Rat Lung Contains a Developmentally Regulated Manganese Superoxide Dismutase mRNA-binding Protein

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Abstract

It has become increasingly clear that RNA-binding proteins play an important role in the regulation of gene expression. The presence in rat lung of a specific, redox-sensitive catalase RNA-binding protein was recently reported (Clerch, L. B., and D. Massaro, 1992. *J. Biol. Chem.* 267:2853). In order to determine if specific manganese superoxide dismutase (MnSOD) RNA-binding proteins exist, we tested whether protein in rat lung extract would bind to ^{32}P-labeled MnSOD RNA. Using a gel mobility shift assay we show rat lung protein forms specific complexes with a 216 b fragment of the 3′ untranslated region of MnSOD RNA and the binding requires the presence of free sulfhydryl groups. Competition studies indicate MnSOD RNA-binding protein is different from catalase RNA-binding protein. Furthermore, unlike catalase RNA-binding protein, rat lung MnSOD RNA-binding protein activity is developmentally regulated; there is less MnSOD RNA-protein binding activity in adult rat lung extract compared to prenatal or neonatal rat lung extracts. We conclude the lung contains developmentally regulated MnSOD mRNA-binding protein that is redox sensitive. (*J. Clin. Invest.* 1993. 92:1278–1281.) Key words: antioxidant enzymes • posttranscriptional regulation • gene expression • redox

Introduction

The antioxidant enzymes including catalase, MnSOD, CuZn-SOD, and glutathione peroxidase (GP),[1] perform an invaluable function by reducing the cellular concentrations of superoxide and hydrogen peroxide (1). These toxic moieties are formed during normal oxidative metabolism but their formation increases during periods of oxidant stress (2–4). An important aspect of antioxidant enzymes is that they are mutually supportive; catalase and GP protect the SODs against inactivation by hydrogen peroxide and, reciprocally the SODs protect catalase and GP against inactivation by superoxide (5). Although the antioxidant enzymes work together in a coordinate way, the mechanisms responsible for the regulation of their gene expression can differ. For example, the concentration of lung MnSOD mRNA, but not that of catalase or GP, increases during exposure of adult rats to 48 h of > 95% O_2 (6). Furthermore, the level of regulation may be developmentally dependent; rat lung catalase mRNA concentration increases during late gestation and during exposure of the neonate to hyperoxia; however, catalase mRNA concentration is regulated at the level of transcription during late gestation but by increased mRNA stability in neonatal rat lung during hyperoxia (7). Since RNA-protein binding can alter mRNA stability, one possible mechanism responsible for altered catalase mRNA stability during exposure of neonatal rats to hyperoxia would be the interaction between catalase mRNA and a catalase mRNA-binding protein. Rat lung has been shown to contain a redox-sensitive protein that specifically interacts with catalase RNA to form RNA-protein complexes (8). In addition, this protein-binding activity inversely correlates with catalase mRNA stability, suggesting that oxidation-induced disassociation of catalase mRNA from its binding protein might result in increased stability of the mRNA (7, 8). That is, the catalase RNA-binding protein could function as a *trans*-acting negative regulator of RNA stability.

Using a gel mobility shift assay, we now show rat lung contains MnSOD RNA–binding protein and that the binding between the RNA and protein requires the presence of free sulfhydryl groups. Competition studies indicate MnSOD RNA–binding protein is different from catalase RNA–binding protein. Furthermore, unlike catalase RNA–binding protein, rat lung MnSOD RNA protein–binding activity is developmentally regulated; our data indicate that the activity is greater in fetal and neonatal rat lung, compared to adult rat lung.

Methods

Animals. We used specific pathogen-free Sprague Dawley rats obtained from Zivic-Miller Labs, Inc. Allison Park, PA, or from Harlan Farms, Indianapolis, IN. They were maintained in our Animal Care Facilities on a 12-h light–dark cycle. Neonatal rats were designated to be 1-d old the day after birth; they were raised 10 per litter, and the litters were adjusted to that size within 12 h of birth. For the hyperoxia study, rats were exposed in 3.4-ft^3 plastic chambers in which O_2 (> 95%), CO_2 (< 0.1%), temperature (22–25°C), and humidity (40–60%) were monitored. Air-breathing rats were exposed in identical chambers in which air flowed from a compressed air generator. To prevent O_2-induced lung damage in the nursing dams, they were alternated daily between litters of pups exposed to air and O_2. Adult rats were allowed food and water ad lib. and pups were allowed free access to the dams. Rats were killed between 9 a.m. and 11 a.m. by intraperitoneal injection of pentobarbital sodium (~ 200 mg/kg), followed by exsanguination.

