A 63 kDa phosphoprotein undergoing rapid dephosphorylation during exocytosis in *Paramecium* cells shares biochemical characteristics with phosphoglucomutase

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We have enriched phosphoglucomutase (PGM; EC 5.4.2.2) ~ 20-fold from *Paramecium tetraurelia* cells by combined fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$, gel filtration and anion-exchange chromatography yielding two PGM peaks. Several parameters affecting PGM enzymic activity, molecular mass and pI were determined. Phosphorylation studies were done with isolated endogenous protein kinases. Like the 63 kDa phosphoprotein PP63, which is dephosphorylated within 80 ms during synchronous trichocyst exocytosis [Höhne-Zell, Knoll, Riedel-Gras, Hofer and Plattner (1992) *Biochem. J.* **286**, 843–849], PGM has a molecular mass of 63 kDa and forms of identical pI. Since mammalian PGM activity depends on the presence of glucose 1,6-bisphosphate (Glc-1,6- P_2) (which is lost during anion-exchange chromatography), we analysed this aspect with *Paramecium* PGM. In this case PGM activity was shown not to be lost, due to *p*-nitrophenyl phosphate-detectable phosphatase(s) (which we have separated from PGM), but also due to loss of Glc-1,6- P_2 . Like PGM from various vertebrate species, PGM activity from *Paramecium* can be fully re-established by addition of Glc-1,6- P_2 at 10 nM, and it is also stimulated by bivalent cations and is insensitive to chelating or thiol reagents. The PGM

which we have isolated can be phosphorylated by endogenous cyclic-GMP-dependent protein kinase or by endogenous casein kinase. This results in three phosphorylated bands of identical molecular mass and pI values, as we have shown to occur with PP63 after phosphorylation *in vivo* (forms with pI 6.05, 5.95, 5.85). In ELISA, antibodies raised against PGM from rabbit skeletal muscle were reactive not only with original PGM but also with PGM fractions from *Paramecium*. Therefore, PGM and PP63 seem to be identical with regard to widely different parameters, i.e. co-elution by chromatography, molecular mass, phosphorylation by the two protein kinases tested, pI values of isoforms, and immuno-binding. Recent claims that PP63 ('parafusin') would not be identical with PGM specifically in *Paramecium* are critically evaluated. Since some glycolytic enzymes are discussed as being associated with the Ca^{2+} -release channel in muscle sarcoplasmic reticulum, and since sub-plasmalemmal Ca^{2+} stores in *Paramecium* closely resemble sarcoplasmic reticulum, a possible function of PP63/PGM in exocytosis regulation is discussed, particularly since dephosphorylation strictly parallels exocytosis.

INTRODUCTION

Recently we have partially characterized an exocytosis-sensitive 63 kDa phosphoprotein, PP63, from *Paramecium tetraurelia* cells [1]. In quenched-flow experiments PP63 is rapidly and selectively dephosphorylated *in vivo* during triggered synchronous (80 ms) trichocyst exocytosis. Within only seconds the dephosphorylated form, P63, is fully rephosphorylated to the phosphorylated form, PP63. Dephosphorylation does not occur when exocytosis-incompetent mutant strains are exposed to the secretagogue aminoethyl-dextran (AED) [2]. PP63 ('parafusin') exists in several isoforms, with pI values between 5.75 and 6.05 [1,3]. All the forms of different pI are extensively dephosphorylated during synchronous exocytosis, including the most intensively phosphorylated form, of pI 5.95, which is also most intensively rephosphorylated after exocytosis [1]. They all are sensitive to alkaline, but insensitive to acid, treatment, thus indicating serine/threonine phosphorylation [1,3]. We have evi-

dence that PP63 isolated after ^{32}P labelling *in vivo* is a substrate for protein serine/threonine phosphatases and kinases (R. Kissmehl, T. Treptau, H. W. Hofer and H. Plattner, unpublished work). Isolation of PP63 [3] and its recent molecular cloning revealed several serine-phosphorylation sites [4]. On the other hand, it was reported that PP63 could be phosphoglycosylated *in vitro* by addition of glucose 1-phosphate (Glc-1- P), catalysed by an α Glc-1- P phosphotransferase utilizing UDP-glucose, to a short chain of mannose residues O-linked to a serine residue [5–7]. Although P63 and PP63 were shown to be cytosolic, its localization by Western blots, from isolated cell-surface fragments, and by immunofluorescence labelling, using a polyclonal antibody against the pI 5.95 isoform, is cortical [1]. This localization, and the strict temporal correlation of its dephosphorylation with exocytosis, suggests a role for exocytosis regulation in *Paramecium tetraurelia*. In this context, the presence of this protein in widely different species, from yeast to mammals [8], implies a mechanism of possibly general importance for

Abbreviations used: AED, aminoethyl-dextran; cGMP, cyclic GMP; IEF, isoelectric focusing; Glc-1- P , glucose 1-phosphate; Glc-6- P , glucose 6-phosphate; Glc-1,6- P_2 , glucose 1,6-bisphosphate; Glc-6- P -DH, glucose-6-phosphate dehydrogenase; P63, dephosphorylated 63 kDa phosphoprotein; PP63, phosphorylated/rephosphorylated 63 kDa phosphoprotein; PGM, phosphoglucomutase; pNPP, *p*-nitrophenyl phosphate; SR, sarcoplasmic reticulum.

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exocytosis regulation. Beyond this, little is known about the nature of this phosphoprotein.

In mammalian, but also in other, cell homogenates UDP-glucose:glycoprotein glucose-1-phosphotransferase (Glc-phosphotransferase) has also been shown to add α Glc-1-*P* to O-linked mannose present on cytoplasmic acceptor proteins of about 63 kDa [9–11]. More recently, such an acceptor protein has been identified in liver [12], PC12 cells and rat synaptosomes [13], as well as in *Saccharomyces cerevisiae* [14,15], as being identical with phosphoglucomutase (PGM, EC 5.4.2.2). However, in *Paramecium* cells, the identity of PP63 with PGM was recently denied [4,7].

PGM catalyses an intramolecular transfer of phosphate between C-1 and C-6 of glucose molecules, and thus has a pivotal role in glycolysis and gluconeogenesis regulation. This enzyme is not only widely distributed in mammalian tissues, but it could also be isolated from flounder and shark [16], from yeast [17], from a line of plant tissues [18–20] and even from *Escherichia coli* [21,22] and *Neisseria* [23]. Depending on the source, the apparent molecular mass of this single-chain monomeric enzyme is in the range 60–65 kDa. Electrophoretic studies also have shown that PGM from mammalian sources is heterogeneous, not only because of coding by different genes [24–26], but also due to different post-translational modifications [27,28]. Recent cDNA cloning and amino acid sequencing of PGM from bacteria [23] and from mammalian species [26,29–31] indicated considerable similarity. Different aspects were reported with regard to phosphorylation and enzymic activation. Treatment of the phospho-form of PGM with an excess of Glc-1-*P* converts the phospho-enzyme almost completely into the enzymically inactive dephosphoform [32]. If the enzyme is partially or completely in the dephospho-form, addition of sufficient glucose 1,6-bisphosphate (Glc-1,6-*P*₂) ensures conversion into the phospho-form [16]. Phosphorylation of PGM at Ser-116 is also well established [29,33,34], and only this form is catalytically active [32,35,36]. An isoform of PGM, localized in junctional sarcoplasmic reticulum (SR) from skeletal muscle, could be demonstrated to be the substrate of a Ca²⁺/calmodulin-dependent protein kinase [36] (see the Discussion section), which has not been isolated from *Paramecium* as yet. A structural and functional equivalent of SR is found in *Paramecium* cells in the form of the sub-plasmalemmal Ca²⁺ stores, the 'alveolar sacs' [37,38].

As pointed out above, there is now some discrepancy between results obtained with *Paramecium* and with a variety of other cell types. Only for *Paramecium* it was claimed that PP63 ('parafusin') could not be PGM. Therefore, we studied in this paper several characteristics of PP63 in relationship to PGM in *Paramecium*, i.e. how it can be phosphorylated *in vitro* and how this phosphorylation compares with phosphorylation *in vivo*. We show that PP63 shares several characteristics with PGM, and that PP63 co-purifies with PGM activity. Additionally, in ELISA the PGM-containing fraction from *Paramecium* binds polyclonal antibodies raised against PGM from rabbit skeletal muscle. Its apparent molecular mass and pI are identical with those of PP63. So far, PP63 and PGM seem to be identical with regard to widely different parameters. Since we can extract two PGM peaks, there might exist isoforms. Furthermore, previous work with *Paramecium* did not take into account inactivation by inappropriate preparation conditions. In addition, we show that PP63/PGM from *Paramecium* can serve as a substrate for endogenous casein kinase and cyclic GMP (cGMP)-dependent protein kinase, and thus yield phosphorylation forms of identical pI values. Of particular importance is the fact that these are identical with those occurring *in vivo*.

