Comparison of in Vivo and in Vitro Phosphorylation of the Exocytosis-Sensitive Protein PP63/Parafusin by Differential MALDI Mass Spectrometric Peptide Mapping†

Martin Kussmann,‡,§,|| Karin Hauser,||,⊥ Roland Kissmehl,⊥ Jason Breed,⊥ Helmut Plattner,⊥ and Peter Roepstorff*,‡

Department of Molecular Biology, Odense University, Campusvej 55, DK-5230 Odense M, Denmark, and Faculty of Biology, University of Konstanz, P.O. Box 5560, D-78434 Konstanz, Germany

Received December 8, 1998; Revised Manuscript Received March 11, 1999

ABSTRACT: PP63 (parafusin) is a 63 kDa phosphoprotein, which exists in at least two different isoforms. It is very rapidly (80 ms) dephosphorylated during triggered trichocyst exocytosis. This occurs selectively in exocytosis-competent *Paramecium tetraurelia* strains. At least two protein kinases isolated from *Paramecium*, casein kinase type II kinase and cGMP-dependent kinase, are able to phosphorylate the two recombinant PP63/parafusin isoforms, both with phosphoglucomutase activity, in vitro. By performing mass spectrometric peptide mapping, we have investigated in vitro phosphorylation of recombinant PP63/parafusin by these kinases in comparison to in vivo phosphorylation of native PP63/parafusin isolated from *Paramecium* homogenates. Low picomolar quantities of proteolytic digests of recombinant and native PP63/parafusin, prior to and following alkaline phosphatase treatment, were directly analyzed by MALDI mass spectrometry. In native PP63-1/parafusin-1, six of 64 serine and threonine residues (S-196, T-205, T-280, T-371, T-373, and T-469) were found definitely, 27 were found possibly phosphorylated, 28 were identified as nonphosphorylated, and three were not covered by mapping. Three of the six certainly phosphorylated amino acids represent consensus phosphorylation sites for casein kinase II or cGMP-dependent protein kinase. In vitro phosphorylation studies of recombinant PP63/parafusin confirm that some of the sites found were used in vivo; however, also significant differences with respect to in vivo phosphorylation of native PP63/parafusin were observed. The two *Paramecium* protein kinases that were used do not preferably phosphorylate expected consensus sites in vitro. Homology structure modeling of PP63/parafusin with rabbit phosphoglucomutase revealed that the majority of residues found phosphorylated is located on the surface of the molecule.

The ubiquity of protein phosphorylation as a means of protein regulation is well-established. Approximately 30% of the intracellular proteins identified to date are phosphorylated. Protein kinases encompass 2% of the eukaryotic genome, and 1% accounts for phosphatases (*1*). The combination of specific endoproteolytic digestion with mass spectrometric peptide mapping has been extended to using enzymes, which remove or transfer posttranslational modifications, rendering also the latter amenable to structural characterization by mass spectrometry (*2*−*4*). This analytical concept has been applied successfully to the determination of the location and structure of glycans in glycoproteins (*2*−*4*) and to the characterization of phosphorylation sites in regulatory proteins (*5*).

We have employed this strategy in investigating phosphorylation sites in the 63 kDa phosphoprotein PP63/parafusin (PP63/pf)[1] of the ciliated protozoan *Paramecium tetraurelia* (*6*, *7*). This protein is heavily enriched in the cell cortex (*8*). It is selectively dephosphorylated within 80 ms during stimulated synchronous exocytosis of trichocysts and fully rephosphorylated within 10 s (*6*, *9*). It is proposed to be involved in the regulation of the exo- and endocytosis cycle since endocytosis rapidly occurs upon phosphorylation of PP63/pf (*10*). Two genes encode two PP63/pf isoforms, i.e., PP63-1/pf-1 and PP63-2/pf-2, which are 98% identical at the amino acid level (*11*). Cloning of the genes for PP63/pf also showed the identity of PP63/pf with phosphoglucomutase (PGM) (*11*).

Regulation of PP63/pf phosphorylation involves several protein kinases and phosphatases (*12*). PP63-1/pf-1 contains

† This work was supported by the Deutsche Forschungsgemeinschaft SFB 156, a grant from Struktur und Funktionssteuerung an zellulären Oberflächen, the Danish Biotechnology Program, and the Center for Experimental Bioinformatics (CEBI), sponsored by the Danish National Research Foundation.

* To whom correspondence should be addressed: Department of Molecular Biology, Odense University, Campusvej 55, DK-5230 Odense M, Denmark. Fax: ++45-65 93 26 61. E-mail: roe@pr-group.ou.dk.

‡ Odense University.

§ Present address: Cerbios-Pharma S. A., Via Pian Scairolo 6, CH-6917 Barbengo, Switzerland.

|| These authors contributed equally to this publication.

⊥ University of Konstanz.

[1] Abbreviations: ACN, acetonitrile; AMP, adenosine monophosphate; AP, alkaline phosphatase; cGMP, cyclic GMP; CK, *Paramecium* casein kinase type II; GMP, guanosine monophosphate; HCCA, 4-hydroxy-α-cyanocinnamic acid; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; PGM, phosphoglucomutase; PKG, cyclic GMP-dependent protein kinase; PP63/pf, 63 kDa phosphoprotein/parafusin.

10.1021/bi982888y CCC: $18.00
Published on Web 05/26/1999

64 and PP63-2/pf-2 65 serine and threonine residues, respectively. There are 18 putative phosphorylation sites for casein- or cyclic GMP/AMP-dependent kinases, 16 of which are consensus sites for cyclic GMP/AMP-dependent protein kinase and seven for casein kinase type II. Apart from these sites, there are three consensus sites for calmodulin-dependent protein kinase and six consensus sites for protein kinase C (*11*).

In this study, native PP63/pf and recombinantly expressed His-tagged His-PP63-1/pf-1 and His-PP63-2/pf-2 proteins were compared in terms of their phosphorylation pattern. Picomolar amounts of PP63/pf were isolated from *P. tetraurelia* in the phosphorylated state and were subjected to differential tryptic and endoprotease Glu-C peptide mapping. The digests of the native protein were analyzed prior to and after alkaline phosphatase (AP) treatment to identify phosphorylation sites in vivo. For further identification of protein kinases putatively involved in the phosphorylation of PP63/pf in vivo, picomolar quantities of two recombinant His-tagged isoforms His-PP63-1/pf-1 and His-PP63-2/pf-2 were assayed in vitro with Ser/Thr-specific protein kinases isolated from *P. tetraurelia* (*12*, *13*) and subsequently analyzed analogously. By using casein kinase type II protein kinase (CK) (*13*) and cyclic GMP-dependent protein kinase (PKG) (*12*), we identified multiple serine and threonine phosphorylation sites in vitro, only some of which correspond to those in vivo. This indicates that in vitro protein phosphorylation by selected kinases does not necessarily reflect the in vivo regulation of the protein substrate.

Molecular homology modeling with PGM from rabbit skeletal muscle shows that almost all of the definitely phosphorylated residues are located on the surface of the molecule and hence are sterically accessible to the kinase molecules.

MATERIALS AND METHODS

Materials

Porcine trypsin was purchased from Promega, and endoprotease Glu-C (*Staphylococcus aureus*) was from Boehringer Mannheim. Both endoproteases were sequencing-grade quality. Alkaline phosphatase (calf intestine) was purchased from Boehringer Mannheim. Pefabloc SC and leupeptin were obtained from Biomol (Hamburg, Germany); pepstatin A was obtained from Serva (Heidelberg, Germany) and aprotinin from Sigma (Deisenhofen, Germany). The matrix 4-hydroxy-α-cyanocinnamic acid (HCCA) was delivered from Sigma-Aldrich (Deisenhofen, Germany) and was recrystallized before being used. For nanoscale reversed phase purification of peptides, Poros R2 material (particle size of ca. 20 μm, retention properties correspond to those of C_{18} reversed phase material) was purchased from PerSeptive Biosystems (Wiesbaden, Germany). All solvents were of the highest quality available. Materials for isolation of protein kinases and for in vivo and in vitro phosphorylation studies were as reported previously (*12*).

Cell Cultures

P. tetraurelia 7S cells were cultured and harvested as described previously (*13*).

Isolation and Purification of Native PP63/pf

P. tetraurelia 7S cells were homogenized on ice in Dryl's solution [1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 2 mM sodium citrate, and 1.5 mM $CaCl_2$ (pH 6.8)], 20 mM NaF, and protease inhibitor cocktail (100 munits/mL aprotinin, 100 μM leupeptin, 15 fM pepstatin A, and 0.2 mM Pefabloc SC) by about 70 hand strokes in a glass homogenizer. Homogenates were centrifuged at 100000*g* for 30 min at 4 °C. PP63/pf was prepared from the 100000*g* supernatant by affinity chromatography using affinity-purified antibodies against recombinant PP63-1/pf-1 (*11*). Antibodies were coupled to CH-activated Sepharose (Pharmacia, Freiburg, Germany) as suggested by the manufacturer and packed into a column (volume of 1.5 mL). Fifteen milliliters of the supernatant of the 100000*g* centrifugation step buffered in PBS [7.8 mM Na_2HPO_4, 2.6 mM KCl, and 136 mM NaCl (pH 7.4)], supplemented with 20 mM NaF, was applied to the column with a flow rate of 200 μL/min. Subsequently, the column was washed with 40 mL of PBS/NaF. PP63/pf was eluted with 0.1 M glycine (pH 2.0) and collected in 1 mL fractions. The protein content of the fractions was monitored at 280 nm, and protein-containing fractions were pooled, neutralized with 1 N NaOH, and dialyzed against PBS.

