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Identification of isoforms of the exocytosis-sensitive phosphoprotein PP63/parafusin in *Paramecium tetraurelia* and demonstration of phosphoglucomutase activity

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PP63 (parafusin) is a 63 kDa phosphoprotein which is very rapidly (within 80 ms) dephosphorylated (to P63) during triggered trichocyst exocytosis; this occurs selectively in exocytosis-competent *Paramecium tetraurelia* strains. In the present work, two cDNAs coding for PP63/parafusin have been isolated, one of which is a new isoform. These isoforms are 99.6 % identical and are derived from two different genes. Similarity searches revealed 43–51 % identity of the deduced amino acid sequences with known phosphoglucomutases from yeast and mammals. The sequences of two proteolytic peptides obtained from PP63/parafusin isolated from *Paramecium* are identical to parts of the amino acid sequence deduced from the major cDNA. The major cDNA was mutated from the macronuclear ciliate genetic code into the universal genetic code and expressed in *Escherichia coli*. The recombinant protein shows the same biochemical and immunological characteristics as the (P)P63/parafusin originally isolated from *Paramecium*. It has the same specific phosphoglucomutase activity as phosphoglucomutase from chicken muscle. We also show that recombinant P63-1/parafusin 1 is a substrate of an endogenous casein kinase from *Paramecium*, as is the originally isolated P63/parafusin. Polyclonal antibodies against recombinant P63-1/parafusin 1 were raised which recognized phosphoglucomutases from different sources. Thus we show that PP63/parafusin and phosphoglucomutase in *Paramecium* are identical.

INTRODUCTION

Exocytosis of trichocysts by *Paramecium tetraurelia* is most probably a defensive response to natural predators such as *Monodinium* or *Dileptus* [1,2]. It also can be triggered by the application of the artificial vital secretagogue aminoethyldextran [3]. Aminoethyldextran triggers the synchronous exocytosis of all docked trichocysts within less than 1 s [3,4]. This synchrony allows the analysis of specific steps, such as the phosphorylation and dephosphorylation of proteins, during triggered exocytosis in *Paramecium*.

Gilligan and Satir [5] described a phosphoprotein of 63 kDa (formerly 65 kDa) which they called parafusin and which is selectively dephosphorylated during trichocyst exocytosis. We have demonstrated that this protein is dephosphorylated within 80 ms after synchronous exocytosis [6], and fully rephosphorylated within a few seconds [4,7], specifically in exocytosis-competent strains [4]. In our terminology this protein is called PP63, for phosphoprotein of 63 kDa. The dephosphorylated form of PP63/parafusin is referred to as P63.

Recently we have shown that PP63/parafusin shares biochemical characteristics with phosphoglucomutase (PGM; EC 5.4.2.2) [7]. PP63/parafusin possesses substantial PGM activity, and it exists in several isoforms with pI values of 5.75 and above [6,8]. PGM from rabbit skeletal muscle and PP63/parafusin are phosphoproteins and have similar molecular masses between 60 and 63 kDa [6,9]. Antibodies raised against commercially available rabbit skeletal muscle PGM recognize PP63/parafusin [7]. We also have evidence that both PP63/parafusin and PGM are substrates for the same serine/threonine phosphatases and kinases [10]. Immunofluorescence labelling studies with polyclonal antibodies against PP63/parafusin revealed a cortical localization of this protein [6,11]. This localization and the strict time correlation of its dephosphorylation with exocytosis suggest a role for this protein in the regulation of exocytosis in *Paramecium*.

Subramanian et al. [12] have isolated a partial cDNA coding for PP63/parafusin. Although this cDNA shows 50 % sequence identity with several known PGM sequences, these authors have denied that PP63/parafusin has PGM activity [12].

In the present study we report the isolation and cloning of a cDNA coding for a new isoform of PP63/parafusin, PP63-2/parafusin 2. Our data allowed us to correct the sequence of PP63-1/parafusin 1 previously published in [12], and we were able to prove clearly the identity of PP63-1/parafusin 1 with PGM by expressing the cloned cDNA for PP63-1/parafusin 1 and by investigating the immunological and biochemical properties of the recombinant protein. Thus we show that recombinant PP63-1/parafusin 1 has PGM activity.

EXPERIMENTAL

Materials

Glucose 1-phosphate, glucose 1,6-bisphosphate, $NADP^+$, glucose-6-phosphate dehydrogenase and PGM from rabbit skeletal muscle were obtained from Boehringer (Mannheim, Germany). Casein and PGM from chicken were from Sigma (Deisenhofen, Germany). Isopropyl β-D-thiogalactopyranoside

Abbreviation used: PGM, phosphoglucomutase.

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The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession nos. Y09969 and Y09970.

was technical grade from Calbiochem (La Jolla, CA, U.S.A.). Ni^{2+}-nitrilotriacetate–agarose was purchased from Qiagen (Hilden, Germany). Restriction enzymes came from New England Biolabs (Beverly, MA, U.S.A.). All other reagents and all solvents used were of analytical grade.

PCR strategy

PCR was performed with 10 μl ($= 1 \times 10^4$ plaque-forming units) of a *P. tetraurelia* 51S cDNA library in λZAP Express (Stratagene G.m.b.H., Heidelberg, Germany) as template. Primers derived from the parafusin sequence [12] were as follows: primer 1, 5′ GTTTAAGTAACATAACCATATGC 3′ (nucleotides 976–998); primer 2, 5′ CTTCTGGAGCAGGAGCACC 3′ (nucleotides 1378–1396). They were used at a final concentration of 0.5 μM. PCR was carried out for 40 cycles for 1 min at 95 °C, 30 s at 48 °C and 1 min at 72 °C, with a final 10 min extension at 72 °C. The PCR products were cloned into the *Eco*RV restriction site of pBluescriptII SK(−). After sequencing of several clones, two relevant plasmids for further investigation were chosen, named p410-7 and p410-9.

