Drosophila PPY, a novel male specific protein serine/threonine phosphatase localised in somatic cells of the testis

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INTRODUCTION

The reversible phosphorylation of proteins on serine and threonine residues is a major mechanism for the regulation of many diverse cellular processes such as signal transduction, mitosis and fertilisation. The level of phosphorylation of key regulatory proteins is controlled through the addition and removal of phosphate by protein kinases and protein phosphatases, respectively. In order to elucidate the mechanisms underlying this type of regulation, the cellular roles of the different kinases and phosphatases need to be defined. Four types of protein serine/threonine phosphatase (PP) have been identified in the cell cytosol from their enzymatic properties, PP1, PP2A and the Ca²⁺-calmodulin regulated protein phosphatase, which structurally comprise one family and the Mg²⁺-dependent protein phosphatase, which belongs to a second family (Cohen, 1989; Cohen et al., 1990; Shenolikar, 1994). PP1 is involved in the regulation of glycogen metabolism by the hormones adrenaline and insulin, Ca²⁺ uptake into the sarcoplasmic reticulum, muscle contractility and exit from mitosis. Despite the wide range of processes regulated by PP1, several novel protein phosphatases have been identified which are structurally related to PP1 but perform distinct cellular functions. These include PPQ, PPZ1 and PPZ2 in Saccharomyces cerevisiae, which have long N-terminal domains and show approximately 60% identity to PP1 in the C-terminal catalytic domain. PPQ plays a role in the regulation of protein synthesis (Chen et al., 1993), while PPZ1 and PPZ2 are involved in the maintenance of cell integrity and osmotic stability (Hughes et al., 1993; Lee et al., 1993; Posas et al., 1993).

A novel phosphatase, termed PPY, was identified by low stringency screening of a D. melanogaster cDNA library using a rabbit PP1 cDNA fragment (Dombrádi et al., 1989a). PPY was similar in size to PP1 and showed 64% amino acid identity. We demonstrate here that, unlike PP1 which is present in all tissues examined, PPY is a testis specific protein phosphatase by analysis of both mRNA and protein distribution. More precise immunolocalisation within the testis, using affinity purified anti-PPY protein and anti-PPY peptide antibodies, shows that PPY is present in somatic cyst cells, which encase the germ cells. The predominant location of PPY is in the nuclei of both head and tail cyst cells throughout the length of the testis except for the apical tip. The distribution of PPY, coupled with its unique biochemical properties, suggests that PPY may be required to prevent cyst cell division, increase transcription for provision of nutrients to the germ cells and/or provide a signal for spermatocyte differentiation.

Key words: cyst cell, Drosophila, protein phosphatase, spermatogenesis

SUMMARY

Drosophila protein phosphatase Y (PPY) displays 64% amino acid identity to protein serine/threonine phosphatase 1 (PP1) and 39% to protein phosphatase 2A (PP2A). Here we show by expression of cDNA in bacteria, that PPY is a protein serine phosphatase and that its biochemical properties are distinct from PP1 in both substrate specificity and regulation by the thermostable inhibitory proteins inhibitor 1 and inhibitor 2. We also demonstrate that PPY is a novel testis specific protein phosphatase by analysis of both mRNA and protein distribution. More precise immunolocalisation within the testis, using affinity purified anti-PPY protein and anti-PPY peptide antibodies, shows that PPY is present in somatic cyst cells, which encase the germ cells. The predominant location of PPY is in the nuclei of both head and tail cyst cells throughout the length of the testis except for the apical tip. The distribution of PPY, coupled with its unique biochemical properties, suggests that PPY may be required to prevent cyst cell division, increase transcription for provision of nutrients to the germ cells and/or provide a signal for spermatocyte differentiation.

