The protein phosphatase 2A (PP2A) phosphatase activator (PTPA) is an essential protein involved in the regulation of PP2A and the PP2A-like enzymes. In this study we demonstrate that PTPA and its yeast homologues Ypa1 and Ypa2 can induce a conformational change in some model substrates. Using these model substrates in different assays with and without helper proteases, this isomerase activity is similar to the isomerase activity of FKBP12, the human cyclophilin A, and one of its yeast homologs Crp7 but dissimilar to the isomerase activity of Pin1. However, neither FKBP12 nor Crp7 can reactivate the inactive form of PP2A. Therefore, PTPA belongs to a novel peptidyl-prolyl cis/trans-isomerase (PPlase) family. The PPlase activity of PTPA correlates with its activating activity since both are stimulated by the presence of Mg\(^{2+}\) ATP, and a PTPA mutant (Δ208–213) with 400-fold less activity in the activation reaction of PP2A also showed almost no PPlase activity. The point mutant Asp\(^{205}\) → Gly (in Ypa1) identified this amino acid as essential for both activities. Moreover, PTPA dissociates the inactive form from the complex with the PP2A methyltransferase. Finally, Pro\(^{190}\) in the catalytic subunit of PP2A (PP2AC) could be identified as the target Pro isomerized by PTPA/Mg\(^{2+}\) ATP since among the 14 Pro residues present in 12 synthesized peptides representing the microenvironments of these prolines in PP2AC, only Pro\(^{190}\) could be isomerized by PTPA/Mg\(^{2+}\) ATP. This Pro\(^{190}\) is present in a predicted loop structure near the catalytic center of PP2AC and, if mutated into a Phe, the phosphatase is inactive and can no longer be activated by PTPA/Mg\(^{2+}\) ATP.

Protein phosphatase 2A (PP2A)\(^4\) represents a major group of Ser/Thr phosphatases involved in the regulation of a plethora of cellular functions. The structure of the holoenzyme comprises a regulatory A scaffolding subunit (PR65) and a catalytic C subunit, forming the dimeric core enzyme (PP2AC). This dimer can further associate with a number of "third" regulatory B subunits (PR55/B, PR61/B, PR72/B, each represented in mammals by at least four isoforms). The regulatory B subunits determine the catalytic properties of PP2A as well as its subcellular localization and substrate specificity (for review, see Ref. 1). The catalytic subunit itself is further regulated by phosphorylation (2, 3) and methylation (4–8). The in vivo role of PP2A is diverse. PP2A is involved in cell growth, intracellular signaling, cell transformation, DNA replication, transcription, protein synthesis, cell differentiation, and apoptosis (1, 9). Besides its Ser/Thr phosphatase activity, PP2A also has a low basal phosphotyrosyl phosphatase activity (10–13) that can be up-regulated in vitro by a protein that was originally named phosphotyrosyl phosphatase activator (PTPA). This protein was renamed phosphatase two A phosphatase activator because recent findings (14–16) suggest that its physiological function is more likely to reactivate the Ser/Thr phosphatase activity of an inactive form of PP2A. This inactive form can be isolated as a complex with PME1, the methyltransferase that specifically demethylates PP2A (14).

PTPA is a well conserved protein that has been found from yeast to human (13, 17, 18). Human PTPA is encoded by a single gene that is mapped to chromosome 9q34 (17). The transcription gives rise to seven different splice variants, four of which are active (18). Basal expression of the gene is dependent on the ubiquitously transcribed factor Yin Yang 1 (19) and functionally antagonized by p53 (20). In yeast, PTPA is encoded by two genes, YPA1 and YPA2. Deletion of both genes is lethal (21, 22), and a single deletion of YPA1 is more severe than deletion of YPA2 (21–23). Deletion of YPA1 leads to an aberrant bud morphology, abnormal actin distribution, and growth defects (21, 23). Genetic evidence identified YPA1 and YPA2 as positive regulators of PP22 (21–23), implicated in the regulation of the TOR pathway (21, 22), and recent studies revealed that Ypa1 physically interacts with the PP2A-like phosphatases Pph3, Sit4, and Ppg, whereas Ypa2 binds to Pph21 and Pph22, the yeast homologues of PP2A. This latter interaction is promoted by Ypa1 (16).

The activation of PP2A by PTPA, Ypa1, or Ypa2 is dependent on the presence of ATP or a hydrolyzable ATP analogue (10, 13) in the presence of Mg\(^{2+}\). Nevertheless, neither a kinase signature was found in the primary structure nor could kinase activity be demonstrated (12). Therefore, the mechanism of activation of PP2A by PTPA is still not known, but it has been suggested that PTPA induces a reversible conformational change in PP2A (10, 14, 15). In this study we investigated whether PTPA can induce a conformational change in some model substrates and whether it has peptidyl prolyl cis/trans isomerase activity similar to the peptidyl prolyl cis/trans isomerase activity of FKBP12.
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(PTPase) activity that could be responsible for a conformational change in PP2A.