Screening of a λZAP Express cDNA library

A total of 1×10^6 plaques of a λZAP Express cDNA library of *P. tetraurelia* 51S from stationary phase were screened according to the Instruction Manual of Stratagene's λZAP Express Cloning Kit. The insert of p410-7 was amplified with primers 1 and 2 by PCR. A sample of 50 ng of the purified PCR fragment was labelled with 50 μCi of [α-^{32}P]dCTP using the Rediprime DNA labelling system from Amersham Life Science (Braunschweig, Germany) and used as a probe. Because of the high A/T content of *Paramecium* DNA [13], hybridization was carried out at 38 °C in 50 % formamide, 0.1 % (w/v) SDS, 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.8), 5 × Denhardt's solution [14] and 100 μg/ml denatured herring sperm DNA. Washing was carried out in 0.1 % (w/v) SDS/1 × SSC for 2×10 min at 38 °C and for 2×30 min at 54 °C. The isolated positive plaques were screened in a second round as described above. Positive plaques were rescued by *in vivo* excision and sequenced. Two clones were chosen for further studies, designated clone 1 and clone 24.

Screening of a genomic DNA library

A total of 300000 primary plaques of a λZAPII (Stratagene) genomic library of *Eco*RI-digested macronuclear DNA from *P. tetraurelia* 51S was screened as described above. A mixture of the inserts from p410-7 and p410-9 amplified by PCR with primer 1 and primer 2 served as probe. The isolated plasmids containing genomic sequences of PP63/parafusin DNA were named pG2 and pG9 respectively.

Site-directed mutagenesis

This was carried out as described by Deng and Nickoloff [15], using the Transformer Site-Directed Mutagenesis Kit from Clontech. Clone 24 was cut with *Eco*RI, *Hin*cII and *Xho*I, resulting in three fragments, *Eco*RI/*Eco*RI, *Eco*RI/*Hin*cII and *Hin*cII/*Xho*I. These fragments were subcloned into pBluescriptII SK(−). The plasmids obtained were mutated separately. After completing the mutagenesis, the plasmid containing the *Eco*RI/*Eco*RI fragment (pC415) was used for adding the missing 5′-end of the coding region (see below). The three fragments were re-joined and the correctness of the sequence was confirmed by sequencing.

Completing the 5′-end of the coding region for PP63-1/parafusin 1 cDNA

Using the sequence information obtained from the genomic sequence pG2, four oligonucleotides were synthesized that covered the sequence from the start ATG codon downstream to the *Nde*I restriction site (CATATG; position 44 in Figure 1A) using the universal genetic code and an additional three restriction sites (*Kpn*I, *Bam*HI and *Xho*I) at the 5′-end which were needed for cloning. The sequences of the four oligonucleotides were as follows: A1, 5′ CGGATCCCTCGAGATGCAACAAGTAATT 3′ (complementary to A2); A2 5′ GGTGCTGGAATTACTTG-TTGCATCTCGAGGGATCCGGTAC 3′; A3, 5′ CCAGCAC-CAAGAGTTCAAGTAACACAACCA 3′; A4, 5′ TATGGTT-GTGTTACTTGAACTCTT 3′ (complementary to A3).

The oligonucleotides were phosphorylated with phosphonucleotide kinase. Samples of 500 pmol of each phosphorylated oligonucleotide were heated for 3 min at 80 °C together in a reaction tube (in phosphonucleotide kinase buffer supplemented by the manufacturer; New England Biolabs) and then cooled to 30 °C within 2 h and placed on ice. Then 10 pmol of this artificial double-stranded DNA was cloned into 0.056 pmol of pC415 cut with *Kpn*I and *Nde*I and sequenced for confirmation.

DNA sequencing

Plasmid DNA was purified using Wizard Plasmid Miniprep (Promega, Madison, WI, U.S.A.) and used as template for sequencing with the T7 Sequencing® Kit (Pharmacia, Uppsala, Sweden) and multiple primers (synthesized by MWG-Biotech G.m.b.H., Ebersberg, Germany). Sequencing was carried out by the dideoxy method [16].

Computer analysis

Homology searches were carried out on the FASTA and BLITZ Server at the EMBL outstation EBI at Hinxton (U.K.) [17] (Internet: http://www.ebi.ac.uk/searches/searches.html). Hydrophobicity plots were calculated with a window of nine amino acids by the method of Kyte and Doolittle [18].

Expression of PP63-1/parafusin 1 cDNA in *Escherichia coli*

After changing all deviant *Paramecium* glutamine codons (TAA and TAG) into universal glutamine codons (CAA and CAG), the PP63-1/parafusin 1 cDNA was cloned into the *Xho*I restriction site of pET 16b expression vector of the pET System from Novagen (Madison, WI, U.S.A.), which employs a His_{10} tag for purification of the recombinant protein.

Purification of recombinant P63-1/parafusin 1

Recombinant P63-1/parafusin 1 was purified by affinity chromatography on Ni^{2+}-nitrilotriacetate–agarose under native conditions, as recommended by the manufacturer (Qiagen). The recombinant P63-1/parafusin 1 protein was eluted with a step gradient in five steps from 100 mM to 500 mM imidazole in 50 mM sodium phosphate, pH 6.0, 300 mM NaCl and 10 % (v/v) glycerol. Fractions collected were analysed on SDS/polyacrylamide gels, and the fractions containing recombinant P63-1/parafusin 1 were pooled and dialysed into the buffer of interest for subsequent experiments.

