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The Protein Phosphatase 2A Phosphatase Activator Is a Novel Peptidyl-Prolyl *cis*/*trans*-Isomerase*[S]

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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 Cpr7 but dissimilar to the isomerase activity of Pin1. However, neither FKBP12 nor Cpr7 can reactivate the inactive form of PP2A. Therefore, PTPA belongs to a novel peptidyl-prolyl *cis*/*trans*-isomerase (PPIase) family. The PPIase 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 PPIase activity. The point mutant $Asp^{205} \rightarrow$ Gly (in Ypa1) identified this amino acid as essential for both activities. Moreover, PTPA dissociates the inactive form from the complex with the PP2A methylesterase. Finally, Pro^{190} in the catalytic subunit of PP2A ($PP2A_C$) 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 $PP2A_C$, 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 $PP2A_C$ 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 ($PP2A_D$). 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 methylesterase 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 ubiquitous transcription 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 PP2A (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 an 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 could 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

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[4] The abbreviations used are: PP2A, protein phosphatase 2A; $PP2A_C$, catalytic subunit of PP2A; $PP2A_i$, inactive form of PP2A; PTPA, PP2A phosphatase activator; FKBP12: FK506-binding protein 12; PME1, phosphatase methyl esterase; PPIase, peptidyl-prolyl *cis*/*trans*-isomerase; MCA, methylcoumarylamine; pNA, *para*-nitroanilide; BSA, bovine serum albumin; HA, hemagglutinin; WT, wild type; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; s and Suc, succinyl.

(PPIase) 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. PPIase was first described by Fischer *et al.* (26). Later, this PPIase 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 PPIase activity (29, 30). Both proteins have an important function in protein folding (31) and are recognized now as representatives of broader families of FKBPs and cyclophilins (32–34). A third family of PPIases is the parvulins, with Pin1 as an important representative (35–37). Cyclophilins and FKBPs 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) as present in mitotic substrates such as Cdc25c, Myt1, Wee1, Plk1, NIMA, Cdc27 (37, 40, 41), p53 (42), and p73 (43).

In this report we demonstrate that PTPA and its yeast homologues Ypa1 and Ypa2 can function as peptidyl-prolyl *cis*/*trans*-isomerases. Their PPIase activity is similar to the activity of FKBP12 and cyclophilin, and unlike the Pin 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*-transfersase-FKBP12 and FKBP12 were expressed and purified according to Bultinck *et al.* (44), Ypa1 and Ypa2 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 in a one-step procedure using Ni^{2+}-Sepharose. The catalytic subunit of PP2A ($PP2A_C$) was purified from bovine heart (46), the dimeric form of PP2A ($PP2A_D$), was from rabbit skeletal muscle (47), and the inactive form of PP2A ($PP2A_i$) was from porcine brain (14) according to published procedures.

Suc-Ala-Ala-Pro-Phe-methylcoumarylamine (s-AAPF-MCA), Suc-Ala-Ala-Pro-Phe-para-nitroanilide (s-AAPF-pNA), Suc-Ala-Ala-Pro-Lys-pNA (s-AAPK-pNA), and Suc-Ala-Glu-Pro-Phe-pNA (s-AEPF-pNA) were purchased from Bachem. Different stock solutions were made in ethanol and diluted in MilliQ water before assay. The PP2A-derived peptides were synthesized in the laboratory with the Fmoc technology (Table 1).

Human $PP2A_{c\alpha}$ was subcloned in the pMB001 vector (48) (a gift of Dr. Aleyde Van Eynde, Division of Biochemistry, Faculty of Medicine, KULeuven, Belgium) to obtain an N-terminal HA-tagged version. Point mutations were introduced with the QuikChange protocol (Stratagene) using Pwo proofreading polymerase (Roche Applied Science). All subcloning steps and point mutations were verified by DNA sequencing.

Recombinant PTPA Purification—Two different types of PTPA preparations were used, recombinant wild type rabbit PTPA and a His-tagged version. 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 vivaspin 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-Ypa1 (D205G) were purified using a one step Ni^{2+}-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 $A_{600} = 0.2–0.3$. The cells were grown at 37 °C until the medium reached an $A_{600} = 0.6$. 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 on 1 ml of a Ni^{2+}-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 × 1 ml of elution buffer (50 mM 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 His tag was removed by an overnight thrombin cleavage at 4 °C (5 units/ml), whereas His-PTPA was still bound to the Ni^{2+}-Sepharose beads. In this case PTPA was eluted with 50 mM Tris, pH 7.5, and the thrombin was removed during the final Superdex-200 gel filtration.

During these purifications PTPA was followed by activity measurements with $PP2A_i$ as described (14), and in neither these nor in the isomerase activity measurements could a substantial difference be observed between the different PTPA preparations. The PTPA inactive

TABLE 1
Pro-containing peptides from PP2A (and 1 from PP1) used to test isomerase activity of PTPA
All peptides were synthesized by the Fmoc technology.

Peptide	Sequence
1	^{47}EVRCPVTVC^{55}
2	^{72}GGKSPDTNY^{80}
3	^{104}KVRYPERIT^{112}
4	^{150}FDYLPLTAL^{158}
5	^{168}GGLSPSIDT^{176}
6	^{186}LQEVPHEGPMCDL^{198}
6-bis	^{186}LQEVPHEGAMCDL^{198}
6-ter	PTDVPDTGLLCD
7	^{199}LWSDPDDRG^{207}
8	^{209}WGISPRGAGY^{218}
9	^{259}IFSAPNYCY^{267}
10	^{287}LQFDPAPRRGE^{297}
11	^{294}RRGEPHVTR^{302}
12	^{302}RRTPDYFL^{309}

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 spectrometry. 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)

PPIase Assay—The routine PPIase assay was essentially based on the protease-coupled method described by Fischer *et al.* (26) and Schutkowski *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 MCA or a chromophore 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 PPIases, resulting in the cleavable *trans* conformation (second phase, slow) (Fig. 1*A*). 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 PPIase 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^{2+}ATP, and the reaction was started by the simultaneous (±1 s) addition of 25 μl of protease solution and 25 μl of PPIase 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 isomer specific protease and s-AAPF-MCA as the substrate (49). Therefore, these conditions were first optimized with PTPA as the potential PPIase. 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. 1*B*).

