Purified Human SUV3p Exhibits Multiple-Substrate Unwinding Activity upon Conformational Change†

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ABSTRACT: Suv3 of *Saccharomyces cerevisiae* has been classified as a mitochondrial RNA helicase. However, the helicase domain in both yeast and human SUV3 varies considerably from the typical RNA helicase motifs. To investigate its enzymatic activities, a homogeneously purified preparation of SUV3 is required. Expression of a processed form of human SUV3 carrying an N-terminal deletion of 46 amino acids (SUV3ΔN46) in a yeast suv3 null mutant, which otherwise fails to grow in a nonfermentable carbon source and forms petite colonies in glucose medium, rescues the null phenotype. Through a five-step chromatographic procedure, an 83 kDa SUV3ΔN46 protein (SUV3−83) and a partially degraded 70 kDa product (SUV3−70) containing amino acids 68−685 were purified to homogeneity. Single- or double-stranded DNA and RNA stimulated ATPase activity of both proteins. SUV3−70, which retains core catalytic domains, can bind and unwind multiple duplex substrates of RNA and DNA with a 5′−3′ directionality over a wide range of pH, while SUV3−83 has helicase activity at only acidic pH. ATP, but not nonhydrolyzable ATP, is essential for the unwinding activity, suggesting the requirement of the energy derived from ATP hydrolysis. Consistent with this notion, suv3 mutants containing alanine (A) or arginine (R) substitutions at the conserved lysine residue in the ATP binding site (K213) lost ATPase activity and also failed to unwind the substrates. Importantly, circular dichroism (CD) spectral analysis showed that SUV3−83, at pH 5.0, adopts a conformation similar to that of SUV3−70, suggesting a conformational change in SUV3−83 is required for its helicase activity. The physiological relevance of the multiple-substrate helicase activity of human SUV3 is discussed.

DNA helicases play a significant role in DNA replication, repair, and recombination (*1*). Likewise, RNA−RNA and RNA−DNA helicases play major roles in transcription, translation, and RNA splicing (*2*, *3*). Consistent with their roles in nucleic acid metabolism, mutations in helicases have been linked to several human genetic diseases, including Werner syndrome (WRN), Bloom syndrome (BLM), and Xeroderma pigmentosum (*2*). The basic biochemical reactions catalyzed by all helicases, namely, nucleic acid binding, NTP[1] binding, and NTP hydrolysis-dependent unwinding of nucleic acids, may be similar. However, their affinity for different nucleic acid substrates, a preference for a particular NTP, and active assembly state (monomer, dimer, or hexamer) can be different (*4*, *5*). WRN, BLM, and RecG are examples of helicases exhibiting specificity toward specialized structures, such as replication forks or Holliday junctions (*6*, *7*). Identification of substrate specificity is an important step toward unraveling the molecular function of helicases.

Suv3 of *Saccharomyces cerevisiae* is a putative ATP-dependent RNA helicase in the DEAD/DExH family that functions in turnover and processing of RNA in mitochondria (*8*−*10*). Human SUV3 was identified on the basis of the homology of its sequence to that of yeast Suv3 (*11*). Sequence homology analysis of databases also identified other putative Suv3 homologue in *Caenorhabditis elegans*, *Arabidopsis thaliana*, and mouse (*11*). Suv3-like proteins have been proposed to form a distinct and conserved family, as they share more homology within the group than with any other family of helicases (*11*, *12*). One biochemical defect detected in yeast suv3 null mutants is a failure to process mitochondrial RNA precursors (*9*, *10*), which results in overaccumulation of excised group I introns. These observations led to the concept that Suv3 plays a major role in RNA metabolism in yeast mitochondria; the absence of a fully functional Suv3 results in mitochondrial genomic loss and respiratory incompetence. A partially purified RNA "degradosome" from yeast mitochondria, consisting of Suv3 and an exoribonuclease, Dss1, possesses RNA helicase activity (*10*). However, whether purified Suv3 alone has any helicase activity is unclear.

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[1] Abbreviations: NTP, nucleoside triphosphate; ds, double-stranded; ss, single-stranded; ssc, single-stranded circular; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; KOAc, potassium acetate; $KO(Cit)_2$, potassium citrate; CD, circular dichroism; UV, ultraviolet; HD, heat-denatured; Sub, substrate; TE, Tris-EDTA buffer; TBE, Tris-boric acid-EDTA buffer; PMSF, phenylmethanesulfonyl fluoride; QFF, Q-Sepharose fast flow.

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In this study, we expressed a processed form of human SUV3, which has an N-terminal deletion of 46 amino acids (SUV3ΔN46), in a yeast suv3 null mutant. Expression of SUV3ΔN46 rescues the SUV3 null phenotype. To investigate their enzymatic activities, a SUV3ΔN46 protein, SUV3−83, and a partially degraded SUV3−70 were purified to homogeneity from the rescued yeast. Both proteins have the ATPase activity that is stimulated by polynucleotide acids. SUV3−70 can bind and unwind multiple duplex substrates of RNA and DNA with a 5′−3′ directionality in a pH-independent manner, while SUV3−83 has the helicase activity at only acidic pH. Significantly, circular dichroism spectral analysis showed that SUV3−83, at pH 5.0, adopts a conformation similar to that of SUV3−70, suggesting that a conformational change in SUV3−83 is required for its helicase activity. Although several other helicases exhibit multisubstrate specificity (*6*, *13*, *14*), this is, to our knowledge, the first helicase in which a conformational change can govern its enzymatic activity.

EXPERIMENTAL PROCEDURES

Yeast Strain and Plasmids. S. cerevisiae strain BWG1 [MAT**a** his134−519 ura3 ade1 leu2 $\omega^+\rho^+$ (*8*)] was from R. A. Butow (University of Texas Southwestern Medical Center, Dallas, TX). A SUV3 cDNA encoding SUV3ΔN46 was constructed by deleting the first 46 amino acids and replacing threonine 47 with methionine by site-directed mutagenesis (Quick Change site-directed mutagenesis kit, Stratagene, La Jolla, CA) using the full-length cDNA as the template. Expression of SUV3ΔN46 in multicopy plasmid pJJ1 was driven by a phosphoglycerate kinase (PGK) promoter to form pJJ1-SUV3ΔN46. To investigate the potential function of the Walker A motif (GXXXXGKT, amino acids 207−214) (*15*), two plasmids, pJJ1-SUV3ΔN46K213A and pJJ1-SUV3ΔN46K213R, carrying the indicated point mutations at lysine 213, were constructed by site-directed mutagenesis using pJJ1-SUV3ΔN46 as the template.

Disruption and Rescue Experiments. Yeast strain BWG1 [MAT**a** his134−519 ura3 ade1 leu2 $\omega^+\rho^+$ (*8*)] was first transformed with pJJ1, pJJ1-SUV3ΔN46, pJJ1-SUV3ΔN-46K213A, or pJJ1-SUV3ΔN46K213R and was plated on synthetic medium lacking leucine (SC-Leu). Individual colonies were picked from the SC-Leu plate, and the corresponding competent cells were then transformed with the yeast Suv3 knockout construct in which URA3 replaced a part of yeast Suv3 (C.-F. Chen, W.-H. Lee, *et al.*, manuscript submitted for publication). Transformants were selected on synthetic media lacking leucine and uracil. Disruptions were confirmed by PCR genotyping using primers outside the target region and inside the URA3 gene. Yeast cells lacking yeast Suv3 and carrying pJJ1, pJJ1-SUV3ΔN46, pJJ1-SUV3ΔN46K213A, or pJJ1-SUV3ΔN46K213R were grown in YP glucose (1% yeast extract, 2% bactopeptone, and 2% glucose) or in YP glycerol (1% yeast extract, 2% bactopeptone, and 2% glycerol).

