Functional Analysis of Conserved Domains in the Phosphotyrosyl Phosphatase Activator. Molecular Cloning of the Homologues from *Drosophila melanogaster* and *Saccharomyces cerevisiae*[†,‡]

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ABSTRACT: Phosphotyrosyl phosphatase activator (PTPA), a 37 kDa cytosolic protein that specifically activates the phosphotyrosyl phosphatase activity of the dimeric form of PP2A, was cloned from *Drosophila melanogaster* and *Saccharomyces cerevisiae*. Sequence alignment of PTPA from yeast to human revealed highly conserved regions including the type B fragment of the putative PTPA ATP binding site. We generated PTPA deletion mutants of these conserved regions as well as point mutations within regions that were suggested to be functionally important. The recombinant proteins were expressed in *E. coli* and subsequently purified. Activity measurements, linked with immunological detection, revealed that most of the well-conserved regions are essential for PTPA activity. However, neither the type A fragment of the putative ATP binding site nor the cysteine-rich region, present in all but the *Drosophila* and yeast homologues, appeared to be essential for PTPA activity. Moreover, we observed that PTPA truncated at glycine$_{266}$ behaves as a dominant negative mutant since it is inhibitory to the wild-type PTPA.

Protein phosphatase type 2A (PP2A)[1] is a family of holoenzymes consisting of a catalytic subunit of 36 kDa and a regulatory subunit of 65 kDa, forming the dimeric core structure ($PP2A_D$). $PP2A_D$ activity is regulated by association or interaction with one of a still growing number of cellular and viral proteins (*1*−*10*). These regulatory proteins largely determine the substrate specificity and activity of PP2A and probably also its cellular localization (*11*, *12*), contributing to the involvement of PP2A in the control of many different cellular processes such as cell division, cell growth, cell differentiation, and cell transformation (*13*, *14*). PP2A is also subject to posttranslational modifications by phosphorylation (*15*−*18*) and methylation (*19*−*23*); two heat-stable specific inhibitors of PP2A were also described recently (*24*).

PP2A is a dual-specificity phosphatase, dephosphorylating Ser/Thr as well as Tyr phosphorylated substrates (*5*, *25*, *26*). In vitro, the phosphotyrosyl phosphatase (PTPase) activity of PP2A is stimulated by a 37 kDa cellular protein, PTPA (phospho-tyrosyl phosphatase activator), in an ATP,Mg^{2+}-dependent reaction (*26*). PTPA has been purified from different eukaryotes including rabbit skeletal muscle, dog liver, porcine brain, *Xenopus* oocytes, and *Saccharomyces cerevisiae* (*26*, *27*). By immunochemical analysis, PTPA has been identified in all tissues and species examined (*27*). The deduced amino acid sequence determined by molecular cloning of PTPA from different vertebrates such as human, rabbit, and *Xenopus* (*28*, *29*) reveals a high degree of similarity among these species. Hence, PTPA is a ubiquitous highly conserved protein, suggesting an important cellular function. The single human PTPA gene is located on chromosome 9 (q34), and its structural organization has been determined (*30*).

PTPA-activated PP2A has a distinct in vitro substrate specificity compared to classical protein phosphotyrosyl phosphatases (*31*), suggesting that PP2A activated by PTPA might dephosphorylate a selective group of tyrosyl phosphorylated substrates in vivo. The cellular concentration of PTPA is estimated in the micromolar range, similar to the concentration of PP2A itself, and after activation, the tyrosyl phosphatase activity of PP2A is only slightly lower than its Ser/Thr phosphatase activity (*26*). However, its biological role in the activation of the phosphotyrosyl phosphatase of PP2A is not yet fully understood. ATP and Mg^{2+} are essential cofactors for PTPA stimulation, and ATP analogues that cannot be hydrolyzed are unable to substitute for ATP. Nevertheless, PTPA is probably not a kinase since neither the PP2A subunits nor PTPA could be phosphorylated. In addition, PTPA could not act as a kinase using diverse substrates (*26*), and the primary sequence of PTPA does not contain the canonical kinase ATP binding site GXGXXG (*28*). In the absence of a kinase motif, the primary sequence of PTPA was screened for ATP binding domains such as those found in proteins involved in other ATP hydrolytic

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[‡] GenBank Accession number for *Drosophila* PTPA: X98401.

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[1] Abbreviations: mT/st, polyoma middle T and small t antigens; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PP2A, protein phosphatase 2A; $PP2A_D$, dimeric form of protein phosphatase 2A; PTPA, phosphotyrosyl phosphatase activator; PTPase, phosphotyrosyl phosphatase.

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processes. These ATP binding sites contain two segments denominated A and B (*32*), and an imperfect A and a nearly consensus B sequence were found in PTPA (*5*).

In this study, we report a mutational analysis of both of the putative ATP binding regions as well as of a cysteine-rich region, thought to be important for the mechanism of action of PTPA. Indeed, a similarity was observed to the PP2A binding site of polyoma middle T and to the catalytic domain of the classical protein tyrosine phosphatases (*5*). We also report the molecular cloning of PTPA from *Drosophila melanogaster* and *Saccharomyces cerevisiae* and present the alignment of these sequences with *Xenopus*, rabbit, and human PTPA. Regions of well-conserved amino acids were found and subjected to functional analysis by constructing deletion mutants. From this study, it is clear that these highly conserved regions are essential for PTPA activity. One further notable mutant, PTPA truncated at glycine$_{266}$, missing two conserved regions, is inactive and behaves as a dominant negative mutant since it is inhibitory to the activity of wild-type PTPA.

EXPERIMENTAL PROCEDURES

Molecular Cloning of Drosophila melanogaster and Saccharomyces cerevisiae PTPA. The cloned part of the *Xenopus* PTPA ORF was generated by PCR with the 5′ sense oligonucleotide GAAGGTGTGAAGAAGCTGACC and the 3′ antisense oligonucleotide TAGCCGGAGGTGACGGGGTGGATGG, labeled by random priming (*33*) and used as a probe to screen a *Drosophila* embryo cDNA library (λZAPII, Stratagene) in order to isolate the *Drosophila* homologue of PTPA (DPTPA). The first yeast PTPA homologue (YPTPA1) was isolated by classical screening of a yeast genomic DNA library (kindly provided by J. Thevelein, Leuven, Belgium) with a yeast PCR product as probe. This PCR probe was generated with oligonucleotides synthesized toward the regions most similar between a PTPA-like yeast ORF (accession number: Z38059) and vertebrate PTPA cDNA as primers (5′ sense: ACAAGATTGGACTACGGCACGGGCC; 3′ antisense: GCCGAACCAGAAATGTTGCACCACGGG) and with yeast genomic DNA (prepared as described in *34*) as template. Prehybridization, hybridization, washing, and plaque purification were performed as described in (*28*). The DNA sequences of isolated clones were analyzed by the automatic laser fluorescence procedure, using the "Autoread Sequencing Kit" (Pharmacia) according to the manufacturer's protocol. To obtain the complete DNA sequence in both directions, we used specific internal primers based on the DNA sequence and used subclones made by restriction enzyme digestion as templates for the sequencing reaction. The second yeast PTPA homologue (YPTPA2) was kindly provided by B. Purnelle, Louvain-la-Neuve, Belgium, as part of a 41.5 kb cosmid, containing the left arm of chromosome XVI (accession number: X96770).

