kDa)) was used to estimate the size of the complexes obtained from RC subunits.

Immunoprecipitations—Fractions from the sucrose gradients containing ATPase subcomplexes were pooled and immunoprecipitated with anti-S2 or anti-S4 polyclonal antibodies. Briefly, aliquots (300 μl) of the pooled fractions were diluted to 500 μl with 2.5 × RIPA buffer (125 mM Tris-HCl, pH 7.4, 375 mM NaCl, 2.5% Nonidet P-40, 0.25% SDS, 5 mM EDTA, 2.5 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml each pepstatin A, aprotinin, antipain, and leupeptin). Antibodies (5 μl) were added to each sample and incubated overnight at 4 °C. Immune complexes were precipitated by incubating the samples with 25 μl of protein A/G PLUS™-agarose for 2 h at 4 °C followed by centrifugation. The sedimented beads were washed five to six times for 5 min with 1 ml of RIPA buffer and applied to SDS-PAGE. Proteins were visualized by autoradiography using Hyperfilm™-βmax.

Partial Dissociation of Purified Regulatory Complexes—Purified human regulatory complexes (1 mg) were incubated in 10 mM Tris-HCl, pH 7.0, containing 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, plus 0, 0.3, 0.9, 2.7, or 3.6 M urea for 1 h at 4 °C. The mixtures were then layered atop 11.5-ml 15–45% sucrose gradients and centrifuged at 39,000 rpm for 19 h at 4 °C using a SW 41 rotor. Gradients were fractionated from the bottom (0.25 ml), and 90-μl aliquots were analyzed by SDS-PAGE. There was little dissociation of the RC after incubation with 0.3 and 0.9 M urea (not shown), whereas intermediate dissociation products were generated after incubation with 2.7 M urea. By contrast, incubation with 3.6 M urea substantially dissociated the regulatory complex into free subunits, which sedimented near the top of the sucrose gradient (data not shown). From