Expression, Isolation, and Purification of Recombinant His-PP63-1/pf-1 and His-PP63-2/pf-2

These procedures were performed as described for His-PP63-1/pf-1 in ref *11*.

Isolation of Endogenous Protein Kinases

The isolation of a cGMP-dependent protein kinase (PKG) and a casein kinase (CK) was carried out as reported previously (*12*, *13*).

In Vitro Phosphorylation of Recombinant PP63/pf-1 and PP63/pf-2.

Phosphorylation by cGMP-Dependent Protein Kinase. The phosphorylation cocktail contained 25 μg of recombinant His-PP63-1/pf-1 or His-PP63-2/pf-2, respectively, 120 μM ATP, 150 nM cGMP, and 5 mM $MgCl_2$ in 250 μL of 20 mM triethanolamine/HCl (pH 7.5). The reaction, started by adding 220 ng of enriched cGMP dependent kinase, was carried out for 60 min at 20 °C and terminated by boiling for 3 min at 95 °C. To monitor incorporation of phosphate, a second reaction was performed in parallel, with [γ-^{32}P]-ATP as the phosphate donor. For controls, assays were also carried out in the absence of either substrate (autophosphorylation) or protein kinase. These samples were subjected to SDS−PAGE and subsequently prepared for autoradiography.

Phosphorylation with Casein Kinase. This was performed in the same way as described for cGMP-dependent protein kinase, apart from the replacement of cGMP-dependent protein kinase by casein kinase (160 ng) and omitting cGMP in the reaction mix.

SDS−PAGE and Autoradiography

SDS−PAGE and autoradiography were performed as described previously (*12*).

Endoproteolytic Digestion of Native and Recombinant His-PP63-1/pf-1 and His-PP63-2/pf-2

Native PP63/pf and the His-tagged His-PP63-1/pf-1 and His-PP63-2/pf-2 isoforms were aliquoted to 5 μg/100 μL portions, dissolved in 20 mM triethanolamine/HCl (pH 7.5). Aliquots of each of them (5 μg $\cong$ 80 pmol) were digested overnight at 37 °C either by 250 ng of trypsin or by 250 ng of endoprotease Glu-C (V8 protease). The molar ratio between enzymes and substrates was 1:20.

Dephosphorylation of Peptide Mixtures

To each sample of a second, identical set of tryptic and V8 proteolytic digests was added 2 μL (ca. 2 units) of alkaline phosphatase (AP) solution. Dephosphorylation was carried out for 2 h at 37 °C.

MALDI Mass Spectrometric Peptide Mapping

Peptide Sample Preparation. The peptide mixtures were purified using a microscale reversed phase cleanup technique (*14*, *15*). Briefly, the microcolumns were prepared by loading 2 μL of a suspension of Poros R2 material in ACN into a GeLoader tip, which was squeezed at the outlet to prevent the loss of column material. The peptide mixtures (ca. 80 pmol in 20 μL of buffer) were acidified with 1 μL of 10% TFA and loaded onto these columns. They were washed with ca. 10 μL of 0.1% TFA and subsequently eluted in ca. 10 μL of ACN/0.1% TFA (80:20). Aliquots of 0.5 μL thereof (ca. 4 pmol) were used in a dried-droplet sample preparation (*16*) with HCCA as the matrix.

Acquisition of Mass Spectra. Positive- and negative-ion mass spectra were acquired in linear and reflector mode on a Voyager Elite MALDI mass spectrometer equipped with a reflectron and delayed-extraction technology. The reflector mode mass spectra provided monoisotopic resolution up to *m*/*z* 3000, whereas the linear mode, with the low mass gate set at *m*/*z* 2000, was applied for maximum sensitivity for high-mass peptides.

Processing of MALDI-MS Data. Differential MALDI-MS peptide mapping before and after treatment of the digests with AP was carried out by overlaying spectra of untreated and dephosphorylated proteolytic peptide mixtures, e.g., by comparison of positive-ion, reflector mode spectra of tryptic digests prior to and after AP addition. The peptide mass lists were imported into the peptide mapping program GPMAW (General Protein Mass Analysis for Windows) (*17*) which compares experimentally obtained peptide masses with a predicted proteolytic digest of a protein of interest. Furthermore, it takes protein modifications, e.g., phosphorylation, into account. Ions corresponding to (multiply) phosphorylated tryptic or Glu-C peptides, which were exclusively detected prior to AP treatment, and ions correlating with unmodified tryptic or Glu-C peptides only appearing after digestion with AP were assigned to (multiply) phosphorylated peptides. A deviation tolerance of 200 ppm between the expected and the theoretical peptide mass was defined as the requirement for unambiguous peptide assignment. The amino acid numbering generally starts at M^1QQ..., i.e., for the native protein at the N-terminus and for the His-PP63-1/pf-1 and His-PP63-2/pf-2 after the His tag with 10 additional amino acids (see the legend of Figure 2 and the text). Hence, serine and threonine positions of recombinant PP63/pfs isoforms and the native PP63/pf correlate.

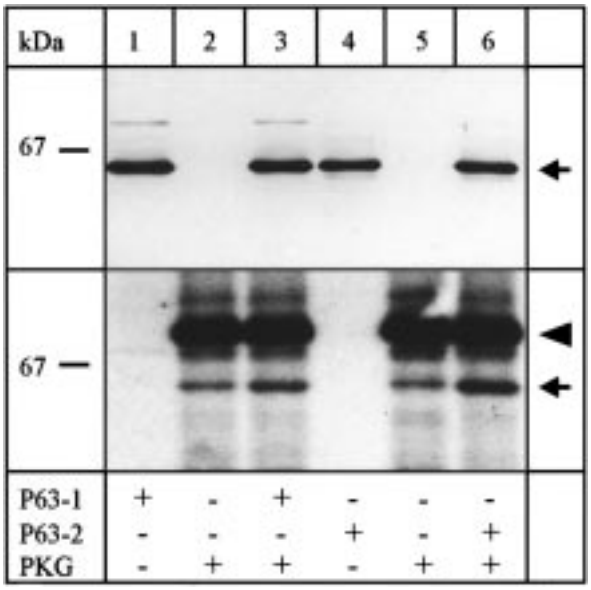

FIGURE 1: In vitro phosphorylation of recombinant His-PP63-1/pf-1 and His-PP63-2/pf-2 with endogenous PKG. The upper panel shows a silver-stained SDS polyacrylamide gel, 0.5 μg of protein per lane. The lower panel shows the corresponding autoradiogram. When assayed under autophosphorylation conditions (lanes 2 and 5), samples already contain a slightly phosphorylated polypeptide at 65 kDa. Recombinant PP63/pfs also appeared at 65 kDa because of their additional His tag. The strong signals in lanes 3 and 6 clearly show that PKG phosphorylates the recombinant PP63/pf polypeptides effectively. Arrows denote PP63/pf, and the arrowhead denotes the autophosphorylation of PKG.

```
MQQVIPAPRVQVTQPYAGQKPGTSGLRKKVSEATQPNYLENFVQSIFNTL   50
RKDELKPKNVLFVGGDGRYFNRQAIFSIIRLAYANDISEVHVGQAGLMST  100
PASSHYIRKVNEEVGNCIGGIILTASHNPGGKEHGDFGIKFNVRTGAPAP  150
EDFTDQIYTHTTKIKEYLTVDYEFEKHINLDQIGVYKFEGTRLEKSHFEV  200
KVVDTVQDYTQLMQKLFDFDLLKGLFSNKDFSFRFDGMHGVAGPYAKHIF  250
GTLLGCSKESLLNCDPSEDFGGGHPDPNLTYAHDLVELLDIHKKKDVGTV  300
PQFGAACDGDADRNMILGRQFFVTPSDSLAVIAANANLIFKNGLLGAARS  350
MPTSGALDKVAAKNGIKLFETPTGWKFFGNLMDAGLINLCGEESFGTGSN  400
HIREKDGIWAVLAWLTILAHKNKNTDHFVTVEEIVTQYWQQFGRNYYSRY  450
DYEQVDSAGANKMMEHLKTKFQYFEQLKQGNKADIYDYVDPVDQSVSKNQ  500
GVRFVFGDGSRIIFRLSGTGSVGATIRIYFEQFEQQQIQHETATALANII  550
KLGLEISDIAQFTGRNEPTVIT
```

FIGURE 2: Primary structure and sequence coverage of native PP63/pf-1 obtained by tryptic and V8 proteolytic peptide mapping. Native PP63/pf consists of isoforms PP63-1/pf-1 and PP63-2/pf-2 with PP63-1/pf-1 being suggested as the dominant form and therefore shown here (*11*). The His-tagged sequence of the recombinant species, which has to be added to the N-terminus of the native protein, is as follows: MGH_{10}SSGHIEGRHMLE. PP63/pf-1 and PP63/pf-2 differ at the following seven positions: S31T (S-31 in PP63-1/pf-1 is replaced by T in PP63-2/pf-2), N37H, Q211S, S232T, R234S, T299A, and Q537E. The total sequence coverage was 88%. Residues in normal font were not covered, and partial sequences in boldface could be covered once; those in bold italic font were found twice, and those marked bold italic and underlined were identified at least three times. S-350, T-353, and S-354 were not covered.