Expression and Purification of Drosophila and Yeast Recombinant Proteins. A *Nde*I restriction site at the start codon of the *Drosophila* and yeast DNA was created by PCR using Ultma DNA polymerase (Perkin-Elmer). The oligonucleotides according to the start region containing the mutation to achieve a *Nde*I restriction site were GAATTC**CATATG**GCGAGTGGCATTAACC (DPTPA), GAATTC**CATATG**TCTCTGGATCGTGTAGATTGGC (YPTPA1), and TCGAATT**CATATG**TTGCCAGAAAAGAG (YPTPA2), respectively. For the stop codon region, elongated with a *Bam*HI restriction site, the oligonucleotides were TG**GGATCC**CTATTCCTTGCTGGGCTTATCC (DPTPA), **GGATCC**TTATCTACGTAGTCTATCTCTTG (YPTPA1), and TC**GGATCC**CTAATCAAAAGGTATTGG (YPTPA2) of the appropriate PTPA DNA. PCR reactions were carried out for 1 cycle of 5 min at 94 °C, 2 min at the appropriate annealing temperature calculated without taking the mutations and restriction sites into account, 3 min at 72 °C, followed by 30 cycles of 80 s at 94 °C, 2 min at the appropriate annealing temperature for the complete oligonucleotide (maximum 60 °C), and 3 min at 72 °C in a total volume of 50 μL containing 1 × buffer (Perkin-Elmer), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 15 pmol of sense and antisense primer, 100 ng of the appropriate DNA in pBluescript as template, and 2.5 units of Ultma DNA polymerase. The final mutated PCR product was subcloned into the pRSET expression vector (InVitroGen) using *Nde*I and *Bam*HI restriction sites present in both the fragments and vector, resulting in a native recombinant protein. The accuracy of the PCR reactions was verified by DNA sequencing of the construct.

BL21(DE3) bacteria were transformed with these plasmid constructs, and after induction of the bacterial culture with 0.4 mM IPTG for 2 h 30 min at 37 °C, the expressed recombinant proteins were purified according to (*27*), including phenyl Sepharose, DEAE Sephacel, monoP, and monoQ chromatography, unless otherwise specified.

In Vitro Transcription and Translation of Drosophila and Yeast PTPA DNA. To linearize the pRSET plasmids containing the complete ORF encoding *Drosophila* and yeast PTPA, respectively, both constructs were digested with *Eco*RI for transcription in the sense direction and *Drosophila* PTPA cDNA was digested with *Xho*I for transcription in the antisense direction.

mRNA was synthesized using the appropriate RNA polymerase (T3 and T7) and capped. Translation of mRNA was performed in 5 μL of rabbit reticulocyte lysate (Stratagene) supplemented with 2 μL of [^{35}S]methionine (15 mCi/mL; 1000 Ci/mmol; ICN) and 3 μg of mRNA. After incubation for 1 h at 30 °C, the reaction was quenched by adding 5 μL of 0.125 M Tris-HCl, pH 6.8, 3% SDS, 35% glycerol, 9% mercaptoethanol, and 0.02% bromophenol and boiled for 5 min. Translation products were separated by SDS−PAGE. Stained gels were dried and exposed to AGFA CURIX RP1 film to visualize the labeled translation products.

Site-Directed Point and Deletion Mutagenesis; Purification of the Recombinant Proteins. Two different methods were used to introduce point mutations and/or deletions in rabbit skeletal muscle PTPA. The point mutations Gly$_{61}$→Glu and Cys$_{165}$→Trp were generated with the "Transformer Site-directed Mutagenesis" kit (Clontech) with full-length rabbit PTPA cDNA in the pRSET expression vector (Invitrogen) as template. Ten picomoles of selection primer and the appropriate mutagenic primer were annealed to 100 ng of denatured double-stranded plasmid DNA (selection primer: AGCAGCCGGATC**T**AGCTTCGAATTCC, mutated in the unique *Hin*dIII site of the pRSET vector; mutagenic primer Gly$_{61}$→Glu: GCTCAGCTTCTTC**T**CCTTCACGCCTTCG; mutagenic primer Cys$_{165}$→Trp: CGATCTTGCACAG**C**-

Table 1: Oligonucleotides Used as Sense and Antisense Primers in the PCR Reaction To Create Point and Deletion Mutants

mutation	sense primer	antisense primer
Cys167→Ser	TGTCTGT**C**CAAGATCGGGGTGCTG	CAGCACCCCGATCTTG**G**ACAGACAGCACAGG
Arg173→Ala	CTG**GC**CGTGGACGACCAGATAGCC	TATCTGGTCGTCCACG**GC**CAGCACCCCGATCTTG
$\Delta M_{201}EPAGS_{206}$	AAAACCTACCGGCAGGGGGTGTGGGGCCTGG	CCACACCCCCTGCCGGGTAGGTTTTCTGGAGC
$\Delta G_{208}VWGLD_{213}$	GCCGGCAGCCAGGATTTCCAGTTCCTGCCC	GAACTGGAAATCCTGGCTGCCGGCGGGCTCC
$\Delta S_{146}TRID_{150}$	TCCGTGGGCAACTACGGCACAGGGCACGAAGC	CCCTGTGCCGTAGTTGCCCACGGACTCC
$\Delta Y_{151}GTGHE_{156}$	ACGCGCATCGACGCAGCCTTTGCCGCTTTCC	GGCAAAGGCTGCGTCGATGCGCGTGGAGTTGCCC

CAGCACAGGAAAGCGG). After second strand synthesis, the DNA was digested with *Hin*dIII and transformed in BMH71-18 *mutS* cells by electroporation. Plasmid DNA was isolated (mini-Qiagen, Westburg), digested with *Hin*dIII, and transformed in JM109 cells. Mutations were confirmed by DNA sequencing.

All other mutations were generated with a two-step PCR procedure: two initial PCR reactions were performed with a sense primer annealing to the start region (start primer: GGCTCCGCGG**CATATG**GCTGAGGG, *Nde*I site in boldface) and an antisense primer at regions to be mutated (see Table 1), and a PCR with an antisense primer annealing to the 3′UTR (UTR primer: GCCCGTCCGCTGCAGGT-**GAGCTC**AGC, *Sac*I site in boldface) and a sense primer annealing to the region to be mutated (see Table 1). For each mutant, these two PCR products were combined in a new PCR reaction with the START and 3′UTR primer. PCR reactions were performed in 50 μL reactions using 2.5 units of *Ultma* DNA polymerase (Perkin-Elmer), 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 15 pmol of primer, and 100 ng of template (rabbit cDNA in the case of the two first PCR reactions; the denatured and reannealed mixture of the initial two PCR products in the final PCR reaction) with 30 cycles of 80 s denaturation at 94 °C, 2 min annealing at the appropriate temperature, and 3 min elongation at 72 °C. This PCR product was digested with *Nde*I/*Sac*I and ligated in a *Nde*I/*Sac*I-digested pRSET vector.

The Δ267−323 truncated form of rabbit PTPA was constructed by deletion of an *Apa*I/*Eco*RI fragment in the PTPA-pRSET construct. *Apa*I cuts in the coding region of rabbit PTPA, resulting in a truncation at $glycine_{266}$.

BL21(DE3) bacteria were transformed with these different plasmid constructs and the recombinant proteins produced and purified as described for the *Drosophila* and yeast recombinant PTPA.

N-Terminal Amino Acid Sequencing. For N-terminal amino acid sequence analysis, 10−30 pmol of protein was loaded on a ProSorb membrane (Applied Biosystems) which was then washed 3 times with 150 μL of 5% CH_3CN in 0.1% TFA to remove the buffer salts. Subsequently, protein sequencing was performed on an Applied Biosystems Procise 492 sequencer operating in the pulsed-liquid mode.

