PIR1, a Novel Phosphatase That Exhibits High Affinity to RNA-Ribonucleoprotein Complexes*

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Protein tyrosine phosphatases are involved in the regulation of important cellular processes such as signal transduction, cell cycle progression, and tumor suppression. Here we report the cloning and characterization of PIR1, a novel member in the dual-specificity phosphatase subfamily of the protein tyrosine phosphatases. PIR1 also contains two stretches of arginine-rich sequences. We have shown that the recombinant PIR1 protein possessed an intrinsic phosphatase activity on phosphoryrosine-containing substrate. A unique feature of this phosphatase is that it binds directly to RNA in vitro with high affinity. In addition, we have found that PIR1 interacted with splicing factors 9G8 and SRp30C, possibly through an RNA intermediate during a yeast two-hybrid screen. PIR1 exhibited a nuclear-staining pattern that was sensitive to RNase A, but not to DNase I, suggesting that PIR1 in the cells are associated with RNA and/or ribonucleoprotein particles. Furthermore, a fraction of PIR1 showed a speckle-staining pattern that superimposed with that of the splicing factor, SC35. Taken together, our data suggest that PIR1 is a novel phosphatase that may participate in nuclear mRNA metabolism.

Protein tyrosine phosphatases, in conjunction with protein tyrosine kinases, regulate the levels of protein tyrosine phosphorylation important for cell growth, differentiation, or transformation (1, 2). Protein tyrosine phosphatases (PTP) can be grouped as classic PTPs (including receptor-like PTPs and cytoplasmic PTPs), dual-specificity phosphatases, and low molecular weight (acid) phosphatases (2). Both classic PTPs and dual-specificity phosphatases contain a conserved signature motif (HCXXGXXRXG), which constitutes the active site in the phosphatase catalytic domain (3). The conserved cysteinyl residue in this motif is required for the formation of a thiol-phosphate intermediate during the phosphate transfer reaction (3).

Dual-specificity phosphatases, a subfamily of protein tyrosine phosphatases, play important roles in signal transduction, cell cycle regulation, and tumor suppression. Although dual-specificity phosphatases contain little primary sequence homology to classic PTPs, they share a similar structural folding, especially at the catalytic site, with classic PTPs (3). Some members of this subfamily of enzymes have been shown to be able to dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine. One well known member of the dual-specificity phosphatases is MKP-1/CL100, a highly selective phosphatase that dephosphorylates and inactivates mitogen-activated protein kinases (4, 5). Another example is Cdc25 (6), which dephosphorylates the inhibitory phosphotyrosine and phosphothreonine residues in Cdc2, a cyclin-dependent kinase required for G2 to M phase transition during cell cycle progression. More recently, PTEN/MMAC1/TPIF1, a novel phosphatase that is encoded by a tumor suppressor locus on chromosome 10q23 and its mRNA level is regulated by transforming growth factor β (7–10), has been added to this subfamily of protein tyrosine phosphatases.

Here we report the cloning and characterization of a novel phosphatase that structurally belongs to the dual-specificity phosphatase subfamily. Interestingly, this novel enzyme can bind to RNA in vitro, and associates with RNA and/or ribonucleoprotein (RNP) complexes in vitro. We have therefore named this novel enzyme PIR1, as phosphatase that interacts with RNA/RNP complex 1.

MATERIALS AND METHODS
cDNA Cloning—The ML1 zAPII cDNA library (9) was screened using the EST clone H60626 as a probe. Twenty positive clones were identified and excised with ExAssist helper phage (Stratagene). By restriction mapping, clone 14 was shown to contain the longest insert. This clone was subjected to DNA sequencing on both strands. The region containing the putative open reading frame was amplified by the polymerase chain reaction (PCR) with a BamHI site added at the ends of both primers. The 1-kilobase BamHI fragment was subcloned into pCGT (11), pGKT9 (CLONTECH), and pAcG1 (PharMingen) and sequenced again. Site-directed mutagenesis was carried out to change cysteine 152 to serine (C → S) using the QuickChange method (Stratagene). The presence of the mutation was confirmed by DNA sequencing. T epitope-tagged PIR1 or PIR1(CS) was amplified from pCGT-PIR1 or pCGT-PIR1(CS) template by PCR with Bgl II linker added at the ends of the primers and subcloned into pVL1393 (PharMingen).

