Purification of a RNA-binding Protein from Rat Liver

IDENTIFICATION AS FERRITIN L CHAIN AND DETERMINATION OF THE RNA/PROTEIN BINDING CHARACTERISTICS*

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In cultured rat hepatocytes the degradation of phosphoenolpyruvate carboxykinase mRNA might be regulated by protein(s), which by binding to the mRNA alter its stability. The 3′-untranslated region of phosphoenolpyruvate carboxykinase mRNA as a potential target was used to select RNA-binding protein(s) from rat liver by the use of gel retardation assays. A cytosolic protein was isolated, which bound to the phosphoenolpyruvate carboxykinase mRNA 3′-untranslated region and other in vitro synthesized RNAs. The protein was purified to homogeneity; it had an apparent molecular mass of 400 kDa and consisted of identical subunits with an apparent size of 245 kDa. Sequence analysis of a tryptic peptide from the 245-kDa protein revealed its identity with rat ferritin light chain. Binding of ferritin to RNA was abolished after phosphorylation with cAMP-dependent protein kinase and was augmented after dephosphorylation with alkaline phosphatase. Weak binding was observed in extracts from okadaic acid-treated cultured hepatocytes compared with untreated cells. Preincubation of ferritin with an anti-phosphoserine or an anti-phosphothreonine antibody attenuated binding to RNA, while an anti-phosphotyrosine antibody generated a supershift indicating that phosphoserine and phosphothreonine but not phosphotyrosine residues were in close proximity to the RNA-binding region. Ferritin is the iron storage protein in the liver. Binding of ferritin to RNA was diminished in the presence of increasing iron concentrations, whereas the iron chelator desferal was without effect. It is concluded that ferritin might function as RNA-binding protein and that it may have important functions in the general regulation of cellular RNA metabolism.

In the regulation of RNA metabolism protein-RNA interactions play a central role. This is well documented for the splicing process, which eliminates intervening sequences from the primary transcript in the ribonucleoprotein particle of the spliceosome (1), for the transport of RNA from the nucleus to the cytoplasm (2), for translational initiation, which is mediated by protein factors recognizing the initiation region on the mRNA and positioning the ribosome to the translational start site (3), for the storage of translationally inactive mRNA (4) or for spatial localization of mRNA in the cell (5). Even if the mechanism and regulation of mRNA degradation are far from being understood, it is clear that RNA-protein interactions have a key role (6, 7). Besides its function in RNA transport and translation the poly(A) tail has been shown to protect poly(A)-carrying mRNAs from rapid degradation by interaction with the poly(A)-binding protein PABP (8, 9). Many mammalian mRNAs contain AU-rich portions in their 3′-untranslated region, which after being occupied by AU-binding proteins render the RNA instable (10–12).

In cultured rat hepatocytes and in rat hepatoma cells the expression of the key control enzyme in gluconeogenesis, phosphoenolpyruvate carboxykinase (PCK), 1 was stimulated at the transcriptional level by glucagon (13) or cAMP (14, 15). Insulin, the glucagon antagonist at large, inhibited the glucagon-(cAMP)-stimulated gene transcription (13, 16). Yet, besides transcriptional regulation the expression of PCK is also regulated at the level of mRNA degradation. In cultured rat hepatocytes insulin and the proinflammatory cytokine interleukin 6 accelerated the degradation of glucagon-induced PCK mRNA (13, 17). The acceleration of PCK mRNA degradation was probably due at least in part to the insulin-enhanced expression of a ribonuclease. 2 In FTO-2B rat hepatoma cells cyclic AMP stabilized PCK mRNA (18, 19). The stability of PCK mRNA seems to be regulated by proteins binding to the 3′-untranslated region of the mRNA. In cultured rat hepatocytes binding of a protein was enhanced after glucagon treatment, which correlated with PCK mRNA accumulation. Insulin prevented the increase in protein binding, which correlated with the decrease in PCK mRNA levels (20, 21). In FTO-2B cells cAMP caused reduced binding of a protein to the 3′-UTR of PCK mRNA (22).

It was the goal of this investigation to identify and characterize protein(s), which might contribute to the regulation of PCK mRNA degradation. By gel retardation assays a cytosolic protein binding to PCK mRNA was identified, which was purified and biochemically characterized. Peptide sequence analysis showed identity of the protein with rat ferritin light chain. The binding of ferritin to RNA was regulated by phosphorylation/dephosphorylation and by iron. Competition experiments showed that ferritin did not bind preferentially to PCK mRNA but to a variety of different RNAs. It is suggested that ferritin may play a regulatory role in PCK mRNA degradation as well as in the general cytosolic mRNA turnover. The identification

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1 The abbreviations used are: PCK, phosphoenolpyruvate carboxykinase; cAMP, cyclic 3′,5′-adenosine monophosphate; UTR, untranslated region; CE, cytosolic protein extract; CHAPS, chomipropyltrimethylammoniumpropane sulfonate; PCR, polymerase chain reaction; IRE, iron response element; IRP, iron response element-binding protein.