Immunoblotting and PTPA Activity Measurement. Western blots were developed by using the affinity-purified anti-rabbit PTPA goat antibodies as described in (*27*). PTPA was assayed as described (*27*, and with many details in *35*), using tyrosyl phosphorylated RCM lysozyme as substrate. The assay is based on the increase in phosphotyrosyl phosphatase activity of the dimeric form of PP2A that is exogenously added. One unit of PTPA activates 50% of the potential PTPase activity of 2−5 units/mL $PP2A_D$. A unit of PTPase catalyzes the release of 1 nmol of phosphate/min using 1 μM RCM lysozyme in a 30 μL assay.

RESULTS

Molecular Cloning of Drosophila melanogaster PTPA. With the *Xenopus* PTPA cDNA as a probe, we screened a *Drosophila* embryo cDNA library and isolated three independent clones. The longest clone, D3.1., with a size of 1.3 kb, was further analyzed and sequenced. It contains a potential ORF of 1196 bp, flanked by a 5′ and 3′ untranslated region of 41 and 99 bp, respectively. The D3.1. cDNA encodes a protein of 399 amino acids with a C-terminal extension of 89 amino acids relative to vertebrate PTPA (Figure 1). The putative start codon for this ORF is located at nucleotides 42−45 of the D3.1. cDNA, but is not preceded by the *Drosophila* consensus sequence of initiation of translation (*36*; C/AAAC/AATG) or by a stop codon in any of the three reading frames. Therefore, it is not excluded that the complete ORF of *Drosophila* PTPA is elongated at the 5′ end. However, all efforts to isolate a 5′ extended *Drosophila* PTPA cDNA by PCR with a 5′ specific antisense oligonucleotide and the T3 sense oligonucleotide as primers were unsuccessful. The deduced amino acid sequence was 47% and 50% identical to the mammalian and *Xenopus* PTPA, respectively. In vitro transcription and translation of the *Drosophila* PTPA cDNA revealed a translation product of 45 kDa and minor bands of 49, 40, 38, 36, and 28 kDa (Figure 2). The predicted molecular mass of *Drosophila* PTPA, based on the deduced amino acid sequence, is 45 285 Da. The lower size translation products are likely to be due to usage of internal initiation sites or perhaps partial degradation. The 49 kDa translation product in fact is the full-length protein (see further below). Expression of the *Drosophila* PTPA cDNA in *E. coli* resulted in a recombinant protein with moderate solubility. The soluble fraction of the expressed protein displayed PTPA activity toward rabbit $PP2A_D$. Purification of the soluble recombinant protein resulted in two peaks of PTPA activity in the last purification step (monoQ chromatography; not shown). One peak of PTPA activity is produced by a pure 36 kDa protein. Amino acid sequencing of the N-terminus revealed the sequence ASGINQA, indicating that the correct initiation site was used by the protein synthesis machinery of *E. coli*, but that the translation product was probably subject to proteolysis at the C-terminal region. The other peak of PTPA activity was a mixture of two proteins of 36 and 49 kDa. These two proteins could be separated from each other on a Superdex 200 gel filtration column, and both displayed PTPA activity (Figure 3). Amino acid sequencing of the N-terminus of the 49 kDa protein revealed the sequence ASGINQA as expected. Therefore, this protein represents the intact *Drosophila* PTPA (DPTPA). Measurement of the PTPA activity of the 49 and 36 kDa proteins in linear conditions revealed a specific activity of 95×10^3 and 80×10^3 units/mg, respectively.

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HPTPA    MAEGERQPPP DSSEEAPPAT QNFIIPKKEI HTVPDMGKWK RSQAYADYIG 50
RPTPA    MAEGERQPPP DSSEETPPAA QNFVIPKKEI HTVPDMGKWK RSQAYADYIG
XPTPA    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DPTPA    ~~~~~~~~~~ ~MASGINQAA GKLPAIAKKV QNLGDMGVWQ KSRAFHDLIG
YPTPA1   ~~~~~~~~~~ MSLDRVDWPH ATFSTPVKRI FDTQTTLDFQ SSLAIHRIKY
YPTPA2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~MLPEKRL LTPDDMKLWE ESPTRAHFTK

HPTPA    FILTLNEGVK GKKLT.FEYR VSE..AIEKL LALLNTLDRW IDETPPVD.. 95
RPTPA    FILTLNEGVK GKKLS.FEYK VSE..AVEKL LALLDTLDRW IDETPPVD..
XPTPA    ~~~~~~RAVK GKKLT.DDYT VSE..VIHKL MALLDTLDRW IDETPPMD..
DPTPA    YINGTSSAIQ GIKTT.DEIF ESE..MLKKL LRLFDALEKL VEQNPPLE..
YPTPA1   HLHKYTTLIS HCSDPDPHAT ASSIAMVNGL MGVLDKLAHL IDETPPLPG.
YPTPA2   FIIDLAESVK GHENSQYKEP ISE..SINSM MNLLSQIKDI TQKHPVIKDA

HPTPA    QPSRFGNKAY RTWYAKLDEE AENLVATVVP THLAAAVPEV AVYLKESVGN 145
RPTPA    QPSRFGNKAY RTWYAKLDEE AEGLVAAVVP AHLAAAVPEV AVYLKESVGN
XPTPA    QPSRFGNKAF RTWYARLDKE AESLVSTVIP VHLSAAVPEV AVYLKESVGN
DPTPA    QPQRFGNKAY RDWAQAMREL LPELLEQLLP DDKKRYQVEL GQYLTESFGN
YPTPA1   .PRRYGNLAC REWHHKLDER LPQWLQEMLP SEYHEVVPEL QYYLGNSFGS
YPTPA2   DSSRFGKVEF RDFYDEVSRN SRKILRSEFP SLTDEQLEQL SIYLDESWGN

HPTPA    STRIDYGTGH EAAFAAFLCC LCKIGVLRV. DDQIAIVFKV FNRYLEVMRK 194
RPTPA    STRIDYGTGH EAAFAAFLCC LCKIGVLRV. DDQIAIVFKV FNRYLEVMRK
XPTPA    STRIDYGTGH EAAFAAFLCC LCKIGVLKV. DDQHAIVFRV FNRYLEVMRK
DPTPA    ATRIDYGTGH ELSFLFFLCS LFKAEILQE. RDIVLGACAE LPRYLEVARQ
YPTPA1   STRLDYGTGH ELSFMATVAA LDMLGMFPHM RG..ADVFLL FNKYYTIMRR
YPTPA2   KRRIDYGSGH ELNFMCLLYG LYSYGIFNLS NDSTNLVLKV FIEYLKIMRI

HPTPA    LQKTYRMEPA GSQGVWGLDD FQFLPFIWGS SQ...LIDHP YLEPRHFVDE 241
RPTPA    LQKTYRMEPA GSQGVWGLDD FQFLPFIWGS SQ...LIDHP FLEPRHFVDE
XPTPA    LQKTYRMEPA GSQGVWGLDD FQFLPFIWGS AQ...LVDHS TLEPRHFVDE
DPTPA    LQRTYNMEPA GSQGVWSLDD FQFVPFIWGS AQ...LAVKS PFDPSKFVDE
YPTPA1   LILTYTLEPA GSHGVWGLDD HFHLVYILGS SQWQLLDAQA PLQPREILDK
YPTPA2   LETKYWLEPA GSHGVWGLDD YHFLPFLFGA FQ...LTTHK HLKPISIHNN
                                                   ↓
HPTPA    KAVNENHKDY MFLECILFIT EMKTGPFAE. HSNQLWNIS. AVPSWSKVNQ 289
RPTPA    KAVNENHKDY MFLECILFIT EMKTGPFAE. HSNQLWNIS. AVPSWSKVNQ
XPTPA    KIVNENHKDY MFLECILFIT EMKTGPFAE. HSNQLWNIS. AVPAWSKVNQ
DPTPA    AIITEYKDHF MFISCIDYIC KVKTGHFGE. HSNQLWSIT. DVPTWAKINA
YPTPA1   SLVREYKDTN FYCQGINFIN EVKMGPF.EE HSPILYDIAV TVPRWSKVCK
YPTPA2   ELVEMFAHRY LYFGCIAFIN KVKSSASLRW HSPMLDDIS. GVKTWSKVAE