Pro is the only amino acid that adopts the cis conformation for a reasonable amount (10–15% in unfolded peptides) (24). Pro isomerization is one of the rate-limiting steps in protein folding (25), but it can also play regulatory roles by subtle conformational changes in native proteins. Prolyl cis/trans isomerization is a spontaneous process, but it can also be catalyzed by different enzymes. PTPase was first described by Fischer et al. (26). Later, this PTPase and the immunosuppressant cyclosporin A-binding protein, cyclophilin, turned out to be the same protein (27, 28). FKBP12, the immunosuppressant FK506-binding protein, also showed PTPase activity (29, 30). Both proteins have an important function in protein folding (31) and are recognized now as representatives of broader families of FKBP and cyclophilins (32–34). A third family of PTPases is the parvulins, with Pin1 as an important representative (35–37). Cyclophilins and FKBP have a broad spectrum of peptide sequences that can be isomerized, whereas Pin1 specifically isomerizes Pro residues, which are preceded by a phosphorylated Ser/Thr (38, 39) and isomerization in protein folding (25), but it can also play regulatory roles by subtle conformational changes in native proteins. Prolyl cis/trans isomerization is a spontaneous process, but it can also be catalyzed by different enzymes. PTPase was first described by Fischer et al. (26). Later, this PTPase and the immunosuppressant cyclosporin A-binding protein, cyclophilin, turned out to be the same protein (27, 28). FKBP12, the immunosuppressant FK506-binding protein, also showed PTPase activity (29, 30). Both proteins have an important function in protein folding (31) and are recognized now as representatives of broader families of FKBP and cyclophilins (32–34). A third family of PTPases is the parvulins, with Pin1 as an important representative (35–37). Cyclophilins and FKBP have a broad spectrum of peptide sequences that can be isomerized, whereas Pin1 specifically isomerizes Pro residues, which are preceded by a phosphorylated Ser/Thr (38, 39). Their PPIase activity is similar to the activity of FKBP12 and cyclophilin, and unlike the Pin1 family (39), a glutamic acid in the –1 position of the prolyl residue inhibits the PPIase activity of PTPA. In addition, we provide evidence that this PPIase activity represents the mechanism by which PTPA may regulate PP2A activity.

EXPERIMENTAL PROCEDURES

Materials, Plasmids, and Site-directed Mutagenesis—Human cyclophilin A, chymotrypsin, and subtilisin were obtained from Sigma, trypsin was from Roche Applied Science, thrombin was from Amersham Biosciences, and BSA was purchased from Serva. Recombinant glutathione S-transferase-FKBP12 and FKBP12 were expressed and purified according to Bultinck et al. (44), Yp1 and Yp2 were purified according to Van Hoof et al. (16), Pin1 was purified according to Winkler et al. (45) with a bacterial expression vector obtained from A. Means (Duke University, Durham, NC), and recombinant Cpr7 was purified as a His-tagged version. His-Ypa1 and His-Ypa2 can function as peptidyl-prolyl cis/trans-isomerases. Their PPIase activity is similar to the activity of FKBP12 and cyclophilin, and unlike the Pin1 family (39), a glutamic acid in the –1 position of the prolyl residue inhibits the PPIase activity of PTPA. In addition, we provide evidence that this PPIase activity represents the mechanism by which PTPA may regulate PP2A activity.

Recombinant PTPA Purification—Recombinant wild type PTPA was expressed and purified as described (12) with some minor modifications to diminish interference in the optical measurements as much as possible. The final concentration step (dialysis against polyethylene glycol and glycerol) was replaced by a visavis centrifugation to obtain a minimal stock solution concentration of 1 mg/ml (27 μM), and dithiothreitol was omitted in the last purification steps. The His-tagged rabbit PTPA and a mutant (Δ208–213) that is 400-fold less active than the wild type (13) and the point mutant His-Yp1 (D205G) were purified using a one step Ni2+-Sepharose column purification. Recombinant His-tagged rabbit PTPA in a pET15b vector was expressed in Escherichia coli BL21 cells. The transformed cells were used to inoculate 200 ml of LB medium containing 100 μg/ml ampicillin with an A600 of 0.2–0.3. The cells were grown at 37 °C until the medium reached an A600 of 6. Expression of the plasmid was induced for up to 4 h at 37 °C by the addition of 0.4 mM isopropyl-thio-β-D-galactopyranoside. The cells were harvested at 4000 × g and stored at least overnight at –80 °C. Thereafter, cells were lysed by the addition of 10 ml of ice-cold lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 2 mg/ml lysozyme, 10 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1 mM pepstatin). The pellet was dispersed and left on ice for 15 min. After vortexing, the suspension was left on ice for another 15 min and centrifuged for 15 min at 13000 × g, and the supernatant was allowed to bind to 1 ml of a Ni2+-Sepharose (Amersham Biosciences) suspension (0.5-ml beads) equilibrated in lysis buffer without lysozyme for 2 h at 4 °C on a rotating wheel. Subsequently the beads were poured in a column and washed with 3 × 10 ml of buffer (50 mM Tris, pH 7.5, 100 mM NaCl). Then the column was washed with 3 × 10 ml of wash buffer (50 mM Tris pH 7.5, 300 mM NaCl, and 10 mM imidazole), and finally the bound proteins were eluted with 7 ml Tris, pH 7.5, 300 mM NaCl, and 250 mM imidazole). The first 3–4 fractions (containing each 10 mg PTPA/ml or more) were individually cleaned up by a Superdex-200 gel filtration column (1 × 100 cm), equilibrated, and eluted with 20 mM Tris, pH 7.5. Fractions of 0.5 ml were collected. The three peak fractions contained about 3 mg/ml, and the four side fractions contained about 1–2 mg/ml. These fractions were separated into aliquots and stored at –20 °C. This purification scheme resulted in 100% pure PTPA as judged by silver staining after SDS-PAGE and by mass spectrometry. In some preparations the PTPA inactive fraction in PP2A (and 1 from PP1) used to test isomerase activity of PTPA