2 T. Heise, A. Krones, A. Nath, K. Jungermann, and B. Christ, unpublished results.
of ferritin as RNA-binding protein is discussed in view of an old hypothesis and recent publications, which proposed ferritin to play a role in the general metabolism of cytosolic RNA (23, 24).

EXPERIMENTAL PROCEDURES

Primary Cultures of Rat Hepatocytes and Induction Experiments—Liver cells were isolated from male fed Wistar rats (200–250 g) by the collagenase perfusion technique. Cells were seeded on 6-cm diameter plastic culture dishes (Greiner, Nürtingen) and maintained in culture in the presence of 100 nM dexamethasone (Sigma, Deisenhofen) and 0.5 mM insulin (Sigma, Deisenhofen) to improve cell viability as described (13). After 48 h of culture, cells were washed twice with medium containing only 100 nM dexamethasone. Fresh medium containing in addition 0.1 mM glucagon (Serva, Heidelberg) was added and the culture was continued for another 2 h. Then insulin at 10 nM and/or the phosphatase inhibitor okadaic acid (ICN, Meckenheim) at 0.5 μM and/or the transcriptional inhibitor actinomycin D at 10 μg/ml (Sigma, Deisenhofen) were added for another 3 h. Finally the cells were harvested for preparation of protein extracts.

Preparation of Cytosolic Extracts from Cultured Rat Hepatocytes—Cells from one culture dish were washed twice with ice-cold phosphate-buffered saline, scraped off in 800 μl of homogenization buffer containing 10 mM Tris/HCl, pH 7.4, 250 mM sucrose, 0.5 mM CaCl2, 0.5 mM MgCl2, 0.1% CHAPS, 0.5 mM dithioerythreitol (Serva, Heidelberg), 0.5 mM phenylmethylsulfonyl fluoride and 0.01% digitonin, and homogenized in a glass homogenizer with a motor-driven Teflon pestle for 6 min at maximal speed. The homogenate was centrifuged at 1,500 × g for 20 min, the resulting supernatant at 5,000 × g for 15 min, and the then resulting supernatant at 120,000 × g for 90 min to obtain a post-polyosomal, cytosolic extract (S120 extract). After heating to 50 °C for 15 min the crude extract was centrifuged at 14,000 × g for 15 min (Fig. 3). The resulting supernatant was brought to 35% saturation with ammonium sulfate and stirred on ice for 30 min. After centrifugation of the slurry for 20 min at 14,000 × g the supernatant was brought to 65% saturation with ammonium sulfate and stirred again on ice for 15 min. The slurry was centrifuged at 14,000 × g for 20 min, the pellet was suspended in dialysis buffer, washed with buffer A containing 200 mM sucrose, 10 mM Tris/HCl, pH 7.4, 0.5 mM dithioerythreitol and phenylmethylsulfonyl fluoride and the extract was dialyzed (cut off 3,500, Spectrum Medical Industries) overnight at 4 °C (cytosolic extract CE) (Fig. 3). Protein concentration was determined by a commercial kit (Bio-Rad, München). The extract was stored at −70 °C. The same procedure was performed for the preparation of cytosolic extracts from whole rat liver or other organs as indicated. In this case tissue was homogenized in 10 volumes of homogenization buffer.

Purification of a RNA-binding Protein from Rat Liver—3.5 g of swolen DEAE Sephadex A-25 (Pharmacia LKB Biotechnologies Inc., Freiburg) pre-equilibrated with buffer A (10 mM Tris/HCl, pH 7.4, and 0.5 mM EDTA) was incubated in a batch procedure for 30 min at 4 °C with 10 ml of cytosolic extract (CE) containing about 100 μg of total protein. Buffer A containing unbound proteins was collected and combined with buffer A resulting from two subsequent washes of the gel. Bound protein was eluted by washing with buffer B (10 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, and 1 M NaCl). Eluted protein and the unbound material were separated by ultrafiltration (Centriplus 100, Amicon Inc., Beverly, MA). The unbound protein fraction called FTI (for historical reasons because this procedure was performed in a column at the beginning of the study) was further processed with fast protein liquid chromatography on the anion exchange matrix Source 30 Q (Pharmacia, Freiburg) in an HR 5/5 column (Pharmacia, Freiburg) (Fig. 3). The column was equilibrated with three successive washes with buffer A. The sample was loaded onto the column and the resin was washed with buffer A until protein was no longer detectable in the flow-through (FT2). Bound protein was eluted in a linear gradient ranging from 0 to 1 M NaCl at a flow rate of 0.5 ml/min. Fractions (1 ml) were assayed for RNA binding activity in the gel retardation assay as described below. RNA binding activity containing fractions were pooled (range of fractions 6–10) (Source extract SO), concentrated, and equilibrated with buffer A by ultrafiltration on Centricron 100 and loaded onto a Superdex column (Column volume, Column volume, V0 = 135 ml) (Pharmacia, Freiburg) buffer A (Fig. 3). Elution volume was performed in buffer A at a flow rate of 0.25 ml/min and 0.5- or 0.6-ml fractions were collected and assayed for RNA binding activity (range of fractions 36–38) (Superdex extract). For molecular mass determination the Superdex column was calibrated with standard proteins in a gel filtration calibration kit (Sigma, Deisenhofen). SDS-polyacrylamide electrophoresis under reducing conditions revealed that the Superdex extract contained one prominent protein band and only minor contaminations. Therefore it was used to raise an antiseraum against the purified RNA-binding protein in rabbits.

