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Drosophila melanogaster protein phosphatase inhibitor-2: identification of a site important for PP1 inhibition

Nicholas R. Helps*, Patricia T.W. Cohen

Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, MSI/WTB Complex, University of Dundee, Dundee DD1 5EH, UK

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Abstract A database search with human protein phosphatase inhibitor-2 (I-2) identified a *Drosophila melanogaster* cDNA that encoded a protein identical in length and sharing 39% amino acid identity (58% similarity) with human I-2. The mRNA encoding this protein is expressed in both sexes and throughout development, unlike *Drosophila* inhibitor-t. The bacterially expressed protein was a specific inhibitor of protein phosphatase 1 with an IC_{50} of < 1 nM, confirming that it is the *Drosophila* homologue of mammalian inhibitor-2. Mutation of Phe residues conserved in I-2 from lower and higher eukaryotes showed that Phe-33 is important for inhibition of PP1c.

Key words: Inhibitor-2; Protein phosphatase; cDNA sequence; *Drosophila melanogaster*

1. Introduction

Protein phosphatase 1 (PP1) is a major protein serine/threonine phosphatase involved in the regulation of numerous processes such as cell division, metabolism, RNA splicing, protein synthesis and muscle contraction (reviewed in [1,2]). The PP1 catalytic subunit (PP1c) is controlled in vivo through its interaction with a diverse group of targetting subunits, which serve to tether PP1c to a particular subcellular location and/or modulate its catalytic activity. In skeletal muscle PP1c is targetted to glycogen and the sarcoplasmic reticulum by a 126 kDa targetting subunit known as G_M [3,4]. This binds to glycogen with high affinity and enhances dephosphorylation of glycogen-bound substrates such as glycogen synthase and phosphorylase. A distinct 33 kDa glycogen targetting subunit (G_L) exists in the liver [5]. In smooth muscle PP1c is targetted to myofibrils by a complex consisting of a 110 kDa and a 21 kDa subunit [6]. This heterotrimer, known as PP1M, has enhanced activity towards smooth muscle myosin compared with PP1c. Striated muscle also contains a PP1M complex that targets PP1c towards skeletal muscle myosin [7]. Many other PP1c targetting subunits have been identified recently, including NIPP1 [8] and PNUTS/p99 [9,10], which target PP1c to RNA and the p53 binding protein 53BP2 [11].

In addition to a wide variety of regulatory subunits, PP1 binds to a number of small cytosolic inhibitor proteins. Classical inhibitors include inhibitor-1 (I-1) and its isoform DARPP-32 that are thought to enhance the effects of cyclic AMP signalling through protein kinase A [1,2] and the structurally distinct inhibitor-2 (I-2), which may promote the correct folding of newly synthesised PP1 [12]. Recently several novel PP1 inhibitor proteins have been isolated, including CPI17, a mammalian smooth muscle-specific PP1 inhibitor [13], the human HCG V gene product (inhibitor-3) [14] and inhibitor-t, a testis-specific PP1 inhibitor from *Drosophila melanogaster* that has 41% sequence similarity to mammalian I-2 [15]. Homologues of I-2 are known from human, rabbit and rat species [16,17] and Glc8p is the likely homologue of I-2 from *Saccharomyces cerevisiae* [18]. Studies performed some years ago showed that an activity possessing the characteristics of I-2 could be identified in *D. melanogaster* [19]. Here we have characterised a protein that possesses this activity in *D. melanogaster* (Dm) and shown that a conserved Phe residue in the N-terminal region of Dm I-2 is essential for potent inhibition of PP1c.