PGM activity assays

PGM activity assays were carried out as described by Treptau et al. [7], with slight modifications. The final volume of the assay

was 200 μl, and activity was assayed in a Titertek Multiscan MCC/340 ELISA Reader (Flow Laboratories) by measuring the increase in A_{340} of NADPH at 20 °C.

Electrophoretic techniques and Western blot analysis

SDS/PAGE was carried out as described by Laemmli [19]. Before electrophoresis, samples were alkylated as described by Westermeier [20]. Gels were either stained with silver or prepared for electrophoretic protein transfer on to nitrocellulose membranes (BA-85), which was performed by a semi-dry blotting technique [21] or in a Bio-Rad Mini Trans-blot cell.

Antibodies raised against chicken PGM and against recombinant PP63-1/parafusin 1 were visualized by a second antibody coupled to alkaline phosphatase (Sigma) using 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as substrates.

Sequencing of proteolytic peptides

A portion of 225 μg of the enriched PGM fraction from *P. tetraurelia* prepared according to [7] was divided into five aliquots, and each aliquot was separated on an SDS/10–20 %-polyacrylamide gradient gel. PP63/parafusin in this fraction was cleaved in the gel with the endoproteinase Lys C by the method of Eckerskorn and Lottspeich [22]. Peptides were separated by reverse-phase HPLC on Supersphere 60 RP (125 mm × 2 mm; Merck, Darmstadt, Germany). The solvent system was 0.1 % (v/v) trifluoroacetic acid in water (solvent A) and 0.1 % (v/v) trifluoroacetic acid in acetonitrile (solvent B). A gradient from 0 to 60 % B in A was performed over 60 min at a flow rate of 300 μl/min. The detection wavelength was 206 nm. Fractions were collected manually and subjected to N-terminal sequence analysis using a 492 protein sequencer (Applied Biosystems/Perkin Elmer, Langen, Germany) according to the instructions of the manufacturer.

Phosphorylation of recombinant P63-1/parafusin 1 *in vitro*

Phosphorylation assays (0.08 ml) contained 2 μg of recombinant P63-1/parafusin 1 in 5 mM $MgCl_2$, [γ-^{32}P]ATP (750 Ci/mmol), 20 mM triethanolamine/HCl, 10 % (v/v) glycerol and 1 mM dithioerythritol, pH 7.5. Assays were performed in the presence of either 2 mM $CaCl_2$ or 1 mM EGTA. Reactions were started by adding 125 ng of casein kinase, and terminated after 20 min at 20 °C by spotting 80 μl of sample buffer [19] into the reaction mixture. Aliquots were subjected to SDS/PAGE and then prepared for autoradiography [10].

Miscellaneous methods

Whole-cell homogenates of *P. tetraurelia* cells were prepared as described in [7]. *Paramecium* casein kinase was enriched from 100000 ***g*** supernatants as described in [10]. Protein concentrations were determined with BSA as a standard [23].

RESULTS

Isolation of two different cDNAs coding for PP63/parafusin

A λZAP Express cDNA library from *P. tetraurelia* cells was screened with a PCR probe which covers the sequence comprising nucleotides 976–1396 of the parafusin sequence [12]. This sequence was chosen as a probe because earlier reports showed that parafusin and PP63 are identical [4,5,7]. After the second round of screening, nine independently derived clones were further investigated, eight of which contained an insert of 1.8 kb. Sequencing revealed two slightly different sequences (Figure 1A). Seven clones contained cDNA sequence PP63-1/parafusin 1 and one clone contained cDNA sequence PP63-2/parafusin 2. All were lacking the start ATG.

Isolation of a genomic fragment coding for PP63/parafusin

To complete the missing 5′-end of the cDNAs, a genomic λZAPII library constructed from *Eco*RI-digested genomic DNA from *P. tetraurelia* was screened with the same probe as above. Again, two highly related sequences were isolated (Figure 1A). They are partial genomic sequences corresponding to the cDNAs PP63-1/parafusin 1 and PP63-2/parafusin 2, and cover the sequence from the 5′-end to the *Eco*RI restriction site at position 517 in Figure 1(A). Using this information, the missing 5′-end of the PP63-1/parafusin 1 cDNA was completed as described in the Experimental section. Figure 1(A) also shows that the two genomic fragments contain two small introns typical of *Paramecium* [24].

PP63/parafusin cDNAs and their deduced amino acid sequences

The open reading frames of the PP63-1/parafusin 1 and PP63-2/parafusin 2 cDNAs each consist of 1716 nucleotides. Since a stop TGA codon is present seven triplets prior to the start ATG, this ATG must be the transcription start. The 3′ non-coding region contains a putative polyadenylation site (underlined in Figure 1A) [25,26] and a poly(A)$^+$ tail. The predicted amino acid sequences are 572 amino acids in length, with a molecular mass of 63.8 kDa for each. This agrees with the apparent molecular mass of the original protein. The calculated pI values are 6.38 for P63-1/parafusin 1 and 6.25 for P63-2/parafusin 2. Our previous data showed pI values between 5.85 and 6.05 for the different isoforms of PP63/parafusin in *P. tetraurelia* [6,7]. The hydrophobicity plots of the two proteins are identical. There are long hydrophilic stretches at the N-terminus (Val-10 to Phe-42), in the first half of the polypeptide (Arg-144 to Asp-181) and at the C-terminus (Ile-434 to Arg-503) (Figure 1B).

