We report the molecular cloning in \textit{Rattus norvegicus} of a novel mammalian enzyme (RnPIP), which shows both 3’-phosphoadenosine 5’-phosphate (PAP) phosphatase and inositol-polyphosphate 1-phosphatase activities. This enzyme is the first PAP phosphatase characterized at the molecular level in mammals, and it represents the first member of a novel family of dual specificity enzymes. The phosphatase activity is strictly dependent on Mg\(^{2+}\), and it is inhibited by Ca\(^{2+}\) and Li\(^{+}\) ions. Lithium chloride inhibits the hydrolysis of both PAP and inositol-1,4-bisphosphate at submillimolar concentration; therefore, it is possible that the inhibition of the human homologue of RnPPIP by lithium ions is related to the pharmacological action of lithium. We propose that the PAP phosphatase activity of RnPPIP is crucial for the function of enzymes sensitive to inhibition by PAP, such as sulfotransferase and RNA processing enzymes. Finally, an unexpected connection between PAP and inositol-1,4-bisphosphate metabolism emerges from this work.

The sulfation reactions in mammals affect many different physiological processes, including deactivation and bioactivation of xenobiotics, inactivation of hormones and catecholamines, structure and function of macromolecules, and elimination of end products of catabolism (1). Sulfation involves the transfer of a sulfate group from 3’-phosphoadenosine 5’-phosphosulfate (PAPS)\(^{1}\) to an acceptor molecule in a reaction that is catalyzed by a family of sulfotransferase enzymes (2). Usually, the obligate co-substrate PAPS donates its sulfuryl group to a functional hydroxyl group: PAPS + R-OH \(\rightarrow\) PAP + R-OSO\(_3^2\). The products are a sulfoconjugate (R-OSO\(_3^2\)) and 3’-phosphoadenosine 5’-phosphate (PAP). PAP is an inhibitory end product in any sulfation reaction because it acts as a competitive inhibitor with respect to PAPS (3). For instance, the M and P forms of human phenolsulfotransferases are potently inhibited by PAP, exhibiting a \(K_I\) value of approximately 0.1 \(\mu\)M (4).

In addition, a toxic effect of PAP on RNA processing enzymes (5’ \(\rightarrow\) 3’ exoribonucleases) has been recently described in yeast (5). This effect might be attributed to the fact that the homologous enzymes in higher eukaryotes might mimic the monomers of a polynucleotide chain and consequently, it might prevent the attack to the phosphodiester bond of RNA processing enzymes. These 5’ \(\rightarrow\) 3’ exoribonucleases are highly conserved in evolution (5), therefore it is very likely that PAP accumulation in mammals has similar inhibitory effects on RNA processing, leading to aberrant changes in the pattern of gene expression. Consequently, a rapid removal of PAP is required both to maintain the activity of sulfotransferase enzymes and to prevent the accumulation of PAP to levels that could be inhibitory for RNA processing enzymes. Thus, it is reasonable to predict the existence of mammalian 5’-phosphoadenosine 5’-phosphate phosphatases, which could rapidly recycle PAP to AMP and inorganic phosphate. Indeed, a preliminary characterization of a 3’,5’-bisphosphate nucleotidase purified from guinea pig liver has been reported (6), although this enzyme showed a very poor affinity for PAP (\(K_m\) 3 mm). No clear role for this enzyme in mammals is known. In this work, we report the first mammalian enzyme to act on the deactivation of PAP.

In this work, we present the first example of the molecular cloning of a mammalian PAP phosphatase. The enzyme was cloned by functional complementation of a \textit{Saccharomyces cerevisiae} \textit{hal2} mutant. \textit{Hal2} is a 3’,5’-bisphosphate nucleotidase that specifically hydrolyzes the 3’-phosphate from PAP (7, 8), thereby preventing the accumulation of PAP. In yeast, PAP is generated as a side product of PAPS reductase, which is a key enzyme in the sulfate assimilation pathway that leads to the synthesis of methionine. PAP accumulation has deleterious effects on the cell because it inhibits PAPS reductase (9), and hence methionine biosynthesis, and RNA processing enzymes (5). The PAP phosphatase activity of \textit{Hal2} is crucial for the function of PAPS reductase, which is reflected in the fact that \textit{hal2} cells are auxotrophic for methionine (7, 10). We took advantage of this phenotype to clone the first mammalian PAP phosphatase by functional complementation of the auxotrophy for methionine of \textit{hal2} cells. Interestingly, the mammalian PAP phosphatase has a dual enzymatic activity, as it is also active as inositol-polyphosphate 1-phosphatase.

