Membrane Association via an Amino-terminal Amphipathic Helix Is Required for the Cellular Organization and Function of RNase II*

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Background: Cellular organization of the Escherichia coli exoribonuclease RNase II is unknown.

Results: Membrane association and self-interactions are both required for the RNase II cellular organization.

Conclusion: Membrane binding facilitates RNase II interactions leading to assembly into organized cellular structures.

Significance: Interplay of membrane association and formation of organized membrane-associated cellular structures could provide a mechanism to organize multiprotein systems within the bacterial cell.

The subcellular localization of the exoribonuclease RNase II is not known despite the advanced biochemical characterization of the enzyme. Here we report that RNase II is organized into cellular structures that appear to coil around the Escherichia coli cell periphery and that RNase II is associated with the cytoplasmic membrane by its amino-terminal amphipathic helix. The helix also acts as an autonomous transplacental membrane binding domain capable of directing normally cytoplasmic proteins to the membrane. Assembly of the organized cellular structures of RNase II required the RNase II amphipathic membrane binding domain. Co-immunoprecipitation of the protein from cell extracts indicated that RNase II interacts with itself. The RNase II self-interaction and the ability of the protein to assemble into organized cellular structures required the membrane binding domain. The ability of RNase II to maintain cell viability in the absence of the exoribonuclease polynucleotide phosphorylase was markedly diminished when the RNase II cellular structures were lost due to changes in the amphipathicity of the amino-terminal helix, suggesting that membrane association and assembly of RNase II into organized cellular structures play an important role in the normal function of the protein within the bacterial cell.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Growth Conditions—E. coli strains were grown in LB medium (6) to which 100 μg/ml ampicillin, 30 μg/ml kanamycin, 30 μg/ml chloramphenicol, or 0.4% (w/v) glucose were added when indicated. Plasmids and strains are listed in Table 1, and the details of their construction are available upon request. Gene knockouts and yfp fusion to the chromosomal rnb gene were constructed by linear DNA recombination using λ Red-mediated gene replacement (7). HA and FLAG-epitope tagging was done as previously described (8). P1-mediated transduction was used to move mutations to different strains (9).

Microscopy—E. coli cells containing plasmids coding for Yfp-labeled proteins were grown at 30 °C in the presence of 10 mM isopropyl β-D-thiogalactoside (IPTG) (10). Yfp-labeled cells were examined by fluorescence microscopy as previously described.
**Cellular Organization of RNase II**

### TABLE 1
#### Plasmids and strains

To allow selection during construction of HA, FLAG, and Yfp chromosomal fusions and deletion strains, a kanamycin-resistance cassette was inserted by linear DNA recombination ("Experimental Procedures").

| Plasmids            | Relevant genotype or description | Reference or source |
|---------------------|---------------------------------|---------------------|
| pMLB1113            | Low copy number vector (18 per cell) | (40 and 41) |
| pAT75               | pSC_yfp::minDA (261–270)         | (27)               |
| pAT80               | pSC_yfp                | This study          |
| pFL1                | pSC-rnb::yfp             | This study          |
| pFL3                | pSC-rnbDA::yfp           | This study          |
| pFL4                | pSC-rnb::yfp             | This study          |
| pFL6                | pSC-rnb::yfp             | This study          |
| pFL7                | pSC-rnb::yfp             | This study          |
| pFL8                | pSC-rnb::yfp             | This study          |
| pFL9                | pSC-rnb::yfp             | This study          |
| pFL10               | pSC-rnb::yfp             | This study          |
| pFL11               | pSC-MBD::rnbDA (1–19)::yfp | This study          |
| pFL12               | pSC-MBD::yfp             | This study          |
| pFL13               | pSC-rnb::yfp             | This study          |
| pFL15               | pSC-rnb::yfp             | This study          |
| pFL16               | pSC-rnb::yfp             | This study          |
| pFL17               | pSC-rnb::yfp             | This study          |
| pFL18               | pSC-rnb::yfp             | This study          |
| pFL20               | pSC-rnb::yfp             | This study          |
| pFL21               | pSC-rnb::yfp             | This study          |
| pFL22               | pSC-rnb::yfp             | This study          |
| pFL23               | pSC-rnb::yfp             | This study          |
| pFL24               | pSC-rnb::yfp             | This study          |
| pFL25               | pSC-rnb::yfp             | This study          |
| pFL26               | pSC-rnb::yfp             | This study          |
| pFL30               | pSC-rnb2::yfp            | This study          |
| pFL31               | pSC-rnb::yfp             | This study          |
| pFL32               | pSC-MBD::rnbDA (1–19)::yfp | This study          |
| pKD4                | kan marker for gene deletion| (7)                 |
| pKD46               | Red recombinase expression plasmid | (7)                  |
| pCP10               | FLP expression plasmid       | (42)                |
| pSU312              | FLAG-kan                | (8)                 |
| pSU315              | HA-kan                  | (8)                 |

| Strains             | Relevant genotype or description | Reference or source |
|---------------------|---------------------------------|---------------------|
| MC1000              | F- araD139 ∆(araABC-leu)7679 galL | (40)               |
| RC1                 | MC1000 ΔinDED·kan             | (43)               |
| AT67                | MC1000 rnb::HA-kan            | This study          |
| AT68                | MC1000 rnb::FLAG-kan          | This study          |
| AT368               | MC1000 rnb::yfp-kan           | This study          |
| AT372               | MC1000 Δmp·cat rnb::yfp-kan   | This study          |
| FL1                 | MC1000 Δrnb·kan              | This study          |
| FL2                 | Δmp·cat                   | This study          |
| FL3                 | Δyfp·cat                  | This study          |
| FL4                 | MC1000 Δrnb·kan Δmp·cat     | This study          |
| TM388               | Δmp·cat                   | This study          |

* Plasmids pAT and pFL are made in pMLB1113 vector.
* Strain not viable unless complemented.