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 PPIase 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 spectrofluorimeter. 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 spectrofluorimeter and later transferred to Sigma-plot 8.1 for mathematical analysis (Fig. 1*A*). 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^{2+}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. 1*A*). 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* value (k_{obs}).

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. 1*B*), chymotrypsin, or trypsin. The autoisomerization rate constant was $k_{obs} = 0.03\ s^{-1}$ at 20 °C.

NMR Measurements—^{1}H NMR measurements were performed on a Varian Unity 500 spectrometer with a 3-mm HCPzgrd 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,

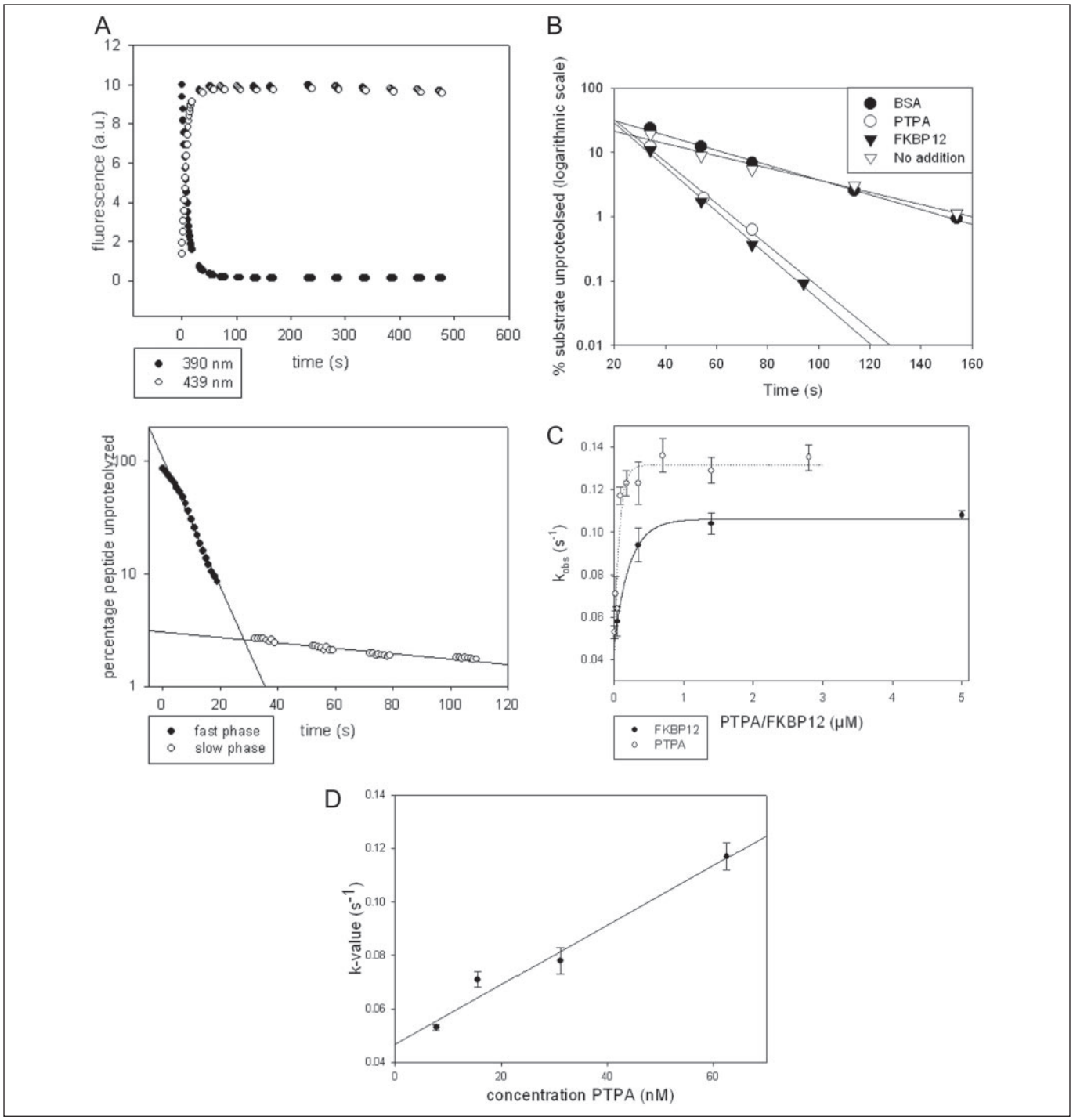

FIGURE 1. **PPIase measurement by the protease based assay.** *A*, *upper panel*, time-resolved fluorescence spectrum. The free (○) and bound MCA (●) are simultaneously detected showing, respectively, an increasing and decreasing graph. *Lower panel*, the data are shown in a semilog plot of % substrate intact *versus* time. Two distinct phases can be observed, the fast phase (●) of the initial cleavage of the peptide, already in the *trans* conformation by the protease, and the slow phase (○) resulting from the isomerization reaction (spontaneous or induced by a PPIase activity). *B*, semilog plot of individual measurements with the addition of either BSA (●), PTPA (○), FKBP12 (▼), or without addition (▽). Experimental conditions: [s-AAPF-MCA] = 7.56 μM, [subtilisin] = 1.82 μM, [Mg^{2+}ATP] = 1 mM/5 mM, [Tris] = 42.5 mM, pH = 7.4, T = 20 °C, [BSA] = 0.4 μM, [PTPA] = 0.5 μM, [FKBP12] = 0.5 μM. The points shown on the graph are the average of the measurements during 10 consecutive seconds. S.E. *bars* are smaller than the size of the symbols and, therefore, not plotted. *C*, graph showing the determination of the k_{obs} value by using different amounts of PTPA (○) or FKBP12 (●) in the assay. Experiment conditions: total sample volume = 500 μl, [Mg^{2+}ATP] = 5 mM/1 mM, [s-AAPF-MCA] = 7.56 μM, [subtilisin] = 1.82 μM, [Tris] = 42.5 mM, pH = 7.4, T = 20 °C, [PTPA] = 2.72, 1.36, 0.68, 0.34, 0.17, 0.085, 0.043, 0.021, and 0.012 μM, and [FKBP12] = 4.9, 1.39, 0.36, and 0.069 μM. Each point represents the mean of three independent measurements. *D*, prolyl isomerase activity of PTPA. The *k* value of the PPIase activity of PTPA is shown in function of the PTPA concentration. Measurements were done in 50 mM Tris, pH 7.4, in the presence of 8.25 μM s-AAPK-pNA and 4.4 μM trypsin. The slope of the line is equivalent to k_{cat}/K_m (n = 3 for each concentration).

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_2O 275-μl 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

TABLE 2

k_{obs} values of different PPIases with some model substrates

k_{obs} values were measured at 20 °C as described under "Experimental Procedures" under the following conditions: [s-AAPF-MCA] = 7.55 μM; [s-AAPK-pNA] = 8.25 μM; [s-AEPF-pNA] = 7.3 μM; [Mg^{2+}ATP] = 1 mM ATP, 5 mM $MgCl_2$; [FKBP12] = 0.5 μM; [Cpr7] = 0.6 μM; [cyclophilin] = 0.2 μM; [PTPA] = 0.5 μM; [Ypa1] = 0.6 μM; [Ypa2] = 0.5 μM; [Pin1] = 0.3 μM; [subtilisin] = 1.8 μM; [trypsin] = 4.4 μM. All experiments were measured in triplicate except the fluorescence measurements, which were measured in triplicate and were doubly analyzed after the formation of free MCA and after the drop in bound MCA (see Fig. 1*A*). The mean values are given ± S.D. All values are presented after subtraction of the blank, measured with the addition of [BSA] = 0.4 μM.

