Identification and cDNA Cloning of a Novel RNA-binding Protein That Interacts with the Cyclic Nucleotide-responsive Sequence in the Type-1 Plasminogen Activator Inhibitor mRNA*

Incubation of HTc rat hepatoma cells with 8-bromo-cAMP results in a 3-fold increase in the rate of degradation of type-1 plasminogen activator inhibitor (PAI-1) mRNA. We have reported previously that the 3’-most 134 nt of the PAI-1 mRNA is able to confer cyclic nucleotide regulation of message stability onto a heterologous transcript. R-EMSA and UV cross-linking experiments have shown that this 134 nt cyclic nucleotide-responsive sequence (CRS) binds HTc cell cytoplasmic proteins ranging in size from 38 to 76 kDa. Mutations in the A-rich region of the CRS both eliminate cyclic nucleotide regulation of mRNA decay and abolish RN-protein complex formation, suggesting that these RNA-binding proteins may be important regulators of mRNA stability. By sequential R-EMSA and SDS-PAGE we have purified a protein from HTc cell polysomes that binds to the PAI-1 CRS. N-terminal sequence analysis and a search of protein data bases revealed identity with two human sequences of unknown function. We have expressed one of these sequences in E. coli and confirmed that the recombinant protein interacts specifically with the PAI-1 CRS. Mutation of the A-rich portion of the PAI-1 CRS reduces binding by the recombinant PAI-1 RNA-binding protein. The amino acid sequence of this protein includes an RGG box and two arginine-rich regions, but does not include other recognizable RNA binding motifs. Detailed analyses of nucleic acid and protein data bases demonstrate that blocks of this sequence are highly conserved in a number of metazoans, including Arabidopsis, Drosophila, birds, and mammals. Thus, we have described a novel RNA-binding protein that identifies a family of proteins with a previously undefined sequence motif. Our results suggest that this protein, PAI-RBPI, may play a role in regulation of mRNA stability.

Regulation of mRNA stability is an important component of the regulation of gene expression and is known to have a significant role in normal physiology and development (1–5). Our understanding of the regulation of message degradation has been enhanced by the identification of consensus cis-acting sequences that are involved in determining message stability and of some proteins that interact with them (4, 6). Although it is known that many stimuli alter mRNA stability and some cis-acting sequences responsible have been identified, in few cases have trans-acting factors been isolated (2, 7–10). In contrast, a broad spectrum of RNA-binding proteins that are involved in RNA processing, cellular localization, and translation have been identified, and structural domains involved in RNA recognition have been described (11, 12). In many cases RNA-binding proteins contain short signature domains that bind RNA and anchor the protein such that functional domains align (13, 14). Much less is known about the mechanisms by which RNA-binding proteins regulate mRNA stability. Plasminogen activators (PAs) are serine proteases that catalyze the conversion of plasminogen to the broad spectrum protease, plasmin. Plasmin is the major fibrinolytic enzyme in blood and also participates in a number of physiological and pathological processes involving localized proteolysis such as tissue remodeling and tumor cell invasion and metastasis (15, 16). PA activity is regulated in large part by type-1 plasminogen activator inhibitor (PAI-1), a 50-kDa glycoprotein found in plasma, platelets, and a variety of cell types (17). PAI-1 expression is regulated by growth factors, cytokines, and hormones, including agents that regulate cellular cAMP levels (18–20).

HTC rat hepatoma cells synthesize tissue-type plasminogen activator (tPA) and PAI-1. These cells respond to cyclic nucleotides with a dramatic (50-fold) increase in tPA activity secondary to a 90% decrease in PAI-1 activity and mRNA. The decrease in PAI-1 mRNA is due primarily to a 3-fold increase in the rate of mRNA degradation (21). By transfecting HTc cells with chimeric constructs containing the β-globin coding sequence and portions of the PAI-1 5’-UTR, we have shown that sequences in the PAI-1 3’-UTR are able to confer cyclic nucleotide regulation onto the heterologous transcript. Analysis of deletion and insertion constructs demonstrated that the 3’-most 134 nt of the PAI-1 mRNA by itself is able to mediate this response. This cyclic nucleotide-responsive sequence (CRS) includes a 75-nt U-rich region at its 5’ end and a 24-nt A-rich

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The abbreviations used are: PA, plasminogen activator; tPA, tissue-type plasminogen activator; PAI-1, type-1 plasminogen activator inhibitor; UTR, untranslated region; CRS, cyclic nucleotide-responsive sequence; RBP, RNA-binding protein; PCR, polymerase chain reaction; nt, nucleotide(s); bp, base pair(s); R-EMSA, RNA electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis, IPTG, isopropyl-β-D-galactopyranoside; RRM, RNA recognition motif; KH, K-homology; CAT, chloramphenicol acetyltransferase; EST, expressed sequence tag.
Identification and Cloning of a PAI-1 mRNA-binding Protein—Here we report the isolation and cloning of one of these proteins and demonstrate its specific interaction with PAI-1 mRNA. Detailed analysis of nucleic acid and protein data bases demonstrates that this PAI-1 mRNA-binding protein includes blocks of sequence that are highly conserved in a number of metazoans. Thus, we have identified and cloned a novel RNA-binding protein that reveals a family of proteins with a previously unidentified domain that may define a new RNA-binding motif. Our results suggest that this protein may play a role in regulation of mRNA stability.