MATERIALS AND METHODS

Materials

Glc-1-*P*, Glc-1,6-*P*₂, NADP⁺, glucose-6-phosphate dehydrogenase (Glc-6-*P*-DH), *p*-nitrophenyl phosphate (*p*NPP) and PGM from rabbit skeletal muscle were obtained from Boehringer (Mannheim, Germany). Casein, histone II-S and Azocoll were from Sigma (Deisenhofen, Germany); HR SDS Precote 10%-polyacrylamide gels and the isoelectric-focusing (IEF) marker proteins were from Serva (Heidelberg, Germany); HPLC_{DTB} 'HeliPrepSystem', Superose 12 HR 16/50, Superose 12 HR 10/30, Mono Q HR 5/5, Mono P HR 5/5, Mono S HR 5/5, IEF gels and the SDS protein molecular-mass markers (LMW) were from Pharmacia (Freiburg, Germany). Protein-assay dye reagent was purchased from Bio-Rad Laboratories (Munich, Germany) and carrier-free [³²P]P_i (PBS 13) was from Amersham-Büchler (Braunschweig, Germany). Polystyrene microtitre plates for ELISA were from Greiner (Solingen, Germany). Other reagents and all solvents used were of analytical grade.

Cell cultures

Paramecium tetraurelia wild-type cells (strain 7S) were grown at 25 °C to early stationary phase in a sterile synthetic medium [39]. Cells were harvested and rinsed twice in 5 mM Pipes/HCl buffer, pH 7.0, supplemented with 1 mM KCl and 0.1 mM CaCl₂ [40]. Cells were washed by centrifugation in this solution for 2 min at 80 *g* at room temperature in a Heraeus Christ (Osterode, Germany) minifuge equipped with a swing-out rotor.

Exocytosis triggering

Synchronous exocytosis was induced by adding 0.01% (w/v) (= 2.5 μ M) AED (40 kDa; one amino group per kDa of dextran) as described previously [41]. To determine the trigger effect of non-cytotoxic AED triggering, samples were additionally triggered with picric acid [42]. Though lethal, picric acid releases residual trichocysts not released with AED. Only cultures with an exocytosis capacity of > 90% in response to AED were used for phosphorylation studies *in vivo*.

³²P labelling of *Paramecium* cells *in vivo*

Methods used for labelling cells *in vivo* were as used previously [1], with slight modifications. Briefly, cells (5 $\times$ 10⁴/ml) were incubated with (2.3–3.0) $\times$ 10⁶ Bq/ml (62–80 μ Ci/ml) H₃³²PO₄ (Amersham-Büchler) for 90 min at room temperature. Under these conditions PP63, but also some other endogenous phosphoproteins, were intensely labelled. Samples were taken either without AED triggering (phosphorylated state) or at 2 s or 1 min after AED triggering, representing the de- and re-phosphorylated state of (P)P63 respectively [43].

Isolation of (P)P63

³²P-labelled untriggered or AED-triggered cells were homogenized by freezing and thawing (to maintain phosphorylation states) by using liquid nitrogen. After two cycles the samples were centrifuged at 100 000 *g* for 30 min (4 °C) in a Beckman Airfuge, rotor A-95. In each case P63 or PP63 was found in the supernatant.

Isolation of PGM

Cells were homogenized in an ice-chilled motor-driven glass Potter homogenizer with a Teflon pestle. The homogenate was

centrifuged at 20000 *g* for 20 min at 4 °C in a Sorvall type OTB Combi ultracentrifuge equipped with an AH 650 swing-out rotor to sediment large subcellular components. The supernatant was centrifuged as indicated above at 100000 *g* for 60 min at 4 °C. (NH₄)₂SO₄ was added slowly with stirring to the supernatant at 0 °C until 35 % saturation was reached. The suspension was stirred for a further 60 min and centrifuged at 30000 *g* for 30 min at 4 °C with the same rotor. The supernatant was adjusted to 75 % saturation with (NH₄)₂SO₄, stirred for a further 60 min, and centrifuged as above. The pellets were resuspended in a minimum volume of 20 mM triethanolamine/HCl buffer, pH 7.5, and centrifuged as above. The supernatant was loaded on a Pharmacia Superose 12 prep grade HR 16/50 column, which had been equilibrated with 20 mM triethanolamine/HCl, pH 7.5, at room temperature and eluted with the same buffer at a flow rate of 1 ml/min with a Pharmacia HPLC system. PGM-active fractions were pooled and applied to a Mono Q HR 5/5 column equilibrated with 20 mM triethanolamine/HCl, pH 7.5. PGM was eluted at a flow rate of 0.5 ml/min with a linear gradient of 0–250 mM NaCl, collected in 1 ml fractions, and assayed for enzyme activity. For further enrichment, PGM-active fractions were applied on to Superose 12 HR10/30. Alternatively, chromatographic fractionation was also performed, i.e. by chromatofocusing (Mono P HR 5/5) or by cation-exchange chromatography (Mono S HR 5/5) using a corresponding Polybuffer system for pH gradients 7–4 (Pharmacia) or 50 mM sodium acetate buffer, pH 5.5, respectively. We tentatively also used other buffer systems, including 20–50 mM Tris/HCl or Pipes/HCl, each at pH 7.5.

Isolation of endogenous protein kinases

We used a new scheme to isolate cGMP-dependent protein kinase and casein kinase (R. Kissmehl, T. Treptau, W. Hofer and H. Plattner, unpublished work), which both are known to occur in *Paramecium* [44,45]. They were both isolated from the *Paramecium* 100000 *g* supernatant by anion-exchange chromatography (DEAE-cellulose). Briefly, hydrophobic exchange chromatography (phenyl-Sepharose CL-4B) followed by affinity chromatography (Affi-Gel Blue) separated a casein kinase activity, whereas cGMP-dependent protein kinase activity could be enriched with affinity chromatography (Affi-Gel Blue) and cation-exchange chromatography (phosphocellulose). cGMP-dependent protein kinase and casein kinase were assayed as described by Thalhoffer et al. [46], with either histone II-S or casein as a substrate.

Protein determination

The concentration of proteins was determined by the method described by Bradford [47], with BSA as a standard.

SDS/PAGE

Protein samples were denatured by boiling for 3 min in 1.3 % (w/v) SDS/0.5 % (w/v) dithiothreitol/20 % (v/v) glycerol/125 mM Tris/HCl, pH 6.8 ('sample buffer') and subjected to electrophoresis on linear gradient (10–20 %) SDS/polyacrylamide gels or on HR SDS Precote 10 %-polyacrylamide gels in the presence of 0.1 % SDS, by using the discontinuous buffer system of Laemmli [48]. Protein standards [low molecular mass, from Pharmacia LKB, with myosin (220 kDa) added] were prepared according to the manufacturer's directions. Gels were silver stained as described by Heukeshoven and Dernick [49]. Before drying, gels were generally incubated for 1 h in 55 % (v/v) methanol with 2 % (v/v) glycerol added.

IEF

Proteins obtained by freeze–thaw solubilization after ³²P labelling were applied to polyacrylamide gels containing Ampholines in a linear gradient of pH 4.0–8.0 (Pharmacia LKB). The calibration kit used (Serva) included trypsin inhibitor (pI 4.55), β-lactoglobulin B (pI 5.13) and bovine carbonic anhydrase (pI 6.0).

Autoradiography

Autoradiography was performed with gels dried on to Cellophane under vacuum. Kodak XRP-5 X-ray film, Kodak direct exposure film (DEF) or Amersham Hyperfilm-MP was used, for 1–3 weeks at –70 °C. Exposure was in Kodak X-Omatic cassettes, with an intensifier screen. The molecular masses of the different phosphoproteins in the autoradiograms were determined as described by Winston [50]. Autoradiograms were evaluated densitometrically using a Quick Scan Densitometer from Desaga (Heidelberg, Germany).

Enzyme assays

The following enzyme assays were used.

PGM activity was measured by determining the rate of Glc-6-*P* formation by using a coupled assay involving Glc-6-*P*-DH and NADP⁺ [36] according to the following scheme:

The standard reaction mixture contained 84 mM triethanolamine/HCl buffer, pH 7.6, 3.5 mM Glc-1-*P*, 0.02 mM Glc-1,6-*P*₂, 0.9 mM EDTA, 1.7 mM MgCl₂, 0.19 mM NADP⁺, 0.47 unit/ml Glc-6-*P*-DH and original PGM or samples isolated from *Paramecium* cells in a final volume of 1 ml. Activity was assayed in a Beckman 35 spectrophotometer by measuring the increase in *A*₃₄₀ of NADPH at 25 °C.