Enzyme	ATP/Mg^{2+}	s-AAPF-MCA	s-AAPK-pNA	s-AEPF-pNA
FKBP12		0.08 ± 0.01 s^{-1}		
	+ATP/Mg^{2+}	0.08 ± 0.01 s^{-1} [a]		
Cpr7			0.062 ± 0.008 s^{-1}	
	+ATP/Mg^{2+}		0.06 ± 0.01 s^{-1} [a]	
Cyclophilin			0.13 ± 0.02 s^{-1}	
	+ATP/Mg^{2+}		0.14 ± 0.03 s^{-1} [a]	
PTPA		0.03 ± 0.01 s^{-1}	0.03 ± 0.01 s^{-1}	0.01 ± 0.01 s^{-1}
	+ATP/Mg^{2+}	0.19 ± 0.02 s^{-1} [b]	0.10 ± 0.02 s^{-1} [b]	0.003 ± 0.006 s^{-1} [a]
Ypa1			0.02 ± 0.01 s^{-1}	
	+ATP/Mg^{2+}		0.07 ± 0.02 s^{-1} [b]	
Ypa2			0.03 ± 0.02 s^{-1}	
	+ATP/Mg^{2+}		0.05 ± 0.03 s^{-1} [a]	
Pin1			0.004 ± 0.002 s^{-1}	0.08 ± 0.02 s^{-1}
	+ATP/Mg^{2+}		0.00 ± 0.01 s^{-1} [a]	0.07 ± 0.01 s^{-1} [a]
Blank (BSA)		0.03 ± 0.01 s^{-1}	0.032 ± 0.003 s^{-1}	0.023 ± 0.002 s^{-1}
	+ATP/Mg^{2+}	0.031 ± 0.007 s^{-1} [a]	0.03 ± 0.01 s^{-1} [a]	0.025 ± 0.003 s^{-1} [a]

[a] Difference between the measurements with and without Mg^{2+}ATP, $p > 0.05$.
[b] Difference between the measurements with and without Mg^{2+}ATP, $p < 0.05$.

added to test the ATP dependence of the PPIase activity of PTPA. 5 μM PTPA, Ypa1, or Ypa2 were added to test the PPIase activity. Tris or 5 μM BSA were used as a negative control, and 5 μM FKBP12 was used as a positive control for the PPIase 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, nnPredict, 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 PP2A with the three-dimensional structure of PP1 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 International). 24 h after seeding into 10-cm plates, cells were transfected with FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. 36 h after transfection, cells (2 × 10 cm plate) were washed with ice-cold phosphate-buffered saline and scraped into 500 μl of ice-cold Tris-buffered saline plus 0.1% Nonidet P-40 supplemented with 50 μg/ml leupeptine, 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 5 μg/ml leupeptine, 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

PPIase Activity of PTPA in Comparison with FKBP12, Cyclophilin A, and Cpr7—Cyclophilins and FKBPs are ubiquitous and highly conserved enzyme families, both characterized as PPIases. Therefore, we compared the PPIase 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 PPIase activity of PTPA was compared with the PPIase activity of FKBP12, a known prolyl *cis*/*trans*-isomerase with a well documented activity for the MCA substrates used. Unless stated otherwise, all PPIase activities of PTPA, Ypa1, and Ypa2 were measured in the presence of a saturating concentration of Mg^{2+}ATP, since these components are proven to be stimulatory also for the PPIase activity (see below). As shown in Fig. 1*B*, the rate of *cis*/*trans* isomerization during the second "slow" phase by PTPA and FKBP12 was very comparable. Second, the k_{obs} was measured for PTPA and FKBP12 for different protein concentrations, and as demonstrated in Fig. 1*C*, the k_{obs} for PTPA and FKBP12 depended on the concentration of PPIases reaching a maximum of, respectively, k_{obs} = 0.13 s^{-1} at 0.25 μM PTPA and 0.11 s^{-1} at 0.5 μM FKBP12 at 20 °C. Therefore, k_{obs} is measured (Table 2) at lower enzyme concentrations where a linear relationship exists between the PPIase concentration and k_{obs} (Fig. 1*D*).