Cell Lines, Northern Blot, and Western Blot Analyses—Cells culture and Northern blot analysis were performed as described previously (9). For Western blot analysis, cell lysates were resolved on 10% SDS-PAGE, and proteins were blotted onto nitrocellulose membrane. Anti-T epitope antibody (Novagen) and 9E10 (Oncogene) were applied in TTBS (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) with 5% nonfat milk. Blots were then washed extensively with TTBS and then incubated with appropriate secondary antibody conjugated with horseradish peroxidase. The immunoreactive proteins were detected with enhanced chemiluminescence (ECL, NEN Life Science Products).

Recombinant Protein Expression—Recombinant baculoviruses were obtained by cotransfecting plasmid pVL1393-T-PIR1 (WT or CS), pAcG1-PIR1 (WT or CS) or pAcG1-MKP1-myc with linear baculovirus DNA (PharMingen) into Sf9 cells according to the standard protocol of PharMingen. Sf9 cells were infected with the recombinant baculoviruses. 72 h post-infection, cells were harvested, resuspended in Buffer A (20 mM Tris, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 5 mM dithiothreitol, 1.0 mM EGTA/1 mM EDTA, pH 7.4), and then disrupted by sonication.

This paper is available online at http://www.jbc.org

* This work was supported by the Donaghue Medical Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF023917.

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Received for publication, October 23, 1997, and in revised form, May 20, 1998.
10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine) and lysed by Dounce homogenization. Lysates were centrifuged at 13,000 rpm for 20 min to remove the insoluble cell debris. The supernatant was then supplemented with either 100 μM KCl (for single-stranded DNA binding assay) or 140 mM KCl (for phosphatase activity assay) and 0.1% Tween 20.

**Protein Tyrosine Phosphatase Activity Assay**—The clarified S9 cell lysates containing glutathione S-transferase(GST)-PIR1 or GST-PIR1(CS) fusion protein (1 ml) were incubated with 100 μl of glutathione-Sepharose beads (Amersham Pharmacia Biotech). After 2 h incubation at 4 °C, beads were collected and washed extensively with buffer A (in mM): 0.5, 1 mM NaCl elution was released by buffer B containing 400 units/ml DNase I, 20 mM vanadyl-ribonucleoside complex and 50 mM NaCl (control). The cells were then extracted with 250 mM (NH4)2SO4 in CSK buffer at room temperature for 10 min and then fixed with 3.7% formaldehyde in CSK buffer. Cells were stained with monoclonal antibody CS35 (ATCC), followed by rhodamine-conjugated donkey antibody to mouse Ig. The coverslips were then incubated with biotinylated anti- mouse IgG antibody (Novagen) followed by fluorescein-conjugated streptavidin (Jackson ImmunoResearch Laboratories). Cells were examined with a Bio-Rad confocal microscope MRC-600 and the imaging data was processed with computer program Adobe Photoshop 4.0.

**RESULTS**

**Cloning of a Novel Member of Dual-specificity Phosphatases**—To identify novel dual-specificity phosphatases, we have used primers corresponding to the conserved sequences HTH-GIN and FEQARGH in the catalytic domain of several dual-specificity phosphatases to amplify gene sequences from human cDNA libraries by polymerase chain reaction. In addition, we have used these sequences to directly search the GenBank™ EST data base. These combined approaches have led us to identify a partial cDNA (EST clone H60626) that potentially encoded a novel phosphatase. To obtain its full-length DNA, we used the EST clone H60626 as a probe to screen a human cDNA library constructed from ML1 cells (a human myeloid cell line). The full-length cDNA sequence consists of 1593 nucleotides that potentially encode a protein of 329 amino acids (Fig. 1A). We have named this novel phosphatase PIR1 (phosphatase that interacts with RNA/RNP complex 1).

