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Ded1p, a conserved DExD/H-box translation factor, can promote yeast L-A virus negative-strand RNA synthesis *in vitro*

Jean-Leon Chong[1], Ray-Yuan Chuang[2], Luh Tung[2] and Tien-Hsien Chang[1,2,*]

[1]Department of Molecular Genetics and [2]Program of Molecular, Cellular and Developmental Biology, The Ohio State University, Columbus, OH 43210, USA

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ABSTRACT

Viruses are intracellular parasites that must use the host machinery to multiply. Identification of the host factors that perform essential functions in viral replication is thus of crucial importance to the understanding of virus–host interactions. Here we describe Ded1p, a highly conserved DExD/H-box translation factor, as a possible host factor recruited by the yeast L-A double-stranded RNA (dsRNA) virus. We found that Ded1p interacts specifically and strongly with Gag, the L-A virus coat protein. Further analysis revealed that Ded1p interacts with the L-A virus in an RNA-independent manner and, as a result, L-A particles can be affinity purified via this interaction. The affinity-purified L-A particles are functional, as they are capable of synthesizing RNA *in vitro*. Critically, using purified L-A particles, we demonstrated that Ded1p specifically promotes L-A dsRNA replication by accelerating the rate of negative-strand RNA synthesis *in vitro*. In light of these data, we suggest that Ded1p may be a part of the long sought after activity shown to promote yeast viral dsRNA replication. This and the fact that Ded1p is also required for translating brome mosaic virus RNA2 in yeast thus raise the intriguing possibility that Ded1p is one of the key host factors favored by several evolutionarily related RNA viruses, including the human hepatitis C virus.

INTRODUCTION

Relative to their host genomes, viral genomes are considerably smaller. They typically encode only a limited number of proteins, which include polymerases for synthesizing viral RNA or DNA, helicases to unwind double-stranded RNA or DNA during replication, and other nucleic acid-modifying enzymes, such as capping enzymes, that add a cap structure to the 5′ end of viral messenger RNAs (mRNAs). Owing to their limited coding capacity, viruses must then borrow host proteins to complete their replication machinery. Prominent examples abound to illustrate how viruses take advantage of the invaluable host resources for their own survival (1–3). One textbook example is the RNA bacteriophage Qβ, whose replication complex includes not only the viral RNA-dependent RNA polymerase (RdRp), but also the host translation elongation factors (EFs) Ts and Tu and the ribosomal protein S1 (4). Interestingly, these borrowed proteins need not play the same role in viral replication as they do in the host. Identification and functional characterization of these host factors thus represents an important area that promises critical insights into the virus–host interaction, which is shaped by the unending co-evolution between the virus and its host.

Several virus-like elements exist permanently in the budding yeast *Saccharomyces cerevisiae*, including the well-studied intracellular double-stranded RNA (dsRNA) viruses L-A and its satellite M_1 (5). The L-A virus contains a single 4.6 kb dsRNA segment encapsidated in a coat made up of 120 copies of the 80 kDa major coat protein (Gag) and an estimated two copies of a minor 180 kDa Gag–Pol fusion protein produced by –1 frameshift translation. The Gag–Pol protein acts as a transcriptase to synthesize the positive-strand RNA, which extrudes from viral particles and serves as mRNA for translation to produce both Gag and Gag–Pol. These proteins then assemble with a viral positive-strand to form new particles, which perform synthesis of the negative-strand on the positive-strand template to form dsRNA. The satellite M_1 virus, which contains two 1.8 kb dsRNA segments encapsidated by both Gag and Gag–Pol produced by the L-A virus, undergoes the same replication cycle as that of the L-A virus, except that the second dsRNA segment is synthesized within the virus particle after completion of the first segment. The genome of the M_1 virus only encodes a 32 kDa killer toxin/immunity precursor protein, which is subsequently processed and secreted as a mature toxin.

A large number of host genes are known to be essential for maintenance, expression or replication of the L-A and the M_1 dsRNA viruses (5). For example, *MAK* genes are required for maintenance of the killer phenotype. In contrast, mutations in *SKI* genes yield the 'superkiller' phenotype, indicating that wild-type *SKI* genes function in controlling (or repressing)

*To whom correspondence should be addressed. Tel: +1 614 688 8678; Fax: +1 614 292 4466; Email: chang.108@osu.edu
Present address:
Ray-Yuan Chuang, Institute for Biological Energy Alternatives, 9700 Great Seneca Highway, Laboratory 174, Rockville, MD 20850, USA

virus replication. Extensive characterization of these host factors over the years by Wickner and co-workers (6–9) has resulted in a great wealth of knowledge regarding how these dsRNA viruses multiply in the cell. Yet, none of these host factors appear to be part of the viral particle. Nor do they appear to directly participate in viral replication to produce viral RNA.