Key words: cyst cell, Drosophila, protein phosphatase, spermatogenesis

MATERIALS AND METHODS

Materials

The baculovirus vector pAcYM1 and the Escherichia coli pCW vectors were generous gifts from Professor David H. L. Bishop.
Expression of PPY from baculovirus and production of antibodies

The ends of the EcoRI fragment containing Drosophila PPY cDNA (nt –5 to 1026) (Dombrádi et al., 1989a) were filled in using the Klenow fragment of DNA polymerase I in the presence of excess dNTPs (Sambrook et al., 1989) and ligated to BamHI linkers. The PPY cDNA was then subcloned into the BamHI site of the baculovirus transfer vector pAcYMI which contained the polyhedrin promoter (Matsuura et al., 1987) giving rise to the recombinant plasmid pAcPPY which was ampliﬁed in E. coli JM109. The correct orientation of PPY was checked by DNA sequencing. Spodoptera frugiperda 9 cells were cotransfected with 25 μg pAcPPY and 1 μg of wild-type baculovirus DNA and polyhedrin negative plagues were selected and puriﬁed. PPY was expressed from the recombinant baculovirus in a predominantly insoluble form as described for PP1 (Berndt and Cohen, 1990). The washed pellet containing PPY was applied to a 18 g of wild-type mammalian cells harvested after 20 hours incubation at 28°C and were resuspended in 0.05 volume (compared to the culture medium) of lysis buffer comprising 50 mM Hepes, pH 7.5, 100 mM KC1, 5% glycerol, 1 mM EDTA, 2 mM MnCl2, 2 mM diithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 0.2 mM benzamidine and 2 mM hydrochloride. Cells were lysed with an ultrasonic processor H104/52 (Fencon, Beds, UK) at medium power (4x 15 seconds on ice) and the insoluble debris was removed by centrifugation at 14,000 g for 10 minutes at 4°C. Expressed PPY was partially puriﬁed by anion-exchange chromatography. The supernatant was ﬁltered through a 0.45 mm ﬁlter and applied to a 10 cm × 16 mm column of Q-Sepharose (Pharmacia Ltd, Milton Keynes, UK) at room temperature in 20 mM triethanolamine, pH 7.5, 0.1 mM EGTA, 5% glycerol, 0.1% 2-mercaptoethanol, 1 mM MnCl2. The column was developed with a 400 g linear gradient from 0-0.5 M NaCl in the same buffer. Fractions containing phosphorylase phosphatase activity were pooled, concentrated using a Centricon-10 microcentrator (Amicon, Beverly, MA), dialysed in 50 mM Tris-HCl, pH 7.5, 50% glycerol, 1 mM MnCl2, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 0.03% Brij-35 and stored at –20°C.

Phosphatase assays

32P-labelled rabbit skeletal muscle glycoenzyme phosphorylase was prepared by phosphorylation by phosphorylase kinase to a stoichiometry of 1 mol phosphate per mol subunit (Cohen et al., 1988a). Cyclic AMP-dependent protein kinase was used to phosphorylate rabbit skeletal muscle phosphorylase kinase to a stoichiometry of 1.7 mol of phosphate/αβγ unit (Stewart et al., 1981) and partially hydrolsed bovine casein to 4.8 mmol phosphate per mg (McGowan and Cohen, 1987). The specific radioactivity of the ATP used in the phosphorylations was 106 cpm/mmol. All protein phosphatase assays were performed as described by Cohen et al. (1988a) in the presence of 0.5 mM MnCl2. The substrate concentrations in the assay were 10 μM for phosphorylase, 1 μM for phosphorylase kinase and 6 μM for casein. Dephosphorylation of the α and β subunits of phosphorylase kinase was quantiﬁed as described by Stewart et al. (1981). One unit of activity is the amount of enzyme which catalyses the release of 1 μmol of [32P]phosphate per minute.