Preparation of cell extracts. The lungs of fetal, neonatal, or adult rats were perfused free of blood, excised, and homogenized in a 25mM Tris buffer, pH7, containing 0.1 mM EDTA, 1% Triton x-100, 40 mM KCl, 10 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.2 U/μl aprotinin. The lung homogenate was centrifuged at 12,000 *g*

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1. *Abbreviations used in this paper:* GP, glutathione peroxidase; 2-ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide; UTR, untranslated region.

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for 15 min at 4°C. The supernatant fluid (hereafter referred to as lung extract) was stored at −70°C. The protein concentration of the lung extract was measured using the Coomassie Plus assay from Pierce Chemical Co., Rockford, IL, with BSA as a standard.

Preparation of labeled and unlabeled cRNA probes. The RNA probe used for MnSOD RNA binding experiments was derived from a StuI-EcoRI restriction fragment of a rat liver MnSOD cDNA-1 kindly provided by Dr. Ye-Shih Ho, Wayne State University, Detroit, MI (9). The 885b StuI-EcoRI restriction fragment, which encompasses the coding region for 47 carboxyl-terminal amino acids and 744 bases of the 3′ untranslated region, was subcloned into a pGEM-7Z vector (Promega Biotec, Madison, WI). The DNA sequence of both strands of this recombinant plasmid (designated RMS-12) was determined using the Sanger dideoxy-nucleotide chain termination method. In order to delimit the regions of MnSOD RNA that are involved in binding protein, the plasmid was made linear using the restriction enzymes, XbaI, PstI, or NdeI, and labeled sense strand transcripts were prepared using SP6 RNA polymerase and [^{32}P]CTP according to Promega Biotec's procedure for in vitro synthesis of high specific activity radiolabeled RNA probes. The resultant MnSOD cRNAs are designated RMS-E, RMS-P, and RMS-N, respectively. Following electrophoresis in a 4% nondenaturing polyacrylamide gel, the transcripts were excised from the gel and eluted overnight in 0.5 M ammonium acetate and 1 mM EDTA. The probe was precipitated by ethanol and resuspended in 20 μl H_2O).

Unlabeled MnSOD cRNA for competition experiments was prepared from the same recombinant plasmid used to prepare the labeled MnSOD cRNA probe (RMS-E) according to Promega Biotec's procedure for the synthesis of large quantities of RNA transcripts. The unlabeled competitors, catalase cRNA and lectin-14K (rat lung β-galactoside lectin, subunit M_r 14,000) cRNA were prepared as described previously from plasmids containing the catalase or lectin-14K cDNA inserts (8). The concentrations of unlabeled cRNA species were measured spectrophotometrically by absorbance at 260 nm and used at a ~ 100-fold molar excess compared to the ^{32}P-labeled MnSOD cRNA probe.

RNA-protein binding assay. The binding assay was performed by incubating lung extract containing 20–30 μg protein with ~ 2×10^4 dpm of ^{32}P-labeled MnSOD cRNA probe in 30 μl of binding buffer containing 5% glycerol, 40 mM KCl, 3 mM $MgCl_2$, 10 mM Hepes, pH 7.6. The solutions were incubated at 25°C for 30 min. T1 RNase (1U) (Gibco BRL, Gaithersburg, MD), which degrades single-stranded RNA that is unprotected because it is not bound to protein, was then added and incubation continued for 10 min. Heparin sulfate (6 μg/μl), which removes nonspecifically bound proteins from RNA, was added and incubation continued for 10 min. A gel loading buffer of 50% glycerol and 1% bromophenol blue was added to the reaction mixtures that then underwent electrophoresis at 14 V/cm in a low crosslinked 4% polyacrylamide gel (acrylamide: bis-acrylamide 60:1) containing 89 mM Tris-borate, pH 8.3, and 2 mM EDTA. After electrophoresis, the gel was transferred to filter paper, dried, and exposed to x-ray film (Hyperfilm; Amersham Corp., Arlington Heights, IL) at −70°C with an intensifying screen. In some experiments densitometry using a scanning densitometer (Image Quant version 3.2; Molecular Dynamics, Inc., Sunnyvale, CA) was used to quantitate binding activity.