Here we report an unexpected finding that Ded1p, a highly conserved DExD/H-box translation factor (10,11), interacts specifically and tightly with the L-A virus, such that functional L-A virus particles can be affinity purified by virtue of this interaction. Further analysis revealed that Ded1p accelerates the rate of negative-strand RNA synthesis *in vitro* and is thus likely to correspond to an as yet uncharacterized host factor activity previously reported to promote *in vitro* viral replication (12). We discuss these data in the context of our present understanding of DExD/H-box protein functions and recent findings that Ded1p is also required for brome mosaic virus (BMV) replication (13) and that the human ortholog of Ded1p interacts with the hepatitis C virus (HCV) core protein (14–16).

MATERIALS AND METHODS

Yeast strains and plasmids

All yeast strains used in this work are isogenic and harbor endogenous L-A virus. Protein A (PA) fusions of Ded1p, Dbp5p and Dbp3p were expressed from *CEN* plasmids in strains that harbor their respective gene deletions on the chromosome. Strains YTC212 (*MAT***a** *ded1*Δ::*TRP1 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1* pRS315-[*DED1-PA*]), YTC433 (*DBP5-PA*) and YTC420 (*DBP5-PA*) have been described (11,17,18). The M_1 virus was transferred from strain YTC598 (*MAT*α *kar1-1his4* [L-A] [M_1]) (kindly provided by R.Wickner) into YTC212 by cytoduction (19) for testing its interaction with Ded1p–PA.

Affinity purification and characterization of Ded1p–PA-associated proteins

Yeast cells collected from 1 l of culture grown to 1 OD_{600} unit were spheroplasted by zymolyase 100T (Seikagaku), resuspended in 15 ml of lysis buffer (150 mM KCl, 20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 0.1% Triton X-100) and kept on ice for 5 min. Cell debris was removed by centrifugation at 16 000 r.p.m. for 20 min in an SS-34 rotor (Sorvall). An aliquot of clarified lysate containing 20 mg of total proteins was mixed with 100 µl of IgG Sepharose 6 Fast Flow (Pharmacia) in a closed Poly-Prep Chromatography column (Bio-Rad) and incubated at 4°C for 2.5 h on a nutator. Following flow-through, the column was extensively washed 10 times, each with 5 ml of lysis buffer, and the bound material was eluted in 1.5 ml of 0.5 M acetic acid (pH 3.4) (20). The eluted proteins were precipitated by trichloroacetic acid (10% final concentration) and analyzed by SDS–PAGE. Alternatively, proteins were eluted in steps by a buffer (20 mM Tris–HCl, pH 7.5, 0.05% Triton X-100) containing increased concentrations of $MgCl_2$ (50, 100, 200 and 500 mM and 1, 2 and 4.5 M) as described (21). To obtain enough material for internal peptide sequencing, the affinity purification procedure was scaled up and the band corresponding to the 80 kDa protein was excised from gel and in-gel digested by trypsin. The resulting peptides were fractionated by high performance liquid chromatography and selected peptides were subjected to chemical sequencing in the Protein Chemistry Laboratory, University of Pennsylvania Medical School. For RNA analysis, IgG-bound materials were extracted by phenol/chloroform, ethanol precipitated and analyzed on 1% agarose gel. To assess the RNase sensitivity of the viral dsRNA in the affinity-purified L-A particles, the bound beads were treated with pancreatic RNase (Sigma) using the condition as described (22). For western blotting to confirm the identities of Gag and Gag–Pol, two antibodies (gifts from Dr R. Wickner) specifically against the Gag and the Pol moieties were used (12).

Purification of L-A viral particles by CsCl gradients

L-A virus particles were purified according to the standard protocol by Esteban and Wickner (23) and advice by Dr R. Wickner (NIH). Cells were harvested from 2 l of culture grown to mid-log phase (for positive-strand-containing particles) or saturation as needed, spheroplasted and broken by French press. Cell debris was removed by low speed centrifugation at 10 000 r.p.m. for 20 min in an SS-34 rotor. Viral particles were collected by high speed centrifugation at 32 000 r.p.m. in a 70Ti rotor (Beckman) for 1 h. The resuspended pellet was then homogenized by 10 strokes in a glass Dounce homogenizer (Kontes) and clarified by low speed centrifugation (see above). The density of the solution was adjusted to 1.35 g/ml by addition of CsCl and viral particles were banded by centrifugation at 38 000 r.p.m. in 70Ti rotor for 20 h. Fractions containing virus particles were dialyzed as described (23), stored at –70°C and used for polymerase assay within 2 weeks.

Electron microscopy

CsCl- and affinity-purified L-A virus particles were negatively stained by 2% uranyl acetate and then imaged on a Philips CM12 transmission electron microscope as described (24). This procedure was done in the Ohio State University Campus Microscopy and Imaging Facility.