Preparative Isoelectric Focusing—200–250 μg of protein in the S120 extracts was incubated in a final volume of 50 μl in focusing buffer (10 mM Tris/HCl, pH 7.4, 2 M urea, 0.1 g glycerol) dialyzed into the precooled (4 °C) Rotofor® cell (Bio-Rad, München). Isoelectric focusing was performed at constant power of 12 watts for 5–6 h. During this time voltage increased from 290 to 900 V and the current decreased from 41 to 13 mA. At the end of the run samples were fractionated as described in the manufacturer’s manual and pH was measured in each sample. Fractions containing separate amphoteres from protein, the samples were incubated in 1 M NaCl for 30 min at 4 °C. Then they were dialyzed against 10 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, and 10% glycerol overnight. Each fraction was assayed for RNA binding activity as described below. Five different focusing buffers were tested containing CHAPS, urea, glycerol, digitonin, and/or Tris/HCl at different concentrations and combinations to minimize denaturation of protein during the focusing procedure. The Tris/HCl buffer described here yielded the best results.

Preparation by PCR of Double-stranded DNA Templates for in Vitro Transcription—Plasmid pBS-PCK722 containing a 722-base pair Pol- EcoRI PCK DNA fragment, which represented 120 translated and 602 untranslated bases of the 3'-portion of PCK mRNA (bases 1890–2611, Ref. 14), was used as the template for the production of PCR products. To obtain various DNA templates for the generation of PCR transcripts of different lengths and regions from the PCK mRNA 3'-UTR the following primers were used for PCR: sense primer 1 and 2 contained at the 5'-end the T7-RNA polymerase promoter sequence (5'-TAATACGACTCACTATAG-3') followed by genuine PCK mRNA sequences from bases 2010 to 2030 and 2265 to 2291 (14). Antisense primers 1 and 2 contained genuine PCK mRNA sequences from bases 2235 to 2259 and 2583 to 2603 (14). The combinatorial use of the primer pairs sense primer 1 and antisense primer 2, sense primer 1 and antisense primer 1, sense primer 2 and antisense primer 2 yielded PCR products PCK1, PCK2, and PCK3, which contained either the total 3'-UTR (bases 2010–2603), the 5'-portion (bases 2100–2290), or the 3'-portion (bases 2256–2603) of the PCK mRNA 3'-UTR (14). PCR was performed with 1 ng of pBS-PCK722 and 90 pmol of each primer in 10 mM Tris/HCl buffer, pH 8.3, containing 50 mM KCl, 1.5 mM MgCl2, 1 μM each of dCTP, dGTP, dATP, and dTTP and finally 1 unit of Taq DNA polymerase. PCR was performed in 35 cycles (45 s at 95 °C, 45 s at 55 °C, 60 s at 72 °C). The resulting PCR products were taken as templates for a second round of PCR under the same conditions. These PCR products were purified by a commercial kit (Qiagen, Düsseldorf) and the DNA content was measured spectrophotometrically.

In Vitro Transcription—The PCR products PCK1, PCK2, and PCK3 were used as templates to generate PCK1, PCK2, and PCK3 transcripts. Transcription reactions were carried out with 10 nM PCR products (see above) in a final volume of 40 μl in transcription buffer (Stratagene, Heidelberg), containing 0.5 mM adenosine-, cytidine-, and guanosine 5'-triphosphate each, 0.025 mM uridine 5'-triphosphate, 55 pmol of ATP, 20 units of RNase-free DNase (Life Technologies, Inc., Bethesda), 10 mM dithioerythreitol, and 25 units of human placental ribonuclease inhibitor (Amersham, Braunschweig). The reaction was started by addition of 25 units of T7 RNA polymerase (Stratagene, Heidelberg). After incubation for 45 min at 37 °C another 25 units of T7 RNA polymerase were added and the reaction was continued for 45 min at 37 °C. The reaction was terminated by adding 10 μg of RNAse A (Boehringer, Mannheim) and 25 units of human placental ribonuclease inhibitor. Subsequently template DNA (PCK1, 2, or 3) was digested with 1 unit of RNase-free DNase I (Boehringer, Mannheim) and 25 units of human placental ribonuclease inhibitor. The resulting RNA was transcribed in a microcentrifuge and the aqueous phase was applied to a Nick column (Pharmacia, Freiburg) to remove unincorporated nucleotides. Human histone H1* and mouse asrosulfatase A transcripts were generated by the same procedure using plasmid DNA kindly provided by Drs. D. Doenecke (Göttingen) and V. Giebel (Potsdam).
on a 5% native polyacrylamide gel (34 × 16 cm) for 2–3 h at 20 mA (about 6 volt/cm) at room temperature. After electrophoresis gels were dried and exposed to Hyperfilm MP (Amersham, Braunschweig) in dry conditions. Many of the bands were detected on the film. Competition experiments were performed by addition of unlabelled competitor RNAs to the binding reaction 1 h prior to the addition of the labeled transcript.