Immunoblot Analysis. Immunoblotting analysis was performed as described previously (*16*) using a mouse anti-SUV3 monoclonal antibody generated against the GST−SUV3 fusion protein (amino acids 144−786) as the probe.

Overexpression and Purification of SUV3−83. A suv3 null BWG1 yeast strain harboring pJJ1-SUV3ΔN46 was cultured in medium lacking leucine and uracil. Detection of the human SUV3 protein was performed by immunoblotting using an anti-SUV3 monoclonal antibody. All the following steps were conducted at 4 °C. Yeast (25 g wet weight) were resuspended in cell breakage buffer [50 mM Tris-HCl (pH 8.0), 5% sucrose, 2.5 mM EDTA, 600 mM KCl, 2 mM DTT, 0.01% Nonidet P-40 with protease inhibitors, including aprotinin, antipain, leupeptin, and pepstatin at 1 μg/mL each, and 1 mM PMSF] at the ratio of 1:5 (w/v) and lysed using a French press at a high pressure. Cellular lysates were clarified by centrifugation (100000*g* for 45 min), and the supernatants were then subjected to ammonium sulfate precipitation at 0.21 g/mL (0−35%). The precipitates were collected by centrifugation (20000*g* for 20 min) and resuspended in ∼50 mL of T buffer [25 mM Tris-HCl (pH 8.0), 5% glycerol, 1 mM EDTA, 2 mM DTT, and protease inhibitors]. After the conductivity was adjusted to the value of buffer A (T buffer with 100 mM KCl), the supernatant was applied to a Q-Sepharose Fast Flow (QFF) column (8 mL) pre-equilibrated with buffer A and developed in a gradient of 10 to 50% buffer B (T buffer with 1 M KCl) over 5 column volumes. The peak of SUV3−83, which eluted at approximately 32% buffer B, was then directly applied to a HiTrap heparin column (5 mL), which was developed in a gradient of 20 to 50% buffer B over 4 column volumes. The peak fractions containing SUV3−83 which eluted around 32% buffer B were pooled and applied to a G-25 Sephadex column (50 mL) to remove the salt, and the protein fractions were then applied to a Mono Q (HR 5/5, 1 mL) column, which was developed in a gradient of 0 to 30% buffer B over 20 column volumes. SUV3−83, which eluted between 16 and 19% buffer B from the Mono Q column, was then applied to a Superdex 200 column (HR 10/30, 24 mL) in buffer A. Peak fractions were pooled and concentrated using Centricon concentrators (models YM-30 and YM-50) and stored at −70 °C.

Purification of SUV3−70. SUV3−70, a stable proteolytic product of SUV3−83, was first detected in QFF fractions in which SUV3−83 and SUV3−70 were eluted together. QFF fractions were loaded onto a HiTrap Blue affinity column (5 mL) and developed in a gradient of 20 to 100% buffer C (T buffer with 2 M KCl) over 4 column volumes. SUV3−70 was eluted at around 46% buffer C whereas SUV3−83 around 75%. The fractions containing SUV3−70 were dialyzed against buffer A and applied to the Mono Q (HR 5/5, 1 mL) column which was developed in a gradient of 0 to 30% buffer B over 20 column volumes. The SUV3−70 was eluted at around 9% buffer B, and the pooled fractions were then applied to a Superdex-200 column. Identification of SUV3−70 was based on its characteristic peptide pattern analyzed by mass spectrometry. The peak fractions from the Superdex-200 column were concentrated and stored at −70 °C.

All the columns and media used in the purification were from Amersham Pharmacia Biotech. The protein concentration was determined by UV absorption using a DU650 spectrophotometer (Beckman Instruments) with molar extinction coefficients for SUV3−83 and SUV3−70 being 67 110 and 61 420 M^{-1} cm^{-1}, respectively. Both SUV3−83 and SUV3−70 were more than 95% pure after Coomassie blue staining of an 8% SDS−PAGE gel (Figure 2C).

Mass Spectrometry and Edman Degradation. SUV3 proteins were digested in-gel using trypsin (Promega), and the resulting peptides were analyzed with Voyager DE-Pro (Applied Biosystems) based on matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry as described previously (*17*). The N-terminal sequence of the purified SUV3−70 was determined by Edman degradation using a PROCISE-cLC instrument (Applied Biosystems) as described previously (*17*)

ATPase Assays. The ATPase assay was carried out in a reaction volume of 20 μL containing ATPase buffer [25 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, 50 mM KCl, and 100 ng of BSA], 0.05 mCi/mL [γ-^{32}P]ATP (Amersham Biosciences), 100 μM ATP, and varying amounts of SUV3 proteins at 37 °C for 60 min as described previously (*12*). The reaction was terminated by adding 2 μL of 0.5 M EDTA. One microliter of each reaction mixture was then spotted onto polyethyleneimine cellulose thin-layer chromatography plates (Selecto Scientific, Suwanee, GA) and developed in 1 M formic acid with 0.5 M LiCl. The amounts of $^{32}P_i$ and [γ-^{32}P]ATP in the reaction mixture were determined using a Storm 320 Phosphor Imager (Molecular Dynamics, Piscataway, NJ) and quantified as picomoles of ATP hydrolyzed.

Factors that could potentially affect the ATPase activity, including cations and polynucleotide acids, were also tested under various conditions. To determine the effect of cations, 0.1 mM EDTA was used to remove contaminating divalent cations, and then $CaCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$ was added to a final concentration of 5 mM. To assay the effect of polynucleotide acids, ssRNA (R40), ssDNA (D40), dsRNA (R40−R14), or dsDNA (D40−D14) was added to the reaction mixture at a concentration of 20−200 μM (nucleotide base). Different pH conditions were also tested for the ATP hydrolysis activity of SUV3−83.

Helicase Substrates. Linear substrates (RNA−RNA, DNA−DNA, RNA−DNA, and DNA−RNA) were prepared by annealing 40-base oligonucleotides with complementary 5′-end-labeled 14-base oligonucleotides as previously described (*16*). The sequences of R14 and D14 were as described previously (*18*); R40 and D40 were identical to R44 and D44 (*18*), respectively, except for omission of four bases at the 3′-end. The shorter oligonucleotide was 5′-end-labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase (Roche Applied Science, Indianapolis, IN). In separate reactions, 5′-end-labeled DNA or RNA was mixed and annealed with a 2-fold excess of unlabeled complementary oligonucleotides in annealing buffer (1× TE with 200 mM NaCl). The annealed substrates were gel purified as described previously (*6*) and named R40−R14, R40−D14, D40−R14, and D40−D14.

The circular DNA helicase substrate was prepared by annealing a complementary 26-base oligonucleotide labeled at the 5′-end to ϕ×174 sscDNA (New England Biolabs, Beverly, MA), thus generating a partial duplex DNA as described previously (*19*).

The substrate for the directionality assay was constructed by hybridizing a 52-mer oligonucleotide (5′-CGA ACA ATT CAG CGG CTT TAA CCG GAC GCT CGA CGC CAT TAA TAA TGT TTT C-3′) to residues 702−753 of ϕ×174 sscDNA (*20*). After *Hpa*II digestion at the partial duplex region, the circular ϕ×174 sscDNA became a linear fragment containing a 22-nucleotide duplex region at its 5′-end and a 30-nucleotide duplex at its 3′-end. To blunt both ends, the linear substrate was filled in with one [α-^{32}P]dCTP and one cold dGTP using the Klenow fragment of DNA polymerase I. The resulting partial duplex substrate contained 24 and 32 bp at the 5′- and 3′-ends, respectively.

