Nuclear Export of the DEAD Box An3 Protein by CRM1 Is Coupled to An3 Helicase Activity*

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We have recently identified the Xenopus laevis An3 protein as a bona fide substrate for the nuclear export receptor CRM1 (Exportin 1). An3 binds directly to CRM1 with high affinity via a leucine-rich nuclear export signal located in the extreme N terminus. An3 is a member of the DEAD box family of RNA helicases, which unwind RNA duplexes. RNA unwinding is coupled to hydrolysis of nucleoside triphosphates by the helicase, and the ATPase activity of several helicases is greatly stimulated by various polynucleotides. Here we report that dATP hydrolysis by An3 is stimulated ~6-fold by total RNA from X. laevis oocytes, whereas poly(U) RNA fails to enhance hydrolysis, suggesting the existence of a specific RNA activator for An3. Kinetic analysis reveals that a mutation within the conserved DEAD box motif reduces the rate of dATP hydrolysis by ~6-fold. In accordance with this, the DEAD box mutant is unable to unwind double-stranded RNA. Microinjection of the An3 DEAD box mutant into X. laevis oocytes nuclei reveals a significantly lower export rate as compared with wild-type An3 protein. This is not because the mutant has lower affinity toward CRM1, nor is it due to altered RNA binding capacity. This suggests that nuclear export of An3 protein by CRM1 is coupled to An3 helicase activity.

The multiple steps in gene expression require that RNA molecules interact with specific RNA and protein partners in a timely and synchronized manner. Some of these changes require alteration of RNA conformation. Chaperones have evolved that either stabilize or destabilize RNA structures and RNA-protein interactions, with RNA helicases being a major class of modulators of RNA base pairings and likely also RNA-RNA-protein interactions, with RNA helicases being a major class of modulators of RNA base pairings and likely also RNA-protein interactions (reviewed in Refs. 1 and 2). RNA helicases contain seven conserved motifs, including the so-called “DEAD box” (from the amino acid sequence Asp-Glu-Ala-Asp). The first two residues of this motif are invariant, while DEAD, DEAH, and DEXH box helicases are categorized in three separate subgroups. The best characterized eukaryotic RNA helicase is the translation initiation factor, eIF-4A,1 which is regarded as a prototype for DEAD box RNA helicases. eIF-4A is thought to play an important role in unwinding RNA secondary structures in mRNA, thereby promoting binding of the small ribosomal subunit (3). Unwinding of RNA duplexes is dependent on the intrinsic ATPase activity of RNA helicases as shown for mammalian (4–6), Xenopus laevis (7) Drosophila (8, 9), Saccharomyces cerevisiae (10–13), and viral enzymes (14–16). Thus, although two Escherichia coli RNA helicases have been reported to disrupt RNA base pairing in the absence of nucleoside triphosphates (17, 18), ATPase-dependent RNA unwinding is expected to be a general feature of DED/H box RNA helicases. Similarly, for most helicases, ATP hydrolysis is greatly stimulated by polynucleotides (e.g. Refs. 6, 11, 12, and 19–27).

The messenger RNA encoding the X. laevis An3 protein was identified by Rebagliati and co-workers (28) in a search for mRNAs that localize to specific parts of unfertilized eggs. An3 is expressed throughout oogenesis and embryogenesis (28, 29), as well as in most adult tissue (30). During the early stages of oogenesis An3 protein is found in both the nucleoli and the cytoplasm, whereas no signal is detected in the nucleus of stage VI oocytes (29, 31). In early embryos An3 protein is still excluded from the nucleus but is later found in both the nucleus and the cytoplasm (31). The central part of the 697-amino acid sequence of An3 contains all the seven characteristic motifs of DEAD box RNA helicases and, furthermore, recombinant An3 protein has been shown to possess ATPase and RNA helicase activity (7).