Homology Modeling of PP63-1/pf-1

Homology models of PP63-1/pf-1 were produced with the structure of rabbit muscle phosphoglucomutase (PDB file

name 3PGM) as a template using MODELLER (*18*). The sequence alignment of PP63-1/pf-1 and phosphoglucomutase used for modeling was produced using CLUSTALW at the World Wide Web site of the European Bioinformatics Institute (http://www.ebi.ac.uk). The two sequences have 50% pairwise identity (*11*).

RESULTS

Biochemical Results

As reported earlier, we have enriched two protein kinases, casein kinase type II (CK) and cGMP-dependent kinase (PKG), from 100000*g* supernatants of *Paramecium* cell homogenates which are able to phosphorylate PP63/pf in vitro (*12*). The scope of this study was to determine whether one of these kinases is involved in the reversible phosphorylation cycle of PP63/pf during regulated exo- and endocytosis in vivo. We compared native PP63/pf, isolated in its phosphorylated state from *Paramecium*, with recombinant His-PP63-1/pf-1 and His-PP63-2/pf-2 phosphorylated in vitro by either CK or PKG, respectively, using mass spectrometric peptide mapping.

In Vitro Phosphorylation of His-PP63-1/pf-1 and His-PP63-2/pf-2. Recombinant His-PP63-1/pf-1 and His-PP63-2/pf-2 were phosphorylated in vitro by CK as reported previously (*11*, *13*). In vitro phosphorylation by PKG was carried out as described in Materials and Methods.

To control the incorporation of phosphate during phosphorylation with ATP, the recombinant proteins were also assayed under similar conditions using radioactively labeled [γ-^{32}P]ATP and analyzed by subsequent autoradiography. Figure 1 shows the silver-stained SDS−polyacrylamide gel (upper panel) and the corresponding autoradiogram (lower panel) from a typical in vitro phosphorylation of recombinant His-PP63-1/pf-1 and His-PP63-2/pf-2 by PKG. Phosphorylation of both isoforms could be detected clearly.

Mass Spectrometric Results

In Vivo Phosphorylation of PP63/pf. Native PP63/pf from *P. tetraurelia* cells exists very likely as a mixture of at least two isoforms (*11*). It was isolated in its phosphorylated state and subjected to differential peptide mapping before and after alkaline phosphatase treatment to identify the phosphorylation sites of the native protein used in vivo. The protein sequence coverage obtained by tryptic and V8 proteolytic peptide mapping is displayed in Figure 2. The total coverage equaled 88%. Multiple coverage of most partial sequences was obtained by the detection of overlapping tryptic and V8 proteolytic peptides. S-350, T-353, and S-354 were the only three threonine and serine residues which could not be covered by tryptic and V8 proteolytic peptide mapping. T-353 represents a putative phosphorylation site for PKG, whereas S-350 and S-354 do not represent consensus sites.

An example of a differential peptide map of native PP63/pf is shown in Figure 3. The negative-ion spectra of the tryptic peptide mixtures before (upper trace) and after (lower trace) alkaline phosphatase treatment are shown. The signals are assigned to the sequence positions of identified tryptic peptides. Most of the nonphosphorylated peptides were observed in both spectra. The monophosphorylated peptide of residues 188−201, encompassing the two putative phosphorylation sites for CK and PKG, T-191 and S-196, was exclusively detected before dephosphorylation with AP. The same observation was made for the doubly phosphorylated peptide of residues 145−176+2P (encompassing T-145, T-154, T-159, T-161, T-162, and T-169, the latter being a putative phosphorylation site for PKG). The exclusive detection of these molecular ions before dephosphorylation and the absence of these signals after AP treatment allow these peptides to be identified as phosphorylated (see also Table 1, columns 6 and 7). At *m*/*z* 1885.00, the isobaric peptides of residues 406−421+P and 10−27 were likely detected simultaneously prior to AP treatment. Since this signal exhibited a decreased abundance after dephosphorylation, the peptide of residues 406−421 (including T-416) was considered possibly phosphorylated.

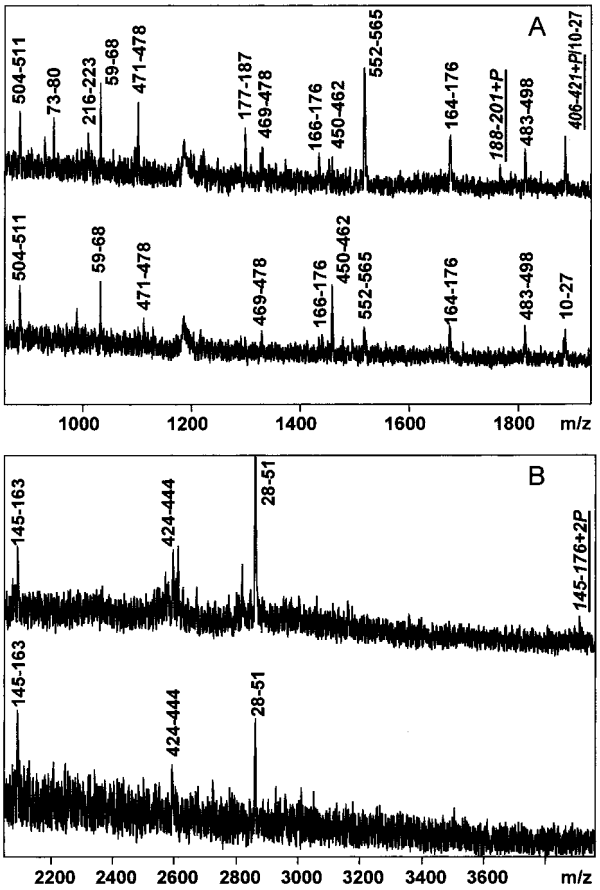

FIGURE 3: Differential peptide map of native PP63/pf. Negative-ion spectra of the tryptic peptide mixtures prior to (upper trace) and after AP treatment (lower trace): (A) lower mass range and (B) upper mass range; sequence positions of found tryptic peptides are denoted. 188−201+P and 145−176+2P indicate mono- and diphosphorylated tryptic peptides. Peptides 406−421+P and 10−27 are isobaric (*m*/*z* 1885.00). The decreased abundance after dephosphorylation renders both peptides likely to be present.

A summary of all peptides found to be phosphorylated in native PP63/pf is given in Table 1. If the number of detected phosphoryl residues, indicated by the mass increment of $n \times 80$ Da relative to the unmodified peptide, equaled the number of serine and threonine residues contained in the peptide, then phosphoryl groups could be directly assigned to certain residues. Often, there were fewer phosphoryl groups found than serines and threonines present in the peptide, and therefore, these residues could only be assigned as potentially phosphorylated. As indicated in column 11,

Table 1: Peptides Identified as Phosphorylated in Native PP63/pf Isolated in Its Phosphorylated State from *P. tetraurelia*[a]

MH^+_f[b]	MH^+_c[c]	MH^-_f[d]	MH^-_c[e]	sequence position	−AP[f]	+AP[g]	PO_3H^-[h]	Ser positions	Thr positions	sites of partial phosphorylation[i]
2987.52	2987.53			1−27	Y	N	1	24	13, 23	13, 23, 24
3781.12	3781.96			28−58	Y	N	1	45	31,[j] 34, 49	34, 45, 49
1791.08	1790.84			41−54	Y	N	1	45	49	45, 49
2333.97	2334.15			145−165	N	Y	0		145, 154, 159, 161, 162	145, 154, 159, 161, 162
		3909.05	3908.73	145−176	Y	N	2		145, 154, 159, 161, 162, 169	145, 154, 159, 161, 162
		2634.56	2634.03	153−171	Y	N	4		154, 159, 161, 162, 169	154, 159, 161, 162
		1784.87	1784.85	188−201	Y	N	1	196	191	191, 196
1710.93	1710.80	1708.87	1708.80	195−208	Y	N	1	196	205	
1791.08	1790.77			195−208	Y	N	2	196	205	
		1784.87	1784.73	202−215	Y	N	2	211[j]	205, 210	
3488.84	3489.44			237−268	Y	N	2	257, 260, 267	252	
1792.18	1792.69			269−284	Y	N	1		280	
3970.35	3970.88			359−393	Y	N	2		371, 373	
1078.51	1078.55			368−376	N	Y	0		371, 373	
1887.00	1886.99	1885.00	1884.99	406−421	Y	N	1		416	
1887.00	1886.99	1885.00	1884.99	10−27	Y	Y	0	−	−	
1097.56	1097.48	1095.58	1095.48	463−470	Y	N	1		469	469
1278.70	1278.56			516−527	Y	N	2	517, 521	519, 525	517, 519, 521, 525
2414.96	2415.26			538−558	Y	N	1	557	542, 544	557

[a] Data were obtained via tryptic and V8 proteolytic peptide mapping before and after AP treatment. [b] Protonated molecular ion, found monoisotopic mass. [c] Calculated monoisotopic mass. [d] Deprotonated molecular ion, found monoisotopic mass. [e] Calculated monoisotopic mass. [f] Before AP treatment (Y is yes and N no) whereby the exclusive detection of a phosphorylated peptide before (Y or N) or the only detection of a dephosphorylated peptide after AP treatment (N or Y) indicates a phosphorylation site. [g] After AP treatment (Y is yes and N no) whereby the exclusive detection of a phosphorylated peptide before (Y or N) or the only detection of a dephosphorylated peptide after AP treatment (N or Y) indicates a phosphorylation site. [h] Number of found phosphoryl residues. [i] Residues found to be partially phosphorylated. [j] Isoform 2 only.