Helicase Assay. Unwinding reactions (20 μL) were carried out in helicase buffer [25 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.1 mg/mL BSA, 2.5 mM $MgCl_2$, and 3 mM ATP] containing 1 nM substrate and an increasing amount of SUV3−83 or SUV3−70 at 37 °C for 60 min. Reactions were terminated by adding 5 μL of "stop mix" loading buffer containing 0.1 M Tris-HCl (pH 7.5), 20 mM EDTA, 0.5% SDS, 0.1% Nonidet P-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 25% glycerol. Aliquots of the reaction were electrophoresed through 15% native polyacrylamide gels in 1× TBE to separate the displaced labeled short strand. Gels were dried, and the radioactivity of the labeled DNA was quantified using a Storm 320 Phosphor Imager (Molecular Dynamics).

The helicase directionality assay was performed under the same conditions except that 0.8 nM of labeled substrate was used. The mixtures were then loaded on an 8% PAGE gel containing 1× TBE, 0.1% SDS, and 3 M urea, and run in 1× TBE buffer. Urea was used to interfere with the potential folding of released fragments.

The effect of pH on the helicase activity of SUV3−83 was analyzed as described above, except that the helicase buffer contained the following different buffer reagents (all at 25 mM): sodium citrate (pH 3.5), sodium acetate (pH 4.5, 5.0, and 5.5), phosphate buffer (pH 6.5 and 7.5), Tris buffer (pH 8.5), and ethanolamine buffer (pH 9.5).

The effect of different salts on the helicase activity was analyzed by preincubating 4 μg of SUV3−83 in 100 μL of 1 M salts [KCl, KOAc, $KO(Cit)_2$, $MgSO_4$, or $(NH_4)_2SO_4$] for 15 min. One microliter of this mixture was then added to the unwinding reaction mixture as described above. The effect of different salt concentrations on helicase activity was measured by varying the concentration of 0−500 mM KCl directly in the helicase reaction mixtures as described above.

Substrate Binding Assays. Gel mobility shift assays on dsRNA and dsDNA were performed exactly as described for the helicase assay except that the nonhydrolyzable ATP analogue ATPγS, instead of ATP, was used. The reaction mixture was incubated with increasing amounts of SUV3−83 for 30 min at 37 °C and the reaction terminated by adding a stop mix (lacking SDS) as described for the helicase assay. SDS (1%) was added separately to an aliquot of reaction mixture with the largest amount of protein to disrupt the binding of protein and nucleic acid to indicate the migration position of the labeled nucleic acids. The reaction mixtures were loaded on 10% native polyacrylamide gels for linear RNA−RNA substrates and 1% agarose gel for circular DNA−DNA substrates and electrophoresed at 4 °C as described above.

Circular Dichroism. Circular dichroism experiments were conducted using an Olis RSM-1000 CD spectrophotometer at 20 °C with continuous nitrogen flow protection in a 1 mm path length cylindrical quartz cuvette. CD spectra were scanned in the far-UV region (195−250 nm) by setting a stepwise increment of 0.5 nm and integrated time of 5 s with a protein concentration of 0.3 mg/mL for SUV3−70 and 0.2

mg/mL for SUV3−83 in 25 mM sodium acetate (pH 5.0) and 25 mM Tris-HCl (pH 7.0). Each datum was collected four times and averaged.

RESULTS

Expression of SUV3ΔN46 Rescues the Growth Defects of suv3 Null S. cerevisiae. Deletion of Suv3 in *S. cerevisiae* is associated with respiratory incompetence (*8*). Cells fail to grow in a nonfermentable carbon source like glycerol and form petite colonies in glucose media (*8*). To test whether human SUV3 can rescue a null phenotype, full-length human SUV3 and an endogenously processed form lacking the 46 N-terminal amino acids, SUV3ΔN46, were expressed in suv3 null yeast from a single-copy plasmid (C.-F. Chen, W.-H. Lee, *et al.*, manuscript submitted for publication). Both forms rescue the lethal phenotype in glycerol growth medium, indicating that human SUV3 is a functional homologue in yeast. To investigate if SUV3 has intrinsic helicase activity, we attempted to overexpress a full-length human SUV3 protein using a pJJ1 vector that contains a 2μ replication origin to facilitate purification. However, this was problematic due to the insolubility and instability of the protein (data not shown). To circumvent this difficulty, SUV3ΔN46, the processed form of SUV3, and its point mutants (SUV3ΔN46-K213A and SUV3ΔN46K213R) carrying A and R substitution at an invariant K213 of the Walker A motif (*15*) were expressed in suv3 null yeast using a 2μ replication origin to test whether they can rescue the null phenotype (Figure 1A). SUV3ΔN46 complemented suv3 null cells as they no longer formed petite colonies on glucose and were also growth-competent in glycerol medium, while the vector alone and the two point mutants failed to rescue the growth defects of the suv3 null yeast (Figure 1B). Because the amounts of expressed protein from SUV3−83, SUV3−83K213A, and SUV3−83K213R were comparable (Figure 1C, lanes 2−4), these results suggest that SUV3−83, expressed from SUV3ΔN46, is a functional homologue of yeast SUV3 and the Walker A motif of SUV3−83 is critical to its function. Hence, we decided to purify SUV3−83 to elucidate the biochemical properties of SUV3.

Identification of SUV3−83 and SUV3−70. While purifying SUV3−83 from yeast (Figure 2A), we observed that SUV3−83 was processed into several truncated forms (Figure 2B, lane 2). The most stable proteolytic form was purified and named SUV3−70 (Figure 2C). Both SUV3−83 and SUV3−70 were identified by mass spectroscopy after trypsin digestion with peptides covering more than 50% of the amino acids being identified in each of these proteins (Figure 3). Identities of both SUV3−83 and SUV3−70 were further confirmed by immunoblotting using an anti-SUV3 antibody.

Since our initial experiments showed that SUV3−70, instead of SUV3−83, contains detectable helicase activity, we decided to characterize SUV3−70 that may serve as a comparison if the deleted region(s) has its influence in its enzymatic activity. N-Terminal sequencing revealed that SUV3−70 initiated at the residue 68 (Figure 3A). The molecular mass of SUV3−70 was determined to be 70 004 Da by mass spectrometry analysis without proteolysis (Figure 3C). On the basis of the molecular mass and the N-terminal sequence, the C-terminal residue was deduced to be amino acid 685 (calculated mass of 70 014 Da). Thus,

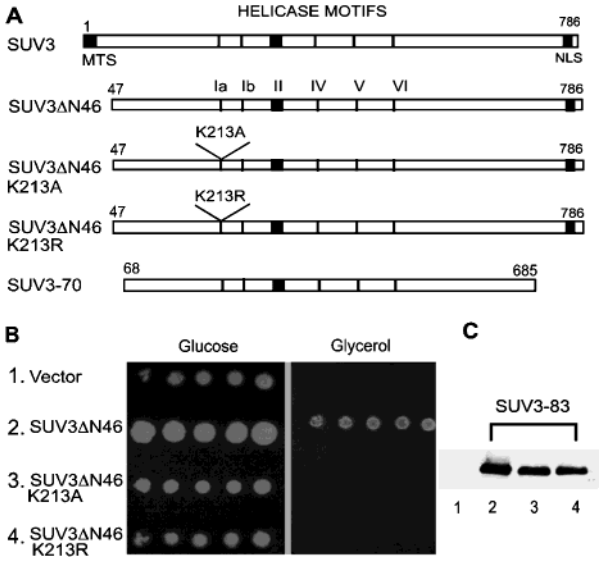

FIGURE 1: Human SUV3−83 is a functional homologue of yeast Suv3. (A) Schematic diagram of amino acid sequences of SUV3 proteins. The black boxes at the N- and C-termini represent the putative mitochondrial targeting signal (MTS) and the putative nuclear localization signal (NLS), respectively. Roman numerals (I−VI) denote the conserved helicase motifs. SUV3ΔN46 lacks the putative mitochondrial targeting signal (the first 21 residues) and an additional 25 amino acids at the N-terminus. SUV3−83K213A and SUV3−83K213R carry a mutation in the conserved lysine residue in motif I. SUV3−70 is a processed form of SUV3−83, which lacks 67 amino acids at the N-terminus and 101 amino acids at the C-terminus. (B) Growth of 5-fold dilutions of suv3 null yeast complemented with vector alone, SUV3ΔN46, SUV3−83K213A, or SUV3−83K213R on glucose- and glycerol-containing media. (C) Detection of SUV3 protein expression in suv3 null yeast transformed with vector alone (lane 1), SUV3ΔN46 (lane 2), SUV3−83K213A (lane 3), and SUV3−83K213R (lane 4) by immunoblotting using an anti-SUV3 monoclonal antibody.