Proteins highly related to An3 have been cloned from human (32), mouse (33, 34), zebrafish (35), S. cerevisiae (36, 37), and Schizosaccharomyces pombe (GenBank accession number AF084222), suggesting that the biological function of An3 is evolutionarily conserved. Since the mouse PL10 gene was the first to be identified we will refer collectively to the aforementioned proteins as members of the “PL10 family.” Through biochemical and genetic analyses Chuang and co-workers (38) showed that the S. cerevisiae Ded1p is required for translation in vivo and in vitro (38). The related human DBX and mouse PL10 proteins each can rescue the growth of yeast cells with a ded1 deletion (38, 39) and it therefore seems plausible that all proteins in the PL10 family are involved in protein synthesis. However, other aspects of gene expression might be regulated by these proteins. In agreement with the immunolocalization experiments showing that An3 is a partly nuclear protein (29, 31), we have recently shown that the An3 protein shuttles between the nucleus and the cytoplasm (40). Nuclear export is mediated by direct binding to the transport receptor CRM1 (41) via an N-terminal leucine-rich nuclear export signal (NES) and, interestingly, an alignment of the PL10 family reveals that the NES sequence is conserved in all members. This suggests that PL10-like proteins may also have a nuclear function distinct from translation.

Transport of proteins and protein-RNA complexes across the nuclear envelope is an active and highly regulated process,
E389Q proteins were prepared in parallel from NES (referred to as RanBP1 in the following) in E. coli BL21 according to standard procedures (Amersham Pharmacia Biotech). Expression and preparation of CRM1, GTP-bound Ran, Rna1p, and RanBP1 has been described previously (45).

RNA Preparation—Strand 1 and 2 RNAs were produced by standard run-off transcription using T7 RNA polymerase (Stratagene) primers 5'-CCGTGCCCCACCGTCAACC and 5'-GATGAGTCATGTGGCCGTGGAAAATG, and pET21a-An3 (40) as template. The fusion protein encoded by this plasmid also contains a recognition sequence for the catalytic subunit of cAMP-dependent heart muscle kinase at the C terminus and a thrombin cleavage site between RanGTP, but not RanGDP, supports formation of a stable heteromeric export complex, the gradients of GDP- and GTP-bound Ran across the nuclear envelope are essential for export of multiple cargos from the nucleus. Conversely, nuclear import complexes consisting of importin β-like proteins are only stable in the absence of RanGTP, i.e., in the cytoplasm. Hence, Ran is a key player in defining directionality in nucleocytoplasmic transport (42).

In this report we show that the ATPase and helicase activities of An3 are coupled to nuclear export. An amino acid substitution within the characteristic DEAD box motif of An3 not only inhibits ATP hydrolysis and RNA unwinding activities in vitro but also diminishes nuclear export of An3 protein in X. laevis oocytes. We demonstrate that the mutant protein is capable of forming a heterotrimeric complex consisting of An3, CRM1, and RanGTP with the same efficiency as the wild-type protein. This excludes the possibility that the mutation leads to an aberrant protein conformation in which the NES of An3 is inaccessible.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The plasmid pGEX-GTH-An3 for expression of An3 fused to the C terminus of glutathione S-transferase (GST) was constructed by inserting a blunt-end PCR fragments into the Smal site of pGEX-GTH (44). The PCR reaction was performed with Pfu polymerase (Stratagene), primers 5'-CCGTGCCCCACCGTCAACC and 5'-GATGAGTCATGTGGCCGTGGAAAATG, and pET21a-An3 (40) as template. The fusion protein encoded by this plasmid also contains a recognition sequence for the catalytic subunit of cAMP-dependent heart muscle kinase at the C terminus and a thrombin cleavage site between the GST and An3 sequences. pGEX-GTH without insert was used as a control. The PCRamplification was performed with Pfu polymerase (Stratagene), primers 5'-GATGAGTCATGTGGCCGTGGAAAATG, and pGEX-GTH (44) as template. This resulted in substitution of An3 (a Glu to Gln substitution), was constructed by whole plasmid PCR amplification using primers 5'-CAAGCAGACAGAATGCTTGAG and pGEX-GTH without insert was used for expression of An3 E389Q by pGEX-GTH in E. coli BL21 according to standard procedures (Amersham Pharmacia Biotech). Expression and preparation of CRM1, GTP-bound Ran, Rna1p, and RanBP1 has been described previously (45).