**EXPERIMENTAL PROCEDURES**

\textit{Chemicals—}All nonradioactive organic compounds were obtained from Sigma. Radioactive compounds were purchased from NEN Life Science Products. PAPS (Sigma) is supplied with 4 mol of lithium/mmol of PAPS. Given the instability of this compound, removal of lithium is not recommended. \[^{35}\text{S}\]PAPS (NEN Life Science Products) is obtained as the triethylammonium salt. Therefore, \[^{35}\text{S}\]PAPS was the substrate.
employed to assay the activity of RnPIP against this compound.

**Yeast Strain and Growth Media**—The Saccharomyces cerevisiae strain used in this study was JRM4 (MATa leu2-3, 112 ura3-251,328,372 hal2::LEU2), kindly provided by Dr. J. R. Murguia (Universidad Politécnica, Valencia, Spain). Complementation of the auxotroph phenotype of the hal2::LEU2 mutant was assayed by adding synthetic glucose medium (SD), 2% glucose, 0.7% yeast nitrogen base without amino acids and 50 mM succinic acid adjusted to pH 5 with Tris. When indicated, the SD medium was supplemented with 100 μg/ml methionine to give SDM medium.

**Cloning of RnPIP**—The yeast strain JRM4 was transformed with a Rattus norvegicus heart cDNA library constructed in the pFL61 vector (11), a yeast expression vector where expression of the rat cDNA is driven by the constitutive phosphoglycerate kinase (PGK) promoter. Transformants were first selected by uracil prototrophy in SDM. 10⁵ primary transformants were obtained and pooled together. To select clones capable of complementing the auxotrophy for methionine of the hal2 mutant, the primary transformants were spread on minimal medium lacking methionine. Four clones (pFL61-PIP) were obtained that conferred growth to the auxotrophine mutant. The yeast shuttle plasmid was recovered by electroporation of E. coli transformants showing that all of them represented the same cDNA.

**Expression of RnPIP in E. coli and Protein Purification**—The coding region of RnPIP was PCR-amplified from pFL61-PIP using Pcoo DNA polymerase (Roche Molecular Biochemicals) and the following primers (XhoI site underlined): upstream, 5′-CCTCTCGAGATGGCTCCAGC; downstream, 5′-CCCTCTGACCCCTTCAGGAGTAGAG.

The PCR-amplified product was subcloned into the pT7-Blue vector (Novagen) to give pT7-PIP. The PIP open reading frame was released with XhoI and subcloned into the XhoI site of pGEX-KG (12), thus generating a fusion protein between glutathione S-transferase (GST) and RnPIP. The pGEX-KG-PIP construct was verified by sequencing with an ABI 377 automatic sequencer, and it was introduced into E. coli DH5α cells. The expression of the recombinant protein was induced with 0.1 mM isopropyl-β-D-galactopyranoside for a period of 1 h at 30°C, and the GST fusion protein was affinity purified on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as indicated by the suppliers. The pGEX-KG-PIP construct contains a thrombin cleavage site and a poly-glycine spacer between the GST and the RnPIP moieties (12). The specificity of the cDNA for the fusion protein could be confirmed by thrombin cleavage. The resulting RnPIP protein behaves identically to the GST fusion protein in the biochemical assays we have performed.

**Enzyme Assay—**PAP phosphatase and inositol-polypophosphate 1-phosphatase activities were assayed by one of the following methods (8). Briefly, phosphatase activity was assayed (assay 1) in a 100-μl mixture containing 50 mM Tris-Cl, pH 7.5, 1 mM magnesium chloride, and the indicated amount of GST fusion protein and substrate. After a 30-min incubation at 30°C, the inorganic phosphate released was quantified by the malachite green method as described previously.

**RESULTS**

**Cloning of RnPIP**—To explore the presence in animals of PAP hydrolytic enzymes, a screening was conducted for mammalian genes that complement the auxotrophy for methionine of the yeast hal2 mutant. A rat cDNA library was constitutively expressed in hal2 cells, and as a result, a cDNA was identified that complemented the auxotrophy for methionine. Sequencing of this cDNA reveals a 927-base pair open reading frame that putatively encodes a protein of 308-amino acid residues with a molecular mass of 33.2 kDa (Fig. 1). The cDNA has 27 base pairs of 5′-untranslated sequence and 961 base pairs of 3′-untranslated region. The protein encoded by this gene, named RnPIP, complements the auxotrophy for methionine of hal2 cells to the same extent as Hal2, as demonstrated by the similar growth rates in methionine-free minimal medium of hal2 cells transformed with RnPIP or SchALS (data not shown).
The RnPIP protein was expressed in E. coli as a GST fusion protein and purified as described under "Experimental Procedures." The phosphatase activity was quantified by the malachite green method (15) as described under "Experimental Procedures." The activity obtained with different substrates (0.2 mM) under conditions of maximal activity, pH 7.5 and 1 mM Mg^{2+}, is expressed as the percent activity observed with 3'-PAP. The specific activity with 3'-PAP of GST-PIP was 0.8 μmol Pi × min⁻¹ × milligram protein⁻¹. The results are the average of two independent experiments, each performed in duplicate. Standard deviations were less than 5%.