In vitro binding of glutathione *S*-transferase (GST)–Ded1p to Gag and to purified L-A virus particles

The protocol and the plasmids for overexpressing GST and GST–Ded1p in *Escherichia coli* and their functional characterization have been described (11). Cell lysate prepared from 1 l of culture was mixed with 3 ml (bed volume) of glutathione–agarose beads (Sigma) and incubated at 4°C for 12 h on a nutator. Beads were washed four times, each with 12 ml of wash buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS) and then washed twice, each with 12 ml of TB buffer (25 mM Tris–HCl, pH 8, 120 mM NaCl). To synthesize Gag *in vitro*, the Gag ORF was PCR amplified and cloned into T-vector (Promega) to yield pGAG1005. ^{35}S-labeled Gag was synthesized in a 50 µl reaction using a coupled transcription/translation rabbit reticulocyte system as instructed by the supplier (Promega). For the binding assay, 15 µl of the translation product was used for binding to 20 µl of the glutathione beads precoated with either GST–Ded1p or GST in a binding buffer (150 mM KCl, 20 mM Tris–HCl, pH 7.5,

5 mM $MgCl_2$, 0.1% Triton X-100) for 2 h at 4°C. After extensive washes with the binding buffer, beads were boiled in SDS–PAGE loading dye. Proteins in the supernatant were separated by SDS–PAGE and the gel was dried and then autoradiographed. To test binding of L-A particles to GST–Ded1p, 15 µl of CsCl-purified L-A particles were used. The binding efficiency was assessed by the recovered L-A dsRNA or the Gag protein.

RNA polymerase reaction and product analysis

In vitro transcription and replication were done in 20 µl reactions with purified L-A particles (5 µl) in the presence of 500 ng of GST or GST°Ded1p using the standard conditions of Fujimura and Wickner (22). The purification of positive-strand RNA-containing L-A particles was done as described (22). Two T-vector (Promega) plasmid clones, pGAG1001 (positive-strand) and pGAG1004 (negative-strand), with the Gag ORF inserted in opposite orientations, were used for preparing single-stranded plasmid DNAs for probing the strandedness of the reaction products by standard Southern blotting procedures.

RESULTS

Specific interaction of Ded1p with the Gag protein

We have previously shown that Ded1p is an essential translation factor (11). To elucidate the role of Ded1p in translation, we sought to identify its interacting proteins by using a yeast strain in which the chromosomal *DED1* gene had been deleted and complemented by a plasmid-born recombinant *DED1–Protein A* gene, in which the Protein A moiety (PA) was fused in-frame to the C-terminus of Ded1p. The growth rate of this strain is indistinguishable from that of the wild-type strain under a variety of conditions, suggesting that the recombinant Ded1p–PA protein is functional (11). Yeast extracts were made from this strain and then incubated with IgG–Sepharose beads. The recombinant Ded1p–PA is expected to bind to IgG via its PA moiety. After extensive washes, Ded1p–PA and its associated proteins were eluted from IgG beads by 0.5 M acetic acid and analyzed by SDS–PAGE (20).

One prominent ~80 kDa protein was found to co-elute with the 94 kDa Ded1p–PA (Fig. 1A, lane 2). Internal peptide sequencing of this 80 kDa protein yielded two peptide sequences, FAYRHALT and VYGDTHGLTK, perfectly matching amino acid sequences 240–247 and 527–536, respectively, of the L-A virus Gag protein. The identity of the 80 kDa protein was confirmed by its specific reaction with the anti-Gag antibody (data not shown). The observed Gag–Ded1p interaction was specific because, using the same experimental protocol, Gag protein could not be recovered from the wild-type yeast extract (Fig. 1A, lane 1), which contained untagged Ded1p, nor from control extracts containing either Dbp3p–PA (lane 3) or Dbp5p–PA fusion proteins (lane 4). The Dbp5p–PA extract was a good control, because Dbp5p is also a cytoplasmic DExD/H-box protein of similar abundance to Ded1p. Experiments using extracts containing N-terminally tagged PA–Ded1p also allowed Gag recovery at the same level (not shown), arguing that the binding of Ded1p to Gag does not simply result from fusion of the PA moiety to the C-terminus of Ded1p.

Because Ded1p is an RNA-binding protein (25), we wondered whether the observed Ded1p–Gag interaction was RNA-dependent. This was not the case, as extensive treatment of the IgG–Sepharose-bound material by RNase did not affect Gag recovery (Fig. 1B). To show that Ded1p directly interacts with Gag, we synthesized ^{35}S-labeled Gag by *in vitro* transcription and translation and used it for binding to glutathione beads precoated with either GST–Ded1p fusion (Fig. 1C, lane 1) or GST alone (lane 2). Binding of Gag to GST–Ded1p was at least 10-fold more efficient than to GST.

We then assessed the binding affinity of Ded1p–PA for Gag by step-eluting the Gag protein using elution buffer containing increasingly concentrated $MgCl_2$ (21). The Gag protein was eluted in a peak at ~0.5 M $MgCl_2$, suggesting high affinity between Gag and Ded1p (Fig. 1D). The same strong interaction was also observed between purified GST–Ded1p and ^{35}S-labeled Gag *in vitro* (not shown). We therefore conclude that Ded1p interacts specifically and directly with Gag with high affinity.