SUV3−70 contains 618 residues from amino acids 68−685 of SUV3 and includes all the essential core helicase motifs (Figure 1A).

We next purified SUV3−83, SUV3−70, SUV3−83K213A, and SUV3−83K213R proteins on a large scale to near homogeneity as described in Experimental Procedures. Purified proteins were loaded on an 8% SDS−PAGE gel and found to be more than 95% pure by Coomassie blue staining (Figure 2C).

SUV3−83 and SUV3−70 Have ATPase Activity. SUV3 contains a Walker A motif at residues 207−214 that is involved in ATP hydrolysis in many other helicases (*21*, *22*). Thus, purified SUV3 proteins were tested for ATPase activity. SUV3−83 and SUV3−70 each exhibited ATPase activity that showed a linear relationship with the amount of enzyme at the given concentration and condition (Figure 4A). However, SUV3−70 exhibited higher activity than SUV3−83 (Figure 4A). In contrast, the Walker A motif mutants, SUV3−83K213A and SUV3−83K213R, were defective in ATP hydrolysis. Thus, SUV3 has intrinsic ATPase activity, which requires K213 within the Walker A motif.

We next investigated the effect of divalent cations on ATPase activity. A strict requirement for certain divalent cations, including Mg^{2+}, Mn^{2+}, and Co^{2+}, was observed, as ATPase activity was abolished in their absence, while replacement with Ca^{2+} and Zn^{2+} reduced the activity by ~60−80% (Figure 4B).

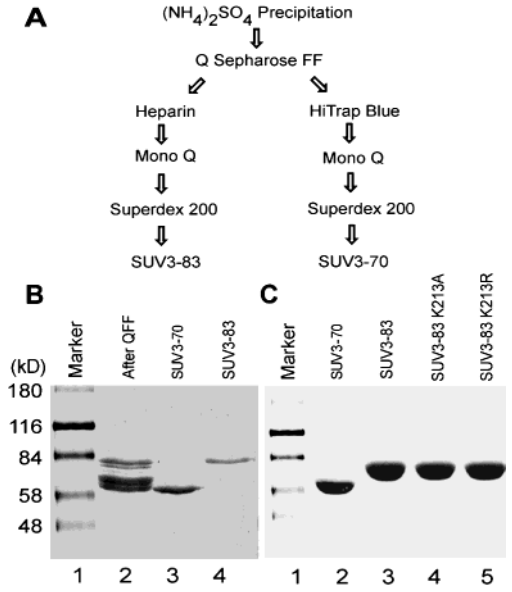

FIGURE 2: Purification of SUV3−83 and SUV3−70 expressed in yeast. (A) Purification schemes for SUV3−83 and SUV3−70 from yeast lysates. (B) Coomassie blue-stained 8% SDS−PAGE gel showing both SUV3−83 and SUV3−70 appear in the same fraction before purification (lane 2) and their subsequent separation: SUV3−70 (lane 3) and SUV3−83 (lane 4). Lane 1 contained molecular mass markers (kilodaltons). (C) SUV3−70 (3 μg, lane 2), SUV3−83 (4 μg, lane 3), SUV3−83K213A (4 μg, lane 4), and SUV3−83K213R (4 μg, lane 5) were analyzed via 8% SDS−PAGE and stained with Commassie blue for their purity. Lane 1 contained molecular mass markers (kilodaltons).

To test whether polynucleotide acids have any affect on the ATPase activity, we added a different amount of ssRNA, ssDNA, dsRNA, and dsDNA to the reaction mixture. At 20 μM (nucleotide base) polynucleotide acids, the ATPase activity of SUV3−70 was enhanced by 4.8-fold with ssRNA and by 1.2-fold with ssDNA (Figure 4C). Similarly, dsRNA and dsDNA were able to increase the ATP hydrolysis activity of SUV3−70, but not that of SUV3−83, at pH 7.5 (Figure 4C). To resolve this distinctive property, the ATPase activity assay was performed at different pHs for SUV3−83 because the enzymatic activity of SUV3−83 is apparently sensitive to pH change (see below). As shown in Figure 4D, dsDNA was able to enhance the ATP hydrolysis significantly at pH 5.0, but not at other pHs. The enhancement by dsDNA (5.4-fold) was even stronger than that of ssDNA (2.0-fold) at 40 μM (nucleotide base, data not shown). These results demonstrate that polynucleotide acids can stimulate the ATPase activity of SUV3.

SUV3−70 Has Multiple-Substrate Helicase Activity. Yeast Suv3 has been suggested to have RNA helicase activity (*9*). However, when compared to the consensus sequence motif for RNA and DNA helicases, the motif in SUV3 appears to be diverged from those found in RNA helicases. Therefore, we tested whether SUV3−83 and SUV3−70 have unwinding activity with a classical DNA substrate, circular single-stranded $\phi\times174$ DNA annealed with 26-mer complementary oligonucleotide labeled at the 5′-end, at pH 7.5. SUV3−70 unwound this substrate efficiently, while SUV3−83 exhibited little to no activity (Figure 5A,B). This suggests that SUV3 can use DNA as a substrate and unwind duplex DNA. To test whether the unwinding activity is ATP-dependent, we repeated the analysis in the absence of ATP or in the presence

