Polyadenylation of RNAs Associated with a Nucleus-localized Phosphorolytic Nuclease

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The exosome is a protein complex consisting of a variety of 3'→5' exoribonucleases that functions both in the processing of rRNA precursors and in the degradation of mRNA. A prokaryotic counterpart of the exosome known as the degradosome exists in bacteria and chloroplasts. Interestingly, RNA polyadenylation has been implicated in degradosome functioning, giving rise to the possibility of a similar role in exosome function. Using phosphorolytic breakdown of RNA as an assay, we have purified an exosome-like activity from pea nuclear extracts. This activity copurifies with at least one Arabidopsis exosome subunit homologue. Recombinant Arabidopsis poly(A) polymerase and purified chloroplast poly(A) polymerase can polyadenylate RNAs that copurify with the exosome-like activity, even though the quantity of this co-purifying RNA is well below the affinity of the PAPs for free RNA. These results suggest a role for polyadenylation in exosome function, perhaps analogous to the role that polyadenylation plays in facilitating RNA breakdown by the bacterial degradosome.

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3'→5' degradation of RNA in eukaryotic cells is performed by a complex of proteins termed the exosome. In yeast, there are at least 11 subunits of the exosome (1); six are related to RNase PH (and more distantly to PNP, both phosphorolytic exonucleases), two to other RNases (RNase R and RNase D, two hydrolytic exonucleases), and three possess characteristic ribosomal protein S1 RNA binding domains (perhaps again suggestive of a functional analogy with PNP, which also has several typical S1 RNA binding domains). In addition to this core of nucleases, exosomes also act in concert with one or more helicases (2-4).

RNA degradation in bacteria also involves a complex of 3'→5' exonucleases (termed degradosomes) (5). The exosome consists minimally of polynucleotide phosphorylase (PNP), a DEAD-box helicase, and RNase E (an endonuclease that seems to serve as a scaffold for the assembly of other degradosome subunits), judging from the ability of these three enzymes to reconstitute degradosome activity in vitro (6). Degradosomes and exosomes are similar in many ways. Thus, these complexes consist largely of one or more 3'→5' exonuclease, including phosphorolytic enzymes. Exosomes or their components have been implicated in RNA turnover and in the biogenesis of ribosomes. For example, in yeast, mutations that affect various exosome subunits lead to defects in rRNA maturation (7, 8). In Escherichia coli, RNase PH- and PNP- negative double mutants are impaired in ribosome metabolism (9), and RNase E and a related nuclease are involved in 16S rRNA maturation (10). It is thus of interest to speculate about the extent to which this similarity extends to other functions.

In our studies of the chloroplast PAP, we noted that nuclear PAPs were able to polyadenylate RNAs associated with chloroplast-derived PNP (11). Given the parallels between degradosomes (which contain PNP as a subunit) and exosomes, it seemed reasonable to speculate about a possible link between plant nuclear PAPs and nucleus-localized exosomes. This study addresses this speculation, asking whether nuclear PAPs can polyadenylate RNAs that co-purify with nucleus-derived exosomes. We report that plants possess a nuclear phosphorolytic nuclease that copurifies with at least one probable exosome subunit, and that a nuclear PAP can polyadenylate RNAs that copurify with this activity. These results add to the list of similarities between prokaryotic and eukaryotic exonucleolytic complexes and suggest that the activity of the exosome may be modulated by polyadenylation, much as is the activity of the prokaryotic degradosome.

Abbreviations: NPN = nuclear phosphorolytic nuclease; PAP = poly(A) polymerase; PNP = polynucleotide phosphorylase.
Materials and Methods