Phosphatase activity was measured as *p*NPP phosphatase activity. Formation of *p*-nitrophenol was monitored at 405 nm. *p*NPP (disodium salt) was dissolved as an aqueous solution just before use. Incubations (volume 200 μl) were performed at 25 °C in a micro-well module containing 20 mM Tris/HCl, pH 7.4, 10 mM NaCl, 20 μg/ml BSA, 1 mM MnCl₂, and 10 μl portions of *Paramecium* extracts after chromatographic fractionation. The reaction was started by adding *p*NPP in a final concentration of 1 mM. The *p*NPP phosphatase activity was monitored spectrophotometrically in a Titertek Multiscan MCC/340 ELISA-Reader (Flow Laboratories) either by the time course of the reaction or after termination by addition of 13 % K₂HPO₄. Controls were run to account for non-enzymic *p*-nitrophenol formation.

Protease activity was determined by using standard assays for Azocoll hydrolysis [51]. Briefly, Azocoll (200 mg) was suspended in 40 ml of buffer (0.05 M Tris/HCl, 1 mM CaCl₂, pH 7.8) and stirred rapidly to a uniform suspension. Portions (10 ml) were transferred into test tubes and allowed to warm for 15 min to 37 °C. Assays were started by adding *Paramecium* extracts before or after chromatographic processing. After different time points, 1 ml samples were taken, put on ice for 3 min and centrifuged in an Eppendorf centrifuge for 2 min. The amount of Azocoll hydrolysed was determined by the increase in *A*₅₂₀ in a Beckman 35 spectrophotometer. For controls, tubes containing Azocoll without *Paramecium* extract were incubated for the same time.

Table 1 Isolation of *Paramecium* PGM from whole cell homogenates

After a 100 000 *g* centrifugation step, further enrichment of PGM from the supernatant was achieved by a fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ (35% and 75%) and a combination of molecular-sieve and anion-exchange chromatography. Samples (5 μl) were used in 0.2 ml assays of PGM activity.

Fraction	Volume (ml)	Total protein (mg)	Total activity (m-units)	Specific activity (m-units/mg of protein)	Purification (fold)	Yield (%)
Homogenate	30.00	179.3	267.6	1.49	1	100
100 000 <i>g</i> supernatant	26.00	85.8	301.9	3.51	2.3	112.8
35% $(\text{NH}_4)_2\text{SO}_4$: supernatant	28.30	40.3	223.3	5.53	3.7	83.4
75% $(\text{NH}_4)_2\text{SO}_4$: pellet	1.00	13.6	159.2	12.15	8.1	59.4
Superose 12 HR 16/50	0.64	2.2	57.3	26.06	17.5	21.4
Mono Q HR 5/5*	0.80	0.84	23.8	28.33	19.0	8.9

* For use of this column, see the Results section. Values indicated are for samples pooled from both peaks occurring in Figure 1(b).

Phosphorylation assays *in vitro*

To analyse phosphorylation of isolated PP63 or PGM-containing fractions *in vitro*, we added endogenous cGMP-dependent protein kinase or casein kinase to samples of P63 or PGM fractions. Some samples were previously denatured by heating (84 °C, 5 min). The phosphorylation assay contained 5 mM MgCl_2 , [γ - ^{32}P]ATP (10–40 nCi/ μl) and 5 mM Pipes/HCl buffer, pH 7.0 (with 1 mM KCl and 0.1 mM CaCl_2 added), or 20 mM triethanolamine/HCl buffer, pH 7.5. Experiments were performed in a final volume of 80 μl for 20 min at room temperature. Assays with cGMP-dependent protein kinase activities additionally contained 0.15 μM cGMP, and those with casein kinase up to 1 mM EGTA. At the end of the incubation, portions were stopped in boiling 'sample buffer', subjected to SDS/PAGE and then processed for autoradiography.

Antisera against PGM

To prepare polyclonal antisera, PGM from rabbit skeletal muscle (Boehringer-Mannheim) was injected subcutaneously and intramuscularly in a cock (strain Lege-Hybrid). The cock was boosted three times at 2-week intervals, and the serum titre against the antigen was determined by ELISA (see below). At 5 days after the last boost, the animal was killed to prepare the serum. For controls, 5 ml of serum was obtained before immunization.

ELISA

For testing the polyclonal antibodies against PGM, the direct ELISA technique was used as described previously [52], with slight modifications. Wells (100 μl) were coated with the antigen samples dissolved in PBS (containing 48 mM Na_2HPO_4 and 18 mM KH_2PO_4 , pH 7.4, with 146 mM NaCl added) overnight at 4 °C. Free binding sites on the polystyrene microtitre plates were saturated by incubation with 5% (w/v) fat-free milk powder in coating buffer (200 μl /well) for 1 h at room temperature. After rinsing with PBS, samples of antisera were serially diluted in PBS containing 1% fat-free milk powder and applied to successive wells (100 μl /well) to interact for 2 h at room temperature. After an extensive wash with PBS, peroxidase-coupled detecting antibodies (rabbit anti-chicken IgG, from Dianova, Hamburg, Germany) diluted 1:3000 in PBS and 1% fat-free milk powder were added to each well (100 μl) and incubated for 1.5 h at room temperature. After six rinses with PBS, we added to each well 150 μl of a freshly prepared peroxidase substrate solution composed of 0.4 mg/ml *o*-phenylenediamine and 0.025% (v/v) H_2O_2

in 0.2 M sodium phosphate/sodium citrate buffer, pH 6.0. After suitable colour development, the reaction was stopped by adding 50 μl of 1 M H_2SO_4 solution, and the absorbance in each well was determined in a Titertek Multiscan MCC/340 ELISA-Reader as the A_{492}/A_{620} ratios.

RESULTS

Isolation of PGM

In *P. tetraurelia* homogenates almost the entire ($\geq 96\%$) PGM activity was found in the soluble fractions by the procedures used, by analogy to PGM isolation from mammalian tissues [53–55]. When we first applied $(\text{NH}_4)_2\text{SO}_4$ (35% satn.) precipitation, almost 85% of the PGM activity was recovered in the supernatant (Table 1). After the following precipitation [75%-satd. $(\text{NH}_4)_2\text{SO}_4$] residual PGM activity was found mainly in the pellet fraction (60%), as shown in Table 1, but only $\sim 7\%$ was identified in the corresponding soluble fraction (results not shown), possibly indicating a rapid inactivation. Thus, a fraction with 8-fold enriched PGM activity was used for further chromatographic separation (Table 1).

Figure 1 illustrates the chromatographic fractionation of *Paramecium* PGM activities by a combination of molecular-sieve (Figure 1a) and anion-exchange chromatography (Figures 1b and 1c). Since PGM activity also depends on the buffer system applied, as seen by the rapid loss of activity with Pipes/HCl or Tris/HCl in the range 20–50 mM (results not shown), we utilized 20 mM triethanolamine/HCl buffer, pH 7.5, during the chromatographic separation. From Superose 12 HR 16/50 at a flow rate of 1 ml/min PGM activity was eluted as a single peak after 50 ml (Figure 1a). By this step PGM could be separated from over 80% of other proteins, as indicated by an increase in specific PGM activity (Table 1).

The subsequent anion-exchange chromatography on Mono Q HR 5/5 partitioned the PGM activity into a first peak eluted by NaCl at approx. 150 mM and a second peak at 180 mM NaCl (Figure 1b). Since at first we found no activity in any of these elution fractions, probably owing to conversion of the active phospho-enzyme into its inactive free dephospho-form, we analysed this aspect with regard to the loss of Glc-1,6- P_2 during anion-exchange chromatography as well as to any possible dephosphorylation by co-eluted phosphatase activity. As with PGM from various vertebrate species [16,32,35], PGM activity in the Mono Q elution fractions could be fully re-established by adding Glc-1,6- P_2 at 10 nM (Figure 1b). Simultaneously, we could separate *p*NPP phosphatase from PGM activity (Figure

Figure 1 Isolation of *Paramecium* PGM

Isolation was carried out from the 100 000 *g* supernatant by using fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ (35–75%) and a combination of molecular-sieve (a) and anion-exchange chromatography (b and c) as described in the Materials and methods section. Samples (5 μl) were used in a 1 ml or a 0.2 ml assay to determine PGM and pNPP phosphatase activity, respectively. In (b) the PGM activity lost (■) could be re-established by addition of 10 nM Glc-1,6- P_2 (▼). In (c) PGM (▼) is effectively separated from phosphatase (◆) activity.

1c). We selected pNPP as a substrate appropriate for most of the Tyr- and Ser/Thr-protein phosphatases, as well as for alkaline and acid phosphatases [56,57]. Any such phosphatase is unlikely to interfere with our analysis.

We have evidence of the existence of two PGM activity peaks in the 100 000 *g* supernatant, as shown by the two peaks in Figure 1(b), probably isoforms. Quantitative evaluation of the two PGM peaks in Figure 1(b) shows approximately equal relative values for protein contents and enzymic activities (Table 2), so that specific activities for both PGM peaks are almost identical.