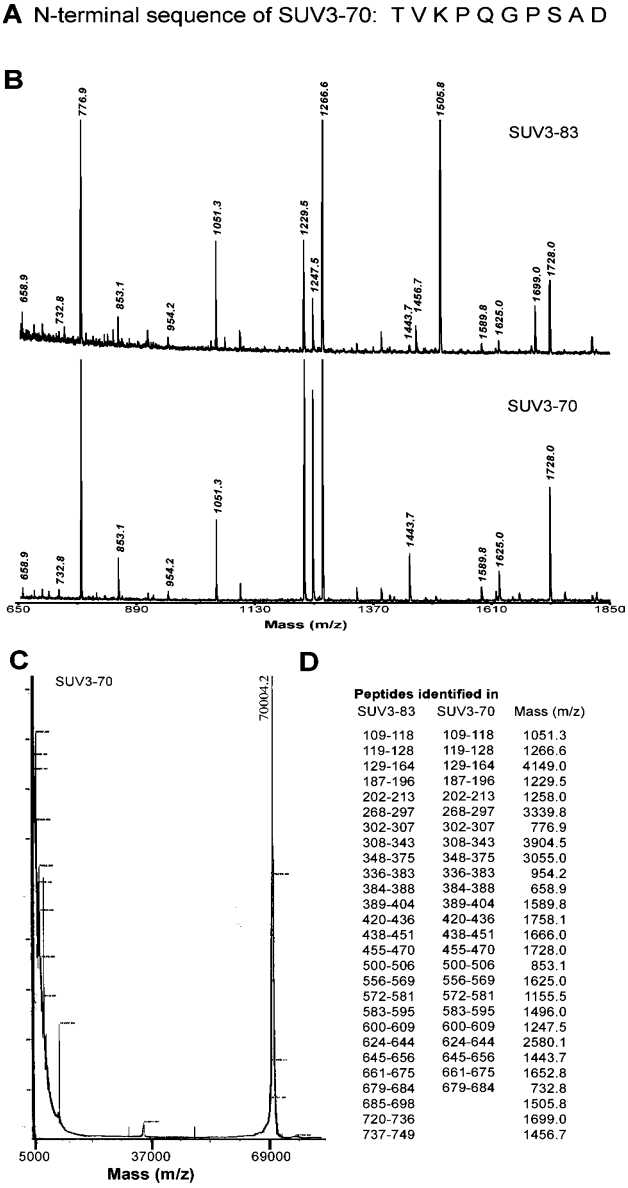

FIGURE 3: Sequence comparison between SUV3−83 and SUV3−70. (A) The N-terminal sequence of SUV3−70 was determined by Edman degradation. The 10 residues listed were obtained. (B) Comparison of the MALDI-TOF mass spectra between trypsin-digested SUV3−83 and SUV3−70. Only a limited *m*/*z* range is shown. (C) The mass of SUV3−70 was determined by mass spectrometry. SUV3−70 exhibited an *m*/*z* value of 70 004.2 in the experiment. (D) Tryptic peptides identified in SUV3−83 and SUV3−70 with their corresponding *m*/*z* values. SUV3−83 has all peptides from SUV3−70 in addition to three peptides at the C-terminal region.

of nonhydrolyzable analogue ATPγS. The results demonstrate that SUV3−70 requires a hydrolyzable form of the ATP for its unwinding activity (Figure 5C).

Next, we tested whether SUV3−70 could unwind linear polynucleotide substrates, including R40−R14, R40−D14, D40−R14, and D40−D14, each of which consisted of a 14 bp duplex region and a 26-nucleotide 5′-overhang. SUV3−70 could efficiently unwind R40−R14, R40−D14, and D40−R14 (Figure 6). In contrast, SUV3−83 could not unwind these substrates under identical conditions (data not shown). Under the same condition, neither SUV3−70 nor SUV3−83 could unwind the D40−D14 substrate (Figure 6D).

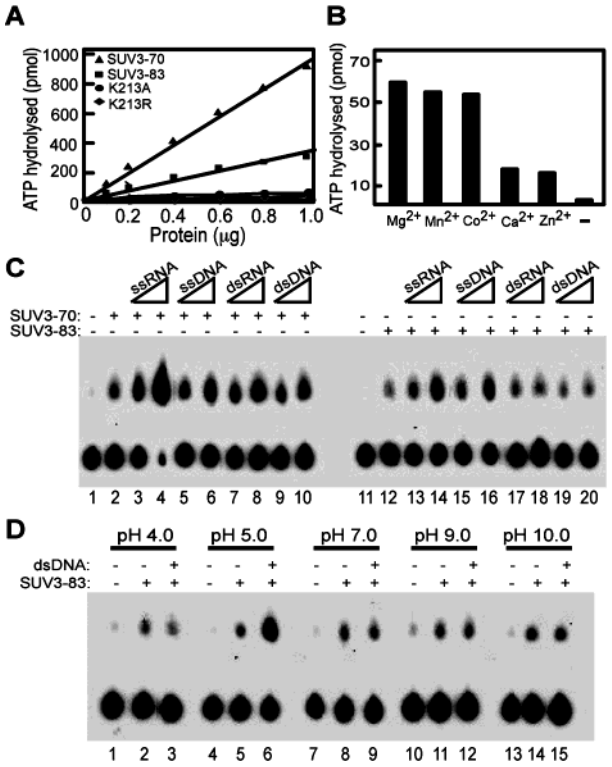

FIGURE 4: Characterization of the ATPase activity of SUV3. (A) Comparison of the ATPase activities of SUV3−83, SUV3−70, and two mutants. The ATPase reaction mixtures consisted of 25 mM Tris-HCl (pH 7.5 and 37 °C), 1 mM DTT, 5 mM $MgCl_2$, 50 mM KCl, and 100 ng/μL BSA. The reactions were carried out at 37 °C for 60 min, including 0.05 μCi/μL [γ-^{32}P]ATP, 100 μM ATP, and varying amounts of SUV3 proteins, including SUV3−83, SUV3−70, SUV3−83K213A, and SUV3−83K213R. SUV3−70 has a higher ATPase activity than SUV3−83. Both Walker A motif mutants lack ATPase activity. (B) Requirement of divalent cations for the ATPase activity of SUV3−83. The ATPase required divalent cations of Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, or Zn^{2+} for its activity and was inactive without it (−). (C) Polynucleotide acids stimulate SUV3 ATPase activity. The reaction was carried out at pH 7.5 as described for panel A, and the mixture contained SUV3−70 (lanes 2−10) or SUV3−83 (lanes 12−20) at 50 ng/μL each, and 20 or 200 μM (nucleotide base) ssRNA, ssDNA, dsRNA, or dsDNA as indicated. Lanes 1 and 11 were control reactions without protein. Polynucleotide acids stimulated both SUV3 ATPase activities, but the SUV3−83 ATPase activity was not enhanced by double-stranded RNA or DNA under the conditions that were used. (D) dsDNA stimulates the ATPase activity of SUV3−83 at pH 5.0. The conditions of the reaction were the same as those for panel A, except the buffer was changed to acetate buffer (pH 4.0 and 5.0), Tris-HCl buffer (pH 7.0), or ethanolamine buffer (pH 9.0 and 10.0). dsDNA [40 μM (nucleotide base)] was added to the mixtures as indicated. Panels C and D show the separation of free phosphate (top spots) from ATP (bottom spots) on the thin layer plates.

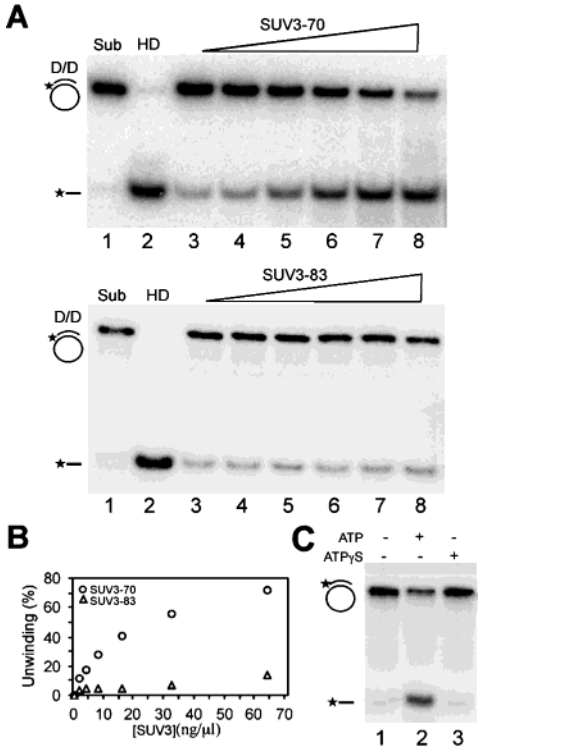

FIGURE 5: Unwinding activity of SUV3−70 and SUV3−83 on a circular DNA substrate. (A) Unwinding of the circular DNA−DNA substrate with increasing amounts of SUV3−70 and SUV3−83. Reactions were carried out in helicase buffer at pH 7.5 (25 mM Tris-HCl, 5 mM DTT, 0.1 mg/mL BSA, 2.5 mM $MgCl_2$, and 3 mM ATP) containing 1 nM circular DNA−DNA substrate. Lane 1 contained the substrate without enzyme and lane 2 the heat-denatured substrate; from lane 3 to 8, the enzyme concentrations were 2, 4, 8, 16, 32, and 64 ng/μL, respectively. (B) Quantitative plot of the unwinding efficiency of SUV3−70 and SUV3−83 at various enzyme concentrations. (C) The unwinding activity is ATP-dependent. The reaction conditions were the same as those described for panel A, except no ATP was present in lane 1 and ATPγS was added in lane 3 instead of ATP.