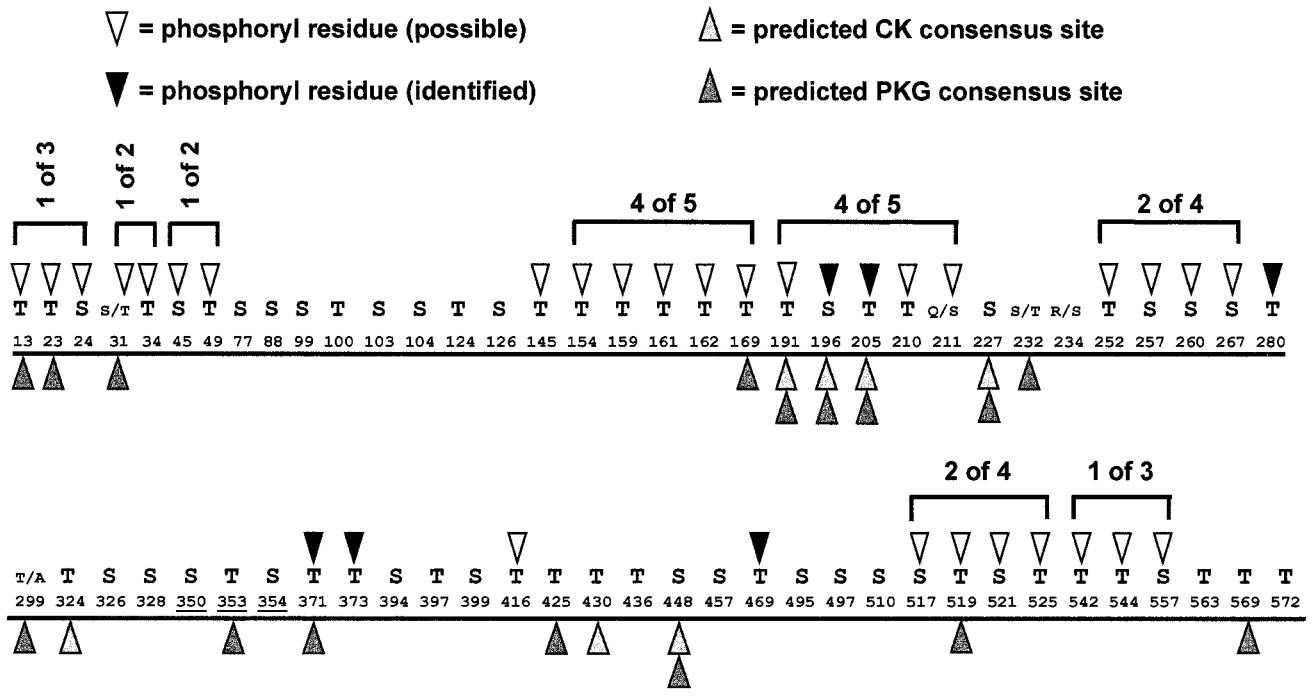

FIGURE 4: Phosphorylation sites of native PP63/pf based on differential peptide mapping data. All serine and threonine positions are given. At positions 31, 211, 232, 234, and 299, different residues for PP63-1/pf-1 and PP63-2/pf-2 are denoted. White triangles denote sites found to be possibly phosphorylated; black triangles denote definitely identified phosphorylation positions. Underlined residue numbers denote serines and threonines not covered by peptide mapping. Below the sequence of the S and T residues, light gray triangles denote the predicted CK, and dark gray triangles denote predicted PKG recognition sites. Terms above brackets show the number of found phosphorylated residues within a range of serine and threonine residues.

several of the potentially or reliably phosphorylated residues were found not to be quantitatively modified. This conclusion was drawn from the fact that, already before AP treatment of the digests, these peptides were detected both with and without phosphoryl groups.

A graphical overview on the in vivo phosphorylation of native PP63/pf is given in Figure 4. The protein is displayed as a sequence of serines and threonines with their positions indicated. Below the sequence of the S and T residues, putative phosphorylation sites recognized by either CK (S/TxxD/E) or PKG [R/Kxx(x)S/T] are marked with triangles (*19*). Many of the potential phosphorylation sites for both CK and PKG have been found to be likely or reliably phosphorylated, i.e., T-13, T-23, T-31 (isoform 2), T-169, T-191, **S-196, T-205, T-371**, and T-519 (definitely identified phosphorylation sites are bold here and are denoted with

black triangles in Figure 4). This result suggests that both CK and PKG are likely to be involved in in vivo phosphorylation of PP63/pf. The putative phosphorylation sites S-227, S/T-232, T-299 (in isoform 1 only), T-324, T-425, T-430, S-448, and T-569 were not found to be phosphorylated. T-353 could not be covered by peptide mapping, and therefore, no conclusion on its phosphorylation status can be drawn. Surprisingly, T-280, T-373, and T-469 were unequivocally identified as being phosphorylated, although they are not located in a potential phosphorylation site for CK or PKG. Note that additional information about possible phosphorylation can be obtained from the indications of how many residues out of a given set are expected to be modified (brackets denoted as "4 of 5", etc.).

In Vitro Phosphorylation of His-PP63-1/pf-1 and His-PP63-2/pf-2. Two recombinant isoforms of PP63/pf were investigated, His-PP63-1/pf-1 and His-PP63-2/pf-2. Both isoforms contain a His_{10} tag at the N-terminus in the following sequence: $^{-24}MGH_{10}SSGHIEGRHMLE^{-1}$. The numbering of amino acids in these recombinant isoforms starts with +1 at the methionine, which corresponds to the start methionine in the native PP63/pf. Thus, the numbering of amino acids in native PP63/pf and in recombinant isoforms correlates. His-PP63-2/pf-2 differs from His-PP63-1/pf-1 in only seven amino acids: S31T (S-31 in His-PP63-1/pf-1 is replaced by T in His-PP63-2/pf-2), N37H, Q211S, S232T, R234S, T299A, and Q537E.

For further identification of protein kinases putatively involved in the regulation of PP63/pf, both recombinant His-tagged isoforms His-PP63-1/pf-1 and His-PP63-2/pf-2 were incubated in vitro with both endogenous CK and PKG, respectively, from *P. tetraurelia* (*12*, *13*) and subsequently analyzed by differential tryptic and V8 proteolytic peptide mapping.

In Vitro Phosphorylation of His-PP63-1/pf-1 by CK. Figure 5 shows the tryptic peptide maps (positive ions) of His-PP63-1/pf-1 incubated with CK, prior to (top) and following AP treatment (bottom). Almost all nonphosphorylated tryptic peptides were detected in both spectra. The monophosphorylated peptide "10-28+P" (m/z_{found} 2094.94, m/z_{calcd} 2095.06), adjacent to but separated by ca. 2 Da from the peptide of residues 145−163 (m/z_{found} 2092.91, m/z_{calcd} 2092.98), was exclusively detected prior to AP treatment. It contains T-13, T-23, and S-24. A weak signal for the triply phosphorylated peptide of residues 10−29, which could not be detected after dephosphorylation, further supports phosphorylation of T-13, T-23, and S-24. The abundant signal "504-527+P" (only detected before AP treatment) assigns the peptide of residues 504−527, which contains the non-consensus phosphorylation sites S-510, S-517, T-519, S-521, and T-525, to a monophosphorylated state. A summary of all peptides found to be phosphorylated in His-PP63-1/pf-1 upon incubation with CK is given in Table 2. As observed in in vivo-phosphorylated PP63, some of the possibly or definitely phosphorylated residues were found to be not quantitatively modified. These residues are listed in the last column. Before AP treatment, these peptides were detected both with and without phosphoryl groups.

The phosphorylation pattern of His-PP63-1/pf-1 after incubation with CK is shown in Figure 6. In contrast to the in vivo phosphorylation, only three potential CK phosphorylation sites for CK were found to be possibly or definitely phosphorylated, namely, T-191, T-205, and S-227. Only CK recognition site S-227 was unequivocally proven to bear a phosphoryl residue. Other CK recognition sites, S-196, T-324, T-430, and S-448, were found to be unmodified. This finding can be explained by the limited access of the endoproteases to this partial sequence of PP63/pf. Remarkably, several S or T residues, which are not located in putative phosphorylation sites for CK, have also been found to be phosphorylated, namely, T-13, T-23, S-24, S-232, T-252, and S-257. Note that additional information about possible phosphorylation can be drawn from the specifications "3 of 6", etc.