Sequence analysis of PIR1 shows that it is closely related to several dual-specificity phosphatases at the amino acid level (Fig. 1B), such as BVP, encoded by the baculovirus Autographa californica (35% identity) (14); two open reading frames identified by the Caenorhabditis elegans genome project T23G7.5 (31% identity) and F54C8.4 (23% identity); and CEL-1 (25% identity). CEL-1 is recently shown to be a phosphatase in C. elegans that can remove 5′-phosphate from newly transcribed RNA molecules and is potentially involved in the mRNA capping reaction (15).

In addition to harboring the protein tyrosine phosphatase signature motif, PIR1 has several interesting features. It contains two stretches of arginine-rich sequences that are found in some of the RNA-binding proteins (Fig. 1A). PIR1 is also rich in lysine and histidine residues, and the protein is predicted to contain 17 basic charges at the neutral pH. PIR1 also carries a proline-rich region, a motif known to interact with the Src homology 3 domain.

We have examined the expression pattern of PIR1 in various human cell lines by Northern blot analysis (Fig. 2). PIR1 was widely expressed with the most abundant messages in HaCaT (human keratinocyte cell line), A431 (human epidermoid cells), and ML1 (human myeloblastic). Interestingly, in several transformed cell lines that lack functional p53 and/or pRb, such as 293 (human embryonic kidney cell line), HeLa (human cervical epithelial carcinoma), and Saos2 cells (human osteosarcoma), the PIR1 level was extremely low. Whether the PIR1 mRNA level may be regulated by p53 or pRb tumor suppressor protein needs to be further studied. No expression of the PIR1 message was observed in PC12 (rat pheochromocytoma) cells, although we cannot rule out the possibility that human PIR1 cDNA probe had failed to recognize its rat homolog.
**A**

![Sequence of PIR1 cDNA. A, nucleotide sequence of PIR1 cDNA and the deduced amino acid sequence. Nucleotides and amino acids are numbered at the ends of each line. The tyrosine phosphatase signature motif is double-underlined, the arginine-rich sequences are underlined, and the proline-rich region is italicized. The first ATG codon is assigned as the putative initiation codon. B, sequence alignment of PIR1 with BVP, CET23G7.5 (GenBank™ accession no. Z68319), CEF54C8.4 (GenBank™ accession no. Z22178), and CEL-1. Alignment was performed using the PILEUP program from the Wisconsin Genetics Computer Group. Identical amino acids are highlighted by the black boxes.](image-url)

**B**

![Sequence alignment of PIR1 with BVP, CET23G7.5, CEF54C8.4, and CEL-1.](image-url)
PIR1 binds to single-stranded DNA or RNA in vitro. PIR1 contains two stretches of arginine-rich regions (Fig. 1A). Because the arginine-rich regions in the viral protein Rev and Tat are known to be involved in binding to RNA (16), we tested whether PIR1 can interact with RNA. Because many RNA-binding proteins can bind to both RNA and single-stranded DNA in vitro, we first tested whether PIR1 can bind to single-stranded DNA. Wild-type PIR1 or PIR1(CS) mutant proteins were expressed as T epitope-tagged forms by recombinant baculoviruses in Sf9 cells. When the cell lysates from such baculovirus-infected Sf9 cells were expressed as T epitope-tagged forms by recombinant E. coli, GST-PIR1 or GST-PIR1(CS) were affinity-purified with glutathione-Sepharose beads. GST-PIR1 displayed protein tyrosine phosphatase activity toward tyrosyl-phosphorylated poly(GluTyr) (Fig. 3). Poly(GluTyr) is a random polymer of glutamate and tyrosine, and the tyrosyl-phosphorylated poly(GluTyr) has been shown to be an excellent in vitro substrate for dual-specificity phosphatases such as PTEN/MMAC1/TEP1 (10). As control, we have assayed in parallel the PIR1 derivative carrying the cysteine 152 to serine mutation (C152S) in the tyrosine phosphatase signature motif. The tyrosine phosphatase activity of GST-PIR1 was abolished by the C152S mutation (Fig. 3). These studies suggest that PIR1 possesses an intrinsic protein tyrosine phosphatase activity. Under similar conditions, we could not detect phosphatase activity using phosphoseryl/threonyl casein as substrate (data not shown). This could be because of the substrate selectivity of PIR1. It remains possible that in vivo PIR1 may dephosphorylate phosphoseryl/threonyl residues in addition to phosphotyrosyl residues with its physiological substrates.