### TABLE I

| Substrates            | RnPIP  |
|-----------------------|--------|
| 3'-PAP                | 100    |
| 2'-PAP                | 33     |
| PAPS*                 | 50     |
| 3'-AMP                | <2     |
| 5'-AMP                | <2     |
| Inositol-1,4-bisphosphate | 45     |
| Inositol-1,3,4-trisphosphate | 20     |
| Inositol-1,4,5-trisphosphate | <2     |
| Inositol-1-phosphate  | <2     |
| Inositol-2-phosphate  | <2     |
| Fructose-1,6-bisphosphate | <2     |
| p-NPP                 | <2     |

* PAPS (Sigma) is supplied with 4 mol of lithium/mol of PAPS. Removal of lithium by cation-exchange chromatography resulted in a significant (15–20%) conversion to PAP, due to the hydrolysis of the high energy phosphoric-sulfuric acid anhydride bond. [35S]PAPS (NEN Life Science Products) is not contaminated with lithium. As RnPIP is very sensitive to inhibition by lithium (IC₅₀ = 0.8 mM), we have compared the activity of RnPIP against 10 μM [35S]PAPS (1–3 Ci/mmol) to an assay with 10 μM PAP. The reaction products were analysed by HPLC and detected either by a RadioFlow detector or by ultraviolet absorption, as described under "Experimental Procedures."

### Biochemical Characterization of the RnPIP Protein

The RnPIP protein was expressed in E. coli as a GST fusion protein. Following induction with isopropyl-1-thio-d-galactopyranoside, the fusion protein was purified by affinity chromatography on glutathione-Sepharose resin. The phosphatase activity of the purified protein with respect to different substrates is summarized in Table I. PAP was the preferred substrate for RnPIP. The Kₘ value for PAP hydrolysis was too low to be determined with the standard colorimetric assay (15) or even using the HPLC-based method where the products are detected by ultraviolet absorption (8). The detection limit of the HPLC analysis for PAP hydrolysis is approximately 2 μM, therefore the Kₘ for PAP must be below 2 μM. PAPS was used as substrate with a 50% efficiency as compared with PAP. No appreciable activity was observed against AMP, 3’-AMP, fructose-1,6-bisphosphate, or pNPP. Interestingly, inositol-1,4-bisphosphate also served as a substrate for the enzyme, although with a 45% efficiency as compared with PAP. In addition, RnPIP hydrolyzed inositol-1,3,4-trisphosphate but not inositol-1,4,5-trisphosphate or inositol monophosphate. Therefore, besides the preferred PAP, RnPIP hydrolyzes the typical substrates of inositol-polyphosphate 1-phosphatase (15).

To determine the position of the phosphate liberated from PAP and inositol-1,4-bisphosphate, we have analyzed by HPLC...
the reaction products of either PAP or inositol-1,4-bisphosphate hydrolysis (Fig. 3). RnPIP converts 3′-PAP to AMP by hydrolysis of the 3′-phosphate (Fig. 3A). The 2′-phosphate from 2′-PAP was also hydrolyzed by the enzyme, although with a lower efficiency. In this respect, RnPIP qualifies as a 3′(2′), 5′-bisphosphate nucleotidase. Inositol-1,4-bisphosphate was converted to inositol-4-phosphate (Fig. 3B), therefore RnPIP catalyzes the removal of the 1′-phosphate, as inositol-polyphosphate 1-phosphatase does.

**Determination of the $K_m$ Values for PAPS and Inositol-1,4-bisphosphate Hydrolysis**

Inositol-1,4-bisphosphate and PAPS hydrolysis were assayed in a reaction mixture containing 0.2 mM potassium Bicine (pH 8.0), 1 mM MgCl$_2$, and different concentrations of [3H]inositol-1,4-bisphosphate (10 C/mm) or [35S]PAPS (1–3 C/mm), respectively. The $K_m$ value for either inositol-1,4-bisphosphate or PAPS hydrolysis was determined by measuring reaction rates at substrate concentrations of 0.055, 0.166, 0.333, and 0.5 mM. The reaction products were analysed by HPLC and detected with a RadioFlow detector as described under “Experimental Procedures.” The reaction product of either [3H]inositol-1,4-bisphosphate or [35S]PAPS hydrolysis is [3H]inositol-4-phosphate or [35S]adenosine 5′-phosphosulfate, respectively. The estimation of the $K_m$ value for PAP is described in the text. The $V_{max}$ values are equivalent to the data presented in Table I. The results are the average of two independent experiments, each performed in duplicate.