EXPERIMENTAL PROCEDURES

Materials—Eagle’s minimal essential medium, calf serum, fetal calf serum, restriction enzymes, RNase T2, and Benchmark® protein markers were purchased from Life Technologies, Inc. Benzamidine, transepoxysuccinyl-l-leucylamido-4-guanidino-butyrate (E64), heparin (sodium salt), RNase T1, and RNA were purchased from Sigma. RNase A, leupeptin, 4-(2-aminoethyl)benzoylguaniferyl fluoride-hydrochloride (F Fabfoll® SC AEBSF), proteinase K, RNase inhibitor, T3 and T7 RNA polymerases, and the high fidelity PCR system were obtained from Roche Molecular Biochemicals. Coomassie® Plus Protein Assay was prepared by published methods (24) using as template either plasmid pET-15b-Vector, BL21(DE3)pLYSs competent Escherichia coli, IPTG, and His-binding resin were purchased from Novagen. SDS-PAGE prestained protein markers were from Bio-Rad.

Cell Culture and Polysome Preparation—Monolayer cultures of HTC cells were maintained in Eagle’s medium with 5% fetal calf serum and 5% bovine serum as described previously (21). Cells were grown to confluence in T-150 flasks and harvested by trypsinization. HTC cell cultures were maintained in Eagle’s medium with 5% fetal calf serum and 5% newborn calf serum as described previously (21).

Preparation of Radiolabeled and Unlabeled RNA—All DNA constructs used as templates to prepare radiolabeled or unlabeled RNA have been described previously (23). 32P-Labeled sense strand RNA was prepared by transcription methods (24). The template either contained DNA linearized with the appropriate restriction enzyme or PCR products, including a T3 RNA polymerase site. The DNA template was incubated for 60 min at 37 °C with T3 or T7 RNA polymerase and the high fidelity PCR system were obtained from Roche Molecular Biochemicals. Coomassie® Plus Protein Assay was prepared by published methods (24) using as template either plasmid pET-15b-Vector, BL21(DE3)pLYSs competent Escherichia coli, IPTG, and His-binding resin were purchased from Novagen. SDS-PAGE prestained protein markers were from Bio-Rad.

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RESULTS

Affinity Purification and Identification of PAI-1 mRNA-binding Protein—To isolate proteins that interact with the rat PAI-1 CRS, we have used an RNA affinity approach, using the 134-nt sequence of the rat PAI-1 CRS shown in Fig. 1. A. HTC cell polyosomal polysomal proteins were incubated with 32P-labeled or unlabeled CRS and separated by non-denaturing polyacrylamide gel electrophoresis. The high molecular weight complex (Fig. 1b, brackets) was excised from the gel, and the proteins were eluted from the gel slices. Proteins from 32 such reactions, representing ~2 mg of starting polysomal protein, were pooled, concentrated by acetone precipitation, and separated by SDS-PAGE. Fig. 1c shows that three protein bands are visible in the Coomassie Blue-stain gel. In parallel experiments, proteins from the SDS-PAGE gel were transferred to polyvinyldifluoride membrane (ProBlott, PE Applied Biosystems, Foster City, CA), and stained with Coomassie Blue. N-terminal amino acid sequencing of the major band (Fig. 1c, arrow) yielded 19 amino acids of N-terminal sequence.

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Redundant protein data base and two entries with 18 identical N-terminal amino acids were found: hypothetical protein (GenBank\textsuperscript{TM} accession number AL080119) and “CGI-55” (GenBank\textsuperscript{TM} accession number AF151819). Both sequences represent human proteins with an as yet unknown function and appear to be splice variants; CGI-55 has a 6-amino acid insertion after position 202 in the hypothetical protein (Fig. 1D). The CGI-55 sequence potentially codes for a 43.2-kDa protein, but is a composite of several ESTs (32) and is not yet cloned as a single sequence. The cDNA for hypothetical protein was cloned by the German Cancer Research Center (Deutsches Krebsforschungszentrum) and was kindly provided to us by the Resource Center and Primary Data base of the German Genome Project (Resource Center and Primary Database, Berlin). While the amino acid sequence does not indicate an RNA recognition motif (RRM) or a K-homology (KH) domain, the sequence does include an RGG box at amino acid positions 343–359, an RG-rich region at amino acid 163–184, and an Arg-rich region at amino acid 126–137, motifs frequently found in RNA-binding proteins (11) (Fig. 1D). In addition, there is a potential protein kinase A phosphorylation site at serine 74, indicating that the protein function could be regulated by cyclic nucleotides.