In Vitro Phosphorylation and Dephosphorylation—In the phosphorylation reaction 40 μg of protein in the CE was treated with 80 units of the catalytic subunit of the cAMP-dependent protein kinase (Sigma) for 30 min at 37 °C in 40 μl of phosphorylation buffer containing 10 mM Tris/HCl, pH 7.4, 4.8 mM MgCl₂, and 0.85 mM ATP. In the dephosphorylation reaction 40 μg of protein in the CE was treated with 7 units of calf intestinal alkaline phosphatase (U. S. Biochemical Corp., Cleveland, OH) for 30 min at 37 °C in 40 μl of dephosphorylation buffer containing 10 mM Tris/HCl, pH 7.4, and 0.01 mM ZnCl₂. Controls were performed without addition of kinase or phosphatase.

Detection of Phosphorylated Amino Acids by Anti-phosphoamino Acid Antibodies—1 μg of protein in the Superdex extract (Fig. 4) was incubated with 0.5 or 1 μg of anti-phosphoserine, anti-phosphothreonine (Biomol, Hamburg), or anti-phosphotyrosine antibody (Amersham, Braunschweig) or with an unrelated antibody in 10 mM Tris/HCl, pH 7.4, and 0.05% Triton X-100 in a final volume of 10 μl at 4 °C overnight. After the incubation the mixture was submitted to the standard RNA binding assay.

Immunoprecipitation of Ferritin with an Anti-ferritin Antibody—40 μg of protein A coupled to Sepharose CL-4B beads (Pharmacia, Freiburg) was swelled in 1.5 ml of TS buffer (15 mM Tris/HCl, pH 7.4, 150 mM NaCl) for 4 h at room temperature. The swelled gel was collected by centrifugation and washed 3 times in TS. The final pellet (200 μl) was resuspended in 200 μl of TS and combined with 20 μl (90 μg) of anti-ferritin antiserum. The mixture was incubated overnight at 4 °C and subsequently for 4 h at room temperature. 60 μl from this mixture were combined with 40 μg of protein in the CE and incubated overnight at 4 °C. Precipitates were pelleted by centrifugation at 14,000 × g. The supernatant was taken off (supernatant 1) and the pellet was washed 2 times with 30 μl of TS. The resulting supernatants were combined with supernatant 1. 25 μg of protein in this ferritin-free CE was applied to the gel retardation assay.

Miscellaneous—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (25). Mass spectroscopy and sequencing of tryptic peptides of the purified protein were conducted by Drs. B. Schmidt and T. Selmer (Göttingen, Germany). Antisera against the purified protein from the Superdex extract was generated in rabbits by Eurogentec (Seraing, Belgium).

RESULTS

Characteristics of Cytosolic Protein Binding to PCK mRNA—To identify and characterize proteins, which might be involved in the regulation of PCK mRNA degradation, a gel retardation assay was established using cytotoxic protein extracts from rat liver and a 32P-labeled transcript, which was produced by in vitro transcription from a PCR-generated DNA fragment containing the entire 3′-untranslated region of PCK mRNA (PCK1 transcript, 594 bases long; position 2010 to 2603 of PCK mRNA). In Vitro Phosphorylation and Dephosphorylation—In the phosphorylation reaction 40 μg of protein in the CE was treated with 80 units of the catalytic subunit of the cAMP-dependent protein kinase (Sigma) for 30 min at 37 °C in 40 μl of phosphorylation buffer containing 10 mM Tris/HCl, pH 7.4, 4.8 mM MgCl₂, and 0.85 mM ATP. In the dephosphorylation reaction 40 μg of protein in the CE was treated with 7 units of calf intestinal alkaline phosphatase (U. S. Biochemical Corp., Cleveland, OH) for 30 min at 37 °C in 40 μl of dephosphorylation buffer containing 10 mM Tris/HCl, pH 7.4, and 0.01 mM ZnCl₂. Controls were performed without addition of kinase or phosphatase.

