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A polysomal ribonuclease involved in the destabilization of albumin mRNA is a novel member of the peroxidase gene family

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ABSTRACT

We have purified an ~60 kDa endoribonuclease from *Xenopus* liver polysomes with properties expected for a messenger RNase involved in the estrogen-regulated destabilization of serum protein mRNAs (Dompenciel et al., 1995, *J Biol Chem* 270:6108–6118). The present report describes the cloning of this protein and its identification as a novel member of the peroxidase gene family. This novel enzyme, named polysomal RNase 1, or PMR-1 has 57% sequence identity with myeloperoxidase, and like that protein, appears to be processed from a larger precursor. Unlike myeloperoxidase, however, PMR-1 lacks N-linked oligosaccharide, heme, and peroxidase activity. Western blot and immunoprecipitation experiments using epitope-specific antibodies to the derived protein sequence confirm the identity of the cloned cDNA to the protein originally isolated from polysomes. The 80 kDa pre-PMR-1 expressed in a recombinant baculovirus was not processed to the 60 kDa form in Sf9 cells and lacks RNase activity. However, the baculovirus-expressed mature 60-kDa form of the enzyme has RNase activity. The recombinant protein is an endonuclease that shows selectivity for albumin versus ferritin mRNA. While it does not cleave at consensus APyrUGA elements, recombinant PMR-1 generates the same minor cleavage products from albumin mRNA as PMR-1 purified from liver. Finally, we show estrogen induces only a small increase in the amount of PMR-1. This result is consistent with earlier data suggesting estrogen activates mRNA decay through a posttranslational pathway.

Keywords: hormone-regulated mRNA degradation; messenger RNase; mRNA degradation; mRNA stability; polysomes; ribonucleases

INTRODUCTION

The term “messenger RNase” was coined by Ross & Kobs (1986) to describe ribonucleases whose primary function is to degrade mRNA. In yeast, at least three enzymes or enzymatic activities are involved in mRNA turnover; a deadenylating nuclease, a decapping pyrophosphatase, and a 5′-3′ exonuclease. De-

adenylation precedes decapping, which is followed by degradation of the body of the mRNA by the 5′-3′ exonuclease Xrn1p (reviewed in Tharun & Parker, 1997). Although endonucleases might also be involved in yeast mRNA decay (Vreken & Raue, 1992), the predominant pathway appears to be exonucleolytic.

There is a growing body of evidence that some vertebrate mRNAs are degraded by a pathway similar to that described for yeast. Many unstable vertebrate mRNAs undergo deadenylation followed by degradation of the mRNA body (reviewed in Ross, 1995; Schoenberg & Chernokalskaya, 1997). Moreover, several poly(A)-degrading exonucleases have been described (Åström et al., 1991; Åström et al. 1992; Caruccio & Ross, 1994; Koerner & Wahle, 1997), although their role, if any, in mRNA decay has not been proved. Although a

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vertebrate decapping pyrophosphatase has yet to be found, the identification of mammalian Xrn1p (Bashkurov et al., 1997) and evidence for decapping (Couttet et al., 1997) indicate that the pathway of deadenylation $\rightarrow$ decapping $\rightarrow$ 5'-3' decay is likely conserved in all eukaryotes.

It is also clear that endonucleases are involved in vertebrate mRNA turnover, particularly for mRNAs whose half-lives fluctuate in response to extracellular stimuli (reviewed in Ross, 1995; Schoenberg & Chernokalskaya, 1997). More is known about endonucleolytic decay pathways than about the endonucleases themselves. In many cases, endonuclease-initiated decay does not involve prior deadenylation. Examples include insulin-like growth factor 2 (Christiansen et al., 1994; Scheper et al., 1996), *gro α , β , γ* (Stoeckle, 1992), *c-myc* (Prokipcak et al., 1994), transferrin receptor (Binder et al., 1994), avian apo-very low density lipoprotein II (Binder et al., 1990), and *Xenopus* maternal homeobox mRNAs (Brown & Harland, 1990; Brown et al., 1993).

We have identified and characterized an estrogen-regulated endoribonuclease that cosediments on *Xenopus* liver polysomes (Pastori et al., 1991b; Pastori & Schoenberg, 1993). The significance of this enzyme relates to the striking regulatory effect of estrogen on the production of serum proteins, such as albumin. In *Xenopus* liver 17 β -estradiol causes a major change in liver protein synthesis; the production of serum proteins (such as albumin) decreases dramatically while there is a similarly dramatic induction in the synthesis of the yolk protein precursor vitellogenin. This is accomplished by the transcriptional induction and stabilization of vitellogenin mRNA (Brock & Shapiro, 1983; Shapiro et al., 1989), and by the destabilization of the mRNAs for the major serum proteins (Pastori et al., 1991a). Our lab has been investigating the mechanism of estrogen-induced albumin mRNA destabilization and have focused on a polysomal ribonuclease activity with properties expected for an albumin mRNAse (Pastori et al., 1991b). Key among these properties were hormone inducibility and substrate selectivity. This enzyme cleaves albumin mRNA in vitro at many of the same sites which are cleaved in vivo following estrogen. Cleavage of the 5' end of albumin within a loop bearing two overlapping copies of the sequence APyrUGA generates a doublet product (Chernokalskaya et al., 1997). This feature was used as a specific assay for the purification of the RNase from liver polysomes (Dompenciel et al., 1995). The purified protein consists of a doublet on SDS-PAGE of $\sim$ 60 kDa, and two-dimensional gel electrophoresis further separates these into a series of isoforms with different isoelectric points (Dompenciel et al., 1995). The present study reports the cloning of this novel endoribonuclease that we call PMR-1 [polysomal RNase-1]. Its GenBank Accession number is U68724. PMR-1 bears no sequence

or structural similarity to the known superfamily of vertebrate ribonucleases. Rather, it is a novel member of the peroxidase gene family which lacks both covalently-bound heme and N-linked oligosaccharide.