Specific interaction of Ded1p with the L-A virus particles

Careful inspection of co-precipitation results (Fig. 1A and B) revealed that a 180 kDa protein of lesser abundance also co-eluted with Ded1p–PA, prompting us to speculate that it could be the Gag–Pol fusion protein (12), which is present in the L-A virus at only one to two copies per particle (12,26). This prediction was validated by western analysis using an antibody (12) recognizing only the Pol moiety (data not shown). These results thus suggest that Ded1p may interact with the L-A virus particle. To test this hypothesis, we asked whether L-A dsRNA could also be co-purified with Ded1p–PA. Indeed, extraction of the IgG-bound material from the Ded1p–PA extract (Fig. 2A, lane 1, top panel) yielded an RNA species with similar gel mobility to that of the cellular L-A dsRNA (lanes 3 and 4). This RNA was not detected in a control experiment using Dbp5p–PA extract (lane 2), despite its presence in both Ded1p–PA and Dbp5p–PA extracts (cf. lanes 3 and 4).

To prove that the co-purified RNA species is indeed the L-A dsRNA, we synthesized ^{32}P-labeled positive-strand and negative-strand probes from an L-A cDNA clone. Both probes hybridized to the RNA species (Fig. 2A, lane 1, middle and bottom panels) and to the L-A dsRNA present in the total cellular RNA (lanes 3 and 4, middle and bottom panels), but not to any corresponding RNA species in the co-purified fraction from the Dbp5p–PA extract (lane 2). Thus, the recovered RNA species is the L-A dsRNA. Because the L-A Gag protein is also used to encapsidate the satellite M_1 dsRNA (5), we checked whether Ded1p also binds to the M_1 virus particle. Similar experiments using extracts prepared from a yeast strain containing Ded1p–PA and M_1 virus particles (see Materials and Methods) showed that M_1 dsRNA, as monitored by northern blotting, was also efficiently recovered (data not shown).

If Ded1p interacts with the L-A virus particles, the L-A dsRNA should be eluted from the IgG beads in a profile identical to that of the Gag protein. This indeed was the case, in that both the L-A dsRNA and the Gag protein were

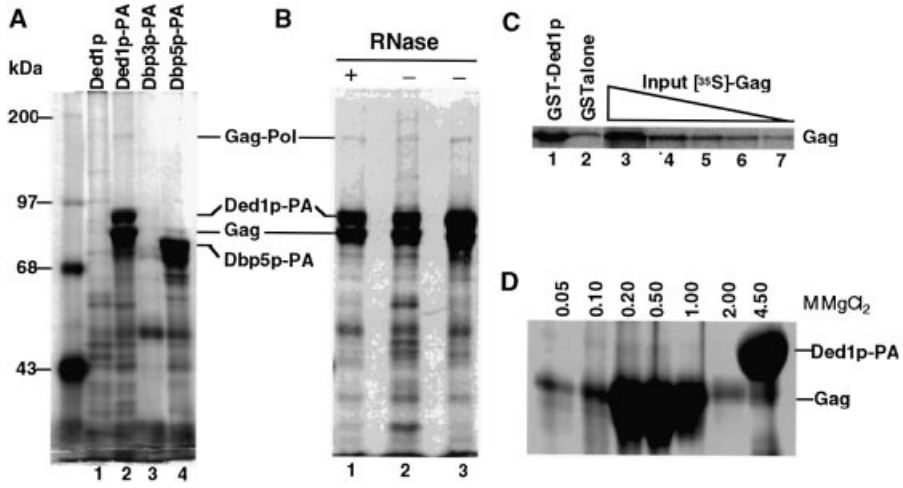

Figure 1. Interaction of the L-A Gag protein with Ded1p. (**A**) Ded1p interacts specifically with Gag. Extracts prepared from yeast strains containing Ded1p (lane 1), Ded1p–PA (lane 2), Dbp3p–PA (lane 3) or Dbp5p–PA (lane 4) were incubated with IgG–Sepharose. After washes, IgG-bound proteins were eluted with acetic acid and analyzed by SDS–PAGE. Molecular size markers are indicated (kDa). (**B**) Resistance of Ded1p–Gag interaction to RNase treatment. Ded1p–PA-bound IgG beads were treated with 10 µl of RNase cocktail (1 mg/ml RNase A and 20 000 U/ml RNase T1; Ambion.) and 10 µl of micrococcal nuclease (1 mg/ml; Worthington Biochemical), incubated at 30°C for 30 min (lane 1) and analyzed as in (A). Lanes 2 (30°C) and 3 (4°C) are Ded1p–PA-bound IgG beads without RNase treatment but incubated for 30 min at the indicated temperatures. (**C**) *In vitro* binding of Ded1p with Gag. *In vitro* synthesized ^{35}S-labeled Gag was incubated with glutathione beads precoated with either GST–Ded1p (lane 1) or GST alone (lane 2). Materials bound were analyzed by SDS–PAGE followed by autoradiography. Lanes 3—7, 4% (lane 3), 1% (lane 4), 0.5% (lane 5), 0.25% (lane 6) and 0.125% (lane 7) of the input ^{35}S-labeled Gag. (**D**) Ded1p interacts strongly with Gag. Extracts from Ded1p–PA-containing strain was incubated with IgG–Sepharose. The IgG-bound proteins were then sequentially eluted with increased concentrations (0.05–4.5 M) of $MgCl_2$ as shown.