HPTPA    GLIRMYKAEC LEKFPVIQHF KFGS.LLPIH PVTSG*~~~~ ~~~~~~~~~~ 323
RPTPA    GLIRMYKAEC LEKFPVIQHF KFGS.LLPIH PVTSG*~~~~ ~~~~~~~~~~
XPTPA    GLIRMYKAEC LEKFPVIQHF KFGS.LLPIQ PVKSG*~~~~ ~~~~~~~~~~
DPTPA    GLVKMYQKEI LSKFPVIQHV YFGE.LMTFE PVSSGTTLSN ALLGHVAPPP
YPTPA1   GLLKMYSVEV LKKFPVVQHF WFGTGFFPWV NIQNGTDLPV FEEKEEESIE
YPTPA2   GMIKMYKAEV LSKLPIMQHF YFSE.FLPC. PDGVSPPRGH IHDGTDKDDE

HPTPA    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
RPTPA    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
XPTPA    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DPTPA    SKRICIGTPN LVPPVPVATA PPPPAESLSI EQNVGDSSSE SSDNSVVLRP
YPTPA1   QANAGSPGRE QTSTRFPTST SMPPPGVPPS GNNINYLLSH QNQSHRNQTS
YPTPA2   CNFEGHVHTT WGDCCGIKLP SAIAATEMNK KHHKPIPFD* ~~~~~~~~~~

HPTPA    ~~~~~~~~~~ ~~~~~~~~~~ ~
RPTPA    ~~~~~~~~~~ ~~~~~~~~~~ ~
XPTPA    ~~~~~~~~~~ ~~~~~~~~~~ ~
DPTPA    STSSSSLVAA AEGSGDKPSK E*
YPTPA1   FSRDRLRR*~ ~~~~~~~~~~ ~
YPTPA2   ~~~~~~~~~~ ~~~~~~~~~~ ~
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FIGURE 1: Alignment of the primary structures of PTPA from different species. The human PTPA sequence is used as a reference for numbering of amino acids. The most conserved regions, subject of deletion mutants in this study, are boxed. Putative functional domains (see text) are underlined, and the amino acids thought to be essential for this function are doubly underlined. The arrow indicates the site of truncation of the Δ267−323 mutant.

Molecular Cloning of the Saccharomyces cerevisiae PTPA Gene. PTPA was previously purified from *Saccharomyces cerevisiae* (*27*). However, attempts to clone PTPA from yeast with vertebrate PTPA as a probe failed. In addition, by Southern blot analysis of fission and budding yeast genomic DNA with vertebrate PTPA cDNA as a probe, no

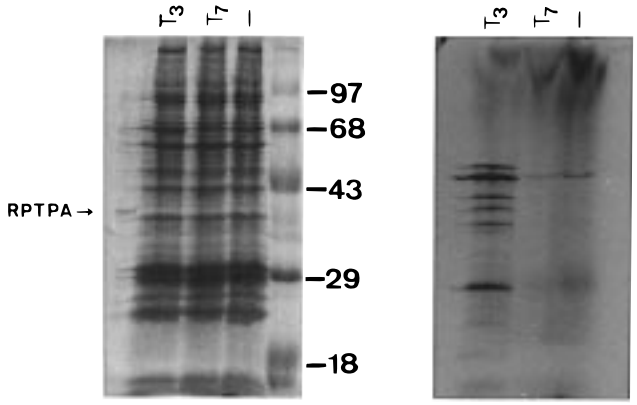

FIGURE 2: In vitro tanscription/translation of *Drosophila* PTPA. Sense (T3), antisense (T7), and no (−) *Drosophila* PTPA mRNA were transcribed in a reticulocyte lysate as described under Experimental Procedures. Methionine-labeled products were analyzed by SDS−PAGE, stained by Coomassie blue (left panel) or autoradiographed (right panel). The 37 kDa rabbit recombinant PTPA (RPTPA) and molecular weight markers were loaded in lanes 1 and 5, respectively.

signal was detected. Screening of the EMBL database with the human PTPA amino acid sequence revealed the presence of a *Saccharomyces cerevisiae* ORF (accession number: Z38059), located at chromosome IX (bp 16227−17408), and encoding a protein of 393 amino acids which is 38% identical to human PTPA. A PCR product was made with oligonucleotides present in conserved regions of the yeast PTPA-like gene and *Saccharomyces cerevisiae* genomic DNA as template. With this probe, we detected by Southern blot analysis of yeast genomic DNA a single PTPA-like gene in budding yeast, whereas fission yeast genomic DNA did not hybridize with this budding yeast specific probe (data not shown). This probe was also used to screen a *Saccharomyces cerevisiae* genomic DNA library, resulting in the isolation of six independent clones with an average size of 10 kb. All clones contained the complete ORF in a *Hin*dIII/*Xho*I fragment of 2 kb which was subcloned in pBluescript for further analysis. DNA sequence analysis of this 2 kb fragment revealed that the ORF contains 1179 bp, encoding a protein of 393 amino acids extending the vertebrate PTPA with a C-terminal amino acid tail of 73 amino acids. The deduced amino acid sequence was 100% identical to the sequence of the database. Yeast PTPA is only 29% identical to *Drosophila* PTPA because of the complete divergence of the two extended C-terminal amino acid tails. Whereas the C-terminal tail of *Drosophila* is proline (14%) and serine (17.5%) rich, the C-terminal extension of yeast PTPA contains a very acidic region (AA327−335). Comparison of both C-terminal regions with the EMBL database revealed no significant similarity with other proteins. Therefore, these C-terminal extensions might assign an additional function to PTPA in these species such as protein−protein interaction (a proline-rich domain is a feature of proteins which can bind to SH3 domains or WW repeats) (*37*) and posttranslational modifications such as phosphorylation or subcellular localization. The ORF of yeast PTPA DNA was subcloned in a prokaryotic expression vector and expressed in *E. coli*. The recombinant yeast protein had PTPA activity as shown in Figure 4. After monoQ chromatography, eluted fractions

