Characterisation of a novel *Drosophila melanogaster* testis specific PP1 inhibitor related to mammalian inhibitor-2: identification of the site of interaction with PP1

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Abstract A novel *Drosophila melanogaster* protein, termed inhibitor-t, that bears 41% sequence similarity to human protein phosphatase inhibitor-2 has been identified using human protein phosphatase 1 (PP1) in the yeast two hybrid system. Inhibitor-t mRNA is detected in adult males, larvae and pupae and the 184 amino acid thermostable protein located only in testis. The gene for inhibitor-t maps to cytological location 86F1 on the third chromosome. Bacterially expressed inhibitor-t specifically inhibits both mammalian and *D. melanogaster* PP1 catalytic subunits with an IC_{50} of approximately 200 nM. A motif -FEX_1X_2RK-, conserved between inhibitor-t, inhibitor-2 and its *Saccharomyces cerevisiae* homologue Glc8, is demonstrated to be required for binding to PP1.

Key words: Protein phosphatase; Inhibitor; Two hybrid system; cDNA sequence; *Drosophila melanogaster*; Testis

1. Introduction

Protein phosphorylation is a ubiquitous mechanism by which eukaryotic cellular functions are regulated, the control of protein phosphatases being an integral part of this process [1,2]. To achieve this regulation, the catalytic subunit of PP1 (PP1c) associates with numerous regulatory subunits in vivo, the function of which is to target PP1c to specific subcellular locations, alter its substrate specificity and to allow its activity to be modified by extracellular signals [3]. Targeting subunits that have been characterised and allow PP1c to regulate the enzymes of glycogen metabolism include the G_M subunit that targets PP1c to both glycogen particles and the sarcoplasmic reticulum in striated muscle [4,5], the G_L subunit that targets PP1c to glycogen in liver [6], as well as two more widely distributed glycogen targeting subunits PP1R5/PTG [7,8] and PP1R6 [9]. Myosin targeting subunits that enable PP1c to regulate myosin contractility have been characterised in skeletal [10] and smooth [11] muscle. Other PP1c interacting subunits that are likely to allow PP1c to regulate a wide variety of processes include the nuclear proteins NIPP-1 [12] and p99/PNUTS [13,14], which may bind to RNA, an RNA splicing factor PSF1 [15], sds22, which is required for exit from mitosis [16], and the retinoblastoma protein [17]. The ribosomal proteins L5 [18] and RIPP1 [19], the p53 binding protein 53BP2 [20], spinophilin, which is present in dendrites [21,22], the microtubule motor KLP38B [23], phosphofructokinase [24] and the g134.5 of *Herpes simplex* virus 1 that influences protein synthesis [25] have also been shown to bind PP1c. Interaction of the different regulatory subunits with PP1c is mutually exclusive, an observation recently explained by the discovery that a short motif -(R/K)(V/I)XF- present in the majority (but not all) of these subunits was sufficient for binding to PP1c [26,27].

In addition to the above regulatory subunits, PP1c binds to a number of small cytosolic inhibitor proteins including inhibitor-1 (I-1), inhibitor-2 (I-2), DARPP-32 (reviewed in [1,28]) and CPI17 [29], which inhibit PP1c activity at nanomolar concentrations. Although I-1 and DARPP-32 bind to PP1c via the sequence -KIQF-, the site of I-2 binding to PP1c is unknown.

In an attempt to uncover novel regulatory subunits of PP1 we have performed yeast two hybrid screens using the human $PP1\gamma_1$ catalytic subunit, which has identified several human proteins including I-2 [30] and 53BP2 [20]. In this communication we describe the identification of a novel *Drosophila melanogaster* testis specific PP1 inhibitor using the yeast two hybrid screen. This inhibitor shares sequence similarity with mammalian I-2 that identifies a novel short motif required for the binding of I-2 and related proteins to PP1.

2. Materials and methods

2.1. Identification of D. melanogaster PP1 interacting proteins using the yeast two hybrid system

Microbial strains and methods for the yeast two hybrid screen using PP1 as bait were as previously described [30]. To isolate *Drosophila* proteins capable of interacting with PP1, the yeast strain Y190 containing a pAS2-$PP1\gamma_1$ plasmid was transformed with DNA from a pACT library containing *D. melanogaster* third instar larval cDNA sequences. After growth on selection medium for 12 days, 10 colonies representing 0.008% of the cells transformed were obtained. The pACT plasmids from all 10 clones were recovered into *Escherichia coli* and their cDNA inserts, termed PP1D1–PP1D10, sequenced.