RESULTS

PMR-1 is a novel member of the peroxidase gene family

The 2,512-bp PMR-1 cDNA was cloned using degenerate primers from the sequences of tryptic peptides of PMR-1 purified from *Xenopus* liver polysomes. The five tryptic peptides sequenced from the purified protein are shown boxed in Figure 1A. Computer searches showed that PMR-1 has considerable homology to myeloperoxidase, lactoperoxidase, and thyroperoxidase, respectively. The highest homology was observed with human myeloperoxidase (hMPO, Fig. 1A).

PMR-1 shows some, but not all of the features of hMPO

Myeloperoxidase is a heme-containing protein unique to myeloid cells, such as neutrophils and polymorphonuclear leukocytes. It is sequestered in lysosome-like azurophil granules, and upon release triggered by bacterial infection, it catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ions. The fully processed protein exists as a heterotetramer of M_r 120–150,000 containing two heavy chains of M_r 60,000, and two light chains of M_r 15,000. Both chains are synthesized from a single 3.2-kb mRNA as an M_r 80,000 precursor protein that is proteolytically processed and glycosylated with four high-mannose oligosaccharide chains (Fenna et al., 1995).

Overall there is 57% sequence identity between PMR-1 and human myeloperoxidase. Comparing these proteins with a local similarity program (SIM, ExPASy) revealed the highest conservation ($>60\%$ identity) with myeloperoxidase begins at PMR-1 amino acid 145 (open arrow in Fig. 1A) and extends to position 685. This corresponds to the light and heavy chain of myeloperoxidase. The sequence identity between amino acids 9 and 144 and the corresponding region of myeloperoxidase is significantly lower (40%), and there is no homology at the N terminus corresponding to the 23 hydrophobic residues of the myeloperoxidase signal sequence. This latter observation is consistent with PMR-1 being an intracellular protein. Both gel filtration of crude polysome extract and SDS-PAGE of purified protein identify the size of PMR-1 as $\sim$ 60 kDa. However, the protein predicted from the translated sequence would be 80 kDa. Suggestive evidence for a larger precursor is seen in an overexposed Western blot of total hepatocyte protein (Fig. 1B) probed with a polyclonal antibody against purified PMR-1 (Dompenciel et al., 1995). Proof for a precursor-product rela-

FIGURE 1. Sequence and structural features of PMR-1. **A:** The sequence of the PMR-1 (GenBank Accession Number U68724) is aligned with human myeloperoxidase (hMPO), with identical residues identified with grey boxes, and the tryptic peptides of purified PMR-1 indicated with open boxes. The black underline denotes the hMPO signal sequence, the open underline the hMPO propeptide, the lightly lined underline the hMPO light chain, and the darkly-lined underline the hMPO heavy chain. The sites of covalent heme attachment to hMPO are shown with an asterisk (*) and the sites that coordinate the heme iron are shown with a black dot (●). An open arrow denotes the proposed proteolytic processing site that generates mature PMR-1. **B:** Ten micrograms of total liver protein obtained 24 h after injection of estradiol were analyzed by Western blot using a polyclonal antibody to purified PMR-1 (Dompenciel et al., 1995). Markers indicate the presumptive 80-kDa precursor, mature PMR-1, and a 40-kDa breakdown product commonly observed on SDS-PAGE (Dompenciel et al., 1995).

tionship between these proteins will require pulse-chase analysis. Because the amino terminus of purified PMR-1 was blocked, the location of the presumptive processing site (open arrow, Fig. 1) was inferred from the size of the purified protein. This falls very close to the proteolytic processing site at the amino terminus of the myeloperoxidase light chain. We have not investigated the potential significance of this finding.

Some, but not all of the core features of hMPO are conserved in PMR-1. Both histidine residues (H261 and H502; Fig. 1A, dots) that coordinate heme iron in myeloperoxidase are conserved between myeloperoxidase and PMR-1, as are two of the residues that form covalent bonds with pyrrole rings (D260 and E408; Fig. 1A, asterisks). However, M409, which is the site of the characteristic sulfonium linkage to the myeloperoxidase heme (Fenna et al., 1995; Andersson et al., 1996) is replaced by glutamine in PMR-1. UV-visible spectroscopy was used to determine whether PMR-1 has heme like hMPO. The UV-visible spectrum of hMPO shows both the characteristic Soret band at 430 nm and a secondary absorbance at 570 (Fig. 2A; Andersson et al., 1996). In contrast, PMR-1 shows no absorbance in the visible spectrum. Thus, PMR-1 lacks covalently-bound heme.