Proteinase, competition, and oxidation-reduction experiments. Proteinase experiments were done by pretreating lung extract with 500 μg/ml proteinase K for 1 h at 37°C. In competition experiments, binding reaction mixtures were incubated with an unlabeled RNA species (MnSOD cRNA, catalase cRNA, lectin-14K cRNA, or yeast tRNA) for 30 min at 25°C before the addition of labeled MnSOD probe. Redox-sensitivity experiments were performed by incubation of reaction mixtures with 100 mM diamide (an oxidizing agent); 10 mM *N*-ethylmaleimide (NEM), an alkylating agent; or 2% 2-mercaptoethanol (2-ME), a reducing agent; for 15 min at 25°C before the addition of labeled MnSOD RNA probe.

Statistical analysis. For densitometry data given in arbitrary units, the values for individual animals were averaged per experimental group and the SEM of the group was calculated. The significance of the difference between two groups was obtained using an unpaired *t* test analysis (10).

Results

Using the gel mobility shift assay, we found a single major band (*arrow*) in the binding reaction between rat lung extract and the 885b ^{32}P-labeled MnSOD cRNA probe designated RMS-E (Fig. 1, lane *1*). Additional bands (*arrowhead*) representing complexes of greater mobility were occasionally observed; these bands appear less intense than the single, consistently observed band indicated by the arrow. The faster migrating bands may represent degradation products of the larger complex. When lung extract was pretreated with proteinase K, the RNA-protein complex was not found, indicating the material bound to the MnSOD RNA is a protein (Fig. 1 *A*, lane *2*). Treatment of lung extract with NEM, an agent that alkylates and therefore blocks free sulfhydryl groups, or with diamide, an agent that oxidizes free sulfhydryl groups to form disulfides, eliminated MnSOD RNA-protein complex formation (Fig. 1 *B*, lanes *2* and *3*, respectively vs. lane *1*, a control reaction in the absence of alkylation or oxidation). The addition of 2-ME, a thiol reducing agent, enhanced band intensity (Fig. 1 *B*, lane *4* vs. lane *1*). These results demonstrate the redox-sensitivity of the MnSOD mRNA–protein interaction. The presence of 2-ME in the reaction mixtures caused restoration of binding activity following its elimination with diamide (data not shown). This sulfhydryl switch mechanism, by which RNA-protein binding is abolished by oxidizing agents but is restored by reducing agents, is similar to that previously observed for the catalase mRNA–protein interaction (8). These results are of particular interest because they indicate the oxidation-reduction state of proteins that bind to antioxidant enzyme mRNAs

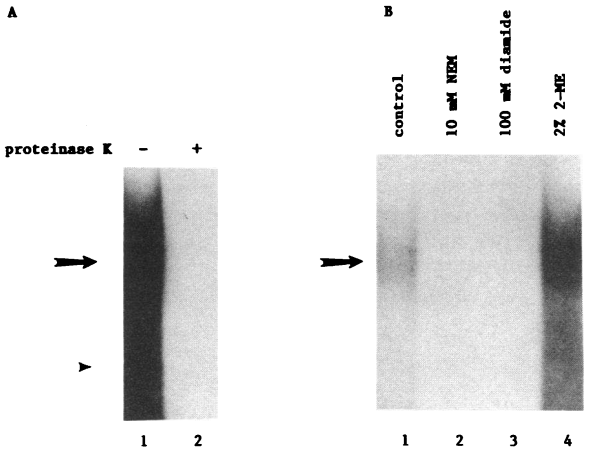

Figure 1. Complex formation between MnSOD RNA and lung protein is sensitive to redox agents. (*A*) One major complex (*arrow*) is formed during the binding reaction of ^{32}P-labeled MnSOD RNA probe (RMS-E) with lung protein extract (lane *1*). The arrowhead points to a less intense complex of faster mobility. Lane *2* shows the lack of complex formation when the lung extract was pretreated with proteinase K (500 μg/ml). (*B*) One major complex (*arrow*) is formed when neither reducing nor oxidizing agent was added to the reaction mixture (lane *1*). Preincubation of the lung extract with either 10 mM NEM (lane *2*) or 100 mM diamide (lane *3*) completely blocked complex formation. In the presence of 2% 2-ME (lane *4*) binding is enhanced.

