SUG1, a Component of the 26 S Proteasome, Is an ATPase Stimulated by Specific RNAs

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SUG1 is an integral component of the 26 S proteasome. Belonging to a novel putative ATPase family, it shares four conserved motifs characteristic of ATP-dependent DNA/RNA helicases. Recombinant rat SUG1 (rSUG1) produced in Escherichia coli was highly purified and characterized in terms of its biochemical properties. The rSUG1 exhibited a Mg\(^{2+}\)-dependent ATPase activity. The K_m for ATP and V_max of rSUG1 were 35 \(\mu\)M and 7 pmol of ATP/min/\(\mu\)g of protein, respectively. Both ATPase activity to release \[^{32}P\]monophosphate and \[^{32}P\]ATP-labeling activity were coordinately affected by core and an ATPase-containing 22 S regulatory complex (3, 4). Cell cycle progression (1, 2). It is composed of the 20 S catalytic subunit with a highly conserved ATPase module with 200 amino acids and a variety of cellular activities) (5). The family members have a putative ATP-dependent RNA/DNA helicases (6–8). We previously reported that mouse SUG1 interacts with various nuclear receptors. Actually, some transcription factors are regulated by proteasomal-dependent proteolysis (1). Biochemical study is required to resolve issues such as how the ATPases are involved in RNA metabolism such as transcriptional regulation. In this study, we report the purification and characterization of rat SUG1. We found that rSUG1 exhibited ATPase activity that was specifically stimulated by particular RNA molecules.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 37, Issue of September 12, pp. 23201–23205, 1997

Experimental Procedures

Expression and Purification of Rat SUG1—SUG1 cDNA was cloned from a rat liver cDNA library as described previously (6). Histidine-tagged rSUG1 was overexpressed in Escherichia coli by use of the pET vector system (16). Insoluble recombinant rSUG1 was purified by Ni\(^{2+}\)-agarose under denaturing conditions according to the instructions supplied by Qiagen. The resulting proteins were further subjected to a preparative SDS-PAGE and recovered from the excised gels. The protein was redissolved in a urea-containing buffer (25 mM Tris-HCl (pH 7.5), 0.3 mM NaCl, 1 mM 2-mercaptoethanol, 0.1% Nonidet P-40, 10% glycerol, and 8 mM urea), and the urea was gradually removed by dialysis. These proteins were analyzed by 10% SDS-PAGE and stained with Coomassie Brilliant Blue.

Assay for ATP Hydrolysis—In the case of Fig. 3A, the ATPase activity was assayed by thin layer chromatography as described (32). Reactions (20 \(\mu\)l) contained 0.5 \(\mu\)M of purified rSUG1 in buffer A (20 mM Tris-HCl (pH 7.5), 70 mM KCl, 2.5 mM MgCl_2, 1.5 mM dithiothreitol, 500 \(\mu\)M ATP, and 1.25 \(\mu\)Ci of \[^{32}P\]ATP). ATP hydrolysis reactions were allowed to proceed at 37 °C for 30 min. Radioactive phosphate released from \[^{32}P\]ATP was separated on a polyethyleneimine plate (Macherey-Nagel) using 1 M formic acid and 0.5 M lithium chloride. The released phosphates were visualized by autoradiography.

In other cases, the ATPase activity was assayed using activated charcoal (Sigma) as described by Armon et al. (17). The purified recombinant SUG1 (0.5 \(\mu\)g) was incubated at 37 °C for 30 min in buffer A. On the basis of \(V_{\text{max}}\), amounts of RNA homopolymers were precisely determined from their A_260. Control reactions without rSUG1 were carried out in parallel tubes, and the control value (radioactivity) was subtracted from each experimental one. Each assay was done in triplicate, and the results were presented as a simple arithmetic average.

ATP Cross-linking Assay—ATP cross-linking assays were performed as described by Pause and Sonenberg (18). Reactions mixture (20 \(\mu\)l) containing 0.5 \(\mu\)g of rSUG1 in a buffer (20 mM Tris-HCl (pH 7.5), 70 mM Mg\(^{2+}\), 3 mM ATP, 500 \(\mu\)M GTP, 35 \(\mu\)Ci of \[^{32}P\]ATP, 10 \(\mu\)Ci of \[^{32}P\]GTP, and 10 \(\mu\)Ci of \[^{32}P\]UTP) were set up in a total volume of 20 \(\mu\)l. The reactions were started by the addition of rSUG1. The reactions were stopped at 0, 15, and 30 min, and the samples were precipitated by addition of acid-precipitable material. After phosphotolysis, the samples were incubated with proteinase K at 37 °C for 20 min and then precipitated with ethanol.