hMPO heavy chain has four N-linked high-mannose oligosaccharide chains (Fenna et al., 1995). To deter-

mine whether PMR-1 is similarly glycosylated, PMR-1 and hMPO were treated with endoglycosidase H, and the reaction products were analyzed by Western blot (Fig. 2B). The panel on the left side shows the effect of such treatment on hMPO. The sulfonium ion bond created by heme attachment to hMPO at M409 can be activated upon heating to produce cleavage of the peptide backbone in the same manner that CNBr cleaves at methionine residues (Taylor et al., 1992). This generates 20- and 40-kDa fragments from the hMPO heavy chain (Fig. 2B, left side, open arrows). Cleavage of the oligosaccharide backbone with endo H resulted in a shift toward faster mobility of all hMPO heavy chain. The endo H susceptibility of hMPO was most easily observed with the 20-kDa autolytic product, whose migration was shifted onto that of the hMPO light chain. No mobility shift was detected upon endo H cleavage of PMR-1 (Fig. 2B, right panel). Moreover, PMR-1 does not bind to concanavalin A-Sepharose whereas hMPO does (data not shown). Therefore, PMR-1 is distinguished from myeloperoxidase by the absence of both heme and N-linked oligosaccharide.

To determine whether hMPO is an endoribonuclease like PMR-1, [32 P]albumin RNA was incubated with a vast excess (1–3 μ g) of hMPO with and without 1 mM $MgCl_2$, which is necessary for activating a number of RNases (Fig. 2C). Under no circumstances did hMPO degrade

the input RNA, whereas the RNA was readily degraded by PMR-1 (lane 10). Thus, generalized RNase activity is not a secondary feature of myeloperoxidase. Finally, hMPO and PMR-1 were tested for peroxidase activity using the chromogenic substrate ABTS (Fig. 2D). Duplicate 100-ng samples of hMPO generated approximately 20 OD₄₀₅ units per μ g protein; however, PMR-1

produced no such product. Therefore, PMR-1 lacks peroxidase activity.

Antibodies to the derived protein sequence are specific for liver PMR-1

We exploited immunoprecipitation and RNase assays with recombinant protein to show that the cDNA we have cloned encodes PMR-1. Immunoprecipitation experiments were performed using epitope-specific antibodies raised to peptides from the derived protein sequence. The locations of the two epitopes chosen for antibody production are shown in Figure 3. Both antibodies identify a single ~60-kDa protein on Western blots of liver polysome extract from estrogen-treated frogs (Fig. 3A, lanes 1 and 2). Proof that these antibodies recognize the 60-kDa protein purified previously from liver polysomes is shown in Figure 3A, lanes 3 and 4. In this experiment, the same polysome extract was immunoprecipitated with either pre-immune serum (Fig. 3A, lane 3) or epitope-specific antibody #1279 (Fig. 3A, lane 4). A Western blot of the recovered protein probed with biotinylated polyclonal anti-serum to purified PMR-1 identified a ~60-kDa protein from the sample recovered with antibody #1279, but not with pre-immune serum. Identical results were obtained with antibody #1277 (data not shown).

The experiment in Figure 3B shows that antibody #1277 is also specific for the ribonuclease activity of purified PMR-1. In this experiment the epitope-specific antibody was used to deplete a specific amount of PMR-1 RNase activity from a solution of purified protein, following which activity was restored by adding purified enzyme to the depleted sample. The results of incubating a [³²P]labeled albumin substrate transcript

FIGURE 2. Comparison of the spectral and biochemical properties of hMPO and PMR-1. **A:** The UV-visible spectra of hMPO (0.2 μ g/mL) and PMR-1 (0.1 μ g/mL) were determined as described in Materials and Methods. **B:** Insensitivity of PMR-1 to endoglycosidase H. One microgram of hMPO or 10 and 20 μ g of partially purified *Xenopus* liver polysome extract were incubated at 37 °C for 1 h with or without 750 units of endoglycosidase H. Extracts were analyzed by Western blot using a polyclonal antibody to hMPO or antibody #1277 against PMR-1. Intact hMPO heavy chain is identified with the filled arrow, and the heavy-chain autolytic cleavage products are identified with the open arrows. The arrowhead on the lower left indicates the position of hMPO light chain, and the positions of size markers are shown on the side of each gel. **C:** Lack of RNase activity of hMPO. One, two or three micrograms of hMPO were incubated for 30 min at 22 °C with a [³²P]labeled albumin substrate transcript in buffer without (lanes 3–5) or with (lanes 7–9) 1 mM MgCl₂. RNA was extracted and electrophoresed on a denaturing 6% acrylamide/urea gel. Lanes 2 and 6 contain transcript incubated with buffer alone, and lane 10 is a reaction containing partially-purified PMR-1. The arrow indicates the position of the characteristic doublet cleavage product. Lane 1 contains molecular size markers (M). **D:** Lack of peroxidase activity of PMR-1. Duplicate 100 ng of hMPO or PMR-1 purified from liver polysomes were assayed for peroxidase activity using ABTS substrate. The data are shown as OD₄₀₅ generated per microgram of protein.

with 10, 20, 30, or 50 units of purified PMR-1 are shown in Figure 3B, lanes 7, 3, 8, and 9, respectively. The amount of input RNA substrate which remained undegraded after each reaction was quantified by phosphorimager analysis, and these data are shown beneath the autoradiogram. Note that 10 units of PMR-1 degraded 57% and 20 units degraded 88% of the input transcript, and that the appearance of the doublet cleavage product (Fig. 3B, arrow) does not relate quantitatively to the relative degradation of the input RNA. The lack of a quantitative precursor-product relationship in this reaction results from further degradation of these initial cleavage products by PMR-1 (Chernokalskaya et al., 1997).