Sequencing of proteolytic peptides of isolated PP63/parafusin

To confirm that the isolated cDNAs contain the sequence coding for PP63/parafusin, two proteolytic peptides of the native PP63/parafusin obtained by cleavage with endoprotease Lys C were sequenced. The resulting sequences matched the deduced amino acid sequence of PP63-1/parafusin 1 cDNA entirely (double underlined in Figure 1A) (Table 1). The residue immediately preceding the cleavage site is a lysine, consistent with the specificity of Lys C.

Analysis of the PP63-1/parafusin 1 amino acid sequence

PP63-1 is identical with *Paramecium* parafusin. The parafusin sequence deduced from its cDNA sequence (EMBL Nucleotide Sequence Database accession no. L12471; [12]) is identical to the C-terminal 566 amino acids of PP63-1 (except for amino acids Lys-56 and Pro-57, which are missing from the parafusin sequence), but varies at the N-terminal end (Figure 2). There is no start methionine in the parafusin amino acid sequence, and it contains 12 additional N-terminal amino acids compared with the PP63 N-terminus. The identity of the two amino acid sequences starts at Ala-19 in parafusin and Ala-7 in PP63 (Figure 2).

In addition to the identity of PP63-1 with parafusin, there is 51.4 % identity with PGM isoform 2 from rabbit skeletal muscle [27] throughout the full amino acid sequence, and 43.7 % identity with PGM isoform 2 from yeast [28]. These data suggest strongly

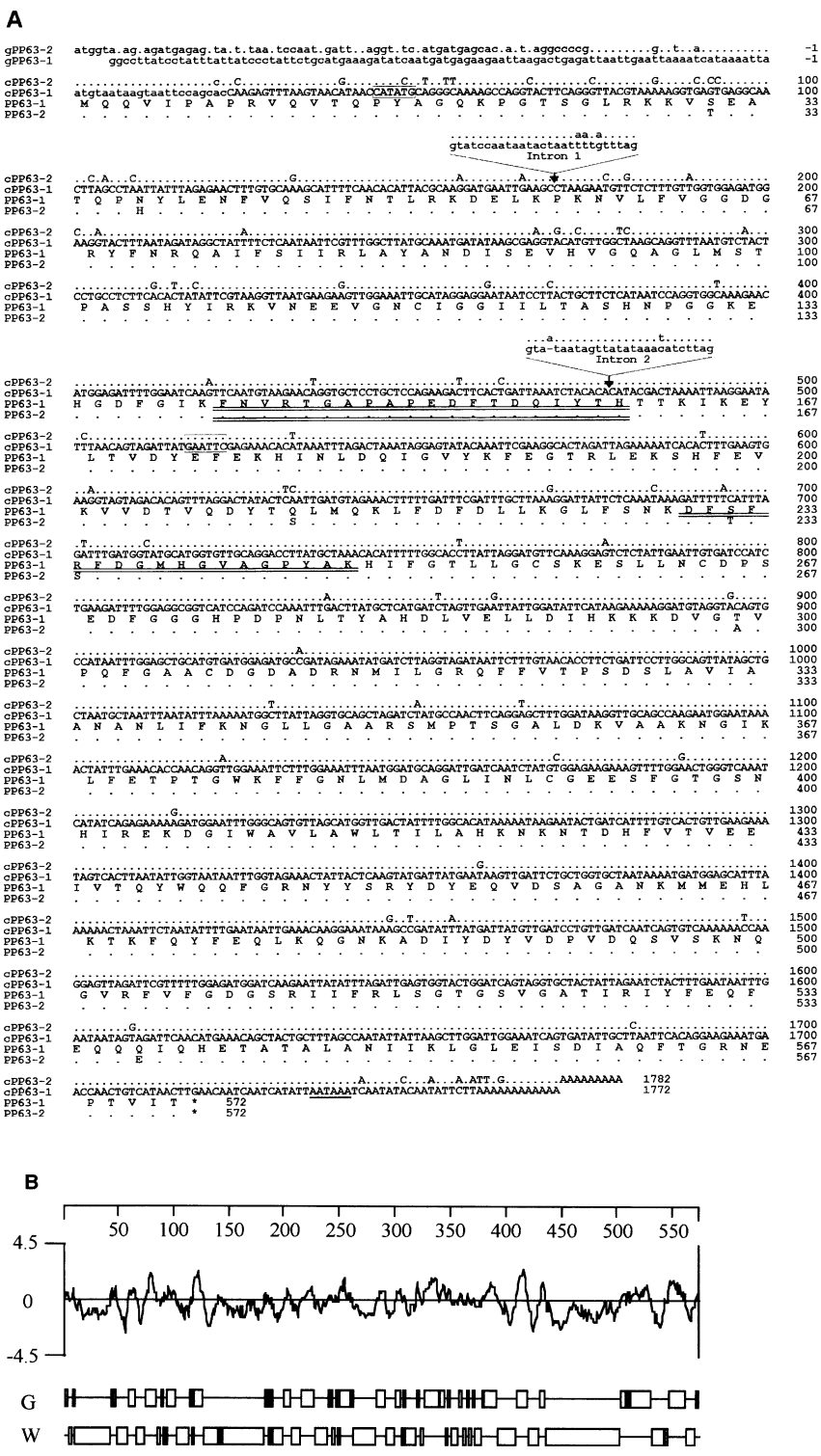

Figure 1 DNA and deduced amino acid sequences of PP63-1/parafusin 1 and PP63-2/parafusin 2

