Helicase Activity Plays a Crucial Role for RNase R Function in Vivo and for RNA Metabolism

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RNase R is a 3' to 5' hydrolytic exoribonuclease that has the unusual ability to digest highly structured RNA. The enzyme possesses an intrinsic, ATP-dependent RNA helicase activity that is essential in vitro for efficient nuclease activity against double-stranded RNA substrates, particularly at lower temperatures, with more stable RNA duplexes, and for duplexes with short 3’ overhangs. Here, we inquire whether the helicase activity was also important for RNase R function in vivo and for RNA metabolism. We find that strains containing a helicase-deficient RNase R due to mutations in its ATP-binding Walker motifs exhibit growth defects at low temperatures. Most importantly, cells also lacking polynucleotide phosphorylase (PNPase), and dependent for growth on RNase R, grow extremely poorly at 34, 37, and 42 °C and do not grow at all at 31 °C. Northern analysis revealed that in these cells, fragments of 16S and 23S rRNA accumulate to high levels, leading to interference with ribosome maturation and ultimately to cell death. These findings indicate that the intrinsic helicase activity of RNase R is required for its proper functioning in vivo and for effective RNA metabolism.

Experimental Procedures

Materials—Mutagenic primers were synthesized and purified by Sigma Genosys. Bacteriophage T4 polynucleotide kinase was purchased from New England Biolabs, Inc., [γ-32P]ATP was from PerkinElmer Life Sciences, nylon membrane was from GE Healthcare Life Sciences, and ExpressHyb hybridization solution was from Clontech. All chemicals were reagent grade.

Bacterial Strains—All strains used in this study were derivatives of E. coli MG1655(Seq)* Δrph, which was considered to be wild type for this study. MG1655(Seq)*, an rph” derivative of MG1655, was constructed by Donald Court (NCI, National Institutes of Health, Bethesda, MD) and provided by Kenneth Rudd (University of Miami, FL). The RNase I” derivatives were

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**TABLE 1**

| Growth rates of RNase R Walker motif mutant strains in rich medium |
|---------------------------------------------------------------|
| **Growth temperature** | **Doubling time (min)** |
|                      | **WT** | **R** | **R736A** | **R D168A,D169A** |
| 42 °C                | 22 ± 2 | 23 ± 2 | 22 ± 3 | 22 ± 2 |
| 37 °C                | 26 ± 2 | 27 ± 2 | 28 ± 2 | 28 ± 3 |
| 31 °C                | 41 ± 2 | 45 ± 2 | 48 ± 3 | 47 ± 3 |
| 25 °C                | 74 ± 3 | 114 ± 4 | 123 ± 4** | 119 ± 4** |
| 20 °C                | 121 ± 3 | 197 ± 4 | 213 ± 5** | 207 ± 4** |
| 15 °C                | 235 ± 5 | 396 ± 5 | 417 ± 6** | 409 ± 6** |

In earlier studies, we found that in vitro the intrinsic helicase activity of RNase R is important for effective nucleolytic activity against dsRNA substrates, particularly at lower temperatures, with duplexes having short 3′ overhangs, and with more stable duplexes (8, 9). The studies presented below examine the role of the helicase activity in RNase R function in vivo.

**Results**

In earlier studies, we found that in vitro the intrinsic helicase activity of RNase R is important for effective nucleolytic activity against dsRNA substrates, particularly at lower temperatures, with duplexes having short 3′ overhangs, and with more stable duplexes (8, 9). The studies presented below examine the role of the helicase activity in RNase R function in vivo.

We also determined the doubling times of WT and mutant strains in M9/glucose minimal medium at different temperatures. As shown in Table 2, all the growth rates were dramatically slowed in minimal medium as compared with rich medium, but the relative rates remained the same. The Walker motif mutants grew the same as the RNase R deletion strain at all temperatures examined. At higher temperatures (37 and 42 °C), the mutant strains had doubling times almost the same as that of the WT strain, but grew more slowly as the temperature decreased, particularly at 25 and 15 °C. Based on these experiments, we concluded that the presence of RNase R is most important at lower temperatures and that the absence of helicase activity is deleterious to cell growth as complete removal of RNase R.

**Growth of RNase R Walker Motif Mutant Strains**

To examine the importance of the RNase R helicase activity, we mutated conserved amino acids within the Walker A (Lys-736) or Walker B (Asp-168 and Asp-169) ATP-binding motifs to alanine residues. These mutations were previously shown to inactivate the helicase activity (8). For initial analysis of the mutant strains carrying each of these mutations in the chromosome, we measured their growth rates in rich medium and compared them with those of the WT strain and with the Walker A or Walker B mutant motifs.