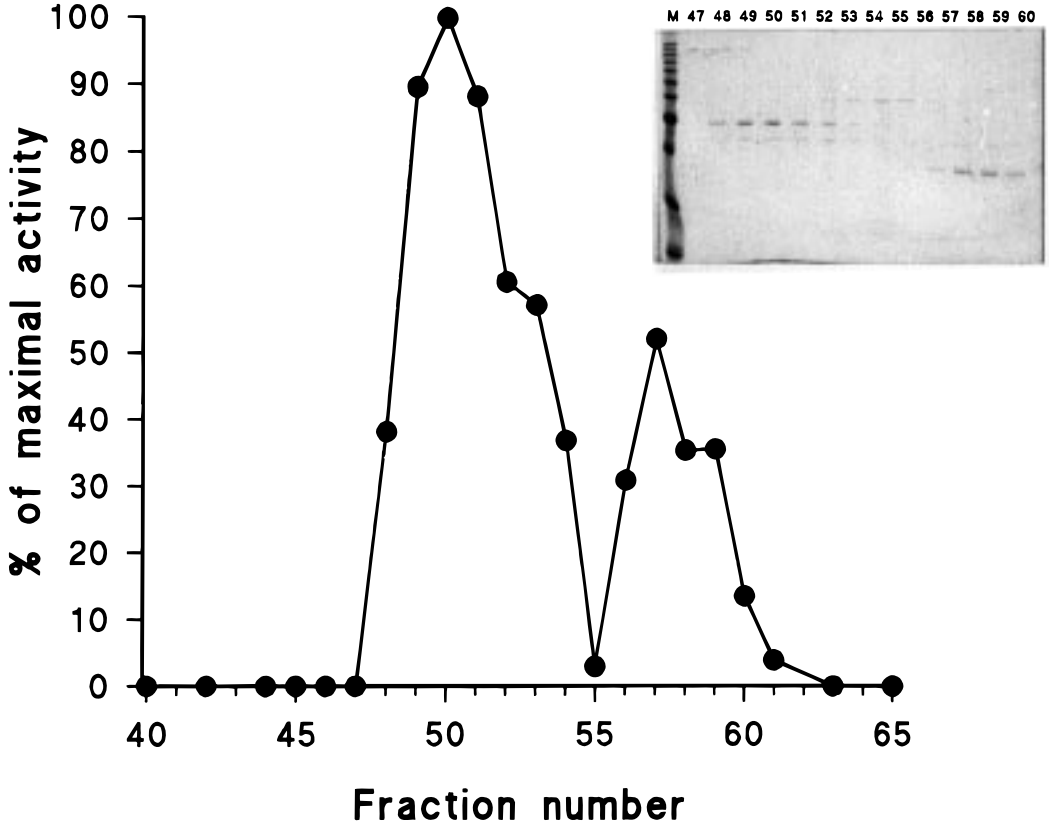

FIGURE 3: Purification and PTPA activity of recombinant *Drosophila* PTPA. The mono Q fraction, containing both a 36 kDa and a 49 kDa *Drosophila* PTPA protein, was separated by gel filtration (Superdex 200) and assayed for PTPA activity after a 200-fold dilution. The insert shows Coomassie-stained SDS−PAGE of the indicated fractions. M represents the 10 kDa marker ladder (Gibco), the most intense band being the 50 kDa marker.

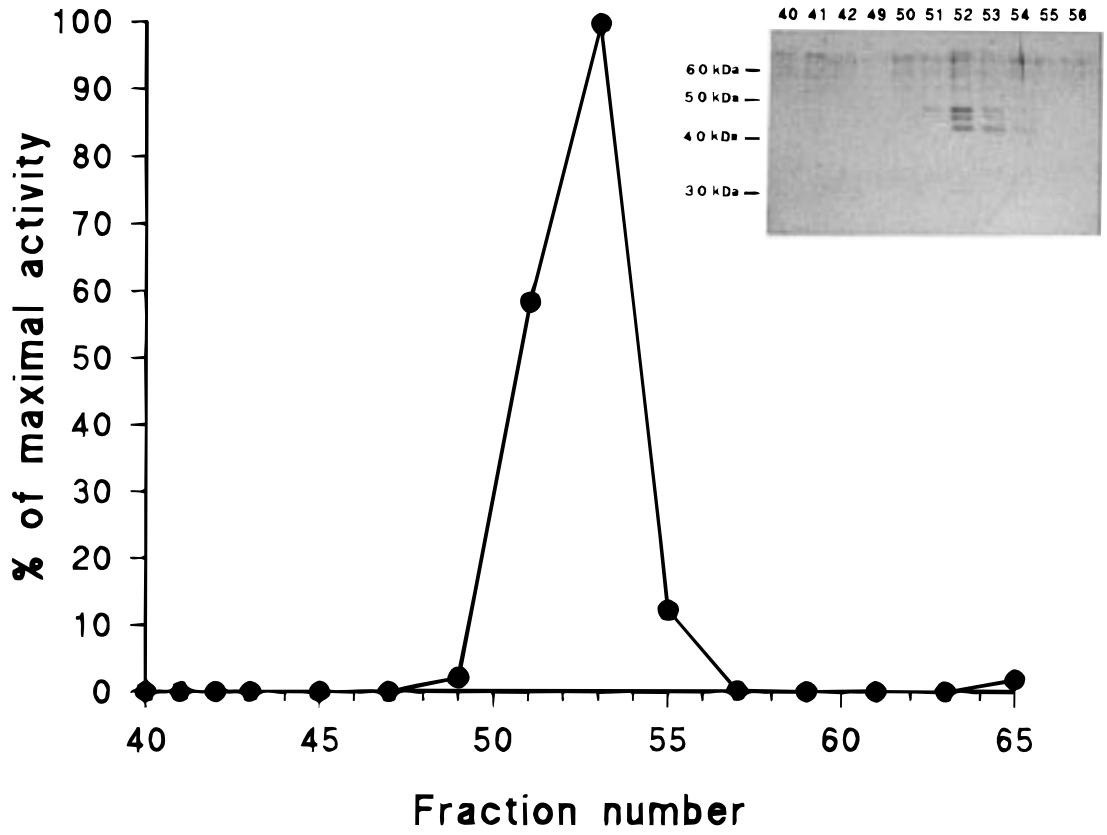

FIGURE 4: Purification and PTPA activity of recombinant yeast PTPA. Recombinant yeast PTPA1 was purified as described under Experimental Procedures, and the elution pattern of PTPA activity (●) and protein pattern (Coomassie-stained SDS−PAGE of indicated fractions, inset) during the last purification step (mono Q) are shown. Activity is expressed as a percentage of the peak activity.

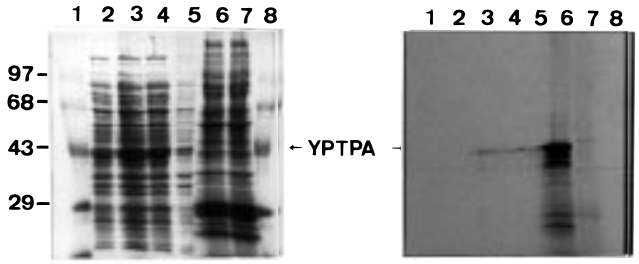

FIGURE 5: In vitro transcription/translation of yeast PTPA. Yeast recombinant PTPA1 was produced in *E. coli* (lanes 2−5) or reticulocyte lysates (lanes 6 and 7) as described under Experimental Procedures. Shown is the Coomassie stain of SDS−PAGE (left panel) and the autoradiogram (right panel) of the same gel. Lanes 1 and 8, molecular weight markers; 2, noninduced total bacterial extract; 3, total extract after IPTG induction; 4, supernatant, and, 5, pellet after a low-speed centrifugation of the induced extract; 6, supernatant of reticulocyte lysate supplemented with YPTPA mRNA; 7, same as 6 without added mRNA.

with PTPA activity contained three proteins with molecular masses of 47, 44, and 40.5 kDa, respectively (Figure 4). All three protein bands probably represent the purified yeast recombinant protein, because they show the same migration profile as the translation products generated by in vitro transcription/translation of the yeast PTPA gene subcloned in the pRSET expression vector (Figure 5). These are probably generated by different sites of initiation of translation or by partial proteolysis. The highest molecular mass form had a molecular mass of 47 kDa, which is in agreement with the predicted molecular mass of the deduced amino acid sequence (45 082 Da); the slightly slower migration in SDS−PAGE is similar to that observed for *Drosophila* PTPA (see above). Measurement of the PTPA activity of the pooled fractions (*51*−*54*, Figure 4) in linear conditions revealed a specific activity of 105×10^3 units/mg, indicating that the three proteins are probably active.