Northern blot analysis of PPY mRNA

Total RNA was puriﬁed from various Drosophila developmental stages and adult tissues which had been stored in liquid nitrogen, as described by Dombrádi et al. (1989b). Northern blotting was performed using the formalddehyde method, essentially as described by Lehrach et al. (1977) using capillary transfer in 3 M NaCl, 0.3 M sodium citrate, pH 7.0, onto Hybond N (Amersham International, Bucks, UK). Hybridisation was carried out overnight in 0.9 M NaCl, 90 mM sodium citrate, pH 7.0, 50% formamide, 10% dextran sulphate, 1× Denhardt’s (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA), 0.1% SDS, 0.25 mg/ml herring sperm DNA at 47°C with a full length PPY cDNA probe labelled with [γ-32P]ATP using a random hexanucleotide priming kit (Boehringer Mannheim UK Ltd, Lewes, UK). Blots were washed at 65°C in 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, 0.1% SDS. Following autoradiography, the PPY probe was removed in 0.5% SDS at 100°C for 5 minutes. The control probe employed subsequently was the full length Drosophila PP1 87B cDNA (Dombrádi et al., 1989b) in the hybridisation and washing conditions described above.

Immunological techniques

Tissues from larvae and 0-5 day adults dissected in Ringer’s solution (111 mM NaCl, 1.8 mM KC1, 2.4 mM NaHCO3, 0.9 mM CaCl2, 0.08 mM Na2HPO4 and stored at ~80°C, were homogenised on ice in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride and centrifuged at 14,000 g for 10 minutes at 4°C. Protein concentrations of the supernatants were determined using Coomassie protein
assay reagent (Pierce, Rockford, IL). Samples were fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis prior to blotting onto nitrocellulose (BA 85; Schleicher and Schuell, Dassel, Germany) as described by MacDougall et al. (1989). The blots were blocked overnight with 5% Marvel dried milk powder (Premier Brands, Birmingham, UK) in PBS (0.137 M NaCl, 2.68 mM KCl, 1.76 mM KH2PO4, 10 mM Na2HPO4, pH 7.4), 0.1% Tween-20, and probed with affinity purified 0.2 μg/ml anti-PPY protein antibodies. Antibody binding was detected using 0.16 μg/ml horseradish peroxidase-conjugated anti-rabbit antibodies (Pierce, Rockford, IL) followed by enhanced chemiluminescence (Amersham International plc, Bucks, UK).

Tests from wild-type Oregon R flies and P-element enhancer trap marker line 600 (Gönczy et al., 1992) were dissected from larvae and 0-5-day-old adults in Drosophila Ringer’s solution, and immediately fixed for 15 minutes in 4% paraformaldehyde in PBS and permeabilised for 15 minutes in 4% paraformaldehyde, 0.1% Triton X-100, 0.1% deoxycholate in PBS. The immunocytochemical procedure was essentially as described by Gönczy et al. (1992), except that the NaCl was increased to 1.2 M during incubation of the larval testis with the primary antibody. Binding of anti-PPY protein antibodies (1 μg/ml) or anti-PPY peptide antibodies (0.5 μg/ml) was detected via Texas Red-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) or 4 μg/ml horseradish peroxidase-conjugated anti-rabbit IgG. Peroxidase activity was visualised using 0.5 mg/ml diaminobenzidine, 0.01% H2O2 in PBS. Anti-β-galactosidase antibodies (11 μg/ml; Sigma) were detected using fluorescein-conjugated anti-mouse antibodies (Jackson ImmunoResearch Laboratories Inc.). Samples were mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) under bridged coverslips. The samples were examined with an Olympus BH-2 microscope and a Bio-Rad MRC-600 laser scanning confocal microscope.

RESULTS

Expression of Drosophila PPY in heterologous systems and characterisation of PPY activity

Expression of Drosophila PPY from recombinant baculovirus in Sf9 cells and from the pT7.7 vector in E. coli gave predominantly insoluble enzyme. However, expression from the tac promoter of the pCW vector in E. coli DH5α produced soluble active PPY, although only at a low level of 0.4 mg PPY per litre of culture. Soluble PPY in E. coli extracts was detected by immunoblotting with anti-PPY antibodies raised against insoluble PPY expressed from recombinant baculovirus (Fig. 1). The soluble PPY was partially purified from E. coli extracts by anion-exchange chromatography on Q-Sepharose, the PPY activity eluting at 250 mM NaCl (data not shown). No phosphatase activity was detected in extracts from bacteria expressing vector alone. After chromatography, fractions containing PPY were pooled and this material (specific activity 30 mU/mg with 32P-labelled phosphorylase as substrate) was used for comparison of the properties of PPY with those of PP1. PPY activity was dependent on the presence of Mn2+ in the assay (optimal concentration 1 mM), with those of PP1. PPY activity was dependent on the presence of Mn2+ in the assay (optimal concentration 1 mM), as observed for bacterially expressed PP1 (Alessi et al., 1993). The specific activity of pure PPY towards 32P-labelled phosphorylase was calculated to be 0.8 U/mg, the protein concen-