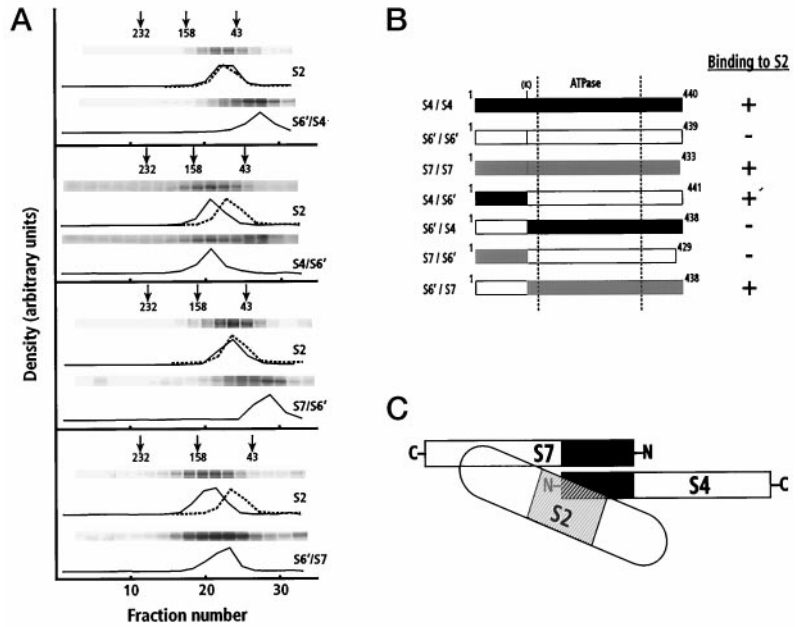

FIG. 4. **Binding of ^{35}S-labeled ATPases and ATPase chimeras to S2.** S2 was co-transcribed and co-translated with S4, S7, S6′, or ATPase chimeras in rabbit reticulocyte lysate containing ^{35}S-labeled methionine. The translation mixtures were then sedimented through 10–30% sucrose gradients. Conditions for sedimentation and analysis of the gradient fractions are given in the legend for Fig. 2. *A*, sedimentation of S2 co-translated with chimeras of S4 and S7. Proteins were visualized by autoradiography and are shown directly above traces obtained by densitometry that indicate the distribution of full-length S2 and ATPase chimeras across the gradients. The distribution of S2 translated alone is depicted by *dashed lines*. The sedimentation of S2 co-translated with chimeras is shown by *solid lines*. Binding of S2 to S4, S7, S4/S6′ (*second panel*), and S6′/S7 (*fourth panel*) resulted in a shift in the distribution of S2 to a position near the aldolase marker (a peak centered around 140 kDa). *B*, schematic representation of ATPases and ATPase chimeras and their binding to S2. *K* represents the *Kpn*I site introduced in each ATPase cDNA to construct the chimeras. *C*, the COOH-terminal portion of S7 (in *white*) and the NH_2-terminal region of S4 (in *black*) associate with S2. The middle portion of S2 (*hatched*) binds S4. Because S2 binds different regions on S4 and S7, we depict these ATPases binding each other in an antiparallel orientation, although, at present, there is no experimental evidence for this proposition (see also legend for Fig. 7*C*).

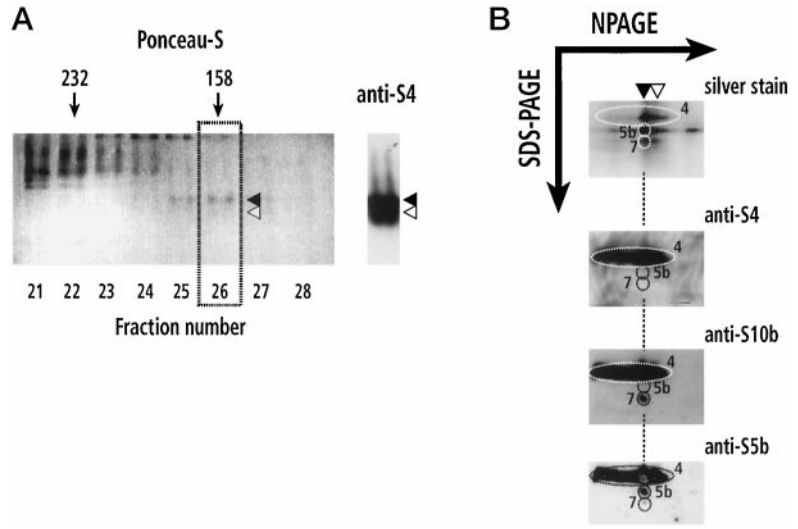

FIG. 5. **Association of S5b with S4 and S7 in a subcomplex generated by urea dissociation of the 19 S RC.** Purified human red blood cell regulatory complexes (1 mg) were partially dissociated in 2.7 M urea as described under "Materials and Methods" and layered atop a 15–45% sucrose gradient (11.5 ml). The gradient was centrifuged at 39,000 rpm for 19 h at 4 °C using a Beckman SW 41 rotor. The gradients was fractionated from the bottom (0.25 ml), and aliquots (90 μl) were applied to nondenaturing gel electrophoresis (*NPAGE*). *A*, proteins were transferred to nitrocellulose, stained with Ponceau S, and immunoblotted with anti-S4 polyclonal antibodies. Fraction 26 contained two major dissociation products that reacted with anti-S4 antibodies after NPAGE (*closed* and *open arrowheads*). *B*, lanes corresponding to this fraction were excised and laid perpendicular to second dimension 10% polyacrylamide SDS gels. The gels were either silver-stained or proteins were transferred onto a nitrocellulose membrane for immunoblotting with anti-RC subunit antibodies as described under "Materials and Methods." The membrane was incubated sequentially with anti-S4, anti-S10b, and anti-S5b polyclonal antisera and stripped between each antibody incubation in 62.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS for 45 min at 50–60 °C. Antibody binding was detected by enhanced chemiluminescence for 15 s (anti-S4) or 10 min (anti-S5b and anti-S10b). Neither anti-S10b nor anti-S5b antisera cross-react with S4. The persistent reactivity at the S4 position is due to incomplete removal of the anti-S4 IgG and long exposure times. The expected position of migration of S4, S5b, and S7 are indicated by the *dotted* areas. We have shown previously that anti-S10b antibodies cross-react with S7 (33). The *dashed lines* indicate the position of migration on the NPAGE gels of the putative S4, S5b, S7 complex. Similar results were also obtained with fractions 24 and 25 of the sucrose gradient (not shown).