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 PPIases, (51, 52) and PTPA showed a PPIase activity in the

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. 1*D*). With a substrate concentration of 8.25 μM and a trypsin concentration of 4.4 μM, we obtained a $k_{cat}/K_m = 1.1\ \mu M^{-1}s^{-1}$. This result can be compared with previous results obtained for cyclophilin ($k_{cat}/K_m = 5.1\ \mu M^{-1}s^{-1}$) and FKBP12 ($k_{cat}/K_m = 0.14\ \mu M^{-1}s^{-1}$) (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. Upon the addition of PTPA it can be seen that the peaks broaden, indicating a PPIase activity of PTPA; this broadening is more pronounced when more PTPA is added to the sample (Fig. 2) and clearly stimulated by the presence of Mg^{2+}ATP. Positive (FKBP12) and negative (BSA) controls validated the conclusion that PTPA can indeed isomerase the prolyl residue from a *cis* to a *trans* conformation in the context of this model substrate and that this PPIase activity is stimulated by Mg^{2+}ATP.

PTPA and Pin1 Have a Different Substrate Specificity—Pin1 is important in cell signaling and regulation of the cell cycle since it can isomerase the prolyl residues preceded by phospho-Ser/Thr. Phosphorylation of these Ser/Thr-Pro motifs is governed by the so-called Pro-directed kinases such as the mitogen-activated protein kinases and the cyclin-dependent kinases, and Pin1 activity is dependent on the phosphorylation of these Ser/Thr-Pro motives (38, 39). Because of this highly selective substrate specificity, it was important to know whether PTPA has a similar substrate selectivity. When the Pin1 substrate, s-AEPF-pNA, where Glu (E) mimics the phospho-Ser/Thr, was used in the PPIase assay of PTPA (0.67 μM) with subtilisin as the *trans*-specific protease, the k_{obs} value was only slightly higher than the blank values. In contrast, Pin1 showed a significant PPIase activity toward this substrate, whereas it had no activity toward the s-AAPK-pNA substrate (Table 2). From this result it is clear that the Glu in the −1 position of the Pro is certainly not a prerequisite for isomerization by PTPA but is, rather, unfavorable.

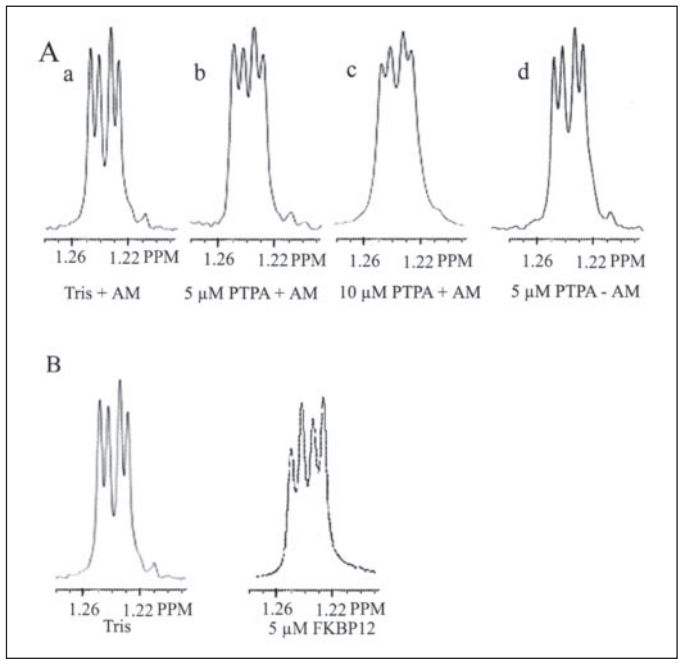

FIGURE 2. **^{1}H NMR measurements of the PPIase activity.** Measurements were conducted in 275 μl of 40 mM Tris, pH 7.1, and 10% D_2O. *A*, 1 mM peptide (s-AAPK-pNA), 2 mM ATP/10 mM Mg^2 (*AM*), and 40 mM Tris (*a*), 5 μM PTPA (*b*), 10 μM PTPA (*a*) at $T = 37$ °C, and 5 μM PTPA without Mg^{2+}ATP added previously (*d*). *B*, control results without Mg^{2+}ATP and PPIase and with 5 μM FKBP12.

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 FKBPs 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 FKBPs 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

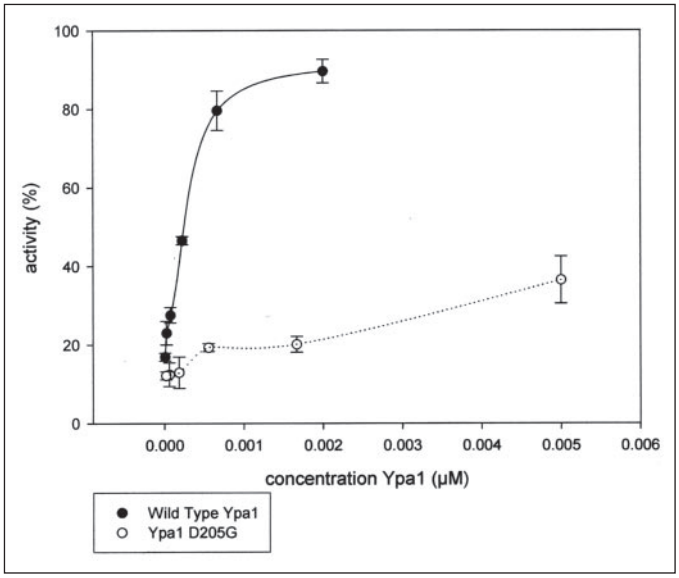

FIGURE 3. **Activation of inactive PP2A by WT and mutant Ypa1.** Activation of inactive PP2A by different concentrations of WT Ypa1 (●) and the Ypa1-mutant D205G (○) is shown. Activity is displayed in % of the activity of inactive PP2A when activated by an excess of PTPA ($n = 2$ for each concentration).