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**Co-immunoprecipitation**—Cells that co-express a chromosomally encoded RNase II-HA and a plasmid-encoded RNase II-Yfp derivative were grown at 30 °C to A<sub>600</sub> 0.4, and then the expression of the indicated Yfp-labeled proteins was induced for 4 h by growth in the presence of 10 μM IPTG.

Cells that express chromosomally encoded RNase II-HA (AT67) or RNase II-Yfp (AT368) from the native P<sub>rnb</sub> promoter were grown under the same conditions except that IPTG was omitted. Equal amounts of AT67 and AT368 cells grown to the same cell density were combined to obtain protein extract containing both chromosomally expressed RNase II-HA and RNase II-Yfp proteins.

The cell pellets were washed twice with cold PBS buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl) and then frozen at −70 °C. Protein extracts were prepared as previously described (12) except that: (i) the cells were broken in a French pressure cell followed by three 20-s bursts of sonication at 4 °C, (ii) EDTA-free protease inhibitor mixture tablets were used in all solutions (Roche Diagnostics), and (iii) the ammonium sulfate pellet was resuspended in immunoprecipitations (IP) buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1% Nonidet P-40, and 1× protease inhibitor mixture). Under these conditions 60–65% of RNase II present in clarified cell lysates was recovered in the ammonium sulfate precipitates as shown by quantitative immunoblotting analysis. Protein concentrations were determined using a BCA assay kit (Pierce). Immunoprecipitations were carried out using protein A/G-agarose beads according to manufacturer’s instructions (Pierce) and polyclonal anti-HA or anti-Yfp antibodies (Santa Cruz Biotechnology) or rabbit IgG (Sigma). Beads were first incubated overnight at 4 °C in the presence of 1 mg of protein extract in 0.5 ml IP buffer and then washed 3 times with 0.5 ml of IP buffer using spin columns. Retained proteins were eluted by incubating the beads for 5 min at room temperature in 50 μl of elution buffer containing primary amine, pH 2.5 (Pierce) and then collected by centrifugation in tubes containing 3 μl of 1 M Tris buffer, pH 9.5, to neutralize the pH. Boiled samples were electrophoresed in 10% SDS mini-polyacrylamide gels and then electropholated to nitrocellulose membrane in a Tris-glycine transfer buffer for 70 min with constant voltage of 100 V. Western blots were done using anti-HA and anti-Yfp (which binds Yfp protein) combined primary antibodies and alkaline phosphatase-conjugated anti-IgG secondary antibody (Sigma). When indicated, cell extracts were preincubated for 15 min at room temperature with 5 μg/ml DNase-free bovine pancreatic RNase (Roche Diagnostics) before overnight incubation with the beads (13). Complete degradation of RNA was obtained within 15 min of incubation at room temperature in IP buffer when RNase activity was tested on protein extracts supplemented with 200 μg/ml of yeast RNA (Sigma).

**Membrane isolation**—Cells were grown as described above for co-immunoprecipitation. Cells were broken in a French pressure cell, and membranes fractions were prepared by two-step sucrose gradient centrifugation as previously described (14). The SG0 fraction containing the total cell membranes was washed once with 10 ml of cold HE buffer (10 mM Hepes, pH 7.4, 5 mM EDTA) and then resuspended in the same buffer.

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**Described** (10). Immunofluorescence experiments were done as previously described (11), except that the initial centrifugation steps were eliminated to avoid possible perturbation of protein localization. Cells were fixed in the growth medium at room temperature for 1 h in the presence of 2% formaldehyde and 0.016% glutaraldehyde and then adsorbed on silane-coated coverslips. Monoclonal mouse anti-HA and polyclonal rabbit anti-FLAG (Sigma) and Alexa fluor 488-conjugated goat anti-mouse and anti-rabbit antibodies (Molecular Probes) were used to detect HA and FLAG-tagged RNase II. Images were not subjected to deconvolution. 200–300 cells were analyzed for each strain, and the shown localization pattern was present in 90–95% of the cells.
To quantitatively assess the RNase II membrane association, membrane and cytoplasm cellular fractions were prepared by ultracentrifugation of cell extracts as previously described (15, 16). Isolated membranes and cytoplasm were brought to the initial volume of the total protein extract, and equal volumes from each fraction were loaded on SDS-polyacrylamide gels and subjected to Western blot analysis using anti-HA and anti-Gfp primary antibodies. Band intensities were proportional to the amount of protein applied to the gels and were quantified by densitometry using the ImageQuant program (Molecular Dynamics).

**Immunoblotting Analyses**—To compare the expression levels of the Yfp-labeled RNase II derivatives, SDS total cell extracts were made using 1 ml of culture from cells that were grown as indicated above for fluorescence microscopy. Cell samples were taken at 2.5, 4.5, 6.5, and 8.5 h after the addition of IPTG. The frozen cell pellets were resuspended in 10 mM Tris buffer, pH 7.5, containing 1 mM EDTA and 4% SDS and then boiled for 5 min. Equal amounts of total protein extracts relative to the A600 of the cultures were then separated on 10% polyacrylamide gels and electrophoresed into nitrocellulose membranes. Band intensities were proportional to the amounts of protein applied to gels. Anti-Gfp primary antibody (which binds Yfp protein) and alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody were used to detect the Yfp-labeled RNase II derivatives.

**RESULTS**

**Cellular Organization of RNase II**—Evidence that RNase II is organized within the cell and not uniformly distributed within the cytoplasm was shown by Yfp-labeling of plasmid-encoded RNase II and by tagging of the *rnb* chromosomal gene with FLAG or HA epitopes followed by fluorescence and immunofluorescence localization studies (Fig. 1, A–D). RNase II-Yfp, RNase II-HA, and RNase II-FLAG proteins are functional proteins, as shown by their ability to restore viability to Δ*rnb* cells that lack polynucleotide phosphorylase and RNase II (below).