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Barths’ saline (15 mM HEPES/NaOH, pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.30 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate). Upon incubation for 0–10 min at 18 °C injected oocytes were manually dissected in D buffer (10 mM HEPES/KOH, pH 7.6, 70 mM NaCl, 7 mM MgCl2, 0.1 mM EDTA, 10 mM KCl) and cytoplasmic oocytes were transferred immediately to TNE (10 mM Tris/HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA) and 96% ethanol, respectively. The fractions of seven oocytes per sample were pooled. Cytoplasmic fractions were homogenized in 250 μl of TNE by repeated pipetting and cleared by centrifugation. The supernatants were mixed with 1 ml of acetone and placed at –70 °C for 1 h along with the nuclear fractions. Cytoplasmic and nuclear proteins were recovered by centrifugation and resuspended in 49 μl of SDS-PAGE sample buffer by vigorous shaking for 15 min and repeated pipetting. Samples were heated to 95 °C for 3 min and 11 μl were loaded on a 10% SDS-PAGE gel. The gels were fixed, equilibrated in Amplified solution (Amersham Pharmacia Biotech), dried, and subjected to autoradiography and PhosphorImager analysis.

**RESULTS**

**ATPase Activities of An3 Protein**—We have recently identified the An3 protein as a nuclear export substrate for the transport receptor CRM1 in *X. laevis* oocytes (40). This suggested that An3 has a nuclear function and in order to test this hypothesis we decided to investigate if a connection exists between An3 helicase function and nuclear export mediated by CRM1. Furthermore, we wished to extend the characterization of the enzymatic activities of recombinant An3. We expressed and purified full-length An3 protein fused to the C terminus of GST using glutathione-Sepharose and cation exchange chromatography. The ATPase activity of An3 has previously been reported to be independent of the presence of polyadenylates (7), which is in contrast to the behavior of most other RNA helicases (e.g. Refs. 6, 11, 12, and 19–27). To re-investigate this we incubated GST-An3 protein from different batches with [γ-32P]dATP and increasing concentrations of total RNA prepared from *X. laevis* oocytes. Following incubation at 37 °C for 1 h the level of hydrolysis was detected by thin layer chromatography and quantified on a PhosphorImager. The combined results from several independent experiments are shown in Fig. 1A. Maximal ATPase activity was obtained with a total concentration of *X. laevis* RNA of 40 ng/μl. At this concentration hydrolysis was stimulated 6-fold as compared with reactions without RNA, whereas at higher RNA concentrations the rate of hydrolysis decreased gradually to basal levels. For comparison we also tested whether poly(U) RNA stimulated the ATPase activity of GST-An3. However, at all concentrations tested poly(U) did not influence the ATPase activity, indicating the existence of a specific An3 activator among the total population of *X. laevis* RNA. To be sure that the stimulation seen upon addition of *X. laevis* RNA is RNA-mediated, we assayed whether prior treatment of the RNA with RNase A would abolish the observed enhancement. Indeed, titration with RNase A-digested RNA samples showed no stimulation of An3 ATPase activity (data not shown), leading us to conclude that this activity is RNA-dependent.

To examine the ability of various nucleoside triphosphates to function as substrates for the ATPase activity of An3 we added a 10-fold molar excess of unlabeled NTPs and dNTPs to standard hydrolysis reactions containing 80 μM [γ-32P]dATP and 100 nm GST-An3 (Fig. 1B). In the absence of competitor nucleotide 21% hydrolysis was obtained while excess ATP and dATP reduced hydrolysis of the radiolabeled dATP by approximately 5-fold (Fig. 1B, compare lane 2 with lanes 3 and 7). In contrast, the six other ribonucleotides and deoxyribonucleotides tested reduced dATP hydrolysis only slightly (Fig. 1B, compare lane 2 with lanes 4–6 and 8–10). Thus, although this assay measures the capacity of the individual nucleoside triphosphates to titrate out hydrolysis of [γ-32P]dATP it presumably reflects their ability to function as nucleotide substrates for An3. We therefore conclude that An3 preferentially hydrolyses ATP and dATP. This notion is in agreement with analysis of nucleotide specificity in RNA unwinding experiments (7).