**Table II**

| Substrates                  | $K_m$ (μM) | $V_{max}$ (μmol Pi × min$^{-1}$ × mg$^{-1}$) |
|-----------------------------|------------|------------------------------------------|
| PAP                         | <2         | 0.8                                      |
| PAPS                        | 1.2 ± 0.3  | 0.4                                      |
| Inositol-1,4-bisphosphate   | 0.2 ± 0.1  | 0.38                                     |

**DISCUSSION**

Biochemical characterization of the RnPIP protein shows that it is a dual enzyme that hydrolyzes both PAP and inositol-1,4-bisphosphate. The $V_{max}$ value for PAP hydrolysis was approximately 2-fold higher than for inositol-1,4-bisphosphate hydrolysis. Although the $K_m$ for PAP could not be accurately determined (<2 μM), we could determine the $K_m$ for the closely related substrate PAPS (1.2 μM) and for inositol-1,4-bisphosphate (0.2 μM), indicating a high affinity for these substrates. The enzyme is very sensitive to Li$^+$ and, therefore, RnPIP is less than 5%.

![Fig. 5. Human ESTs share high similarity with RnPIP.](image)

**Fig. 5. Human ESTs share high similarity with RnPIP.** A schematic diagram showing the amino acid alignment of RnPIP with human ESTs (HsEST) corresponding either to the N or C terminus of the protein is shown. The accession numbers for the EST1 and EST2 are H97426 and AA643182, respectively.
the fourth animal enzyme that is inhibited by Li⁺ ions in the therapeutic range, the other three being inositol monophosphate phosphatase (IMPPases), and PAP phosphatase (PAPases) enzymes (17). RnPIP is 20, 27, 30, 90, 31, 30, and 25% identical to AtFBP, BtIPP1, MnIPP, HsEST, AtSAL1, ScHal2, and ScIpml, respectively. The PILEUP program was employed to align the amino acid sequence of the following proteins and ESTs: AtFBP (A. thaliana, Swiss-Prot P25851), BtFBP (Brassica napus, Swiss-Prot Q07204), BtIPP1 (Bos taurus, Swiss-Prot P21327), HsIPPF (Homo sapiens, Swiss-Prot P49441), MnIPPF (Mus musculus, Swiss-Prot P49442), RnPIP (R. norvegicus, EBI AJ000347), MnEST (M. musculus, GenBank™ AA008240), HsEST (H. sapiens, GenBank™ H97426), Sc-EST (S. cerevisiae, EBI Z84066), DmEST (Drosophila melanogaster, GenBank™ AA990707), AtSAL1 (A. thaliana, GenBank™ U40433), At-AL2 (A. thaliana, EBI Z83312), OsRHL (Oryza sativa, GenBank™ U33283), AaALH1 (A. thaliana, GenBank™ 155200), ScHal2 (S. cerevisiae, Swiss-Prot P21719), and EcCysQ (E. coli, Swiss-Prot P26264), Scmp1 (S. cerevisiae, PIR S70117), Scmp2 (S. cerevisiae, Swiss-Prot P93710), AnQuG (Aspergillus nidulans, Swiss-Prot P25416), HsIPPH (H. sapiens, EBI S28900), BtIMP (B. taurus, EBI J05394), XIMP (Xenopus laevis, EBI X55153), LeIMP1 (Lycopersicon esculentum, Swiss-Prot P54926), and EcSubb (E. coli, Swiss-Prot P22783).

In summary, we propose that the PAP phosphatase activity of RnPIP, by preventing accumulation of PAP, is necessary to maintain active sulfotransferase (6, 21) and RNA processing enzymes (5). RNA processing is crucial in cell metabolism. The importance of sulfation in animals is illustrated by the phenotype of brachymorphic mice, which have abnormal hepatic detoxication, bleeding times, and postnatal growth (22). This phenotype is because of a missense mutation in SK2 (sulfurylase kinase 2) (22), a member of the gene family encoding the bifunctional enzymes that synthesize the universal sulfate donor, PAPS. In addition, the inositol-polyphosphate 1-phosphatase activity of RnPIP could play a role in the phosphoinositide-signaling pathway. Finally, the unexpected connection between PAP and inositol-1,4-bisphosphate metabolism remains to be investigated.

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