Table 2 Comparison of PGM-containing fractions, peaks 1 and 2, from Figure 1(b)

PGM activity of peaks 1 and 2 (from Mono Q HR 5/5) was measured by determining the rate of Glc-6-*P* formation by using a coupled assay as described in the Materials and methods section. The reaction mixture used included 84 mM triethanolamine/HCl buffer, pH 7.6, 3.5 mM Glc-1-*P*, 0.02 mM Glc-1,6- P_2 , 0.19 mM NADP⁺, 0.47 unit/ml Glc-6-*P*-DH, 1.7 mM MgCl_2 and 5 μl of the *Paramecium* PGM samples, in a final volume of 1 ml.

Fractions from Mono Q HR 5/5 chromatography	Protein (%)	PGM activity (%)
PGM peak 1 (33–37)	48.1	51.2
PGM peak 2 (37–47)	51.9	48.8

Table 3 Influence of metal-chelating reagents and bivalent cations on *Paramecium* PGM activity

PGM activity of the 100 000 *g* supernatant was measured by determining the rate of Glc-6-*P* formation by using a coupled assay as described in the Materials and methods section. The reaction mixture used included 84 mM triethanolamine/HCl buffer, pH 7.6, 3.5 mM Glc-1-*P*, 0.02 mM Glc-1,6- P_2 , 0.19 mM NADP⁺, 0.47 unit/ml Glc-6-*P*-DH and 1.7 mM MgCl_2 . In some assays MgCl_2 was replaced by either 1.7 mM CaCl_2 or double-distilled water as indicated. Activity was assayed 10 min after addition of molecules to be analysed for their potential effect on PGM activity (see the Results section).

Reagent	Concentration (mM)	PGM activity (%)
Cysteine	5.1	100
GSH	5.1	98
Histidine	10.2	89
Imidazole	10.2	88
EDTA	0.9	96
Water + MgCl_2	1.7	100
Water + CaCl_2	1.0	62
Water		58

The activity of each of the two PGM-containing fractions from Figure 1(b) strongly decreased within a few hours, independent of the addition of Glc-1,6- P_2 , when they were tentatively subjected to Superose 12 HR 10/30 chromatography (results not shown). Any possible interference by proteases was analysed with Azocoll (results not shown), an insoluble protein-dye conjugate widely used for the assay of proteolytic enzymes [51,58]. Although we did not detect any protease activity, neither in the 100 000 *g* supernatant nor after its chromatography, we cannot exclude any proteolytic effects which would not be recognized by the Azocoll assay.

When we tried further chromatographic fractionation, i.e. by chromatofocusing (Mono P HR 5/5 Pharmacia) or by cation-exchange chromatography (Mono S HR 5/5, Pharmacia), this resulted either in complete loss of PGM activity, due to the essential polybuffer system or, as in the case of Mono S HR 5/5, only a few proteins, but no PGM, could be adsorbed in the presence of 50 mM sodium acetate buffer, pH 5.5. Therefore, to analyse enzymic characteristics of *Paramecium* PGM, we used PGM fractions obtained after Mono Q HR 5/5 chromatography, especially since in the presence of sufficient Glc-1,6- P_2 (10 nM) the activity remained stable for several weeks. By this separation procedure total PGM activity was enriched 19-fold and its yield was about 9% (Table 1).

Figure 2 (a) Labelling of P63 *in vivo*; (b) IEF autoradiogram from PP63 labelled *in vivo*

(a) Autoradiogram after SDS/PAGE of a 100 000 *g* supernatant (5 µg) after ^{32}P labelling of cells (0.4 mCi/5 ml of cell suspension) *in vivo*. Incubation with $[\gamma\text{-}^{32}\text{P}]\text{P}_i$ was as follows: lane 1, 2 min; lane 2, 4 min; lane 3, 8 min; lane 4, 16 min; lane 5, 32 min; lane 6, 90 min. Lanes 7 and 8 were as lane 6, but followed by rapid (2 s) AED-induced dephosphorylation of PP63 (arrowhead, lane 7) and subsequent rephosphorylation of P63 after 4 s (lane 8). Note the intense labelling of some other proteins in the 36–43 kDa and 12–20 kDa regions, which, however, are not sensitive to exocytosis stimulation, in contrast with a faint 31.5 kDa band. (b) IEF of 5 µg of protein after 90 min of incubation with ^{32}P (124 µCi/2 ml). S marks the starting point of the IEF gel electrophoresis, and arrows 1, 2 and 3 show the different isoforms of PP63, with pI values of 5.85, 5.95 and 6.05, respectively.

Characterization of PGM activity

Rabbit muscle PGM is active in the presence of metal-chelating reagents, including cysteine [53], histidine [59,60], imidazole [61–63], EDTA [64] or glutathione [65]. As shown in Table 3, activity of *Paramecium* PGM was not affected by these reagents, whereas the presence of MgCl_2 was essential. CaCl_2 could not substitute for MgCl_2 , as described also for mammalian PGM [66,67]. Data in Table 3 were obtained with the soluble fraction (100 000 *g* supernatant) of whole cell homogenates, but very similar results (not shown) were also obtained with the different PGM fractions after Mono Q HR 5/5 chromatography.

Phosphorylation properties of PP63 and PGM

Since several characteristics of PGM from most of the cell types investigated resemble those of PP63 from *Paramecium* (see the Introduction), we isolated and compared both the content of PGM activity and ^{32}P labelling of PP63 from the 100 000 *g* supernatant in order to establish their possible identity. We compared the phosphorylation pattern, *in vivo* and *in vitro*, of both PP63- and PGM-containing fractions (Figures 2–5). After freezing (to maintain phosphorylation states) and thawing in two

Figure 3 Phosphorylation of P63 by endogenous casein kinase *in vitro*

Lack of autophosphorylation of casein kinase was analysed in the absence (lane 1) or presence of MgCl_2 (lane 2) and in presence of $\text{MgCl}_2 + \text{CaCl}_2$ (lane 3). Lane 4 shows P63 included in the 100 000 *g* supernatant (obtained by phosphorylation *in vivo* and subsequent AED-triggered PP63 dephosphorylation) directly after heat treatment (84 °C, 5 min). PP63 phosphorylation was assayed in lanes 5 and 6, each containing heat-treated 100 000 *g* supernatant, *Paramecium* casein kinase, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, MgCl_2 , CaCl_2 and, for lane 6, additionally EGTA. The phosphorylation assays were performed in a volume of 80 µl with casein kinase (320 ng) and 100 000 *g* supernatant (6.35 µg). The concentrations used were 5 mM MgCl_2 , 100 µM CaCl_2 , 1 mM EGTA and 3 µCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After 20 min at 25 °C, 100 µl samples were subjected to SDS/PAGE (10% polyacrylamide gel), and the ^{32}P -labelled polypeptides were detected by autoradiography. Note phosphorylation of P63 (arrowhead) by casein kinase, with some additional phosphorylation bands at 93 kDa and 31.5 kDa.

cycles, PP63 could be recognized as a soluble phosphoprotein, in its phosphorylated, dephosphorylated or rephosphorylated form (Figure 2a). In detail, this was analysed in the following way. Some labelling *in vivo* already occurred after 4 min incubation with ^{32}P (lane 2), and maximal phosphorylation occurred after 30 min (lane 5), thus indicating a rapid turnover of phosphorylation. We finally used ^{32}P labelling for 90 min (lane 6) for further analysis. During synchronous exocytosis induction by AED, PP63 was totally dephosphorylated (lane 7) and 4 s later was already fully rephosphorylated (lane 8). Except for a 31.5 kDa phosphoprotein, other phosphoproteins in the range 36–43 kDa, as well as between 12 and 20 kDa, were not affected by AED triggering. In Figure 2(b) we confirmed previous data [1] showing that PP63 labelled *in vivo* exists in three isoforms, with pI values of 5.85, 5.95 and 6.05.

We have found that P63 is phosphorylated *in vitro* by a casein kinase (Figure 3) or a cGMP-dependent protein kinase (results not shown) which we isolated from *Paramecium*. The dephosphorylated state, P63, was obtained as indicated in the Materials and methods section. To ascertain that endogenous enzymes in the P63-containing fraction were not involved in the phosphorylation assay *in vitro*, the 100 000 *g* supernatant fraction was inactivated by heat (84 °C, 5 min). After this treatment, neither proteolytic digestion nor loss of the ^{32}P label was seen when phosphorylated or rephosphorylated PP63 was analysed by SDS/PAGE (results not shown).

