In Vivo Analysis of the Major Exocytosis-sensitive Phosphoprotein in *Tetrahymena*

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Abstract. Phosphoglucomutase (PGM) is a ubiquitous highly conserved enzyme involved in carbohydrate metabolism. A number of recently discovered PGM-like proteins in a variety of organisms have been proposed to function in processes other than metabolism. In addition, sequence analysis suggests that several of these may lack PGM enzymatic activity. The best studied PGM-like protein is parafusin, a major phosphoprotein in the ciliate *Paramecium tetraurelia* that undergoes rapid and massive dephosphorylation when cells undergo synchronous exocytosis of their dense-core secretory granules. Indirect genetic and biochemical evidence also supports a role in regulated exocytotic membrane fusion. To examine this matter directly, we have identified and cloned the parafusin homologue in *Tetrahymena thermophila*, a ciliate in which protein function can be studied in vivo. The unique *T. thermophila* gene, called *PGM1*, encodes a protein that is closely related to parafusin by sequence and by characteristic post-translational modifications. Comparison of deduced protein sequences, taking advantage of the known atomic structure of rabbit muscle PGM, suggests that both ciliate enzymes and all other PGM-like proteins have PGM activity. We evaluated the activity and function of *PGM1* through gene disruption. Surprisingly, ΔPGM1 cells displayed no detectable defect in exocytosis, but showed a dramatic decrease in PGM activity. Both our results, and reinterpretation of previous data, suggest that any potential role for PGM-like proteins in regulated exocytosis is unlikely to precede membrane fusion.

THE functions of proteins that are secreted via regulated exocytosis are as wide-ranging as tissue coordination by hormones in metazoans and defense from predators in ciliated protists (Harumoto and Miyake, 1991). In contrast, the cytosolic machinery involved in vesicle targeting and membrane fusion appears to be highly conserved throughout eukaryotes (Bennett and Scheller, 1993). The mechanism of exocytosis must involve a set of proteins that can respond rapidly to changes in cytosolic signals, frequently involving a rise in the concentration of cytosolic Ca^{2+} (Calakos and Scheller, 1996). The activity of some of these proteins may be modulated by reversible modification. In particular, phosphorylation or dephosphorylation-linked regulation of exocytosis has been suggested in disparate systems, though in many cases the modified proteins have not been identified (Sudhof and Jahn, 1991; Calakos and Scheller, 1996).

In *Paramecium tetraurelia*, microinjection of protein phosphatases, including calcineurin, triggers exocytosis of dense-core secretory granules in vivo. This also occurs in a cell-free system. Furthermore, phosphatase inhibitors and antibodies to calcineurin inhibit exocytosis (Momayezi et al., 1987). A potential target for dephosphorylation was identified as parafusin (a.k.a. PP63, PP65) (Subramanian et al., 1994). Parafusin is a major phosphoprotein in resting *Paramecium* cells and is dramatically dephosphorylated when cells are stimulated with secretagogues (Gilligan and Satir, 1982). Because exocytosis is rapid and synchronous in this organism, stop-flow methods could be used to determine that dephosphorylation occurs within 80 ms of stimulation, a similar time-scale to that of membrane fusion (Höhne-Zell et al., 1992). After exocytosis, rephosphorylation begins within 5 s, and the protein returns to its resting state in ~20 s (Zieseniss and Plattner, 1985). Dephosphorylation was only observed in *Paramecium* capable of exocytosis; it was blocked by exocytosis-inhibiting drugs, and was not seen in exocytosis-deficient mutants (Gilligan and Satir, 1982; Zieseniss and Plattner, 1985). In addition, microinjection of antibodies to parafusin inhibited both exocytosis and dephosphorylation in vivo (Stecher et al., 1987).

Parafusin is phosphorylated by several mechanisms in vitro, but it is not clear which are physiologically relevant. The most unusual is the attachment of phosphoglucose to the

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protein by the enzyme UDP-Glc:glycoprotein Glc-1-phosphotransferase (glucose phosphotransferase)[1]. Parafusin is the only detectable protein acceptor for this enzyme in vitro (Satir et al., 1990). More conventional phosphorylation by kinases has also been demonstrated, but no functional role has yet been established for any posttranslational modifications of parafusin (Kissmehl et al., 1996).

Cloning of parafusin (Subramanian et al., 1994) revealed that it is related to PGM (EC 2.7.5.1). This ubiquitous enzyme catalyzes the interconversion of glucose-1-phosphate and glucose-6-phosphate, an important step in glycogen metabolism, trehalose synthesis, galactose metabolism, and glycoprotein synthesis (reviewed in Boles et al., 1994). Although the enzyme has no known role in signal transduction or secretion, a related protein in PC12 cells and rat synaptosomes was shown to undergo posttranslational modification concurrent with exocytosis (Veyna et al., 1994). It has been argued that parafusin does not have PGM activity (Andersen et al., 1994; Subramanian et al., 1994). However, this claim has recently been cast into doubt by the demonstration of PGM activity in transgenically expressed and purified parafusin (Hauser et al., 1997). This activity may explain at least one source of phosphorylation in vivo, since PGM in other species is phosphorylated on its active site serine.

Another disputed issue has been the localization of parafusin. Proteins that are involved in rapid exocytotic responses are expected to be localized near the site of fusion at the plasma membrane. Immunolocalization of parafusin has been complicated by uncertainty over whether the protein was indeed PGM, and potential interference by antigenically related proteins (Murtaugh et al., 1987; Stecher et al., 1987; Satir et al., 1989; Höhne-Zell et al., 1992; Subramanian and Satir, 1992; Wyroba et al., 1995; Hauser et al., 1997). Indeed, recent cloning results indicate that at least two closely related isoforms are present in *Paramecium* (Hauser et al., 1997).

Although a number of issues remain unresolved, parafusin is the best-studied of a group of PGM-like proteins that have been proposed, based on their localization or reversible posttranslational modification, to play roles that are unrelated to the namesake enzymatic activity of PGM. Adaptation of abundant metabolic enzymes for other specific functions is well known, for example in the case of the lens crystallins (Wistow et al., 1987). To address these issues for a PGM-related protein, we have turned to another ciliate, *Tetrahymena thermophila*. Like *Paramecium*, *Tetrahymena* is capable of synchronous fusion of its dense-core secretory granules. A major experimental advantage in *T. thermophila* is that techniques for gene disruption are well-developed, so that the in vivo role of identified proteins can be evaluated. Importantly, regulated exocytosis is nonessential for laboratory growth, so any mutants disrupted in this pathway, as might be expected in the case of parafusin mutations, are expected to remain viable. Disruption analysis is facilitated by the fact that *Tetrahymena* appears frequently to have maintained genes as single micronuclear copies, where *Paramecium* has duplicated copies or gene families. We here describe the identification and functional analysis of a parafusin homologue in *Tetrahymena thermophila*.

1. *Abbreviations used in this paper*: PGM, phosphoglucomutase; Glc, glucose; glucose phosphotransferase, UDP-Glc:glycoprotein Glc-1-phosphotransferase.

Materials and Methods

Restriction enzymes were from New England Biolabs (Beverly, MA). All other reagents were from Sigma Chem. Co. (St. Louis, MO), unless otherwise noted.

Cells and Cell Culture

Cells were grown at 30°C with agitation in 1% proteose peptone, 0.1% yeast extract (both from Difco, Detroit, MI), 2% glucose, and 0.003% ferric EDTA. *Tetrahymena thermophila* strains derived from inbred strain B were used in this work, and are listed in Table I. Strain B2086 is considered to be wild-type, and all initial strains are wild-type with respect to regulated exocytosis. Strains CU428.1 and B2086 were obtained from Peter Bruns (Cornell University, Ithaca, NY).