The data in Figure 3B, lanes 4 and 5, assay the activity remaining in solution after incubating 20 units of PMR-1 with protein A-Sepharose beads bound with either pre-immune serum (Fig. 3B, lane 4) or antibody #1277 (Fig. 3B, lane 5). Treatment with beads bearing pre-immune serum had no effect on substrate transcript degradation (compare Fig. 3B, lanes 3 and 4). However, approximately half (10 units) of the input RNase activity was depleted by beads bound to antibody #1277 (Fig. 3B, lane 5). In Figure 3B, lane 6, 10 units of purified PMR-1 was added back to the immunodepleted sample from lane 5. This addition quantitatively restored RNase activity to the level observed in both the enzyme and pre-immune serum controls (Fig. 3B, lanes 3 and 4). These data rule out the possibility that the #1277-bound protein A beads introduced an inhibitor of PMR-1 activity, and prove that the epitope-specific antibody is selective for PMR-1 enzymatic activity. Taken together, the data in Figure 3 indicate that peptide antibodies prepared to the sequence of the cloned cDNA Western-blot a protein of the same size as the one we purified from liver polysomes, immunoprecipitate a protein that cross-reacts with antibodies to the purified polysomal RNase, and immunoprecipitate albumin mRNA-selective RNase activity present in a preparation of purified PMR-1. These data therefore confirm the identity of the cloned cDNA as PMR-1.

Ribonuclease activity of recombinant PMR-1

To determine whether the cDNA encoded a ribonuclease, we first attempted to express the protein in bacterial cells. However, PMR-1 expression plasmids were

FIGURE 3. Immunoprecipitation of PMR-1 by epitope-specific antibodies. **A:** Identification of the 60-kDa protein. Antibodies were prepared in rabbits to KLH conjugates of the 15 residue peptide epitopes indicated in white on the derived sequence of PMR-1. Lanes 1 and 2: Western blots of *Xenopus* liver polysome extract were probed with each epitope-specific antiserum. Lanes 3 and 4: polysome extract was immunoprecipitated with either pre-immune serum or antibody #1279, and the recovered proteins were analyzed by Western blot using biotinylated polyclonal antiserum to purified PMR-1. Antibody complexes were detected by enhanced chemiluminescence using HRP-coupled streptavidin. **B:** Immune depletion of RNase activity. 150 fmol of a 160-nt [³²P]labeled albumin substrate transcript was incubated with 10 (lane 7), 20 (lane 3), 30 (lane 8), or 50 (lane 9) units of purified PMR-1. In lanes 4 and 5 a solution containing 20 units of PMR-1 was immunoprecipitated with protein A-Sepharose beads bound to either pre-immune serum or antibody #1277. The supernatant was then assayed for RNase activity as in lanes 3, 7, 8, and 9. The sample in lane 6 consists of the immunoprecipitated solution from lane 5 supplemented with 10 units of purified PMR-1. The undegraded input substrate transcript is shown in lane 2. The arrow indicates the position of the characteristic doublet product resulting from cleavage at adjacent APyrUGA elements in the substrate RNA (Chernokalskaya et al., 1997). The percentage of intact input transcript remaining in each sample was quantified by phosphorimager analysis and is indicated under the figure.

consistently lost or rearranged in bacterial cells in which protein expression was attempted, suggesting that PMR-1 is toxic to these cells. However, we were able to express it in a recombinant baculovirus. Two constructs were prepared that expressed full-length 80-kDa PMR-1 precursor and the 60-kDa mature form of the enzyme. As noted above, the N-terminus of purified PMR-1 was blocked. Therefore, T147 was chosen as the N-terminus to produce a protein approximating mature PMR-1 in size. Figure 4A is a Western blot using antibody #1277 for protein expressed in Sf9 cells infected with two recombinant baculovirus expressing 60-kDa protein (PMR60; lanes 2 and 3), the baculo-

virus expressing pre-PMR-1 (PMR80; lane 4), or cells infected with nonrecombinant virus (AcPNV; lane 5). The recombinant viruses direct the expression of protein products of the anticipated size from the PMR60 clones, and only an 80-kDa product was obtained from cells infected with PMR80. This result suggests that the enzymatic pathway involved in processing the larger precursor to mature PMR-1 is not present in these cells. The smaller cross-reacting protein seen in cells infected with nonrecombinant virus is also seen to some extent in cells infected with recombinant PMR60 and in uninfected cells (data not shown). The significance of this is not known.

Both PMR80 and PMR60 were primarily found in association with the insoluble pellet fraction of infected cells. In Figure 4B this fraction from cells infected with PMR80 was tested for RNase activity using a uniformly labeled albumin substrate transcript. There was minimal transcript degradation, indicating both that recombinant PMR-1 precursor protein lacks enzymatic activity and that there is little background RNase activity present in this fraction from Sf9 cells.