Figure 4 Phosphorylation *in vitro* of PGM fraction from *Paramecium*

Isolation was performed as indicated in Figure 1, including final Mono Q HR 5/5 chromatography. (a) SDS/PAGE autoradiogram after phosphorylation *in vitro* by cGMP-dependent protein kinase. All phosphorylation assays (80 μ l) were performed under conditions in which isolated *Paramecium* cGMP-dependent protein kinase (465 ng) was highly stimulated ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$, cGMP, MgCl_2). The concentrations used in the assays were 0.804 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 5 mM MgCl_2 and 0.15 μM cGMP. Autophosphorylation assays of either cGMP-dependent protein kinase (lane 3) or PGM fraction with (lane 4) or without (lane 5) heat treatment were performed in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, cGMP and MgCl_2 . Under the same conditions PGM phosphorylation by cGMP-dependent protein kinase was assayed in lanes 1 and 2 containing non-denatured (lane 2) or heat-treated (lane 1) PGM fraction. After incubation, 15 μ l samples were subjected to SDS/PAGE (Serva HR SDS-Precote 10%-polyacrylamide gel) and then the ^{32}P -labelled polypeptides were revealed by autoradiography. Under conditions of autophosphorylation (20 min at 25 $^\circ\text{C}$), in the fraction containing cGMP-dependent protein kinase no P63 phosphorylation was observed (lane 3), in contrast with the PGM-containing fractions (lane 5). The autophosphorylated form of cGMP-dependent protein kinase is shown as a phosphoprotein of about 80 kDa (small arrowhead). Addition of cGMP-dependent protein kinase to the non-denatured PGM fraction (11.3 μg of protein) causes an increase in ^{32}P incorporation into a 63 kDa phosphoprotein (lane 2). No phosphorylation at 63 kDa (large arrowhead) was detectable in heat-treated PGM fractions, whether cGMP-dependent protein kinase was present (lane 1) or absent (lane 4). (b) IEF autoradiogram after phosphorylation *in vitro* by endogenous casein kinase. PGM-containing fraction (5 μg) was phosphorylated by casein kinase (320 ng) by incubation in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.8 μCi) and MgCl_2 (5 mM) for 20 min at 25 $^\circ\text{C}$. The meaning of S and arrows 1, 2 and 3 is as for Figure 2.

Figure 3 shows the phosphorylation of P63 *in vitro* by endogenous casein kinase. Casein kinase does not undergo autophosphorylation (Figure 3, lanes 1–3). It was also not contaminated by endogenous P63 when it was probed with 3 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence (lane 1) or presence of 5 mM MgCl_2 (lane 2) or of 5 mM MgCl_2 + 0.1 mM CaCl_2 (lane 3). When the 100000 *g* supernatant (containing some slightly phosphorylated P63; lane 4) was incubated with casein kinase under the same conditions as in lane 3, a strong phosphorylation of P63 was observed (lane 5). Since EGTA (1 mM) did not affect the phosphorylation of P63 (lane 6), we assume a Ca^{2+} -independent phosphorylation. The slightly increased phosphorylation of some other phosphoproteins, of 93 kDa and 31.5 kDa, still remains to be investigated. When we used isolated endogenous cGMP-dependent protein kinase, this was also able to phosphorylate P63 in the 100000 *g* supernatant (results not shown). IEF autoradiograms of PP63 samples phosphorylated *in vitro* either by casein kinase or by cGMP-dependent protein kinase revealed the same three isoforms for PP63, with pI values

Figure 5 Specification of polyclonal antisera against PGM as analysed by direct ELISA

Binding of the polyclonal chicken anti-rabbit PGM antiserum (H37) to PGM from rabbit skeletal muscle (0.5 μg of protein/well) (■). Controls were performed under identical assay conditions, but PGM antiserum was either replaced by preimmune serum (+) to H37 or omitted (■): application of rabbit anti-chicken IgG-POX only).

Table 4 Direct ELISA analysis of cross-reactivity of the *Paramecium* PGM-containing fraction with polyclonal chicken antiserum against PGM from rabbit skeletal muscle

A polyclonal antiserum H37 raised against PGM from rabbit skeletal muscle (diluted 1:100) was assayed in a direct ELISA test for immuno-binding to PGM from rabbit skeletal muscle (0.5 μg of protein/well) as well as to the *Paramecium* PGM fraction (Mono Q HR 5/5) (also 0.5 μg of protein/well). Controls were performed under identical assay conditions, with the exception of replacing the PGM antiserum either by the preimmune serum to H-37 (1:100) or by applying the second antibody (rabbit anti-chicken IgG-POX) only.

	PGM from rabbit skeletal muscle (A_{492}/A_{620})	PGM from <i>Paramecium</i> (A_{492}/A_{620})
Without first antibody	0.002	0.007
Preimmune serum	0.021	0.057
PGM antiserum H37	1.754	0.631

of 5.85, 5.95 and 6.05, as was shown in Figure 2(b) to occur after labelling *in vivo* (results not shown).

Similar tests were made with PGM fractions isolated from *Paramecium* by chromatography on Mono Q HR 5/5. A typical result is shown using cGMP-dependent protein kinase (Figure 4a) and casein kinase (Figure 4b). Under conditions in which isolated *Paramecium* cGMP-dependent protein kinase was highly stimulated ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$, MgCl_2 and cGMP), autophosphorylation of cGMP-dependent protein kinase occurred (Figure 4a, lane 3), as indicated by the presence of a phosphoprotein of about 80 kDa. This probably represents the autophosphorylated form of cGMP-dependent protein kinase, since in *Paramecium* this protein kinase has been shown to have a molecular mass in the same range [44]. Again, this kinase was not contaminated by PP63 (lane 3). In contrast, the PGM-enriched fraction seems to be contaminated by an endogenous protein kinase activity, since under the same autophosphorylation conditions a 63 kDa protein was partially phosphorylated (Figure 4a, lane 5). Any identity of this kinase

activity with cGMP-dependent protein kinase is unlikely, however, since no ^{32}P -labelled 80 kDa phosphoprotein was detectable. This activity might be due to a co-eluted casein kinase, since in *Paramecium* this protein kinase, on the one hand, does not undergo autophosphorylation (see above) and, on the other hand, does not require autophosphorylation for its enzyme activity (T. Treptau, R. Kissmehl, H. W. Hofer and H. Plattner, unpublished work). In assays using a heat-treated PGM fraction (Figure 4a, lanes 1 and 4) a ^{32}P -labelled 63 kDa protein was not detected, either in the presence (lane 1) or in the absence (lane 4) of *Paramecium* cGMP-dependent protein kinase. Evidently heat-denatured PGM cannot be phosphorylated by cGMP-dependent protein kinase. When the native PGM-enriched fraction was incubated with cGMP-dependent protein kinase, the most intense incorporation of ^{32}P into the 63 kDa protein was observed (Figure 4b, lane 2).

When we prepared gels of the PGM-enriched fraction after treatment by the endogenous casein kinase *in vitro*, we also obtained a 63 kDa phosphoprotein band (results not shown), and IEF autoradiograms revealed the same three isoforms, with pI values of 5.85, 5.95 and 6.05, as was shown for PP63 labelled *in vivo* (compare Figure 4b with Figure 2b). An analogous investigation with cGMP-dependent protein kinase showed the same three isoforms (results not shown). In conclusion, as far as analysed, phosphorylation is quite similar with PP63 and PGM, and the phosphorylation achieved is similar with endogenous casein kinase as well as with cGMP-dependent protein kinase.

Immuno-binding assays

These experiments were performed to analyse in more detail whether PP63 might be identical with PGM. We raised polyclonal antisera against commercially available PGM from rabbit skeletal muscle. Antibodies were produced in chicken. Figure 5 illustrates the specificity of polyclonal antisera in ELISA tests using the original antigen. The chicken antiserum H37 against PGM from rabbit skeletal muscle recognized the antigen used for immunization (Figure 5). After subtraction of the background value obtained with preimmune serum or with the second antibody only, the actual titre was determined to be in the range of 1:10000. ELISA tests were then performed to analyse cross-reactivity with the *Paramecium* PGM fraction. In Table 4 we probed the PGM-enriched fractions from *Paramecium* with chicken antiserum H37 against PGM from rabbit skeletal muscle. The positive control with original PGM from rabbit skeletal muscle exhibited strong immuno-binding to original PGM, but we also observed considerable cross-reactivity with *Paramecium* PGM. Specificity was again confirmed by control assays either using the preimmune serum or omitting the first antibody.