PIR1 interacts directly with single-stranded DNA or RNA in vitro. To examine whether PIR1 shows binding preference for RNA or single-stranded DNA, we performed the filter binding assay using either 32P-labeled RNA or 32P-labeled single-stranded DNA as probes, referred to as Northwestern blot and Southwestern blot analysis, respectively. The RNA and DNA probes used were of defined sequences and were both generated with the same set of DNA templates. To increase the sequence complexity of the defined probes, we have used a mixture of its association with nucleic acid-binding proteins, we therefore performed Southwestern blot analysis to examine whether PIR1 can interact with single-stranded DNA directly. In this assay, GST-PIR1 or its CS derivative was affinity-purified, resolved on SDS-PAGE, and blotted to nitrocellulose. The proteins on the filters were subjected to denaturation and renaturation and then incubated with 32P-labeled single-stranded DNA probe derived from the salmon sperm DNA template. After washing, the bound radiolabeled probe was detected by autoradiography. As shown in Fig. 4B, both GST-PIR1 or GST-PIR1(CS) bound to single-stranded DNA with comparable affinity in this assay. To examine the specificity of the binding, we used the GST-MKP-1 protein as a control. No single-stranded DNA binding was observed for GST-MKP-1, although Coomassie Blue staining confirmed that all GST-fusion proteins were present in about equal amounts (Fig. 4B). Our subsequent studies showed that similar single-stranded DNA binding could be achieved even when the denaturation and renaturation step was omitted (data not shown), suggesting that the native conformation of these proteins may not be required for the nucleic acid binding. In addition, this assay showed that both the wild type PIR1 and the catalytically inactive CS mutant protein bound to RNA with comparable affinity, suggesting that the PTP activity of the enzyme is not required for the binding.

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PIR1, a Novel Phosphatase Binding to RNA/RNP

FIG. 4. Binding of PIR1 to single-stranded DNA or RNA in vitro. A, both the WT and CS mutant of PIR1 binds tightly to single-stranded DNA agarose beads. Lysates from Sf9 cells expressing either T epitope-tagged PIR1 (w.t.) or C152S (CS) mutant were incubated with single-stranded DNA agarose beads, washed, and step-eluted with the indicated salt solution. PIR1 or CS proteins in fractions of input, flowthrough (FT), 0.25, 0.5, and 1 M salt eluates, and the proteins retained on the beads after 1 M salt elution (Beads) (which were then released by boiling in Laemmli sample buffer) were analyzed by Western blot analysis with anti-T epitope antibody followed by ECL detection. B, GST-PIR1 fusion proteins binds to single-stranded DNA directly in vitro. GST-PIR1 wild type (WT) or GST-PIR (CS) mutant, and GST-MKP-1 were expressed in Sf9 cells and purified by glutathione-Sepharose beads. The purified proteins were examined by SDS-PAGE and Coomassie Blue staining (left panel). All GST-fusion proteins were shown to be present in comparable amounts. Lysate from uninfected Sf9 cells were analyzed in parallel to serve as a control. Molecular standards are indicated on the left. The purified proteins were also analyzed by Southwestern blot analysis (right panel) in which a parallel set of proteins from SDS-PAGE gel were transferred to nitrocellulose filter. The filter was incubated with a 32P-labeled single-stranded DNA probe derived from the salmon sperm DNA template. The bound probe was detected by autoradiography. C, PIR1 binds preferentially to RNA rather than to single-stranded DNA in vitro. Affinity-purified GST-PIR1(WT) proteins were analyzed by filter binding assay with either 32P-labeled RNA probe (Northwestern blot) or 32P-labeled single-stranded DNA probe (Southwestern blot) analysis. Both the RNA and the DNA probes were derived from the same pool of cDNA templates and were of the same specific activity and concentration (see “Material and Methods”). The protein amount loaded in each lane is indicated.