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When the binding reaction was performed in the absence of RNase A two dominant RNA-protein complexes (complex 1 and 2) formed with PCK1 transcript and cytotoxic protein(s) from rat liver. While formation of complex 2 did not require the presence of Mg²⁺ complex 1 formed only in the presence of both Mg²⁺ and EDTA (Fig. 1A, lanes 3–5 versus lanes 4–6). Addition of RNase A yielded the specific formation of only complex 1 along with only in the presence of equimolar concentrations of a divalent cation (Mg²⁺ or Mn²⁺ or Ca²⁺ or Zn²⁺) and EDTA as shown representatively for Mg²⁺/EDTA (Fig. 1A, lane 10 versus lanes 7–9). This complex was only obtained with protein from rat liver cytosol and not with bovine serum albumin or with nuclear protein extracts (Fig. 1A, lanes 11 and 12). Complex formation required incubation at 37 °C for at least 15 min (Fig. 1B, lanes 4–6) and did not occur at 4 °C (Fig. 1B, lanes 1–3), indicating that a temperature-sensitive step was involved in RNA-protein complex formation. Heating the cytosolic extract to 70 °C abolished complex formation (not shown). Pretreatment of the extracts with proteinase K did not destroy the RNA binding activity of the protein (Fig. 1B, lane 11). Pretreatment of the extracts with a protease mixture containing proteinase K, trypsin, and Pronase E prevented complex formation (not shown). These data show that complex 1 was due to an interaction of protein(s) with PCK1 transcript.

RNA-protein interactions can be expected to be dependent on salt concentration and pH. Binding of the rat liver cytosolic protein to PCK1 transcript was still observed at 0.4 M KCl but was lower compared with 0.1 M KCl (Fig. 1B, lane 6 versus 7). Protein binding to PCK1 transcript was stronger at pH 4.0 compared with pH 9.0 (Fig. 1B, lanes 8–10). Preparative isoelectric focusing (IEF, “Experimental Procedures”) enriched the RNA binding activity in fractions between pH 4.0 and pH 5.2, which showed that the RNA-binding protein was acidic.

The binding activity containing IEF fractions were used for an estimate of the binding affinity of the protein to RNA. Increasing the amount of protein from 10 to 100 μg at a constant amount of the PCK1 transcript (16 fmol) increased complex formation in a sigmoidal manner. Half-maximal protein binding was reached at 38 μg of protein (not shown). The binding protein from rat liver (which was identified as rat ferritin light chain, see below) was en-
FIG. 2. Competition of various RNAs and DNAs with the 3′-UTR of PCK mRNA for binding of cytosolic protein from rat liver. The binding reaction was carried out under standard conditions. In A and C, 5 ng of 32P-radiolabeled RNA transcript PCK1; in B, radiolabeled transcripts representing the 3′-UTRs of PCK, histone H1* (HIS), and arylsulfatase A (ASA) were used in the gel retardation assay. In A and B, CE from rat liver, in (C) cytosolic extracts from rat liver (LI), kidney (KI), spleen (SP), and lung (LU) were used. Unlabeled competitor RNAs were added 3 min prior to the addition of PCK1 transcripts. Homoribopolymers poly(A), poly(C), poly(G), and poly(U) were added in 500- and 1000-fold excess by weight. Escherichia coli tRNA was added in a 2000- and 4000-fold molar excess. Unlabeled transcripts of PCK1 (bases 2010–2603), PCK2 (bases 2010–2259), and PCK3 (bases 2265–2603) were applied in a 150- and 500-fold, 400- and 1200-fold, and a 300- and 900-fold molar excess. DNA competitor PCR products 1, 2, and 3 were applied at a 500-fold excess by weight.

Specificity of Cytosolic Protein Binding to RNA—Binding of cytosolic protein to PCK1 transcript should be hindered in the gel retardation assay by RNA molecules, which share sequence and/or structure similarities with PCK1 transcript. To study the binding preference of the protein for different RNAs, the gel retardation assay with PCK1 transcript was performed in the presence of single-stranded RNA homopolyribonucleotides (poly(A), poly(C), poly(G), and poly(U)) (Fig. 2A, upper panel). In the presence of poly(U) (500 and 1000-fold excess by weight) binding of protein to PCK1 transcript was abolished and with tRNA (2000 and 4000-fold molar excess) binding was effectively attenuated. Poly(A) competed for protein binding to a lesser extent than poly(U), whereas poly(C) exhibited weak and poly(G) no competition for binding. Binding to labeled PCK1 transcript was also investigated in the presence of unlabeled transcripts of PCK1, PCK2, and PCK3, which contained either the entire 3′-UTR of PCK mRNA (PCK1 bases 2010–2603) or the 5′ (PCK2 bases a 150- and 500-fold, 400- and 1200-fold, and a 2603) (14) part of it in a 150–1200-fold molar excess (Fig. 2B, bottom part). With all three unlabeled transcripts binding of cytosolic protein to PCK1 transcript was effectively attenuated. However, when the PCR DNA fragments, which were used as templates for the generation of the transcripts, were included in a 500-fold excess by weight in the binding reaction, no interference with protein binding was observed.

Protein binding to PCK1 transcript was compared with binding to the 3′-UTR of the human histone 1* (HIS) mRNA, which encodes a nuclear protein, and a portion of the 3′-UTR of the mouse arylsulfatase A mRNA, which encodes a lysosomal protein. With cytosolic protein from rat liver HIS and arylsulfatase A transcripts formed a complex, which had the same electrophoretic mobility as the complex with PCK1 transcript (Fig. 2B).