may have an important role regulating antioxidant enzyme gene expression during the lung's response to oxidant stress.

To determine the specificity of the RNA–protein interaction we performed competition studies in which lung extract was preincubated with unlabeled RNA before the addition of radiolabeled MnSOD RNA. In the absence of competitor, the radioautograph shows one major complex (*arrow*) was formed after incubation of radiolabeled MnSOD RNA with lung protein extract (Fig. 2, lane *1*). The MnSOD RNA-protein complex demonstrated in lane *1* was specifically inhibited by competition with unlabeled MnSOD RNA (Fig. 2, lane *2*) but complex formation was not eliminated by preincubation with tRNA, catalase cRNA, or lectin-14K cRNA (Fig. 2, lanes *3*, *4*, and *5*, respectively). In cross-competition experiments using radiolabeled catalase RNA and unlabeled excess MnSOD RNA, we found MnSOD did not inhibit formation of catalase RNA-protein complexes (data not shown). The results of the competition studies indicate that binding observed with MnSOD RNA is specific and the MnSOD RNA-binding protein is different from the catalase RNA-binding protein.

We used unique restriction endonuclease sites (PstI and NdeI) within the StuI-EcoRI fragment of MnSOD cDNA-1 to delimit the region of MnSOD RNA involved in protein binding. The size and orientation of the MnSOD cRNA probes are depicted in the diagram in Fig. 3 *A*. Of the three ^{32}P-labeled cRNA probes generated by in vitro transcription, we found RMS-E and RMS-P, but not RMS-N, contain *cis* elements that bind lung protein (Fig. 3 *B*). The protein binding activity to both RMS-E and RMS-P was enhanced in vitro by the addition of 2-ME (Fig. 3 *B*). These findings indicate protein binding occurs in a 216-base fragment of the 3′ untranslated region (3′ UTR) between the NdeI and PstI restriction endonuclease sites. This region, which we designate RMS-NP, is located 111 bases downstream of the translation stop codon (Fig. 3 *A*). RMS-NP is contained within the 339-base NdeI-EcoRV fragment of 3′ UTR shown by Hurt et al. to be present in four rat MnSOD RNA species (3.8, 2.7, 2.2, and 1.3 kb) that originate as a result of alternate polyadenylation (11).

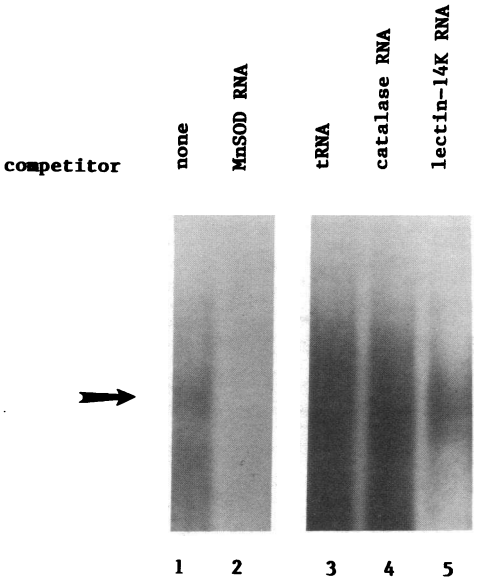

Figure 2. MnSOD RNA–binding protein is different from catalase RNA–binding protein. One major MnSOD RNA–protein binding complex (*arrow*) is formed when rat lung extract is incubated with MnSOD cRNA (RMS-E) in the absence of competitor RNA (lane *1*). MnSOD RNA–protein binding is inhibited by incubation with excess unlabeled MnSOD cRNA (lane *2*). There is no inhibition of binding in the presence of nonspecific yeast tRNA (lane *3*) or in the presence of excess unlabeled catalase cRNA or lung lectin-14K cRNA (lanes *4* and *5*, respectively).