(**A**) The top two lines in each group show the DNA sequences, and the bottom two lines show the amino acid sequences (single-letter code). Dots indicate identical nucleotides or amino acids between the two sequences. Nucleotides shown in lower case are derived from the genomic sequences (g); those shown in upper case are from the cDNAs (c). The features indicated are: boxed, restriction sites for *Nde*I (position 44; CATATG) and *Eco*RI (position 517; GAATTC); double underlined, amino acid sequences derived by microsequencing of two proteolytic peptides of PP63/parafusin (cf. Table 1); underlined, putative polyadenylation signal AATAAA. The asterisk indicates the stop codon. The two introns are inserted at positions 169 and 477. (**B**) Hydrophobicity plot of the deduced amino acid sequence of PP63-1/parafusin 1 cDNA. Shown are hydrophobic (G) and hydrophilic (W) regions of the polypeptide. The scale at the top gives the amino acid numbering. The hydrophobicity plot for the deduced amino acid sequence of PP63-2/parafusin 2 cDNA (results not shown) is identical to that for PP63-1/parafusin 1.

that PP63/parafusin is identical to PGM of *P. tetraurelia*, and extends our recent finding that PP63/parafusin is biochemically identical with PGM [7].

PGM is a glycolytic enzyme which catalyses the transfer of a phosphate group between C-1 and C-6 of glucose [27,29]. A computer search for motifs revealed the PGM/phosphomanno-

Table 1 Peptide sequences of proteolytic peptides from isolated PP63/parafusin

Shown are the amino acid sequences of two peptides formed after cleavage of isolated PP63/parafusin with the endoproteinase Lys C, and their positions in the deduced amino acid sequences of the PP63-1/parafusin 1 and PP63-2/parafusin 2 cDNAs.

	Amino acid sequence	Amino acid nos.
Peptide 1	FNVRTGAPAPEDFTDQIYTHT	141–161 in PP63-1 and PP63-2
Peptide 2	DFSFRFDGMHGVAGPYAK	230–247 in PP63-1*

* In PP63-2 the underlined serine and arginine are replaced by threonine and serine respectively.

Table 2 Enzyme activities of different PGMs

Enzyme activity tests were performed as described in the Experimental section. Data are means ± S.D. from five independent experiments.

Enzyme	Specific activity (μmol/min per mg)
Recombinant P63-1/parafusin 1	4.3 ± 0.9
Chicken PGM	3.6 ± 0.4
Rabbit PGM	13.2 ± 1.5

mutase signature sequence from amino acid 120 to 135, which corresponds to the active centre of the enzyme. Ser-126 coincides with Ser-116 of human PGM, which is transiently phosphorylated during the enzymic reaction [29].

Expression of PP63-1/parafusin 1 cDNA in *E. coli*

To verify the identity of PP63/parafusin with PGM, the PP63-1/parafusin 1 cDNA was expressed in *E. coli*. Because *Paramecium* employs a divergent genetic code by using the universal stop codons TAA and TAG for glutamine [13], 16 TAA and six TAG codons in the PP63-1/parafusin 1 cDNA were mutated into the universal glutamine codons CAA and CAG. The mutated cDNA was cloned into the pET16b expression vector and expressed in *E. coli* with a His_{10} tag at the N-terminus. Approx. 50% of the recombinant protein was found in the soluble fraction and 50% in the insoluble fraction of the *E. coli* lysate. The protein was purified from the soluble fraction by Ni^{2+}-affinity chromatography.