Having found an optimal concentration of oocyte RNA we next determined the kinetic parameters of GST-An3. In the presence of 40 ng/μl *X. laevis* RNA and various concentrations of dATP the rate of hydrolysis was determined as before. The combined results of five independent experiments are shown in Fig. 1C, and in the double reciprocal Lineweaver-Burk plot in Fig. 1D. From these data the *Km* and *kcat* values for dATP as substrate for GST-An3 were calculated to 65 μM and 9 min⁻¹, respectively.

**A Mutation within the DEAD Box Motif of An3 Reduces ATPase Activity**—The existing mutational and structural data on RNA helicases clearly show that the DE/H box motif (also called the Walker B motif or motif II) is essential for ATP binding and hydrolysis (e.g. Refs. 4, 19, 22, 48–50). We therefore decided to replace the glutamate residue at position 389 with glutamine (DEAD → DQAD), giving rise to a mutant protein that we name An3 E389Q. The mutant GST-An3 E389Q protein has the same properties as the wild-type proteins with regard to expression and purification from *E. coli* (see Fig. 5A). To compare the ATPase activities of the wild-type and the mutant protein we performed time course experiments with fixed concentrations of protein and dATP. Again, multiple experiments were performed using independent protein preparations and at different protein and dATP concentrations. In the presence of either wild-type GST-An3 or GST-An3 E389Q the amount of ADP produced increased as a linear function of incubation time, but the rate whereby GST-An3 E389Q hydrolyzed dATP was reduced about 6-fold relative to GST-An3 (Fig. 2A). Thus, as expected, the DEAD box motif of An3 is important to ATP hydrolysis.

**Unwinding of Double-stranded RNA by An3 Requires ATP Hydrolysis**—To test if the ATPase activity of An3 is required for unwinding of RNA duplexes we constructed two plasmids from which *in vitro* transcription produces RNA molecules that can form extensive intermolecular base pairings (Fig. 3A). When incubated with GST-An3 and ATP ~9% of the heterodimeric RNA was unwound to RNA monomers (Fig. 3B, lane 3), whereas the same amount of GST-An3 E389Q produced almost no detectable monomeric RNA (Fig. 3B, lane 4). ATP is an absolute prerequisite for RNA unwinding as no monomeric RNA was detected when ATP was omitted (Fig. 3B, lane 5). The absolute level of unwinding seen in the presence of GST-An3 is relatively low, which might be due to inefficient association of GST-An3 with the RNA substrate employed. The unwinding
The ATPase activity of GST-An3 was investigated using experiments (slope shows a linear regression of values from poly(U) RNA obtained in five independent experiments with 800 nM of the indicated nucleotides (lanes 3–10)). The actual percentage activity of RNA helicases requires that the substrate is partially single-stranded, presumably to provide an initial binding site from which helicase movement can proceed (e.g., Refs. 6, 11, and 51). Hence, if the putative RNA structure proposed in Fig. 3A is correct, only short unpaired regions are available for initial binding of An3, perhaps making this a limiting step in RNA unwinding.