The abbreviations used are: rSUG1, rat SUG1; PAGE, polyacrylamide gel electrophoresis.

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Printed in U.S.A.
RESULTS

Expression and Purification of Recombinant Rat SUG1—We cloned rat SUG1 (rSUG1) cDNA and found it to encode a protein of 406 amino acids (Fig. 1) and to have exactly the same structure as human p45 (21), the human homolog of yeast SUG1. The central regions of rSUG1 and the other four proteasomal ATPases share greater than 60% identity and are designated as the ATPase module (Fig. 1). This domain contains a putative ATP-binding motif, GXXGKT, and ATP hydrolysis motif, DEID, which is analogous to the DEID box protein domains including many ATP-dependent RNA/DNA helicases (22–25). Two additional sequences in rSUG1, SAT and (H/Q)R, are also characteristic motifs for RNA/DNA helicases (Fig. 1).

To investigate the enzymatic nature of rSUG1, we subcloned its cDNA into the pET3a vector and expressed rSUG1 as a fusion protein with a histidine-tag appended to its N terminus in E. coli. Although rSUG1 protein was able to be overexpressed in E. coli (Fig. 2, lane 2), most of the protein was obtained in an insoluble fraction. We attempted to dissolve the insoluble protein in a urea-containing buffer and to purify it under denaturing conditions. Purification by Ni\textsuperscript{2+}-agarose resulted in >40% pure protein as judged by inspection of the Coomassie Brilliant Blue-stained SDS-acrylamide gel (Fig. 2, lane 3). To further remove contaminating E. coli proteins, we subjected the protein sample to a preparative SDS-PAGE and subsequently excised the rSUG1 and extracted it from the gel. The recovered proteins were dissolved in a urea-containing buffer and then refolded by dialysis to gradually remove urea (see “Experimental Procedures”). As expected, the final preparation was apparently pure as judged by SDS-PAGE (Fig. 2, lane 4).

rSUG1 Protein Exhibits ATPase Activity—The ATPase activity of the purified rSUG1 was tested by its ability to release radioactive phosphate from [γ-\textsuperscript{32}P]ATP as described under “Experimental Procedures.” A commercial ATPase from dog kidney was used as a positive control (Fig. 3A, lane 1). We found that rSUG1 hydrolyzed ATP to release monophosphate (Fig. 3A, lane 2) but that the enzyme activity could not be detected in a parallel sample prepared from control E. coli (data not shown).

Fig. 2. Expression and purification of rSUG1. Histidine-tagged rSUG1 was overproduced and purified from E. coli. Lane 1, E. coli extract; lane 2, E. coli extract (induction by isopropyl-1-thio-β-D-galactopyranoside); lane 3, purification by Ni\textsuperscript{2+}-agarose under denaturing conditions; lane 4, rSUG1 recovered from preparative SDS-PAGE. Each sample was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue.

Fig. 3. ATPase activity of rSUG1. A, assay for rSUG1 ATPase. The ATPase activity was measured by thin layer chromatography as described under “Experimental Procedures.” B, nucleoside triphosphate specificity of rSUG1. The ATPase activity was assayed by the charcoal method described under “Experimental Procedures.” Cold nucleoside triphosphates were added to the mixture at a final concentration of 5 mM. Relative ATPase activities are shown.
determined the $K_m$ for ATP to be 35 $\mu$M and the $V_{max}$ to be 7 pmol of ATP/min/μg of protein (Table I).

To examine the nucleotide specificity for the SUG1-catalyzed hydrolysis, we employed unlabeled nucleotides as competitors. Expectedly, we found ATP was the most potent competitor; however, CTP competed little with ATP for hydrolysis. GTP also highly stimulated by poly(U) and poly(C), but not by poly(A).