DISCUSSION

We have previously shown by quenched-flow that PP63 is rapidly (80 ms) and selectively dephosphorylated during AED-induced synchronous exocytosis [1] and rephosphorylated within seconds, a process confined to exocytosis-competent *Paramecium* strains [2]. Several findings suggested an involvement of a protein phosphatase/kinase system in *Paramecium*. (i) Exocytosis could be inhibited *in vivo* (by microinjection) as well as *in vitro* (using isolated cortex fragments) by polyclonal antibodies against the Ca^{2+} /calmodulin-dependent protein phosphatase 2B (calcineurin) from bovine brain [68]. Recently the involvement of a calcineurin-mediated dephosphorylation step in exocytosis has been ascertained also with exocrine pancreatic cells [69]. (ii) In *Paramecium* an activated Ca^{2+} -calmodulin-calcineurin complex

is able to trigger exocytosis *in vivo* and *in vitro* [68], i.e. with the use of isolated cell-surface complexes ('cortices'). These findings are supported by (iii) the identification of an endogenous protein phosphatase of this type and (iv) our observation that PP63 is a substrate for this phosphatase (R. Kissmehl, T. Treptau, H. W. Hofer and H. Plattner, unpublished work). (v) We now also show PP63 to be a substrate for casein kinase and for cGMP-dependent protein kinase. (vi) The latter finding is supported by a several-fold increase in cGMP concentration observed after stimulation of exocytosis [70], i.e. within the time where rephosphorylation takes place. Thus cGMP appears of possible relevance for rephosphorylation of P63 *in vivo* by endogenous cGMP-dependent protein kinase, which we now show to be active *in vitro*.

It also was reported that PP63 could be phosphoglycosylated *in vitro* by addition of Glc-1-P, catalysed by a Glc-phosphotransferase utilizing UDP-glucose, to O-linked mannose residues [5-7]. Such an acceptor protein, of 63 kDa, has also been shown to occur in mammalian tissues and in other cells [9-11, 71, 72].

The aim of the present study was to investigate whether there exists any relationship between this exocytosis-sensitive 63 kDa phosphoprotein in *P. tetraurelia*, (P)P63, and a protein of the same molecular mass, which has been identified in various other cell types as being identical with PGM [12-15]. However, the identity of PP63 with PGM in *Paramecium* has been recently denied [4, 7].

Biochemical implications

Since at the beginning of our work only little, if any, information was available on the presence of PGM in protozoa, we chose an isolation procedure described for mammalian PGM [53-55, 73, 74] and we used an established assay *in vitro* for the determination of PGM activity as generally used for mammalian cells [36]. Thus we were able to enrich most of the PGM activity from the soluble fraction. However, as the various purification schemes also exhibit differences not only in individual isolation steps but also in the choice of separation media [frequently involving precipitation by pH, heat or $(\text{NH}_4)_2\text{SO}_4$, followed by an anion- or cation-exchange chromatography, with a final molecular-sieve chromatography on Sephadex], this may partly explain some discrepancy in the specific enzyme activity of PGM in the published literature [32]. The existence of several isoforms with partly different enzymic characteristics would also contribute to the diversities reported [24, 55, 73, 74]. Therefore, any direct relationship of our enzymic data of *Paramecium* PGM activity to any of these mammalian isoforms is difficult to establish, and we had to check different conditions for *Paramecium* activity. Nevertheless, several characteristics remain for direct comparison. These include the requirement for Glc-1,6- P_2 and for MgCl_2 , as well as for appropriate buffer systems for the conservation of PGM activity. These conditions are quite similar to those reported for mammalian PGM from different sources [16, 32].

We show here that P63 can be phosphorylated *in vitro* by two endogenous protein kinases, casein kinase and cGMP-dependent protein kinase, as revealed by SDS/PAGE and by IEF. We also show that the same bands (63 kDa, three forms of pI 5.85, 5.95, 6.05) occur in three instances, i.e. not only (i) after phosphorylation *in vitro*, but also (ii) after phosphorylation *in vivo* and (iii) after enrichment of PGM activity. Furthermore (iv), we show by ELISA that PGM in our enriched fractions is recognized by anti-PGM antibodies. Therefore, we wondered whether in *P. tetraurelia* PP63 and PGM are identical.

Functional Implications

In a chromaffin PC12 cell line, as well as in rat cortical synaptosomes, a cytosolic form of PGM of 63 kDa was also postulated to be involved in stimulus–secretion coupling when the extent of its phosphorylation was compared with Ca^{2+} fluxes [13]. In response to stimulation, an increase in cytoplasmic free Ca^{2+} concentration was accompanied by an increase in radioactivity incorporated, i.e. in presence of $[\beta\text{-}^{35}\text{S}]\text{UDP-glucose}$. The label incorporated was assumed to reside in an oligosaccharide terminating in phosphodiester-linked glucose, by analogy with similar experiments with yeast [14]. In rat cortical synaptosomes, incorporation was maximal at 5 s after depolarization and returned to baseline within 25 s [13]. This will not directly reflect the situation in *Paramecium*, since here the exocytosis-sensitive phosphoprotein PP63 has been shown to lose phosphate within 80 ms upon stimulation, as analysed by quenched flow [1], and to regain its phosphate within seconds thereafter ([2]; the present study). An additional difference concerns the accessibility of P63 from *Paramecium* to protein kinases, as we have shown. Finally, PP63 is sensitive to alkaline treatment, but insensitive even to strong acid treatment [1], a characteristic of serine/threonine phosphorylation sites. In contrast, the product analysed in PC12 cells is sensitive even to mild acid treatment [13]. Nevertheless, the results of Veyna et al. [13] provide an example not only of reversible and rapidly regulated PGM but also of a possible involvement of PGM in stimulus–secreting coupling, although they analysed glycophosphorylation, whereas we analysed protein phosphorylation, and both might occur at the same time in PC12 cells.

The first evidence of an isoform of PGM to be a phosphoprotein phosphorylated by a Ca^{2+} /calmodulin-dependent protein kinase was reported by Lee et al. [36]. Such an enzyme could be purified and cloned as a CHAPS-soluble phosphoprotein of about 60 kDa from SR vesicles containing five putative phosphorylation sites for Ca^{2+} /calmodulin-dependent protein kinase. It was hypothesized that this phosphoprotein might be involved in the regulation of Ca^{2+} release, since the extent of its phosphorylation paralleled the inhibition of Ca^{2+} release from the SR [75,76]. The specific association not only of PGM, but also of other glycolytic and glycogenolytic enzymes, with SR membranes, as well as their participation in striated-muscle excitation–contraction coupling, has been demonstrated by various groups [77,78], as reviewed by Meissner [79]. This could involve several functional aspects. One function proposed was the glycolytic delivery of ATP to the Ca^{2+} -ATPase [80–82]. Another function proposed was the specific binding of $\text{Ins}(1,4,5)\text{P}_3$ to aldolase, although the participation of InsP_3 in the regulation of Ca^{2+} release from skeletal-muscle triads [83,84] is the subject of considerable debate [79]. Finally another glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, is also located in the gap between the terminal cisternae and the transverse tubules.

How does this compare to the situation in *Paramecium* cells? In *P. tetraurelia* the structural and functional organization of the sub-plasmalemmal Ca^{2+} stores, which presumably are involved in the regulation of exocytotic membrane fusion, closely resembles the situation in skeletal-muscle triads ([37,85]; S. Lange, N. Klauke and H. Plattner, unpublished work). Hence, there might be a functional relationship between PP63 and the phosphoprotein of about 60 kDa identified as an isoform of PGM in skeletal-muscle SR [36]. Although PP63 could be isolated as a cytosolic phosphoprotein, independently of its phosphorylation state, its localization is clearly cortical, according to cell fractionation and immunofluorescence labelling

[1,43]. This localization and the strict correlation of its dephosphorylation with exocytosis (see above) suggest a role in the regulation of this Ca^{2+} -dependent process. Most of the evidence emerging now for SR-associated glycolytic enzymes indicates non-glycolytic functions, as reviewed by Meissner [79]. PGM is non-ionically bound to SR by a N-terminal hydrophobic stretch [36], and SR binding decreases PGM activity [36,86]. This makes any involvement in glycolytic energy supply unlikely. More likely is a modulatory role in Ca^{2+} release, for the following reasons. As discussed above, phosphorylation of the 60 kDa SR protein, identified as a PGM isoform [36], inactivates Ca^{2+} release [87]. By analogy with SR, in *Paramecium* initial PP63/PGM dephosphorylation could activate Ca^{2+} release from sub-plasmalemmal stores, and rephosphorylation could abolish this process. This is as speculative as similar assumptions for SR, and therefore other functions remain open. Remarkably, non-glycolytic functions are also discussed for other glycolytic enzymes associated with SR, e.g. glyceraldehyde-3-phosphate dehydrogenase. Again, its association entails decreased enzymic activity [78], and functions proposed include a role in the assembly of SR–cell-membrane connections [88] or in stimulus–contraction coupling [89]. A similar role for PGM in Ca^{2+} regulation has recently been discussed, where it could be isolated in fully glycosylated or underglycosylated forms [15]. With several glycolytic enzymes, including PGM, the situation is, however, even more complicated. Several may also exert non-enzymic structural functions, as reviewed by Fothergill-Gilmore and Michels [90]. This is true for PGM in the zonula occludens [91], as well as in Z-discs and in non-junctional acetylcholine-receptor clusters [92]. Therefore, a role in the assembly of the exocytotic machinery also has to be envisaged. The actual function of PP63/PGM de- and re-phosphorylation in *Paramecium* therefore remains to be established.