these observations we infer that subcomplexes of RC subunits do not reform on the sucrose gradient. Aliquots (90 μl) from the 2.7 M urea-treated RC gradient were analyzed by NPAGE. Specific lanes from fractions corresponding to 158–232 kDa were excised and further analyzed on second dimension 10% SDS gels. Proteins were either stained using the Silver Stain Plus® kit or transferred to nitrocellulose for

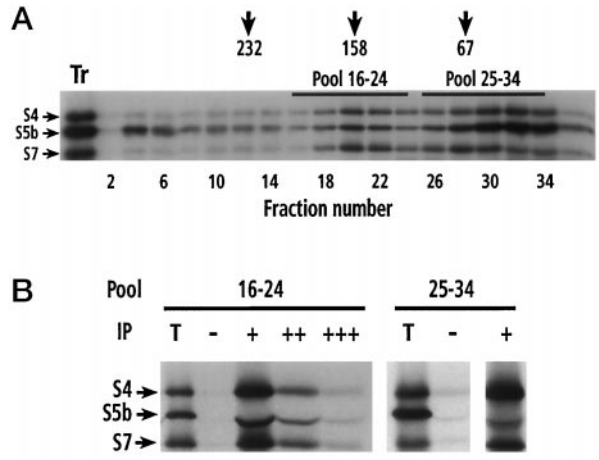

FIG. 6. **Sucrose gradient sedimentation of co-translated S4, S5b, and S7.** *A*, S4, S5b, and S7 were transcribed and translated in lysate containing radiolabeled methionine and layered atop a 10–30% sucrose gradient. Conditions for sedimentation and analysis of the gradient fractions are given in the legend for Fig. 2. BSA (67 kDa) was used as a sizing standard instead of ovalbumin. A sample of the translation mixture is designated as *Tr*. *B*, fractions were pooled as indicated based on the distribution of S4 across the gradient and subjected to immunoprecipitation (*IP*) analyses. A pool of fractions 16–24 was subjected to three consecutive rounds of immunoprecipitation with anti-S4 polyclonal antibodies (+, ++, +++). As a control, fractions were also precipitated with preimmune IgG (−). A sample of the pooled fractions before immunoprecipitation (*T*) is shown for comparison. The stoichiometry of each subunit in the immunoprecipitates was calculated by densitometry using the NIH Image version 1.61 software and corrected for the number of methionine residues in each protein. Setting the amount of S4 as 1.0, the ratio of S5b to S4 was 0.7, 0.9, and 1.2, and the ratio of S7 to S4 was 0.9, 1.2, and 1.4 for the +, ++, and +++ immunoprecipitates, respectively. Although S4, S5b, and S7 were also immunoprecipitated with anti-S4 from a pool of fractions 25–34, the abundance of each protein was significantly different. S4 and S7 were highly abundant with only very small amounts of S5b being recovered in the precipitate. The presence of S7 in the precipitate can be attributed to the formation of S4-S7 dimers that sediment near the BSA marker (33).

immunoblotting with anti-RC subunit antibodies as described previously (33).

RESULTS

Binding of ^{35}S-Labeled S2 to Regulatory Complex Subunits—We used far Western blots to examine binding of S2, the second largest human RC subunit (100 kDa), to other components in the regulatory complex. Subunits of purified human regulatory complexes were separated by SDS-PAGE, transferred to nitrocellulose, and the membrane was incubated with ^{35}S-labeled S2 translated *in vitro*. S2 bound two ATPases, S4 and S7 (Fig. 1*A*), which migrate on SDS gels with apparent molecular masses of 56 and 47 kDa, respectively; this assignment was confirmed by two-dimensional PAGE (Fig. 1*B*). In both analyses, radiolabeled S2 also bound, albeit more weakly, to S1 (M_r = 110,000) (Fig. 1, *A* and *B*). However, association of S2 with S1 was not detected by sucrose gradient sedimentation after the two subunits were co-synthesized in reticulocyte lysate (data not shown).