FIG. 5. Specific interaction of PIR1 with splicing factors in yeast two-hybrid system. Using PIR1 as a bait, positive clones were isolated that encode 9G8 or SRp30C. The plasmid expressing 9G8 or SRp30C (in pGADGH vector) was retransformed into yeast YRG2 strain either together with PIR1 or with CDK6 bait plasmid (in pGBT9 vector). Two independent colonies from each transformation were streaked out on histidine deficient plates. 9G8 (upper panel) or SRp30C (lower panel) interacted specifically with PIR1 but not with CDK6.

PIR1 Interacts with RNPs in the Yeast Two-hybrid System—To gain an insight into the cellular processes that are regulated by PIR1, we have employed the yeast two-hybrid screen method to identify proteins that may interact with PIR1. By screening the HeLa cDNA yeast two-hybrid library with PIR1 as a bait, we have identified ~100 positive clones in both histidine prototroph and β-galactosidase assay, and 20 of them were chosen for further analysis. These plasmids were recovered from yeast colonies. To test the specificity of the interaction with the bait protein, each plasmid was then transformed into the yeast strain together with a bait plasmid encoding either PIR1 or CDK6. The plasmids that conferred interactions only with PIR1, but not with CDK6 control, were then sequenced. Two of the cDNA clones were found to encode splicing factors 9G8 or SRp30C, both as an in-frame fusion with the upstream Gal4 transcription activation domain (Fig. 5). Both 9G8 and SRp30C belong to the SR family splicing factors, which share a domain containing serine and arginine repeats and are components of the mRNA-splicing complexes (21). So far, we have not been able to detect physical association between PIR1 and splicing factors in mammalian cells. It is possible that the interaction of PIR1 with splicing factors 9G8 or SRp30C is mediated through an RNA intermediate, as PIR1 itself can bind to RNA directly, and our coimmunoprecipitation method may not be sensitive enough to detect such interaction.

Immunolocalization of the Ectopically Expressed PIR1—To examine the cellular localization of PIR1, we expressed the T epitope-tagged PIR1 in HeLa cells by the transient transfection method, followed by immunofluorescence staining with anti-T epitope antibody. PIR1 was localized to the nuclei with the exclusion of the nucleolus, and no staining was observed when cells were transfected with the vector alone (Fig. 6, A and B). To determine whether PIR1 in cells is associated with certain nucleic acids or protein complexes involved in nucleic acids metabolism, we subjected the permeabilized cells to DNase I or RNase A treatment before fixation. Although PIR1 staining was not affected by pretreatment with DNase I or RNase A treatment before fixation. Although PIR1 staining was not affected by pretreatment with DNase I, it was greatly diminished by pretreatment with RNase A (Fig. 6, C and D). The sensitivity of PIR1 staining to RNase A suggests that PIR1 in mammalian cells is associated with RNA and/or RNP complexes involved in nuclear mRNA metabolism.

Because PIR1 interacts with splicing factors in the yeast two-hybrid system, we examined whether PIR1 is colocalized with proteins involved in mRNA splicing in mammalian cells. Several splicing factors, including SC35, are known to exhibit a speckled immunofluorescence pattern (22). Speckles are be-
Endogenous splicing factor SC35 was detected with antibody SC35 followed by rhodamine-conjugated secondary antibody (panel B). We wondered if removal of the diffused PIR1 staining would allow us to detect certain PIR1-associated subnuclear structures. We modified the immunostaining procedure so that the cells were permeabilized in the absence of RNase inhibitor to allow limited RNase digestion to take place. Under this condition, the ectopically expressed PIR1 was observed in speckles, which was reminiscent of the pattern known for the distribution of SC35 (22). We therefore performed a double immunostaining experiment in the PIR1-transfected HeLa cells and examined whether a fraction of PIR1 colocalizes with SC35 by confocal fluorescence microscopy. As shown in Fig. 7, both the exogenous PIR1 and endogenous SC35 exhibited the speckled pattern, which was superimposable, suggesting that PIR1 partially colocalizes with SC35 in these cells. These studies provide further support that PIR1 is associated with RNA and/or RNP in the mRNA processing or maturation process.