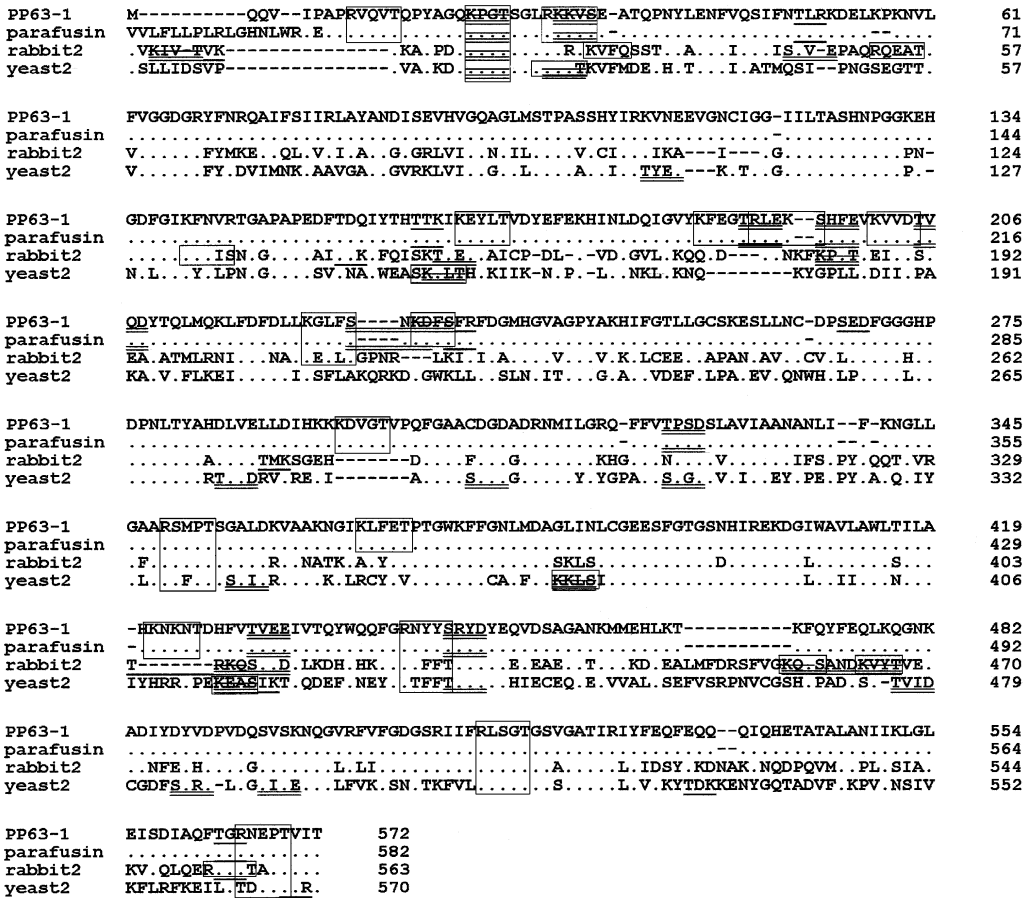

Figure 2 Alignment of the deduced amino acid sequences of PP63-1/parafusin 1 cDNA, parafusin and PGMs from rabbit and yeast

Amino acids are shown in single-letter code. Dots indicate amino acids identical to those in PP63-1/parafusin 1. Potential phosphorylation sites are shown: underlined, protein kinase C; double underlined, casein kinase type II; double underlined and crossed (XX), Ca^{2+}/calmodulin-dependent protein kinase; boxed, cAMP- and cGMP-dependent protein kinases.

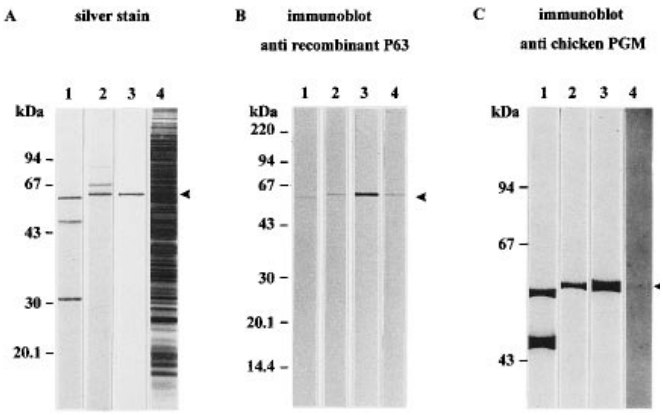

Figure 3 Cross-reactivity of antibodies raised against PGMs and recombinant P63-1/parafusin 1

(**A**) Silver-stained SDS/polyacrylamide (10–20%) gradient gel containing 500 ng of chicken PGM (lane 1), 500 ng of rabbit skeletal muscle PGM (lane 2), 350 ng of recombinant P63-1/parafusin 1 (lane 3) and 50 μg of the 100000 ***g*** supernatant from *Paramecium* whole-cell homogenate (lane 4). (**B**) Western blot of the gel in (**A**) detected with antibodies raised against recombinant P63-1/parafusin 1. (**C**) Western blot from a 10% polyacrylamide gel with lanes 1–4 as in (**A**), detected with an antiserum raised against chicken PGM. Controls run with the corresponding preimmune serum did not show any cross-reactivity (results not shown). The arrowheads indicate the 63 kDa band of PGM, recombinant P63-1/parafusin 1 or original PP63/parafusin. Note that chicken PGM is slightly smaller (62 kDa) than rabbit PGM and P63-1/parafusin 1, and that commercial PGM samples contain some contaminating proteins of other molecular masses.

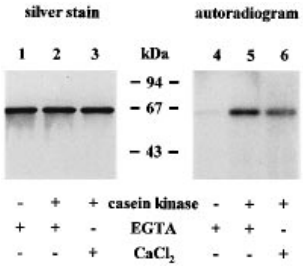

Figure 4 Phosphorylation of recombinant P63-1/parafusin 1 by endogenous casein kinase *in vitro*

Phosphorylation assays were conducted as described in the Experimental section. Aliquots of 1 μg of *in vitro*-phosphorylated recombinant P63-1/parafusin 1 were subjected to SDS/PAGE and then stained with silver (lanes 1–3) or processed for autoradiography (lanes 4–6). Note that marked phosphorylation of recombinant P63-1/parafusin 1 occurs in the presence of casein kinase and 1 mM EGTA (lane 5), but is much less obvious in the presence of 2 mM $CaCl_2$ (lane 6).

Recombinant P63-1/parafusin 1 displayed PGM activity comparable with that of PGM from chicken or rabbit skeletal muscle (Table 2).

Antibodies raised against recombinant P63-1/parafusin 1 also cross-reacted with PGM from chicken and rabbit skeletal muscle. Figure 3(A) shows a silver-stained gel, and Figures 3(B) and 3(C) show Western blots. Both PP63/parafusin and PGM were detected by polyclonal antibodies raised against recombinant P63-1/parafusin 1 (Figure 3B) and against chicken PGM (Figure 3C), i.e. both antibodies recognized both their original antigen and the corresponding proteins from all sources.

Recombinant P63-1/parafusin 1 was subjected to phosphorylation by an enriched *Paramecium* casein kinase fraction, with and without Ca^{2+}, and subsequently separated by SDS/PAGE (Figure 4). The recombinant protein was strongly phosphorylated in absence of Ca^{2+}, but much less so in presence of Ca^{2+}, like its original form [10].