These studies showed that carboxyl-terminal-labeled RNase II-Yfp was localized within cellular structures that appeared to coil around the cell periphery, extending for considerable distances along the long axis of the cell (Fig. 1A). Immunofluorescence microscopy of cells expressing chromosomally encoded RNase II-FLAG or RNase II-HA using anti-FLAG or anti-HA

The amino acid sequence of the NTH domain with its secondary structure is shown. K, shown is Western blot analysis of a membrane fraction isolated by sucrose gradient centrifugation (14) (panel 1) and membrane and cytoplasm fractions isolated by ultracentrifugation (15, 16) (panels 2–6) using monoclonal antibodies anti-HA and anti-Gfp (panels 1–4) or purified polyclonal antibodies anti-MinD (panel 5) or anti-MreB (panel 6), and the indicated cellular fractions from cells that co-expressed chromosomally encoded HA-tagged RNase II and plasmid-encoded Yfp-labeled RNase II mutants are shown. Panels 1 and 2 and panels 5 and 6, AT67/pFL13 cells (mbr::HA/lanp::mbrΔ**/**-yfp). Panel 3, AT67/pFL13 cells (mbr::HA/lanp::mbrΔ**/**-yfp). Panel 4, AT67/pFL13 cells (mbr::HA/lanp::mbrΔ**/**-yfp). Isolated cellular fractions were brought to the same volume as the protein extract, and then equal volumes from total protein extract (lanes 1–3), membranes (lanes 4–6), and cytoplasm (lanes 7–9) were loaded as indicated. Panels 1, 2, 4, or 8 μl from each sample were loaded in lanes 1 and 4 and lanes 2, 3, 5, or lanes 3 and 6, respectively. Arrows indicate positions of protein bands of Yfp-labeled RNase II mutants (green), HA-tagged RNase II (white), and MinD (black). The arrowhead indicates MreB bands, and mbr** indicates mbr mutants.

**FIGURE 1. Role of the amino-terminal amphipathic helix in membrane binding and cellular organization of RNase II.** A–H, micrographs of Yfp fluorescence of the plasmid-encoded RNase II proteins in the indicated strains and immunofluorescence of the chromosomally encoded FLAG or HA-tagged RNase II are shown. Panel A, FL1/pFL3 cells (Δmbr/lanp::mbr-yfp). Panel B, AT68 (mbr::FLAG). Panel C, AT67 (mbr::HA). Panel D, FL1/pFL1 (Δmbr/lanp::yfp-mbr). Panel E, FL1/pFL13 (Δmbr/lanp::mbrΔ**/**-yfp). Panel F, MC1000/pAT80 (wt/lanp::yfp). Panel G, FL1/pFL4 (Δmbr/lanp::mbrΔ**/**-yfp). Panel H, FL1/pFL30 (Δmbr/lanp::mbrΔ**/**-yfp). Scale bar = 1 μm. 200–300 cells were analyzed for each strain, and the shown localization pattern was present in 90–95% of the cells (‘‘Experimental Procedures’’). I, an x-ray structure of RNase II shows the amino-terminal helix (red), the nucleic acid binding oligonucleotide/oligosaccharide binding (OB)-fold domains (blue), and the ribonuclease catalytic domain (gray). The image generated according to Refs. 19 and 20 using the PyMOL molecular graphics system (39) and RNase II protein (protein data bank accession number 2ID0). J, shown is a helical wheel projection of the RNase II amino-terminal helix using the HeliQuest program (30). Nonpolar (yellow), polar noncharged (black), and polar charged residues (blue) are shown.
primary antibodies, respectively, showed similar organized cellular structures (Fig. 1, B and C). In contrast, amino-terminal-labeled Yfp-RNase II showed a diffuse localization pattern presumably because Yfp obstructed binding of the protein to the membrane (see below) (Fig. 1D).

The cellular localization pattern of RNase II was not an artifact of Yfp-labeling (17) or cell fixation as similar RNase II structures were observed both by fluorescence microscopy of live RNase II-Yfp cells and immunofluorescence microscopy of fixed cells that expressed RNase II-HA or RNase II-FLAG. The organized cellular structures were not due to RNase II overexpression because similar localization patterns were obtained with cells that expressed higher than normal levels of plasmid-encoded RNase II-Yfp and cells that expressed chromosomally encoded RNase II-HA or RNase II-FLAG under control of the native Prnb promoter (Figs. 1, A–C and 4F2).

Taken together the data show that RNase II exists within the cell as organized structures that appear associated with the cell periphery. These RNase II cellular structures resemble previously described long range ordered structures of other E. coli proteins (11, 18).

RNase II Contains an Amino-terminal Amphipathic Helix That Acts as a Membrane Binding Anchor—We noted that the previously reported x-ray structure of RNase II shows an amino-terminal helical domain that extends at roughly right angles from the body of the protein (Fig. 1). The amino-terminal helix (NTH) is not part of the adjacent oligonucleotide/oligosaccharide binding-fold cold shock domain 1, and its function has not previously been elucidated (19, 20). A helical wheel projection of the amino acid sequence of the NTH revealed an amphipathic character of the helical surfaces, with hydrophobic residues clustered on one face and polar residues on the other face (Fig. 1I). Because amphipathic helices act as membrane binding anchors for other proteins (21–26), we asked whether the NTH of RNase II might play a role in binding the protein to the cytoplasmic membrane and/or in the formation of the organized cellular structures of the protein.

Evidence that RNase II is a membrane-associated protein and that the NTH domain is required for its membrane binding came from cell fractionation studies. The strain AT67/pFL4 co-expressed a chromosomally encoded RNase II-HA protein that showed an organized cellular pattern and a plasmid-encoded RNase IIΔYfp protein that lacked the NTH helix domain and showed a diffuse cytoplasmic distribution pattern (see below). The total membrane fraction isolated by sucrose gradient sedimentation (14) was analyzed by Western blot using anti-Gfp (which interacts with Yfp) and anti-HA to identify RNase IIΔ19, Yfp and RNase II-HA, respectively. As shown in Fig. 1K1, the membrane fraction contained the full-length HA-tagged RNase II but not the derivative that lacked the amphipathic helix domain. This shows that RNase II is a membrane-associated protein and that determinants in the NTH domain are required for the binding of the protein to the membrane.