**Retention of An3 E389Q in the Nucleus of X. laevis Oocytes—** Since An3 is a nucleocytoplasmic shuttling protein we speculated that this property may be coupled to its activities as an RNA helicase. To test this, we prepared An3 and An3 E389Q proteins by in vitro translation in rabbit reticulocyte lysate, followed by microinjection into the nuclei of *X. laevis* oocytes. Upon incubation for up to 100 min the oocytes were dissected manually and nuclear and cytoplasmic fractions were recovered. As a control for nuclear integrity and proper fractionation of the two cellular compartments GST protein was co-injected. Although the molecular mass of GST in principle is small enough to allow passive diffusion through the nuclear pore complexes, the majority of the protein remains in compartment of injection (Fig. 4), presumably because of multimerization. Using this method we have previously demonstrated that An3 is exported from the nucleus in a CRM1-dependent manner (40). To further study the rate of export we dissected the oocytes as early as 20 min after injection and found that nearly 50% of the injected wild-type An3 protein had left the nucleus (Fig. 4A, lanes 3 and 4, Fig. 4B), revealing a high export rate of An3. The percentage of An3 protein exported to the cytoplasm increased to approximately 90% after 100 min (Fig. 4, A and B). The amount of control GST protein in the cytoplasm was ≤ 12% at all time points. In contrast to the efficient export of wild-type protein, we repeatedly observed a significant lower export rate of the An3 E389Q mutant. 90 min after injection into the nucleus only 25% of the An3 E389Q protein had left the nucleus, as compared with ~80% export of wild-type protein (Fig. 4C, compare lanes 3 and 4 and lanes 5 and 6, Fig. 4D). An An3 mutant in which 2 leucine residues at positions 19 and 21 of [α-32P]dATP hydrolysis (after subtraction of spontaneous dATP decomposition in the absence of GST-An3 (lane 1)) is written below. C and D, the rate of hydrolysis (nM dATP hydrolyzed per hour per nm GST-An3) observed at various concentrations of GST-An3 and dATP plotted as rate of hydrolysis versus the concentration of dATP (C, only concentrations up to 350 μM dATP are depicted) and in a double reciprocal Lineweaver-Burk plot (D). Assuming Michaelis-Menten kinetics we calculated the $k_{cat}$ and $K_m$ values (indicated in panel C by dotted and full lines, respectively), and from these values the curve corresponding to the theoretical rate of hydrolysis ($V = \frac{[dATP] * V_{max} / [K_m + [dATP]]}$) was generated (panel C).

**Fig. 2.** GST-An3 E389Q is impaired in ATPase activity. Fixed concentrations of GST-An3 (○) and GST An3 E389Q (○) (ranging from 50 to 130 nt between experiments) were incubated with 50 or 150 μM dATP and 40 ng/μl X. laevis RNA. At various time points the amount of hydrolysis was determined as described in the legend to Fig. 1. Hydrolysis is expressed as nanomole of dATP hydrolyzed per nmol of protein allowing data from differential experimental conditions to be combined in one graph.

**Fig. 1.** Biochemical characterization of GST-An3 ATPase activity. The ATPase activity of GST-An3 was investigated using [α-32P]dATP as substrate and analyzed by thin layer chromatography and PhosphorImager quantification. The data in A–C were obtained from several experiments using different batches of GST-An3 and RNA. A, various amounts of total RNA from *X. laevis* oocytes (∙) or poly(U) RNA (●) was incubated with 190 nM GST-An3 and incubated at 37 °C for 1 h. The level of hydrolysis is represented as average values relative to samples without RNA. Error bars indicate the standard deviation obtained in five independent experiments with *X. laevis* RNA. The straight line shows a linear regression of values from poly(U) RNA experiments (slope = 0). B, hydrolysis of 80 μM dATP by 100 nM GST-An3 in the presence of 40 ng/μl *X. laevis* RNA (lane 2) was competed by 800 μM of the indicated nucleotides (lanes 3–10). The actual percentage of hydrolysis was determined as described in the legend to Fig. 1. Hydrolysis is expressed as nanomole of dATP hydrolyzed per nmol of protein allowing data from differential experimental conditions to be combined in one graph.
within the NES are replaced by alanines (An3 L19A/L21A) was restricted to the nucleus (Fig. 4C, lanes 7–8; Fig. 4D), confirming that translocation to the cytoplasm requires an intact NES. Similarly, a triple mutant, An3 L19A/L21A/E389Q, was retained in the nucleus upon microinjection (data not shown).