Conserved Regions in PTPA from Yeast to Human Reveal a Second Yeast PTPA Homologue. Alignment of the amino acid sequences of PTPA from yeast to human revealed only a few regions that are highly conserved in the PTPA protein (Figure 1). Comparison of these conserved regions with protein sequences from the NCBI database, using the Blast program, resulted in one extra putative ORF which contains all of these conserved regions. This sequence was part of a 55 kb fragment on the left arm of chromosome XVI of *Saccharomyces cerevisiae* and was sequenced by Purnelle et al. (*38*) as part of the yeast genome sequencing project (accession number: X96770). This second homologue of PTPA in yeast, hereafter referred to as YPTPA2, has an ORF of 1074 bp, encoding a protein with 358 amino acid residues and a predicted molecular weight of 41 452. YPTPA2 is 27 and 36% identical to *Drosophila* and vertebrate PTPA, respectively. However, YPTPA2 shows only 25% analogy with its yeast homologue previously described in this report (YPTPA1). YPTPA2 has a C-terminal extension relative to vertebrate PTPA, which does not show any homology with the C-terminal extensions of YPTPA1 or DPTPA. The ORF of yeast PTPA2 was subcloned in a prokaryotic expression vector and expressed in *E. coli*. The recombinant yeast protein was purified according to standard methods (see Experimental Procedures), but no PTPA activity could be detected in the crude extract or during the purification. However, in the absence of a specific detection method of

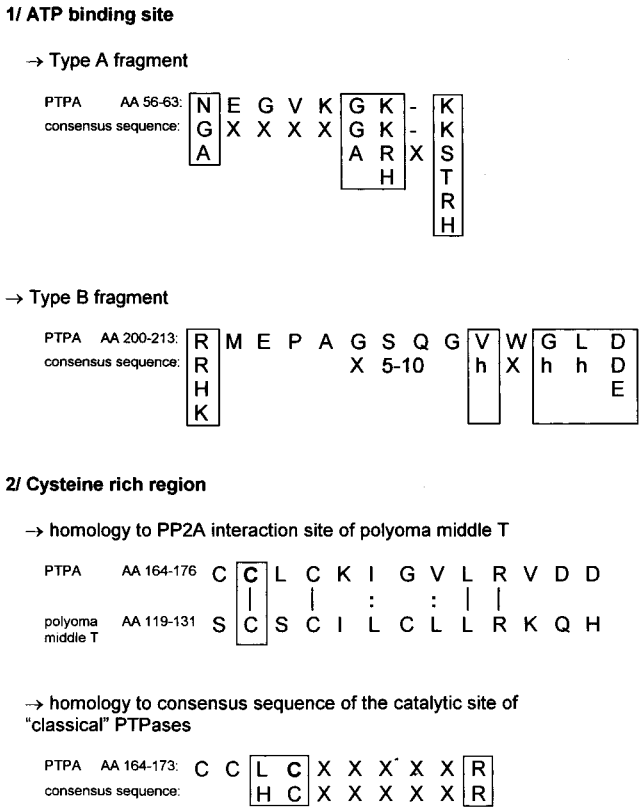

FIGURE 6: Comparison of putative functional domains of mammalian PTPA with the appropriate consensus sequences. The essential amino acid residues defining the consensus sequences are indicated with boxes.

YPTPA2 protein, this is not conclusive evidence that YPTPA2 has no PTPA activity, even if a protein of the correct size is expressed in reticulocyte lysates (data not shown).

Mutational Analysis of Highly Conserved Regions of PTPA. Sequence alignment of PTPA from yeast to human reveals highly conserved stretches of amino acids (Figure 1). The high conservation of these stretches suggests an important role in the function of PTPA. Therefore, different deletion mutants were constructed and their functionality investigated.

One of the most highly conserved regions is R_{200}-MEPAGSQGVWGLD$_{213}$, previously identified as being related to the consensus type B fragment of the putative ATP binding site of PTPA (*5*; see Figures 6 and 7). Only the basic residue at the N-terminus of this motif (R_{200}) is less well conserved (see Figure 1). This B fragment can be divided into a variable region consisting of an amino acid stretch of 5−10 amino acids (M_{201}EPAGS_{206}, in the context of PTPA) that separates this basic residue from the more stringent hydrophobic region, followed by an acidic residue (G_{208}VWGLD$_{213}$). Deletion of either of these two regions in the context of the rabbit recombinant PTPA affected PTPA activity. The Δ201−206 PTPA mutant shows a 7-fold reduction in PTPA activity (Figure 7A), whereas the Δ208−213 PTPA mutant displayed 400-fold less activity (Figure 7B). These results suggest that this region is important for PTPA activity. The fact that deletion of the more stringent hydrophobic region of the type B fragment has more severe effects than deletion of the variable, PTPA-specific residues (defined as X in the consensus sequence; see Figure 6)

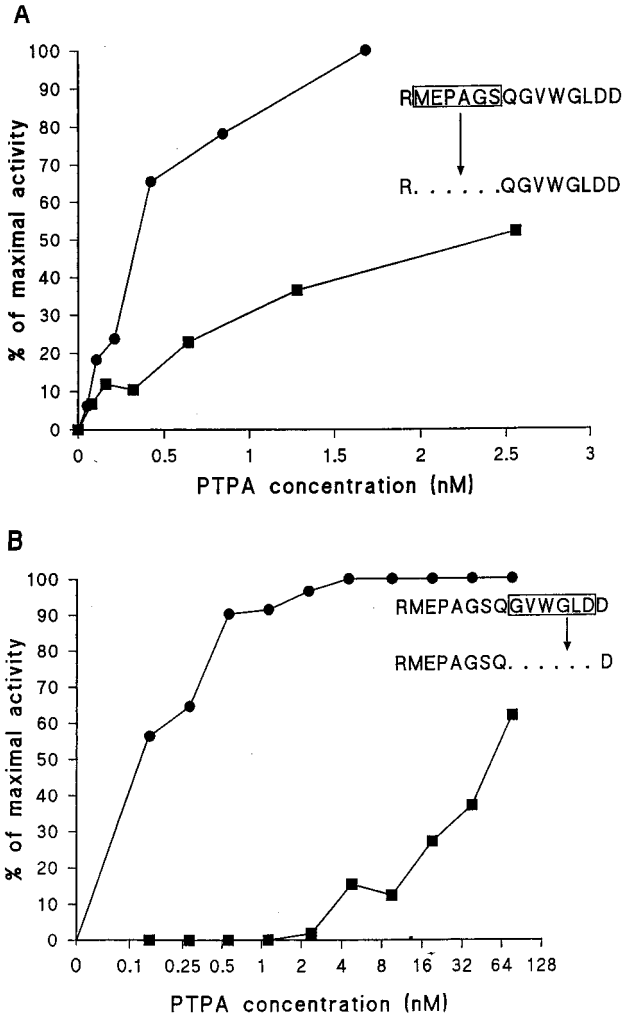

FIGURE 7: Comparison of PTPA activity of wild type, ΔM_{201}-EPAGS_{206}, and ΔG_{208}VWGLD_{213}. In panel A, PTPA activities of different concentrations of purified wild type (●) and ΔM_{201}-EPAGS_{206} (■) were compared using 10 nM $PP2A_D$ in the assay. In panel B, wild-type PTPA (●) was compared with ΔG_{208}-VWGLD_{213} (■), using 20 nM $PP2A_D$.

suggests that there is a relation between the homology of this region with the type B fragment of the ATP binding site and the requirement of ATP in the activation of $PP2A_D$ by PTPA.