To assess the RNase II membrane association, total membrane and cytoplasmic fractions were isolated by ultracentrifugation (15, 16) from the strain that co-expressed a chromosomally encoded RNase II-HA protein and a plasmid-encoded RNase IIΔ19-Yfp protein and then quantitatively analyzed by Western blot using anti-Gfp and anti-HA antibodies. As illustrated in Fig. 1K2, the membrane fraction contained ~50% of the full-length HA-tagged RNase II but undetectable amounts of the derivative that lacked the amphipathic helix domain.

Parallel analyses of membrane and cytoplasmic fractions using antibodies against the known membrane-associated proteins MinD and MreB, which bind the membrane through an amphipathic helix, also showed ~50% of the proteins in the membrane fraction (Fig. 1K, 5 and 6). The presence of a portion of the protein in the cytoplasmic fraction may reflect partial dissociation of the peripheral membrane proteins during the process of cellular breakage and fractionation or dynamic membrane association of proteins within the cell.

Evidence that the NTH domain RNase II(1–19) is able to act as a transplanta membrane anchor that can direct normally cytoplasmic proteins to the membrane was obtained from localization studies of the Yfp-labeled NTH domain (RNase II(1–19)-Yfp) and cell fractionation studies. Expression of the Yfp-labeled NTH domain in Δrnb cells that lack endogenous RNase II showed a peripheral localization pattern characteristic of membrane-associated proteins (Fig. 1I). As expected, expression of free Yfp resulted in diffuse localization of Yfp throughout the cytoplasm (Fig. 1F).

Fractionation of cells that expressed RNase II(1–19), Yfp showed a similar fractionation pattern as the full-length RNase II protein and the membrane associated proteins MinD and MreB; that is, 51% of RNase II(1–19)-Yfp was recovered with the membrane fraction (Fig. 1K3). This demonstrates that the NTH domain of RNase II can impart membrane binding activity to the normally cytoplasmic Yfp protein. The NTH domain is, therefore, an autonomous membrane binding domain.

The Amino-terminal Helix Is Required for the RNase II Cellular Organization—To determine whether the membrane binding domain of RNase II plays a role in the cellular organization of the protein, we carried out localization studies of plasmid-encoded Yfp-labeled RNase II derivatives in cells that lacked the endogenous mbb gene. Deletion of the first 19 residues of RNase II, including the entire amphipathic helix, led to loss of the organized structures observed with full-length RNase II. Instead, the RNase IIΔ19-Yfp was diffusely distributed within the cell, indicating a cytoplasmic localization (Fig. 1G). In contrast, deletion of the three residues that precede and lie outside of the amphipathic helix did not affect the ability of RNase II to assemble within the structures (Fig. 1H), indicating that these residues are not required for RNase II membrane binding and cellular organization.

Cellular fractionation studies further confirmed the subcellular localization of RNase IIΔ3 and RNase IIΔ19. RNase IIΔ19 was exclusively found in the cytoplasmic fraction (Fig. 1K2), whereas RNase IIΔ3 was associated with the membrane fraction, similar to full-length RNase II (Fig. 1K4). Taken together with the fact that RNase II(1–19)-Yfp was peripherally localized with no evidence of organized structures (Fig. 1I), the data indicate that membrane binding of RNase II is required but is not sufficient for assembly of the organized membrane-associated cellular structures that are seen with full-length RNase II.

Role of Amphipathicity of the RNase II Amino-terminal Helix—We next used site-directed mutagenesis to address the role of
the amphipathic character of the NTH in the membrane association and cellular organization of RNase II. To disrupt the amphipathicity of the helix within the full-length protein, four leucines of the hydrophobic face of the helix (Fig. 2A) were individually replaced by the polar amino acid aspartic acid. In all cases the Yfp-labeled L7D, L8D, L11D, and L15D mutant proteins were diffusely distributed throughout the cytoplasm (Fig. 2, B, F, J, and N), indicating that the mutant proteins were incapable of binding the membrane and assembling into organized cellular structures. Diffuse localization patterns were also obtained when the mutations L7D, L8D, L11D, or L15D were introduced into the RNase II(1–19)Yfp protein, which contains the membrane binding NTH domain in the absence of the rest of the RNase II protein (Fig. 2, C, G, K, and O), confirming that interference with the amphipathic nature of the NTH results in loss of membrane binding of its NTH domain.

In contrast, when the leucines were individually replaced by other hydrophobic amino acids (valine or isoleucine), the RNase II-Yfp L7V, L8V, L11V, and L15V mutant proteins were organized into structures that were indistinguishable from the cellular structures formed by wild type RNase II-Yfp (Fig. 2, D, H, L, P, and Q). Similarly, replacement of polar amino acids with other polar amino acids in RNase II(1–19)Yfp and RNase II(1–19)L15D-Yfp did not affect the ability of the proteins to assemble into organized structures (Fig. 2, E and J). Consistent with the observation that deletion of the residues that precede the amphipathic helix did not interfere with the normal cellular localization and organization of RNase II (Fig. 1, H and K4), replacement of the second residue phenylalanine by lysine did not alter the cellular organization of RNase II (Fig. 2M). Quantitative Western blot analysis showed similar cellular concentrations of all of the wild type and mutant proteins, indicating that the differences in localization pattern among the RNase II mutants and derivatives did not come from variations in cellular levels of the proteins (data not shown).

Consistent with the fluorescence localization studies of the RNase II mutants, cell fractionation experiments confirmed that the membrane association was completely abolished in L7D RNase II and RNase II(1–19)Yfp-labeled mutant proteins (Fig. 2R, I and 2) and was maintained similar to the wild type in L7V and K12E RNase II-Yfp mutants (Fig. 2R, 3 and 4). Taken together, the data indicate that the amphipathic nature of the NTH is required for RNase II membrane association and for the organization of RNase II within the cell.