TABLE 1

| Peptide | Sequence |
|---------|----------|
| 1       | 47VRCPYTVY^25 |
| 2       | 7GGSKPTDNYV^61 |
| 3       | 16KVYRVPETIP^112 |
| 4       | 10FDYIPLTAL^138 |
| 5       | 16GG3LPSNTF^176 |
| 6       | 16LOSEPHEPMCDL^186 |
| 6-bis   | 16LOVEPHEGAMCDL^198 |
| 6-ter   | PTDVDPGLICD^197 |
| 7       | 17WSDPDDG^207 |
| 8       | 20WGISPRGAG^218 |
| 9       | 22IFSPNPYC^247 |
| 10      | 25QOFQAPAQRGE^297 |
| 11      | 29BRGEHPIVTR^302 |
| 12      | 30RRTDPYV^309 |

All peptides were synthesized by the Fmoc technology.
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Mutant (Δ208–213) and Ypa1 D205G mutant were followed during their purification spectrophotometrically at 280 nm and by SDS-PAGE. In addition, activity of the protein was measured after the last purification step.

Mass Spectrometry—Mass spectrometry was performed on an API-3000 (Applied Biosystems) triple quadrupole mass spectrometer. The PTPA sample was analyzed with nanospray mass spectrometer, and the mass spectrum was deconvoluted making use of the Biotoolbox algorithms provided with the instrument and proved to be a pure homogeneous population of a protein of 36,869 Da. This represents the mass of PTPA (36,456 Da) plus 3 amino acids (GSH), remaining after the thrombin cleavage of the His tag. Purity of the protein was also proven by SDS-PAGE (Supplemental Fig. 1).

PPlase Assay—The routine PPlase assay was essentially based on the protease-coupled method described by Fischer et al. (26) and Schuttowski et al. (38). Basically, the assay consists of a conformation-specific proteolysis by an endopeptidase such as subtilisin, trypsin, or chymotrypsin of a substrate peptide, carrying a C-terminal fluorophore or chromophore with a different physical property in the bound and unbound forms.

Substrates are of the type Suc-Ala-X-Pro-Y-Z. X and Y denote variable amino-acyl residues, and Z denotes a fluorophore such as para-nitroaniline. In solution, such substrates are largely (80–90%) in the trans conformation and can be cleaved by the protease. In the presence of sufficient amounts of protease in the reaction mixture, the trans population is rapidly cleaved, whereas the cis population remains intact (first phase, rapid). The subsequent slow isomerization reaction is accelerated by PPlases, resulting in the cleavable trans conformation (second phase, slow) (Fig. 1A). A disadvantage of this protease-coupled assay is the requirement for high concentrations of helper proteases to obtain the two phases. This assay could, therefore, only be used for proteins that proved to be relatively resistant to the protease in assay, at least for the duration of the experiment. These parameters have to be determined for each PPlase in combination with the substrate and the protease. In practice, the substrate was pre-equilibrated in the cuvette at 20 °C in 450 μl of 50 mM Tris, pH 7.4, with or without the addition of Mg<sup>2+</sup>ATP, and the reaction was started by the simultaneous (±1 s) addition of 25 μl of protease solution and 25 μl of PPlase solution. As a positive control for the prolyl isomerase assay, we used the activity of recombinant FKBP12. This enzyme was measured in the past with subtilisin or chymotrypsin as the isomerase, and s-AAPF-MCA as the substrate (49). Therefore, these conditions were first optimized with PTPA as the potential PPlase. The optimal concentration for chymotrypsin and subtilisin was, respectively, 1.92 and 1.73 μM. Lower concentrations resulted in a slower proteolysis of the substrate and produced lower calculated kinetic constants. Higher concentrations of chymotrypsin or subtilisin also resulted in lower calculated kinetic constants, probably due to proteolysis of PTPA or FKBP12 during the second phase of the assay. Indeed, SDS-PAGE of samples taken at different time intervals during an experiment with the optimal protease concentration revealed that after 10 min only 20–30% of the original amount of PTPA (1.4 μM) remained intact. Therefore, it was obligatory to do these experiments with relatively high concentrations of PTPA and short assay times (standard conditions less than 3 min). In principle the proteolytic breakdown products of the protein could interfere in the assay, but a control with 2.5 μM BSA showed that this is not the case (Fig. 1B).