**Complementation Analysis**

E. coli MG1655ΔΔrrn, E. coli MG1655ΔΔrrnΔδhelicase, and E. coli MG1655ΔΔrrnΔΔδhelicase were used. Plasmid pET44 containing RNase R WT and its mutant derivatives were used for the complementation analysis. Leaky expression of the inserted genes in this vector system in the absence of isopropyl-1-thio-β-D-galactopyranoside was used to keep overexpression low. After transformation, each strain was streaked on LB plates and incubated at different temperatures for various time periods, as indicated in the figures.
data, it appears that the intrinsic helicase activity is an important component for the proper functioning of RNase R in vivo, and that its role becomes more pronounced at lower temperatures, consistent with our previous findings that the helicase activity is essential for efficient degradation of double-stranded RNA in vitro (8, 9).

**Growth of RNase R Walker Motif Mutant Strains Also Lacking PNPase**—In *E. coli*, in addition to RNase R, PNPase also participates in degradation of structured RNA (2, 18). Cells become inviable only when both nucleases are absent. Thus, to more accurately assess the specific role of the helicase activity for RNase R function, we introduced a PNP− mutation into the strains already mutated in their Walker A or Walker B motifs, and then measured growth of the resulting strains (Table 3). Cells lacking PNPase grew more slowly than those lacking RNase R at all temperatures examined, and the growth differential became more pronounced at lower temperatures, indicating that PNPase is required for normal cell growth, especially at temperatures below −25 °C. However, removal of the RNase R helicase activity by mutation of either Walker motif together with the absence of PNPase had a profound effect on cell growth. Cells were able to grow only at temperatures at or above 34 °C, and they grew extremely slowly as compared with cells lacking just PNPase; at 31 °C or below, cells were unable to grow. It should be noted that cells lacking both RNase R and PNPase are inviable even at the elevated temperatures (3). These data clearly demonstrate that the helicase activity is required for the proper functioning of RNase R in vivo. Even at higher temperatures, conditions in which RNase R lacking helicase activity functions relatively normally in vitro (8), the role of RNase R is severely compromised in vivo.

We also examined the doubling times of the strains lacking PNPase together with a Walker motif mutation in M9/glucose minimal medium and compared their growth with an RNase R deletion mutant strain and with a PNPase deletion mutant strain (Table 4). As before, cells grew more slowly in minimal medium, and as was observed in rich medium, cells containing a non-functional Walker motif, and also lacking PNPase, were unable to grow at 31 °C or below. Moreover, they grew more slowly than cells lacking RNase R or PNPase, even at 37 and 42 °C, temperatures more optimal for growth. From these observations, it is apparent that the RNase R helicase activity is essential for normal growth and viability of cells lacking PNPase, and therefore, these data also support the conclusion that the intrinsic helicase activity is an essential component for RNase R function in vivo.

**RNA Metabolism in Cells Lacking PNPase and Mutated in an RNase R Walker Motif**—In previous studies, we found that strains lacking PNPase and RNase R are inviable because they accumulate large amounts of 23S and 16S rRNA fragments (3). Here, we examined whether the poor growth observed for strains missing PNPase and carrying an RNase R Walker motif mutation was also due to an inability to degrade rRNA fragments. To examine this point, cells were grown in LB medium to an A600 = 0.6 at either 37 °C or 37 °C and then switched to 31 °C, a temperature at which these cells cease growth. Total RNA was isolated and analyzed on an agarose gel. As shown in Fig. 1, A and B, several new RNA fragments, detectable by ethidium bromide staining, appeared in cells lacking PNPase and also containing an RNase R Walker motif mutation that was absent in the WT, RNase R deletion mutant, or PNPase deletion mutant strains.

More detailed examination was carried out by Northern analysis using multiple probes complementary to 16S or 23S RNA. As shown in Fig. 1, C and D, the RNA molecules that accumulated were, in fact, fragments of both 16S and 23S RNA, and were present at a much lower level in the WT, the RNase R deletion mutant, and the PNPase deletion mutant strains as compared with cells lacking both PNPase and the RNase R helicase activity. Based on these data, we conclude that...
cells lacking PNPase and mutated in either Walker motif behave essentially the same with regard to accumulation of rRNA fragments as cells that lack both PNPase and RNase R activity (3). These findings support the conclusion that in the absence of its RNA helicase activity, RNase R is relatively inactive 

in vivo.

Helicase-deficient RNase R Does Not Complement Cells Lacking Other RNA Helicases—As reported earlier (3), cells require either active RNase R or active PNPase to maintain cell viability, and as shown above, growth cannot be maintained by RNase R alone at temperatures of 31 °C or below if the enzyme lacks its intrinsic helicase activity. A similar situation occurs with PNPase. Thus, in cells lacking RNase R, in which growth is dependent on the presence of active PNPase, growth ceased at temperatures of 31 °C or lower when the six known RNA helicases in E. coli were also absent (Fig. 2A, 6Hel−, R− strain). Cells lacking only RNase R or only the six RNA helicases could grow at all the temperatures tested (Fig. 2A). These data show that PNPase also requires RNA helicase activity to function in vivo.