![Fig. 1. Expression of PPY in bacteria. (A) Schematic diagram of the PPY/pCW expression construct, driven by a tandemly repeated tac promoter. (B) Immunoblot analysis of PPY expressed from E. coli strain DH5α. The samples are 10 μg of soluble extract from E. coli transformed with the pCW vector only and E. coli transformed with the PPY/pCW expression construct; 50 ng PPY expressed from baculovirus was used as a control. Sizes of molecular mass markers ×10^3 are indicated.](image-url)

The following table compares the properties of PPY with those of other protein serine/threonine phosphatases.

<table>
<thead>
<tr>
<th>Property</th>
<th>PPY (PP1*)</th>
<th>PP2A (PP2*)</th>
<th>PP5 (PP5*)</th>
<th>PP2B (Ca2+/calmodulin regulated PP, calcineurin, PPP3*)</th>
<th>PP2C (Mg2+ dependent PP MPP1*)</th>
<th>References</th>
</tr>
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<tr>
<td>Phosphorylase phosphatase</td>
<td>0.8</td>
<td>30</td>
<td>5</td>
<td>Very low</td>
<td>Very low</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Chen et al., 1994</td>
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<tr>
<td>Casein phosphatase</td>
<td>0.7</td>
<td>PP1γ 1.2</td>
<td>PP1c 0.3</td>
<td>nd</td>
<td>Very low</td>
<td>Cohen et al., 1989</td>
</tr>
<tr>
<td>specific activity (U/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>McGowan and Cohen, 1987</td>
</tr>
<tr>
<td>Preference for α or β subunit</td>
<td>No preference</td>
<td>β</td>
<td>α</td>
<td>α</td>
<td>α</td>
<td>Cohen, 1989</td>
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<tr>
<td>of phosphorylase kinase</td>
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<td></td>
<td></td>
<td></td>
<td>M. X. Chen and P. T. W. Cohen, unpublished data</td>
</tr>
<tr>
<td>Inhibition by I1 and I2</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Cohen et al., 1989</td>
</tr>
<tr>
<td>IC50 for okadaic acid</td>
<td>10 nM</td>
<td>10 nM</td>
<td>&lt;0.2 nM</td>
<td>1.4 nM</td>
<td>5 μM</td>
<td>Cohen et al., 1989</td>
</tr>
</tbody>
</table>

*Nomenclature employed for the human protein phosphatases (Cohen, 1994). Bacterially expressed PP1 catalytic subunit isoform, termed PP1γ (Barker et al., 1993) and PP1 catalytic subunit purified from skeletal muscle (PP1c) possessed similar activity towards all substrates and inhibitors tested except for casein. nd, not determined.
The specific activity of PPY is approximately 40-fold lower than the specific activity of PP1 against the same substrate (30 U/mg), while the specific activity of PPY against casein was similar to PP1 (Table 1).

Phosphorylase kinase is phosphorylated on two serine residues, one on the \( \alpha \)-subunit and the other on the \( \beta \)-subunit. PP1 dephosphorylates the \( \beta \)-subunit rather than the \( \alpha \)-subunit of phosphorylase kinase (Cohen et al., 1988), whereas other protein serine/threonine phosphatases including PP2A, PP5, PP2B (PP3, calcineurin) and the \( \text{Mg}^2+ \) dependent protein phosphatase (MPP1, PP2C) preferentially dephosphorylate the \( \alpha \)-subunit of phosphorylase kinase compared to the \( \beta \)-subunit. In contrast, PPY dephosphorylates both the \( \alpha \)- and \( \beta \)-subunit at similar rates (Fig. 2).