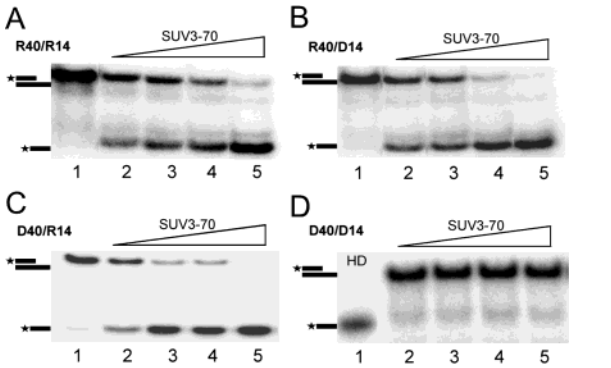

FIGURE 6: Unwinding activity of SUV3−70 on linear substrates. Reactions were carried out in helicase buffer at pH 7.5 with increasing concentrations of SUV3−70, at 2, 4, 8, and 16 ng/μL in lanes 2−5, respectively. Different substrates, including (A) R40−R14, (B) R40−D14, (C) D40−R14, and (D) D40−D14, were used in each reaction.

Similar results were seen when the experiments were performed using R26−R14, R26−D14, D26−R14, and D26−D14 linear substrates which had an eight-nucleotide 5′-overhang and a four-nucleotide 3′-overhang (data not shown). Collectively, these results indicate that SUV3 is a multiple-substrate helicase.

SUV3−70 Displays 5′−3′ Unwinding Activity. To unwind duplex DNA or RNA efficiently, helicases usually initiate the reaction at single-strand regions adjacent to the duplex (*23*). To determine the directionality of SUV3, we used a long linear DNA substrate containing partial duplex regions at either end blunted (Figure 7A). The release of a 24-mer fragment indicates movement in the 3′−5′ direction, while release of a 32-mer fragment indicates movement in the 5′−3′ direction. When this substrate was incubated with increasing amounts of SUV3−70, the 32-mer was preferentially displaced in an ATP-dependent manner (Figure 7B, lanes 5−9), indicating that SUV3−70 prefers the 5′−3′ direction (Figure 7B,C), although a slight displacement of 24-mer was also observed.

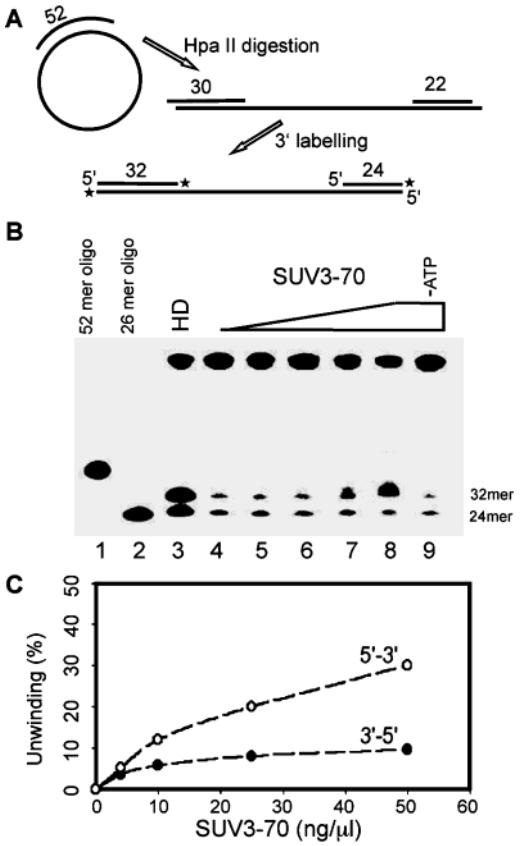

FIGURE 7: Determining the directionality of SUV3 helicase. (A) Methods of construction of the partial duplex substrate with blunted ends used to determine directionality. Details are given in Experimental Procedures. (B) Directionality assay of SUV3−70 helicase activity. Lanes 1 and 2 contained the labeled 52- and 26-mer as markers, respectively. Lanes 3 and 4 contained the heat-denatured substrate (HD) and native substrate, respectively. Lanes 5−8 show reactions with increasing amounts of SUV3−70 (4, 10, 25, and 50 ng/μL, respectively). Lane 9 had the same conditions as lane 8 but without ATP. (C) Quantitative plots of the unwinding percentage in panel B. An increasing amount of 32-mer from the reaction indicated that SUV3 helicase has a 5′−3′ directionality.

Optimizing SUV3−83 Helicase Activity. Although it contains the same helicase motif as SUV3−70, SUV3−83 did not display helicase activity under standard reaction conditions (pH 7.5). It is possible that SUV3−83 may require conditions different from those needed by SUV3−70 for the activity. Addition of salts can stimulate the strand exchange activity of human Rad51 (*24*) and ATPase activity of yeast Mot1 (*24*). Consequently, we tested the effect of various salt concentrations on the helicase activity of SUV3−83. The activity of SUV3−83 on the circular DNA−DNA substrate remained undetectable despite the presence of 0−500 mM KCl in the helicase reaction mixture (data not shown). Changing the order of treatment by preincubation of SUV3−83 in different salts before addition to the reaction mixtures also had no effect on stimulating helicase activity.

Next, we tested whether the SUV3−83 helicase activity on a circular DNA−DNA substrate was pH-dependent. Interestingly, SUV3−83 exhibited DNA−DNA helicase activity at only acidic pHs ranging from 4.5 to 5.5 but not at neutral or basic pHs (Figure 8). For example, SUV3−83 unwinds circular DNA−DNA substrates at pH 5.0 in a dose-dependent manner (Figure 8B). To further test whether ATPase activity is essential for the helicase activity, two