2.2. General methods

Oligonucleotides were synthesised by Miss Audrey Gough (University of Dundee) on an Applied Biosystems model 394 DNA synthesiser. DNA sequencing was performed on an Applied Biosystems 373A automated DNA sequencer using Taq dye terminator cycle sequencing. Screening of 1.5×10^5 pfu of a *D. melanogaster* Canton S genomic library constructed in bacteriophage λ EMBL-3 SP6/T7 vector (Clontech, Palo Alto, CA) with a 600 bp *Eco*RI fragment of from the 5′ end of the PP1D6 cDNA was performed under high stringency conditions. A 4 kb *Eco*RI fragment and a 2.2 kb *Eco*RI-*Xho*I fragment present in three of four positive genomic clones identified were subcloned into pBluescript KS and sequenced. Total RNA was purified from

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D. melanogaster (Oregon R strain) developmental stages and subjected to formaldehyde gel electrophoresis and Northern blotting as described in [31] using 'Rapid-hyb' buffer (Amersham Life Science, Bucks, UK).

D. melanogaster protein extracts were prepared and immunological analyses performed as in [31]. Extracts were heated at 100°C as described for *E. coli* extracts (Section 2.3).

2.3. Bacterial expression and purification of inhibitor-t and production of antibodies

The 2.2 kb genomic *Eco*RI-*Xho*I fragment in pBluescript KS which contained the entire open reading frame (ORF) of inhibitor-t was used as a template in a polymerase chain reaction (PCR) with the oligonucleotide primers 5′-GCGCCATATGCATAAGAACTTCAAAAGTCTG-3′ and 5′-GCGCGTCGACTAACCGCCAAAACCCTTGG-3′ that incorporate *Nde*I and *Sal*I restriction endonuclease sites (underlined) at the initiating and terminating codons of the ORF respectively. The PCR product was cleaved with these enzymes, inserted into the same sites of the *E. coli* expression vector pT7-7 [32] to generate plasmid pT7-inhibitor-t and sequenced. Following expression in *E. coli* in the presence of 100 μM IPTG for 4 h, the bacteria were treated as for I-2 [30], except that buffer A was 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 0.02% (v/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol plus 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM benzamidine. The supernatant obtained was applied to a Mono S HR 5/5 column (Pharmacia Ltd, Milton Keynes, UK), which was developed with a 25 ml 0–400 mM linear NaCl gradient in buffer A at room temperature. Inhibitor-t, which eluted at 290 mM NaCl, was concentrated using a Centricon 10 (Amicon, Beverly, MA) and stored at −70°C. To generate a fusion between I-2 and inhibitor-t, the human I-2 cDNA was used in a PCR with primers 5′-GCGCCATATGGCGGCCTCGACGGCCTC-3′ and 5′-CCGGCCGCGGCCTCGTCGACATTCCCACGGGGCTG-3′ that generated *Nde*I and *Sac*II sites (underlined) at the initiating methionine and downstream from amino acid 38 respectively. The *Sac*II containing primer enabled mutation of an endogenous *Sac*II site within the I-2 cDNA. The PCR product was cloned into the pT7-inhibitor-t construct using these sites and sequenced. Expression and purification of the fusion protein was performed as for inhibitor-t. A single peak of I-2/I-t fusion protein eluted at 180 mM NaCl. Phe^{133} within the I-2/I-t protein was mutated to Ala using the 'QuikChange' (Stratagene) site-directed mutagenesis kit and the mutated protein purified as described for I-2/I-t.

Antibodies against inhibitor-t were raised in sheep at the Scottish Antibody Production Unit (Carluke, Lanarkshire, UK) using the full length protein expressed in *E. coli* or synthetic peptides (HKNFKSLQNTNPSNC and CDQNNIKFSKGFGG) conjugated to keyhole limpet haemocyanin. Antibodies raised against the whole protein were affinity purified using either the full length protein or two glutathione *S*-transferase (GST) fusion proteins containing amino acids 1–92 or 120–184 of inhibitor-t and antibodies against synthetic peptides were affinity purified as previously described [33].