As an additional test of the importance of the RNase R helicase activity for RNase R function, we examined the ability of RNase R to complement the growth of the 6Hel−, R− strain at lower temperatures. As shown in Fig. 2, A and B, growth was restored to the 6Hel−, R− strain when the WT RNase R gene was introduced. However, growth did not occur if the complementing RNase R gene was mutated in either its Walker A or its Walker B motif (Fig. 2B), which eliminates the intrinsic helicase activity. These data confirm that RNase R does not function in vivo at 31 °C or below when its RNA helicase activity is absent. Moreover, cells do not grow at 31 or 25 °C when the complementing RNase R gene lacks nuclease activity (D272N) despite the fact that this RNase R still retains helicase activity (8). Thus, the helicase activity of RNase R, by itself, is unable to complement the six missing helicases.

Discussion

The studies presented here clearly show that the intrinsic RNA helicase activity of RNase R is essential for the proper functioning of RNase R in vivo, even at a temperature (37 °C) at which its catalytic activity is relatively unaffected in vitro (8, 9). Thus, in the absence of the helicase activity, cells grow extremely poorly, especially when PNPase is also missing such that growth is dependent on a fully active RNase R. Cells lacking both PNPase and RNase R are unable to grow, and accumulate massive amounts of rRNA fragments (3). The fact that cells lacking PNPase, and containing a helicase-deficient RNase R, exhibit the identical phenotype shows that RNase R without its helicase activity is essentially non-functional in vivo. The slow rate of growth exhibited by these cells at 34–42 °C is likely due to a low level of residual RNase R nuclease activity combined with the greater “breathing” of RNA duplexes at the higher temperatures. However, growth is so slow under these conditions that any residual nuclease activity must be quite low.
It is interesting that in vitro the nuclease activity of helicase-deficient RNase R is relatively normal at 37 °C (8), whereas it is markedly deficient in vivo at this temperature. We believe that this difference is due to the much wider range of potential duplex substrates present in vivo as compared with the few synthetic substrates used to assess catalytic activity in the in vitro experiments. Moreover, duplex RNAs are probably stabilized in vivo as a consequence of their association with proteins, polyamines, and divalent cations as well as the very different ionic conditions that exist in cells as compared with those in the in vitro assays.

Based on our findings, it is reasonable to conclude that exoribonucleases would require the assistance of an RNA helicase in vivo to digest through structured RNA. This helicase activity may be intrinsic to the nuclease, as in the case of RNase R, or it may be a distinct protein as occurs with PNPase in its association with components of the RNA degradosome (19, 20). Interestingly, although bioinformatic analysis indicated that most RNase Rs contain Walker A and Walker B motifs indicative of an intrinsic helicase activity, the RNase Rs of some organisms lack these motifs (8). For example, RNase Rs in thermophilic bacteria do not contain Walker motifs, suggesting that they lack an intrinsic RNA helicase activity, and raising the possibility that the activity is not necessary for cells living in a high temperature environment in which duplex RNA structures might be less stable. On the other hand, the RNase R of the psychrophilic bacterium, Pseudomonas syringae, also lacks Walker motifs, and this nuclease is part of a degradosome containing an RNA helicase (21). Perhaps an intrinsic RNA helicase is insufficient to open the stable RNA duplexes expected to be present in an organism surviving at low temperatures, and only an energy-driven helicase suffices. Further work will be needed to answer these questions.

One aspect of the RNase R helicase activity that is not yet fully understood is its ability to complement the cold shock function of CsdA, a DEAD-box helicase (11). CsdA is essential at low temperatures, and we believe this is because it associates with PNPase and is required for PNPase to function under these conditions (22, 23). In the absence of CsdA, PNPase would be unable to function properly, thereby leading to the same impaired growth as is observed for a PNP− strain. The single chromosomal copy of rnr is unable to provide sufficient activity to overcome the absence of PNPase activity, but RNase R can complement when overexpressed (10). This scenario would explain the earlier complementation data. What has not been easy to explain is how RNase R lacking its nuclease activity is still able to complement a csaD deletion (11). We have now found in vitro that at high levels, nuclease-deficient RNase R can complement because it converts a duplex substrate to single strands, which then are easily digested by PNPase and RNase R (data not shown). If a similar situation occurs in vivo, such a mechanism would allow PNPase and the WT RNase R present in the strain to function in the absence of CsdA and lead to the apparent complementation by nuclelease-deficient RNase R that was observed (11).

The work presented in this study greatly increases our appreciation of the importance of the RNA helicase activity to RNase R function. Taken together with our recent study (9) explaining how the helicase facilitates RNase R action on structured RNA, we now have a much better understanding of the mechanism and role of RNase R in RNA metabolism.

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