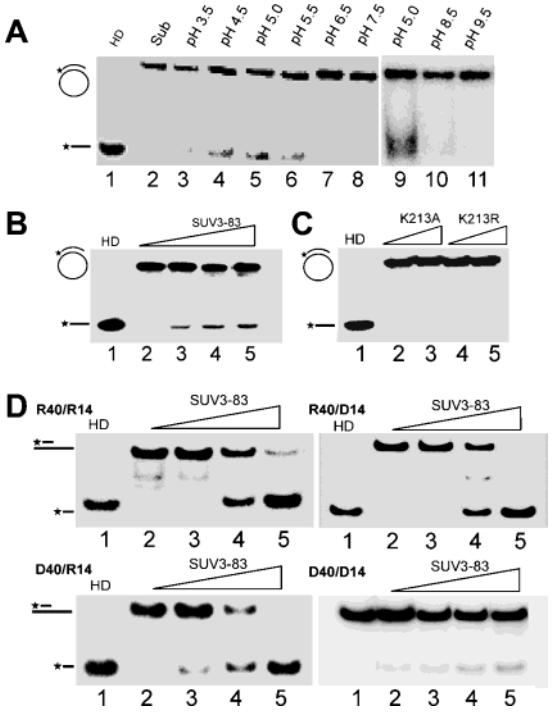

FIGURE 8: SUV3−83 exhibits helicase activity under acidic conditions. (A) Reaction mixtures containing 4 ng/μL SUV3−83 and 1 nM circular DNA substrate were incubated in 25 mM helicase buffers at different pHs: sodium citrate at pH 3.5 (lane 3), sodium acetate at pH 4.5 (lane 4) and pH 5.0 (lanes 5 and 9), phosphate buffer at pH 5.5 (lane 6), pH 6.5 (lane 7), and pH 7.5 (lanes 8), Tris-HCl buffer at pH 8.5 (lane 10), and ethanolamine buffer at pH 9.5 (lane 11). (B) Helicase reactions at pH 5.0 (25 mM sodium acetate, 5 mM DTT, 0.1 mg/mL BSA, 2.5 mM $MgCl_2$, and 3 mM ATP) with increasing amounts of SUV3−83 (2, 4, 8, and 16 ng/μL for lanes 2−5, respectively) and 1 nM partial duplex circular DNA substrate. (C) SUV3−83K213A and SUV3−83K213R have no detectable helicase activity. Reactions were carried out at pH 5.0 as described for panel B. Lane 1 contained the heat-denatured substrate. Lanes 2 and 3 contained 8 and 16 ng/μL SUV3−83K213A, respectively. Lanes 4 and 5 contained 8 and 16 ng/μL SUV3−83K213R, respectively. (D) Reaction mixtures contained increasing amounts of SUV3−83 (2, 4, 8, and 16 ng/μL in lanes 2−5, respectively) were incubated with R40−R14, R40−D14, D40−R14, or D40−D14 substrates in the pH 5.0 helicase buffer.

Walker A motif mutants, SUV3−83K213A and SUV3−83K213R, which were deficient in ATPase activity (Figure 4A), were used for an unwinding assay with a circular DNA−DNA substrate at pH 5.0. Both mutants were inactive (Figure 8C), consistent with the notion that hydrolysis of ATP is required for the SUV3 helicase activity.

Other linear substrates used in the helicase assay for SUV3−70 were also tested for SUV3−83 at pH 5.0. As shown in Figure 8D, SUV3−83 unwound linear RNA−RNA, RNA−DNA, and DNA−RNA substrates efficiently, although only minimal activity was detected using a linear DNA−DNA substrate. These results indicate that SUV3−83 has multiple-substrate helicase activity under acidic conditions.

Effect of pH on Substrate Binding of SUV3−83. Many helicases first bind substrate then translocate along the substrate template to unwind the duplex (*23*). The inability of SUV3−83 to unwind duplex substrates could be due to its low affinity with these substrates at pH 7.5. Therefore, we compared the binding of SUV3−83 to an RNA−RNA substrate at pH 7.5 and 5.0 by incubating various concentra-

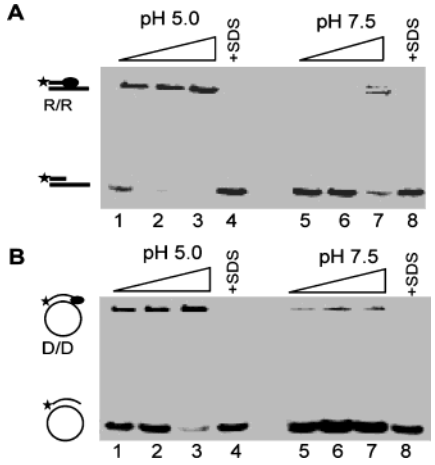

FIGURE 9: SUV3−83 preferentially binds its substrates at acidic pH. (A) Binding assay of SUV3−83 with the R40−R14 substrate at pH 5.0 (lanes 1−3) and pH 7.5 (lanes 5−7) with increasing amounts of protein (4, 8, and 16 ng/μL). Lane 4 was the same as lane 3 but was treated with 1% SDS; lane 8 was the same as lane 7 but was treated with 1% SDS. (B) Binding assay of SUV3−83 with a circular DNA−DNA substrate (1 nM) at pH 5.0 (lanes 1−3) and pH 7.5 (lanes 5−7) with increasing amounts of SUV3−83 (4, 8, and 16 ng/μL). Lane 4 was the same as lane 3 but was treated with 1% SDS; lane 8 was the same as lane 7 but was treated with 1% SDS.

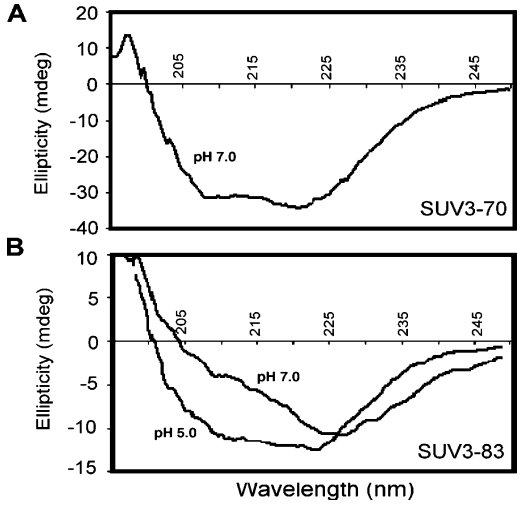

FIGURE 10: Circular dichroism spectra of SUV3−70 and SUV3−83 at different pHs. The far-UV CD spectrum was recorded for (A) SUV3−70 (0.3 mg/mL) in 25 mM Tris-HCl buffer (pH 7.0) and (B) SUV3−83 (0.2 mg/mL) in 25 mM sodium acetate buffer (pH 5.0) or in 25 mM Tris-HCl buffer (pH 7.0).

tions of SUV3−83 with the substrate in the presence of nonhydrolyzable ATP analogue ATPγS. SUV3−83 bound the RNA−RNA substrate strongly at pH 5.0, while it bound weakly to the same substrate at pH 7.5 (Figure 9A). Similarly, SUV3−83 bound the circular DNA−DNA substrate with greater affinity at pH 5.0 than at pH 7.5 (Figure 9B). Thus, substrate binding of SUV3−83 is pH-dependent, which is consistent with the observation that SUV3−83 has unwinding activity at acidic pHs.

Conformational Change Regulates the Helicase Activity of SUV3. The difference in the helicase activity of SUV3−83 and SUV3−70 can be ascribed to the sequences missing from both the N- and C-termini of SUV3−83 (Figure 1A). These sequences may affect the conformation of SUV3−83 by altering the affinity for substrates. In the case of SUV3−70, these putative regulatory domains are missing, and hence, the catalytic site is readily accessible under all conditions. On the basis of this rationale, an acidic pH would be expected to induce a conformational change in SUV3−83 to expose its active sites. To test this possibility, we used far-UV CD spectroscopy to detect the conformational changes in the secondary structure of these proteins. As shown in Figure 10A, the CD spectra of SUV3−70 suggest that it is an α-helix rich protein (*26*−*28*) which did not change in shape significantly when the pH was decreased to 5.0 (data not shown). The CD spectrum of SUV3−83 at pH 7.0 is much different from that of SUV3−70 (Figure 10B). However, when the pH was decreased to 5.0, the spectrum closely resembled that of SUV3−70 (Figure 10B). This result indicated that SUV3−83 underwent a significant conformational change at pH 5.0 and adopted a conformation similar to that of SUV3−70.