Because it is known that PTPA is resistant to relatively high concentrations of trypsin (10), s-AAPK-pNA was the substrate by preference because it could be hydrolyzed by trypsin. By using this protease (4.4 μM) and this substrate, lower PTPA concentrations could be used in the PPlase assays. The resistance of PTPA to trypsin was confirmed by SDS-PAGE of PTPA samples, taken at different time intervals after incubation with trypsin even at a concentration 10-fold higher than used in the assay. After 10 min there was no detectable difference in PTPA concentration.

Fluorescence Measurements—Time-dependent fluorescence intensities were measured on a Photon Technology International spectrophotofluorimeter. Photobleaching was prevented by a shutter, programmed to open for 10 s at various time intervals. The fluorescence signal was simultaneously detected every second on two different detectors set to detect at different emission wavelengths. Samples were allowed to equilibrate for 10 min in the cuvette holder. Fluorescence data were acquired with the FELIX computer program that accompanied the PTI spectrophluorimeter and later transferred to Sigma-plot 8.1 for mathematical analysis (Fig. 1A). The optimal excitation and emission wavelengths for the MCA substrate were confirmed for the intact (respectively, 320 and 390 nm) as well as for the free MCA molecule (respectively, 352 and 439 nm). To allow a simultaneous measurement of the leftover (intact) substrate and the formed product (free MCA) and to minimize reciprocal interference as much as possible, emission was measured at 370 and 460 nm simultaneously using an excitation wavelength at 336 nm. Every experiment was repeated three times so we obtained six graphs per assay condition, three after the intact substrate and three after the free MCA after proteolysis.

Absorbance Measurements—The pNA substrate time-resolved absorbance measurements at 400 nm were performed on a Shimadzu UV-160 double-beam spectrophotometer using 50 mM Tris, pH 7.5, as the reference solution. At this wavelength we followed the formation of free para-nitroaniline, where less than 0.5% absorbance is due to intact peptide, protein, or Mg<sup>2+</sup>ATP. The intact peptide could not be followed because of the overlap between intact peptide and the other components of the reaction. Absorbance was measured every second, and results were transferred to Sigma-plot 8.1 for mathematical analysis.

Calculation of the Kinetic Constants—The reaction is biphasic, with an initial fast phase (the initial proteolysis of the substrate in the trans-conformation) and a subsequent slow phase (the cis/trans isomerization of the substrate, after which it is proteolyzed). The two phases could be distinguished when the data were fitted in a semilog plot (Fig. 1A). The second part of the reaction fit the formula.

\[
y = y_0 + ae^{-bx} \quad \text{(Eq. 1)}
\]

\[
y = y_0 + (1 - ae^{-bx}) \quad \text{(Eq. 2)}
\]

Equation 1 is used when monitoring the intact substrate, and Equation 2 is used when following the free MCA or pNA, wherein \( a \) equals the amplitude of the isomerase reaction, and \( b \) is the observed \( k_{obs} \) value.

Autoisomerization Activity—The rate of autoisomerization of the substrate was determined in the absence of a peptidyl-prolyl cis/trans-isomerase. This autoisomerization was measured both in the presence and absence of BSA, a protein without an isomerase activity. The results of the experiments in the presence or absence of BSA are comparable, proving that the addition of a protein as such has little or no effect on the hydrolysis rate of the substrate by subtilisin (Fig. 1B), chymotrypsin, or trypsin. The autoisomerization rate constant was \( k_{obs} = 0.03 \text{s}^{-1} \) at 20 °C.

NMR Measurements—<sup>1</sup>H NMR measurements were performed on a Varian Unity 500 spectrometer with a 3-mm HCPzwprb probe at 27 and 37 °C. Water-suppressed one-dimensional proton spectra with a spectral width of 4560 Hz were measured by accumulating 100 transients,
each using 3-s presaturation delay followed by a 90° (angle) observation pulse and 1-s acquisition time. Processing was performed with VNMR 6.1 software.