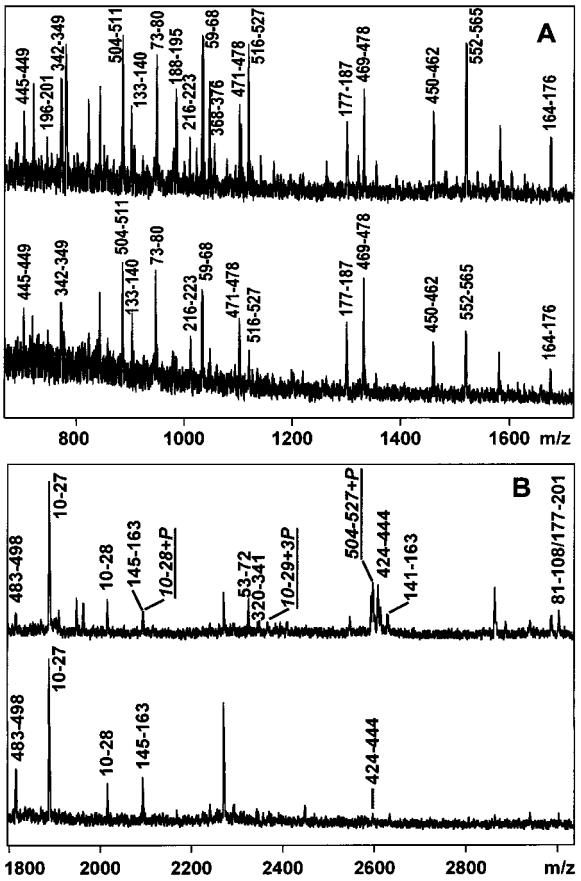

FIGURE 5: Differential peptide map of His-PP63-1/pf-1. Positive-ion spectra of His-PP63-1/pf-1, phosphorylated with CK in vitro, before (top) and after AP addition (bottom): (A) lower mass range and (B) upper mass range; sequence positions of found tryptic peptides are denoted. 10−28+P, 504−527+P, and 10−29+3P denote singly and triply phosphorylated tryptic peptides.

In Vitro Phosphorylation of His-PP63-1/pf-1 with PKG. Similar analyses were performed with the in vitro PKG-phosphorylated His-PP63-1/pf-1. Figure 7 shows the low-mass ranges of the tryptic peptide maps (positive ions) of His-PP63-1/pf-1 assayed with PKG, before (upper trace) and after AP treatment (lower trace). All nonphosphorylated tryptic peptides could be detected in both spectra. The peptide of residues 224−229 was exclusively found after AP treatment. This indicates that either one or both of the two PKG consensus sites S-227 and S-232 are phosphorylated. Table 3 summarizes all the His-PP63-1/pf-1 peptides, which were found to be phosphorylated in vitro by PKG. Peptides identified as partially phosphorylated, i.e., found both modified and unmodified prior to AP treatment of the digests,

Table 2: Peptides Identified as Phosphorylated in His-PP63-1/pf-1 upon in Vitro Phosphorylation by CK[a]

MH^+_f[b]	MH^+_c[c]	MH^-_f[d]	MH^-_c[e]	sequence position	−AP[f]	+AP[g]	PO_3H^-[h]	Ser positions	Thr positions	sites of partial phosphorylation[i]
2094.94	2095.06			10−28	Y	N	1	24	13, 23	13, 23,24
2382.72	2383.09			10−29	Y	N	3	24	13, 23	13, 23, 24
2092.91	2092.98	2091.12	2090.98	145−163	N	Y	0		145, 154, 159, 161, 162	145, 154, 159, 161, 162
3499.79	3499.67			167−194	Y	N	1		169, 191	191
1809.79	1809.80			205−218	Y	N	1		205, 210	205
		2354.29	2354.04	219−236	Y	N	2	227, 232		
1134.74	1134.64			221−230	N	Y	0	227		
3411.56	3411.43			231−259	Y	N	3	232, 257	252	
2593.25	2593.32			504−527	Y	N	1	510, 517, 521	519, 525	510
3510.26	3510.69			542−572	Y	N	3	557	542, 544, 563, 569, 572	
2101.08	2101.85			556−572	Y	N	3	557	563, 569, 572	557, 563, 569, 572

[a] Data were obtained via tryptic and V8 proteolytic peptide mapping before and after AP treatment. The exclusive detection of a phosphorylated peptide before (Y or N) or the exclusive detection of a dephosphorylated peptide after AP treatment (N or Y) indicates a phosphorylation site. [b] Protonated molecular ion, found monoisotopic mass. [c] Calculated monoisotopic mass. [d] Deprotonated molecular ion, found monoisotopic mass. [e] Calculated monoisotopic mass. [f] Before AP treatment (Y is yes and N no) whereby the exclusive detection of a phosphorylated peptide before (Y or N) or the only detection of a dephosphorylated peptide after AP treatment (N or Y) indicates a phosphorylation site. [g] After AP treatment (Y is yes and N no) whereby the exclusive detection of a phosphorylated peptide before (Y or N) or the only detection of a dephosphorylated peptide after AP treatment (N or Y) indicates a phosphorylation site. [h] Number of found phosphoryl residues. [i] Residues found to be partially phosphorylated.

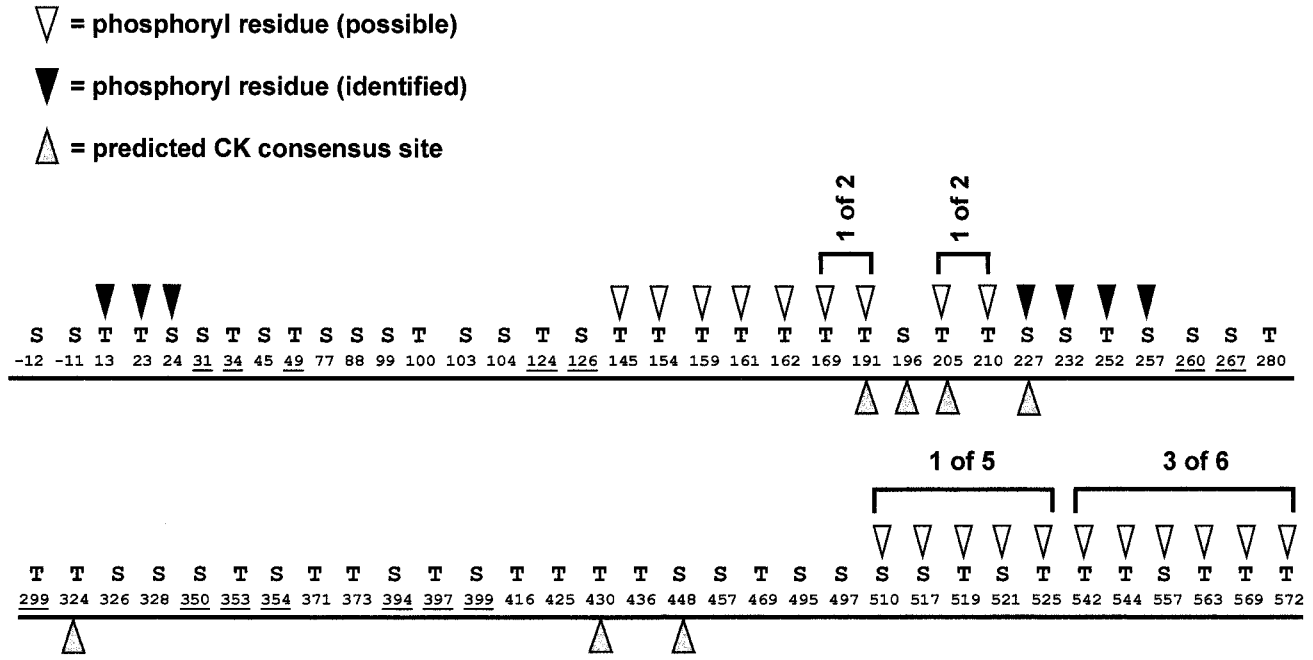

FIGURE 6: Phosphorylation pattern of His-PP63-1/pf-1 after in vitro phosphorylation with CK. Gray triangles below the sequence denote the predicted CK phosphorylation sites. Other symbols are like those shown in Figure 4.

are listed in the last column of Table 3. A corresponding graphical summary is given in Figure 8. The consensus site T-191 was identified as phosphorylated. The same is valid for either one or both of the two PKG consensus sites S-227 and S-232. Moreover, the consensus sites S-196, T-205, S-227, S-232, T-353, T-371, T-425, and T-569 are possibly bearing a phosphoryl residue (note that additional information about possible phosphorylation can be drawn from the specifications "1 of 2", etc.). The potential PKG phosphorylation sites S-31, T-169, T-299, S-448, and T-519 were identified as unmodified. T-416 was shown to be phosphorylated, although not identified as a predicted PKG recognition site. The PKG recognition sites T-13 and T-23 were not covered by peptide mapping.