Sedimentation of S2 Co-translated with S4 and S7—Translated alone, S2 and S4 sediment as monomers, whereas S7 forms oligomers (33). When S2 was co-translated with S4 and S7, the three proteins co-sedimented in a complex the size expected for a heterotrimer (Fig. 2*A*). The apparent trimer, fractions 18–24 in Fig. 2*A*, was incubated with anti-S2 or anti-S4 polyclonal antibodies, and both antibodies precipitated the three subunits (Fig. 2*B*). Although almost equal amounts of S2, S4, and S7 were present in the anti-S2 precipitate, less S2 was present in the anti-S4 precipitate (see Fig. 2 legend). The sedimentation of S2 was also altered when co-translated with either S4 or S7 alone, forming what appears to be a dimer with each ATPase. However, anti-S4 precipitated little S2 from fractions containing the putative dimers (data not shown). As expected, S2 was not precipitated by anti-S4 (Fig. 2*C*), nor did we observe interactions between S2 and the remaining four ATPases alone or in pairs (S6-S8 and S6′-S10b). These experiments show that S2 forms an immunoprecipitable trimer with S4 and S7, but not with other ATPase pairs. S2 also forms dimers with S4 or S7, which are apparently not stable enough to detect by immunoprecipitation.

The Central Region of S2 Binds S4—NH_2 terminal regions in S4-like ATPases and the sequence Ala^{36}–Gly^{70} of S2 are predicted to form coiled coils (52, 53). The sequence of human S2 (46) also contains a KEKE motif (Lys^{623}–Glu^{641}). Both of these motifs have been proposed to mediate protein-protein interactions (53–55). To determine which regions of S2 are responsible for its association with S4 and S7, PCR was used to generate synthetic genes encoding three S2 segments. These three pieces are designated S2-NT (Met^1–Met^{416}) which contains the putative coiled coil, S2-I (Ile^{417}–Asn^{701}) containing the KEKE motif, and S2-CT (Pro^{702}–Leu^{908}), which lacks known motifs in the PROSITE database (see Fig. 3*A*). Each fragment of S2 was synthesized in reticulocyte lysate containing [^{35}S]methionine (Fig. 3*B*) and tested for its ability to bind S4 or S7 on nitrocellulose filters. S2-NT bound weakly to a variety of RC subunits, including S1, S4, S5, S7, S8, and S9 (Fig. 3*C*). The widespread, but low level, binding presumably reflects nonspecific association with RC subunits that could result from misfolding of the S2-NT fragment. The S2-I fragment specifically bound to S4 indicating that residues 417–701 of S2 contain a major binding site for this ATPase. S2-CT bound weakly and presumably nonspecifically to S4 and S5. None of the S2 fragments bound S7. Because full-length ^{35}S-labeled S2 efficiently binds to S7 after SDS-PAGE and electroblotting (Fig. 1), these results suggest that either the S2-NT and S2-CT fragments do not fold properly upon synthesis in lysate or that the binding site for S7 spans at least two of the S2 segments.

S2 Binds the NH_2-terminal Region of S4 and the COOH-terminal Portion of S7—As part of a separate project to identify functional regions in the RC ATPases, we have constructed a series of ATPase chimeras in which the NH_2-terminal 100–150 residues have been exchanged among the six ATPases.[2] The chimeras allowed us to test whether S2 binds NH_2- or COOH-terminal regions in S4 and S7. For these experiments, we used chimeras between S4 or S7 and the S6′ ATPase since S2 does not bind S6′. The chimeric ATPases were co-translated with S2 and analyzed on 10–30% sucrose gradients (Fig. 4). Following co-translation of S6′/S4 with S2, each protein sedimented as a monomer (Fig. 4*A*, *top panel*). By contrast, the S4/S6′ chimera formed a stable complex with S2 that sedimented as an apparent dimer (Fig. 4*A*, *second panel from top*). Thus, the NH_2-terminal region of S4, which mediates its association with S7 (33), is also involved in interaction with S2. A fraction of S6′/S7 molecules co-sedimented as an apparent heterodimer upon co-translation with S2, whereas the S7/S6′ chimera sedimented as a monomer under the same conditions (Fig. 4*A*, *two bottom panels*), indicating that the COOH-terminal two-thirds of S7 mediates its association with S2. These experiments not only confirm the binding of S4 and S7 to S2, they identify regions in the ATPases (NH_2-terminal for S4, COOH-terminal for S7) responsible for their association with S2 (Fig. 4*C*).

Subunit 5b Forms a Trimer with S4 and S7—The S5b sub-

[2] C. Gorbea, D. Taillandier, and M. Rechsteiner, manuscript in preparation.