DISCUSSION

We describe the cloning of two cDNAs coding for the exocytosis-sensitive phosphoprotein PP63/parafusin in *Paramecium* [4,6,7]. One of them, PP63-1/parafusin 1 cDNA, is virtually identical to the partial parafusin cDNA sequence published by Subramanian and co-workers [12]. Our findings allowed us to correct and complete the 5′-end of this sequence. The second cDNA, PP63-2/parafusin 2 cDNA, is a new isoform of PP63/parafusin. We also prove that PP63-1/parafusin 1 is identical with the glycolytic enzyme PGM.

Sequence analysis

PP63-1 cDNA is almost identical with the previously described parafusin cDNA [12]. The main difference between the PP63-1 cDNA and the parafusin cDNA is at the 5′-end. The published parafusin cDNA sequence seems to carry an incorrect 5′-end, because it does not contain an ATG start codon within the open reading frame. It is very likely to represent a fusion product of two cDNAs, of which the first part shows identity to elongation factor α and only the second part codes for parafusin [12]. Further evidence to support this assumption comes from our isolated cDNA clones. The sequences of these clones all start between 27 and 32 nucleotides after the initiation codon GTA (nucleotide 913 in [12]) of the parafusin cDNA, and do not contain any sequence of the part that is similar to elongation factor α.

It has been found that *Paramecium* mRNA possesses relatively short untranslated 5′-sequences, which might be lost together with the first few coding nucleotides during construction of the cDNA library (J. Linder, unpublished work). Taking these aspects into account, together with similarity searches for the deduced amino acid sequences of the open reading frames of the PP63/parafusin cDNAs, we assumed that the missing 5′-end could not consist of more than 40 or less than 10 nucleotides. The isolation of partial genomic sequences coding for PP63/parafusin proved this assumption to be correct. The genomic sequences show that the cDNAs lack only 22 nucleotides compared with the complete coding sequence (Figure 1A). They contain one initiation codon ATG, which must be the correct start codon since a TGA stop codon occurs 21 nucleotides upstream in-frame. Comparisons of the deduced amino acid sequence of the PP63-1/parafusin 1 cDNA with known protein sequences also confirms this ATG as the correct initiation codon. The correct N-terminal amino acid sequence does not show any hydrophobic characteristics, in contrast with the N-terminal sequence found for parafusin [12]. No extended hydrophobic region can be seen in the polypeptide. This contradicts the theory of membrane attachment of PP63/parafusin [5,12,30], but agrees with the actual solubility properties of the protein [6,7,10].

There is a second minor difference between our PP63-1/parafusin 1 cDNA and the published parafusin cDNA sequence [12]. Two codons of the PP63-1/parafusin 1 sequence, coding for Lys-56 and Pro-57, are missing from the parafusin sequence described in [12]. These two additional codons have been confirmed by examining the genomic DNA, and they are also present in PP63-2/parafusin 2 cDNA and genomic DNA (Figure 1A). Interestingly, the coding sequence for Lys-56 and Pro-57 is interrupted by an intron in the genomic DNA.

The second cDNA, PP63-2/parafusin 2, is closely related to PP63-1/parafusin 1. On the nucleotide level the two sequences

are 95.4% identical. On the amino acid level the identity is 98.6%. There are seven amino acid exchanges over the full length of the two polypeptides, four of which are conservative exchanges (Figure 1A). Taking this into account, the similarity of the two sequences is 99.6%.

The two cDNAs are derived from two different genes. For each cDNA a genomic DNA fragment could be isolated. They cover the first 522 nucleotides from the 5′-end of the cDNA to the *Eco*RI restriction site GAATTC (Figure 1A). Within this part of the genomic sequence there are two short introns typical of *Paramecium* [24]. These occur at identical sites in the two sequences. Intron 1 is 27 nucleotides in length in both sequences, and the length of intron 2 is 26 nucleotides in PP63-1/parafusin 1 DNA and 27 nucleotides in PP63-2/parafusin 2 DNA.

The amino acid sequences of two proteolytic peptides from the original PP63/parafusin protein (with PGM activity [7]) obtained after cleavage with the endoproteinase Lys C were identical to amino acids 141–161 in both PP63/parafusin polypeptides and to amino acids 230–247 in PP63-1/parafusin 1 respectively (Figure 1A and Table 1). This additionally proves the molecular identity of PP63/parafusin with PGM. It also shows that PP63-1/parafusin 1 is the major isoform expressed.

P63-1/parafusin 1 has PGM activity

Similarity searches revealed approx. 50% identity between PP63-1/parafusin 1 and mammalian PGMs, and 43% identity with yeast PGM (Figure 2). This is consistent with our previous results, which suggested very strongly that PP63/parafusin and PGM in *P. tetraurelia* are identical [7]. We decided to express the PP63-1/parafusin 1 cDNA in *E. coli*, since this cDNA seems to be expressed in *Paramecium* to a much greater extent than PP63-2/parafusin 2 cDNA. In the cDNA library, 87% of the PP63/parafusin cDNA was PP63-1/parafusin 1 (only one clone out of eight was PP63-2/parafusin 2), and the amino acid sequences of the two proteolytic peptides show that they were derived from PP63-1/parafusin 1 (Table 1). Expression of PP63-1/parafusin 1 cDNA in *E. coli* yielded a recombinant protein with an apparent molecular mass of 63 kDa, which corresponds to the molecular mass of the original PP63/parafusin. This protein showed specific PGM activity comparable with the specific activities of chicken muscle PGM and rabbit skeletal muscle PGM (Table 2).