In Vitro Phosphorylation of His-PP63-2/pf-2 by CK and PKG. To summarize the results of in vitro phosphorylation of His-PP63-2/pf-2, serine and threonine residues identified as reliably (underlined) or possibly (not underlined) phosphorylated are listed below, with those corresponding to a putative recognition site in bold: for His-PP63-2/pf-2 and CK, **S** 126, 234, 260, 267, 326, 457, and 557; and **T** 124, 232, 280, **324**, 469, 542, and 544.

Peptide mapping of His-PP63-2/pf-2 in vitro phosphorylated with CK yielded only a sequence coverage of ca. 60%. Hence, no conclusions about the phosphorylation status of the following residues could be drawn: T-31, T-34, S-45, T-49, S-77, S-88, S-99, T-100, T-169, S-227, T-252, S-257, S-350, T-353, S-354, T-371, T-373, S-394, T-397, S-399, S-517, T-519, S-521, and T-525. The reason for the poor coverage only observed in this particular case remains unclear. Remarkably, only one consensus site (T-324) could be shown to be definitely used in vitro: for His-PP63-2/pf-2 and PKG, **S** 24, 77, 88, 99, 103, 104, **227**, 234, 260, 267, 326, **448**, 495, 497, and 510; and **T** **13**, **23**, 100, **169**, **232**, 280, 324, **371**, 373, and 416.

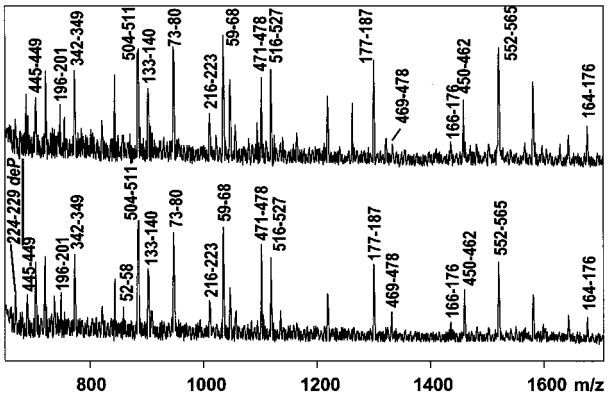

FIGURE 7: Differential peptide map of His-PP63-1/pf-1. Positive-ion spectra of His-PP63-1/pf-1, phosphorylated in vitro with PKG, prior to (upper trace) and after AP treatment (lower trace). Sequence positions of found tryptic peptides are denoted; 224−229 deP denotes a peptide exclusively detected after dephosphorylation with AP.

In peptide mapping studies with His-PP63-2/pf-2 in vitro phosphorylated by PKG, T-252, S-257, S-394, T-397, and T-399 could not be covered.

The observation of a phosphorylation pattern, which only partially corresponds to the consensus sites used by mammalian homologues of CK and PKG, and which furthermore reveals several nonrecognition sites as phosphorylated, is also valid for the in vitro phosphorylation studies with His-PP63-2/pf-2.

Molecular Modeling Results

Location of Phosphorylation Sites in Models of PP63-1/pf-1. Serine and threonine residues unequivocally proven to be modified in in vivo-phosphorylated PP63/pf were analyzed for their surface accessibility in a homology-based computer model of PP63-1/pf-1. This model was derived from rabbit PGM isoform 2, which is 50% identical to PP63-1/pf-1 (*11*). Residues S-196, T-205, T-280, T-371, T-373, and T-469 were found to be definitively phosphorylated. Surface accessibility calculations suggest that (a) T-371 is buried (exposed surface area of <10%) and (b) T-205 has an intermediate level of exposure (10−30% exposed surface area) and that residues S-196, T-280, T-373, and T-469 are exposed (>30% exposed surface area). Thus, the majority of exposed PP63-1/pf-1 residues are surface-accessible kinase targets in our model. Twenty-two of 28 definitely nonphosphorylated residues are buried or only partially exposed (data not shown). The locations of phosphorylated serine and threonine residues in a model of PP63-1/pf-1 are shown in Figure 9. T-280 and T-373 are found at the entrance to and inside the active site cleft, respectively. T-373 is located at the wall of the cleft opposite the catalytic serine, S-126.

DISCUSSION

Methodical Implications of Mass Spectrometric Analysis. The interpretation of protein modification data based on mass spectrometric peptide mapping requires the discussion of intrinsic possibilities and limitations of mass spectrometric analyses of peptide mixtures. The combination of selective enzymatic chemistry with optimized sample preparation and the high accuracy in the mass determinations of modern (MALDI) mass spectrometers allows for specific peptide identification and for characterization of peptide modifications. In this study, these analytical features were realized by using the specific enzyme alkaline phosphatase, application of a micropurification technique, and data acquisition on a MALDI mass spectrometer equipped with a reflectron (*20*) and delayed-extraction (*21*) technology providing high mass resolution and accuracy.

A major limitation of direct mixture analysis by MALDI-MS is ion suppression; i.e., the absence of a molecular ion of a peptide does not indicate that it does not exist. Rather, one peptide may generate an abundant molecular ion in one and may not be detected at all in another mixture analysis. This can be partially circumvented by using different matrixes, sample preparations, and acquisition modes, as applied in this study by detection of positive and negative ions in linear and reflector mode.

Moreover, mass spectrometric techniques are per se nonquantitative analytical tools. This is in particular valid for MALDI mass spectrometric analysis of peptide mixtures. The abundance of a molecular ion strongly depends on the type of peptide. A phosphoryl peptide often yields a less abundant molecular ion than its dephosphorylated analogue. This aspect is relevant with regard to parallel detection of both species.

The positive evidence of a peptide and its phosphoryl group (i.e., the detection of the phosphorylated species before dephosphorylation, possibly supplemented by detection of the corresponding dephosphorylated peptide after AP treatment) can be regarded as a specific identification. Since the negative result of an absent signal of a peptide does not allow for conclusions about its existence, the exclusive detection of a dephosphorylated peptide after AP treatment can only be interpreted as a strong indication of phosphorylation. The dephosphorylated species could also have been present before AP addition but may have been suppressed.

Only in very few cases, two pieces of evidence for a phosphorylpeptide were observed, namely, the detection of both the phosphoryl peptide and the corresponding dephosphorylated species after addition of alkaline phosphatase. By far, most of the phosphoryl peptides did not yield a molecular ion of the dephosphorylated species. This finding can be partially explained by the intrinsic limitations of direct peptide mixture analyses by MALDI-MS. The removal of a phosphoryl group dramatically alters the ionization and desorption properties of a peptide so that the unmodified peptide may be suppressed. A counter argument is the frequent observation that nonphosphorylated peptides yield higher ion abundances than phosphoryl peptides. Another reason for the absence of many ion signals of dephosphorylated peptides may be the reversed phase micropurification or the adsorption of the dephosphoryl peptides to surfaces. The removal of the phosphoryl group renders the peptide significantly more hydrophobic, so the retention of the dephosphopeptide on reversed phase material and the adsorption to hydrophobic surfaces may be enhanced and hence lead to a reduced peptide recovery.

However, in many cases, this limitation was largely offset by the detection of sequence-overlapping, dephosphorylated peptides after AP incubation. Although not identical, these peptides encompassed the same phosphorylation sites, which were found to be modified in similar phosphoryl peptides prior to AP treatment.

Table 3: Peptides Found To Be Phosphorylated in His-PP63-1/pf-1 after in Vitro Phosphorylation by PKG[a]

MH^+_f[b]	MH^+_c[c]	MH^-_f[d]	MH^-_c[e]	sequence position	−AP[f]	+ AP[g]	PO_3H^-[h]	Ser positions	Thr positions	sites of partial phoshorylation[i]
2911.22	2911.26			−24 to 0[j]	N	Y	0	−12, −11[j]		
1059.37	1059.48			188−195	Y	N	1		191	
1710.87	1710.8			195−208	Y	N	1	196	205	196
665.34	665.35			224−239	N	Y	0	227, 232		
1861.17	1860.87			313−327	Y	N	1	326	324	324, 326
		1004.14	1004.48	350−359	N	Y	0	350, 354	353	
2812.24	2812.47			359−383	N	Y	0		371, 373	
3429.06	3428.73			405−433	Y	N	1		416, 425, 430	
1886.93	1886.99	1885.00	1884.99	406−421	Y	N	1		416	
2602.62	2602.23			454−475	N	Y	0	457	469	469
1546.82	1546.82			559−572	N	Y	0		563, 569, 572	563, 569, 572

[a] Data were obtained via tryptic and V8 proteolytic peptide mapping before and after AP treatment. The exclusive detection of a phosphorylated peptide before (Y or N) or the exclusive detection of a dephosphorylated peptide after AP treatment (N or Y) indicates a phosphorylation site. [b] Protonated molecular ion, found monoisotopic mass. [c] Calculated monoisotopic mass. [d] Deprotonated molecular ion, found monoisotopic mass. [e] Calculated monoisotopic mass. [f] Before AP treatment (Y is yes and N no) whereby the exclusive detection of a phosphorylated peptide before (Y or N) or the only detection of a dephosphorylated peptide after AP treatment (N or Y) indicates a phosphorylation site. [g] After AP treatment (Y is yes and N no) whereby the exclusive detection of a phosphorylated peptide before (Y or N) or the only detection of a dephosphorylated peptide after AP treatment (N or Y) indicates a phosphorylation site. [h] Number of found phosphoryl residues. [i] Residues found to be partially phosphorylated. [j] His tag positions.