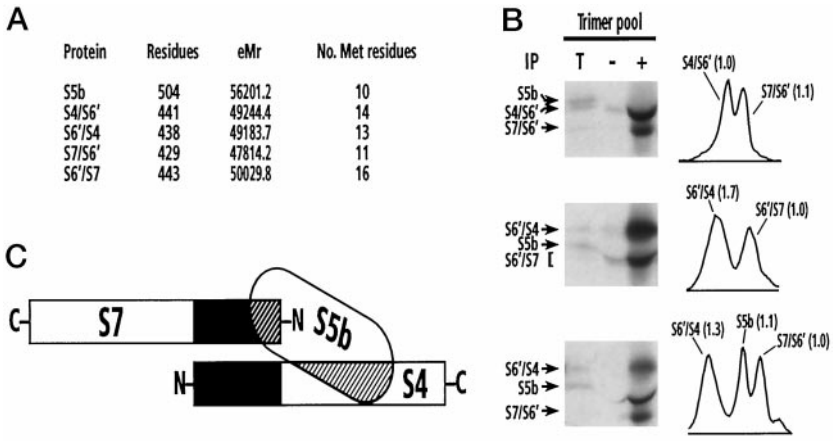

FIG. 7. **Association of S5b with chimeric ATPases.** Subunit 5b of the regulatory complex was co-transcribed and co-translated with pairs of S4 and S7 chimeras in reticulocyte lysate containing [^{35}S]methionine. The radiolabeled proteins were sedimented through 5 ml 5–20% sucrose gradients, fractionated, and analyzed as described in the legend for Fig. 2. The radiolabeled proteins were visualized by exposure to a PhosphorImager screen or x-ray film. *A*, properties of S5b and S4 and S7 chimeras. *eMr*, calculated molecular mass (daltons). *B*, *left*: fractions sedimenting around the position of the aldolase marker (158 kDa), where chimeras/S5b trimeric complexes would be expected to sediment, were pooled and immunoprecipitated (*IP*) with either preimmune IgG (−) or anti-S4 polyclonal antibodies (+). Immune complexes were analyzed by SDS-PAGE (10% polyacrylamide) and exposed to x-ray film for 7 days. A sample of the pooled fractions (*T*) is shown for comparison. The *bracket* depicts S6′/S7, which is synthesized as a doublet in reticulocyte lysate. *Right*, the intensity of each protein band in anti-S4 immunoprecipitates was quantitated by densitometry using the NIH Image version 1.61 software. The integrated areas (arbitrary units) were then corrected for the number of Met residues present in each protein (*A*) and used to calculate the relative abundance of each species in the precipitate (*numbers* in *parentheses*). The S4/S6′ and S6′/S7 chimeras neither bound to each other nor to S5b and sedimented as oligomers and monomers, respectively (data not shown). Both S4/S6′ plus S7/S6′ and S6′/S4 plus S6′/S7 pairs of chimeras formed immunoprecipitable complexes (*top* and *middle panels*). However, only S5b, S6′/S4, and S7/S6′ formed a complex containing stoichiometric amounts of each protein (*bottom* panel). This putative trimer was only evident upon immunoprecipitation and not after sucrose gradient sedimentation (not shown). *C*, model of binding of S5b to the NH_2- and COOH-terminal portions of S7 and S4, respectively. We have shown that the NH_2-terminal region of S4, which contains a putative coiled coil, is required for binding to S7 (33). Since S6′ is monomeric following synthesis (33), the S4/S6′ and S7/S6′ complex demonstrates that the NH_2-terminal regions of S4 and S7 are sufficient to mediate the interaction of S6′ molecules. However, because the S6′/S4 and S6′/S7 chimeras also bind to one another, and we have not shown that removal of the NH_2-terminal coiled coil region of S7 abrogates its binding to S4, binding of S4 and S7 via their corresponding NH_2-terminal regions remains hypothetical.