A 10% D$_2$O 275-$\mu$l standard solution of 40 mM Tris, pH 7.1, and 1 mM concentrations of different substrates were used (final concentrations). 2 mM ATP/10 mM Mg$^{2+}$ or Tris 40 mM (final concentrations) were
added to test the ATP dependence of the PPlase activity of PTPA. 5 μM PTPA, Ypa1, or Ypa2 were added to test the PPlase activity. Tris or 5 μM BSA were used as a negative control, and 5 μM FKBP12 was used as a positive control for the PPlase activity.

Sequence Homology and Three-dimensional Structure Predictions—
Alignments were calculated with BLAST. Homology between the different isomerase families was calculated with MULTALIN, LALIGN, DIALIGN, and MUSCA. The secondary structure was predicted using Prof, GOR IV, HNN, jPred, nPredict, PSA, and PSIpred. Results of these algorithms were compared and condensed in a secondary structure with the highest probability. We used SwissModel (swissmodel.expasy.org), three-dimensional Jigsaw (www.bmm.icnet.uk/servers/3djigsaw), and ESyPred3D (www.fundp.ac.be/urbm/bioinfo/esypred) as algorithms to model the structure of PPA2 with the three-dimensional structure of PPI1 as a template.

Cell Culture, Transfections, and Immunoprecipitation—
COS7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 1 g/liter glucose (BioWhittaker) supplemented with 2 mM L-glutamine (BioWhittaker), 100 units/ml penicillin (BioWhittaker), 100 mg/ml streptomycin (BioWhittaker), and 10% fetal calf serum (Sera Laboratories Whittaker). This suspension was briefly vortexed, and after 15 min of incubation on ice, it was centrifuged at 16,000 × g for 15 min at 4°C. The HA-tagged fusion proteins were immunoprecipitated from the supernatant with 6 μl of monoclonal anti-HA antibodies (Sigma) and 80 μl of 50% protein G-Sepharose beads (Amersham Biosciences). Immunoprecipitates were washed 3 times with Tris-buffered saline buffer plus 0.1% Nonidet P-40 supplemented with 50 μg/ml leupeptin, 20 μg/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 0.1 mM N-α-Tosyl-l-lysine chloromethyl ketone, and 10 mM dithiothreitol. This suspension was briefly vortexed, and after 15 min of incubation on ice, it was centrifuged at 16,000 × g for 15 min at 4°C. The HA-tagged fusion proteins were immunoprecipitated from the supernatant with 6 μl of monoclonal anti-HA antibodies (Sigma) and 80 μl of 50% protein G-Sepharose beads (Amersham Biosciences). Immunoprecipitates were washed 3 times with Tris-buffered saline buffer plus 0.1% Nonidet P-40 supplemented with 50 μg/ml leupeptin, 2 μg/ml pepstatin, 0.1 mM phenylmethanesulfonyl fluoride, and once in Tris/dithiothreitol (20 mM Tris-HCl, 0.1 mM dithiothreitol, pH 7.4). As controls, the empty vector was also transfected, and the non-transfected cells were also immunoprecipitated. Finally, the beads were resuspended in 100 μl of Tris/dithiothreitol and assayed for phosphatase activity or subjected to Western blotting after the addition of SDS sample buffer and boiling and SDS-PAGE.

Western Blot Analysis—After separation on SDS-PAGE, the proteins were transferred to a polyvinylidene difluoride membrane. The Western blots were preincubated in phosphate-buffered saline supplemented with 0.1% Tween 20 and 5% skimmed milk powder. Subsequently, Western blots were incubated overnight with the indicated primary antibodies at 4°C. Mouse immunoglobulins coupled to horseradish peroxidase (Dako) were used as secondary antibodies. After extensive washing, Western blots were visualized using the ECL plus Western blotting detection system (Amersham Biosciences).

RESULTS

PPlase Activity of PTPA in Comparison with FKBP12, Cyclophilin A, and Cpr7—Cyclophilins and FKBP8 are ubiquitous and highly conserved enzyme families, both characterized as PPIases. Therefore, we compared the PPlase activity of PTPA with a representative of each family; mammalian FKBP12, mammalian cyclophilin, and Cpr7, a Saccharomyces cerevisiae homologue of cyclophilin 40 (50).

First the PPlase activity of PTPA was compared with the PPlase activity of FKBP12, a known prolyl cis/trans-isomerase with a well-documented activity for the MCA substrates used. Unless stated otherwise, all PPlase activities of PTPA, Ypa1, and Ypa2 were measured in the presence of a saturating concentration of Mg2+ ATP, since these components are proven to be stimulatory also for the PPlase activity (see below). As shown in Fig. 1B, the rate of cis/trans isomerization during the second “slow” phase by PTPA and FKBP12 was very comparable. Second, the kobs was measured for PTPA and FKBP12 for different protein concentrations, and as demonstrated in Fig. 1C, the kobs for PTPA and FKBP12 depended on the concentration of PPlases reaching a maximum of, respectively, kobs = 0.13 s⁻¹ at 0.25 μM PTPA and 0.11 s⁻¹ at 0.5 μM FKBP12 at 20 °C. Therefore, kobs is measured (Table 2) at lower enzyme concentrations where a linear relationship exists between the PPlase concentration and kobs (Fig. 1D).