The Impaired Export of An3 E389Q Is Not Due to Inefficient Interaction with CRM1—Although we have mapped the NES of An3 to the N terminus, the reduced export rate of An3 E389Q might still be caused by an inefficient interaction with the nuclear export receptor CRM1, e.g. because of misfolding of the An3 E389Q protein. To exclude this possibility we examined whether wild-type GST-An3 and GST-An3 E389Q can form ternary complexes with CRM1 and RanGTP using purified recombinant proteins only. Equal amounts of GST-An3 and GST-An3 E389Q protein (see Fig. 5A) were bound to glutathione-Sepharose beads and incubated with CRM1 and RanGTP. GST protein was included as a control for specificity of CRM1 retention. Following extensive washes to remove unbound protein, retained CRM1 and RanGTP was released from the columns by challenging the complexes with RanBP1 and an NES peptide from the minute virus of mice NS2 protein (40). RanBP1 destabilizes RanGTP-containing export complexes while the excess NES peptide competes for binding to CRM1, thereby preventing re-association of released CRM1 with GST-An3. This approach has a major advantage, in that it specifically detects heterotrimeric An3-CRM1-RanGTP complexes mimicking export complexes. As is evident from Fig. 5B, GST-An3 and GST-An3 E389Q supported to a similar extent formation of CRM1- and Ran-containing complexes that could be disassembled by RanBP1 and NES2 NES peptide (Fig. 5B, lanes 4 and 5). In contrast, neither CRM1 nor Ran was released from the control GST column (Fig. 5B, lane 6). The CRM1 and Ran present in Fig. 5B, lane 9 reflects unspecific binding to the matrix or to GST itself. As seen in Fig. 5B, lanes 7 and 8, not all CRM1 and Ran was released from the GST-An3 columns upon incubation with RanBP1 and NES peptides, probably reflecting that free NES peptides are recognized less efficiently as compared with NES-containing polypeptides (40). Alternatively, residual binding might be due to protein-protein interactions not involving the NES of An3. To further substantiate the direct binding of CRM1 to GST-An3 and GST-An3 E389Q the pull-down experiment was repeated, but this time complexes were disassembled by triggering hydrolysis of Ran-bound GTP with RanBP1 and the Ran GTPase activating protein, Rna1p (40). As before, this approach specifically detects ternary An3-CRM1-RanGTP complexes. Fig. 5C shows that equal amounts of CRM1 and Ran were released from columns containing GST-An3 (lane 1) and GST-An3 E389Q (lane 2) by the concerted action of RanBP1 and Rna1p. We therefore conclude that wild-type An3 and An3 E389Q are recognized by CRM1 with the same efficiency.

Another possible explanation for the nuclear retention of An3 E389Q could be that the mutant protein has a dramatically higher affinity toward nucleic acids and therefore aggregates nonspecifically with RNA or DNA within the cell nucleus. To examine this possibility we compared the binding of 3′-end labeled X. laevis RNA to GST-An3 versus GST-An3 E389Q by nitrocellulose filter binding as well as co-precipitation on glutathione-Sepharose columns. However, in either assay we could not detect a significant difference in RNA affinity of the two proteins (data not shown). The use of X. laevis oocyte extracts in co-precipitation experiments also failed to reveal any differences in retention of protein or RNA (data not shown). Thus, the impaired export of An3 E389Q from the nucleus is not due to a general increase in nucleic acid affinity, but rather is linked to an association with at least unidentified specific nuclear RNA or protein target.

**DISCUSSION**

In this report, we have investigated the biochemical properties of the X. laevis DEAD box RNA helicase An3. An3 preferentially hydrolyzes ATP and dATP, and a 6-fold stimulation of dATP hydrolysis is seen with total RNA from X. laevis oocytes whereas poly(U) RNA has no effect. Intriguingly, a single amino acid substitution within the DEAD box motif of An3 that impedes the ATPase and RNA unwinding activities also leads to inhibition of nuclear export of An3 in X. laevis oocytes.