unit of the human regulatory complex (48) is not encoded in the yeast genome, and its function is unknown. However, it has low homology with p55, the bovine homolog of the Rpn5 subunit of *S. cerevisiae* lid subcomplexes (1, 56). As an alternate method to mapping RC subunit interactions by assembly, we have partially dissociated purified regulatory complexes in urea and subjected the resulting subcomplexes to sucrose gradient sedimentation. Electrophoretic analyses of fractions co-sedimenting with aldolase (~160 kDa) revealed that one of the RC dissociation products is a trimer of S4, S7, and S5b (Fig. 5, *A* and *B*, *solid arrowheads*). In light of the S4-S5b-S7 trimer seen following urea dissociation of RCs, we asked whether the three proteins would co-assemble upon synthesis in reticulocyte lysate. For these experiments, S5b was co-translated with pairs of ATPases (*e.g.* S4-S7, S6-S8, and S6′-S10b) previously shown to associate *in vitro* (33), and the translation products were analyzed by sucrose gradient centrifugation. The results shown in Fig. 6*A* demonstrate that S5b forms a ternary complex with S4 and S7. Because co-translation of S4 and S7 yields tetramers that, like the S4-S5b-S7 trimer, sediment on sucrose gradients near the aldolase marker (not shown), we performed three consecutive rounds of immunoprecipitation with anti-S4. Each immunoprecipitate contained the three subunits in equal amounts (Fig. 6*B*), indicating that virtually all copies of each protein were present as heterotrimers. It is notable that in the presence of S5b, the S4 and S7 ATPases formed, at most, a few tetramers (Fig. 6). Because S5b did not form a heterodimer with either S4 or S7 (not shown), assembly of the S4-S7 dimer appears to be required for subsequent formation of the S4-S5b-S7 trimer. Interactions were not observed between S5b and S6′-S10b or S6-S8. Also, ^{35}S-labeled S5b did not bind S4 or S7 in far Western assays (not shown).

S5b Binds the NH_2-terminal Region of S7 and the COOH-terminal Portion of S4—We used S4 and S7 chimeras to determine which regions in these ATPases are required for binding to S5b. Different pairs of S4 and S7 chimeras were co-translated with S5b, and the products were analyzed on 5–20% sucrose gradients. Immunoprecipitation with anti-S4 antibodies of pooled fractions from the sucrose gradients revealed that S5b formed a complex with the S6′/S4 and S7/S6′ chimeras, whereas it did not interact with either S4/S6′ plus S6′/S7 (not shown), S6′/S4 plus S6′/S7, or S4/S6′ plus S7/S6′ pairs of chimeras (Fig. 7). Thus, in the trimeric complex, S5b contacts the NH_2-terminal region of S7 and the COOH-terminal portion of S4.

S2 and S5b Form a Tetramer with S4 and S7—Because the S4 and S7 ATPases formed ternary complexes with both S2 and S5b, we co-translated both S2 and S5b non-ATPases with S4 and S7 to determine whether they compete for binding the ATPase dimer. As shown in Fig. 8, the four proteins sedimented as expected for a tetramer (*panel A*). Furthermore, anti-S2 and anti-S4 immunoprecipitates contained S2, S4, S7, and S5b in nearly equal amounts (Fig. 8*B*). S4, S7, and the two non-ATPases were also present as smaller oligomers near the 160-kDa aldolase marker (Fig. 8*A*). These lighter fractions presumably contain a mixture of two trimers, one composed of S2, S4, and S7, and another containing S5b plus S4 and S7. The two ATPases, S4 and S7, were the only proteins recovered in anti-S4 immunoprecipitates from fractions sedimenting with or slower than the BSA marker (Fig. 8*B*). Co-translation of S2 and S5b, followed by sucrose gradient sedimentation provided evidence that these two subunits do not interact directly (Fig. 8*C*).

DISCUSSION

Interactions between ATPases and non-ATPase Subunits—In determining interactions between human regulatory complex subunits, we have used far Western blotting and *in vitro* assembly assays similar to those employed to identify S5a as a