Cloning of PGM1 cDNA and Genomic DNA

Based on known codon usage in *Tetrahymena thermophila* (Martindale, 1989), degenerate primers (Integrated DNA Technologies, Coralville, IA) were designed to amplify parafusin homologues via the polymerase chain reaction. Primers were designed to correspond to regions conserved between *GAL5* (*Saccharomyces cerevisiae*), *PGM1* (*Homo sapiens*), and *parafusin* (*P. tetraurelia*). A total of four forward and four reverse primers were designed and used in all 16 possible combinations. The primers correspond to residues 126-133, 142-149, 321-327, 339-344, 388-384, 369-362, 410-403, and 427-421 of the deduced Pgm1p protein sequence (see Fig. 4). PCR was performed on a *Tetrahymena thermophila* λgt10 cDNA library generously provided by Tohru Takemasa (University of Tsukuba, Japan), or on *Tetrahymena thermophila* strain CU428.1 genomic DNA. The 50-μl reactions contained 10^8 plaque-forming units or 10 ng genomic DNA, 2.5 U Taq polymerase (Promega Corp., Madison, WI), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM each dNTP, and 1 μM each primer. 35 cycles of 92°C, 0.5 min; 40, 45, 50, or 55°C, 1 min; 72°C, 0.5 min, were performed. A total of 12 bands of the expected sizes were cloned into pCRII (Invitrogen, San Diego, CA) and sequenced; 5 products were unrelated to PGM, the other 6 (3 amplified from genomic DNA, 3 from cDNA, using 6 of the 8 primers in all) were related to various PGMs. These were determined to be identical in overlapping regions and derived from a single gene.

The largest PCR product, 1,265 bp (corresponding to bases 559-1824 of Fig. 2) was used to screen a cDNA phage library using the Genius system (Boehringer Mannheim, Indianapolis, IN), which resulted in the isolation of a single clone containing a 1.7-kB insert. After subcloning into pBluescript SK (+) (Stratagene, La Jolla, CA), sequencing revealed that the 5′ end was truncated. On the assumption that some full-length clones were present in the library, we conducted PCR on the cDNA library using a λgt10 primer and a primer complementary to the portion of the gene we had already sequenced. Cloning and sequencing of the resultant 900-bp product revealed that it contained a putative start codon.

Genomic sequences 5′ of the cDNA were obtained by inverse PCR (Ochman et al., 1988) on genomic DNA digested with EcoRI. The forward primer corresponded to bases 306-275, and the reverse primer to bases 1250-1273 of Fig. 2. PCR was performed on 200 ng of circularized genomic DNA using the same reagents and cycling times as above, but with a 1.5-min extension step. These reactions produced a single product which was the expected size (3 kB) based on Southern blotting. This was cloned into pCRII and partially sequenced. The *PGM1* sequence reported represents the consensus obtained from at least three independent templates, each sequenced on both strands.

Table I. Strains Used in This Work

Strain	Genotype
B2086	(II)
CU428.1	*mpr1-1/mpr1-1* (mp-s, VII)
ΔPGM1	*MPR1/mpr1-1*; *PGM1/PGM1* (*pgm1-1::neo2*; pm-r, mp-r)

Southern and Northern Blotting

Probe templates were prepared by PCR amplification of cloned sequences followed by purification on QIAprep columns (Qiagen, Chatsworth, CA). Probes labeled with either digoxigenin (Boehringer Mannheim; for Southern analysis) or ^{32}P (DuPont NEN, Wilmington, DE; for Northern analysis) were then prepared by randomly primed DNA synthesis.

Genomic DNA was prepared as described (Gaertig et al., 1993) except that after isopropanol precipitation, DNA was further purified by dissolving in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 μg/ml RNaseA for 10 min at 65°C, and then separated from contaminants on a Q-100 column (Qiagen, Chatsworth, CA) according to the manufacturer's directions. DNA was quantified by spectroscopic analysis (A_{260}/A_{280} ratios were typically 1.75–1.85). Southern blotting was according to manufacturer's instructions (Genius, Boehringer Mannheim) loading 20 μg of DNA per lane. For low-stringency blots, hybridization was conducted in 50% formamide, 5× SSC, 2.0% DIG blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, 0.2% sodium dodecyl sulfate, at 20°C for 16 h. This was followed by four 15-min washes in 2× SSC containing 0.1% SDS, also at 20°C. RNA purification and Northern blotting were performed as previously described (Chilcoat et al., 1996).

Disruption of PGM1

PGM1 was disrupted by replacement of the genomic sequences −76 to +2182 relative to the start codon (resulting in removal of the first 486 residues) with a construct containing the *neo2* resistance cassette (Chilcoat et al., 1996; Gaertig et al., 1994), which confers paromomycin resistance in *Tetrahymena*. Electroporation (ECM 600; BTX Corporation, San Diego, CA) was used to transform mating pairs of B2086 and CU428.1, as previously described (Gaertig et al., 1995). Initial drug selection was with 120 μg/ml paromomycin, added 6 h after transformation. 3–6 transformants were obtained per transformation (n = 3). Because only some of the macronuclear *PGM1* copies were replaced initially, cells were grown in increasing concentrations of paromomycin for stringent selection of the *pgm1-1::neo2* allele. Several transformants were cloned and grown in paromomycin (up to 4 mg/ml) for 400 generations.

Enzyme Assay

To measure PGM activity, 500-ml cultures were grown to 1–2 × 10^5 cells/ml, then rapidly chilled and pelleted at 200 *g* for 5 min in conical bottles. All subsequent steps were performed cold. Cells were washed once in 10 mM Hepes-KOH, pH 6.9, and the pellet volume measured. The pellet was then washed and suspended in 2 vol of Buffer A (0.3 M sucrose, 10 mM Hepes-KOH, pH 6.9, 36 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA; Fluka, Ronkonkoma, NY) with the protease inhibitors leupeptin (0.5 μg/ml; Boehringer Mannheim), antipain (12.5 μg/ml), E-64 (10 μg/ml), and chymostatin (10 μg/ml). The resuspended pellet was homogenized by passage through a ball-bearing cell cracker (Hans Issel, Palo Alto, CA) with a nominal clearance of 0.0005 in. In some cases this crude lysate was fractionated by centrifuging in a Beckman Optima TLX ultracentrifuge with a TLA120.2 rotor at 890 *g* for 10 min (to generate S1 and P1 fractions), 16,000 *g* for 15 min (S2 and P2) and 300,000 *g* for 15 min (S3 and P3). Pellets were washed and suspended in the starting volume of Buffer A. *Paramecium tetraurelia* was grown as a monoxenic culture, and the bacteria were depleted by a brief period of cell starvation before cell homogenization (Sonneborn, 1970). *Paramecium* homogenates were prepared identically to those of *Tetrahymena,* and *S. cerevisiae* (strain GPY60) lysates were prepared as described (Baker et al., 1990).

PGM activity was determined using an assay based on (Joshi, 1982). 200-μl reaction volumes contained a final concentration of 2 mM Glc-1-P, 7.9 nM Glc-1-6-biP, 0.5 mM NADP, 5 mM $MgSO_4$, 40 mM imidazole-HCl, pH 7.4, and 0.2 U Glc-6-*P* dehydrogenase. Ribose-5-phosphate was added to some reactions to a final concentration of 2 mM. After all reaction components were assembled they were incubated at 4°C for 5 min to ensure complete activation of PGM. Samples were then warmed to 30°C; after a 2-min equilibration the absorbance at 340 nm was recorded every 10 s for 12 min on a microplate reader (Bio-Tek 340, Highland Park, VT). This resulted in linear plots of absorbance vs. time for all measurements used. This assay was found to be linear for solutions of 4–400 mU/ml PGM. Each assay was calibrated with purified rabbit PGM equilibrated at 4°C for 30–120 min in 40 mM imidazole, pH 7.4, 2 mg/ml BSA (Calbiochem-Novabiochem, La Jolla, CA). Protein concentrations were determined using bicinchoninic acid (Pierce, Rockford, IL).