**ATPase Activity**—Most commonly, hydrolysis of nucleoside triphosphates by RNA helicases is dramatically stimulated by a variety of polynucleotides including homogenous polymers (e.g. Refs. 6, 11, 12, 19–21, and 24–27). However, An3, human CAP-Rf, and Drosophila Vasa proteins have been reported to display RNA-independent ATPase activity (7, 9, 52). The data presented here contradicts such observation in that dATP hydrolysis by An3 can be stimulated 6-fold by total RNA from X. laevis oocytes. At higher RNA concentrations the stimulatory effect decreases and finally disappears. Therefore, since Gururajan and Weeks (7) also tested total X. laevis RNA, but observed no effect, we suggest that the discrepancy between our and their data is caused by differences in RNA concentrations. The observation that poly(U) RNA fails to stimulate An3 ATPase activity indicates that a specific An3 activator is present in the total population of X. laevis RNA. Some RNA specificity in stimulation of ATP hydrolysis has also been demonstrated for a putative yeast homologue of An3, Ded1p (23) (see below), while the ATPase activity of E. coli DpbA and S. cerevisiae Prp5 is specifically stimulated 2400-fold by ribosomal...
An3 E389Q has a diminished nuclear export rate. Nuclear export of An3, An3 E389Q (An3 EQ), and An3 L19A/L21A (An3 LLAA) proteins was investigated by injection of 35S-labeled proteins into the nuclei of X. laevis oocytes. Cytoplasmic and nuclear fractions were subsequently recovered and analyzed by SDS-PAGE. In all experiments 35S-labeled GST protein was included as an internal control for proper injection and fractionation. A, a time course experiment of nuclear export of wild-type An3 protein was performed by dissecting the oocytes 0–100 min after injection as indicated above the lanes. C and N denote cytoplasmic and nuclear fractions, respectively. The migration of An3 and GST is indicated on the right. B, the experiment shown in panel A was quantified by PhosphorImager analysis. Percentage of protein exported was calculated as the amount of protein located in the cytoplasmic fraction as compared with the combined signal from the cytoplasmic and nuclear fraction. Gray columns represent An3 protein, while GST is indicated by hatched columns. C, a comparison of nuclear export of wild-type An3 (lanes 3 and 4) to the export of An3 E389Q (lanes 5 and 6) and An3 L19A/L21A (lanes 7 and 8) mutants. Lanes 1 and 2 show the distribution immediately after injection, while the remaining lanes represent the cytoplasmic (C) and nuclear (N) fractions 90 min after injection. D, the average nuclear export observed in three independent experiments as the one shown in panel C. Calculations and representation of data are as described for panel B. Error bars indicate standard deviations.

Characterization of an An3 ATPase Mutant—Substitution of the conserved glutamate residue within the DEAD box of An3 by glutamine (An3 E389Q) reduces the ATPase activity 6-fold. Crystallographic data of RNA and DNA helicases reveal that the first, invariant aspartate residue of the helicase motif II (the DEAD box) is involved in co-ordination of a magnesium ion that contacts the terminal phosphate group of ATP (48, 56–57). Furthermore, it is proposed that the succeeding glutamate residue activates the attacking water molecule during hydrolysis of ATP (56–58). Due to the high degree of conservation of helicase motifs I–VI in An3 as compared with other helicases it is reasonable to assume that a similar mechanism is true for An3. In agreement with its reduced ATPase activity, An3 E389Q is severely impaired in unwinding of double-stranded RNA. We found that an excess of wild-type An3 protein is able to dissociate a stable RNA heterodimer into RNA monomers in an ATP-dependent process. The requirement for excess protein over RNA was also observed by Gururaj and Weeks (7) based on similar experiments with GST-An3, as well as in studies with most other RNA helicases (e.g. Ded1p (23), eIF-4A (4, 21, 51), Prp16p (11), Prp22p (12, 24), p68 (5), Vasa (9), NS3 (59)). In fact, catalytic unwinding activity with subamounts of protein to RNA has so far only been demonstrated for RNA helicase A (6) and vaccinia virus NPH-II (14). The mechanism behind RNA unwinding is not well understood and perhaps it may differ among individual RNA helicases. In nearly all cases examined ATP hydrolysis is necessary for RNA unwinding, most likely reflecting that disruption of base pairings is an active, energy-dependent process (4–16). A possible explanation of the requirement for excess protein in in vitro assays could then be that the helicase has to occupy the unwound single-stranded RNA to prevent reannealing of the RNA, and that several proteins are bound to each RNA molecule. Similarly, E. coli CsdA and DpbA, which both have been reported to unwind RNA in the absence of ATP (17, 18), might function by binding to single-stranded RNA regions appearing due to “breathing” of the RNA duplex and thereby trapping the RNA in an unwound conformation (23).