We next examined RNase activity from cells infected with the two PMR60 constructs, as well as uninfected cells and cells infected with a wild-type baculovirus. In the experiment shown in Figure 5 recombinant PMR-1 expressed from both PMR60 constructs degraded >90% of the input albumin substrate transcript within 10 min, and no input RNA remained after 20 min of reaction. Unlike protein purified from liver polysomes (Fig. 5, lane 2), the recombinant protein did not generate a doublet cleavage product (see below). A small amount of contaminating ribonuclease activity was observed in cells infected with wild-type virus but not in mock-infected cells. This activity was more labile than PMR-1, as evidenced by its disappearance within the first 10 min of the reaction. The fact that PMR60 has ribonuclease activity whereas PMR80 does not suggests that proteolytic processing of the 80-kDa PMR-1 precursor is a necessary step in the production of enzymatically active protein.

FIGURE 4. Baculovirus expression of recombinant PMR-1. **A:** Western analysis of baculovirus-expressed PMR-1. Western blots were performed with antibody #1277 on 2×10^4 cells infected with two different baculovirus expressing 60-kDa PMR-1 (PMR60, lanes 2 and 3), a baculovirus expressing the 80-kDa pre-PMR-1 (PMR80, lane 4), or cells infected with nonrecombinant baculovirus (lane 5). **B:** RNase assay of recombinant pre-PMR-1. The insoluble pellet fraction of Sf9 cells infected with PMR80 was incubated with a 160-nt [32 P]labeled albumin RNA substrate transcript bearing the mapped PMR-1 cleavage sites for 0–45 min at 22 °C (lanes 4–8) in 3 μ L of 40 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol. The input RNA is in lane 2, and lane 3 contains a standard reaction performed with purified PMR-1. The arrow indicates the position of the PMR-1 doublet cleavage product. The reactions were terminated by adding an equal volume of formamide sample buffer and heating at 90 °C for an additional 3 min. The resulting samples were separated on a 6% acrylamide/urea gel.

Recombinant PMR-1 is a sequence-selective endonuclease

Ferritin mRNA remains undegraded *in vivo* during the estrogen-stimulated destabilization of albumin mRNA (Pastori et al., 1991a). A hallmark of the RNase activity identified as PMR-1 is its *in vitro* selectivity for albumin versus ferritin mRNA (Pastori et al., 1991b, Dompenciel et al., 1995). To determine whether recombinant PMR-1 retained substrate selectivity, a PMR60 preparation was incubated with total liver RNA, followed by Northern blot and phosphorimager analysis to quantify the degradation of full-length albumin and ferritin mRNA. The results in Figure 6 show that albumin mRNA is rapidly degraded by recombinant PMR-1 with a half-life

FIGURE 5. RNase activity of baculovirus-expressed 60-kDa PMR-1. The pellet fractions from cells infected with two recombinant baculovirus expressing mature PMR-1 (PMR60), cells infected with wild-type virus (AcNPV), or mock-infected cells (Sf9) were incubated with a [32 P]labeled albumin RNA substrate transcript as described in the legend to Figure 4B. Controls for input transcript and transcript cleaved with purified PMR-1 are shown in lanes 1 and 2, respectively. The arrow indicates the position of the PMR-1 doublet cleavage product.

of 2.5 min. Ferritin mRNA was only slowly degraded, with greater than half of the input mRNA remaining intact after 30 min. Like PMR-1 purified from liver polyosomes (Dompenciel et al., 1995), recombinant PMR-1

FIGURE 6. Substrate selectivity of recombinant PMR-1. Ten-microgram samples of total liver RNA were incubated for the indicated times with 1 μ g of recombinant PMR-1 recovered in the pellet fraction from baculovirus-infected cells. The recovered RNA was analyzed by Northern blot hybridized to a mixed probe of albumin (filled circles) and ferritin (open circles) cDNAs and quantified by phosphorimager analysis.

lost activity in the course of the reaction at 23 °C. The cause of this inactivation is unknown.

The endonuclease activity of liver PMR-1 was previously characterized using 5'-end-labeled albumin substrate transcripts (Pastori et al., 1991b, Dompenciel et al., 1995, Chernokalskaya et al., 1997). The same approach was used in Figure 7 to examine the activity of recombinant PMR60. We previously demonstrated that purified PMR-1 preferentially cleaved at 3 APyrUGA sites in a albumin 5' substrate transcript, and at a number of additional sites, not all of which have been mapped (Chernokalskaya et al., 1997). These cleavages are seen in Figure 7, lane 2, with the products of cleavage at APyrUGA elements identified by arrows. The minor cleavage products described previously are also evident (dots), and most of these products were also generated by recombinant PMR60. These data confirm that recombinant PMR60 has endonuclease activity like the protein purified from liver polyosomes, and cleaves albumin mRNA at many of the same sites as the purified protein. We speculate that the ability to generate the doublet cleavage product requires specific folding of the protein in a manner that is not achieved in Sf9 cells.