2. Materials and methods

2.1. Identification and sequence analysis of the cDNA encoding *D. melanogaster* I-2

The National Center for Biotechnology Information (NCBI) expressed sequence tag database (dbEST) was searched with the human I-2 protein sequence using the TBLASTN algorithm [20]. Five cDNA sequences from two different *D. melanogaster* libraries were identified that encoded portions of a polypeptide with significant sequence identity to human I-2. One of these (clone ID GH10368) was obtained from the Berkeley *Drosophila* Genome Project (DBGP) via Research Genetics, Inc. (Huntsville, AL, USA) and digested with *Eco*RI and *Xho*I to release the cDNA insert. A complex pattern of DNA fragments with a total length of approximately 4.5 kb (not shown) was obtained. The cDNA encoding I-2 was analysed by primer-walking on the presumed coding strand. Sequencing was performed by Dr A. Hill (MSI/WTB complex central DNA sequencing service, University of Dundee) on an Applied Biosystems model 373AStretch automated DNA sequencer using BigDye terminator chemistry.

2.2. General methods and reagents

Oligonucleotides were synthesised by Miss K. Maxwell (University of Dundee) on an Applied Biosystems model 394 DNA synthesiser. Synthetic peptides were synthesised on an Applied Biosystems 430A peptide synthesiser and purified by Mr F.B. Caudwell. Total RNA was purified from *D. melanogaster* (Oregon R strain) and subjected to formaldehyde gel electrophoresis and Northern blotting as described in [21] using 'Rapid-hyb' buffer (Amersham Life Science, Bucks, UK).

2.3. Bacterial expression and purification of *D. melanogaster* I-2

The 615 nt open reading frame (ORF) encoding the entire inhibitor-2 protein (205 amino acids) was amplified in a polymerase chain reaction (PCR) using the 1503 nt Dm I-2 cDNA as a template. The oligonucleotide primers GCCCAT*ATG*CAGAACAATCCCAGCC and GCTCTAGA*CTA*GTTATTCGATGGCTCCAG incorporate *Nde*I and *Xba*I sites (underlined) at the initiating and terminating

*Corresponding author. Fax: (44)-1382-223778.
E-mail: n.r.helps@dundee.ac.uk

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codons (italicised) respectively. The PCR product was cloned into the plasmid pCR2.1 using the TOPO cloning system (Invitrogen) and the sequence of the insert confirmed. Due to technical difficulties encountered when we attempted to clone the ORF into the pT7-7 vector [22] using the *Nde*I and *Xba*I sites, we instead utilised a *Hin*dIII site in the pCR2.1 plasmid multiple cloning site to clone the Dm I-2 ORF into pT7-7 on *Nde*I and *Hin*dIII sites. Following sequence confirmation, the construct was transformed into the *Escherichia coli* strain BL21(DE3)pLysS and transformants selected on LB plates containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml). Expression and purification of Dm I-2 was then performed as for human I-2 [17]. The peak I-2 fraction from Mono-Q chromatography, which eluted at 230 mM NaCl, was collected and stored at −70°C.

2.4. Mutagenesis of the D. melanogaster I-2 cDNA

F33 within the Dm I-2 protein was changed to A33 using the complementary oligonucleotides GCAAAAGTGCCAAGGCCGATGAGCTGAACG and CGTTCAGCTCATCGGCCTTGGCACTTTTGC (mutated codon underlined) in a QuikChange (Stratagene) mutagenesis reaction on the pT7-7 expression construct. F119 was mutated similarly using the complementary oligonucleotides CGGCGCGAAGCTGAGCGACGCC and GCGTCGCTCAGCTTCGCGCCGC (mutated codon underlined). The mutations were confirmed by sequence analysis.

2.5. Protein phosphatase and inhibitor assays

Phosphatase assays were performed in the presence of 0.5 mM Mn^{2+} (PP1-87B) or in the absence of divalent cations ($PP2A_1$) using ^{32}P-labeled rabbit skeletal muscle glycogen phosphorylase [23]. One unit of phosphatase activity is that amount of enzyme that catalyses the release of 1 μmol [^{32}P]phosphate/min from phosphorylase *a*. I-2 was assayed as in [24]. One unit of I-2 is that amount that inhibits the dephosphorylation of 0.01 mU of PP1 by 50% in the standard 30 μl assay.