PP1 can also be distinguished from other protein phosphatases by its sensitivity to the thermostable proteins, inhibitor 1 and inhibitor 2. These PP1 inhibitors had no effect on PPY activity using \(^{32}\text{P}\)-labelled phosphorylase as substrate, even at concentrations which completely inhibited PP1 (Fig. 3). However, both PPY and PP1 exhibited similar inhibition characteristics in the presence of okadaic acid with apparent IC\(_{50}\) values of 10-12 nM. PPY was also inhibited by stoichiometric amounts of microcystin as observed for PP1.

**Developmental and tissue expression of PPY in Drosophila**

The expression of PPY mRNA was examined by northern blot analysis of total RNA from stages of the *Drosophila* life cycle, using the PPY cDNA as a probe (Fig. 4A). A single 1.4 kb transcript was observed in males, pupae and at a low level in larvae. PPY transcripts could not be detected in embryos or adult female flies even after prolonged exposure of the northern blots. To control for variation in loading of the samples, the PPY probe was removed and the same blot hybridised with PP1 87B cDNA (Fig. 4B) which is expressed at a constant level during the *Drosophila* life cycle (Dombrádi et al., 1989b). Since the gonads constitute a significant portion of the adult fly body, we analysed the expression of PPY in total RNA purified from testes and ovaries dissected from adult flies. Fig. 4C shows that PPY mRNA is abundantly expressed in the testis, but is not present in the ovary. In order to examine whether PPY was a testis specific transcript during development, we dissected testes from larvae and adult male flies and isolated RNA from the dissected tissue and from the remaining parts of the larvae and adults. Fig. 5A shows that the PPY mRNA is expressed...
3371PPY, a male specific protein phosphatase in the larval and adult testis, but not detected in the rest of the larvae or adult male even after prolonged exposure of the northern blot. No PPY transcript was detected in male heads (data not shown). Since PPY was originally isolated from a D. melanogaster head cDNA library (Dombrádi et al., 1989a), the absence of PPY mRNA from male and female head tissues indicates that the occurrence of PPY cDNA in the head library is likely to have arisen from contamination of heads with some bodies before preparation of the mRNA used in construction of the library.

In order to investigate whether PPY protein had the same distribution as the PPY mRNA, we performed immunoblotting of Drosophila extracts with the affinity purified anti-PPY antibodies. An immunoreactive band, which comigrated with the baculovirus expressed PPY, was only observed in testis extracts (Fig. 6A), with >70% of the PPY being found in the soluble fraction (data not shown). No PPY band was detected in extracts made from whole adult male flies (lane 1) or from extracts made from abdomens and thoraxes (lane 5) suggesting that the level of PPY is below the detection limit of the antibodies in these extracts. The level of PPY in testis was estimated by immunoblotting to be approximately ~0.03% of total protein (Fig. 6B).

Fig. 4. PPY mRNA levels during the Drosophila life cycle and in Drosophila tissues. (A) Total RNA from each developmental stage was fractionated on a denaturing agarose gel, blotted onto nylon membrane and hybridised with the full length PPY cDNA. (B) The blot was stripped and rehybridised with PP1 87B cDNA to control for loading variation (PP1 87B transcripts are expressed at a constant level during development; Dombradi et al., 1989b). E1, 0-4 hour-old embryos; E2, 4-24-hour-old embryos; L, larvae; P, pupae; F, female adult flies; M, male adult flies. (C) Total RNA was isolated from dissected ovaries (O) and testes (T) visualised by ethidium bromide staining and hybridised with PPY and PP1 87B cDNA. Transcript sizes are calculated from their mobility relative to that of RNA size markers and are indicated by arrowheads.

in the larval and adult testis, but not detected in the rest of the larvae or adult male even after prolonged exposure of the northern blot. No PPY transcript was detected in male heads (data not shown). Since PPY was originally isolated from a D. melanogaster head cDNA library (Dombrádi et al., 1989a), the absence of PPY mRNA from male and female head tissues indicates that the occurrence of PPY cDNA in the head library is likely to have arisen from contamination of heads with some bodies before preparation of the mRNA used in construction of the library.