A Nuclear Function of An3—We have recently shown that An3 shuttles continuously between the nucleus and the cytoplasm of X. laevis oocytes (40). Moreover, probing of early-stage oocytes as well as embryonic cells with anti-An3 antibodies gives rise to both nuclear and cytoplasmic staining (29, 31). Export from the nucleus is mediated by the export receptor.
CRM1, requiring an N-terminal NES (40) (Fig. 4C). Alignment of putative An3 homologues from multiple species within the PL10 family (see Introduction) reveals that they all contain a similar motif with characteristic spacing of four hydrophobic residues at their N termini (40). Thus, we expect that other PL10 family members are nucleocytoplasmic shuttling proteins as well. Biochemical and genetic data have assigned an essential role of Ded1p in translation initiation (38). Human DBX and mouse PL10 can both substitute for Ded1p in a genetic complementation assay, which suggests that a function in translation initiation is conserved in all PL10-like proteins (38, 39). However, due to the shuttling behavior of An3, we anticipate that PL10 family members may have a nuclear function as well. Based on localization studies, Weeks and colleagues have suggested that An3 is involved in rRNA processing (29, 31), but there is no direct evidence available that might provide clues as to which function An3 fulfills in the nucleus. This led us to look for a correlation between nuclear export and the enzymatic activities of An3. Interestingly, we consistently observed a reduction in the export rate of An3 E389Q as compared with wild-type protein, indicating that such a connection does exist. The similar mutation in eIF-4A gives rise to protein that acts as a dominant negative over wild-type protein in in vitro translation (50). We therefore speculated that nuclear injection of GST-An3 E389Q might inhibit the function of wild-type An3 in X. laevis oocytes. However, preliminary experiments have shown no effect on the nuclear export of several classes of RNA (data not shown). A likely explanation for this is the fact that we have been unable to obtain active protein preparations of higher concentrations than 0.5 μM and consequently only 10 fmol of protein could be injected per nuclei. In comparison, a concentration of 50–100 μM of the human Dbp5 RNA helicase harboring a corresponding E389Q mutation was needed in order to detect an RNA export phenotype in similar oocyte injections (60); see below.

In addition to An3, RNA helicase A has been found to shuttle between the nucleus and the cytoplasm (61). However, in contrast to An3, export of RNA helicase A is not mediated by CRM1 and the nuclear export signal has no homology to known NESs (62). RNA helicase A associates with viral RNA transport elements in vivo and influence the expression of reporter genes regulated by these elements (63). Overexpression of RNA helicase A stimulates expression of genes encoded within introns harboring these RNA elements (i.e. where nuclear export of intron-containing pre-mRNA is required), while injection of anti-RNA helicase A antibodies reduces their expression (63). The mechanism underlying these effects remains unknown, although it has been suggested that RNA helicase A might stimulate nuclear export of RNA either directly (62), or by releasing the pre-mRNA from spliceosomes (63). In yeast, the Dbp5p RNA helicase was found by two groups to be essential for mRNA export (10, 64). Conditional dbp5 mutant strains show a rapid accumulation of poly(A)⁺ RNA in the nucleus when shifted to the restrictive temperatures, concomitant with a decrease in the cytoplasmic poly(A)⁺ RNA level. Pre-mRNA splicing, RNA stability, and translation are unaffected upon the temperature shift, indicative of a direct role of Dbp5p in mRNA export (10, 64). At normal growth conditions, Dbp5p is located in the cytoplasm and accumulates on the cytoplasmic side of the nuclear envelope (10, 64). A similar distribution was recently found for the human homologue of Dbp5p, and is apparently mediated by an interaction with the nucleoporin CAN/Nup159p, which is part of the cytoplasmic fibrils of the nuclear pore complex (60, 65). Interestingly, when mutant Dbp5p RNA helicase containing an amino acid substitution corresponding to the An3 E389Q mutation was injected into the cytoplasm of X. laevis oocytes it resulted in an inhibition of mRNA export (60). In addition, a more severe mutant inhibited export of U small nuclear RNA as well (60). Based on the accumulation of Dbp5p at the nuclear envelope, Dbp5p is proposed to act by releasing mRNA from export factors once the export complexes reaches the cytoplasmic face of the nuclear pore complexes. Likewise, as An3 ATPase and unwinding activities are required for An3 protein export, a nuclear RNA unwinding function of An3 may be coupled to transport through the nuclear pore complex. We speculate that An3 might be involved in stripping nuclear RNA processing factors off the RNA molecules and/or in melting of RNA secondary structures during the translocation process. However, future studies are required to elucidate to nuclear function of An3 and other PL10 family members.

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