2.4. Expression of PP1 catalytic subunits in E. coli

Human PP1γ_1 was expressed and purified as described in [34]. *Drosophila* PP1–87B catalytic subunit was expressed using the PP1–87B cDNA in pBluescript KS as a template in a PCR with oligonucleotide primers 5′-GCGCCATATGGGCGACGTGATGAATATC-3′ and T7 to introduce a *Nde*I site (underlined) at the initiating methionine, then cloned into the plasmid pCR2.1 using the 'TOPO' cloning kit (Invitrogen) and sequenced. The construct was cleaved with *Nde*I and *Xba*I (present in the polylinker of pCR2.1) and inserted into the same sites of plasmid pCW to generate the construct pCWPP1–87B. PP1–87B was expressed in *E. coli* and purified as in [34]. Homogeneous PP1–87B had a specific activity of 48 U/mg against phosphorylase *a* in the presence of 0.5 mM Mn^{2+} ions.

2.5. Protein phosphatase and inhibitor assays

Phosphatase assays were performed in the presence of 0.5 mM Mn^{2+} (PP1γ_1 and PP1–87B) or in the absence of divalent cations (PP2Ac) using ^{32}P-labelled rabbit skeletal muscle glycogen phosphorylase [35] or ^{32}P-phosphorylated myosin P-light chains [26]. One unit of phosphatase activity is that amount of enzyme that catalyses the release of 1 μmol [^{32}P] phosphate/min from phosphorylase *a*. Microcystin-LR, okadaic acid, inhibitor-2 and inhibitor-t were assayed as in [36].

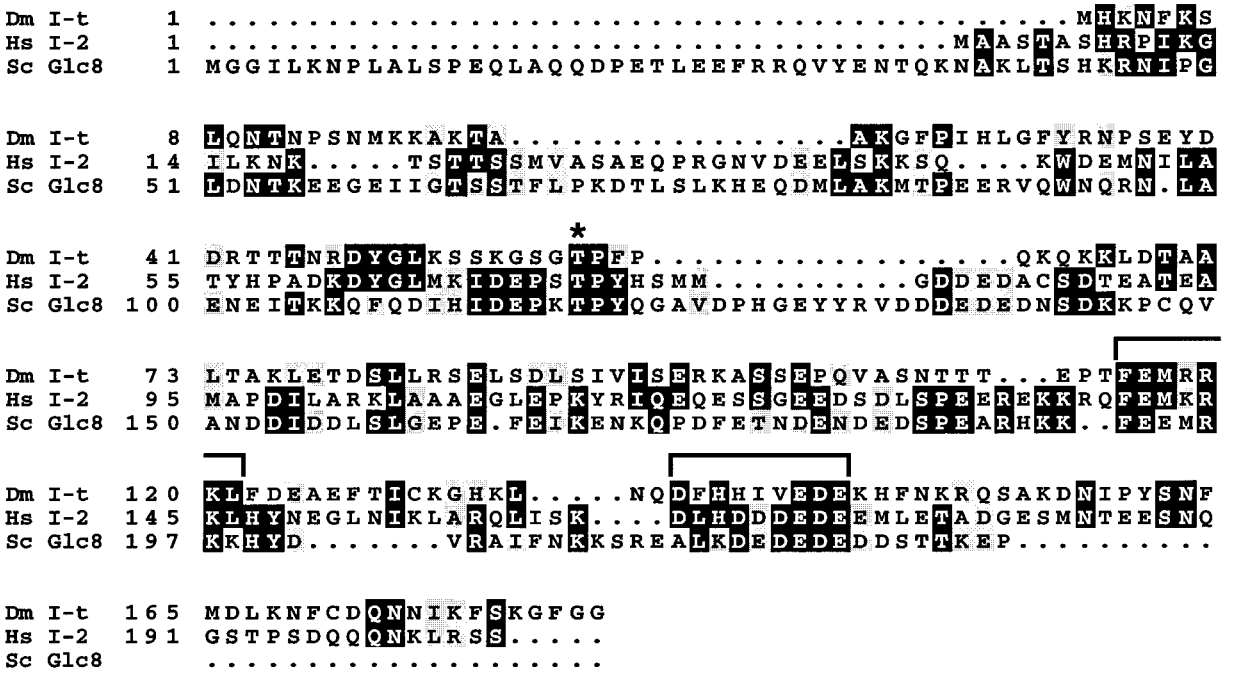

Fig. 1. Comparison of *D. melanogaster* inhibitor-t (top), human I-2 (middle) and *S. cerevisiae* Glc8 (bottom) protein sequences. Identical residues are boxed in black, while conserved residues are boxed in grey. Threonines 59 of inhibitor-t, 72 of human I-2, and 118 of Glc8 are indicated (*), while other areas of sequence conservation are indicated by brackets. The comparison was created using the program PILEUP (Genetics Computer Group, Madison, WI, USA) and shading was performed using the program BOXSHADE (v3.21, K. Hofmann and M. Baron).