Purification of NPN — Pea nuclear extracts were prepared and fractionated on DEAE-Sepharose as previously described (11). Fractions were assayed for 3'->5' exonuclease activity, and the active fractions were pooled, dialyzed and further fractionated by chromatography on spermine-agarose. Fractions containing the nuclease activity (typically, 500 µg of protein) were pooled, dialyzed for 4 h in dialysis buffer (40 mM KCl, 25 mM HEPES-KOH pH 7.9, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol), and added to 1 ml of spermine agarose (Sigma) that had been equilibrated with NEB40 (40 mM KCl, 25 mM HEPES-KOH pH 7.9, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM PMSF, 10% glycerol). The samples were rocked on ice for 1 h, the matrix collected by centrifugation, and then washed five times with 10 ml of NEB40. The matrix was then collected and resuspended in 1 ml of NEB40. Bound proteins were eluted with 1 ml of NEB2000 (2 M KCl, 25 mM HEPES-KOH pH 7.9, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM PMSF, 10% glycerol) per 0.5 ml of NEB40 by shaking on ice for 15 min. The elution step was done twice. The eluted protein was pooled and dialyzed for 4 h in NEB40. The various fractions were again assayed for 3'->5' exonuclease activity. Active fractions were pooled and applied to a Mono Q HR5/5 column (Pharmacia LKB). This column was developed with a linear gradient of KCl in NEB (40-500 mM KCl in 25 mM HEPES-KOH pH 7.9, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM PMSF, 10% glycerol) as described before (12) and analyzed for 3'->5' exonuclease activity. Fractions with activity were pooled and dialyzed against NEB40 for further studies.

Enzyme assays — Phosphorolytic exonuclease activity was assayed as described by Li et al (13), except that the final phosphate concentration used in the assays was 5 mM. Poly(A) polymerase was assayed as described (12), except that the reactions were carried out in the presence of 1.66 mM ATP, and in the absence of added RNA. Recombinant Arabidopsis nuclear PAP was prepared as described (11).

To analyzed the labeled products of the poly(A) polymerase reactions, reactions were extracted using phenol:chloroform and precipitated with glycogen and absolute ethanol. The resulting pellet was dissolved in RNAse-free water and aliquots treated with 1 unit DNase I at 30°C for 30 min, or with 100 ng RNase A at 37°C for 30 min. One aliquot was not treated. After these treatments, samples were extracted with phenol: chloroform and RNAs were precipitated with glycogen and ethanol. Recovered nucleic acids were analyzed on 10% sequencing gels. After separation, labeled molecules were visualized using a Molecular Dynamics Phosphorimager-System. Note that, under the conditions used for electrophoresis, molecules larger that 500 nts focus as a discrete band (as indicated by the lack of separation of the largest size standards); this makes for easier visual inspection of longer RNAs as well as short ones.

Immunoblot analysis — Protein fractions from the different columns and the purified NPN were electrophoresed in SDS-PAGE gels and transferred on to nitrocellulose membranes for studies by western blotting. The antibodies used were against PNP (13), for checking the co-purification of this protein with NPN, and AtRrp41 (14).

Results

Based on prior observations (11), it was hypothesized that one or more activities similar to polynucleotide phosphorylase would exist in plant nuclear extracts. Accordingly, pea nuclear extracts were assayed for phosphorolytic nuclease activity after fractionation on DEAE-Sepharose. As shown in Fig. 1A, an activity capable of producing ADP from RNA that had been labeled with ATP was readily discernible. This activity was further purified by chromatography on spermine agarose and

![Fig. 1. Purification and characterization of NPN. (A) Pea nuclear extracts were fractionated on DEAE-Sepharose and 20 µl of selected column fractions assayed for phosphorolytic nuclease activity using in vitro-synthesized RNA that had been labeled with 32P-α-ATP. Standards show the migration of each species in the TLC system. Lane 1 shows the results of an assay of a sample lacking extract. Lane 2 shows the results of an assay of the crude extract before chromatography, and lane 3 the results of an assay of the material that was not bound to the DEAE column. The remaining lanes are results of assays of fractions that eluted with increasing concentrations of KCl. The production of ADP is diagnostic of phosphorolytic nuclease activity. That part of the column that was pooled for subsequent steps of the purification are shown above the autoradiogram ("nuclease"); (B) Nuclease that had been purified on DEAE-Sepharose, spermine-agarose, and MonoQ was assayed for phosphorolytic nuclease activity in the presence or absence of inorganic phosphate. The lanes are labeled with their respective samples: "Standards" (the identities of ATP, ADP, AMP, and the labeled RNA are indicated on the left), "-Pi", showing the results of an assay of an 20 µl (ca. 60 ng of protein) of purified NPN in the absence of phosphate; "-Pi", showing the results of an assay of a 20 µl of purified NPN in the presence of phosphate; and "no extract", showing the results of an assay with no extract (includes phosphate).]