Could PP63 be identical with PGM?

We showed in *P. tetraurelia* the existence of two PGM peaks (Figure 1b) with approximately equal specific activities (Table 2), where we also found PP63 to occur. In contrast, Andersen et al. [7] claim the occurrence of only one PGM form different from PP63 ('parafusin'). They also show partial sequence similarity of *Paramecium* 'parafusin' to mammalian PGM [4]. However, they conclude 'parafusin' to be different from PGM [4,7]. How can our view be reconciled with theirs?

(i) In any eukaryotic system analysed so far, from yeast [35,55,93], flowering plants [18,20], insects [94], fish [16] to mammals [24,27,28,54,73,74,95], at least two PGM isoforms occur, as we found with *Paramecium*. (ii) We found PGM in *Paramecium* to have some characteristics not previously tested (see the Results section). We also observed inactivation by Tris/HCl or by Polybuffer (chromatofocusing), in accordance with previous literature on yeast [35] and mammalian PGM [96]. However, during the isolation of 'parafusin' by DEAE-cellulose chromatography, gel filtration and chromatofocusing, either Tris/HCl or Polybuffer was used in the work with *Paramecium* mentioned [3,7]. (iii) 'Parafusin' is an acceptor for Glc-1-P transferred from UDP-glucose by Glc-phosphotransferase [5–7]. However, this is also true for PGM in yeast [14,15], PC12 cells and synaptosomes [13] and liver [12]. Lack of Glc-1-P incorporation into PGM of *Paramecium* [7] might be explained simply by the absence of Glc-phosphotransferase from the eluted fractions. Up to now this enzyme has not been isolated from *Paramecium*. (iv) Serine/threonine phosphorylation, as we showed for P63 in *Paramecium*, also occurs with rabbit skeletal-muscle PGM [36]. Several serine-phosphorylation sites were also

seen in the amino acid sequence deduced from the cDNA of 'parafusin' [4]. (v) Antibodies against 'parafusin'-derived peptides showed no immunoreactivity with the PGM fraction isolated from *Paramecium* [7]. However, surprisingly, blots were prepared from elution fractions far from the PGM activity peak. (vi) The sequence similarity between 'parafusin' from *Paramecium* and mammalian PGM, shown by Subramanian et al. [4], is high, just intermediate between bacterial and mammalian PGM [23]. Similarity would even appear much more pronounced if one would take into account the numerous conservative amino acid exchanges.

Conclusions

The evidence that 'parafusin' is not a form of PGM is inconclusive, on the basis of our own data and in relationship to the literature currently available on PGM. Considerable sequence similarity between 'parafusin' from *Paramecium* and mammalian PGM forms [4] may underscore their possible identity, rather than diversity. Further studies on the biochemical and molecular characteristics of PP63 in *P. tetraurelia* may therefore provide insight to the following aspects. (i) Is PGM (which we assume to be equivalent to PP63) phosphorylated in different ways *in vivo*, i.e. by glycophosphorylation and/or by serine phosphorylation at different sites? (ii) How do these modifications affect enzyme activity? (iii) Is enzymic activity and/or phosphorylation of PGM relevant for exocytosis regulation? (iv) In which step of exocytosis regulation might it be involved?

We thank Dr. Massoud Momayezi for raising the polyclonal antisera. Michael Knochel for assistance in the ELISA experiments, and Edda Dassler and Doris Bliestle for photographic work. This study was supported by grant SFB156/B4 from the Deutsche Forschungsgemeinschaft.

REFERENCES

- Höhne-Zell, B., Knoll, G., Riedel-Gras, U., Hofer, W. and Plattner, H. (1992) *Biochem. J.* **286**, 843–849
- Zieseniss, E. and Plattner, H. (1985) *J. Cell Biol.* **101**, 2028–2035
- Murtaugh, T. J., Gilligan, D. M. and Satir, B. H. (1987) *J. Biol. Chem.* **262**, 15734–15739
- Subramanian, S. V., Wyroba, E., Andersen, A. P. and Satir, B. H. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9832–9836
- Satir, B. H., Srisomsap, C., Reichman, M. and Marchase, R. B. (1990) *J. Cell Biol.* **111**, 901–907
- Subramanian, S. V. and Satir, B. H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11297–11301
- Andersen, A. P., Wyroba, E., Reichman, M., Zhao, H. and Satir, B. H. (1994) *Biochem. Biophys. Res. Commun.* **200**, 1353–1358
- Satir, B. H., Hamasaki, T., Reichman, M. and Murtaugh, T. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 930–932
- Srisomsap, C., Richardson, K. L., Jay, J. C. and Marchase, R. B. (1988) *J. Biol. Chem.* **263**, 17792–17797
- Srisomsap, C., Richardson, K. L., Jay, J. C. and Marchase, R. B. (1989) *J. Biol. Chem.* **264**, 20540–20546
- Marchase, R. B., Richardson, K. L., Srisomsap, C., Drake, R. R. and Haley, B. E. (1990) *Arch. Biochem. Biophys.* **280**, 122–129
- Auger, D., Bounelis, P. and Marchase, R. B. (1993) in *Molecular Mechanisms of Membrane Traffic* (Morré, D. J., Bergeron, J. J. M. and Howell, K. M., eds.), pp. 289–292, Springer-Verlag, New York
- Veyna, N. A., Jay, J. C., Srisomsap, C., Bounelis, P. and Marchase, R. B. (1994) *J. Neurochem.* **62**, 456–464
- Marchase, R. B., Bounelis, P., Brumley, L. M., Dey, N., Browne, B., Auger, D., Fritz, T. A., Kulesza, P. and Bedwell, D. M. (1993) *J. Biol. Chem.* **268**, 8341–8349
- Dey, N. B., Bounelis, P., Fritz, T. A., Bedwell, D. M. and Marchase, R. B. (1994) *J. Biol. Chem.* **269**, 27143–27148
- Hashimoto, T. and Handler, P. (1966) *J. Biol. Chem.* **241**, 3940–3948
- Hirose, M., Sugimoto, E., Sasaki, R. and Chiba, H. (1970) *J. Biochem. (Tokyo)* **68**, 449–457
- Takamiya, S. and Fukui, T. (1978) *Plant Cell Physiol.* **19**, 759–768
- Salvucci, M. E., Drake, R. R., Broadbent, K. P., Haley, B. E., Hanson, K. R. and McHale, N. A. (1990) *Plant Physiol.* **93**, 105–109
- Pan, D., Strelow, L. I. and Nelson, O. E. (1990) *Plant Physiol.* **93**, 1650–1653
- Joshi, J. G. and Handler, P. (1964) *J. Biol. Chem.* **239**, 2741–2751
- Hanabusa, K., Dougherty, H. W., Del Río, C., Hashimoto, T. and Handler, P. (1966) *J. Biol. Chem.* **241**, 3930–3939
- Zhou, D., Stephens, D. S., Gibson, B. W., Engstrom, J. J., McAllister, C. F., Lee, F. K. N. and Apicella, M. A. (1994) *J. Biol. Chem.* **269**, 11162–11169
- Dawson, D. M. and Mitchell, A. (1969) *Biochemistry* **8**, 609–614
- Harris, H. and Hopkinson, D. A. (1976) *Handbook of Enzyme Electrophoresis in Human Genetics*, North-Holland, Amsterdam
- March, R. E., Putt, W., Hollyoake, M., Ives, J. H., Lovegrove, J. U., Hopkinson, D. A., Edwards, Y. H. and Whitehouse, D. B. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10730–10733
- Hopkinson, D. A. and Harris, H. (1971) *Annu. Rev. Genet.* **5**, 5–32
- Fisher, R. A. and Harris, H. (1972) *Ann. Hum. Genet.* **36**, 69–77
- Ray, W. J., Jr., Hermanson, M. A., Puvathingal, J. M. and Mahoney, W. C. (1983) *J. Biol. Chem.* **258**, 9166–9174
- Whitehouse, D. B., Putt, W., Lovegrove, J. U., Morrison, K., Hollyoake, M., Fox, M. F., Hopkinson, D. A. and Edwards, Y. H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 411–415
- Rivera, A. A., Elton, T. S., Dey, N. B., Bounelis, P. and Marchase, R. B. (1993) *Gene* **133**, 261–266
- Ray, W. J., Jr. and Peck, E. J., Jr. (1972) *Enzymes 3rd Ed.* **6**, 407–477
- Dai, J.-B., Liu, Y., Ray, W. J., Jr. and Konno, M. (1992) *J. Biol. Chem.* **267**, 6322–6337
- Deng, H., Ray, W. J., Jr., Burgner, J. W. and Callender, R. (1993) *Biochemistry* **32**, 12984–12992
- Joshi, J. G. (1982) *Methods Enzymol.* **89**, 599–605
- Lee, Y. S., Marks, A. R., Gureckas, N., Lacro, R., Nadal-Ginard, B. and Kim, D. H. (1992) *J. Biol. Chem.* **267**, 21080–21088
- Knoll, G., Grässle, A., Braun, C., Probst, W., Höhne-Zell, B. and Plattner, H. (1993) *Cell Calcium* **14**, 173–183
- Erxleben, C. and Plattner, H. (1994) *J. Cell Biol.* **127**, 935–945
- Kaneshiro, E. S., Beischel, L. S., Merkel, S. J. and Rhoads, D. E. (1979) *J. Protozool.* **26**, 147–158
- Glas-Albrecht, R. and Plattner, H. (1990) *Eur. J. Cell Biol.* **53**, 164–172
- Plattner, H., Stürzl, R. and Matt, H. (1985) *Eur. J. Cell Biol.* **36**, 32–37
- Pollack, S. (1974) *J. Protozool.* **21**, 352–362
- Stecher, B., Höhne, B., Gras, U., Momayezi, M., Glas-Albrecht, R. and Plattner, H. (1987) *FEBS Lett.* **223**, 25–32
- Miglietta, L. A. P. and Nelson, D. L. (1988) *J. Biol. Chem.* **263**, 16096–16105
- Walczak, C. E., Anderson, R. A. and Nelson, D. L. (1993) *Biochem. J.* **296**, 729–735
- Thalhofer, H. P., Daum, G., Harris, B. G. and Hofer, H. W. (1988) *J. Biol. Chem.* **263**, 952–957
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Heukeshoven, J. and Dernick, R. (1986) in *Elektrophorese Forum '86* (Radola, B. J., ed.), pp. 22–27, Technische Universität München, Freising-Weihenstephan
- Winston, V. (1989) *Electrophoresis* **10**, 220–222
- Chavira, R., Jr., Burnett, T. J. and Hageman, J. H. (1984) *Anal. Biochem.* **136**, 446–450
- Momayezi, M., Kersken, H., Gras, U., Vilmart-Seuwen, J. and Plattner, H. (1986) *J. Histochem. Cytochem.* **34**, 1621–1638
- Najjar, V. A. (1955) *Methods Enzymol.* **1**, 294–299
- Yankeelov, J. A., Jr., Horton, H. R. and Koshland, D. E., Jr. (1964) *Biochemistry* **3**, 349–355
- Joshi, J. G., Hooper, J., Kuwaki, T., Sakurada, T., Swanson, J. R. and Handler, P. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **57**, 1482–1489
- Brautigan, D. L. (1989) in *Receptor Phosphorylation* (Moudgil, V. K., ed.), pp. 89–111, CRC Press, Boca Raton, FL
- Takai, A. and Mieskes, G. (1991) *Biochem. J.* **275**, 233–239
- Cheng, Y.-S. E. and Aronson, A. I. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1254–1258
- Sutherland, E. W. (1949) *J. Biol. Chem.* **180**, 1279–1284
- Bodansky, O. (1958) *J. Biol. Chem.* **232**, 859–866
- Robinson, J. P. and Najjar, V. A. (1960) *Biochem. Biophys. Res. Commun.* **3**, 62–66
- Harshman, S., Robinson, J. P., Bocchini, V. and Najjar, V. A. (1965) *Biochemistry* **4**, 396–400
- Robinson, J. P., Harshman, S. and Najjar, V. A. (1965) *Biochemistry* **4**, 401–405
- Brown, D. H. (1953) *J. Biol. Chem.* **204**, 877–889
- Lehmann, H. (1939) *Biochem. J.* **33**, 1241–1244
- Ray, W. J., Jr. (1969) *J. Biol. Chem.* **244**, 3740–3747
- Peck, E. J., Jr. and Ray, W. J., Jr. (1969) *J. Biol. Chem.* **244**, 3754–3759