3. Results

3.1. Identification of the cDNA encoding D. melanogaster inhibitor-2

Sequence analysis of a cDNA from the NCBI EST database identified an ORF encoding a polypeptide of 205 amino acids with regions of high sequence identity to human I-2 (Fig. 1). The sequence at the presumed initiating codon (CAGGATG) conforms reasonably well to the consensus for *D. melanogaster* translation initiation of (C/A)AA(A/C)ATG [25]. Although there is one other ATG codon preceding the presumed initiating codon, this is followed by two stop codons. The 1503 bp cDNA contains 249 bp of 5′ untranslated region (UTR) and 636 bp of 3′ UTR and has been deposited in the GenEMBL database under the accession number AJ133246. 1503 bp is much smaller than the size of the insert DNA (4.5 kb) revealed by restriction digest analysis of the EST clone. This discrepancy is explained by the presence of a second cDNA insert cloned into the vector downstream of and in the opposite orientation to the I-2 cDNA. The identity of this cDNA has not been investigated.

3.2. Comparison of the predicted D. melanogaster I-2 with other I-2 sequences

Fig. 1 shows a comparison of the predicted Dm I-2 protein with human I-2, the *S. cerevisiae* Glc8 protein and a putative *Caenorhabditis elegans* I-2 protein. The *C. elegans* I-2 protein sequence (Wormpep ID: Y32H12A_69.A) was identified by a search of the *C. elegans* Wormpep database (The Sanger Centre, Hinxton Hall, Cambridge, UK) using the human I-2