In Vivo Labeling of PGM1

An overnight culture of cells was pelleted for 5 min at 200 *g*, then washed and suspended in Buffer H (5 mM Hepes-KOH, pH 7.5, 0.65 mM $CaCl_2$, 0.1 mM $MgCl_2$) for overnight starvation at 30°C without shaking. Starved cells are optimal for labeling and secretion. Cells were concentrated by centrifugation to a final concentration of 2–9 × 10^6 cells/ml and labeled by addition of 1/9 vol [^{32}P]orthophosphoric acid (DuPont-NEN, 8500-9120 Ci/mmol), to a final concentration of 1 mCi/ml, and incubated at room temperature for 45 min. To induce secretion, 1/5 vol of saturated picric acid was added, followed within ~2 s by the addition of 2 vol of 95°C SDS-PAGE sample buffer (190 mM Tris-HCl, pH 6.95, 15% glycerol, 6% SDS, 15 mM EDTA, 0.002% bromophenol blue). Samples were heated to 95°C for 10 min, separated by SDS-PAGE, and electrophoretically transferred to nitrocellulose. Membranes were then washed (3 × 1 min) with 1× TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl), dried, and exposed to film (Kodak XOMAT AR) for 12–60 h or to PhosphorImager screens (Molecular Dynamics, La Jolla, CA) overnight. After exposure, membranes were stained for total protein using Ponceau S. All experiments were performed at least three times, with similar results.

In Vitro Glucose Phosphotransferase Assay

Cells were grown to 3–6 × 10^5/ml, pelleted for 5 min at 200 *g*, washed and suspended in Buffer B (50 mM Bis-Tris-HCl, pH 6.8, 105 mM NaCl, 5 mM $CaCl_2$, and 1 mM NaH_2PO_4), and homogenized as for PGM assays. 25 μg of this lysate was incubated in Buffer B with 2.8 μCi [β-^{35}S]UDP-Glc (2.8 mCi/ml, 345 Ci/mmol; DuPont NEN, Wilmington, DE) and 0 or 200 μM glucose-1-phosphate, plus 0 or 200 μM UDP-glucose in a total volume of 50 μl for 30 min at room temperature (Marchase et al., 1993). 2 vol of 95°C SDS-PAGE sample buffer were added, and samples were visualized as described above.

Exocytosis Assays

Cells were grown to 3–6 × 10^5/ml, washed and suspended in DMC (0.2 mM sodium phosphate, pH 6.9, 0.17 mM sodium citrate, 0.1 mM $MgCl_2$, 0.65 mM $CaCl_2$) at a concentration of 10^6/ml, and incubated for 6–9 h at room temperature. Aliquots of cells were stimulated by the addition of 1/4 vol Alcian Blue 8 GX in water (concentrations given in text) for 15 s. Cells were then fixed and processed for immunofluorescence using mAb 4D11, which recognizes the granule core protein p80, as previously described (Turkewitz and Kelly, 1992). Cells in randomly chosen fields were scored as either unstimulated or stimulated based on the presence or absence of docked granules.

Results

In both *Paramecium tetraurelia* and *Tetrahymena thermophila*, synchronous exocytosis of dense-core secretory granules can be triggered by a transient increase in intracellular calcium. In *Paramecium,* exocytosis has been correlated with dephosphorylation of parafusin, an abundant phosphoprotein. To see whether a similar protein exists in *Tetrahymena*, we labeled cells with [^{32}P]orthophosphate. One major phosphoprotein of the same apparent molecular mass as parafusin (~60 kD) was observed in unstimulated cells, and was massively dephosphorylated in stimulated cells (Fig. 1). This suggested that a parafusin homologue with a conserved function could be studied in *Tetrahymena*.

To clone the gene encoding the putative homologue as well as any related proteins, we identified protein sequences conserved between *Paramecium* parafusin, human PGM, and yeast PGM, and designed eight degenerate PCR primers corresponding to those regions. PCR amplification using combinations of these primers, in conjunction with *Tetrahymena* cDNA or genomic DNA, generated six products encoding a PGM-like protein. Each overlapped with all of the others, and overlapping sequences were identical, indicating that they were derived from a single gene.

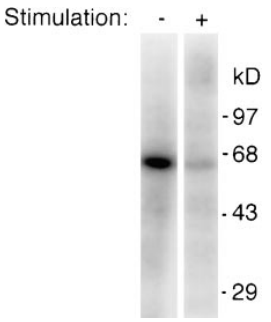

Figure 1. Sensitivity of *Tetrahymena* phosphoproteins to exocytotic stimulation. Cells were metabolically labeled with [^{32}P]orthophosphate and SDS lysates prepared just before, or 2 seconds after, exocytotic triggering. A major phosphoprotein of ~60 kD is present before exocytosis, but is dephosphorylated in stimulated cells. After autoradiography, membranes were stained with Ponceau S to verify that lanes contained equal amounts of protein.

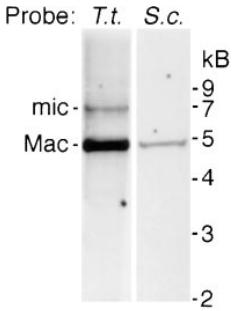

Figure 3. Low stringency Southern blot of wild-type *Tetrahymena* genomic DNA probed with *Tetrahymena PGM1* cDNA (*T.t.*) or an *S. cerevisiae* PGM gene *GAL5* (*S.c.*). A single macronuclear fragment of 4.9 kB is seen using both probes. The more sensitive *PGM1* probe also detects a micronuclear allele of ~7 kB.

The largest product hybridized to a 1.7-kB clone from a cDNA library, whose sequence was identical to that of the gene identified by PCR. We extended the search for potential homologues with low stringency Southern blots of genomic DNA, using probes made both from *S. cerevisiae* PGM (*GAL5*) as well as the isolated *Tetrahymena* cDNA clone. Both probes detected a single macronuclear gene (see Fig. 3). Based on these lines of evidence, it appears that *Tetrahymena* has only a single gene homologous to PGM and parafusin, which we named *PGM1* for reasons described below. Northern blots (see Fig. 6 *C*) were also consistent with a single gene.

Sequence Analysis

Both cDNA and genomic DNA sequences were determined. The *PGM1* sequence displayed characteristics of a *Tetrahymena thermophila* gene: several short introns and the 5′ and 3′ UTRs were rich in AT-rich sequence, and codon usage showed the expected bias (Fig. 2) (Horowitz et al., 1987; Martindale, 1989). Southern blotting of total *Tetrahymena* DNA indicated two bands hybridizing with *PGM1*, which differed in intensity 20-fold (Fig 3). This is the expected difference between macronuclear and micronuclear alleles, since the macronucleus contains 45 copies of each gene, while the micronucleus is diploid. The micronuclear allele appeared to contain a ~2.0 kB insertion, known as an IES. This was confirmed by direct cloning of the IES, which was determined to lie within intron 4 (Fig. 2). As expected, this micronuclear copy persisted in clones whose macronuclear alleles were disrupted (not shown).