PPIases, its biochemical and structural characteristics are sufficiently distinct to catalog PTPA as a new family of prolyl *cis*/*trans*-isomerases (see also "Discussion").

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, ^{208}GVWGLD^{213}, 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 $PP2A_i$ 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 ($k_{obs} = 0.008\ s^{-1}$), not significantly higher than the autoisomerization reaction. By changing Asp^{205} into Gly in the Ypa1 context (Asp^{213} in mammalian PTPA), we could identify this amino acid as essential both for the activation of PP2Ai (Fig. 3) and for the PPIase activity of Ypa1 (Table 3). Therefore, Asp^{205} 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 PPIase activity of PTPA and the activation of PP2A, it is reasonable to assume that both activities are linked and that the PPIase activity of PTPA is not accidental but essential for the activation reaction of PP2A.

TABLE 3

k_{obs} values of PTPA mutants

k_{obs} values were measured at 20 °C as described under "Experimental Procedures" with the following conditions: [s-AAPK-pNA] = 8.25 μM; [Mg^{2+}ATP] = 1 mM ATP, 5 mM $MgCl_2$; [trypsin] = 4.4 μM; [PTPA (Δ208–213)] = 0.5 μM; [Ypa1 (D205G)] = 0.5 μM. The mean values are given ± S.D. All values are presented after subtraction of the blank, measured with the addition of [BSA] = 0.4 μM (see Table 1).

Enzyme	ATP/Mg^{2+}	s-AAPK-pNA
PTPA (Δ208–213)		0.006 ± 0.002 s^{-1}
	+ATP/Mg^{2+}	0.008 ± 0.001s^{-1} [a]
Ypa1 (D205G)		0.011 ± 0.004 s^{-1}
	+ATP/Mg^{2+}	0.016 ± 0.002s^{-1} [a]

[a] Difference between measurements with the mutants and blank measurement, $p > 0.05$.

Which Proline in PP2A Is the Target for PTPA PPIase Activity?—Because no three-dimensional structure is yet available either for the active or for the inactive forms of PP2A, we do not have direct proof for which proline in PP2A might be the target for a *cis*/*trans* isomerization reaction. In $PP2A_C$, 14 Pro are present, and we reasoned that if the PPIase activity of PTPA could be responsible for the activity change of PP2A, at least one of these prolines should be a substrate in the isomerization reaction. Therefore, we synthesized 12 peptides representing the microenvironment of these 14 Pro residues in PP2A (Table 1). To exclude a potential influence of the length of the peptides in the isomerization reaction and to provide a minimal length, possibly necessary for specificity, 9-mers were synthesized with Pro in the middle. This rule could not be followed in peptide 6 and 10 since 2 Pro were too close; therefore, we chose for a minimal of 4 amino acids after the second Pro. This reasoning is proven to be fruitful. The only peptide that showed a peak broadening in its NMR spectrum by adding PTPA/Mg^{2+}ATP was peptide 6 (fig. 4) (see Supplemental Fig. 2 for the results with the 11 other peptides). Because peptide 6 contains Pro^{190} and Pro^{194}, potentially both could undergo a *cis*/*trans* isomerization. Therefore, an additional peptide (6-bis) was synthesized with the second Pro mutated into a Ala. Also, this peptide showed a peak broadening in the presence of PTPA/Mg^{2+}ATP, clearly demonstrating that it is Pro^{190} that is the substrate for the PPIase activity of PTPA. To further substantiate the specificity of the isomerization reaction by PTPA/Mg^{2+}ATP, we synthesized peptide 6-ter, representing the peptide taking a similar position in PP1. Also, the Pro in this peptide is not converted into the *trans* configuration by PTPA/Mg^{2+}ATP.

Because PP1 and PP2A have a high degree of similarity (55) and the three-dimensional structure of PP1 is known (56, 57), PP1 can be used as template to predict the structure of $PP2A_C$. By doing so, the position of peptide 6 could also be predicted. This peptide is on the surface of $PP2A_C$ and is part of a loop structure connecting α helix 6 and β strand 6 near the catalytic center. This is a strategic position for influencing the activity of PP2A since changing the conformation of the Pro in this loop will also change the conformation of the loop and, therefore, the accessibility of the PP2A catalytic site or the efficiency of the catalysis.