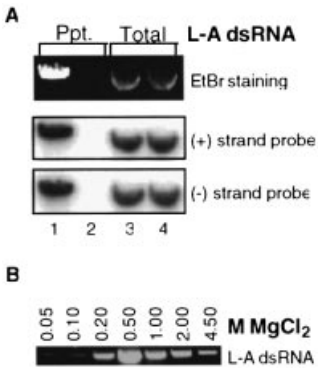

Figure 2. L-A dsRNA co-precipitates with Ded1p–PA. (**A**) L-A dsRNA co-precipitates specifically with Ded1p–PA. Extracts made from yeast strains harboring Ded1p–PA (lane 1) or Dbp5p–PA (lane 2) were incubated with IgG–Sepharose. The bound RNAs were extracted with phenol/chloroform, precipitated (Ppt.) by ethanol, separated by agarose gel electrophoresis and visualized by ethidium bromide (EtBr) staining (top panel, lanes 1 and 2). Total RNAs (Total) prior to immunoprecipitation were extracted and used as positive controls (lanes 3 and 4). The identity of the L-A dsRNA was verified by northern blotting using either ^{32}P-labeled positive-strand probe (middle panel) or negative-strand probe (bottom panel). (**B**) The L-A dsRNA co-elutes with Gag. The experiment was done as in Figure 1D, except that the RNA was extracted from $MgCl_2$-eluted fractions, separated by agarose gel electrophoresis and then visualized by EtBr staining.

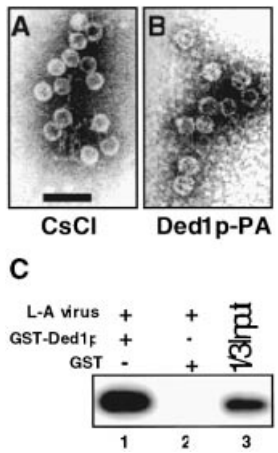

Figure 3. Electron microscopy imaging of purified L-A particles and their interaction with GST–Ded1p. (**A**) L-A particles were purified from a wild-type strain by a conventional CsCl gradient method or (**B**) from a Ded1p–PA-containing strain by affinity purification, in which L-A particles were eluted in one step by 2 M $MgCl_2$. Virus particles were then imaged by transmission electron microscopy. Bar, 100 nm. (**C**) Direct interaction of Ded1p with L-A particles. CsCl-purified L-A particles were incubated with glutathione beads precoated with GST–Ded1p (lane 1) or GST (lane 2). Lane 3 represents 33% of the input L-A particles. After incubation and washes, beads were analyzed as described in Figure 2A.

co-eluted in a peak at ~0.5 M $MgCl_2$ (Figs 1D and 2B). To further show that L-A virus particles interact with Ded1p, the IgG-bound material was eluted and subjected to electron microscopy imaging. A population of particles measuring ~40 nm in diameter was observed (Fig. 3B). These particles were identical in shape and dimension to the L-A virus particles (24) purified from a wild-type yeast strain using a conventional CsCl purification protocol (Fig. 3A).

To demonstrate that Ded1p is capable of interacting with the purified L-A virus *in vitro*, we used the CsCl-purified L-A virions for binding to either GST–Ded1p- or GST-coated glutathione beads. GST–Ded1p allowed recovery of >50% of the input L-A virions, judging from the amount of L-A dsRNA recovered (Fig. 3C, lane 1). In contrast, little, if any, L-A dsRNA was recovered from GST-coated beads (lane 2). Western analysis using anti-Gag antibody yielded the same

result (data not shown). This higher yield of recovery, in comparison to the recovery of the *in vitro* synthesized Gag by GST–Ded1p (Fig. 1C), raised the possibility that the conformation of the L-A virion is critical for its effective binding to Ded1p.

Affinity-purified L-A virions are transcriptionally active

It is possible, although it seems unlikely, that Ded1p–PA interacts only with either damaged or defectively assembled L-A particles, which could be functionally inactive. To address this issue, we first assessed the RNase susceptibility of the L-A dsRNA molecules in the affinity-purified material. Incubation of the IgG-bound material in the presence of an internal control, i.e. a ^{32}P-labeled L-A transcript, with pancreatic RNase (22) resulted in complete destruction of the added L-A transcript, yet still permitted quantitative recovery of the L-A dsRNA (Fig. 4A). Since the RNase treatment was shown to readily degrade naked L-A dsRNA (22) these results suggest that the affinity-purified L-A particles were sufficiently intact to shield the encapsidated L-A dsRNA from RNase attack.