3. Results

3.1. Identification and sequence of PP1 inhibitor-t

Ten yeast colonies positive on selective medium were obtained from a yeast two hybrid analysis using human PP1γ_1 to screen a *D. melanogaster* larval cDNA expression library. Plasmids from two of these colonies, designated PP1D6 and PP1D10, contained overlapping cDNA sequences encoding 181 and 174 amino acids respectively of a novel protein. Since the PP1D6 and PP1D10 cDNAs did not contain the full coding sequence of the novel protein, genomic clones were identified from a *D. melanogaster* genomic library by screening with a probe from the 5′ end of the PP1D6 cDNA. A 2.2 kb *Eco*RI-*Xho*I genomic fragment contained the full open reading frame of the gene, which has been submitted to the GenEMBL database under the accession number AJ006867. The complete open reading frame encodes a protein of 184 amino acids with a predicted molecular weight of 20.5 kDa. A consensus *Drosophila* transcription start site sequence (GTCAGTT [37]) is found between nucleotides −61 and −67 with respect to the initiating methionine and is preceded by a number of TATA promoter elements. Within the 3′ untranslated region of PP1D6 and PP1D10 cDNAs as well as in the gene sequence there exist three consensus polyadenylation signals.

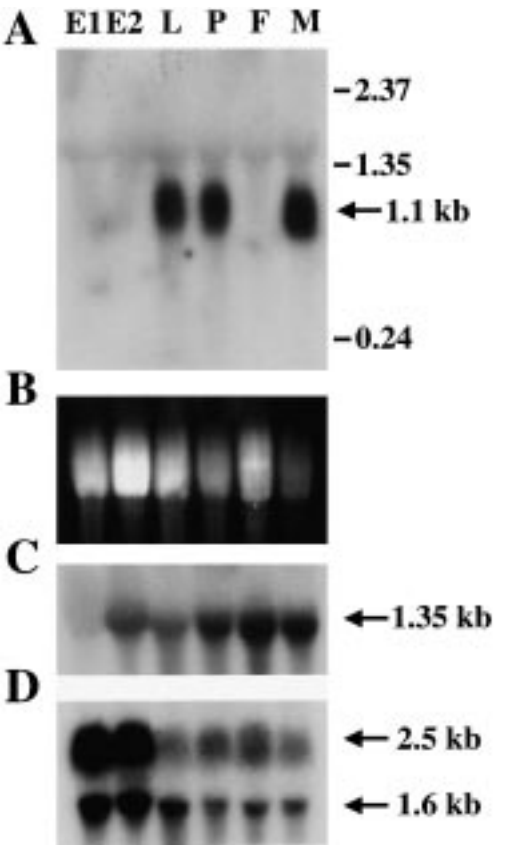

Fig. 2. Expression of *inhibitor-t* mRNA during the *Drosophila* life cycle. 20 μg of total RNA from the developmental stages shown was separated on a denaturing 1% agarose gel and transferred to nylon membrane. The membrane was probed with ^{32}P-radiolabelled 600 bp *Eco*RI fragment from the *PP1D6* cDNA (A). It was subsequently probed with cDNAs for human β-actin (Clontech) (C) and *D. melanogaster* protein phosphatase 1–87B [40] (D), since these mRNAs are expressed fairly uniformly during development. B: RNA stained with ethidium bromide and visualised by ultraviolet light. Lanes are: E1, 0–4 h embryos; E2, 4–24 h embryos; L, third instar larvae; P, pupae; F, adult female; M, adult male. Sizes are in kb. Transcripts are indicated (arrows).