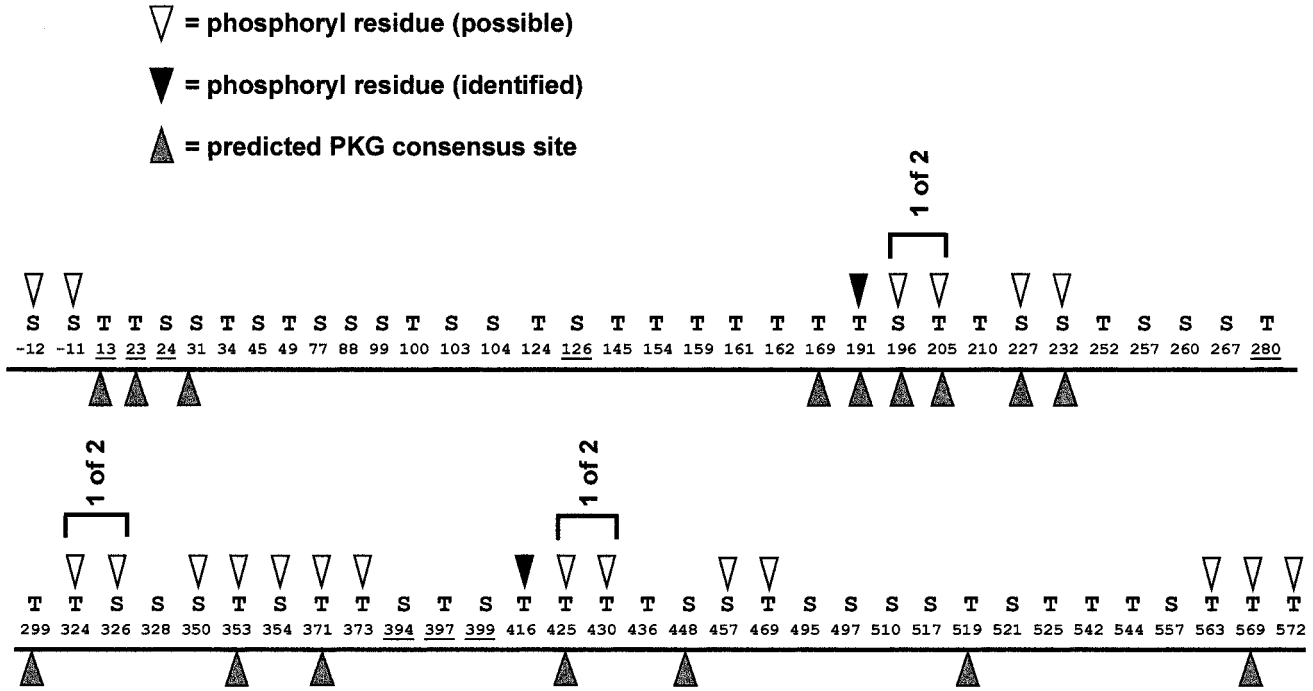

FIGURE 8: Phosphorylation pattern of His-PP63-1/pf-1 after in vitro phosphorylation with PKG. Gray triangles below the sequence denote the predicted PKG phosphorylation sites. Other symbols are like those shown in Figure 4.

In this study, we compared phosphorylation sites of the native phosphoprotein PP63/pf from *P. tetraurelia* with phosphorylation sites used by enriched *Paramecium* CK or PKG, respectively, when assayed in vitro with recombinant isoforms of His-tagged PP63/pf as the substrate. Table 4 summarizes the results for phosphorylation analyses of native PP63/pf and the two in vitro-phosphorylated isoforms of recombinant His-PP63/pf after incubation with endogenous CK and PKG, respectively. Indicated are definitely (dark gray) and possibly (light gray) phosphorylated serine and threonine residues and the putative phosphorylation sites (boxed) for the two protein kinases.

A partial correlation between the results obtained from the native PP63/pf and the His-PP63-1/pf-1 was found. Many of the possibly phosphorylated residues in native PP63/pf are also probably phosphorylated in recombinant PP63-1/pf-1 that has been incubated with CK, e.g., all threonine residues within the peptide from T-145 to T-191 and all serine and threonine residues contained in the peptide from S-517 to S-557. This can be interpreted in a way that PP63-1/pf-1 may be the predominant isoform in vivo as previously discussed in ref *11*. However, several differences in the phosphorylation patterns of native PP63/pf and the recombinant isoforms phosphorylated by endogenous kinases show that in vitro mimicry of in vivo regulation by kinase assays is difficult and does not necessarily reflect the cellular situation.

Except in the case of the in vitro phosphorylation of His-PP63-2/pf-2 with CK, many if not most of the serines and threonines can be regarded as nonquantitatively modified (see Tables 1−3). This result is based on the fact that, in the non-AP-treated digests, the peptides identified as phospho-

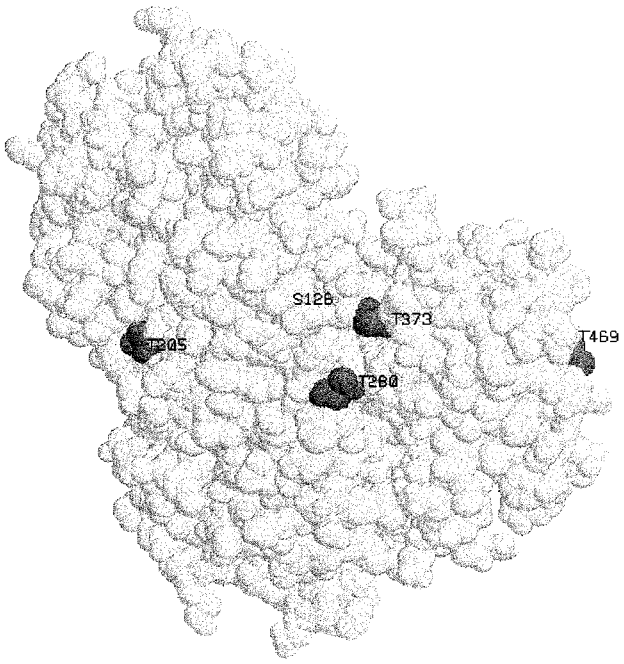

FIGURE 9: Space-filling representation of the homology model of PP63-1/pf-1. Residues identified as definitely phosphorylated are shaded darkly and labeled as is the catalytic serine, S-126.

rylated were often observed in both forms, i.e., unmodified and modified.

Conclusions about the relative amounts of modified versus unmodified peptides cannot be drawn at this stage. Semiquantification in MALDI-MS can be achieved with internal standards, e.g., by spiking the analyte mixture with a known amount of a compound, which is chemically very similar to the molecule of interest and which yields approximately the same ion abundance when present at the same concentration. Because of small sample quantities, this has not been carried out in the approach described here.

In many regions of possible in vitro and in vivo phosphorylation, the putative sites can be narrowed further (as indicated in Figures 4, 6, and 8), and some serine and threonine residues could be unequivocally identified as phosphorylated. On the other hand, a large number of "theoretical" sites could definitely be excluded from modification by kinases. The more accurate characterization of phosphorylation sites requires (phospho)peptide sequencing and is the subject of further studies. The methods of choice are MS/MS techniques (CID, collision-induced dissociation, and PSD, post-source decay) and carboxypeptidase treatment. Because of the limited sample amounts, these approaches were not pursued at this stage. However, a detailed comparison of in vitro and in vivo phosphorylation of PP63/parafusin could be demonstrated by differential MALDI mass spectrometric peptide mapping.

Possible Biological Implications. The peptide mapping data of native PP63/pf indicate that this protein is multiply phosphorylated and that both protein kinases, CK and PKG, are most likely involved in in vivo phosphorylation of this protein. Several of the CK and PKG consensus sites were found (likely or definitely) to be phosphorylated.