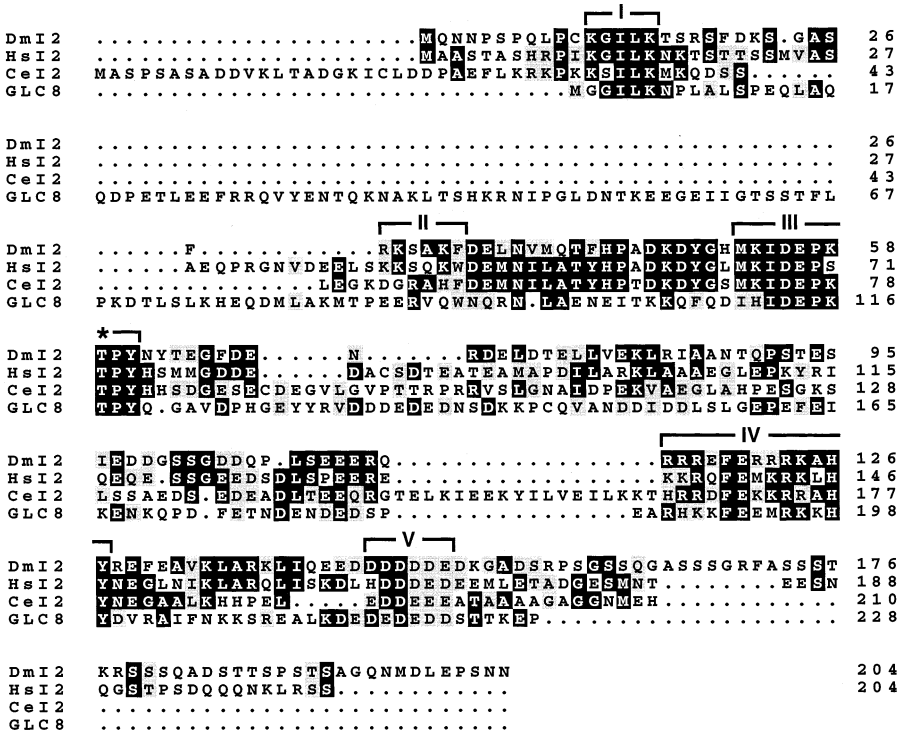

Fig. 1. Comparison of the predicted *D. melanogaster* inhibitor-2 protein (DmI2) with human inhibitor-2 (HsI2), the putative *C. elegans* inhibitor-2 protein (CeI2) and the *S. cerevisiae* GLC8 protein. Numbering of the amino acids is from the residue following the initiating Met, which is cleaved from the mature protein. The five conserved regions of sequence (I–V) are indicated by brackets and threonines 59 of DmI2, 72 of HsI2, 79 of CeI2 and 117 of GLC8 are indicated (*). The comparison was created using the program PILEUP (Genetic Computer Group, Madison, WI, USA) followed by manual optimisation of the alignment and shading was performed using the program BOXSHADE (v3.21, K. Hofmann and M. Baron).

protein sequence. Dm I-2 has 39% identity (58% similarity if conservative substitutions are included) to human I-2 and 41% identity (61% similarity) to the predicted *C. elegans* I-2. These figures are comparable to the 37% identity (53% similarity) between human I-2 and the predicted *C. elegans* I-2 protein. As expected, lower levels of identity are seen between all of these I-2 proteins and Glc8p.

Fig. 1 reveals highly conserved sections of sequence among the proteins. Residues between amino acids 52 and 61 of Dm I-2 (conserved region III) are almost totally conserved between all four proteins. Four other regions of conservation (conserved regions I, II, IV and V; Fig. 1) are also apparent. Regions IV and V consist largely of basic and acidic residues respectively. It is also of interest that, despite *D. melanogaster*, *C. elegans* and human I-2 all being very similar sizes (in fact Dm I-2 is identical in length to human I-2; 205 amino acids), the optimal alignment leads to substantial insertions and deletions in certain areas of the proteins. These are presumably regions of the proteins not essential for a common function.

3.3. Expression of the Dm I-2 mRNA during D. melanogaster development

Fig. 2A shows that an mRNA species of 1.6 kb can be detected at all stages of development. This size of mRNA is consistent with the length of Dm I-2 cDNA (1.5 kb) isolated. Reprobing the blot with the cDNA for PP1-87B (Fig. 2B), which is reasonably evenly expressed during *D. melanogaster* development [26], demonstrates that the level of the Dm I-2 mRNA is also fairly constant. The one possible exception is in 0–4 h embryos (Fig. 2A, lane 1) where the Dm I-2 mRNA appears more abundant than at other stages of development. The two PP1-87B mRNAs also appear more abundant in these early embryos than at later stages of development, as has been observed previously [15]. Since PP1-87B represents the most abundant PP1 isoform in *D. melanogaster* [27], it is likely that the level of I-2 mirrors that of PP1-87B in order that PP1 is folded and/or it activity controlled efficiently.