Since CsCl-purified L-A virions are known to synthesize both positive- and negative-strand RNAs *in vitro* (22), we next examined the affinity-purified L-A virions for such activities. Affinity-purified L-A virions were bound to the Ded1p–PA-conjugated IgG beads and incubated under standard *in vitro* transcription conditions. The production of several major RNA species was readily detected by denaturing agarose gel electrophoresis (Fig. 4B, lane 1). The 4.6 kb species, because of its apparent size, most likely corresponds to a full-length L-A transcript, whereas the shorter species may represent either truncated or degraded products of *in vitro* transcription. As expected, the control experiment using Dbp5p–PA extract yielded no detectable RNA synthesis (Fig. 4B, lane 2). To determine the strandedness of the synthesized products, we gel-purified the ^{32}P-labeled 4.6 kb species and used it as a probe for Southern analysis. This probe hybridized to two single-stranded DNA clones harboring either the positive or the negative strand of the L-A dsRNA (Fig. 4C, lanes 3 and 4) and to a double-stranded L-A cDNA clone (lane 2), but not to an empty vector (lane 1). These data suggest that Ded1p interacts with at least two functional forms of the L-A virion: one that contains the L-A dsRNA, which can synthesize the positive-strand transcript, and the other that contains the positive-strand RNA, which can produce the negative-strand transcript.

Ded1p promotes L-A negative-strand RNA synthesis

Although a number of chromosomal *MAK* genes are essential for viral dsRNA, especially M_1, replication, their gene products are not known to stably associate with the virus particles (5). The fact that Ded1p interacts specifically and strongly with the L-A particle thus raises the possibility that it may participate in the L-A life cycle. Since earlier data suggest that a host factor(s) in a 0–50% ammonium sulfate fraction of a crude extract is required for negative-strand RNA synthesis *in vitro* (12), we wondered whether Ded1p could be a part of this activity. To test this hypothesis, we purified a fraction of the L-A particles that contain predominantly, if not exclusively, the positive-strand RNA from mid-log phase yeast cultures (22). When incubated with GST alone, these

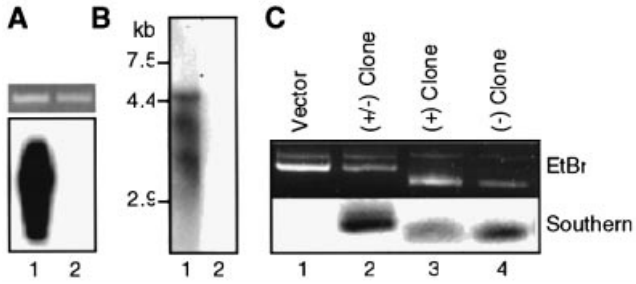

Figure 4. Affinity-purified L-A particles are active in synthesizing L-A RNA. (**A**) The co-precipitated L-A dsRNA is resistant to RNase treatment. Ded1p–PA-bound IgG beads were incubated with pancreatic RNase in the presence of a ^{32}P-labeled L-A transcript as described in Materials and Methods. After incubation, RNAs were recovered and run on an agarose gel, which was stained with ethidium bromide (top panel) and autoradiographed (bottom panel). (**B**) *In vitro* synthesis of the L-A transcript. L-A particles were bound to IgG beads precoated with Ded1p–PA (lane 1) or Dbp5p–PA (lane 2) for *in vitro* RNA polymerase activity assay. The ^{32}P-labeled RNA products were separated on a denaturing agarose gel and autoradiographed. RNA size markers (kb) are indicated to the left. (**C**) Production of both positive (+) and negative (–) RNA by affinity-purified virions. The virus-synthesized RNA from lane 1 of (B) was used as a probe to hybridize to vector DNA alone (lane 1), vector DNA containing L-A cDNA (lane 2), single-stranded DNA containing the (+)-strand L-A cDNA (lane 3) and single-stranded DNA containing the (–)-strand L-A cDNA (lane 4). (Top panel) Ethidium bromide (EtBr) staining; (bottom panel) autoradiogram of the Southern blotting result.

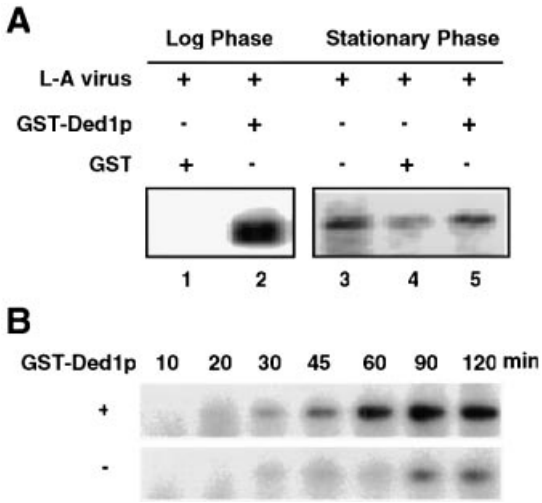

Figure 5. Ded1p accelerates the rate of *in vitro* negative-strand RNA synthesis. (**A**) Ded1p promotes negative-strand RNA synthesis but not positive-strand RNA synthesis. Positive-strand RNA-containing (Log Phase) or dsRNA-containing (Stationary Phase) L-A particles were incubated in the presence of either GST (lanes 1 and 4) or GST–Ded1p (lanes 2 and 5). Lane 3, dsRNA virus alone. After incubation, RNAs were extracted and analyzed by denaturing agarose gel electrophoresis (lanes 1 and 2) and autoradiographed. (**B**) Time–course study of Ded1p-promoted negative (–) RNA synthesis. Aliquots of the reaction in (A) using the positive (+)-strand RNA-containing L-A particles (Log Phase) were withdrawn at various time points and analyzed in native agarose gel electrophoresis.