The longest ORF in *PGM1* is predicted to encode a 587-residue protein with a calculated mass of 65.5 kD. This product, Pgm1p, is closely related to *P. tetraurelia* parafusin (60% identity) and known eukaryotic PGMs (40–50% identity). We note that *T. thermophila* and *P. tetraurelia*, while both ciliates, diverged at least several hundred million years ago (Wright and Lynn, 1997). To evaluate the significance of the observed differences between these proteins, we used the known crystal structure of rabbit muscle PGM (Dai et al., 1992) as a framework upon which to compare a group of PGM-related proteins. These included *Paramecium* parafusin and *H. sapiens* PGM5, both of which have been speculated to lack enzymatic activity in part on the basis of sequence analysis (Andersen et al., 1994; Subramanian et al., 1994; Moiseeva et al., 1996) and

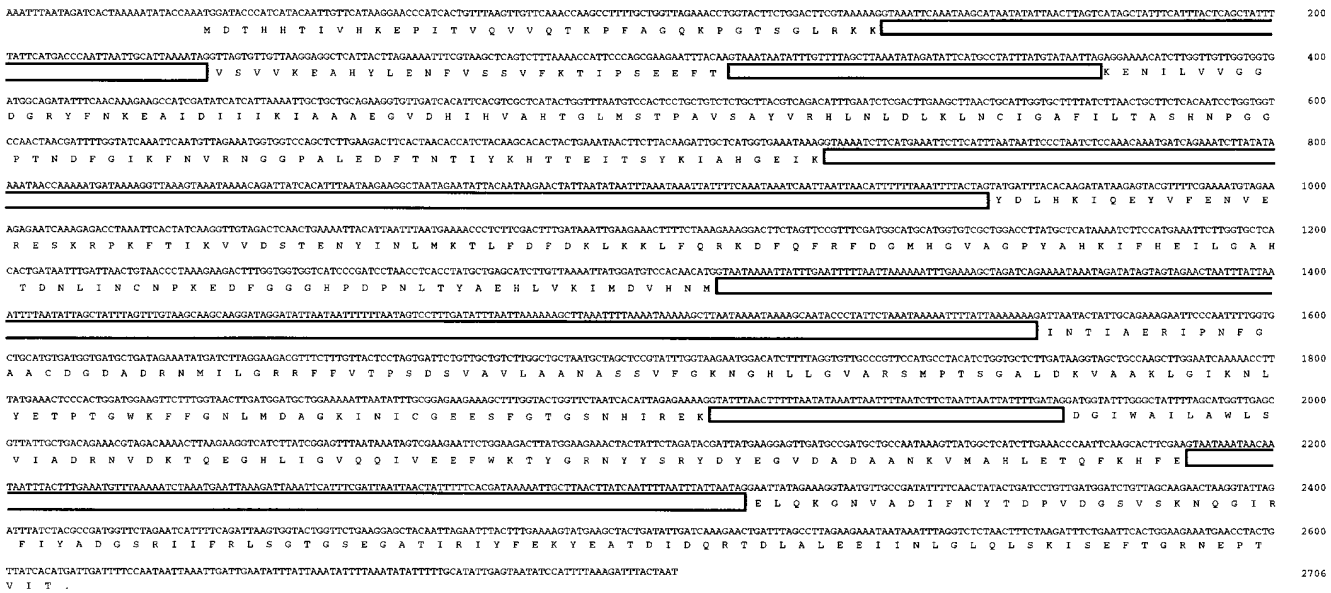

Figure 2. Sequence of *PGM1*. The deduced amino acid sequence is shown below the corresponding nucleotide sequence. Introns are indicated by boxes beneath the nucleotide sequence. The site of an IES in the micronuclear chromosome is indicated by a line. These sequence data are available from Genbank/EMBL/DDBJ under accession number AF020726.

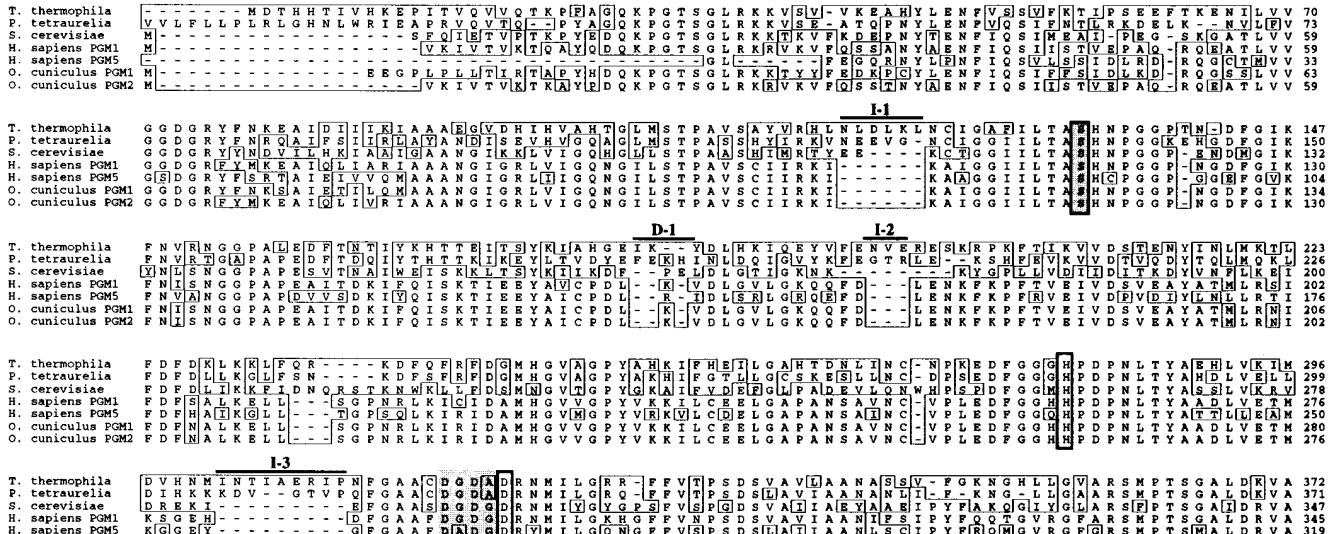

Figure 4. Amino acid sequence alignment of PGMs. Residues differing from the consensus are lightly boxed. Residues located in the active site of the three-dimensional structure, which are predicted to be necessary for enzymatic function, are heavily boxed. Residues that form a magnesium binding pocket in the active site are shaded. The active site phosphoserine is heavily boxed and shaded. Thick bars mark four insertions and two deletions in parafusin, relative to mammalian PGMs, which were previously identified (Subramanian et al., 1994). Sequences were aligned using Lasergene software (DNAStar, Madison, WI) and the clustal method with the PAM250 residue weight table. Genes and Genbank accession codes are *T. thermophila* (*PGM1*, AF020726), *P. tetraurelia* (*Parafusin*, L12471), *S. cerevisiae* (*GAL5,* U09499), *H. sapiens* PGM1 (M83088), *H. sapiens* PGM5 (L40933), *O. cuniculus* PGM1 (M97663), and *O. cuniculus* PGM2 (M97664).

O. cuniculus PGM2, a sarcoplasmic reticulum-associated phosphoprotein (Lee et al., 1992). Strikingly, all six residues predicted to be involved in substrate binding and enzymatic catalysis in conventional PGM (Dai et al., 1992) are completely conserved in these genes (Figs. 4 and 5), arguing that all are enzymatically active. As has been previously noted, parafusin contains several insertions and deletions relative to mammalian PGMs (overlined in Fig. 4); it has been hypothesized that these differences account for a non-PGM activity in parafusin (Subramanian et al., 1994). *Tetrahymena* Pgm1p contains insertions and deletions at the same positions. However, neither the size nor the sequence of the insertions is conserved between *Tetrahymena* and *Paramecium*. Moreover, molecular modeling predicts that all insertions and deletions in the ciliate proteins relative to the mammalian enzyme occur at the end of helices or in regions of random coil, and are located at the periphery of the molecule (Fig. 5). Thus, residues predicted to be involved in enzyme catalysis are maintained in both parafusin and Pgm1p, and variable regions lie in parts of the molecule that are not likely to be important for either folding or enzymatic activity.