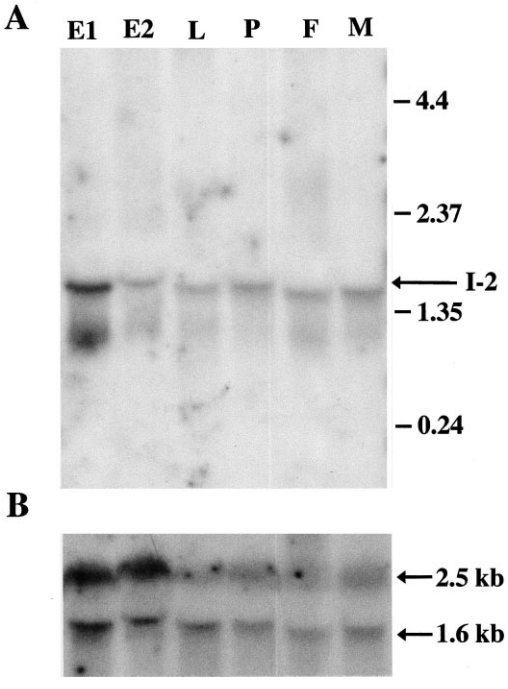

Fig. 2. Expression of I-2 mRNA during *D. melanogaster* development. 25 μg of total RNA from the developmental stages shown was separated on a denaturing 1% agarose gel and transferred to nylon membrane. The membrane was probed with a 600 bp ^{32}P-radiolabelled *Nde*I-*Xba*I fragment from the plasmid pCR2.1 containing the entire open reading frame for Dm I-2 (A). The membrane was subsequently probed with a ^{32}P-radiolabelled fragment of *D. melanogaster* protein phosphatase 1-87B [26] to control for loading (B). 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).

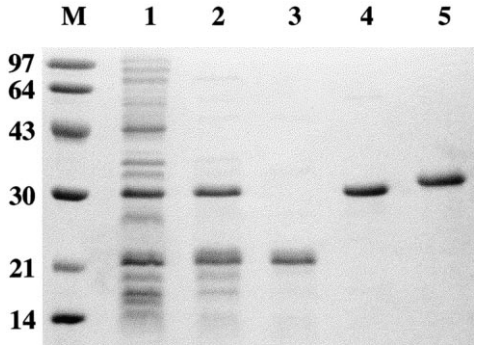

Fig. 3. Purification of *D. melanogaster* I-2 protein expressed in *E. coli*. Protein samples from the stages of purification shown were separated on a 10% SDS-PAGE and stained with Coomassie brilliant blue R250 to visualise the proteins. Lanes are: M, markers; 1, 10 μg of whole cell soluble extract from *E. coli* expressing I-2; 2, supernatant from 10 μg of *E. coli* extract heated to 100°C and centrifuged; 3, 5 μg of flow through from Mono Q column; 4, 2 μg of Dm I-2 eluted from Mono Q; 5, 2 μg of human I-2. Marker sizes are in kDa.

3.4. Purification and assay of D. melanogaster I-2 expressed in E. coli

Expression of the Dm I-2 cDNA in *E. coli* produced a 30 kDa band visible in the initial extract (Fig. 3, lane 1) that, after heating at 100°C, was found in the soluble fraction (Fig. 3, lane 2). Chromatography on Mono Q resulted in purification of the 30 kDa I-2 protein (Fig. 3, lane 4), which was tested on *D. melanogaster* PP1-87B [15] using ^{32}P-labeled glycogen phosphorylase as substrate. Fig. 4A shows that Dm I-2 inhibits PP1-87B with a K_i of less than 1 nM in the standard assay. This is identical to the inhibition observed with human I-2 (data not shown). As expected for an I-2 protein, the polypeptide did not significantly inhibit the phosphatase activity of $PP2A_1$ (Fig. 4A). We have previously shown that mutagenesis of a conserved Phe within the C-terminus of the *D. melanogaster* inhibitor-t protein, which is related to I-2, disrupts the ability of this protein to inhibit PP1 [15]. To determine whether this was also the case for Dm I-2, we mutated Phe-119 to Ala and purified the mutant protein to homogeneity as for the wild type protein. When this protein was added to PP1-87B in a standard assay, it inhibited PP1-87B phosphatase activity as effectively as wild-type Dm I-2 (Fig. 4A).

3.5. Identification of a site on D. melanogaster I-2 that interacts with PP1-87B

Previous studies using short synthetic peptides derived from the G_M regulatory subunit of PP1 have identified a hydrophobic groove on the surface of human PP1c that is responsible for binding many PP1 regulatory subunits [28]. In order to determine whether PP1-87B also binds *D. melanogaster* I-2