A Pro^{190} Mutant of $PP2A_C$ Cannot Be Activated by PTPA—To prove the importance of Pro^{190} in PP2A for activation by PTPA/Mg^{2+}ATP, we mutated Pro^{190} in the context of $PP2A_C$ into a Phe or an Ala, expressed them as HA-tagged versions in COS 7 cells, immunoprecipitated the constructs, and attempted to determine the possibility of activating this PP2A by PTPA/Mg^{2+}ATP. As controls, a HA-tagged version of wt $PP2A_c$ was used, and the same construct with Pro^{263} mutated into an Ala. As can be seen in Fig. 5*A*, the amount of precipitated PP2A is very similar for the four constructs. PP2A was specifically measured in the

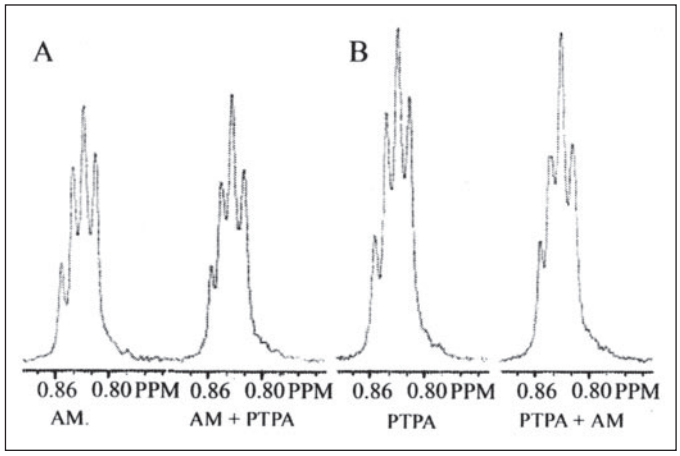

FIGURE 4. **^{1}H NMR spectra of a PP2A-derived peptide.** Shown is a region of a one-dimensional NMR proton spectrum with signals from methyl groups in the LQEVPHEG-PMCDL peptide. Measurements were conducted in 275 μl of 40 mM Tris, pH 7.1, 10% D_2O, and 1 mM peptide (peptide 6 in Table 1) at 27 °C and 2 mM ATP/10 mM Mg^{2+} (*AM*) and/or 5 μM PTPA. In *A* and *B*, the order of addition of the components was alternated as indicated in the figure.

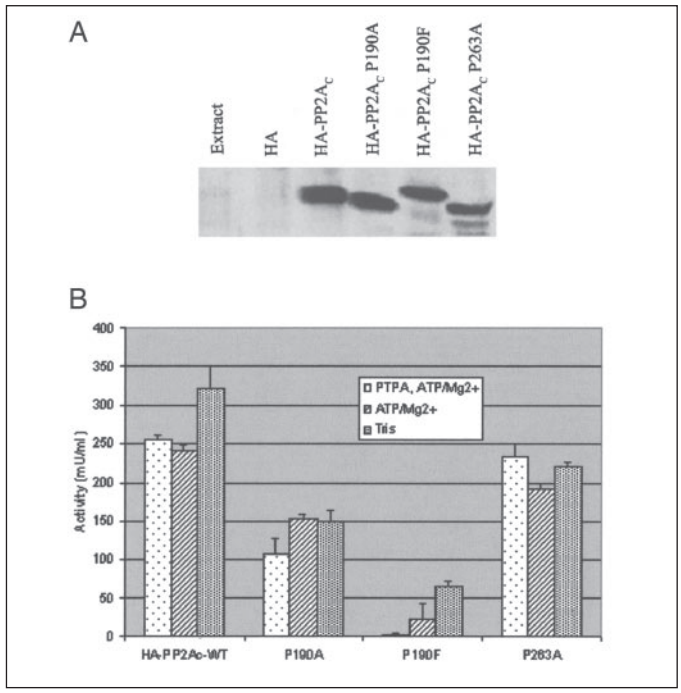

FIGURE 5. **Importance of Pro^{190} for activity of PP2A$_C$.** Cos7 cells were not transfected (*lane 1*), transfected with pMB001 (empty vector, *lane 2*), or transfected with PP2A$_C$WT-pMB001, PP2A$_C$P$_{190}$A-pMB001, PP2A$_C$P$_{190}$F-pMB001, and PP2A$_C$P$_{263}$A-pMB001 and immunoprecipitated. These immunoprecipitations were further analyzed by Western blotting after SDS-PAGE with anti-HA antibodies (*A*) or phosphatase activity measurements (*B*) with or without activation by PTPA/Mg^{2+} ATP as indicated ($n = 2$). Background activity as measured in two control conditions (less than 20% of the WT conditions) was subtracted. For more details see "Experimental Procedures."

presence of protamine sulfate (16) plus or minus activation by a saturating amount of PTPA/Mg^{2+}ATP, and as can be seen in Fig. 5*B*, PP2A$_c$ wt was spontaneously active and could not be further activated by PTPA/Mg^{2+}ATP, PP2A$_c$ (P190A) was partially active and not activable by PTPA/Mg^{2+}ATP, and PP2A$_c$ (P190F) was almost completely inactive and not activable either, whereas the PP2A$_c$ (P_{263}A) mutant was almost as active as WT and also not activable. These results clearly show the importance of Pro^{190} for the activity of PP2A$_c$. The fact that PP2A$_C$ WT is fully active in this experiment is probably due to the fact that in COS7 cells, PTPA is highly expressed[5] and, therefore, is not the limiting factor for all PP2A activity. If Pro^{190} is mutated into a Phe, a conformational change will be induced that inactivates PP2A and that can no longer be reversed by PTPA/Mg^{2+}ATP. Replacement of Pro^{190} by Ala results in a partially active PP2A$_C$ (>50% of WT activity), but that can also not be activated by PTPA/Mg^{2+}ATP, indicating that Pro^{190} 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 indeed 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 Pro^{190} 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 PP2A$_C$ mutant with Pro^{190} changed into a Phe was inactive and could not be activated by PTPA/Mg^{2+}ATP, strongly arguing for the importance of Pro^{190} in the activation reaction. Although we cannot rule out other explanations for the inability of PTPA/Mg^{2+}ATP to activate the PP2A$_C$ (P190F) inactive mutant, the most plausible hypothesis would be that Pro^{190} 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 methylesterase PME1 (54), suggesting that some interacting surfaces are different in the active and this inactive PP2A. Also, by purifying PME1 from tissues, an inactive form of PP2A was found associated with PME1. After incubation with PTPA and Mg^{2+}ATP, PP2A was activated and dissociated from PME1 (14). These observations strongly argue for the existence *in vivo* of two conformations of PP2A, one of them favored by PTPA.