Disruption of PGM1

To analyze the in vivo role of *PGM1*, a disruption vector (Gaertig et al., 1994) was used to target the *PGM1* gene. The vector was designed so that its recombination with the *PGM1* locus eliminated the first 80% of *PGM1*, including the start codon (Fig. 6 *A*). Disruption occurs by replacement of macronuclear *PGM1* sequences with *neo2*, which confers resistance to paromomycin. *Tetrahymena* have both a micro- and a macronucleus, but only the latter is transcribed during vegetative growth. The macronucleus contains ~45 identical copies of most genes (Bruns, 1986). Immediately after transformation, vector integration results in replacement of a small number of the macronuclear copies. Transformed clones are then grown in increasing concentrations of paromomycin, to select for cells containing a progressively higher fraction of disrupted alleles,

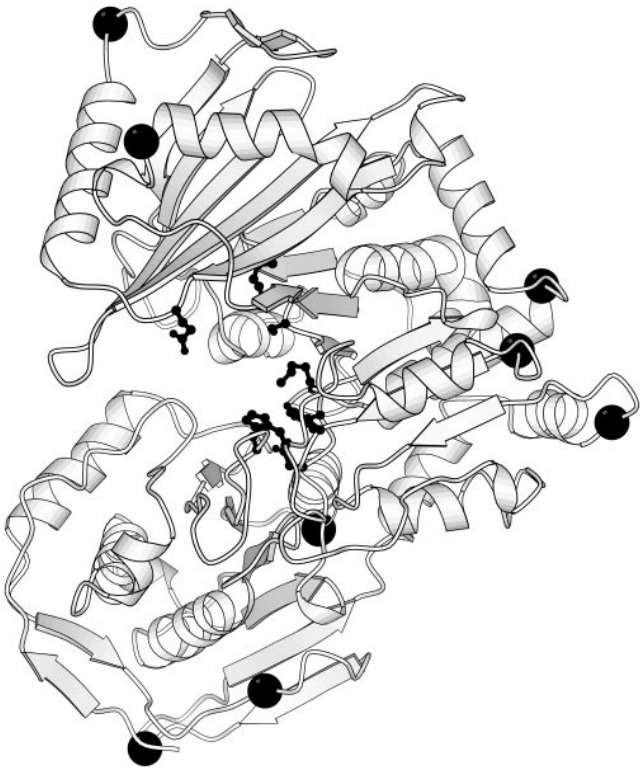

Figure 5. Mapping of universally conserved active site residues versus insertion/deletions in the ciliate sequences, using the crystal structure of rabbit PGM. Small dark side-chains indicate active site residues (found in all eukaryotic PGMs, and boxed in Fig. 4). Large dark spheres indicate the alpha-carbon positions at sites of insertions and deletions in the ciliate proteins, compared with other PGMs. These occur exclusively on the periphery of the molecule, and primarily at the end of helices or in regions of random coil. The coordinate dataset used here was described in Dai et al. (1992) and was obtained from the Brookhaven protein database. MOLSCRIPT was used for molecular modeling (Kraulis, 1991).

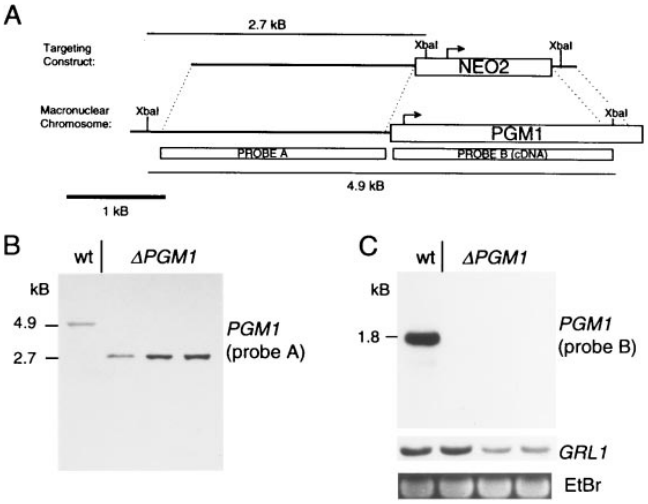

Figure 6. Genomic structure of *PGM1* and Southern and Northern analysis of ΔPGM1 transformants. (*A*) *neo2*, which confers paromomycin resistance in *Tetrahymena* (Gaertig et al., 1994; Chilcoat et al., 1996), was used to replace the 5'-most 80% of the *PGM1* gene as shown. (*B*) Genomic DNA was prepared from wild-type and ΔPGM1 transformants and digested with XbaI. Southern blotting using a probe made from a region upstream of the *PGM1* gene (*probe A*) was performed as described in Materials and Methods. Successful targeting resulted in elimination of a band at 4.9 kB, concurrent with the appearance of a band at 2.7 kB. Upon overexposure, the ~7 kB micronuclear allele could be seen in all lanes. (*C*) Northern blotting was performed on total RNA prepared from wild-type (*wt*) or ΔPGM1 strains using a *PGM1* cDNA probe (*probe B*), and a probe derived from *GRL1* cDNA (Chilcoat et al., 1996). Ethidium bromide staining of rRNA is also shown. ΔPGM1 cells contain normal levels of *GRL1* transcript and total RNA, but have no detectable *PGM1* transcript.

through a process called phenotypic assortment (Bruns, 1986). After phenotypic assortment, we verified the complete replacement of macronuclear *PGM1* through Southern and Northern blotting (Fig. 6, *B* and *C*). A second disruption strategy was also used, in which *neo2* was inserted in the 5′ NspV site of *PGM1* (not shown). Transformants created using either construct showed identical phenotypes. Furthermore, Southern blotting using a probe derived from *neo2* demonstrated that the targeting construct had not integrated elsewhere in the genome (not shown). After complete gene disruption, cells were transferred to drug-free media for subsequent analysis. ΔPGM1 cells displayed no noticeable defects in growth or morphology.

Pgm1p Phosphorylation In Vivo and In Vitro

A well-characterized biochemical property of parafusin in *Paramecium* lysates is that it serves as the acceptor of a glucose phosphotransferase (Satir et al., 1990). To determine whether this property was shared by *Tetrahymena* Pgm1p, we prepared cell lysates and incubated them with the donor molecule for this reaction, [β-^{35}S]UDP-glucose. A single band with the mobility predicted for Pgm1p was labeled in lysates prepared from wild-type cells (Fig. 7 *A*). In contrast, no band was labeled in parallel experiments done with ΔPGM1 lysates.

Labeling of PGM in this reaction mix could also be due to the presence of a breakdown product, [α-^{35}S]Glc-1-P, which can label the active site of PGM (Marchase et al., 1987). To eliminate this possibility, cell lysates were incubated with [β-^{35}S]UDP-glucose in the presence of a 200-fold excess of either unlabeled UDP-Glc or Glc-1-P (Fig. 7 *B*). UDP-Glc, but not Glc-1-P, nearly abolished the labeling, indicating that glucose phosphotransferase activity and not active site labeling was primarily being observed.

Our initial observation was that a ~60-kD phosphoprotein underwent dephosphorylation in vivo upon exocytosis induction in *Tetrahymena*. To establish this parafusin homologue as Pgm1p, the experiment was repeated with ΔPGM1 cells. As anticipated, the parafusin homologue at that position was no longer present (Fig. 7 *C*). In summary, similarity between parafusin and Pgm1p is seen both in sequence and in two kinds of characteristic phosphorylation. No other protein in *T. thermophila* had this combination of qualities.