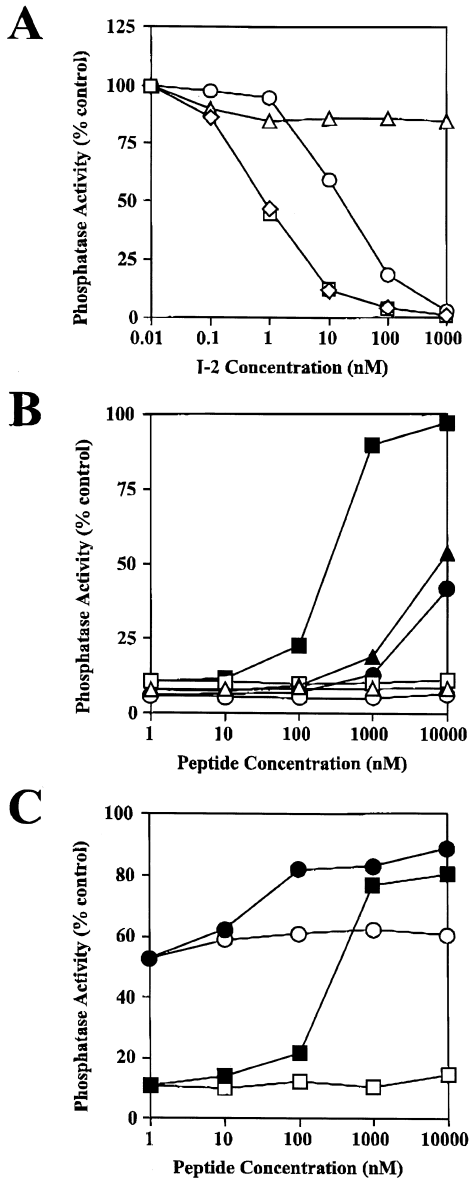

Fig. 4. Effect of I-2 proteins on the phosphorylase phosphatase activity of PP1-87B and $PP2A_1$. Phosphatase activity is given as a percentage of the maximum obtained in the absence of any inhibitors. A: The PP1-87B catalytic subunit was incubated with wild type (□), F119A (◇), F33A (○) Dm I-2. $PP2A_1$ was incubated with wild type Dm I-2 (△). B: Relief of Dm I-2 inhibition of PP1-87B by synthetic peptides. PP1 activity was measured in the presence of 10 nM Dm I-2 and increasing amounts of peptides. ■, 53BP2 peptide (GQVSLPPGKRTNLRKTGSERIAMGMRVKFNPLALLLDSC); ▲, NIPP1 peptide (KRKNSRVTFSED); ●, p99 peptide (GRKRKSVTWPEEGKLR); □, 53BP2 control peptide (GQVSLPPGKRTNLRKTGSERIAMGMRVKANPLALLLDSC); △, NIPP1 control peptide (REKPQTLPSAVKGD); ○, p99 control peptide (GRKRKSVTAPEEGKLR). The consensus PP1 binding site is underlined in the peptide sequences. In all assays the maximal release of ^{32}P from phosphorylase *a* in the absence of inhibitors was 10%. C: Effect of peptides on the inhibition of PP1-87B by wild type (□,■) and F33A (○,●) Dm I-2. Closed symbols, 53BP2 peptide (GQVSLPPGKRTNLRKTGSERIAMGMRVKFNPLALLLDSC); open symbols, 53BP2 control peptide (GQVSLPPGKRTNLRKTGSERIAMGMRVKANPLALLLDSC).
←