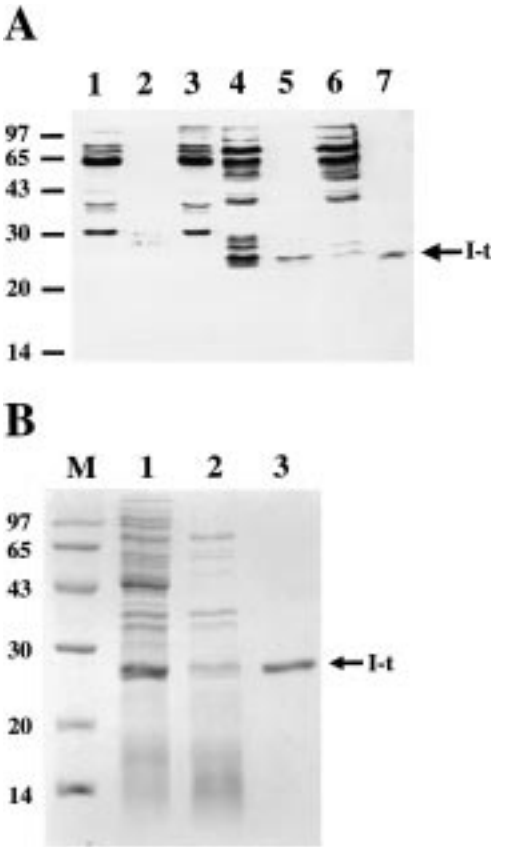

Fig. 3. Immunoblotting of *D. melanogaster* protein extracts and purification of inhibitor-t expressed in *E. coli*. A: *D. melanogaster* extracts were separated on a 12.5% acrylamide SDS-PAGE, transferred to nitroclellulose membrane and probed with affinity-purified anti-inhibitor-t whole protein antibody at a concentration of 200 ng/ml, followed by anti-sheep-HRP conjugate. Signal was detected using enhanced chemiluminescence. Lanes are: 1, 40 μg adult male *D. melanogaster* whole body minus testis soluble extract; 2, supernatant from 40 μg of this extract after heating at 100°C and centrifugation; 3, pellet from the heated extract; 4, 40 μg of adult male *D. melanogaster* testis soluble extract; 5, supernatant from 40 μg of this extract after heating at 100°C and centrifugation; 6, pellet from the heated extract; 7, 100 pg of inhibitor-t expressed in *E. coli*. Inhibitor-t is indicated (arrow). B: Samples from the stages of purification shown were separated on a 12.5% SDS-PAGE and stained with Coomassie brilliant blue R250 to visualise the proteins. Lanes are: M, markers; 1, 20 μg of whole cell soluble extract from *E. coli* expressing inhibitor-t; 2, 2.5 μg of supernatant from the extract heated at 100°C; 3, 2 μg of inhibitor-t eluted from Mono S. Marker sizes are in kDa. Inhibitor-t is indicated (arrow).

Database searching revealed no proteins with high sequence identity to the 20.5 kDa *Drosophila* protein. However, it did show 21% identity (41% similarity) to PP1 inhibitor-2 (I-2) [30,38] and 18% identity (34% similarity) to its yeast homologue Glc8 [39] (Fig. 1) in pairwise comparisons using the GAP algorithm (Genetic Computer Group, Madison, WI, USA). These figures are comparable to those for I-2 and Glc8 (26% identity and 43% similarity). The novel 20.5 kDa *Drosophila* protein was therefore termed PP1 inhibitor-t (I-t) since, unlike I-2, it was also shown to be testis specific (see below).