PGM Activity and PGM1

To determine whether Pgm1p was enzymatically active, we measured PGM activity in crude cell lysates prepared from

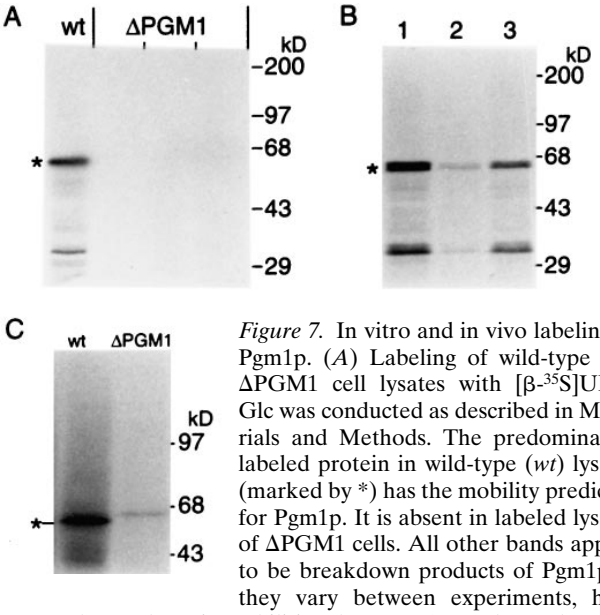

Figure 7. In vitro and in vivo labeling of Pgm1p. (*A*) Labeling of wild-type and ΔPGM1 cell lysates with [β-^{35}S]UDP-Glc was conducted as described in Materials and Methods. The predominantly labeled protein in wild-type (*wt*) lysates (marked by *) has the mobility predicted for Pgm1p. It is absent in labeled lysates of ΔPGM1 cells. All other bands appear to be breakdown products of Pgm1p as they vary between experiments, have faster electrophoretic mobilities than Pgm1p, and are absent in ΔPGM1 lysates. (*B*) Excess unlabeled UDP-Glc specifically reduces labeling by [β-^{35}S]UDP-Glc. Wild-type lysates were labeled with [β-^{35}S]UDP-Glc alone (lane *1*) or with unlabeled UDP-Glc (lane *2*), or unlabeled Glc-1-P (lane *3*). (*C*) In vivo labeling of wild-type (*wt*) and ΔPGM1 cells with [^{32}P]orthophosphate. A major phosphoprotein (marked by *) in wild-type cells, of the mobility predicted for Pgm1p, is absent in ΔPGM1 cells. A minor phosphoprotein of slightly lower mobility is observed in both. The generally lower background in the ΔPGM1 cells was reproducible, and may be due to a role of PGM metabolites in macromolecular synthesis.

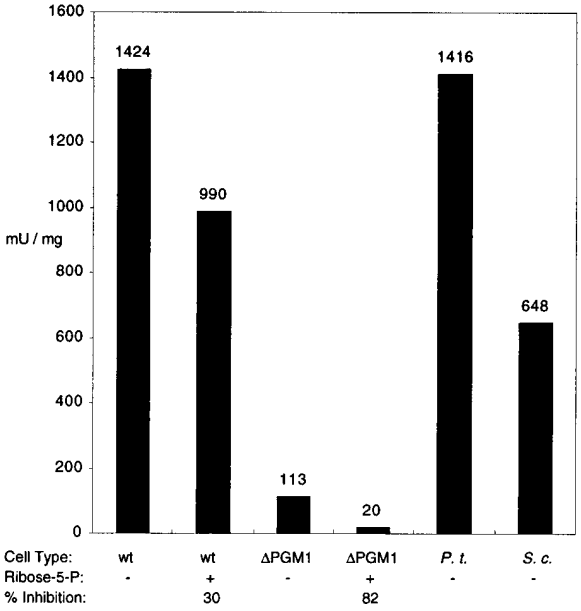

Figure 8. PGM activity in whole cell lysates prepared from wild-type (*wt*) and ΔPGM1 *T. thermophila*, and from *S. cerevisiae* and *P. tetraurelia*. Assays were performed as described in Materials and Methods in the absence or presence of ribose-5-phosphate. All *Tetrahymena* samples were measured on at least four different occasions, with similar results.

wild-type or ΔPGM1 cells (Fig. 8). PGM activity in ΔPGM1 cells was reduced 92% (±1.8%) relative to wild-type cells, indicating that Pgm1p accounts for most of the PGM activity in *T. thermophila.* Since data presented above argue that *T. thermophila* does not contain other PGM-like genes, the residual PGM activity observed in ΔPGM1 cells might be accounted for by unrelated phosphomutase(s) with broad specificity. Similarly, *S. cerevisiae* contains proteins other than PGMs that can catalyze interconversion of glucose-1-P and glucose-6-P (Boles et al., 1994; Hofmann et al., 1994). We asked whether various monosaccharide phosphates, known to act as competitive inhibitors for unrelated phosphomutases, would affect the apparent PGM activity in wild-type or ΔPGM1 lysates. Supporting our hypothesis, ribose-5-phosphate was found to be a more potent inhibitor of the residual activity present in ΔPGM1 cell lysates (82% inhibition) than of the activity in wild-type lysates (30% inhibition; Fig. 8) or purified rabbit PGM (35% inhibition; not shown). Similar differential inhibition was seen with mannose-1-P (not shown). This argues that the residual PGM activity in ΔPGM1 cells comes from enzymes with substantially different properties from classically defined PGMs. Candidates include phosphopentomutase, phosphomannomutase, and *N*-acetylglucosamine phosphomutase.

Localization

To examine the localization of Pgm1p in *Tetrahymena*, cell homogenates were fractionated by centrifugation, and PGM activity was determined. Less than 0.1% of the total PGM activity could be pelleted (Table II). It remains possible that posttranslational modification of Pgm1p induces a weak or transient membrane attachment. A second potential complication is that a membrane-localized population of PGM might have a latent activity, as has been reported for a sarcoplasmic reticulum-associated PGM (Lee et al., 1992). However, a variety of treatments which had been shown to activate latent PGM activity in that system, including nonionic detergents, high salt, and denaturants, failed to uncover latent activity in *Tetrahymena* membranes. Therefore, Pgm1p appears to be a soluble protein with no detectable membrane association.

Effect of PGM1 Disruption on Exocytosis

Tetrahymena contain a large number of dense-core granules, called mucocysts, whose exocytosis can be easily triggered and monitored. Virtually all granules in resting cells are docked in positions from which they can fuse and re-

Table II. Phosphoglucomutase Activity in Subcellular Fractions

Fraction	mU/mg
Wild-type supernatant	3488
Wild-type pellet	<4
ΔPGM1 supernatant	262
ΔPGM1 pellet	<1

Cell lysates were prepared and fractionated as described in Materials and Methods. P1, P2, and P3 fractions were assayed separately, and the total pelletable activity calculated. A total of 110–120% of starting activity was recovered in each of four independent experiments.