Because PTPA is resistant to trypsin, the kinetic parameters were preferentially measured with the s-AAPK-pNA substrate since trypsin is the stereospecific protease for this substrate. When measured with s-AAPK-pNA as substrate, human cyclophilin A and yeast Cpr7, both known as PPlases, (51, 52) and PTPA showed a PPlase activity in the
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same range (Table 2). Also, the yeast homologues of PTPA, Ypa1 and Ypa2 (named also RRD1-YIL153w and RRD2-YPL152w) (15, 22), displayed a similar PPIase activity with this substrate.

We measured the $k_{cat}/K_m$ values by varying the PTPA concentration (Fig. 1D). With a substrate concentration of 8.25 μM and a trypsin concentration of 4.4 μM, we obtained a $k_{cat}/K_m = 1.1 \mu M^{-1} \text{s}^{-1}$. This result can be compared with previous results obtained for cyclophilin ($k_{cat}/K_m = 5.1 \mu M^{-1} \text{s}^{-1}$) and FKBP12 ($k_{cat}/K_m = 0.14 \mu M^{-1} \text{s}^{-1}$) (52).

PPIase Activity of PTPA Is Stimulated by Mg$^2+$-ATP—Because the activation of PP2A by PTPA is dependent on the presence of hydrolysable Mg$^2+$-ATP during the activation of the tyrosyl phosphatase activity (10) and also during the activation of the Ser/Thr phosphatase activity of the inactive form of PP2A (14), we tested whether the PPIase activity is also dependent on the presence of Mg$^2+$-ATP. PTPA showed an isomerase activity in the absence of Mg$^2+$-ATP, but the addition of Mg$^2+$-ATP increased this activity at least 3-fold. An $A_{50}$ value for ATP was determined as 0.2 mM in the presence of 5 mM MgCl$_2$. This should be compared with the 0.12 mM found for the stimulation of the tyrosyl phosphatase activity (10) and to the 3 μM found for the activation of the inactive form of PP2A (14). On the other hand, an excess of ATP over Mg$^2+$, resulting in “free” ATP, was inhibitory in both the activation and PPIase reaction (results not shown). No Mg$^2+$-ATP stimulation was found with the other PPIases (Table 2), whereas the stimulation of the PPIase activity of Ypa2 was somewhat less in comparison with Ypa1 or PTPA.

Protease-free Measurement of the PPIase Activity of PTPA—We sought independent evidence for the PPIase activity of PTPA by dynamic proton NMR spectroscopy using the band-shape analysis technique (53). This allows proof of isomerization of the prolyl peptide bond independent of any protease activity. The basic theory behind this type of NMR measurement is the acceleration of the cis to trans transition, which is translated into a broadening of the peaks of the methyl groups of Ala preceding the Pro. Although only about 10% of the peptide is in the cis conformation, one can distinguish some small peaks next to the large peak resulting from the trans conformation. The identity of these peaks was confirmed by two-dimensional NMR. The $k_{cat}/K_m$ values by varying the PTPA concentration (Fig. 1D). With a substrate concentration of 8.25 μM and a trypsin concentration of 4.4 μM, we obtained a $k_{cat}/K_m = 1.1 \mu M^{-1} \text{s}^{-1}$. This result can be compared with previous results obtained for cyclophilin ($k_{cat}/K_m = 5.1 \mu M^{-1} \text{s}^{-1}$) and FKBP12 ($k_{cat}/K_m = 0.14 \mu M^{-1} \text{s}^{-1}$) (52).

PTPA Is a New Family of Prolyl cis/trans-Isomerase

Other PPIases Cannot Activate PP2A—From the current data one could hypothesize that PTPA can activate PP2A by a conformational change induced by its PPIase activity. Therefore, it was investigated whether other PPIases such as cyclophilins or FKBP1s could replace PTPA in the activation reaction. Recombinant FKBP12 and cyclophilin Cpr7 were tested in several concentrations with and without 1 mM ATP and 5 mM MgCl$_2$ in the activation assay of inactive PP2A. No effect of these proteins could be detected (data not shown). Because the immunosuppressive and toxic effects of cyclophilins and FKBP1s are mediated by binding to cyclosporin A and the macrolide FK506 (27–30) or rapamycin, respectively, the activation of PP2A by PTPA, FKBP12, or Cpr7 was also measured in the presence of these effectors. No effects on the activation were observed (data not shown). These data indicate that if the PPIase activity of PTPA is indeed causal for a conformational change in PP2A, it is specific for PTPA since other PPIases cannot do the same.