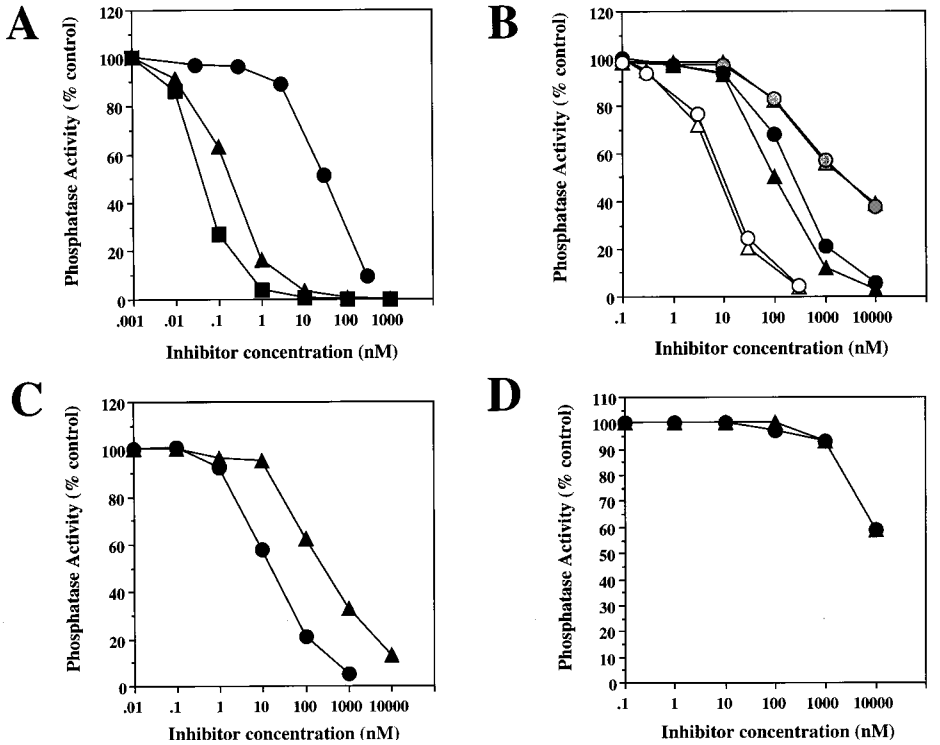

Fig. 4. Properties of expressed PP1-87B phosphatase and effect of inhibitor-t on activity of various phosphatase catalytic subunits compared with known inhibitors of PP1γ_1 and PP1–87B. Phosphatase activity is given as a percentage of the maximum obtained in the absence of any inhibitors. A: Effect of human I-2 (triangles), okadaic acid (circles) and microcystin-LR (boxes) on the phosphorylase *a* phosphatase activity of PP1–87B catalytic subunit. B: Inhibition of phosphorylase *a* phosphatase activity of PP1γ_1 (circles) and PP1–87B (triangles) by inhibitor-t (closed symbols), I-2/I-t wild type fusion protein (open symbols) and I-2/I-t Phe^{133}Ala mutant fusion protein (shaded symbols). C: Inhibition of phosphorylase *a* (triangles) and myosin P-light chain (circles) phosphatase activity of PP1γ_1 by inhibitor-t. D: Inhibition of phosphorylase *a* phosphatase activity of PP2Ac by I-2 (circles) and inhibitor-t (triangles). In all assays the maximal release of ^{32}P from substrates in the absence of inhibitors was 10%.

3.2. Expression of PP1 inhibitor-t mRNA and protein in D. melanogaster

Analysis of RNA during the *D. melanogaster* life cycle revealed that an inhibitor-t cDNA probe hybridised with a 1.1 kb mRNA transcript that could be detected in larvae, pupae and adult males (Fig. 2A). Longer exposures failed to detect this transcript in either embryos or adult females. Since larvae and pupae were not sexed prior to extracting RNA, it is likely that the signal seen at these developmental stages represents a mRNA that is male specific as in the adult. Approximately equal amounts of RNA were present in all lanes (Fig. 2B).

Antibodies against the inhibitor-t protein failed to detect a band of the correct size in *D. melanogaster* extracts made from whole male flies (data not shown), but when separate extracts were prepared from testes and the remainder of the male flies, the inhibitor-t antibodies recognised a protein of 25 kDa in the testis extract (Fig. 3A, lane 4) and not in the extract made from the other tissues (Fig. 3A, lane 1). Since inhibitor-2 is known to be thermostable, we examined whether endogenous inhibitor-t also possessed this property by heating extracts at 100°C. The 25 kDa band seen in whole testis extract was present in the soluble fraction after heating (Fig. 3A, lane 5). Proteins that crossreacted with the anti-inhibitor-t antibodies, which were abundant as judged by Ponceau S staining, were not seen since they were insoluble after heat treatment (Fig. 3A, lane 6). Comparison of the immunostaining intensity for inhibitor-t in the heat treated testis supernatant (Fig. 3A, lane 5) with the positive control (Fig. 3A, lane 7) indicates that inhibitor-t represents only approximately 0.0025% of the total testis protein. Even if the level of inhibitor-t is estimated from the band migrating at that position in the total soluble extract prior to heat treatment (Fig. 3A, lane 4) this estimate only rises to 0.005% of the total protein. To confirm that the 25 kDa band seen in testis extracts was inhibitor-t, anti-inhibitor-t antibody was pre-incubated with a 20-fold molar excess of bacterially expressed inhibitor-t prior to adding to the immunoblot. This treatment completely abolished the signal in the testis extract (not shown).

Although endogenous inhibitor-t migrates with an apparent molecular mass of 25 kDa, which is larger than was expected from its sequence (20.5 kDa), the apparent size is identical to that of the bacterially expressed inhibitor-t (Fig. 3A, lane 7). It should also be noted that mammalian I-1 and I-2 migrate anomalously on SDS-PAGE [1,38]. A specific subcellular immunolocalisation of inhibitor-t in *D. melanogaster* testis could not be obtained, presumably due to the low abundance of inhibitor-t.