The requirement of Mg^{2+}ATP in the activation reaction of PP2A is still intriguing. Also, for the PPIase activity of PTPA, the presence of Mg^{2+}ATP seems to be stimulatory but less stringent since also in the absence of Mg^{2+}ATP a low but measurable PPIase activity could be detected. This is not so surprising since small peptides are used as substrate 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 PPIase activity of PTPA is probably causal for the activity change in PP2A, we measured the PPIase activity in a mutant of PTPA (Δ^{208}GVWGLD^{213}) that is almost completely inactive in the phosphotyrosyl phosphatase activation reaction of PP2A (13) and in the activation of $PP2A_i$ (this study).This mutant was also inactive as PPIase. Therefore, the activation of PP2A and the PPIase 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^{213} in PTPA, shows a similar correlation, corroborating its implication in the catalysis of the PPIase.

So far three families of peptidyl-prolyl isomerases are known: cyclophilins, FKBPs, and parvulins. Cyclophilins and FKBPs were originally discovered as the intracellular binding targets of immunosuppressive drugs. Almost simultaneous, these proteins were characterized as PPIases. These enzymes have long been thought to play an exclusive role in protein folding, but since in yeast all cyclophilins (eight) and FKBPs (four) are individually and collectively dispensable for viability in *S. cerevisiae* (33), it was concluded that each cyclophilin and FKBP probably regulates a restricted number of unique partner proteins that still remain to be identified (33). In the genome of the same organism only one parvulin is present. ESS1, also known as PTF1 (processing/termination factor), is the homologue of the mammalian Pin1, and this gene was found to be essential (33, 59). Remarkably, the yeast orthologues of PTPA, Ypa1 and Ypa2, also have PPIase activity (this study) and also cover an essential function since a double deletion strain is not viable (21–23). PTPA and Pin1 have different substrate specificities as PPIases (this study), differentiating PTPA as different from the parvulins. PTPA is also the only PPIase known so far that shows a stimulatory effect of Mg^{2+}ATP. Furthermore, only PTPA could activate inactive PP2A, whereas recombinant FKBP12 and Cpr7 had no effect in the presence or absence of FK506 and cyclosporin A, respectively. Finally, similarity studies showed only a minor similarity in the primary structure as well as in the secondary structure of PTPA with the other PPIases. For all these reasons, PTPA can be catalogued as a separate, fourth family of PPIases, targeting PP2A and the PP2A-like phosphatases.