positive-strand RNA particles produced little negative-strand RNA (Fig. 5A, lane 1). In sharp contrast, addition of GST–Ded1p greatly stimulated negative-strand synthesis to at least 10-fold (lane 2). Time–course studies further revealed that Ded1p significantly accelerated the rate of negative-strand synthesis (Fig. 5B). Notably, this stimulation is specific, as

Prp28p (27), a DExD/H-box splicing factor, and a mutant Ded1p with altered NTP-binding pocket (DEAD-to-DAAD) known to be devoid of ATPase and RNA-winding activities (25), both failed to appreciably promote negative-strand RNA synthesis (not shown). We confirmed that the Ded1p-dependent product is indeed the negative-strand RNA by strand-dependent Southern analysis (not shown). Finally, to test whether Ded1p can also promote positive-strand RNA synthesis, L-A dsRNA-containing virus particles purified from stationary phase yeast cultures (22) were used in the same reaction. Neither GST nor GST–Ded1p was found to stimulate production of the positive-strand RNA (Fig. 5A, lanes 4 and 5). Taken together, we conclude that Ded1p is likely to be a host factor recruited by the L-A virus to promote its negative-strand synthesis.

DISCUSSION

To successfully multiply in the cell, RNA viruses, such as the positive-strand RNA and the dsRNA viruses, must overcome a series of challenges. One of the immediate tasks is to compensate for their lack of a mRNA 5′ cap and/or 3′ poly(A) tail, which in theory would severely handicap viral mRNAs in terms of their stability and translatability. RNA viruses are known to have evolved a variety of strategies to circumvent this problem. For example, poliovirus uses its virus-encoded proteinase 2A to cleave the translation initiation factor 4G (eIF4G) (28) and poly(A)-binding protein (29,30), thereby inhibiting host cell mRNA translation. Yet, poliovirus RNA translation proceeds in a cap-independent fashion by recruiting ribosomes via its internal ribosome entry site (IRES) in the absence of intact eIF4G. The L-A virus, on the other hand, uses its Gag protein to covalently bind to and cleave the cellular mRNA 5′ cap, thereby yielding cap-less decoys to shield the also cap-less L-A transcript from RNase attack (31). Once the virus-encoded RdRp and other viral proteins are produced, viruses, owing to their gene-poor nature, face another layer of challenge in effectively organizing an RNA replication complex by recruiting host factors to produce negative-strand RNA from the positive-strand template. Classic examples of this borrowing act include the bacteriophage Qβ, which incorporates EF-Ts and EF-Tu and the ribosomal protein S1 into its replicase (4) and vesicular stomatitis virus, whose RdRp binds strongly to EF-1 (homologous to bacterial EF-Tu), which in turn binds EF-1β and 1 (both homologous to EF-Ts) (32). Remarkably, these and other examples, including those of BMV (13,33–35) and tobacco mosaic virus (36,37), appear to indicate that components of the host translation machinery are favored targets for viral recruitment. In this work, we provide a novel example that appears to be consistent with this general proposal.

We showed that Ded1p, an evolutionarily conserved DExD/H-box translation factor, binds specifically and tightly to the yeast L-A virus particle and, in doing so, promotes L-A negative-strand RNA synthesis *in vitro*. Although work is in progress to demonstrate that Ded1p is required for L-A replication *in vivo*, several lines of evidence have already implicated Ded1p in some aspects of the viral role. First and foremost, a recessive *ded1* allele (*ded1-18*), isolated from a genetic screen aiming to identify host factors required for BMV replication, represses BMV replication in yeast by selectively inhibiting BMV RNA2 translation without impacting on general cellular translation or translation of BMV RNA1 (13). Thus, Ded1p must play a role in promoting BMV RNA2 translation, at least in yeast. Since Ded1p is highly conserved among eukaryotes, its involvement in BMV replication in plants is almost certainly expected. Second, DBX (DDX3 or CAP-Rf), the human ortholog of Ded1p (54% sequence identity), was found by three independent studies (14–16) to interact directly and strongly with the HCV core protein, reminiscent of the interaction of Ded1p with the Gag protein. While the physiological significance of this interaction remains to be elucidated, the apparent evolutionary relatedness among positive-strand RNA viruses and dsRNA viruses argues for the case. For example, an evolutionary link between dsRNA viruses and flaviviruses, including HCV, has been proposed on the basis of the striking similarity in the catalytic side chain positions, overall molecular architecture and topology and other shared biochemical properties of their respective RdRp (38). In addition, recent studies on the assembly of the BMV replication complex by Schwartz *et al*. (39) clearly demonstrated that positive-strand RNA viruses, dsRNAs viruses and retroviruses share fundamental similarities in replication and, therefore, may have common evolutionary origins. The fact that Ded1p is implicated in all classes of viruses that replicate through mRNA intermediates raises the tantalizing possibility that it may be widely favored among these RNA viruses and therefore may represent a viable target for antiviral therapeutic intervention.