3.3. Properties of inhibitor-t

Bacterially expressed inhibitor-t was purified utilising its thermostable nature and fast protein liquid chromatography on Mono S, eluting as a single peak of protein that was homogeneous as judged by SDS-PAGE (Fig. 3B). Inhibitor-t was found to inhibit both human PP1γ_1 and *D. melanogaster* PP1-87B (the major PP1 catalytic subunit isoform [40]) with an IC_{50} of approximately 200 nM when mammalian phos-

phorylase *a* was used as a substrate (Fig. 4B). Inhibitor-t was unable to inhibit the catalytic subunit of human PP2A (Fig. 4D), or the catalytic subunit of the *D. melanogaster* testis specific PP1-like phosphatase PPY (data not shown) [31] over the concentration range that inhibited PP1c, indicating that the inhibition was specific for PP1c. When phosphorylated myosin P-light chains were used as a substrate, inhibitor-t was found to inhibit PP1 more potently with an IC_{50} of approximately 20 nM (Fig. 4C), suggesting that the degree of inhibition is partially substrate dependent. Previous deletion studies indicate that the amino-terminal 35 residues of I-2 are involved in the inhibition of PP1 [41]. From the sequence comparison (Fig. 1), it can be seen that residues 1–20 of inhibitor-t are aligned with residues 1–38 of I-2. We therefore replaced the amino-terminal 20 residues of inhibitor-t with 38 residues from the amino-terminus of human I-2. Fig. 4B shows that the I-2/I-t fusion protein is over 10-fold better than inhibitor-t at inhibiting both PP1γ_1 and PP1–87B, with an IC_{50} of approximately 10 nM when phosphorylase *a* is the substrate.

Comparison of inhibitor-t with I-2 and Glc8 shows that a group of basic residues and a region of acidic residues towards the C-termini of all three proteins are highly conserved (marked in Fig. 1). Previous results have shown that residues after Leu^{146} of I-2 are not required for inhibition of PP1 [41], thus eliminating the acidic region as essential for inhibition of or binding to PP1. We therefore chose to mutate the conserved Phe^{133} residue (Phe^{115} in inhibitor-t) in the I-2/I-t fusion protein to Ala and then to study the efficacy of this protein as a PP1 inhibitor. Fig. 4B shows that the mutant I-2/I-t protein exhibited an IC_{50} of about 2 μM against PP1γ_1 when phosphorylase *a* was used as a substrate. This is 200-fold higher than for the wild type protein and indicates that this Phe residue within inhibitor-t, and therefore probably also within I-2 and Glc8, is important for inhibition or binding of PP1. Immunoprecipitation experiments with pure PP1γ_1 and the I-2/I-t fusion proteins showed that, whilst the wild type I-2/I-t fusion protein was efficiently co-immunoprecipitated with PP1γ_1, the Phe^{133}Ala mutant failed to be recovered in the pellet (data not shown). This result shows that Phe^{115} of inhibitor-t and the corresponding residues of I-2 and Glc8 are likely to be important for binding of these proteins to PP1.

I-2 can be phosphorylated on Thr^{72} by glycogen synthase kinase-3 (GSK-3) [42], which results in activation of a complex of PP1c and I-2 termed PP1I. Since inhibitor-t contains sequence around Thr^{59} that show high conservation to that surrounding Thr^{72} in I-2, we examined the phosphorylation of the former. GSK-3 phosphorylated inhibitor-t up to a stoichiometry of 1 mol phosphate/mol inhibitor-t and protein sequencing identified a single phosphorylated residue that was Thr^{59} (data not shown).

3.4. Localisation of the inhibitor-t gene on D. melanogaster chromosomes

In situ hybridisation to *D. melanogaster* polytene chromosomes revealed that the *inhibitor-t* gene is present at 86F1 on the third chromosome (data not shown). A search of the FlyBase database (University of Indiana) did not reveal any candidate mutants at this location and analysis of a panel of P element insertions mapping to this location (kindly provided by Dr P. Deak, University of Dundee) failed to identify an insertion event within the *inhibitor-t* gene.

4. Discussion

We have previously used the yeast two hybrid system to identify mammalian proteins capable of interacting with human PP1c [20,30]. Given the high level of sequence identity between mammalian and *D. melanogaster* PP1cs [40], it seemed likely that a mammalian PP1c would interact with *D. melanogaster* PP1c binding proteins in the yeast two hybrid system. Two out of the 10 *Drosophila* clones identified in the screen with human PP1γ_1 contained overlapping sequences, indicating that they encoded a bona fide PP1 binding protein. The corresponding gene sequence specified a novel protein with sequence similarity to mammalian I-2 and its *Saccharomyces cerevisiae* homologue Glc8. I-2 is a specific inhibitor of PP1c that has been identified in a wide range of eukaryotic organisms [1,38]. However, inhibitor-t is not the *D. melanogaster* homologue of I-2, since we have shown that inhibitor-t is testis specific, while I-2 has been identified in all mammalian tissues so far examined [43,44]. In addition, I-2 activity has been detected in *D. melanogaster* head extracts as well as in the whole organism [45]. Inhibitor-t may therefore represent a specialised form of I-2 that is required for some aspect of PP1c regulation in the testis. A testis-specific isoform of mammalian PP1, termed PP1γ_2, has been identified [46]. PP1γ_1 and PP1γ_2 are produced from the same gene by alternative mRNA splicing, resulting in them possessing different C-termini, but PP1γ_1 has a wide tissue distribution [46]. *D. melanogaster* possesses four isoforms of PP1c [40], although it remains to be seen whether any of these are enriched in testis.