The three other well-conserved regions among PTPA proteins of all species (Figure 1) did not show any similarity with other proteins or motifs present in the EMBL database. Their functional significance for PTPA activity was investigated by constructing 3 different deletion mutants: ΔS_{146}-TRID_{150}, ΔY_{151}GTGHE_{156}, and Δ267−323. ΔS_{146}TRID_{150} and ΔY_{151}GTGHE_{156} represent two deletion mutants in the first conserved domain, whereas Δ267−323 represents a C-terminally truncated PTPA, missing the two last conserved regions. If induced at 20 °C, the three recombinant proteins are expressed partially as soluble protein as detected by Western blot analysis. In none of the crude extracts could PTPA activity be detected. Therefore, these proteins could not be purified by following their activity. Using Western blot as the detection method (see Figure 8 and not shown), these deletion mutants were partially purified through the mono P step (except the Δ151−156 mutant that was employed after the DEAE Sephacel step). None of these mutants displayed PTPA activity at concentrations up to 100

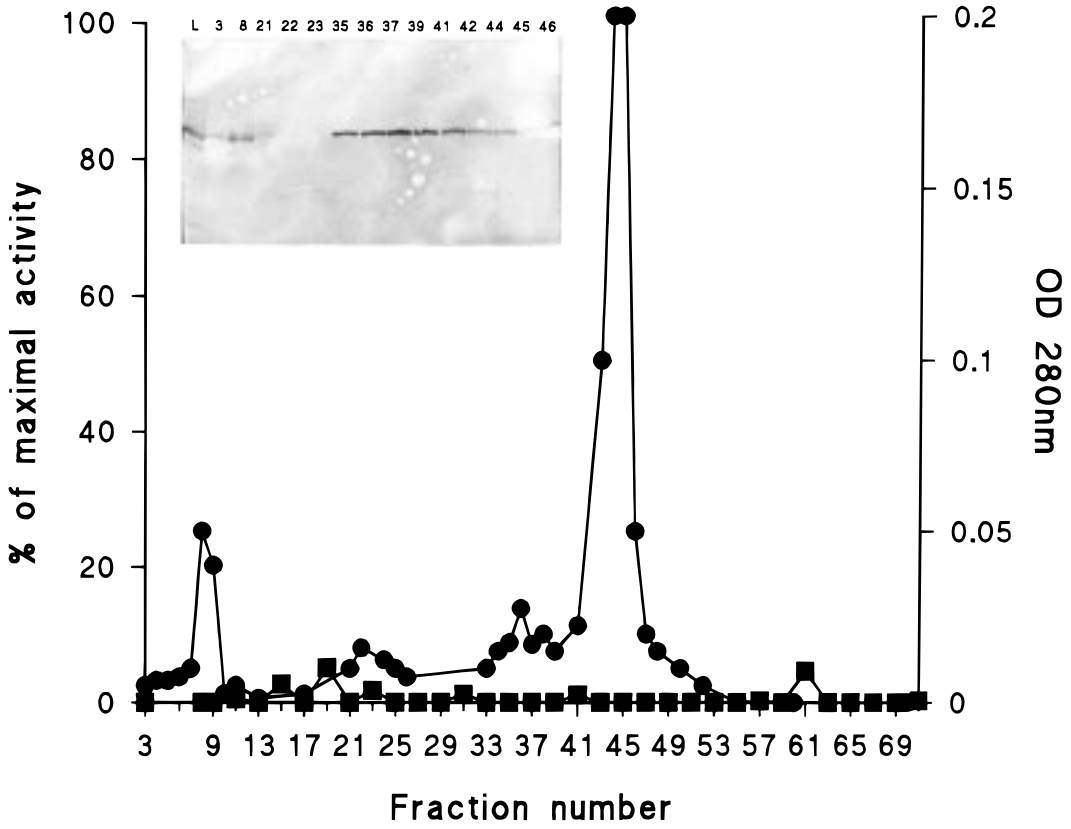

FIGURE 8: Mono P chromatography of the $\Delta S_{146}TRID_{150}$ recombinant mutant PTPA. Mutant PTPA was purified as described under Experimental Procedures. The elution profile of the protein (OD_{280}, ●) and activity measurements (■) of the indicated fractions are shown. Activity is expressed as a percentage of a control assay where $PP2A_D$ was maximally activated by wild-type PTPA. The inset shows the Western blot of the indicated fractions and the column load (L), developed with anti-rabbit PTPA antibodies.

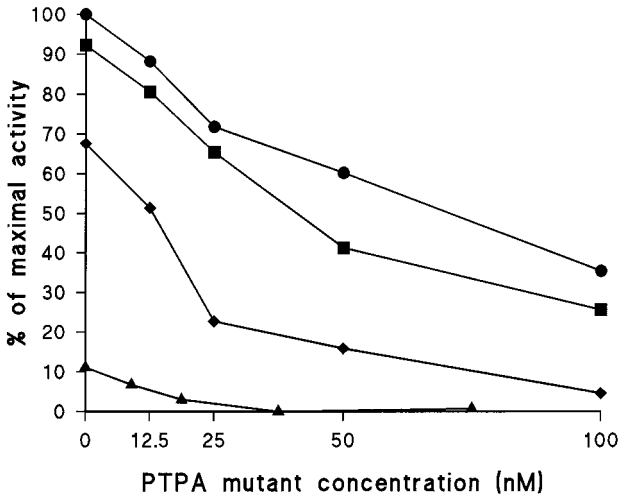

FIGURE 9: Inhibition of wild-type PTPA by the Δ267−323 PTPA deletion mutant. The indicated concentrations of mutant PTPA were included in the standard PTPA assay where 6.6 nM (●), 2.2 nM (■), 0.7 nM (◆), or no (▲) wild-type PTPA was used to activate the PTPase activity of 20 nM PP2A, in the presence of 0.6 mM ATP and 3 mM $MgCl_2$.

nM (Δ146−150), 50 nM (Δ151−156), or 300 nM (Δ267−323). The mutants were further tested for their ability to inhibit wt PTPA. The Δ267−323 mutant was found to act competitively as an inhibitor (Figure 9).

PTPA Point Mutations in the A Fragment of the Putative ATP Binding Domain and in the Cysteine-Rich Region. Previous studies (*5*, *28*) suggested two domains as being potentially important for the activity of PTPA: a putative ATP binding site, consisting of a type A and a type B fragment (*32*); and a cysteine-rich region (C_{164}**C**L**C**KIGV-L**R**$_{173}$), showing some similarity to the polyoma middle T−PP2A interaction site (*5*, *39*) and to the consensus sequence of "classical" PTPases (Figure 6). We examined these domains for their functional significance by site-directed mutagenesis.

First, mutation in the putative A fragment $N_{56}EGVKGKK_{63}$ of the essential glycine$_{61}$ into an acidic glutamic acid does not result in any change of PTPA activity or K_m for ATP (data not shown). Second, Cys_{165} in the cysteine-rich region was mutated into a Trp, since it is known that such mutation in the context of polyoma mT (see Figure 6) disrupts the interaction of mT with the 65 kDa regulatory subunit of PP2A (*39*). Direct determination of the association of PTPA with PP2A was not possible, since PP2A binds with very low affinity to PTPA (*27*). Therefore, the impact of this mutation on the PTPA−PP2A interaction was measured as a change in PTPA activity. Mutation of Cys_{165} affects the solubility of this protein, since it is deposited in inclusion bodies when expressed at 37 °C. However, when the bacteria were induced at lower temperature (25 °C), a significant fraction of the expressed protein remains in the soluble fraction after lysis of the bacteria. During purification of the mutant protein, the Cys_{165} mutant behaved differently compared to wild-type PTPA. While the wild-type protein eluted from the phenyl-Sepharose with 50% ethylene glycol, the mutant PTPA (±90%) remained bound to the hydrophobic resin under these conditions. The mutant PTPA could be partially recovered by further elution with 50% glycerol.

Hence, the Cys_{165}→Trp mutation of PTPA appears to change the hydrophobicity of PTPA, suggesting that the cysteine cluster of PTPA might be important for the conformation of the protein. We compared the PTPA activity of the partially purified mutant (following phenyl-Sepharose and DEAE-Sephacel chromatography) with the PTPA activity of pure wild-type protein and found no significant change in PTPA activity (data not shown).