**Specificity of RNase II Amino-terminal Membrane Anchor—** To investigate the sequence specificity of the NTH in directing the membrane binding and cellular organization of RNase II, we replaced the RNase II NTH domain with the heterologous membrane binding amphipathic helical domain of the MinD protein (MBDMinD) (Fig. 3A). MBDMinD was previously shown capable of acting as a membrane anchor when linked to Gfp or several cytoplasmic proteins (22, 23, 27). As opposed to the amino-terminal membrane binding domain of RNase II, MBDMinD is located at the carboxyl end of the MinD protein. Replacing the RNase II NTH with MBDMinD, therefore, reverses the relative orientation of the MBDMinD amino acid sequence and its connectivity to MinD protein. Because of the possibility that the relative orientation of MBDMinD to RNase II protein affects...
membrane binding, we studied the localization patterns of RNase II derivatives in which MBD\textsuperscript{MinD} or the reversed sequence of MBD\textsuperscript{MinD} (DBM\textsuperscript{MinD} Fig. 3A) replaced the RNase II NTH domain. As shown in Fig. 3, B and C, MBD\textsuperscript{MinD}-RNase II\textsuperscript{Δ19-Yfp} and DBM\textsuperscript{MinD}-RNase II\textsuperscript{Δ19-Yfp} were localized into organized cellular structures independently of the orientation of the MinD membrane binding domain. The cellular structures formed by the RNase II-MinD chimeras closely resembled the structures of the native RNase II protein (Figs. 1, A–C, and 3, B and C). We conclude that the ability to support the membrane association and the assembly of RNase II into organized cellular structures is not restricted to the RNase II NTH domain.

We also asked whether the RNase II NTH domain could substitute for the MBD\textsuperscript{MinD} in supporting MinD membrane association and formation of the MinD cytoskeletal-like helical structures (18). As expected from previous studies, deletion of the last 10 residues of MinD, coding for the MBD\textsuperscript{MinD}, abrogated the membrane binding and the cytoskeletal-like organization of the MinD protein (Fig. 3E) (21, 28). This was reversed by adding the RNase II NTH domain. Thus, the chimeric Yfp-MinD\textsuperscript{1–260}-NTH\textsuperscript{RNase II} was organized into cellular structures that coiled around the cell periphery and extended along the length of the cell (Fig. 3F). These structures were similar to the normal MinD cytoskeletal-like helical structures (18).

These experiments show that the RNase II and MinD membrane domains are interchangeable in supporting the membrane binding and higher order cellular organization of the RNase II and MinD proteins. To further investigate the specificity of the membrane anchor for the membrane association and cellular organization of RNase II, we replaced the RNase II NTH domain with the carboxyl-terminal amphipathic membrane binding helix of the cell division protein FtsA (MBD\textsuperscript{FtsA}) (Fig. 3A). The MBD\textsuperscript{FtsA} was previously shown to act as an independent membrane anchor when appended to other proteins (23, 29).

The chimeric MBD\textsuperscript{FtsA}-RNase II\textsuperscript{Δ19-Yfp} protein was localized at the cell periphery but without any higher order organized structures of the protein (Fig. 3G). Thus, although the RNase II, MinD, and FtsA membrane anchors were functionally interchangeable in recruiting RNase II to the membrane, only the RNase II and MinD MBDS were compatible with the assembly of RNase II into its characteristic organized cellular structures. The properties of these different membrane binding domains are further discussed below.

Role of the Membrane Anchor in RNase II Self-interaction—Why might membrane association be required for formation of the RNase II organized cellular arrays? Here we consider the possibility that membrane association of RNase II facilitates self-interaction of the protein that plays a role in the assembly of the RNase II long range ordered cellular structures.

Evidence for RNase II self-association came from co-immunoprecipitation of differentially labeled RNase II proteins from protein extracts of cells that co-expressed chromosomally encoded RNase II-HA and plasmid-encoded RNase II-Yfp. IPs were obtained by using protein A/G beads coupled to anti-HA, anti-Gfp, or IgG antibodies. The immunoprecipitates were then analyzed by Western blots using anti-HA and anti-Gfp antibodies simultaneously. This showed the presence of both RNase II-HA and RNase II-Yfp in the IP fractions obtained with anti-HA beads (Fig. 4, A1, right panel). Similarly, RNase II-HA and RNase II-Yfp were both present in the IP fractions obtained by immunoprecipitation with anti-Gfp beads (Fig. 4A2). In contrast, no RNase II bands were detected in the IP fraction obtained with the IgG control beads (Fig. 4, A1, left panel). The observed co-immunoprecipitation of RNase II-HA and RNase II-Yfp was consistent with RNase II-RNase II interactions within the cell extract.

Silver staining of the IP fraction obtained with anti-HA beads showed RNase II-HA and RNase II-Yfp as the only major bands in addition to the IgG bands (data not shown), consistent with direct RNase II-RNase II interactions. However, the possibility that one or more other proteins mediated the observed RNase II-RNase II interactions cannot be excluded.

Evidence that the RNase II interaction was not mediated by an RNA bridge (i.e. substrate-mediated association) came from immunoprecipitation of cell extracts that were pretreated with
bovine pancreatic RNase. RNase II-HA and RNase II-Yfp bands were both present in the IP fractions obtained from RNase-treated extracts using anti-HA beads (Fig. 4A1), indicating that the degradation of RNA in the protein extracts did not alter the RNase II-RNase II interactions. This suggests that RNA does not play a role in the observed RNase II-RNase II interactions.