PTPA Is a New Family of Prolyl cis/trans-Isomerase—Similarity studies of the primary structure of PTPA with the cyclophilins, the FKBP-like proteins, and the parvulins further demonstrated that PTPA belongs to a new class of PPIases. PTPA yielded a maximum sequence similarity of 7.14%, probably not significant since the similarities are scattered over the protein. In addition, using a BLAST search, no other proteins were found with any homology to PTPA, as previously noticed (12).

A prediction study of the secondary structure of PTPA was performed using different databases and algorithms. This secondary structure was compared with the secondary structures of the three other known families of prolyl cis/trans-isomerases. Also in the secondary structure, there was no similarity found between PTPA and any of the other families. The crystal structure of PTPA is not yet available to compare possible three-dimensional structures and domains among the different families of prolyl cis/trans-isomerases and PTPA.

Although we cannot yet rule out the possibility that the spatial configuration of the PPIase catalytic site of PTPA might be similar to other

![FIGURE 2](image-url)
PTPA Is a cis/trans-Isomerase

The Catalytic Center of PTPA—The catalytic center of the PPIase in PTPA is not known, but highly conserved domains were found and tested for their importance in the activation reaction (13). By deleting one such well conserved domain, 208GVWGLD213, PTPA was about 400-fold less active than the wild type in the phosphotyrosyl phosphatase activation reaction of PP2A (13). We confirmed this by the activation of PP2A, with the purified His-tagged version of this mutant; also in this assay, about 400-fold more protein was needed to obtain the same activity as the wild type. As shown in Table 3, the \( k_{obs} \) that was measured with this mutant was very low (\( k_{obs} = 0.008 \text{ s}^{-1} \)), not significantly higher than the autoisomerization reaction. By changing Asp\(^{105} \) into Gly in the Ypa1 context (Asp\(^{213} \) in mammalian PTPA), we could identify this amino acid as essential both for the activation of PP2A (Fig. 3) and for the PPIase activity of Ypa1 (Table 3). Therefore, Asp\(^{105} \) probably participates in the catalysis of both reactions, further substantiating the correlation between both activities.

PTPA Induces a Conformational Change in PP2A—Many reasons exist to assume that PTPA can induce a conformational change in PP2A.
1) Because after activation PP2A can dephosphorylate phosphotyrosyl substrates apparently by the same catalytic center, a conformational change was assumed (10, 12).
2) Inactivating mutations of some amino acids in PP2A, essential for catalysis, changed the affinity of PP2A for some proteins such as PME1 (15, 53) and PTPA (15).
3) When PME1 was purified from tissues, an inactive form of PP2A was found associated in a complex with PME1, and PTPA could activate this form of PP2A, leading to a dissociation of the complex (14). Taken together, it is clear that PTPA not only changes the catalytic center of PP2A but also some interacting surfaces.
4) In a yeast strain wherein both Ypa1 (RRD1) and Ypa2 (RRD2) were deleted and lethality was rescued by a different genetic background, PP2A\(_c\) was not only less stable but was also altered in substrate specificity and metal ion dependence (15), indicating that in the absence of PTPA a PP2A form was synthesized with a different conformation.
Because we now found a PPIase activity in PTPA and its yeast homologues Ypa1 and Ypa2 and Mg\(^{2+} \) ATP plays a role both in the

TABLE 3
\( k_{obs} \) values of PTPA mutants

| Enzyme                  | ATP/Mg\(^{2+} \) | s-AAPK-pNA |
|-------------------------|-----------------|------------|
| PTPA (Δ208–213)         | +ATP/Mg\(^{2+} \) | 0.006 ± 0.002 s\(^{-1} \) |
| Ypa1 (D205G)            | +ATP/Mg\(^{2+} \) | 0.011 ± 0.004 s\(^{-1} \) |

\( ^a \) Difference between measurements with the mutants and blank measurement, \( p > 0.05 \).
PTPA Is a cis/trans-Isomerase

FIGURE 4. 1H NMR spectra of a PP2A-derived peptide. Shown is a region of a one-dimensional NMR proton spectrum with signals from methyl groups in the LQEVPHG-PMCDL peptide. Measurements were conducted in 275 μl of 40 mM Tris, pH 7.1, 10% D2O, and 1 mM peptide (peptide 6 in Table 1) at 27 °C and 2 mM ATP/10 mM Mg2+ (AM) and/or 5 μM PTPA. In A and B, the order of addition of the components was alternated as indicated in the figure.

presence of protamine sulfate (16) plus or minus activation by a saturating amount of PTPA/Mg2+ ATP, and as can be seen in Fig. 5B, PP2A wt was spontaneously active and could not be further activated by PTPA/Mg2+ ATP, PP2Awt (P190A) was partially active and not activable by PTPA/Mg2+ ATP, and PP2Awt (P190F) was almost completely inactive and not activable either, whereas the PP2Awt (P305A) mutant was almost as active as WT and also not activable. These results clearly show the importance of Pro190 for the activity of PP2Awt. The fact that PP2Awt WT is fully active in this experiment is probably due to the fact that in COS7 cells, PTPA is highly expressed5 and, therefore, is not the limiting factor for all PP2A activity. If Pro190 is mutated into a Phe, a conformational change will be induced that inactivates PP2A and that can no longer be reversed by PTPA/Mg2+ ATP. Replacement of Pro190 by Ala results in a partially active PP2Awt (P190A) and could not be activated by PTPA/Mg2+ ATP, indicating that Pro190 is essential for an activation to take place.