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Fig. 5. Expression of PPY mRNA in larval and adult testis. Lane 1, 50 μg of total RNA from larvae from which testes had been removed; lane 2, 10 μg of total RNA from larval testis; lane 3, 50 μg of total RNA from adult male flies from which testes had been removed; lane 4, 10 μg of total RNA from adult testis. (A) The blot was hybridised with full length PPY cDNA and (B) PP1 87B cDNA. Transcript sizes are calculated from their mobility relative to that of RNA size markers and are indicated by arrowheads.

Fig. 6. Immunoblot analysis of PPY protein in Drosophila. (A) 10 μg of soluble protein from Drosophila extracts were separated by SDS-PAGE, blotted onto nitrocellulose membrane and probed with anti-PPY antibodies. Lane 1, adult male; lane 2, adult female; lane 3, male head; lane 4, female head; lane 5, male body; lane 6, female body; lane 7, testis; lane 8, ovaries; lane 9, 1 ng of PPY; lane 10, 50 ng of PPY. (B) Estimation of PPY by immunoblotting of testis (lane 1) and ovary (lane 2) extracts (6 μg of each). Lanes 3 and 4, 2 ng and 1 ng of pure PPY protein, respectively. The blot was probed with anti-PPY antibodies.
Immunolocalisation of PPY in *Drosophila* testis

The adult *Drosophila* testis is essentially a coiled tubular structure through which spermatogenesis occurs in a series of discrete steps (Lindsley and Tokuyasu, 1980). Since different stages of spermatogenesis, which include mitosis, meiosis and differentiation, occur at defined regions of the testis, localisation studies may yield information on the stage at which a particular protein functions. We examined the subcellular localisation of PPY in adult testis using affinity purified anti-PPY protein antibodies. Detection of PPY, using antibodies coupled to peroxidase staining, demonstrated that PPY was predominantly in the nuclei of cells distributed along the length of the testis, although staining was weak in the apical region (Fig. 7A). No staining was observed using an affinity purified antibody fraction which had been adsorbed by passage through Sepharose to which PPY had been covalently coupled (Fig. 7B). Examination of the PPY staining at higher magnification revealed the existence of two distinct nuclear morphologies, the first having a round form (Fig. 7C) and the second having a long thin form (Fig. 7D).

The staining pattern observed for PPY is reminiscent of that observed for enhancer trap lines which label the nuclei of cyst cells in the testis. The morphology of these cells is such that the nuclei comprise most of the cell content while the cytoplasmic region appears small but highly elongated in order to surround the developing germ cells. The enhancer trap line 600 (Gönczy et al., 1992) expresses β-galactosidase, modified to include a nuclear localisation signal, in cyst cells. In order to test whether PPY co-localises with expressed β-galactosidase in the nuclei of this enhancer trap line, testes were double stained with mouse anti-β-galactosidase and rabbit anti-PPY antibodies, binding being detected via fluorescein-conjugated anti-mouse and Texas Red-conjugated anti-rabbit antibodies, respectively (Fig. 8). No cross reactivity was observed between the fluorescein-conjugated antimouse antibodies and the rabbit anti-PPY antibodies, or the Texas Red-conjugated anti-rabbit antibodies and the mouse anti-β-galactosidase antibodies (data not shown). Examination of the immunofluorescent double staining pattern by confocal microscopy showed that PPY co-localised with β-galactosidase in the nuclei of cyst cells in the enhancer trap line 600 and at high magnification it can be seen that both β-galactosidase and PPY are excluded from the nucleoli. The immunofluorescent analysis also shows that PPY is present in the cytoplasm of the cyst cells, but at a much lower level. Anti-PPY peptide antibodies gave essentially the same results as the anti-PPY protein antibodies.

The localisation of PPY was also examined by immunofluorescence in larval testis dissected from marker line 600. Double labelling with anti-PPY peptide antibodies and anti-β-galactosidase antibodies showed that PPY co-localised with β-galactosidase in the cyst cell nuclei of larval testis, while also being present in the cytoplasm (Fig. 8).