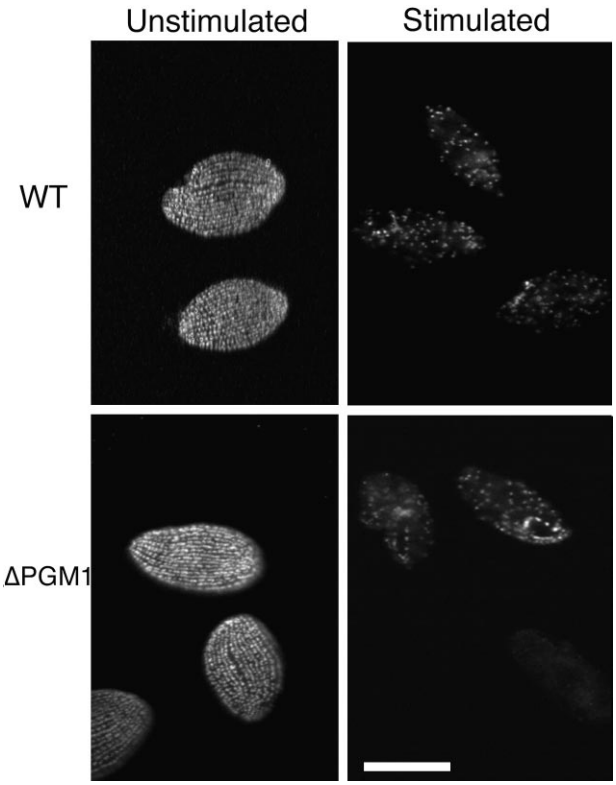

Figure 9. Immunolocalization of the secretory granule protein p80. Samples were prepared from wild-type (*wt*) and ΔPGM1 cells, both before and 15 s after exocytotic stimulation with 0.002% Alcian Blue. Before stimulation, both wild-type and ΔPGM1 cells contain large numbers of granules; these form a distinctive immunofluorescence pattern that reflects their docking at specific sites beneath the plasma membrane. After stimulation the cells are largely devoid of labeling since virtually all granules have undergone exocytosis. All figures are the same magnification. Bar, 40 μm.

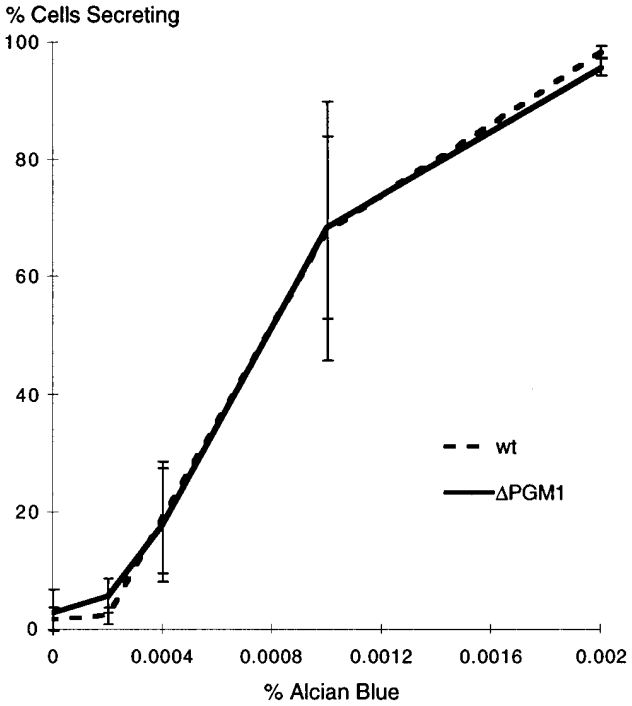

Figure 10. Titration of stimulation threshold. Exocytosis was induced by a 15-s incubation of cells with various concentrations of Alcian Blue. Samples were then processed for immunofluorescence as in Fig. 9, and cells in randomly selected fields were scored as either unstimulated or having undergone secretion. Error bars represent the standard deviation of four independent experiments.

lease their contents within seconds of stimulation. This dramatic release can be detected by immunofluorescence visualization of docked granules using a mAb against a secreted granule protein. Before stimulation, both wild-type and ΔPGM1 cells displayed an identical pattern of docked granules, indicating that granule synthesis, targeting, and docking had taken place (Fig. 9). Cells were then visualized after 15 s of stimulation, and individual cells in randomly chosen fields were scored as either unstimulated or having undergone extensive exocytosis. Under optimal stimulation conditions, ΔPGM1 cells displayed the same response as wild-type cells, both showing virtually complete exocytosis in >97% of the cells (Fig. 10). A number of suboptimal stimulation conditions (lower concentrations of secretagogue) were tested to see whether the sensitivity of the ΔPGM1 cells might be altered. Again, the responses of ΔPGM1 and wild-type cells were indistinguishable. In summary, ΔPGM1 cells appeared identical to wild-type in both the extent of exocytosis and the stimulation threshold.

Several other tests were performed to assess the ΔPGM1 exocytotic response. At higher concentrations of secretagogue, >90% of both wild-type and ΔPGM1 cells were seen to become encapsulated, an assay that has previously been used to indicate extensive and synchronous secretion (Tiedtke, 1976). We tested the response of ΔPGM1 cells to other secretagogues including dibucaine, the calcium ionophore A23187, electrical shock, and picric acid. Secretion was evaluated by several methods: immunofluorescence as previously described; observing cell behavior (secreted protein causes cells to clump in a distinctive fashion); isolating secreted proteins (Turkewitz et al., 1991); and electron microscopy (Chilcoat et al., 1996). In all cases, the responses of ΔPGM1 cells were indistinguishable from those of wild-type (data not shown).

Discussion

Parafusin has been the subject of a large number of papers, and its potential role in regulated exocytosis has been widely cited. The most significant evidence supporting such a role includes the demonstration of dramatic dephosphorylation on a <80-ms time scale, indicating that this modification occurs rapidly enough to be playing a regulatory role in membrane fusion, and that the dephosphorylation does not occur in exocytosis-deficient mutant cells (Zieseniss and Plattner, 1985). The potential significance of these findings was underscored by the finding that a related protein undergoes posttranslational modification upon depolarization of rat brain synaptosomes, suggesting that this step represented an evolutionarily maintained aspect of regulated exocytosis for both dense-core granules and synaptic vesicles (Veyna et al., 1994). This model also fits with data obtained in numerous systems that indicate a role for

protein phosphatases during exocytotic triggering (Verhage et al., 1995; Raufman et al., 1997). Nonetheless, these data can be broadly described as circumstantial, and no direct evidence has established a functional role for parafusin or its dephosphorylation.

We approached these questions by taking advantage of an organism whose physiological and experimental features make it highly advantageous for studies of regulated exocytosis. Like *Paramecium*, *Tetrahymena* can undergo rapid synchronous exocytosis of dense-core secretory granules under controlled conditions. In addition, recent methodological advances facilitate targeted gene disruption, thereby allowing in vivo analysis of protein function. We have identified and cloned the parafusin homologue in *T. thermophila*. Results of gene disruption demonstrate that the *PGM1* gene encodes enzymatically active PGM. The data do not support a role for Pgm1p in regulated exocytosis.

A parafusin homologue was identified using a variety of approaches, including degenerate PCR, library screening, low stringency Southern blotting, Northern blotting, PGM activity assays, in vivo stimulus-dependent dephosphorylation, and in vitro glucosylphosphotransferase assays. All of these approaches identified *PGM1*, but no other related genes. This experimental redundancy implies that we have identified the parafusin homologue, demonstrates that it encodes active PGM, and indicates that there are no other PGM-like proteins in *Tetrahymena*. The gene product displays several highly characteristic features of parafusin.

While *Tetrahymena* contains a single PGM, multiple isoforms of the enzyme are found in other eukaryotes. In mammals, isoforms of metabolic enzymes with apparently identical functions are commonly observed, sometimes with differences in tissue distribution or gene regulation. Such is the case with human PGM (reviewed in Whitehouse et al., 1992), and a similar situation may account for the number of isoforms in rabbit. The two isoforms of PGM in the generally compact genome of *S. cerevisiae* result from an ancient large-scale chromosomal duplication (Boles et al., 1994). In *P. tetraurelia*, where at least two isoforms of parafusin exist, multiple isoforms are the norm rather than an exception (Madeddu et al., 1995). Our results strengthen the impression that single-copy genes are common in *T. thermophila*.