Expression of PAI-1 RNA-binding Protein in E. coli—The coding region of the cDNA for hypothetical protein was amplified by PCR and subcloned into the pET-15b vector downstream from the sequence coding for a histidine tag and a thrombin cleavage site, and the plasmid was expanded in E. coli AG-1. The subclone was verified by PCR and DNA sequencing and transformed into E. coli BL21(DE3)pLysS. Expression was induced by 2 mM IPTG and the protein partially purified by Ni\textsuperscript{2+} chelation as described under “Experimental Procedures.” Proteins in the column eluate were separated by SDS-PAGE, and as shown in Fig. 2 (lane 3), the major product appears as a triplet. To determine which protein band represents hypothetical protein, we carried out a thrombin digestion, which is expected to cleave at the thrombin site and remove the N-terminal His-tag and associated amino acids. Thrombin digestion resulted in approximately a 2000-dalton decrease in the size of each band in the triplet (Fig. 2, lane 4), demonstrating that all three bands represent the hypothetical protein. The different sizes most likely are the result of premature translation termination due to differences in codon usage frequency between bacteria and eukaryotes (24, 33). Western analysis with anti-His antibody confirmed that all three bands carry the His-tag. NorthWestern analysis demonstrated that a protein that migrates at the same location binds \textsuperscript{32}P-labeled PAI-1CRS (data not shown).

Binding Activity of Recombinant Hypothetical Protein—To confirm that the recombinant human hypothetical protein binds to the rat PAI-1 CRS, we have carried out both R-EMSA and UV cross-linking experiments. Purified recombinant protein was incubated with \textsuperscript{32}P-labeled PAI-1 CRS and R-EMSA carried out as described under “Experimental Procedures.” Fig. 3A illustrates that the recombinant protein binds in a concentration-dependent fashion (lanes 1–5). Binding is competed by increasing amounts of identical unlabeled sequence (lanes 6–8) but not by a 100-fold molar excess of the bacterial CAT RNA sequence (lane 11). The U-rich region of the PAI-1 CRS (nt 2926/3024), which by itself does not form the major R-EMSA complex with HTC cell cytoplasmic proteins (23), competes only...
weakly for CRS interaction with the recombinant protein (lane 9). More significantly, a portion of the rat PAI-1 mRNA (nt 2125/2296) that does not confer cyclic nucleotide regulation of message stability (22) also fails to compete for binding (lane 10).

Similar results were obtained by UV cross-linking experiments. As shown in Fig. 3B, the RNA-protein complex migrates on SDS-PAGE as a triplet, forms only a single UV cross-linked complex with PAI-1 CRS, suggesting that the smaller, C-terminal truncated forms are not able to bind.

Delineation of Sequences Involved in Binding to Recombinant Human PAI-1 RNA-binding Protein—To further define the sequence within the PAI-1 CRS required for binding to the PAI-RBP1, we carried out binding experiments with portions of the CRS. Fig. 4A shows diagrammatically the regions of the CRS used to generate radiolabeled probes. In R-EMSA the 3′ portion of the CRS (nt 2926–3034, lane 9), a portion of the PAI-1 3′-UTR that does not confer cyclic nucleotide responsiveness (nt 2125–2296, lane 10), and bacterial CAT RNA (lane 11) were each added at 100-fold molar excess.

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Protein Sequence Alignment and Identification of a Family of Proteins—The initial search of the protein data base using the 19-amino acid sequence produced only the two sequences with the identical N-terminal amino acids. The data base has grown.
considerably since, and we have now done an extensive search, as described under “Experimental Procedures,” for similarity to the entire hypothetical protein and DKFZp564M2423 cDNA sequence. This search has revealed several other proteins with a high degree of similarity, particularly in the C-terminal portion. These similar proteins are found in nine different species, including Arabidopsis, Drosophila, chicken, mouse, and rat (Fig. 5). The availability of new members of the family permitted us to generate a multiple alignment and to identify their common motifs; the alignment revealed that these proteins share several blocks of conserved sequence (Fig. 5). This domain, which we propose has a function in RNA binding, is located at the C-terminal of all the proteins in this family and, apart from a relatively short stretch of RGG repeats, appears to be a compact entity. The C-terminal RGG box is located between the last two sequence blocks and the RG-rich and Arg-rich regions are N-terminal to the conserved blocks. A number of the other proteins in the family have the RGG box. Thus, PAI-RBP1/hypothetical protein is a member of a family of proteins that share a putative novel RNA binding motif.