To determine whether the observed RNase II self-interaction was due to RNase II-Yfp overexpression, we compared parallel co-immunoprecipitations of differentially labeled RNase II proteins from protein extracts of cells that expressed RNase II-Yfp from a plasmid under the control of the \( P_{\text{lac}} \) promoter or from the chromosomal native \( P_{\text{rmb}} \) promoter. The cell extract that contains both of the chromosomally expressed RNase II-HA and RNase II-Yfp proteins was obtained by mixing equal amounts of AT67 and AT368 cells that expressed individual proteins, respectively. These showed the presence of equivalent amounts of RNase II-Yfp in IP fractions obtained with anti-HA beads (Fig. 4F1), although the cellular level of the chromosomally expressed RNase II-Yfp was 10-fold lower compared with the level of plasmid-encoded protein (Fig. 4F2) as determined by quantitative Western blots. The fact that similar extents of co-immunoprecipitation of RNase II-HA and RNase II-Yfp were obtained whether or not RNase II-Yfp was overexpressed shows that the RNase II interactions shown here were not artifacts of protein overexpression.

To determine whether the RNase II self-interaction required membrane association, we asked whether the interaction is dependent on the presence of the NTH domain. We performed the immunoprecipitation procedure on protein extracts from cells that co-expressed RNase II-HA, which contains the NTH domain, and RNase II-Yfp. The proteins were precipitated using anti-HA beads, and the bands were analyzed using Western blots. The results showed that RNase II-HA and RNase II-Yfp were precipitated together, indicating that the interaction is not dependent on the NTH domain.

**FIGURE 4. RNase II self-association.** Co-immunoprecipitation with anti-HA (A1–E1 (right side) and F1), anti-Gfp (A2–E2 (right side)), or IgG (A–E (left side)) beads of protein extract from cells that expressed the indicated RNase II derivatives is shown. Protein extracts (PE (3 μg)) and immunoprecipitation fractions, flow-through (FT), and IP, obtained with the indicated IgG, anti-HA, or anti-Gfp beads, were subjected to Western blots using combined anti-HA and anti-Gfp polyclonal (panels A–E) or monoclonal (panel F1) antibodies. Drawings depict beads used to obtain the fractions analyzed in the adjacent gel. A, AT67/pFL3 (\( rnb::HA/P_{\text{lac}}::rnb::yfp \)). B, AT67/pFL4 (\( rnb::HA/P_{\text{lac}}::rnb::yfp \)). C, AT67/pFL8 (\( rnb::HA/P_{\text{lac}}::rmb::yfp \)). D, AT67/pFL11 (\( rnb::HA/P_{\text{lac}}::MBD\text{ftsA}::rmb::yfp \)). E, AT67/pFL32 (\( rnb::HA/P_{\text{lac}}::MBD::rnb::yfp \)). F, AT67/pFL3 (\( rnb::HA/P_{\text{lac}}::rmb::yfp \)) (left), AT67 (\( rnb::HA \)) + AT368 (\( rnb::yfp \)) (right). To determine the extent of overexpression of the plasmid-encoded RNase II-Yfp same (1X) or three-time (3X) protein amounts of cell extracts from AT67/pFL3 and AT67 + AT368 cells were applied to gels and immunoblotted with monoclonal (F2 top panels) or polyclonal (F2 bottom panels) antibodies as indicated. When indicated, protein extracts were pretreated with RNase from bovine pancreas as described under “Experimental Procedures” (panels A1 and D2). Empty arrows show the positions of Yfp-tagged RNase II derivatives, and black arrows show the positions of HA-tagged RNase II bands.
and forms organized cellular structures (Fig. 1C), and RNase IIΔ19-Yfp, which fails to form organized structures and is located in the cytoplasm (Fig. 1, G and K2). This showed that only a single band of the cognate RNase II-HA or RNase IIΔ19-Yfp was detected in the IP fractions obtained by immunoprecipitations with anti-HA or anti-Gfp beads, respectively (Fig. 4B, right panels). We obtained similar results (Fig. 4C) from cells that co-expressed RNase II-HA and RNase IIΔL8D-Yfp, which failed to form organized membrane-associated structures (Fig. 2F). Thus, the RNase II-RNase II interaction was lost when one of the interacting partners lacked the NTH domain (RNase IIΔ19-Yfp) or the amphipathicity of its NTH domain was disrupted (RNase IIΔL8D-Yfp). These results show that the RNase II self-interaction requires the presence of functional membrane binding domains on both interacting proteins, implying a requirement for RNase II membrane binding by both partners. This suggests that the interactions occur subsequent to entry of both partners into the membrane. Membrane anchors may also interact with small membrane vesicles that remained in cell extracts or with lipid-detergent micelles within the amphiphilic media used for preparation of cell extracts and the immunoprecipitation experiments. The data indicate that assembly of the RNase II organized cellular structures is associated with the ability of the protein to bind the membrane and to interact directly or indirectly with itself.

To ask whether the RNase II interaction is specific for the RNase II NTH domain, immunoprecipitation experiments were performed on extracts from cells that co-expressed RNase II-HA and a plasmid-encoded RNase II derivative in which the NTH domain was replaced by the membrane binding domain of MinD or FtsA. As shown in Fig. 4D, RNase II-HA and MBDMinD-RNase IIΔ19-Yfp were both found in the IP fractions obtained with anti-HA or anti-Gfp beads but not detected in the IP fractions obtained with IgG control beads. The maintenance of interactions between RNase II derivatives that carry these different membrane anchors indicates that RNase II self-interaction does not occur via homotypic interactions between the membrane binding anchors.

In contrast, no interaction was found when MBDFtsA replaced the native membrane anchor of RNase II-Yfp. As shown in Fig. 4E, only a single protein band of RNase II-HA or MBDFtsA-RNase IIΔ19-Yfp was detected in the IP fractions obtained with anti-HA or anti-Gfp beads, respectively. The lack of interaction between RNase II and MBDFtsA-RNase IIΔ19 indicates that membrane binding of RNase II, although required, is not sufficient for the RNase II-RNase II interactions in cell extracts.