Many RNA viruses encode DExD/H-box or helicase-like proteins, such as NS3 (HCV), 2C (poliovirus) and 1a (BMV), in their genomes. These helicase-like proteins are thought to participate in viral replication via their putative RNA helicase activities because some can unwind short RNA duplexes *in vitro*. Yet, their precise roles in the viral life cycle remain largely a mystery. For example, NS3 turns out to be a highly processive helicase on DNA, but a poor helicase on RNA, thus prompting speculation on its role in influencing host DNA (40). Perhaps this offers a partial explanation as to why viruses may need to recruit another DExD/H-box protein, such as Ded1p, for their replication. What, then, could possibly be the role of Ded1p in RNA virus replication? Previous work by Noueiry *et al.* (13) on BMV replication provides some interesting clues. They showed that the dependency of BMV RNA2 translation on Ded1p is correlated with the presence of a 31 nt region found only in RNA2. Thus, a logical prediction would be that Ded1p exerts its function on this region by resolving a putative inhibitory RNA structure. This model fits well with the conventional view that DExD/H-box proteins bind directly to and unwind specific RNA duplexes and that eIF4A, the prototypical DExD/H-box translation factor, is responsible for unwinding stable RNA structures in the 5′ untranslated region to promote mRNA translation (41). In this light, a plausible model for the role of Ded1p in L-A replication would be that Ded1p modulates the positive-strand RNA structure to facilitate negative-strand RNA synthesis. Implicit in this model is the notion that Ded1p must be able to reach the positive-strand L-A RNA molecule encapsidated in the virus particle. Structural studies of the L-A virus (42,43) revealed that the capsid wall of the particle is perforated by openings of 18 Å in diameter at the icosahedral 5-fold axes.

These openings are thought to function as molecular sieves to allow the exit of transcript and the exchange of metabolites, while retaining dsRNA and excluding degradative enzymes. Although we have yet to completely rule out the possibility that a fraction of purified L-A particles were damaged, thereby allowing Ded1p to access the L-A RNAs, the fact that the affinity- and CsCl-purified particles are resistant to RNase treatment (Figs 1B and 4A) and that the CsCl-purified particles, which were used for structural analysis by other groups, bind to GST–Ded1p (Fig. 3C) argues that Ded1p most likely binds to the virus particle externally. If so, it would be difficult to envisage how Ded1p directly touches the L-A RNAs.

An alternative model would be to evoke a recently propounded 'RNPase' hypothesis (44,45) built on several lines of experimental evidence. First, biochemical studies of eIF4A suggest that the DExD/H-box proteins may perform functions distinct from RNA unwinding, which include mediating large-scale RNA structural rearrangements, disrupting protein–RNA or protein–protein interactions and functioning as fidelity sensors in RNA–RNA interactions and rearrangements (46,47). Second, it was demonstrated that the essential requirement of two DExD/H-box splicing factors, Prp28p (27) and Sub2p (48), can be eliminated by specific mutations that, by genetic definition, define their corresponding *in vivo* protein targets. Third, the DExD/H-box protein NPH-II from vaccinia virus can displace the protein U1A from RNA in an active ATP-dependent fashion (49). On the basis of these studies, it was proposed that DExH/D proteins may act as 'RNPases' to reorganize or 'remodel', the structures of ribonucleoprotein assemblies. Thus, it is tempting to speculate that recruitment of Ded1p by the positive-strand RNA-containing L-A particles may result in conformational changes of the viral particles, which in turn influence the L-A Gag–Pol activity to favor negative-strand synthesis. Alternatively, Ded1p may specifically modify Gag–Pol alone to yield the same outcome. To differentiate these possibilities, works are in progress to map the contact points and to determine the stoichiometric ratio between Ded1p and the L-A particle.

Finally, it remains formally possible that Ded1p may also have a role in promoting L-A translation, because it is a general translation factor (10,11). Our recent biochemical analysis suggests that Ded1p plays a critical role prior to formation of the 43S translation initiation complex, because depletion of Ded1p results in a dramatic build-up of mRNPs (J.-L.Chong, R.-Y.Chuang and T.-H.Chang, in preparation). Critically, Ded1p appears to function in a cap- and poly(A) tail-independent manner (J.-L.Chong, R.-Y.Chuang and T.-H.Chang, in preparation). This latter observation is of particular interest and may provide an underlying rationale for the specific recruitment of Ded1p, among other equally abundant cytoplasmic DExD/H-box proteins, by L-A, because L-A transcript possesses neither 5′ cap nor 3′ poly(A) tail. Future insights into the mechanistic role of Ded1p in translation initiation may shed new lights on its viral role.

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