**DISCUSSION**

PPY is a protein serine/threonine phosphatase with biochemical properties distinct from those of PP1

The sequence of *Drosophila* PPY determined from the cDNA demonstrated that PPY belonged to the family of protein serine/threonine phosphatases that include PP1 (Dombrádi et al., 1989) Although more similar to PP1 than any other known protein phosphatase, PPY shows only 60-64% identity to the *Drosophila* PP1 isoforms, termed PP1 9C, PP1 13C, PP1 87B and PP1 96A, which share more than 80% identity to one another (Dombrádi et al., 1990, 1993). This suggests that PPY may not simply be an isoform of PP1, but may perform a distinct cellular function.

![Fig. 7. Immunolocalisation of PPY in *Drosophila* testis. Testes were dissected from young adult males, fixed in 4% paraformaldehyde and permeabilised in 0.1% Triton X-100 + 0.1% sodium deoxycholate. Affinity purified anti-PPY antibodies were detected using horseradish peroxidase-conjugated anti-rabbit antibodies. (A) testis stained with anti-PPY antibodies. The apical tip is on the left and the terminal region and seminal vesicle is on the right (B) testis stained following immuno-depletion of anti-PPY antibodies on PPY-Sepharose. (C) head cyst cell nuclei and (D) tail cyst cell nuclei stained with anti-PPY antibodies. Bars: (A and B), 100 μm; (C and D) 10 μm.](image-url)
Expression of soluble PPY in E. coli generated a functional protein phosphatase that was capable of dephosphorylating various [32P]-labelled substrates (Table 1). Two main criteria have been used for defining type 1 protein phosphatases, namely preference for dephosphorylation of the β rather than the α subunit of phosphorylase kinase and the inhibition of phosphatase activity by inhibitor 1 and inhibitor 2. PPY differed from PP1 by both these criteria. The expressed PPY dephosphorylated both the α and β subunits of phosphorylase kinase at the same initial rate and PPY activity was unaffected by inhibitor 1 and inhibitor 2, even at concentrations which completely blocked PP1 activity. Therefore PPY not only has a different substrate specificity from PP1 but also differs in its interaction with modulatory proteins, indicating that PPY is likely to have a different cellular role and mode of regulation from PP1. Although it is possible PPY may exist as the free catalytic subunit, it would seem likely by comparison with PP1 and PP2A, that PPY will be complexed with its own distinct regulatory subunit(s) in vivo. The overall enzymatic analyses (Table 1) also indicate that PPY is distinct from all other characterised protein serine/threonine phosphatases.

**PPY is male specific and is abundantly expressed in the testis**

Analysis of PPY mRNA expression in the *Drosophila* life cycle shows that a single transcript is present in larvae, pupae and adult males, suggesting that PPY is a male specific protein phosphatase. Tissue distribution studies of the transcript in larvae and adult males show that PPY mRNA is abundantly expressed in testis and not in other tissues. PPY protein detected by immunoblotting with affinity purified anti-PPY protein antibodies identified a single immunoreactive protein (Mr 36,000), which also was only present in testis extracts. These results demonstrate that PPY is a testis specific protein phosphatase and suggest that the enzyme is likely to play a role in some aspect of spermatogenesis.

Testis-specific forms of protein phosphatase catalytic subunits have been reported in mammalian systems, but none of them would appear to be the mammalian homologue of PPY. The γ2 isoform of PP1 has been shown to be abundantly expressed in rat testis (Sasaki et al., 1990) but it is associated with the nuclei of germ cells (Shima et al., 1993), unlike PPY which is found in somatic cells. Molecular cloning has identified testis-specific isoforms of PP2B (Muramatsu et al., 1992) and the Mg2+ dependent protein phosphatase (Hou et al., 1994), but these have low (40%) or no sequence identity, respectively, to PPY. It is also of interest that a testis specific regulatory subunit of PP2B (Chang et al., 1994) has been identified and there may also exist a testis specific regulatory subunit of PP2A (Hatano et al., 1993). However, PPY is the first novel testis specific protein serine/threonine phosphatase to be identified. The occurrence of PPY and testis specific isoforms of other protein phosphatases suggest that protein serine/threonine phosphorylation and dephosphorylation is particularly important for the regulation of cellular functions specific to the testis.