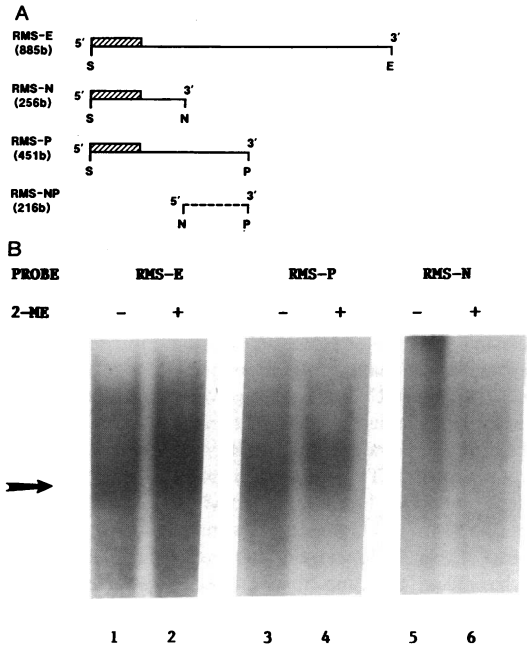

Figure 3. The MnSOD RNA–protein binding element is located in a 216-base fragment of the 3′ UTR. (*A*) Diagram of MnSOD cRNA probes generated by in vitro transcription after linearization of plasmid RMS-12 by restriction endonucleases. The designated name and length of the probe is given on the left. The 5′ to 3′ orientation is shown and the MnSOD coding region is depicted by the cross-hatched bar. RMS-NP (*dashed line*) represents the deduced protein binding *cis* element of the MnSOD 3′ UTR. S, StuI; E, EcoRI; N, NdeI; P, PstI. (*B*) Gel mobility shift assay using RMS-E (lanes *1* and *2*), RMS-P (lanes *3* and *4*), and RMS-N (lanes *5* and *6*). The binding reaction was done in the absence of 2-ME (lanes *1*, *3*, and *5*) and in the presence of 2% 2-ME (lanes *2*, *4*, and *6*). The major complex formed between 30 μg of neonatal lung protein and RMS-E and RMS-P, but not RMS-N, is indicated by the arrow.

MnSOD mRNA protein binding appears to be developmentally regulated. We found similar band intensity on the radioautograph of the binding reaction between MnSOD RNA and lung extracts from fetal and neonatal rats (Fig. 4 *A*, lanes *1* to *4*). Complex formation with adult lung extract (Fig. 4 *A*, lane *5*) appears as a band of much less intensity than the bands formed with perinatal extracts (Fig. 4 *A*, lane *5* vs. lanes *1* to *4*). We found no difference in RNA-protein binding activity between lung extract from 7-d-old rats exposed to 72 h > 95% O_2 and air-breathing 7-d-old (1.97±0.20 SE, $n = 4$, and 1.89±0.22 SE, $n = 3$, respectively). Fig. 4 *B* shows the complexes formed using lung and brain extracts from three 7-d-old neonatal rats and three adult rats. Using scanning densitometry, we found significantly greater MnSOD RNA–binding activity in neonatal lung extract than in adult lung extract (2.58±0.26 SE vs. 0.73±0.01 SE, $P < 0.005$). We did not find a significant difference in MnSOD RNA–binding activity between neonatal rat brain extract and adult brain extract (5.57±1.50 SE and