Estrogen induces only a moderate increase in PMR-1

One of the key pieces of data linking PMR-1 to the estrogen-regulated destabilization of serum protein mRNAs was the appearance of its activity on polyosomes following estrogen administration (Pastori et al., 1991b). The destabilization of serum-protein mRNAs occurs between 9 and 12 h after estrogen administration (Schoenberg et al., 1989, Pastori et al., 1991a). A

FIGURE 7. Endonuclease activity of recombinant PMR-1. Albumin substrate transcript was 5'-end labeled by transcription in the presence of γ -[32 P]GTP. It was incubated for 20 min at 23°C with buffer (lane 2), purified PMR-1 (lane 3), or with increasing amounts of protein from the pellet fraction from cells infected with baculovirus-expressed PMR60 (lanes 4–9). The products generated by purified PMR-1 cleavage are shown in lane 3. Cleavage at the overlapping APyrUGA elements in the loop corresponding to residues 158–166 of albumin mRNA (sites 1 and 2, Chernokalskaya et al., 1997) are identified with the filled arrow. Cleavage at the upstream APyrUGA element (site 5, Chernokalskaya et al., 1997) is identified with an open arrow. The minor cleavage products generated by both purified and recombinant PMR-1 are indicated by the filled dots on the right side of the figure.

time-course Western blot of total liver protein (Fig. 8) shows estrogen induces a 1.5-fold increase in PMR-1 by 6 h, and PMR-1 returned to the unstimulated level by 24 h following hormone administration. We previously showed that the pathway of estrogen-induced mRNA decay does not require new protein synthesis (Moskaitis et al., 1991). The fact that PMR-1 is present

FIGURE 8. Western blot analysis of PMR-1 following estrogen stimulation. Ten microgram samples of hepatocyte protein isolated at the indicated times following estrogen stimulation were analyzed by Western blot using a polyclonal antiserum to PMR-1. Lane 1 is a standard of 1 ng of purified PMR-1. A parallel blot of 100 ng of hepatocyte protein per lane was probed with sheep antiserum to vitellogenin as a control for estrogen stimulation.

at time 0 and mRNA decay initiates 3 h after the moderate induction of PMR-1 seen at 6 h implies posttranslational steps may be involved in activating mRNA decay. This is discussed below.

DISCUSSION

Most of the vertebrate ribonucleases identified and characterized to date are structurally related members of a large family of enzymes related to RNase A (D'Alessio & Riordan, 1997). In contrast, the data in Figure 1 show that the polysomal endonuclease thought to be involved in the estrogen-regulated destabilization of albumin mRNA is a new and unique member of the peroxidase gene family. The sequence similarity between PMR-1 and myeloperoxidase, its closest relative, is striking. Overall, there is 57% amino acid identity, and the primary translation products of both proteins are similar in length. However, several properties of PMR-1 clearly distinguish it from myeloperoxidase. First, PMR-1 is an intracellular protein, and, as one might expect, lacks the first 23 hydrophobic residues that constitute much of the signal peptide of myeloperoxidase. Second, PMR-1 lacks the unique hMPO heme attachment site at M409, and both covalently bound heme and peroxidase activity. Third, hMPO is a glycoprotein whereas PMR-1 is not. Fourth, PMR-1 behaves under all of the conditions examined to date as a ~60-kDa monomer, whereas hMPO is a 120–150-kDa heterotetramer. The sequence of PMR-1 cDNA and the West-

ern blot in Figure 1 provide suggestive evidence that, like hMPO, PMR-1 is made as a larger precursor and is proteolytically processed to the form identified on polysomes. It may or may not be a coincidence that the site of this putative processing event occurs at a position similar to that which separates the propeptide and light chain of hMPO.

Expression of the full-length PMR-1 cDNA in a recombinant baculovirus (Fig. 4) yielded the 80-kDa protein (PMR80) expected from the sequence, but this was not processed to the 60-kDa form isolated from liver polysomes and lacked enzymatic activity. These data indicate both that Sf9 cells lack the enzymatic pathway by which PMR-1 is proteolytically processed, and that processing is required to generate a catalytically active form of the enzyme. In contrast, the recombinant 60-kDa form of PMR-1 estimated to represent the protein isolated from polysomes (PMR60) is an active endonuclease (Figs. 5–7). PMR-1 was originally identified by its ability to generate a doublet cleavage product from a portion of the 5' end of albumin mRNA (Pastori et al., 1991b). This was later mapped to consensus overlapping APyrUGA elements in a single-stranded loop region (Chernokalskaya et al., 1997). That study also demonstrated that the purified enzyme also cleaved albumin mRNA at a number of sites that did not correspond to this consensus sequence. Recombinant PMR60 did not cleave albumin mRNA at consensus APyrUGA elements (Fig. 7). However, the recombinant enzyme generated much the same pattern of nonconsensus cleavages as the purified enzyme, indicating both that it is an endonuclease and that it is indeed PMR-1. The reason recombinant PMR60 does not cleave at APyrUGA elements is not known, although we suspect it is related to folding of the protein, because a similar result was obtained with purified PMR-1 that was renatured after treatment under denaturing conditions (data not shown).