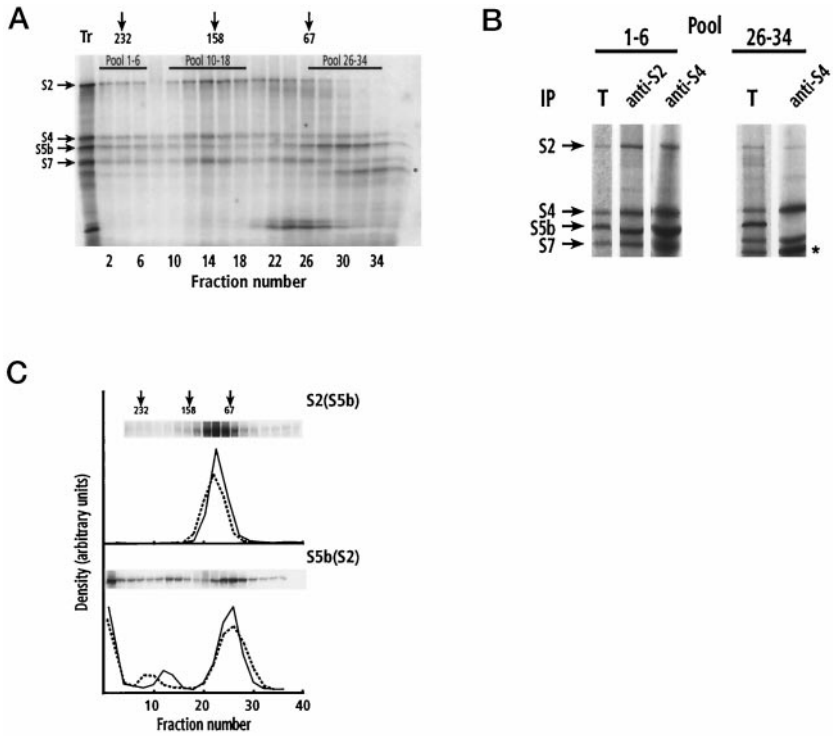

FIG. 8. **Sucrose gradient sedimentation of co-translated S2, S4, S7, and S5b.** The four RC subunits, S2, S4, S7, and S5b, were translated in the presence of [^{35}S]methionine as described under "Materials and Methods" and sedimented on a 5–20% sucrose gradient at 39,000 rpm for 18 h at 4 °C using a SW 50.1 rotor (*A*). Conditions for fractionation and analysis of the gradient fractions are given in the legend for Fig. 2. *B*, fractions were pooled as indicated and subjected to immunoprecipitation (*IP*) analyses with anti-S2 or anti-S4 polyclonal antibodies. For comparison, aliquots of the pooled fractions before immunoprecipitation (*T*) are shown next to SDS-PAGE analyses of the anti-S2 and anti-S4 immunoprecipitates (+). The starting materials used in both anti-S2 and anti-S4 immunoprecipitation experiments were identical. However, anti-S4 antibodies were the better immunoprecipitating reagent so shorter exposure times were thus required to visualize ^{35}S-labeled subunits. Anti-S2 and anti-S4 immunoprecipitates of fractions 10–18 (*A*) yielded the four subunits in nonstoichiometric amounts (not shown). Presumably, this is because of the presence of a mixture of ternary complexes (*i.e.* predominantly S2-S4-S7 but also S4-S5b-S7). S2 and S5b do not interact directly upon co-translation and sucrose gradient sedimentation (*C*); only S2 was recovered in anti-S2 immunoprecipitates (not shown). Sizing markers for the gradients were catalase (232 kDa), aldolase (158 kDa), and BSA (67 kDa). Samples of the translation products are designated *Tr*. The *asterisks* denote a smaller S4 translation product in pool 26–34 that was precipitated by anti-S4 antibodies.

polyUb binding subunit and to demonstrate specific pair formation among ATPase subunits (33, 41). One of the central findings from the studies presented here, that S2 interacts with the S4-S7 dimer, is supported by results from both assays. Binding of ^{35}S-labeled S2 to S4 and S7 on nitrocellulose filters was unambiguous (Fig. 1*A*), and in solution the three proteins formed a trimer stable to immunoprecipitation (Fig. 2). Whereas anti-S2 precipitated S2, S4, and S7 in roughly equal amounts, full-length S2 was underrepresented in the anti-S4 precipitate. There are several possible explanations for the reduced amount of S2 in the anti-S4 precipitate. Newly synthesized S4 and S7 also form a tetramer which sediments near the aldolase marker. If these tetramers were present in the gradient pool, they would only be precipitated by anti-S4. It is also possible that S2 was lost from the anti-S4 precipitate either by dissociation from the S2-S4-S7 trimer during washing or by proteolysis, which was evident (see Fig. 2*C* legend).