**DISCUSSION**

We report here the identification of a novel rat cytoplasmic protein isolated based on its ability to form R-EMSA complexes with the rat PAI-1 mRNA cyclic nucleotide-responsive sequence. N-terminal sequence data reveal identity with a human sequence, hypothetical protein (GenBank™ accession number AL080119) of unknown function. We have expressed the human sequence in bacteria and analyzed its binding properties. Our results demonstrate that the human protein is a PAI-1 mRNA-binding protein; we now refer to this gene product as PAI-1 RNA-binding protein or PAI-RBP1.

Recombinant human PAI-RBP1 binds the A-rich portion of the rat PAI-1 CRS and fails to interact with the isolated U-rich portion. Mutations of the A-rich sequence severely decrease binding. In addition, the specificity of PAI-RBP1 binding is demonstrated by its failure to form a complex with either the bacterial CAT RNA or an upstream, noncyclic nucleotide-responsive region of the PAI sequence. We have reported previously that the PAI-CRS forms complexes with HTC cell cytoplasmic proteins ranging in size from 38 to 76 kDa (23). The binding specificity and the size of the complex seen in Fig. 3 indicate that PAI-RBP1 is similar to the 50–53-kDa HTC cell cytoplasmic protein and may, in fact, be the human homologue of that rat protein. Because the PAI-1 mRNA binding site is a cyclic nucleotide-responsive sequence, it would be reasonable to expect that binding activity of PAI-RBP1 may be influenced by its phosphorylation state. The recombinant protein was expressed in bacteria and, therefore, not posttranslationally processed as it might be in mammalian cells, possibly explaining the high concentration of protein required for binding.

As seen in Fig. 2, the partially purified protein migrates on SDS-PAGE as a triplet. The size of the smaller products is

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**Fig. 5. Protein sequence alignment.** An iterative search of the nonredundant protein data base was carried out with PSI-BLAST, and the multiple sequence alignment was produced using Gibbs sampling function of PROBE (28). Some family members were added from an alignment generated by HMMER (29). The figure was made using ALSCRIPT (46). Numbers on the left are GenBank™ identifier codes for proteins. The first and last amino acid residues of the aligned region are numbered preceding the first block and following the last block, respectively. Numbers in parentheses indicate the size of gaps between blocks. Common protein names from their data base annotations are shown at the end of the alignment, followed by species names. The sequence from Rattus Norvegicus (clone C426 intestinal epithelium) has question marks in place of amino acids numbers because the sequence is incomplete. Amino acids are colored differently from the background when at least 90% of the residues conform to a consensus. The following color scheme was used: hydrophobic residues (ACFGHKLMRTVWY) are dark blue; aliphatic residues (ILV) are green; polar residues (CDEHKNQRST) are red; small residues (ACDGNPSTV) are purple; charged residues (DEHKR) are orange. Individual residues with more than 90% identity across the whole alignment are highlighted in yellow.
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consistent with a C-terminal truncation at the beginning of or within the RGG box. One of the arginines and four of the glycines in this region are coded by rare codons in bacteria and could cause premature termination (24, 33). Only a single RNA-protein complex is detected in UV cross-linking experiments (Fig. 3A), strongly suggesting that the most C-terminal portion is required for RNA recognition.

A number of proteins that interact with RNA transcripts have been identified and structural domains involved in RNA recognition described (11, 12). While the majority of known RNA-binding proteins function in RNA processing or translation, several proteins that interact with mRNAs influence on mRNA stability or processes requiring interaction with mRNA (2, 42). Vigilin is a KH-domain protein that is induced in a wide range of tissues, including pancreas, liver, lung, muscle, ovary, and brain. These findings strongly suggest that PAI-RBP1 has two RRMs, as well as a C-terminal RGG that appears to be important for binding (38–40). c-CP-1 and c-CP-2 are KH domain (41) poly(rC)-binding proteins and play an important role in regulation of α-globin and tyrosine hydroxylase mRNA stability (2, 42). Vigilin is a KH-domain protein that is induced in Xenopus oocytes by estrogen and binds to and stabilizes vitellogenin mRNA (7).

PAI-RBP1 does not have a recognizable RRM or KH domain. It does, however, have an RGG box at amino acid positions 343–359 (Fig. 1D), as well as an RG-rich (amino acids 126–137) and an Arg-rich (amino acids 163–184) motif, which places it in the general category with RNA-binding proteins (11, 12). It is of particular interest that this protein, which may be involved in a cyclic nucleotide-mediated regulation event, has a potential RNA binding properties and/or the function of the RNA-protein complex. Experiments are in progress to construct each of these variants and to analyze their RNA binding properties.

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