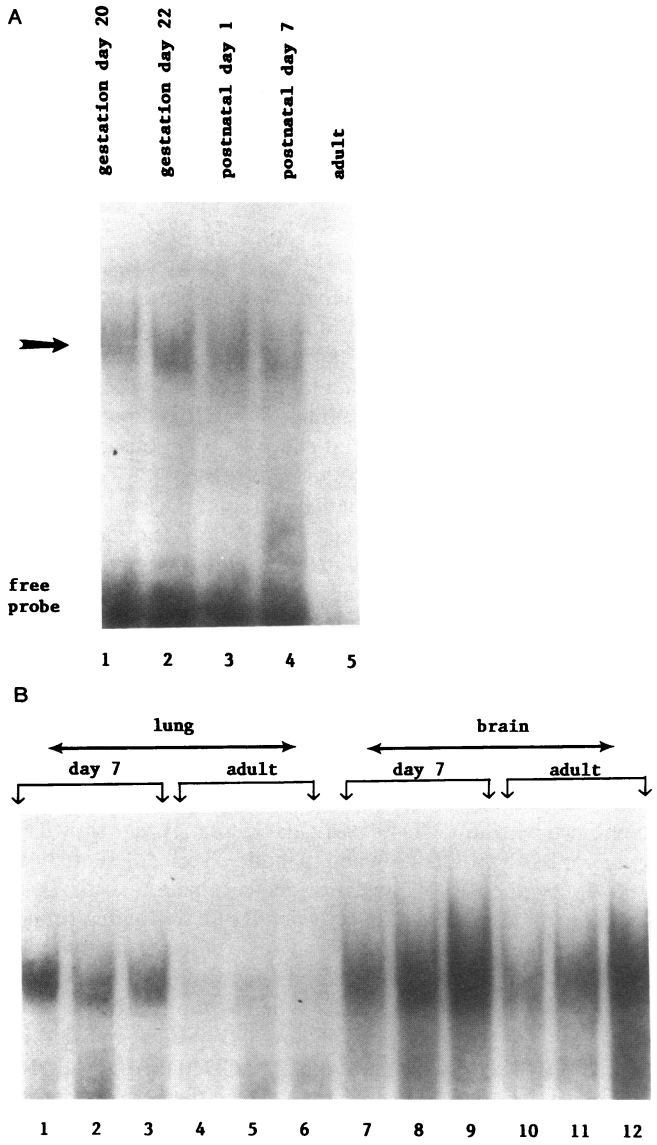

Figure 4. Rat lung MnSOD RNA–protein binding activity is developmentally regulated. (*A*) The MnSOD RNA binding reaction was performed using the RMS-P probe with 30-μg lung protein extract from rats at gestation day 20, gestation day 22, postnatal day 1, postnatal day 7, and adulthood (250–300 g) (lanes *1* to *5*, respectively). The arrow points to the major RNA-protein complex. The position of free unbound probe is indicated. (*B*) MnSOD RNA–protein binding activity is developmentally regulated in rat lung but not in rat brain. The MnSOD RNA binding reaction was performed using the RMS-P probe and 30 μg of protein extract from 7-d-old rat lungs (lanes *1–3*), adult rat lungs (lanes *4–6*), 7-d-old rat brain (lanes *7–9*), and adult rat brain (lanes *10–12*).

5.60±1.97 SE, respectively). These data indicate rat lung MnSOD RNA–protein binding activity is developmentally regulated and this developmental regulation is, at least in part, tissue specific. The cause and significance of the decreased binding activity observed in adult lungs compared to perinatal lungs is unknown. It is possible that there is an inactivation of binding activity in adult extracts, perhaps due to oxidation of the binding protein. Other possible explanations for less MnSOD RNA-binding activity in adult lungs include: (*a*) decreased concentration of the binding protein in adult lung compared to perinatal lungs and (*b*) decreased RNA-binding affinity of the protein in adult extracts compared to neonatal extracts. We have not distinguished between these possibilities at this time.

In order to determine the in vivo function of the MnSOD RNA-binding protein, further studies are required. However, we speculate that MnSOD RNA-binding protein may have a role during development as a negative regulator of MnSOD mRNA stability. We base this speculation on previous published work showing that lung MnSOD RNA in adult rats has a longer half-life (8.2 h±0.8 S.E.) than MnSOD RNA in neonatal lung (5.5 h±0.1 SE, $P < 0.025$) (6, 12). The increased stability of adult rat lung MnSOD mRNA correlates with decreased RNA-binding protein activity, suggesting the binding protein may be a *trans*-acting negative regulator of stability. Future work will be focused on isolating the MnSOD RNA-binding protein to directly test its function.

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