The tissue distribution of the cytosolic RNA-binding protein was studied. Cytosolic protein extracts from rat liver (LI), kidney (KI), spleen (SP), and lung (LU) were applied to the gel retardation assay with the PCK1 transcript. Whereas rat liver extracts exhibited strong protein binding to the PCK1 transcript, extracts from kidney, spleen, and lung showed weak but significant binding forming a complex with slightly higher mobility in the case of spleen and lung (Fig. 2C).

These data show that the cytosolic RNA-binding protein was prevalent in rat liver and that the protein bound specifically to all transcripts investigated but not to DNA. The fact that poly(U) and to a lesser extent poly(A) competed for protein binding might indicate that single-stranded A- and/or U-rich sequences were involved in the contact of the protein to RNA, which are present in multiple regions in the 3′-untranslated part of PCK mRNA (14).

Purification of the RNA-binding Protein and Identification as Ferritin—The RNA-binding protein was purified by sequential chromatography of cytosolic protein of rat liver on the weak anion exchange resin DEAE 25, the strong anion exchange material Source Q, and the final gel filtration over Superdex 200 pg column (Fig. 3). The enrichment of the RNA-binding protein from the CE was assessed in the gel retardation assay with PCK1 transcripts by counting the radioactivity in the
RNA-protein complex using a PhosphorImager. Whereas after
the second anion exchange chromatography on Source Q the
RNA binding activity was enriched in the range of fractions
7–15 about 10-fold over the cytosolic protein extract, it was
enriched only about 5-fold after gel filtration on Superdex
200-μg column in the range of fractions 36–38. The RNA-
binding protein eluted from the Superdex column in a molecular
mass range of about 400 kDa. SDS-polyacrylamide gel
electrophoresis analysis of this material under reducing condi-
tions revealed that this fraction contained one prevalent pro-
tein of 24,500 Da. Two further protein bands of molecular
masses of 59,500 Da and of about 150,000 Da were visible in
the SDS-polyacrylamide gel electrophoresis. However, after in-
cubation for 20 min at 95 °C in β-mercaptoethanol these two
bands vanished and only the 24.5-kDa protein appeared (not shown).
This indicated that the RNA-binding protein had a
native molecular mass of about 400 kDa and consisted of iden-
tical subunits with a molecular mass of 24.5 kDa each. Mass
spectroscopy of the purified protein revealed a molecular mass
of 21.2 kDa.

The Superdex-purified native protein was processed for se-
quencing by carboxymethylation under reducing conditions
and was subsequently digested with 1% trypsin. After fraction-
ation of the tryptic digest by high performance liquid chroma-
tography a suitable peptide was chosen for sequencing. Se-
quencing was terminated after completion of 9 amino acids.
The resulting peptide sequence ALFQDVQKF was used for a
homology search in the Swiss protein sequence data bank. The search revealed sequence identity with amino acids 76–84 in
the rat ferritin light chain (Swiss-Prot AC: P02793).

Gel retardation assays were performed with commercial fer-
titin (Sigma; 99% pure) and PCK1 transcript and compared with
gel retardation assays with the protein purified here from
rat liver. Both protein preparations formed a protein-RNA com-
plex with PCK1 transcript, which had the same electrophoretic
mobility. Complex formation with the commercial ferritin re-
quired the same divalent cation, EDTA, and pH conditions as
the purified ferritin from rat liver (data not shown). The puri-
ified protein was used to generate a polyclonal antibody. In
Western blots this antibody detected both the commercial ferr-
titin and the ferritin purified in this study.

The antibody was incubated overnight at 4 °C with the CE or
the purified protein preparation (Fig. 4A, lanes 1–4). In gel
retardation assays with the preincubated protein preparations
and with PCK1 transcript, complex 1 did not form, indicating
that the ferritin antibody prevented protein binding to PCK1
transcript. To show that this was not due to an interaction of
the antibody with PCK1 transcript, which could unspecifically
prohibit complex formation, ferritin was immunoprecipitated
with Sepharose CL-4B protein A-coupled antibody from the
CE. Depletion of ferritin from CE by immunoprecipitation with
protein A abolished complex formation with the PCK1 tran-
script (Fig. 4B, lanes 1 and 2) demonstrating an interaction of
ferritin with PCK1 transcript.