DISCUSSION

S. cerevisiae Suv3 has been classified as a mitochondrial RNA helicase. In work yet to be published, we showed that SUV3 is processed by protease at the mitochondria to remove 46 amino acids from the N-terminus, and it is the major functional component of SUV3 ubiquitously expressed in different types of mammalian cells (C.-F. Chen, W.-H. Lee. *et al*., manuscript submitted for publication). Consistent with this finding, expression of SUV3ΔN46 in the yeast suv3 null mutant restores their ability to grow in a nonfermentable carbon source and form healthy colonies in glucose medium, suggesting that SUV3−83 is a functional homologue of yeast Suv3 *in vivo*. Through a five-step chromatographic procedure, both SUV3−83 and SUV3−70 were purified to more than 95% homogeneity. Both SUV3−83 and SUV3−70 have moderate ATPase activities that can be stimulated by polynucleotide acids. At neutral pH (7.5), SUV3−70 can bind and unwind multiple substrates, including homo- and heteroduplexes of RNA and DNA, while SUV3−83 has little helicase activity. At acidic pHs (4.5−5.5), SUV3−83 unwinds multiple substrates, like SUV3−70. Consistent with this observation, SUV3−83, at pH 5.0, adopts a conformation similar to that of SUV3−70. These results indicate that SUV3−83 is a multiple-substrate helicase that can be modulated by conformational change.

ATPase Activity of SUV3−83 and SUV3−70. Purified SUV3−83 and SUV3−70 each displayed ATPase activity, but SUV3−70 exhibited activity higher than that of SUV3−83 when assayed at neutral pH. The reason for the lower activity of SUV3−83 is currently unclear. The ATPase activity exhibited strict cation dependence and was supported almost equally by Mg^{2+}, Mn^{2+}, and Co^{2+}, with Ca^{2+} and Zn^{2+} being less effective. Two mutants with amino acid substitution in the conserved Walker A motif completely lost ATPase activity, indicating that the Walker A motif is required for ATP hydrolysis.

At neutral pH, single-stranded RNA or DNA at >20 μM (nucleotide base) stimulated the ATPase activities of both SUV3−83 and SUV3−70, while double-stranded RNA and DNA significantly enhanced the ATPase activity of SUV3−70 but not of SUV3−83. After the assay condition had been changed to pH 5.0, both double-stranded RNA and DNA significantly stimulated SUV3−83 ATPase activity. These

results are consistent with the known observation that polynucleotide acids stimulate the ATPase activity of helicase (*15*). Although it remains to be verified how these polynucleotide acids stimulate the ATPase activity of SUV3, it is likely that binding to these polynucleotide acids may facilitate the ATP hydrolysis by these two proteins, which is consistent with the result that SUV3−83 bound strongly to dsRNA or dsDNA at only acidic pHs (Figure 9).

Substrate Specificity of SUV3 Helicase. Suv3 of *S. cerevisiae* has been categorized as a putative ATP-dependent mitochondria RNA helicase member of the DEAD/DExH family (*8*−*10*). On the basis of our results, we conclude that human SUV3 is an ATP-dependent multiple-substrate helicase. In addition to dsRNA and dsDNA unwinding activity, SUV3−70 and SUV3−83 can unwind heteroduplexes of RNA and DNA. The latter substrate specificity of SUV3 provides an ample opportunity for executing its biological function. It was recently reported that SUV3 preferentially unwinds duplex DNA over duplex RNA (*12*). However, we did not find any such preferential unwinding of DNA by either SUV3−83 or SUV3−70. The SUV3 protein used in this previous report had 22 amino acids deleted at the N-terminus, as opposed to 46 amino acids deleted in the SUV3−83 described here. Because the proteolytic processing at the mitochondria is critical for the biological function of SUV3 (C.-F. Chen, W.-H. Lee, *et al.*, manuscript submitted for publication), SUV3 with 22 amino acids deleted from the N-terminus is not able to complement suv3 null yeast (*12*), perhaps because of its inability to be processed correctly at mitochondria. Thus, the biochemical properties exhibited by SUV3−83 are more likely to reflect *in vivo* function. Alternatively, the preferential unwinding of DNA could be attributed to the nature of the DNA substrate used in their assay. In our study, unwinding activity was seen on only substrates with partial duplex circular DNA or long partially duplex linear DNA, but not on substrates with short linear duplex DNA. The shorter linear duplex DNAs, including D40−D14 and D26−D14 used in this study, were poor substrates despite the fact that they had a short nucleotides overhang, while the substrate used by Minczuk *et al.* (*12*) had a forked structure at the 5′-end. It is possible that SUV3, like BLM and WRN (*6*), may have a higher affinity for specialized DNA structures such as forked DNA or X-junctions.

Conformation Change Induced by pH. It is known that pH is a critical factor influencing helicase activity (*29*, *30*). RepA, a DNA helicase from *Escherichia coli*, exhibits optimal helicase activity and DNA binding activity at pH 5.5, while at pH 7.5, these activities are greatly diminished (*29*). Similarly, SUV3−83 was unable to engage in productive dsDNA and dsRNA binding and unwinding activity at pH 7.5; however, it exhibited optimal binding and unwinding activities at pH 5.0. How the conformational change induced by pH affects the enzyme activity is very intriguing. SUV3−83 adopts different secondary structures as observed by its CD spectra at two distinctive pH conditions. On the basis of this information, it is reasonable to assume that the protein conformation changes at acidic pH may modulate the active site and the nucleotide binding domain, thus facilitating its polynucleotide binding, ATP hydrolysis, and duplex unwinding function. It is noted that SUV3−70, which lacks 21 amino acids at the N-terminus and 101 amino acids at the C-terminus, in contrast to SUV3−83, has a helicase activity at neutral and acidic pH, suggesting that these residues that are absent in SUV3−70 may have a regulatory role in SUV3 protein folding.

Physiological Relevance of the SUV3−83 Helicase Activity. The pH-induced conformational change of SUV3−83 observed *in vitro* could be mimicked *in vivo* by a post-translational modification or by interaction with an accessory protein. For example, phosphorylation and acetylation of p53, a tumor suppressor and transcription factor, has been shown to stimulate its specific DNA binding activity (*31*, *32*). In the case of ATM, a DNA damage checkpoint protein, autophosphorylation was shown to result in a conformational change, which, in turn, activated its kinase activity (*33*). Whether SUV3 is subjected to secondary modification by phosphorylation is not yet known. Alternatively, binding to other proteins may activate the helicase activity of SUV3−83. It was reported that Mre11 and the Mre11−Rad50 complex exhibit differential nuclease activity (*34*), indicating that the binding of Rad50 affects Mre11 activity. Our preliminary result showing that binding to Mre11 activated SUV3 helicase activity supports this possibility.

Although SUV3 has been classified as a mitochondrial helicase, our preliminary results suggest that SUV3 exists in two compartments of the cell, mitochondria and nucleus (C.-F. Chen, W.-H. Lee, *et al*, manuscript submitted for publication). SUV3−83 is rapidly processed from full-length SUV3 at mitochondria and then translocates to nucleus and mitochondria matrix. Yeast Suv3 has been shown to play a very important role in mitochondrial RNA stability and has been postulated to be responsible for the degradation of aberrant RNAs (*10*). It is currently unclear whether the RNA−RNA helicase activity displayed by SUV3 has any role in the nucleus. It is also possible that in these two different organelles SUV3 recognizes different substrates as dictated by the environment and its binding partners. The RNA−DNA helicase activity of SUV3 could point toward its role in transcription, lagging strand synthesis, mitochondrial DNA replication, and/or telomere maintenance where such RNA−DNA hybrid structures are commonly encountered. The DNA−DNA helicase activity exhibited by human SUV3 could be employed in DNA recombination as suggested by our preliminary results (C.-F. Chen, W.-H. Lee, *et al*, manuscript submitted for publication). Thus, SUV3 may serve as a critical helicase for many physiological functions because of its substrate diversity and highly regulated unwinding activity.

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