It has been shown that at least a part of the PP63/pf pool in the cell is membrane-associated, i.e., associated with cortical structures (*8*). This membrane association and

Table 4: Summary of Phosphorylation Sites Detected in His-PP63-1/pf-1, His-PP63-2/pf-2 Phosphorylated in Vitro by either CK or PKG, and Native PP63/pf[a]

recombinant PP63-1/pf-1 and CK	recombinant PP63-1/pf-1 and PKG	native PP63/pf	recombinant PP63-2/pf-2 and CK	recombinant PP63-2/pf-2 and PKG
T-13	[(T-13)]	[*T-13*]	T-13	**[T-13]**
T-23	[(T-23)]	[*T-23*]	T-23	**[T-23]**
S-24	(S-24)	*S-24*	S-24	**S-24**
(S-31)	[S-31]	[*S-31/T-31*]	(T-31)	[T-31]
(T-34)	T-34	*T-34*	(T-34)	T-34
S-45	S-45	*S-45*	(S-45)	S-45
(T-49)	T-49	*T-49*	(T-49)	T-49
S-77	S-77	S-77	(S-77)	*S-77*
S-88	S-88	S-88	(S-88)	*S-88*
S-99	S-99	S-99	(S-99)	*S-99*
T-100	T-100	T-100	(T-100)	*T-100*
S-103	S-103	S-103	S-103	*S-103*
S-104	S-104	S-104	S-104	*S-104*
(T-124)	T-124	T-124	**T-124**	T-124
(S-126)	(S-126)	S-126	**S-126**	S-126
T-145	T-145	*T-145*	T-145	T-145
T-154	T-154	*T-154*	T-154	T-154
T-159	T-159	*T-159*	T-159	T-159
T-161	T-161	*T-161*	T-161	T-161
T-162	T-162	*T-162*	T-162	T-162
T-169	[T-169]	[*T-169*]	(T-169)	**[T-169]**
[*T-191*]	**[T-191]**	[*T-191*]	[T-191]	[T-191]
[S-196]	[*S-196*]	**[S-196]**	[S-196]	[S-196]
[*T-205*]	[*T-205*]	**[T-205]**	[T-205]	[T-205]
T-210	T-210	*T-210*	T-210	T-210
Q-211	Q-211	*Q-211/S-211*	S-211	S-211
[S-227]	[*S-227*]	[S-227]	[(S-227)]	**[S-227]**
S-232	[*S-232*]	[S-232/T-232]	*T-232*	**[T-232]**
R-234	R-234	R-234/S-234	*S-234*	**S-234**
T-252	T-252	*T-252*	(T-252)	(T-252)
S-257	S-257	*S-257*	(S-257)	(S-257)
(S-260)	S-260	*S-260*	**S-260**	*S-260*
(S-267)	S-267	*S-267*	**S-267**	*S-267*
T-280	(T-280)	**T-280**	**T-280**	*T-280*
(T-299)	[T-299]	[T-299/A-299]	A-299	A-299
[T-324]	*T-324*	[T-324]	**[T-324]**	*T-324*
S-326	*S-326*	S-326	**S-326**	*S-326*
S-328	S-328	S-328	S-328	S-328
(S-350)	*S-350*	(S-350)	(S-350)	S-350
(T-353)	[*T-353*]	[(T-353)]	(T-353)	[T-353]
(S-354)	*S-354*	(S-354)	(S-354)	S-354
T-371	[*T-371*]	**[T-371]**	(T-371)	[*T-371*]
T-373	*T-373*	**T-373**	(T-373)	*T-373*
(S-394)	(S-394)	S-394	(S-394)	(S-394)
(T-397)	(T-397)	T-397	(T-397)	(T-397)
(S-399)	(S-399)	S-399	(S-399)	(S-399)
T-416	**T-416**	*T-416*	T-416	**T-416**
T-425	[*T-425*]	[T-425]	T-425	[T-425]
[T-430]	*T-430*	[T-430]	[T-430]	T-430
T-436	T-436	T-436	T-436	T-436
[S-448]	[S-448]	[S-448]	[S-448]	**[S-448]**
S-457	*S-457*	S-457	*S-457*	S-457
T-469	*T-469*	**T-469**	**T-469**	T-469
S-495	S-495	S-495	S-495	**S-495**
S-497	S-497	S-497	S-497	**S-497**
S-510	S-510	S-510	S-510	**S-510**
S-517	S-517	*S-517*	(S-517)	S-517
T-519	[T-519]	[*T-519*]	(T-519)	[T-519]
S-521	S-521	*S-521*	(S-521)	S-521
T-525	T-525	*T-525*	(T-525)	T-525
T-542	T-542	*T-542*	*T-542*	T-542
T-544	T-544	*T-544*	*T-544*	T-544
S-557	S-557	*S-557*	*S-557*	S-557
T-563	*T-563*	T-563	T-563	T-563
T-569	[*T-569*]	[T-569]	T-569	[T-569]
T-572	*T-572*	T-572	T-572	T-572

[a] Sequences of proteins are shown as a list of serine and threonine residues. At positions 31, 211, 232, 234, and 299 of native PP63/pf, amino acids of both isoforms are indicated. Definitely phosphorylated residues are shown in bold and possibly phosphorylated residues in italic. Brackets denote residues within potential phosphorylation sites for the corresponding protein kinase. Underlined residues in parentheses are not covered by peptide mapping.

additional proteins clustered around the PP63/pf molecule may mask some of the potential phosphorylation sites for CK and PKG, and therefore, these sites cannot be phosphorylated even if they are located on the surface of the molecule.

The finding of phosphorylated serines and threonines, which do not represent potential phosphorylation sites of CK or PKG, suggests that phosphorylation in vitro does not precisely reflect the in vivo situation. This may be because of conformational properties of the protein in vivo. Furthermore, one has to consider differences between kinases of *P. tetraurelia* versus those from higher eukaryotes to which consensus sites refer. It may also be possible that in vivo further kinases are participating in phosphorylation of PP63. For instance, recently a novel protein kinase type with calmodulin-type repeats has been reported in *P. tetraurelia* (*22*).

It has also been shown that PP63/parafusin is identical with *Paramecium* PGM (*11*, *23*). It is 50% identical to rabbit PGM isoform 2. We have used homology molecular modeling of PP63-1/pf-1 with the structure of rabbit PGM to visualize the implications of the MS data and to provoke ideas for further experiments. All but one of the definitely phosphorylated residues in in vivo-phosphorylated PP63/pf are located at the surface of the molecule. This is encouraging since it shows that the results of differential phosphopeptide mapping agree well with the probable structure of the investigated molecule.

It has been suggested that PP63/pf dephosphorylation may play a role in processes downstream of exocytotic membrane fusion (*24*). However, it remains to be determined how and at which steps during the exo- and endocytosis cycle de- and rephosphorylation of PP63/pf may play a role, e.g., for an increase of the ATP supply, etc. This finding of multiple phosphorylation by different protein kinases is seminal to further analyses of the function of the different phosphorylation states during the exo- and endocytosis cycle.

REFERENCES

1. Hunter, T. (1995) *Cell 80*, 225−236.
2. Andersen, J. S., Svensson, B., and Roepstorff, P. (1996) *Nat. Biotechnol. 14*, 449−457.
3. Burlingame, A. L., and Carr, S. A., Eds. (1996) *Mass Spectrometry in Biological Sciences*, Humana Press, Totowa, NJ.
4. Kussmann, M., and Roepstorff, P. (1998) *Spectroscopy 14*, 1−27.
5. Bean, M. F., Annan, R. S., Hemling, M. E., Mentzer, M., Huddleston, M. J., and Carr, S. A. (1995) in *Techniques in Protein Chemistry* (Crabb, J., Ed.) pp 107−116, Academic Press, San Diego, CA.
6. Zieseniss, E., and Plattner, H. (1985) *J. Cell Biol. 101*, 2028−2035.
7. Gilligan, D. M., and Satir, B. M. (1982) *J. Biol. Chem. 257*, 13903−13906.
8. Kissmehl, R., Hauser, K., Gössringer, M., Momayezi, M., Klauke, N., and Plattner, H. (1998) *Histochem. Cell Biol. 110*, 1−8.
9. Höhne-Zell, B., Knoll, G., Riedel-Gras, U., Hofer, W., and Plattner, H. (1992) *Biochem. J. 286*, 843−849.
10. Knoll, G., Braun, C., and Plattner, H. (1991) *J. Cell Biol. 113*, 1295−1304.
11. Hauser, K., Kissmehl, R., Lindner, J., Schultz, J. E., Lottspeich, F., and Plattner, H. (1997) *Biochem. J. 323*, 289−296.
12. Kissmehl, R., Treptau, T., Hofer, H. W., and Plattner, H. (1996) *Biochem. J. 317*, 65−76.
13. Kissmehl, R., Treptau, T., Hauser, K., and Plattner, H. (1997) *FEBS Lett. 402*, 227−235.
14. Gobom, J., Nordhoff, E., Ekman, R., and Roepstorff, P. (1997) *Int. J. Mass Spectrom. Ion Processes 169*, 153−163.
15. Kussmann, M., Nordhoff, E., Rahbek-Nielsen, H., Haebel, S., Røssel-Larsen, M., Jakobsen, L., Gobom, J., Mirgorodskaya, E., Kroll-Kristensen, A., Palm, L., and Roepstorff, P. (1997) *J. Mass Spectrom. 32*, 593−601.
16. Karas, M., and Hillenkamp, F. (1988) *Anal. Chem. 60*, 2299−2301.
17. Højrup, P. (1998) *GPMAW*, version 3.15, Lighthouse Data, Engvej 35, DK-5230 Odense M, Denmark.
18. Šali, A., and Blundell, T. L. (1993) *J. Mol. Biol. 234*, 779−815.
19. Pearson, R. B., and Kemp, B. E. (1991) *Methods Enzymol. 200*, 63−81.
20. Cotter, R. J. (1992) *Anal. Chem. 64*, 1027A.
21. Brown, R. S., and Lennon, J. J. (1995) *Anal. Chem. 67*, 1998−2004.
22. Kim, K., Messinger, L. A., and Nelson, D. L. (1998) *Eur. J. Biochem. 251*, 605−612.
23. Treptau, T., Kissmehl, R., Wissmann, J.-D., and Plattner, H. (1995) *Biochem. J. 309*, 557−567.
24. Chilcoat, N. D., and Turkewitz, A. P. (1997) *J. Cell Biol 139*, 1197.

BI982888Y