**PPY predominantly localises to nuclei of somatic cyst cells in the testis**

Since PPY maps to 55A1-3 (Dombrádi et al., 1989a), we examined two male sterile mutants, *halley* (Castrillon et al., 1993) and P571, in an attempt to define the function of PPY. Both mutants carry P elements at 55A, but analyses of PPY mRNA in these mutants reveal no differences from controls in size or concentration. An immunological approach was therefore taken to investigate the function of PPY. Immunohistochemical analyses, using two different anti-PPY anti-
bodies, indicate that PPY is located to somatic cyst cells in the larval and adult testes. Subcellularly, PPY is predominantly present in the nuclei of these cells. Cyst cells are derived from somatic cyst progenitor cells which are anchored at the apical tip of the testis. At the onset of spermatogenesis two somatic cyst progenitor cells divide asymmetrically to yield two cyst cells which encase a single germ cell. The cyst cells remain associated with the developing germ cells throughout germ cell division and differentiation to mature spermatocytes, which occurs in an apical to terminal progression along the coiled length of the testis. The cyst cells do not themselves undergo cell division during spermatogenesis but differentiate into head cyst cells which remain small and surround the sperm head nuclei and tail cyst cells which become elongated and surround the growing sperm tails. In parallel, the nuclei of the head cyst cells remain small and rounded, while the nuclei of the tail cyst cells become thin and elongated. At the completion of spermatogenesis, the cyst cells are phagocytosed by specialised terminal epithelial cells and the mature spermatoozoa are released into the seminal vesicles. Since PPY is absent from the apical tip of the testis where the cyst cells divide, but is present in the more distal regions of the testis when cyst cell division has ceased, it is possible that the predominantly nuclear PPY might play a role in the arrest of cyst cell division.

The precise function of the cyst cells in Drosophila testes is not known, but there is evidence that they are required for spermatogenesis. Mutation of the gene encoding β3 tubulin, a specialised isoform of tubulin that is found in cyst cells but not germ cells (Kimble et al., 1989), results in a male sterile phenotype (Kimble et al., 1990). Therefore β3 tubulin, and by implication cyst cell function, is essential for normal spermatogenesis. Head cyst cells play a role in the process by which mature spermatoozoa are encased in a membrane before being released into the seminal vesicle (Fuller, 1993). However, since tail cyst cells are not involved in this function, it is unlikely that the main function of PPY, located in both head and tail cyst cells, is to participate in this process. A major role of cyst cells is to act as a support role for the developing germ cells (Fuller, 1993) perhaps supplying nutrients to them. A possible function of PPY in the nucleus might therefore be to increase transcription of genes required for a continuous supply of nutrients.

Studies employing germ cell transplantation techniques showed that germ cell development in male and female Drosophila is controlled by signals from the somatic cells as well as the germ cells themselves (Steinmann-Zwicky, 1989). When XX pole cells (precursor of germ cells) from wild-type embryos are transplanted into XY oskary301 embryos, which fail to produce any viable pole cells, the transplanted pole cells undergo spermatogenesis, indicating that inductive signals from the somatic environment are influencing sexual identity. In contrast, XY germ cells undergo spermatogenesis in an XX host ovary indicating that XY cells contain autonomous information concerning their sexual identity. However, the XY cells only develop as far as the primary spermatocyte stage in an XX host, which suggests that although they possess the appropriate information to undergo mitosis, they require some inductive signal from the testis soma to enable completion of spermatogenesis. Since cyst cells are intimately associated with the germ cells, they would seem to be the ideal somatic candidate for such a signal (Fuller, 1993). The temporal expression of PPY in the cyst cells is consistent with the timing of a putative somatic signal leading to spermatocyte differentiation.

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