Although inhibitor-t does specifically inhibit PP1c, its IC_{50} of 200 nM with phosphorylase *a* as substrate is around two orders of magnitude higher than that of I-2 ($IC_{50} \approx 2$ nM). However, the IC_{50} of Glc8 was found to be at least one order of magnitude higher than that measured for I-2 [47]. In addition, given that inhibitor-t is not the *D. melanogaster* homologue of mammalian I-2 and may play an alternative role in the regulation of PP1c function from I-2, the higher inhibition constant is not necessarily of concern. The effect of inhibitor-t on PP1c activity is also substrate dependent, a much lower IC_{50} ($\sim$20 nM) being measured when phosphorylated myosin P-light chains were used as substrate.

Previous truncation studies have suggested that the N-terminal 1–35 amino acids of I-2 are important for potent inhibition of PP1c [41], although an alteration in the truncated I-2 conformation could not be ruled out. We show that a chimaeric protein consisting of the first 38 amino acids of I-2 fused to residues 19–184 of inhibitor-t is a much better inhibitor of PP1 than inhibitor-t itself, demonstrating that these residues of I-2 are indeed crucial for potent inhibition and may possess a low affinity binding site. However, the N-terminal 38 residues of I-2 are insufficient to maintain binding of the I-2/I-t Phe-Ala mutant to PP1.

Comparison of the structures of inhibitor-t, I-2 and Glc8 is of interest because conservation of amino acids across evolutionarily diverse species may identify functionally important regions. The region immediately around Thr^{72} of I-2 that is phosphorylated by GSK-3 [42] and MAP kinase [48], is conserved in inhibitor-t and Glc8. This phosphorylation event is critical for the activation of an inactive complex of PP1 and I-2 (termed PP1I), which is postulated to represent a pool of PP1 maintained ready for targeting subunits to interact with when required [1,2]. More recently, it has been demonstrated

that complex formation of PP1 and I-2 followed by phosphorylation of Thr^{72} by GSK-3 is required for the correct folding of bacterially expressed PP1, suggesting that it may be required for the folding of newly synthesised PP1 into its native conformation in vivo [34,49]. Phosphorylation of Thr^{72} by MAPK has been postulated to play a role in the activation of cytosolic PP1 by growth factors [48]. Here, we have shown that inhibitor-t can be phosphorylated on Thr^{59} (corresponding to Thr^{72} in I-2) by GSK-3 in vitro. It therefore seems likely that inhibitor-t will be phosphorylated on Thr^{59} in vivo and that this will have implications for its function.

Other regions that are conserved between inhibitor-t, I-2 and Glc8 might be expected to be important in binding to PP1 and/or weak inhibition of PP1. Two regions exist towards the C-terminus of inhibitor-t that are also conserved in I-2 and Glc8 (Fig. 1). Previous studies on I-2 indicate that the second, acidic, region is not necessary for inhibition of PP1, but may be important for activation of PP1/I-2 complexes by GSK-3 [41]. This suggests that the first conserved region (residues 140–145 in I-2) could be a PP1 binding motif with the consensus sequence -FEX_1X_2RK-. Mutation of the conserved Phe residue within this motif in the I-2/I-t fusion protein resulted in almost complete loss of inhibition of PP1 by this chimaeric inhibitor protein. Furthermore, we have shown that mutated I-2/I-t is unable to bind to PP1, thus explaining the loss of inhibition and more importantly implicating the -FEX_1X_2RK- motif in the association of PP1 with I-2, inhibitor-t and Glc8, although there may be some restrictions on the amino acids that can be tolerated at X_1 and X_2. Over half the known PP1 binding proteins interact with PP1 via an -(R/K)(V/I)XF- motif or slight variation thereof [13]. It will be interesting to determine if the -FEX_1X_2RK- motif is used by some other PP1 binding proteins.

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