The identification of a novel PGM from the highly divergent ciliate kingdom led to some interesting sequence-based observations. PGM is an ancient enzyme, highly conserved even between prokaryotes and eukaryotes (Lu and Kleckner, 1994), and we asked if intron/exon boundary sites are conserved between *T. thermophila PGM1*, *H. sapiens PGM1*, and *P. tetraurelia parafusin*. Except for the first intron of *Paramecium* and the second of *Tetrahymena*, no boundary sites were conserved (Putt et al., 1993; Hauser et al., 1997). In none of these genes do intron/exon junctions coincide with polypeptide domain boundaries (as determined by Dai et al., 1992).

A number of recently sequenced PGM-like genes have been predicted to lack enzymatic activity because of the perceived divergence of important amino acid residues. The suspected absence of PGM activity was indeed one of the strongest arguments that these proteins have novel roles. As a divergent PGM, the *Tetrahymena* protein is especially useful for evaluating these predictions. When the deduced product of *PGM1* was aligned with these proteins, it was apparent that active site residues were universally conserved. This is in contrast to what was originally stated for parafusin (Subramanian et al., 1994), but is in agreement with the recently observed activity of parafusin expressed in vitro (Hauser et al., 1997). The short insertions and deletions present in both parafusin and Pgm1p, relative to mammalian PGMs, are predicted by molecular modeling to lie in regions where small changes will have little or no effect on enzyme activity. The lack of sequence conservation between the *Tetrahymena* and *Paramecium* proteins in these regions does not support the proposal that they endow parafusin with a specialized role in exocytosis, as previously proposed (Subramanian et al., 1994). *H. sapiens PGM5* (also known as aciculin), is a dystrophin- and utrophin-associated protein that was suggested, based in part on sequence analysis, to lack enzymatic activity (Belkin et al., 1994; Moiseeva et al., 1996). Nonetheless, it shows complete conservation of active site residues. Similarly, while a Gly-to-Ala substitution in the Mg^{2+}-binding hairpin of aciculin was perceived as incompatible with enzymatic activity, both the ciliate sequences predict an Ala-to-Gly substitution in a symmetric position of this hairpin. Given these observations, the reported existence of PGM-like proteins lacking PGM activity may deserve reconsideration.

If phosphorylation plays a regulatory function, the sites of phosphorylation might be conserved among divergent family members. In vivo data suggest that (Ser/Thr) kinases/phosphatases may be involved (Momayezi et al., 1987; Verhage et al., 1995; Raufman et al., 1997), and parafusin can be phosphorylated in vitro by casein kinase and dephosphorylated by calcineurin (Kissmehl et al., 1996, 1997). Disallowing the active site, only 4 of 11 Ser/Thr residues conserved throughout eukaryotes are expected from the crystal structure to be on or near the surface of the protein: residues 30, 286, 584, and 587 (*Tetrahymena* Pgm1p numbering). Only Thr30 and Thr286 are on the same face of the enzyme as the active site, and molecular modeling suggests that phosphorylation of Thr30 would block entry of substrate.

Effect of PGM1 Disruption

We evaluated the function of *PGM1* by generating three independent cell lines in which all macronuclear copies of *PGM1* were disrupted. Lysates prepared from ΔPGM1 cells contain a dramatically reduced level of PGM activity, and the residual activity appears to be the result of an unrelated enzyme(s). The specific activity of PGM in wild-type *Tetrahymena* was two- to fivefold higher than that usually reported in *S. cerevisiae*, plants, or liver (Hanson and McHale, 1988; Oh and Hopper, 1990; Mithieux et al., 1995), but was identical to that we observed in *Paramecium*.

To test if *PGM1* was required for regulated secretion, we induced exocytosis using a variety of stimulatory conditions. Wild-type and ΔPGM1 cells displayed indistinguishable sensitivity to stimulation, and extent of release. Since Pgm1p became dephosphorylated under stimulation conditions, our results appear to uncouple these two previously correlated phenomena and indicate that this protein

is not essential for exocytosis. Two alternative interpretations deserve mention, although we consider both to be unlikely. First, the *Tetrahymena* protein may have lost a function conserved in other eukaryotes; however, it is then necessary to explain why rapid dephosphorylation has been maintained. Second, although we stimulated cells using a variety of different conditions, our quantitative measure was based on cells fixed 15 s after stimulation. Because this period is long relative to the minimum time required for membrane fusion, it is possible that ΔPGM1 and wild-type cells have different kinetics of release, which cannot be detected by our assay. In this case, one would have to postulate that Pgm1p acts as a kinetic effector of exocytosis that has no effect on either the threshold or extent of release.

The lack of an observable phenotype in a gene disruption experiment may be due to suppression by an offsetting second site mutation to maintain an advantageous trait. For the results reported here, this could theoretically involve selection for PGM activity or regulated exocytosis. Both seem highly unlikely for the following reasons. In a variety of organisms, PGM mutants with only mild phenotypes have been obtained (Hanson and McHale, 1988; Boles et al., 1994; Lu and Kleckner, 1994; Zhou et al., 1994), indicating that PGM is not an essential enzyme under many growth conditions. Similarly, regulated exocytosis in *Tetrahymena* appears completely dispensable for growth in the laboratory: a variety of exocytosis-deficient mutants have been obtained that have no growth defects and are nonreverting (Maihle and Satir, 1985; Turkewitz et al., 1991; Gutierrez and Orias, 1992). Finally, indistinguishable results were obtained with three independent transformants.

In summary, our results indicate, in contradiction to the central hypothesis of previous work on parafusin, that Pgm1p is not required upstream of membrane fusion during regulated exocytosis. Some of the earlier evidence can easily be reinterpreted in light of these findings. The absence of parafusin dephosphorylation in exocytosis mutants or in the presence of exocytosis inhibitors (Gilligan and Satir, 1982; Zieseniss and Plattner, 1985) was originally interpreted as evidence that the genetic or pharmaceutical blocks are epistatic to dephosphorylation of parafusin, which was postulated to be epistatic to exocytosis. However, the data are equally consistent with a model in which exocytosis is epistatic to dephosphorylation, leaving open the possibility that parafusin may function in recovery from exocytosis. The time course of dephosphorylation established in *Paramecium* may suggest that it is not membrane fusion *per se* that triggers dephosphorylation, but some earlier event.

Possible Functions of Pgm1p Dephosphorylation

The family of PGM-like proteins, some of which have been shown to undergo changes in phosphorylation, includes a calmodulin-dependent kinase substrate in sarcoplasmic reticulum (Lee et al., 1992), a yeast PGM which undergoes phosphoglucosylation upon heat shock (Dey et al., 1994), and a dystrophin/utrophin-associated PGM found in adherens junctions (Moiseeva et al., 1996). A possible link between these proteins may be their sensitivity to fluctuations in intracellular calcium. Modification appears to occur under conditions of increased free cytosolic calcium associated with secretion in regulated secretory cells and contraction in muscle (Clapham, 1995; Erxleben et al., 1997). In addition, localization of calcium-sensitive proteins to the adherens junction suggests that it is also a site of calcium flux (Burridge et al., 1988). Therefore, PGM-like proteins may respond (via kinases/phosphatases or phosphoglucose-transferases/esterases) to calcium levels. What is the nature and function of this response? Given the rapidity and extent of the modifications, we considered that the dephosphorylated enzyme may play a role in demobilization of the high calcium required to trigger exocytosis, for example by increasing the rate of calcium uptake into the vesicular stores that underlie the plasma membrane of ciliates, or into the ER or SR in mammalian cells. Recent experiments in mammalian cells suggest that levels of glucose can influence calcium transport, which could mean that modulation of PGM activity affects free calcium levels (Darbha and Marchase, 1996). Therefore, one might expect to see effects from perturbation of calcium homeostasis in ΔPGM cells, but these have not been reported in either *S. cerevisiae* (Boles et al., 1994) or in *Tetrahymena* (Chilcoat, N.D., unpublished observation). Further development of these or other in vivo systems may help to address this issue.

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