Effects of Polyribopyrimidines on the rSUG1 Functions—Because SUG1 contains a DEID motif, the protein is considered to belong to a subfamily of the DEAD-box proteins. This family includes more than 100 proteins with diverse functions, and some of these proteins have an RNA-stimulated ATPase activity (27, 28). So we investigated whether or not nucleic acids would affect the ATPase activity of rSUG1. The enzyme activity was not affected by single-stranded or double-stranded DNA, whereas the rSUG1 ATPase activity was stimulated 6-fold by poly(U) (Fig. 5A). When the other RNA homopolymers were tested, we observed significant enhancement of the ATPase activity by poly(A) and poly(U). It is noteworthy that the homoribopolymer stimulation of the rSUG1 ATPase activity is different from that of Yhs4p, whose activity is enhanced by single stranded-DNA, double-stranded DNA, and RNA (26).

We also determined the kinetic parameters of the rSUG1 ATPase in the presence of poly(U). The $K_m$ and $V_{max}$ values showed similar affinity ($K_m$) for ATP in the hydrolysis reaction with or without poly(U) (Table I). In contrast, addition of 1 μg of poly(U) increased the $V_{max}$ 4-fold (Table I). When poly(C) was used, similar results were obtained (data not shown). The increased ATP cross-linking of rSUG1 by poly(U) and poly(C) correlated with their higher rate of ATP hydrolysis, but not with their ATP binding ability, since the $K_m$ values of rSUG1 were similar in the presence or absence of the polyribopyrimidines. We suppose that the cross-linking assay used here detects two molecular species, i.e. ATP-bound rSUG1 and ADP-bound rSUG1. The ATP cross-linking of rSUG1 is suggested to reflect the abilities to both bind and hydrolyze ATP.

**Stimulation of rSUG1 ATPase Activity by Particular Types of RNA**—On the basis of the above results, it was important to examine whether natural RNA can stimulate the ATP hydrolysis reaction of rSUG1. Cellular total RNA, poly(A)$^+$ RNAs, and poly(A)$^-$ RNAs were added to the ATPase reaction. As shown in Fig. 6A, total RNA slightly stimulated the ATPase activity (1.5-fold). It was most striking that the ATPase activity was highly enhanced (6-fold) by poly(A)$^+$ RNA, whereas poly(A)$^-$ RNA showed little effect on the ATPase activity of rSUG1. The stimulation of rSUG1 ATPase activity by poly(A)$^+$ RNAs is consistent with their higher rate of ATP hydrolysis, but not with their ATP binding ability, since the $K_m$ values of rSUG1 were similar in the presence or absence of the polyribopyrimidines. We suppose that the cross-linking assay used here detects two molecular species, i.e. ATP-bound rSUG1 and ADP-bound rSUG1. The ATP cross-linking of rSUG1 is suggested to reflect the abilities to both bind and hydrolyze ATP.
poly(A) RNA exhibited apparently no effect (Fig. 6A). This finding suggests that the rSUG1 activity is specifically stimulated by mRNA.

We therefore examined the effect of various kinds of mRNA-type RNAs on the ATP hydrolysis. Four kinds of cDNAs encoding rat SUG1, MSS1 (6), HTF (30), and TIP120 (31) in pBluescript were transcribed by T7 or T3 RNA polymerase in vitro to produce sense and antisense RNAs as described under "Experimental Procedures." Each kind of RNA was then added to the reaction mixture as indicated. Each point represents mean ± S.E. of five experiments.

**DISCUSSION**

In this paper, we have provided evidence that rat SUG1 exhibits ATPase activity. Our rSUG1 preparation was apparently pure, and S9 cell-expressed soluble rSUG1 protein also showed the same enzyme properties (data not shown) as obtained with the renatured bacterially expressed protein. This agreement suggests that the nature of rSUG1 presented in this study reflects intrinsic enzyme activities. Importantly, biochemical characterization of the recombinant rSUG1 yielded the following significant findings. (i) The rSUG1 ATPase activity is specifically stimulated by particular types of RNAs including poly(C) and poly(U), but not by single-stranded and double-stranded DNAs. (ii) Polyribopyrimidines also enhance the labeling of rSUG1 with [γ-32P]ATP. (iii) Cellular poly(A) RNAs and in vitro transcribed specific RNAs enhance ATP hydrolysis.

Although several DEAD-box family proteins show ATPase activity facilitated by RNA, the observation that rSUG1 exhibits a specific RNA requirement is particularly interesting. rSUG1 is the first example of a proteasomal ATPase having such a requirement. Yhs4p, a yeast homolog of human S4, is activated by unspecified RNAs and DNA commonly (26). It is of interest to investigate the effect of nucleic acids on the ATPase activity of human S4 and to compare the results between the human and yeast ATPases. Other mammalian pro-

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