An Additional Factor Besides Ferritin Was Necessary for
Efficient RNA-Protein Complex Formation—Ferritin formed a
RNA-protein complex with PCK1 transcript. This complex was
stronger with binding activity containing fractions from the anion
exchange chromatography compared with the purified
Superdex 200-μg fraction, indicating the loss of a putative
factor, which promoted complex formation between ferritin and
the PCK1 transcript (Fig. 3). 15 μg of protein of cytosolic
protein extract were subjected to the gel retardation assay with
PCK1 transcript and increasing amounts of Superdex extract
fractions 36–38 (Fig. 5A). The combination of 15 μg of protein
CE with 4.8 μg of protein Superdex extract stimulated complex
formation 10-fold compared with Superdex extract alone. This
implies that the CE contained a component, which was lost
during gel filtration. Therefore the gel retardation assay using
PCK1 transcript and 1 μg of protein Superdex extract was
completed with fractions from the Superdex chromatography
following the Superdex extract fraction 37 (Fig. 5B). Addition of
an aliquot derived from the seventh fraction after the ferritin-
containing fraction (i.e. fraction 44 of total fractions) increased
complex formation 3-fold. This fraction corresponded to a mol-
ecular mass range of about 180,000 Da. It obviously contained
a factor capable of stimulating complex formation between
ferritin and PCK1 transcript. In the absence of ferritin, protein
from this fraction alone did not form any complex with PCK1
transcript. The nature of the factor remains at present un-
known because it had not been further purified and character-
ized. It is, however, clear that the factor does not act on ferritin
binding to RNA by increasing the amount of binding protein
but by enhancing its affinity or its activity. This can be con-
cluded because protein binding of the combined 4.8 μg of Su-
perdex extract and of 15 μg of CE was not additive to protein
binding of each of the fractions alone (Fig. 5A). Moreover, in the
experiment described in Fig. 5B the amount of protein in Super-
dex extract was kept constant (1 μg) and only the addition of
fraction 44 increased protein binding.

In Vitro Phosphorylation/Dephosphorylation Regulated
Complex Formation between Ferritin and RNA—If ferritin
should play a role in the regulation of mRNA turnover, it is
reasonable to assume that its binding to RNA should be regu-
related by phosphorylation/dephosphorylation. To confirm this assumption, the purified ferritin was treated \textit{in vitro} in the presence of ATP with the catalytic subunit of CaMP-dependent protein kinase prior to the binding reaction. This treatment abolished protein binding to PCK1 transcript (Fig. 6A, \textit{lunes 1 versus 2}). Treatment with alkaline phosphatase prior to the binding reaction enhanced binding of the purified ferritin to PCK1 transcript (Fig. 6A, \textit{lunes 3 versus 4}). The loss of RNA binding activity of ferritin after treatment with the catalytic subunit of CaMP-dependent protein kinase could be restored by subsequent treatment with alkaline phosphatase (not shown). The data show that the RNA binding activity of ferritin purified from rat liver was regulated by phosphorylation/dephosphorylation.

To specify which amino acid residues were phosphorylated, the purified ferritin was incubated overnight with specific antibodies against phosphoserine, phosphothreonine, and phosphotyrosine or with an unrelated antibody. After this incubation the gel retardation assay was performed with the PCK1 transcript. Incubation with the anti-phosphoserine antibody generated a "supershift" (Fig. 6B) indicating that the ferritin contained phosphorylated tyrosine residues. Binding of the antibody to phosphotyrosines did not interfere with protein binding to RNA. In contrast, preincubation with the anti-phosphoserine or anti-phosphothreonine antibodies decreased complex formation (Fig. 6B) indicating that binding of the antibodies to phosphorylated serine and threonine residues in the protein interfered with the binding to RNA. Apparently, binding of the antibody to phosphoserine or phosphothreonine masked the RNA-binding region. Unrelated antibodies did not affect protein binding (not shown).

\textbf{Inhibition of Protein Phosphatases Decreased Binding of Ferritin to RNA in Vivo—}In cultured rat hepatocytes glucagon increased PCK mRNA levels transiently to a maximum after 2 h. When insulin was given at the maximal increase at 2 h it accelerated PCK mRNA degradation (13). The serum/threonine phosphatase inhibitor okadaic acid given at the maximal increase at 2 h inhibited the degradation of PCK mRNA in the absence and presence of insulin, indicating the involvement of dephosphorylation events in the control of PCK mRNA degradation. Therefore, to demonstrate that phosphorylation and dephosphorylation may also play a role in the regulation of RNA binding activity of ferritin \textit{in vivo}, extracts were prepared from 48-h cultured rat hepatocytes treated for 2 h with glucagon and thereafter in addition for another 3 h with insulin in the absence and presence of okadaic acid. Ferritin in cytosolic extracts from okadaic acid-treated cells, irrespective whether treated simultaneously with insulin or not, possessed lower binding activity to the PCK1 transcript than protein from non-okadaic acid-treated cells (Fig. 7). This implies also that \textit{in vivo} phosphorylation of the protein decreased and dephosphorylation increased binding to the PCK mRNA. Ongoing gene transcription did not play a role in the regulation of protein binding to PCK1 transcript, because addition of the transcriptional inhibitor actinomycin D to the hepatocytes did not affect formation of complex 1. The half-life time of ferritin in the liver was determined at 50–70 h (27). Therefore, it is unlikely that under the chosen experimental conditions, i.e. preparation of extracts 3 h after application of insulin, okadaic acid, or actinomycin D, changes in binding activity were due to changes in ferritin content of the hepatocytes. It can be assumed that the decrease in protein binding to PCK1 transcript was rather due to the decrease in the affinity or activity of ferritin binding to RNA.