PGM is involved in the conversion of glucose 1-phosphate into glucose 6-phosphate [27,29]. It is a well known enzyme and has been thoroughly studied in many systems. In all eukaryotic organisms investigated so far, i.e. in yeast [31–34], plants [35,36], *Drosophila* [37], fish [38] and mammals [39–44], at least two isoforms of PGM are present. We also were able to show the existence of at least two PGMs in *P. tetraurelia* [7]. Since the two deduced amino acid sequences of the PP63/parafusin cDNAs are nearly identical and are derived from two separate genes, they might encode different isoforms of PP63/parafusin which correspond to the two isoforms of PGM in *P. tetraurelia* [7].

Immunological identity

Figure 3 demonstrates that polyclonal antibodies raised against chicken PGM recognized original PP63/parafusin as well as recombinant P63-1/parafusin 1 and PGM from rabbit skeletal muscle (Figure 3C). Polyclonal antibodies against recombinant P63-1/parafusin 1 also cross-reacted with PGM from chicken and rabbit muscle and with original PP63/parafusin (Figure 3B). However, the two antisera did not show equivalent staining of the proteins. In Figure 3(B) the antibodies against recombinant P63-1/parafusin 1 showed the strongest reaction with their original antigen (lane 3), whereas the reactions with the heterologous PGMs from chicken (lane 1) and rabbit (lane 2) gave a weaker signal. Lane 4 contains proteins from the 100000 ***g*** supernatant from homogenized *Paramecium* cells. Since only approx. 0.1% of the total protein in this fraction is PP63/parafusin, lane 4 contains seven times less PP63/parafusin than lane 3. This could explain the weak staining by the antibody in this lane. The same applies to Figure 3(C). Polyclonal antibodies against PGM from rabbit skeletal muscle reacted equally with PGM from chicken, with original PP63/parafusin [7] and with recombinant P63-1/parafusin 1 (results not shown). These cross-reactivities again demonstrate the close relationship between PGM and PP63/parafusin.

Phosphorylation of P63/parafusin

PP63/parafusin is rapidly dephosphorylated during triggered trichocyst exocytosis (within 80 ms) and is fully rephosphorylated within a few seconds [6], and then separates on isoelectric focusing gels into at least three different isoforms. *In vitro*, P63/parafusin is phosphorylated by an endogenous casein kinase [7,10], resulting in the same three isoforms on isoelectric focusing. The same phosphorylation pattern occurs when a PGM fraction from *P. tetraurelia* is phosphorylated by this kinase [7]. Here we show that this casein kinase is able to phosphorylate recombinant P63-1/parafusin 1. Previous studies showed that the phosphorylation of original P63/parafusin from *P. tetraurelia* by this casein kinase is not stimulated, but rather is inhibited, by Ca^{2+}. Phosphorylation of recombinant P63-1/parafusin 1 by this casein kinase shows the same effect (Figure 4). Further investigations revealed that this casein kinase is closely related to casein kinase type II [44a] A search of the PP63-1/parafusin 1 amino acid sequence for potential phosphorylation sites revealed seven phosphorylation sites for casein kinase type II (S/TXXD/E) [45] (double underlined in Figure 2). Four of these sites are unique to PP63-1/parafusin 1 (at positions 191, 196, 205 and 227) compared with the other two PGM sequences shown.

Several further potential phosphorylation sites are present in PP63-1/parafusin 1. Six of these could be specific for protein kinase C (S/TXR/K; underlined in Figure 2), although whether this protein kinase occurs in *Paramecium* is unknown. Based on the consensus sequence R/KXX(X)S/T [45], there are 16 potential phosphorylation sites for cAMP- or cGMP-dependent protein kinases present in the PP63-1/parafusin 1 sequence (boxed in Figure 2). *In vitro*, original P63/parafusin is a substrate for endogenous cGMP-dependent kinase [10]. Similarly, recombinant P63-1/parafusin 1 can be phosphorylated by endogenous cGMP-dependent protein kinase (results not shown). Lee et al. [9] have shown that an isoform of PGM in the sarcoplasmic reticulum of rabbit skeletal muscle could be phosphorylated by a Ca^{2+}/calmodulin-dependent kinase. However, such a kinase has not been identified in *Paramecium* so far, and our results show that P63-1/parafusin 1 can be phosphorylated in the absence of Ca^{2+} (Figure 4). The PP63-1/parafusin 1 amino acid sequence contains three potential phosphorylation sites for a Ca^{2+}/calmodulin-dependent protein kinase (K/RXXS/T; Figure 2).

Possible function of P63/PP63/parafusin/PGM in exocytosis

The rapid dephosphorylation of PP63/parafusin/PGM in *Paramecium* (selectively in exocytosis-competent strains [4]), in synchrony with the occurrence of exocytosis [6], strongly suggests a role for this protein in triggered exocytosis. One possibility

concerns mobilization of Ca^{2+} from subplasmalemmal pools, the 'alveolar sacs', during exocytosis. Alveolar sacs remotely resemble sarcoplasmic reticulum in skeletal muscle [46,47]. Lee and co-workers [9] found an isoform of PGM in the heavy sarcoplasmic reticulum fraction of rabbit skeletal muscle where Ca^{2+}-release channels are enriched. In the sarcoplasmic reticulum the enzyme activity of this PGM was considerably decreased when associated with membranes, but could be partially recovered by detergent treatment. In this system a role for PGM in the regulation of Ca^{2+} dynamics has been discussed, since the extent of phosphorylation of PGM coincided with Ca^{2+} release from the sarcoplasmic reticulum [48,49]. Finally, the fact that glycolytic enzymes are frequently involved in unexpected functions other than glycolysis [50] suggests that PGM dephosphorylation during exocytosis may have even more complex regulatory functions.

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