The MBDFtsA-RNase IIΔ19 derivative, in contrast to MBDMinD-RNase IIΔ19, failed to assemble into organized structures despite its membrane association (Fig. 3, B and G). The FtsA MBD differs from the MBDS of RNase II and MinD in the higher hydrophobicity of its non-polar face. Thus, the hydrophobic moments of the RNase II, MinD, and FtsA membrane binding domains were 0.373, 0.554, and 0.716, respectively (30). The FtsA MBD also contains more positively charged residues on its hydrophilic surface (Fig. 3A), increasing its amphipathic character. The possible role of these differences on the self-interaction properties of RNase II and the ability of the protein to assemble high order membrane-associated structures is discussed below.

Role of RNase II Cellular Organization in Normal Cell Function—RNase II is essential for the viability of cells that lack polynucleotide phosphorylase, the other major exoribonuclease of the cell (3). This provided a means to evaluate the functional significance of the RNase II membrane association and cellular organization. We compared the ability of the plasmid-encoded RNase II derivatives to support normal growth in Arnb Δrnp cells (FL4) that lacked the chromosomally encoded RNase II derivatives in the presence of 10 μM IPTG or RNase II-Yfp under the control of the P rmb chromosomal promoter are, respectively, shown. The presence of higher IPTG concentrations (50 and 100 μl) gave similar growth. White circles, AT372 Δrnp/pFL21 (Arnb Δrnp/P rmb-yfp); black circles, FL4/pFL3 (Arnb Δrnp/P rmb-yfp); black squares, FL4/pFL21 (Arnb Δrnp/P rmb-L8V-yfp); stars, FL4/pFL7 (Arnb Δrnp/P rmb-L7V-yfp); black triangles, FL4/pFL8 (Arnb Δrnp/P rmb-L7V-yfp).

FIGURE 5. Effect of the RNase II mutations on cell growth. Complementation assays compare the ability of the RNase II derivatives to rescue FL4 cells (Δrnp). A, mid-log phase cultures of FL4 strains harboring the indicated plasmids were adjusted to the same optical density (A 600) and serially diluted (10−1–10−6), and then 1 μl of each culture was spotted on agar plates containing 50 μM IPTG or 0.4% glucose. Similar results were obtained when the plates contained 150 μM IPTG or when mb-HA and mb-FLAG replaced the chromosomal copy of mb gene in FL2 (Δrnp mb::HA) and FL3 (Δrnp mb::FLAG) cells, respectively (data not shown). B, growth curves of FL4 and AT372 cells expressing under P lac control the indicated plasmid-encoded RNase II-Yfp derivatives in the presence of 10 μM IPTG or RNase II-Yfp under the control of the P rmb chromosomal promoter are, respectively, shown. The presence of higher IPTG concentrations (50 and 100 μM) gave similar growth. White circles, AT372 Δrnp/pFL3 (Arnb Δrnp/P rmb-yfp); black circles, FL4/pFL3 (Arnb Δrnp/P rmb-yfp); black squares, FL4/pFL21 (Arnb Δrnp/P rmb-L8V-yfp); stars, FL4/pFL7 (Arnb Δrnp/P rmb-L7V-yfp); black triangles, FL4/pFL8 (Arnb Δrnp/P rmb-L7V-yfp).
of RNase II or RNase II-Yfp expression by growth in the presence of glucose blocked cell growth (Fig. 5A, first and second lanes (+glucose)). Disruptions of the amphipathicity of the RNase II NTH that lead to cytoplasmic localization of the protein (RNase II^{LTV}, RNase II^{LAV}) were associated with a significant decrease in growth rate of Δrnb Δpnp cells (Fig. 5, A, second to fourth lanes, and B). In contrast, conservative mutations of the NTH of RNase II that maintained the normal cellular organization pattern of the wild type protein (RNase II^{LTV}, RNase II^{LAV}) supported normal growth in Δrnb Δpnp cells (Fig. 5, A, fifth and sixth lanes, and B). This suggested that the ability of RNase II to associate with the membrane and/or to assemble into higher order organized cellular structures is required for the maintenance of normal growth in cells that lack polynucleotide phosphorylase.

Evidence that the ability of RNase II-Yfp to maintain viability of Δpnp Δrnb cells was not due to overexpression of the plasmid-encoded RNase II-Yfp came from comparison of growth rates of Δpnp Δrnb cells whose rnb gene was replaced by a plasmid-encoded rnb::yfp under P_{lac} control (FL4/pFL3) and Δpnp cells whose rnb gene was replaced by a chromosomally encoded rnb::yfp under the native P_{rnb} promoter (AT372). As shown in Fig. 5B, both strains grew at similar growth rates independently of the expression levels of RNase II-Yfp (Fig. 4F2). Thus, the observed complementation of Δpnp Δrnb cells was not due to overexpression of RNase II-Yfp.

The chimeric RNase II protein in which the MBD^{MinD} replaced the RNase II NTH domain, which exhibited the normal organization pattern of RNase II, behaved similarly to wild type RNase II in its ability to restore normal growth to the Δrnb Δpnp strain (Fig. 5A, lane 7). In contrast, the MBD^{FixA}·RNase IIΔ^{19}·Yfp protein that shows peripheral localization but fails to assemble the normal organized cellular patterns failed to restore growth in the Δrnb Δpnp cells. This was shown by the inability to produce colonies in the presence of a wide range of concentrations of IPTG after the attempted introduction of a Δpnp-cat mutation into the Δrnb/P_{lac}·MBD^{FixA}·:rnbΔ^{19}·:yfp cells by P1 transduction or by Λ Red-mediated gene replacement (7, 9). This was done under conditions that gave a large number of Δpnp colonies in parallel experiments using Δrnb/ P_{lac}·rnb or Δrnb/P_{lac}·rnb::yfp recipient cells. This indicates that RNase II membrane association alone is insufficient to restore normal cellular function. Taken together, the data suggest that the RNase II higher order cellular organization is required for normal cell growth in Δpnp cells.