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REFERENCES

1. Janssens, V., and Goris, J. (2001) *Biochem. J.* **353,** 417–439
2. Chen, J., Martin, B. L., and Brautigan, D. L. (1992) *Science* **257,** 1261–1264
3. Guo, H., and Damuni, Z. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2500–2504
4. Lee, J., and Stock, J. (1993) *J. Biol. Chem.* **268,** 19192–19195
5. Xie, H., and Clarke, S. (1993) *J. Biol. Chem.* **268,** 13364–13371
6. Xie, H., and Clarke, S. (1994), *Biochem. Biophys. Res. Commun.* **203,** 1710–1715
7. Lee, J., Chen, Y., Tolstykh, T., and Stock, J. (1996) *Biochem. Biophys. Res. Commun.* **93,** 6043–6047
8. De Baere, I., Derua, R., Janssens, V., Van Hoof, C., Waelkens, E., Merlevede, W., and Goris, J. (1999) *Biochemistry* **38,** 16539–16547
9. Van Hoof, C., and Goris, J. (2003) *Biochim. Biophys. Acta* **1640,** 97–104
10. Cayla, X., Goris, J., Hermann, J., Hendrix, P., Ozon, R., and Merlevede, W. (1990) *Biochemistry* **29,** 658–667
11. Van Hoof, C., Cayla, X., Bosch, M., Merlevede, W., and Goris, J. (1994) *Eur. J. Biochem.* **226,** 899–907
12. Cayla, X., Van Hoof, C., Bosch, M., Waelkens, E., Vandekerckhove, J., Peeters, B., Merlevede, W., and Goris, J. (1994) *J. Biol. Chem.* **269,** 15668–15675
13. Van Hoof, C., Janssens, V., Dinishiotu, A., Merlevede, W., and Goris, J. (1998) *Biochemistry* **37,** 12899–12908
14. Longin, S., Jordens, J., Martens, E., Stevens, I., Janssens, V., Rondelez, E., De Baere, I., Derua, R., Waelkens, E., Goris, J., and Van Hoof, C. (2004) *Biochem. J.* **380,** 111–119
15. Fellner, T., Lackner, D.H., Hombauer, H., Piribauer, P., Mudrak, I., Zaragoza, K., Juno, C., and Ogris, E. (2003) *Genes Dev.* **17,** 2138–2150
16. Van Hoof, C., Martens, E., Longin, S., Jordens, J., Stevens, I., Janssens, V., and Goris, J., (2005) *Biochem. J.* **386,** 93–102
17. Van Hoof, C.,Sayed Aly, M., Garcia, A., Cayla, X. Cassiman, J. J., Merlevede, W., and Goris, J. (1995) *Genomics* **28,** 261–272
18. Janssens, V., Van Hoof, C., Martens, E., De Baere, I., Merlevede, W., and Goris, J. (2000) *Eur. J. Biochem.* **267,** 4406–4413
19. Janssens, V., Van Hoof, C., De Baere, I., Merlevede, W., and Goris, J. (1999) *Biochem. J.* **344,** 755–763
20. Janssens, V., Van Hoof, C., De Baere, I., Merlevede, W., and Goris, J. (2000) *J. Biol. Chem.* **257,** 20488–20495
21. Van Hoof, C., Janssens, V., De Baere, I., De Winde, J. H., Winderickx, J., Dumortier, F., Thevelein, J. M., Merlevede, W., and Goris, J. (2000) *J. Mol. Biol.* **302,** 103–119
22. Rempola, B., Kaniak, A., Migdalski, A., Rytka, J., Slonimski, P. P., and di Rago, J.-P. (2000) *Mol. Gen. Genet.* **262,** 1081–1092
23. Van Hoof, C., Janssens, V., De Baere, I., Stark, M. J. R., De Winde, J. H., Winderickx, J., Thevelein, J. M., Merlevede, W., and Goris, J. (2001) *Exp. Cell Res.* **264,** 372–387
24. Brandts, J. F., Halvorson, H. R., and Brennan, M. (1975) *Biochemistry* **22,** 4953–4963
25. Brandts, J. F., and Lin, L.-N. (1986) *Methods Enzymol.* **131,** 107–126
26. Fischer, G., Bang, H., and Mech, C. (1984) *Biomed. Biochim. Acta* **43,** 1101–1111
27. Takahashi, N., Hayano, T., and Suzuki, M. (1989) *Nature* **337,** 473–475
28. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F.X. (1989) *Nature* **337,** 476–478
29. Siekierka, J. J., Hung, S. H. Y., Poe, M., Lin, C. S., and Sigal, N. H. (1989) *Nature* **341,** 755–757
30. Harding, M. W., Galat, A., Ueling, D. E., and Schreiber, S. L. (1989) *Nature* **341,** 758–760
31. Schmid, F. X., Mayr, L. M., Mücke, M., and Schönbrunner, E. R. (1993) *Adv. Protein Chem.* **44,** 25–66
32. Lu, K. P. (2000) *Prog. Cell Cycle Res.* **4,** 83–96
33. Dolinski, K., Muir, S., Cardenas, M., and Heitman, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 13093–13098
34. Bukrinsky, M. I. (2002) *Trends Immunol.* **23,** 323–325
35. Lu, K. P., Hanes, S. D., and Hunter, T. (1996) *Nature* **380,** 544–547
36. Rahfeld, J. U., Schierhorn, A., Mann, K., and Fischer, G. (1994) *FEBS Lett.* **343,** 65–69
37. Yaffe, M. B., Schutowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Rahfeld, J.-U., Xu, J., Kuang, J., Kirschner, M. W., Fischer, G., Cantley, L. C., and Lu, K. P. (1997) *Science* **278,** 1957–1960
38. Schutkowski, M., Bernhardt, A., Zhen Zhou, X., Shen, M., Reimer, U., Rahfeld, J.-U., Lu, K. P., and Fischer, G. (1998) *Biochemistry* **37,** 5566–5575
39. Hani, J., Schelbert, B., Bernhardt, A., Domdey, H., Fischer, G., Wiebauer, K., and Rahfeld, J. U. (1999) *J. Biol. Chem.* **247,** 108–116
40. Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. (1998) *Genes Dev.* **12,** 706–720
41. Crenshaw, D. G., Yang, J., Means, A. R., and Kornbluth, S. (1998) *EMBO J.* **17,** 1315–1327
42. Zacchi, P., Gostissa, M., Uchida, T., Salvagno, C., Avolio, F., Volinia, S., Ronai, Z., Blandino, G., Schneider, C., and Del Sal, G. (2002) *Nature* **419,** 853–857
43. Mantovani, F., Piazza, S., Gotissa, M., Strano, S., Zacchi, P., Mantovani, R., Blandino, G., and Del Sal, G. (2004) *Mol. Cell* **14,** 625–636
44. Bultinck, G., De Smet, P., Rossi, D., Callewaert, G., Missiaen, L., Sorrentino, V., De Smedt, H., and Parys, J.B. (2001) *Biochem. J.* **354,** 413–422
45. Winkler, K. E., Swenson, K. I., Kornbluth, S., and Means, A. R. (2000) *Science* **287,** 1644–1647
46. Ramachandran, C., Goris, J., Waelkens, E., Merlevede, W., and Walsh, D. A. (1987) *J. Biol. Chem.* **262,** 3210–3218
47. Waelkens, E., Goris, J., and Merlevede, W. (1987) *J. Biol. Chem.* **262,** 1049–1059
48. Gijsbers, R., Ceulemans, H., Stalmans, W., and Bollen, M. (2001) *J. Biol. Chem.* **276,** 1361–1368
49. Bächinger, H. P. (1987) *J. Biol. Chem.* **262,** 17144–17148
50. Duina, A. A., Marsh, J. A., and Gaber, R. F. (1996) *Yeast* **10,** 943–952
51. Mayr, C., Richter, K., Lilie, H., and Buchner, J. (2000) *J. Biol. Chem.* **275,** 34140–34146
52. Schiene, C., Reimer, U., Schutkowski, M., and Fischer, G. (1998) *FEBS Lett.* **432,** 202–206
53. Hübner, D., Drakenberg, T., Forsén, S., and Fischer, G. (1991) *FEBS Lett.* **284,** 79–81
54. Ogris, E., Du, X., Nelson, K., Mak, E., Yu, X., Lane, W., and Pallas, D. (1999) *J. Biol. Chem.* **274,** 14382–14391
55. Baton, G., Cohen, P. T. W., and Barford, D. (1994) *Eur. J. Biochem.* **220,** 225–237
56. Egloff, M.-P., Cohen, P. T. W., Reinemer, P., and Barford, D., (1995) *J. Mol. Biol.* **254,** 942–959
57. Goldberg, J., Huang, H.-B., Kwon, Y. G., Greengard, P., Nairn, A. C., and Kuriyan, J. (1995) *Nature* **376,** 745–753
58. Evans, D., Myles, T., Hofstege, J., and Hemmings, B. A. (1999) *J. Biol. Chem.* **274,** 24038–24046
59. Hani, J., Stumpf, G., and Domdey, H. (1995) *FEBS Lett.* **365,** 198–202

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