\textbf{Iron Decreased Cytosolic Protein Binding to RNA in Vitro—}Ferritin is the iron storage protein in the liver. This might indicate that complex formation with the purified protein and RNA can be regulated by iron. Therefore the gel retardation assay with the PCK1 transcript was performed with purified rat liver ferritin (Superdex column fractions 36–38) after preincubation for 30 min at 37 °C with the catalytic subunit of cAMP-dependent protein kinase A under the chosen experimental conditions, i.e. preparation of extracts 3 h after application of insulin, okadaic acid, or actinomycin D, changes in binding activity were due to changes in ferritin content of the hepatocytes. It can be assumed that the decrease in protein binding to PCK1 transcript was rather due to the decrease in the affinity or activity of ferritin binding to RNA.

\textbf{DISCUSSION}

\textit{Ferritin as RNA-binding Protein—}The expression of PCK is in part regulated at the level of mRNA degradation (13, 18, 19). To find regulatory proteins which might be involved in this process, the 3′-UTR of PCK mRNA was used as target for the selection of RNA-binding proteins from rat liver. By this procedure a RNA-binding protein was purified and identified as rat ferritin light chain. The RNA-protein complex formed not only with transcripts containing PCK mRNA 3′-UTR sequences but with a variety of different mRNA 3′-UTR species (Fig. 2). Computer alignment with these different RNAs revealed no sequence homologies, which could be the common target sequence for protein binding. This indicates that complex, so far unidentified, RNA structures might be necessary for RNA-protein complex formation. Also searching the SWISS protein data bank for consensus peptide motifs in ferritin, which could be putative contact sites for RNA, revealed no similarities with known RNA-binding peptide motifs, e.g. with the RNP motifs in heterogeneous nuclear ribonucleoproteins (28). However, it is clear that ferritin functions as a RNA-
binding protein with the potency to bind to a broad range of different RNA species.

RNA binding properties of ferritin and ferritin-homologs have already been described. A prosome-like particle, which was believed to play a role in protein degradation, was isolated and turned out to be composed of identical apoferitin subunits. This particle was associated with small RNA molecules, probably various tRNA species (24). These RNAs were capable of hybridizing with globin mRNA (24, 29). From Artemia cysts the protein artemin was purified, which shared homology with vertebrate ferritins and probably functions as iron storage protein (30). In addition, artemin belongs to a class of RNA-binding proteins and was proposed to maintain the integrity of the dehydrated cyst (31, 32).

Ferritin is the iron storage protein in liver, which protects cells against cytotoxic effects of reactive oxygen species derived from iron-catalyzed free radical reactions (for review, Ref. 33). Three different types of ferritin were described (apoferritins), the L (light) and two variants of H (heavy) chains, of which the L type is prevalent in rat liver. The apoferritins form a holoferritin complex, which consists of 24 subunits forming a very stable shell for the storage of about 4500 iron ions as Fe3+[OH] inside the complex (34).

Early experiments using transcriptional inhibitors showed that after an iron challenge the increase in cellular ferritin concentrations were not due to an increase in gene transcription but rather to the increase in the translation of ferritin mRNA (23). Under normal, non-iron-depleted conditions ferritin concentrations remain fairly constant because of the stabil-
(27). The half-saturation concentration determined may be in
the range of “free” ferritin. Therefore, it is feasible that binding
of ferritin to RNA is of physiological relevance.

Some evidence exists that ferritin via its RNA-binding ca-
pacity might play a role in the general metabolism of RNA. For
example, addition of ferritin-containing prosome-like particles
to an in vitro translation system inhibited the translation of
globin mRNA, indicating that ferritin functioned as a transla-
tional repressor (24, 29). This had also been suggested in ear-
lier reports, which claimed that ferritin bound to the 5’-UTR of
its own mRNA and stored the mRNA in a translational incom-
petent form (42). Due to its similar amino acid composition and
immunological cross-reactivity the RNA-binding protein from
Artemia cysts had been suggested to be related to the elonga-
tion factor eEF-Ts, which, however, remains to be experimen-
tally established (31). These data suggest that ferritin, besides
its function as iron storage protein, may not have a unique but
rather a general function in the regulation of RNA metabolism
at various levels (23, 24).

The present study had the goal to identify protein(s), which
might be involved in the regulation of PCK mRNA degra-
dation. Ferritin was isolated and its RNA binding characteris-
tics determined (Figs. 1–4). Because no specific binding to PCK
mRNA but rather a general binding to mRNAs was detected, it
seems likely that ferritin is not only involved in PCK mRNA
metabolism but in general processes, which, however, at pres-
cent cannot be defined. It might be relevant that in the present
study RNA-protein complex formation was inhibited by iron
(Fig. 8). This might link the iron loading status of the cell to the
regulation of general RNA degradation.

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