It is unclear how a putative messenger RNase evolved from the peroxidase gene family. Myeloperoxidase lacks detectable ribonuclease activity (Fig. 2C), indicating this family of proteins does not have a secondary enzymatic function. Perhaps some insight into this may be found in the dual function of several metabolic enzymes as RNA-binding proteins. IRP-1 functions as a regulator of iron metabolism through its role in the translational repression of ferritin mRNA (Gray & Hentze, 1994) and stabilization of transferrin receptor mRNA (Binder et al., 1994). Iron binding to the active site of this protein converts IRP1 from its RNA-binding form to cytoplasmic aconitase (Hirling et al., 1994). Glyceraldehyde-3-phosphosphate dehydrogenase is a well-characterized hydrolytic enzyme which can bind AU-rich RNA sequence elements through its NAD(+) binding site (Nagy & Rigby, 1995). Glutamate dehydrogenase was identified as the protein whose binding to the 3' UTR of cytochrome c oxidase mRNA correlates

with liver-specific mRNA stabilization (Preiss et al., 1995), and AUH, an ARE-binding protein, has enol-CoA hydratase activity (Nakagawa et al., 1995). Thus, it seems possible that a number of enzymes previously identified for their role in metabolic processes may also function in mRNA metabolism. The results of molecular modeling done by fitting the sequence of PMR-1 to the atomic coordinates of hMPO heavy chain (Peitsch, 1996) suggest that the active site of PMR-1 may correspond structurally to the heme binding cleft of myeloperoxidase. Site-directed mutagenesis experiments are in progress to test this hypothesis.

The destabilization of serum protein mRNAs by estrogen is blocked by 4-hydroxytamoxifen (a pure anti-estrogen in *Xenopus*) indicating that this process is dependent on the action of the estrogen receptor (Riegel et al., 1987). However, blocking protein synthesis by >95% with cycloheximide has no effect on estrogen-induced mRNA destabilization (Moskaitis et al., 1991), indicating that the synthesis of new protein products is not a prerequisite for estrogen-induced mRNA decay. Work from other labs has demonstrated posttranslational activation of both adenylate cyclase (Aronica et al., 1994) and the *c-src/p21^{ras}/MAP* kinase pathway (Migliaccio et al., 1996) by estrogen acting through an estrogen receptor alpha-dependent pathway. PMR-1 is present in control liver and is only slightly induced by estrogen (Fig. 8) prior to the disappearance of albumin mRNA at 9 h (Schoenberg et al., 1989). A hypothesis consistent with these observations is that estrogen initiates mRNA destabilization through a posttranslational mechanism. PMR-1 purified from liver of estrogen-treated frogs separates into 3 peptides of the same molecular weight on two-dimensional gel electrophoresis (Dompenciel et al., 1995), and alkaline phosphatase alters the mobility of PMR-1 in a manner consistent with the removal of phosphate residues (M. Hanson, unpublished). The derived protein sequence of the mature 60-kDa PMR-1 peptide has seven consensus casein kinase II sites, two protein kinase C sites, and two JNK/MAP kinase sites. Experiments are in progress to identify which sites on PMR-1 are phosphorylated, to determine whether estrogen alters the phosphorylation of PMR-1, and the functional consequences of this modification.

MATERIALS AND METHODS

The purification of PMR-1, preparation of plasmids, transcripts and liver polysome extracts, assay for ribonuclease activity, preparation of transcripts, and gel analyses were performed as described previously (Dompenciel et al., 1995). The handling of experimental animals, preparation of total RNA, and Northern blots were performed as described by Schoenberg et al. (1989). Myeloperoxidase and antibodies to myeloperoxidase were obtained from Dako, Inc. Peroxidase activity was assayed using 2,2'-azino(3-ethyl-benzthiazoline sulfonate) (ABTS, Amresco). One hundred nanograms of

hMPO or PMR-1 purified from liver polysomes were added to 200 μ L of 1 mM ABTS in 50 mM sodium citrate, 150 mM sodium phosphate, pH 4.4, containing 0.01% H₂O₂. The mixture was incubated for 15 min at 25 °C, following which the absorbance was measured at 405 nM.

Epitope specific antibodies

Epitope-specific antisera were prepared in New Zealand white rabbits by Genosys Biotechnologies to keyhole limpet hemocyanin conjugates of the sequences NQFRRARDGDRFYEE (#1277), corresponding to amino acids 637–652, and KIPPNDPRISNQSD (#1279), corresponding to amino acids 267–281, respectively, of the translated sequence of PMR-1 cDNA. These peptides were chosen based on computer modeling for regions exposed on the protein surface which were not in stable secondary structures. For the experiment in Figure 3B, 50 μ L of antibody #1277 was incubated for 5 h at 2 °C with 100 μ L of protein A–Sepharose in a total volume of 500 μ L of 50 mM Tris-HCl, pH 7.4. Unbound protein was removed by four washes with three volumes each of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% (v/v) Triton X-100. After centrifugation, 50 mM Tris-HCl, pH 7.4, plus 100 units of purified PMR-1 were added to the packed beads to achieve a final volume with beads of 300 μ L (1 unit of PMR-1 is the amount of enzyme needed to completely degrade 7 fmol of albumin substrate transcript in 30 min at 23 °C). After incubating for 15 h at 2 °C the beads were removed by centrifugation. The supernatant was divided into five equal aliquots, four of which were mixed with 150 fmol of [³²P]labeled 164-nt albumin substrate transcript bearing the mapped PMR-1 cleavage sites (Chernokalskaya et al., 1997). The sample shown in Figure 3B, lane 5 received no additional enzyme, whereas the samples in lanes 6–8 received 10, 30 and 50 additional units of PMR-1 respectively. Reactions were performed in a final 25 μ L volume as described previously (Chernokalskaya et al., 1997). The samples in Figure 3B, lanes 9–11 were identical to those in lanes 6–8 except that the equivalent volume of reaction buffer was added in place of the supernatant from the immunoprecipitation. Immunoprecipitation with pre-immune serum (Fig. 3B, lane 4) was performed under the same conditions as above, but using one fifth the volume and amounts of reagents. Reactions were terminated by adding an equal volume of formamide loading solution followed by heating at 90 °C for 2 min and electrophoresis on a 6% urea/acrylamide gel. Degradation of input transcript was quantified on a Molecular Dynamics phosphorimager.