**DISCUSSION**

Our studies demonstrate that PIR1 is a novel member of the dual-specificity subfamily of protein tyrosine phosphatases that exhibits high affinity to RNA. PIR1 bound directly to the in vitro transcribed RNAs derived from cDNA templates. Using PIR1 as a bait, we have isolated cDNA clones encoding accessory splicing factors 9G8 and SRp30C that showed specific interactions with PIR1 in the yeast two-hybrid system. When ectopically expressed in HeLa cells, PIR1 manifested a nuclear staining pattern, and the staining was removed by pretreatment of cells with RNase A but not DNase I. Furthermore, a fraction of PIR1 was colocalized with the splicing factor SC35 in speckles. PIR1 is the first member in the protein tyrosine phosphatase family that shows high affinity for RNA both in vitro and in vivo. Our studies suggest that PIR1 may participate in nuclear mRNA metabolism.

Increasing evidence suggests the participation of protein kinases and phosphatases in mRNA processing (21, 23). For example, a dual-specificity kinase Clk/Sty is found to be a partner with SR splicing factors in a yeast two-hybrid screen, and a catalytic inactive form of this kinase is colocalized with SR splicing factors in speckles in transfected cells (24). Serine/threonine phosphatase 1, or a serine/threonine phosphatase-1-like activity, has been shown to affect the subnuclear localization of the splicing factors (25). More recently, CEL-1, a phosphatase in *C. elegans* was shown to be a 5' 3'-phosphatase for RNA molecules and was suggested to be involved in the mRNA capping reaction (15). Whether PIR1 is involved in regulating pre-mRNA splicing or other aspects of mRNA metabolism such as capping, polyadenylation, stability, or transport awaits further studies.

One interesting question arising from our studies is which region or domain in PIR1 mediates its interaction with RNA. Our observations that both the wild type PIR1 and its catalytically inactive C152S mutant bind to RNA with comparable affinity, and our data that binding can take place with denatured phosphatases raises the interesting possibility that interaction of PIR1 with RNA may not require the catalytic center nor the native conformation of the enzyme. One possibility is that PIR1 binds to RNA through its arginine-rich

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**Fig. 6.** Association of PIR1 with RNA- but not DNA-containing complexes in HeLa cells. HeLa cells were transfected with either pCGT vector alone (panel A) or with pCGT-PIR1 plasmid (panel B-D) and then processed for immunostaining with anti-T epitope antibody followed by rhodamine-conjugated secondary antibody. PIR1 immunostaining was observed in the nucleus of cells transfected with the PIR1 plasmid (panel B) but not in cells with the control vector (panel A). The PIR1 staining was not affected by treatment of permeabilized cells with DNase I (panel C) but was greatly diminished by treatment with RNase A before fixation (panel D).

**Fig. 7.** Colocalization of a fraction of PIR1 with SC35 by confocal fluorescence microscopy analysis. HeLa cells transfected with pCGT-PIR1 plasmid were permeabilized in the absence of the RNase inhibitor to allow limited RNase digestion before fixation. Exogenous T epitope-tagged PIR1 was revealed with biotinylated anti-T epitope antibody followed by fluorescein-conjugated streptavidin (panel A, green). Endogenous splicing factor SC35 was detected with antibody SC35 followed by rhodamine-conjugated secondary antibody (panel A, green). The superimposed images of panels A and B are shown in panel C (yellow), indicating that PIR1 is colocalized with SC35.
sequences. Several specific RNA binding proteins, such as Rev and Tat, are known to contain an arginine-rich motif (16). Rev, a protein encoded by HIV, can bind and facilitate nuclear export of intron-containing viral RNA. Tat, also encoded by HIV, is involved in the regulation of transcription by binding to viral mRNA. Very little identity is found between the arginine-rich motif sequences except for the richness of arginine residues. Further studies are required to determine whether the arginine-rich regions in PIR1 mediate its binding to RNA.

Acknowledgments—We would like to thank Drs. Susan Baserga and Hui Zhang for helpful discussions and critical reading of the manuscript, Peer Bork and Anne Marie Quinn for assistance with the computer sequence analysis.

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