- 68 Momayezi, M., Lumpert, C. J., Kersken, H., Gras, U., Plattner, H., Krinks, M. H. and Klee, C. B. (1987) *J. Cell Biol.* **105**, 181–189
- 69 Groblewski, G. E., Wagner, A. C. C. and Williams, J. A. (1994) *J. Biol. Chem.* **269**, 15111–15117
- 70 Knoll, G., Kerboeuf, D. and Plattner, H. (1992) *FEBS Lett.* **304**, 265–268
- 71 Hiller, A. M., Koro, L. A. and Marchase, R. B. (1987) *J. Biol. Chem.* **262**, 4377–4381
- 72 Marchase, R. B., Saunders, A. M., Rivera, A. A. and Cook, J. M. (1987) *Biochim. Biophys. Acta* **916**, 157–162
- 73 Fazi, A., Piacentini, M. P., Piatti, E. and Accorsi, A. (1990) *Prep. Biochem.* **20**, 219–240
- 74 Piatti, E., Accorsi, A., Piacentini, M. P. and Fazi, A. (1990) *Biochem. Int.* **21**, 155–163
- 75 Campbell, K. P. and MacLennan, D. H. (1982) *J. Biol. Chem.* **257**, 1238–1246
- 76 Kim, D. H. and Ikemoto, N. (1986) *J. Biol. Chem.* **261**, 11674–11679
- 77 Pierce, G. N. and Philipson, K. D. (1985) *J. Biol. Chem.* **260**, 6862–6870
- 78 Caswell, A. H. and Corbett, A. M. (1985) *J. Biol. Chem.* **260**, 6892–6898
- 79 Meissner, G. (1994) *Annu. Rev. Physiol.* **56**, 485–508
- 80 Entman, M. L., Kaniike, K., Goldstein, M. A., Nelson, T. E., Bornet, E. P., Futch, T. W. and Schwartz, A. (1976) *J. Biol. Chem.* **251**, 3140–3146
- 81 Entman, M. L., Keslensky, S. S., Chu, A. and Van Winkle, W. B. (1980) *J. Biol. Chem.* **255**, 6245–6252
- 82 Cuenda, A., Nogues, M., Gutiérrez-Merino, C. and De Meis, L. (1993) *Biochem. Biophys. Res. Commun.* **196**, 1127–1132
- 83 Thieleczek, R., Mayr, G. W. and Brandt, N. R. (1989) *J. Biol. Chem.* **264**, 7349–7356
- 84 Heilmeyer, L. M. G., Jr., Han, J.-W., Thieleczek, R., Varsanyi, M. and Mayr, G. W. (1990) *Mol. Cell. Biochem.* **99**, 111–116
- 85 Plattner, H., Lumpert, C. J., Knoll, G., Kissmehl, R., Höhne, B., Momayezi, M. and Glas-Albrecht, R. (1991) *Eur. J. Cell Biol.* **55**, 3–16
- 86 Putt, W., Ives, J. H., Hollyoake, M., Hopkinson, D. A., Whitehouse, D. B. and Edwards, Y. H. (1993) *Biochem. J.* **296**, 417–422
- 87 Leddy, J. J., Murphy, B. J., Qu-Yi, Doucet, J.-P., Pratt, C. and Tuana, B. S. (1993) *Biochem. J.* **295**, 849–856
- 88 Corbett, A. M., Caswell, A. H., Brandt, N. R. and Brunschwig, J. P. (1985) *J. Membr. Biol.* **86**, 267–276
- 89 Han, J.-W., Thieleczek, R., Varsányi, M. and Heilmeyer, L. M. G., Jr. (1992) *Biochemistry* **31**, 377–384
- 90 Fothergill-Gilmore, L. A. and Michels, P. A. M. (1993) *Prog. Biophys. Mol. Biol.* **59**, 105–235
- 91 Belkin, A. M., Klimanskaya, I. V., Lukashev, M. E., Lilley, K., Critchley, D. R. and Kotliansky, V. E. (1994) *J. Cell Sci.* **107**, 159–173
- 92 Belkin, A. M. and Burridge, K. (1994) *J. Cell Sci.* **107**, 1993–2003
- 93 Oh, D. and Hopper, J. E. (1990) *Mol. Cell Biol.* **10**, 1415–1422
- 94 Cicchetti, R., Argentin, G. and Nicoletti, B. (1990) *Biochem. Genet.* **28**, 247–255
- 95 Accorsi, A., Piatti, E., Piacentini, M. P., Gini, S. and Fazi, A. (1989) *Prep. Biochem.* **19**, 251–271
- 96 Tedokon M., Suzuki, K., Kayamori, Y., Fujita, S. and Katayama, Y. (1992) *Clin. Chem.* **38**, 512–515