Cloning and expression of PMR-1 cDNA

Tryptic fragments of the purified PMR-1 were sequenced at the William Keck Foundation Biotechnology Resource Lab at Yale University. The 3' half of the cDNA was cloned using CloneAmp pAMP1 System (Life Technologies) and protocols provided with that kit. The first strand cDNA was synthesized using adapter-primer 5'-GGCCACGCGTCGACTAGTAC(T₁₇) and mRNA from 24-h estrogen-treated frogs. cDNA amplification used a degenerate gene-specific primer RDU based on the sequence of peptide 2HP831-121 (located closest to the amino terminus in Fig. 1) and containing 5' extension for digestion by uracil-D-glycosidase. The antisense amplification primer was 5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC. PCR was performed by heating the reaction mixture for 5 min at 94 °C, followed by 30 rounds of 94 °C $\times$ 1 min, 50 °C $\times$ 1 min, 72 °C $\times$ 1 min. The process was terminated by incubation at 72 °C for 15 min. The products of this reaction were digested with 1U/ μ L of uracil-D-glycosidase and annealed with plasmid pAMP1. One of the clones (UDG14) contained all five of the peptide sequences within a 1.2-kb insert. The sequence of UDG14 was used to prepare three overlapping primers, EC38, 39 and 40, which were used for amplification of the 5' portion of the RNase from a unidirectional cDNA library from liver of 24-h estrogen-treated frogs prepared in the pSPORT plasmid (Life Technologies). The first round of amplification was performed with EC40 (see Table 1 for position), and a primer to the T7 promoter. The products of this reaction were reamplified with EC39 and the T7 promoter primer and were then cloned into pCRII (Invitrogen). Positive colonies were selected by hybridization with 5'-labeled EC38. The largest insert found was 1.2 kb and contained 200 bp of overlapping sequence with UDG14. This sequence was joined to UDG14 at a single shared *Sac*I site. The 5' end of the cDNA was obtained by 5' RACE using primer EC58 for first strand synthesis (Table 1).

Recombinant baculovirus expressing PMR-1 were generated by inserting either full-length PMR-1 cDNA or a subclone spanning from T147 to the stop codon into *Eco*R I + *Not* I-digested pVL1393 (Pharmingen). All subsequent steps followed the protocols provided by the supplier. Sf9 cells were harvested 5 days after infection with recombinant PMR-1 baculovirus. The cells were washed with 10 mM Tris, pH 7.4, 1 mM EDTA, suspended in 1 mL of 50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, homogenized and assayed for RNase activity as described previously (Dompenciel et al., 1995) using a 160-nt albumin mRNA transcript bearing the mapped PMR cleavage sites (Chernokalskaya et al., 1997).

TABLE 1. Primers used for cloning PMR-1.

Primer	Sequence	Position in cDNA
RDU	CAUCAUCAUGA(A/G)CA(A/G)CCNGGCTNACNGC	1219–1228
EC38	GAAAGCAGTAAGGCCAG	1242–1226
EC39	GTAACGCGCTGCTATG	1288–1272
EC40	CTTGCGAGCTTCCTG	1341–1327
EC54	GCTGTTCCCTGCACTGAG	143–126
EC57	TTGTTATCCAGTTCTTCAAC	182–163
EC58	AACCAGATGCTTTGCTTCT	222–205

In the experiment in Figure 6, 5'-end-labeled transcript was incubated with 250 ng (lane 4), 500 ng (lane 5), 1 μ g (lane 6), 2.5 μ g (lane 7), 5 μ g (lane 8) and 8 μ g (lane 9) of pellet fraction from cells expressing recombinant PMR60.

Spectral analysis of hMPO and PMR-1

UV-visible spectra were performed on a 0.1 μ g/mL solution of PMR-1 purified as described previously (Dompenciel et al., 1995) or a 0.2 μ g/mL solution of hMPO dissolved in 50 μ L of 40 mM Tris-HCl, pH 7.5, 2 mM DTT using a Beckman DU 640 spectrophotometer.

Endoglycosidase H cleavage

One microgram hMPO or 10 μ g of PMR-1 fractionated on a QAE column were incubated at 100 °C for 10 min in a 25- μ L volume containing 0.5% SDS and 1% β -mercaptoethanol. The samples were cooled to 25 °C, and 2.5 μ L of 0.5 M sodium citrate, pH 5.5, plus 750 units of endoglycosidase H (Endo H, New England Biolabs) were added. Each reaction mixture was incubated at 37 °C for 1 h and analyzed by Western blot using antibody #1277 to PMR-1 or a polyclonal antibody to hMPO.

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