DISCUSSION

Localization studies of RNase II, the major exoribonuclease of E. coli, using fully functional Yfp-labeled and epitope-tagged RNase II proteins, showed that the protein is not diffuse within the cytoplasm but rather organized as long range cellular structures that appear to coil around the cell periphery. The present work also shows that an amino-terminal amphipathic helix (NTH) of RNase II acts as a membrane binding anchor that is required for the assembly of RNase II into organized cellular structures. Assembly of the RNase II cellular structures is dependent on the ability of the protein to bind the cytoplasmic membrane via the amphipathic anchor, as shown by the observation that deletion of the RNase II NTH domain or perturbation of its amphipathicity abrogated membrane association and assembly of the organized cellular structures.

It is also clear that assembly of the RNase II cellular structures requires determinants outside of the membrane anchor. This was shown by the inability of the NTH domain of NTH-Yfp to form organized cellular structures in the absence of other RNase II domains despite the fact that the NTH domain alone was capable of recruiting Yfp to the membrane.

Proteins of the E. coli MreBCD and MinCDE cytoskeletal-like systems also rely on amphipathic helix membrane anchors for their cellular organization. The amino-terminal helix of MreB and carboxyl-terminal helix of MinD, respectively, are required for assembly of MreB cellular structures (24), which are essential for cell shape maintenance, or for formation of the helically organized MinD structures that are required for proper placement of the division site at midcell (31). In these systems the membrane anchor is located on the surface of the membrane, oriented parallel to the bilayer surface. The membrane association is mediated by interactions of lipid acyl chains of the bilayer with nonpolar amino acids chains that extend from one face of the amphipathic helix into the bilayer structure (24, 28, 32). A similar mechanism for membrane binding is likely for the NTH domain of RNase II, as this successfully replaced the MBD^{MinD} in supporting membrane association and assembly of the MinD cytoskeletal-like structures.

Assembly of the organized RNase II cellular structures is also dependent on the ability of the protein to interact directly or indirectly with itself, as shown by co-immunoprecipitation studies. The RNase II interactions require the presence of a membrane binding domain on both interacting RNase II partners, implying that membrane association is required for the RNase II self-interactions. It is possible that these interactions may also involve interactions between adjacent membrane anchor domains. However, the x-ray structure of RNase II shows that the NTH domains of adjacent molecules are oriented away from each other and from the subunit interfaces of the protein (20), arguing against the likelihood that direct interaction between NTH domains mediates the observed RNase II self-interaction.

The results are compatible with a model in which membrane binding of RNase II is associated with a change in conformation that leads to RNase II self-association that plays a role in its subsequent assembly into membrane-associated organized cellular structures. The RNase II self-association shown by co-immunoprecipitation from cell extracts could reflect either direct RNase II-RNase II interactions or the association of RNase II molecules via interactions with other components of the RNA processing and degradation machinery.

The RNase II cellular structures were maintained when the unrelated MinD MBD replaced the RNase II NTH membrane anchor. In contrast, assembly of the RNase II organized structures was lost when the FtsA MBD directed the protein to the membrane. The FtsA MBD differs from the MBDS of RNase II and MinD by the higher amphipathicity of its helix. MBD^{FixA} is, therefore, likely to bind more tightly to the membrane surface. This could affect the conformation or orientation of the membrane-associated protein, thereby hindering RNase II interac-
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...tions that are required for assembly of its characteristic organized cellular structures.

It is of interest that many of the proteins involved in RNA processing and degradation also contain potential membrane binding domains. Thus, the endoribonuclease RNase E contains an internal amphipathic helix that can act as a membrane anchor to associate the protein with lipid vesicles (33), PAP I contains a potential membrane binding domain within its first 24 amino acids (34, 35), Hfq is localized in close proximity to the cytoplasmic membrane in electron microscopic studies (36), and the endoribonuclease RNase III and RNase P were found in inner membrane fractions in cell fractionation studies (37). Whether other components of the RNA processing and degradation are also membrane associated and whether membrane-association of these proteins is required for their proper cellular organization and function, as is the case for RNase II, remains to be determined.

What might be the biological role of the membrane association and cellular organization of RNase II? Mutations of the NTH that interfered with formation of the RNase II organized structures were associated with a significant defect in the growth of cells that lack polynucleotide phosphorylase and RNase II. This suggests that the cellular organization of RNase II might be required for the enzyme to carry out essential cellular functions. The growth defect could come from loss of the cellular organization of RNase II or/and from a direct effect of the NTH mutations on the intrinsic catalytic activity of the enzyme. Arguing against the second possibility are previous studies showing that RNase II derivatives with deletions of the first 38 or 84 residues of the protein, which remove the NTH membrane binding domain, were as efficient as full-length RNase II in degrading RNA substrates in vitro (38). Nonetheless, it remains to be directly determined whether the mutations described here, which are restricted to individual residues within the NTH domain, interfere with the exoribonuclease activity of the isolated proteins.

There are several possible biological roles for the assembly of RNase II into its characteristic organized membrane-associated structures. (i) The membrane association may play a regulatory role by modulating the enzymatic activity of the protein. (ii) The cytoplasmic membrane may act as a matrix to facilitate interactions of RNase II with itself or with other components of the RNA processing and degradation machinery by increasing the local concentration due to confinement to the two-dimensional membrane milieu or by altering the conformation of the protein. (iii) The higher order organization of the protein might sequester RNase II and other components of the cellular RNA processing and degradation, such as the RNA degradosome, within a common compartment. This could serve as a mechanism to bring in close proximity protein partners that act cooperatively on their RNA substrates and/or keep the proteins away from the cytoplasm to prevent premature degradation of RNA substrates. These possibilities are not mutually exclusive. Further work will be needed to define the nature of the apparent higher order RNase II structures in the cell and the role of the membrane association and cellular organization of the protein in the essential RNA degradation and processing functions of the bacterial cell.

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