Finally, the primary sequence C_{167}KIGVLR$_{173}$ resembles an imperfect PTPase signature lacking the His in front of the essential Cys (see Figure 6) (*40*). It is possible that Cys_{167} and Arg_{173} create "in trans", with the catalytic subunit of PP2A, a PTPase catalytic site. To test this hypothesis, Cys_{167} was mutated into a Ser, and Arg_{173} was mutated into an Ala. Neither mutation affected the PTPA activity (data not shown).

DISCUSSION

In this paper, we present a biochemical analysis of mutations and deletions in putative functional domains of PTPA and their conservation among PTPA from different species after molecular cloning of PTPA from *Drosophila* and *Saccharomyces cerevisiae*. *Drosophila* and budding yeast PTPA are 47% and 38% (YPTPA1) or 36% (YPTPA2) identical to vertebrate PTPA, respectively. YPTPA1 and YPTPA2 are the most divergent proteins among all PTPA proteins cloned, with only 25% identity. This might explain why only one single gene was detected in the *Saccharomyces cerevisiae* genomic DNA using YPTPA1 as probe. The PTPA-like proteins from both species have C-terminal extensions with completely different features. This results in identities between yeast and *Drosophila* PTPA of only 29% (YTPA1) or 27% (YPTPA2). The role of these extensions in the function of PTPA is not clear, but seems to be unrelated to the activity of PTPA in vitro. This conclusion is based on several observations: (1) PTPA from vertebrates has no C-terminal extension but displays a similar PTPA activity as DPTPA [120×10^3 units/mg for rabbit muscle PTPA (*27*) compared to 95×10^3 units/mg for DPTPA]; (2) the 36 kDa form of PTPA from *Drosophila*, lacking the largest part, if not the total C-terminal extension, has a PTPA activity (80×10^3 units/mg) that is very similar to the PTPA activity of the intact 49 kDa protein (with the C-terminal extension, see Figure 3); (3) YPTPA1 has a completely different C-terminus compared to DPTPA, but a similar PTPA activity (105×10^3 units/mg). When DPTPA and YPTPA1 were expressed in *E. coli*, both recombinant proteins displayed PTPA activity toward rabbit $PP2A_D$. Therefore, *Drosophila* and also *Saccharomyces cerevisiae* have at least one functional homologue of mammalian PTPA. The second yeast PTPA homologue, YPTPA2, does not display PTPA activity under our experimental conditions. In the absence of a specific detection method to follow the YPTPA2 protein, this conclusion is equivocal. If, however, this protein does not display PTPA activity, it would mean that the conserved regions, that seem to be necessary for PTPA activity (see further below), are not sufficient. Further research will be necessary to clarify this issue.

While this paper was in preparation, the PTPA homologue of *S. pombe* (accession number: Z98980) was deposited in the database. In addition, an EST sequence of *Ricinus communis* (accession number: T14877), encoding a PTPA-like protein from AA47−173 with 43% identity to human PTPA, was also found by database screening. The first highly conserved region (Figure 1) is present in this sequence. Therefore, the highly conserved regions found by alignment of PTPA from different species (Figure 1) represent new motifs for an essential cellular function.

ATP is an essential cofactor in the activation of PP2A by PTPA, and the primary structure of vertebrate PTPA reveals an imperfect consensus sequence of a type A and a type B fragment of a potential ATP binding site. Therefore, we investigated the functional significance of these domains by site-directed mutagenesis. Mutation of a Gly_{61} into a glutamic acid would destroy ATP binding and therefore PTPA activity. However, this point mutation did not change PTPA activity nor ATP dependency, indicating that this residue is not essential for the function of PTPA. Hence, the potential type A fragment of the ATP binding site is probably not functional and certainly not essential for PTPA activity. This conclusion is further confirmed by sequencing PTPA from *Drosophila* and *Saccharomyces cerevisiae*, since in both cases PTPA does not contain this type A fragment. In contrast, the type B fragment of the potential ATP binding site is well conserved in all species, with the exception of the basic residue at the N-terminus (R200) of this motif which is replaced by Asn in *Drosophila* and Thr in yeast (see Figure 1). Deletion of M_{201}EPAGS_{206}, or G_{208}-VWGDL_{213}, results in a PTPA protein with 7- and 400-fold, respectively, less activity. This region is obviously important for PTPA activity. Nevertheless, further research is required to determine whether this region is responsible for the ATP dependency of PTPA activity.

While only the vertebrate sequences of PTPA were known, the cysteine-rich region (L_{163}CCLCR_{168}) was considered to be important for PTPA activity. In part this is because the sequence is unusual but also because there is some similarity with a cysteine cluster present in polyoma mT. The mT cysteine cluster was shown to be essential for the interaction with PP2A, since amino acid substitution of Cys_{120} by Trp prevents binding of PP2A to mT (*39*). An equivalent replacement of Cys_{165} of PTPA by Trp resulted in a dramatic change in the hydrophobicity/conformation of the PTPA protein, so that it was present exclusively in inclusion bodies on induction at 37 °C. A soluble mutant protein, obtained by low-temperature induction, showed a distinct behavior during the first purification step compared to the wild-type protein, but had a rather similar PTPA activity. Hence, the PTPA activity was not abolished by the Cys_{165}→Trp substitution. Therefore, the cysteine cluster might not be directly implicated in the interaction of PTPA with PP2A, but would be an essential structural determinant. The absence of the cysteine cluster in lower organisms is further evidence that it is not important for the function of PTPA.

An arginine residue is located six amino acids C-terminal to Cys_{167}. One could argue that this is at the right distance to form the consensus sequence of the catalytic site of a "classical" PTPase (*40*). The lack of a histidine residue at position −1 of Cys_{167} into PTPA could be delivered in trans by PP2A, resulting in an active PTPA−PP2A complex displaying PTPase activity. However, amino acid substitutions of Cys_{167} and Arg_{173} in PTPA showed that these residues are not essential for PTPA activity, indicating that

the catalysis of the PTPase activity is quite different from classical PTPases. The absence of the cysteine-rich region in *Drosophila* and in both yeast PTPA proteins supports these results. In summary, we can conclude that none of the point mutations that were introduced were critical for ATP binding or catalysis.

We performed deletions in the most conserved regions of PTPA observed after alignment of the amino acid sequence of all different species. Comparison of these domains with proteins and motifs present in the EMBL database did not provide any further insights. However, deletion of short stretches ($\Delta S_{146}TRID_{150}$ or $\Delta Y_{151}GTGHE_{156}$) in one of these regions or omission of the C terminus, containing two other highly conserved regions, resulted in complete loss of function. These mutant proteins were partially soluble. During purification, they could be followed by Western blot analysis and behaved as the native protein. Therefore, the global conformation of these proteins does not seem to be affected drastically, while the effects of the deletions are specific. Interestingly, the truncated PTPA Δ267−323 can inhibit native PTPA activity and therefore behaves as a dominant negative mutant. This indicates that this deletion mutant can compete probably with the binding of PTPA to $PP2A_D$, resulting in an inactive complex. This also indicates that the global conformation of this PTPA is retained. Furthermore, since this mutation is dominant negative, it might inhibit the PTPA activity in vivo and may be used to examine the physiological role of PTPA.

Finally, we can conclude that the cloning of *Drosophila* and the two yeast PTPA homologues revealed highly conserved regions that are essential but probably not sufficient for PTPA activity. Deletion mutants in these regions resulted in less active or inactive PTPA. One of these regions matches a region previously identified as the B fragment of a potential ATP binding site and is therefore probably important for that function.

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