DISCUSSION

Because PTPA has a PPIase activity that is comparable with previously characterized PPIases such as FKBP12 and cyclophilin, it is reasonable to assume that this PPIase activity is an essential element for its cellular function. The substrate specificity of this PPIase is not yet known and might have a broader activity spectrum than just PP2A and the PP2A-like enzymes, but so far only this group of protein phosphatases is shown to be regulated by PTPA. Biochemical (10–15), genetic (20–23), and physical (15, 16) evidences exist for this regulation, and it was already suggested that PTPA accomplishes this function by inducing a conformational change in PP2A (10, 14, 15). However, direct evidence for such a change was missing. In this study we demonstrate with different techniques that PTPA has PPIase activity and, therefore, could induce a conformational change due to a prolyl isomerization reaction. Evidence for a specific prolyl residue of PP2A that can be isomerized by PTPA is presented by the NMR results of the different PP2A-specific peptides of which only one peptide containing Pro190 can be isomerized by PTPA. This peptide is situated in an easily accessible region of PP2A, nearby the catalytic site. The primary structure of this peptide is conserved among PP2A and its yeast homologues PPH21 and PPH22 and the PP2A-like proteins Ppg, Sit4, and Pph3. Although this peptide is situated in an area with a large identity with PP1, the latter is not activated by PTPA, confirming the specificity of the PPIase reaction on PP2A. A PP2Awt mutant with Pro190 changed into a Phe was inactive and could not be activated by PTPA/Mg2+ ATP, strongly arguing for the importance of Pro190 in the activation reaction. Although we cannot rule out other explanations for the inability of PTPA/Mg2+ ATP to activate the PP2Awt (P190F) inactive mutant, the most plausible hypothesis would be that Pro190 will indeed undergo a conformational change induced by the PPIase activity of PTPA. Further studies will be necessary to delineate the exact structural change(s) induced in the complete PP2A by the PPIase activity of PTPA. No crystals are available yet for the active and inactive forms of PP2A, but the high similarity of PP1 and PP2A at the primary structure level allowed predictions for amino acids that are essential for catalysis (58). Mutations of some of these essential amino acids renders PP2A inactive, and these inactive PP2As associate with the PP2A-specific histidine kinase Dpb15 (59), suggesting that some interacting surfaces are different in the active and this inactive PP2A. Also, by purifying Dpb15 from tissues, an inactive form of PP2A was found associated with PPI1. After incubation with PTPA and Mg2+ ATP, PP2A was activated and dissociated from PPI1 (14). These observations strongly argue for the existence in vivo of two conformations of PP2A, one of them favored by PTPA.

The requirement of Mg2+ ATP in the activation reaction of PP2A is still intriguing. Also, for the PPIase activity of PTPA, the presence of Mg2+ ATP seems to be stimulatory but less stringent since also in the absence of Mg2+ ATP a low but measurable PPIase activity could be detected. This is not so surprising since small peptides are used as substrates in the PPIase reaction. Inducing a conformational change of a prolyl residue in a whole protein might need to overcome interactions of other side chains to allow the prolyl cis/trans isomerization. To overcome these resistances, the addition of extra energy might be necessary.

5 S. Longin, unpublished results.
To further substantiate the hypothesis that the PPlase activity of PTPA is probably causal for the activity change in PPA2, we measured the PPlase activity in a mutant of PTPA ($\Delta^{208}$GVWGLD$^{213}$) that is almost completely inactive in the phosphotyrosyl phosphatase activation reaction of PPA2 (13) and in the activation of PPA2, (this study). This mutant was also inactive as PPlase. Therefore, the activation of PPA2 and the PPlase activity of PTPA are correlated. The fact that a low activity could be measured in this mutant seems to exclude the possibility that it is completely misfolded. Moreover, the point mutant D205G in Ypa1, equivalent to Asp$^{215}$ in PTPA, shows a similar correlation, corroborating its implication in the catalysis of the PPlase.

So far three families of peptidyl-prolyl isomerases are known: cyclophilins, FKBP, and parvulins. Cyclophilins and FKBP were originally discovered as the intracellular binding targets of immunosuppressive drugs. Almost simultaneously, these proteins were characterized as PPIases, targeting PP2A and the PP2A-like phosphatases.

These enzymes have long been thought to play an exclusive role in cell signaling, corroborating its implication in the catalysis of PPIases. These enzymes have long been thought to play an exclusive role in cell signaling, corroborating its implication in the catalysis of PPIases.