Association between S2 and S4 was maintained when chimeric ATPases or fragments of S2 were used in the assays (Figs. 3 and 4). In fact, these experiments revealed that S2 binds the COOH-terminal two-thirds of S7 and that a central portion of S2 binds the NH_2-terminal region of S4 (Fig. 3). S4 and S7 also bound a second non-ATPase subunit, S5b (Figs. 5 and 6). Although association of S5b with S4-S7 was only observed in the co-assembly assay, the existence of the putative trimer is substantiated by immunoprecipitation and by the appearance of an S4-S5b-S7 trimer among the urea dissociation products of the human regulatory complex. Association of S5b with S4 and S7 required the COOH-terminal portion of S4 and the NH_2-terminal region of S7 (Fig. 7). The observation that S2 and S5b bind different regions on S4 and S7 can explain why a S2-S4-S5b-S7 tetramer readily forms (Fig. 8).

Evidence Supporting the Proposed Associations of S2 with Specific ATPases—In previous studies that demonstrated pairing of RC ATPases *in vitro* (33), we argued that the inherent specificity of binding among the ATPases provided evidence that the observed associations reflect *bona fide* contacts among RC components. This argument can be extended in light of the results presented here. If S4 and S7 were not immediate neighbors, it seems unlikely that S2 and S5b would form a tetramer with these two ATPases and fail to interact with the other four ATPases. In a sense, the results in Figs. 1, 2, 5, 6, and 8 provide support for the original proposition that S4 and S7 bind one another directly. Association of S4 with S7 and S2 with S4 is also supported by studies on fission yeast 26S proteasomes. Gordon *et al.* (9) have found that overexpression of mouse S7 suppresses a temperature-sensitive mutation in *Schizosaccharomyces pombe* S4, and Wilkinson *et al.* (12) have demonstrated direct interaction between *S. pombe* S2 and S4. The results from fission yeast and the highly specific interactions between S2, S4, S5b, and S7 demonstrated here provide substantial evidence that the four subunits form a cluster within the human regulatory complex. Ferreira *et al.* (57) have recently found that the cyclophilin-like domain of Ran-binding protein 2 mediates its association with a subcomplex of RC components that includes S1, S2, S3 and S6. Thus, in addition to the S4-S7 pair, S2 may physically interact with S1. In fact, the far Western blot in Fig. 1*B* shows weak binding of S2 to S1.

On the Location of S5a and S5b within the RC—The polyUb-binding subunit, Rpn10 or S5a, does not seem to be essential for either assembly of the 26 S proteasome or for most physiological functions of the enzyme since deletion of the *RPN10* gene is not lethal in yeast (43). Human S5a did not bind any RC components in far Western assays, and to date, we have not detected its interaction with other RC components using the co-translation and sedimentation approach. Presumably, S5a binds to S1 and/or S2 or the regulatory complex ATPases because it is found in the base subcomplex of the yeast 26 S proteasome (1). S5b of the human RC does not have an ortholog in *S. cerevisiae*. For this reason, it is not clear whether it should be considered a component of the RC lid or base. As the sequence of S5b does not contain PCI or MPN domains characteristic of lid components (58, 59), we assume that S5b is a component of the base subcomplex in higher eukaryotes. It clearly binds tightly to other base components.

Amino acid sequences are known for all 18 subunits in the mammalian RC. With the exception of the six ATPases, this information has not provided insight into their functions. We clearly need to know what each RC component does during the degradation of protein substrates. It is also important that we localize subunits within the regulatory complex since this may provide clues as to their function. The experiments presented above are a step in that direction. We believe that there is good evidence that S2 and S5b bind the S4-S7 ATPase pair. Nonetheless, the subunit associations inferred from our studies will require confirmation by other techniques such as dissociation of regulatory complexes, electron microscopy of antibody decorated 26 S proteasomes and, ultimately, crystallography. Although x-ray diffraction has produced detailed pictures of 20 S proteasomes (28, 29), solving a crystal structure for the 26 S proteasome will prove more difficult due to the subunit complexity of the regulatory complex and its asymmetry (32). In the meantime, the information presented here on the arrangement of subunits within the base complex should prove useful for further structural analyses on the RC and may provide insights concerning enzymatic mechanisms.

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