through this groove, peptides that disrupt PP1c–regulatory subunit interactions were utilised. These peptides effectively relieved the inhibition of PP1c phosphatase activity caused by I-2 in standard phosphatase assays (Fig. 4B). Control peptides, containing F → A or W → A mutations in the -(K/R)(V/I)-X(F/W)- motif were ineffective at relieving I-2 inhibition (Fig. 4B). In order to ascertain whether I-2 might contain a sequence similar to the -(K/R)(V/I)X(F/W)- motif, we searched the aligned I-2 sequences from mammals and lower eukaryotes for conserved F/W residues (Fig. 1). Only two such sites exist, which are F33 and F119 in Dm I-2. Mutation of F33A increased the IC_{50} of Dm I-2 for PP1 approximately 30-fold (from 0.6 nM to 17 nM), while mutation of F119A did not affect the inhibition of Dm I-2 for PP1 (Fig. 4A). In the phosphatase standard assay, wild type Dm I-2 at 10 nM gave 90% inhibition of PP1c, while Dm I-2(F33A) at 10 nM showed only 45% inhibition of PP1c (Fig. 4C). The concentrations of wild type Dm I-2 and Dm I-2(F33A) were quantitated both by Bradford assay and by Coomassie staining after gel electrophoresis. The 53BP2 peptide relieved the residual inhibition of PP1c by the Dm I-2(F33A) mutant at a 10-fold lower peptide concentration (100 nM) than that (1 μM) required to relieve the inhibition of PP1c by wild type I-2 (Fig. 4C).

4. Discussion

Several lines of evidence demonstrate that the *D. melanogaster* sequence we present here is the bona fide homologue of mammalian I-2. The mRNA is expressed at all stages of *D. melanogaster* development and is present at equal levels in adults of both sexes, as observed for the mRNA encoding the major isoform of *D. melanogaster* PP1. In contrast, the *D. melanogaster* PP1 inhibitor protein we recently identified (termed I-t) is only expressed in testis [15]. Dm I-2 shares much higher sequence identity with human I-2 (39%) than I-t exhibits (21%) and both inhibitor proteins possess the conserved threonine residue (Thr-59 in Dm I-2) necessary for activation of an inactive complex of PP1c and I-2 (termed PP1I) by glycogen synthase kinase-3 (GSK-3) [1,2]. However, a residue corresponding to Ser-86 of human I-2 which, when phosphorylated by casein kinase II, increases phosphorylation of Thr72 by GSK-3 [29], does not appear to be present within the *D. melanogaster* inhibitor proteins. The bacterially expressed Dm I-2 specifically inhibits the activity of PP1c at nanomolar concentrations in standard phosphatase assays, while I-t is a much less potent inhibitor of PP1. Both inhibitors share additional features common to inhibitor proteins from other species, including thermostability and aberrant migration on SDS-PAGE.

Comparison of the human and Dm I-2 sequences with *S. cerevisiae* Glc8p and with a protein likely to represent *C. elegans* I-2 (Fig. 1) reveals some interesting features. The amino-terminal regions of the proteins are highly divergent, but encompass a short section (conserved region I) containing the motif (G/S)ILK. Deletion studies have identified the amino-terminus of I-2 as essential for PP1c inhibition [30,31] and mutagenesis studies have elucidated a number of key residues for inhibitor-2 function, including some of those present within the (G/S)ILK motif [30]. We have shown that residues within the amino-terminus of I-2 contribute significantly to the inhibitory capacity of I-2 through generation of a fusion protein between human I-2 and the *D. melanogaster* I-t. This

chimaeric protein, which contains conserved region I of human I-2, has significantly greater inhibitory activity than the native I-t protein that lacks such a motif [15].

Conserved region III contains the Thr residue that is phosphorylated by GSK-3, resulting in activation of the inactive PP1I complex [32]. Conserved regions IV and V are also important for I-2 function. We have previously shown that mutation of the Phe residue in conserved region IV of a fusion between human I-2 and *D. melanogaster* I-t results in a dramatic reduction in its inhibitory capability [15]. This is not the case for human I-2 [30] and Dm I-2, possibly because conserved region IV of I-2 makes more extensive contacts with PP1c than the corresponding region of I-t. Alternatively, the Phe to Ala mutation may convert I-2/I-t chimera (but not I-2) into a conformation that is unable to bind PP1 (see below). The two clusters of basic amino acids in this region that are required for nuclear accumulation [33] are conserved in *D. melanogaster* I-2 (amino acids 115–117 and 121–123). Previous deletion studies have shown that the area of I-2 containing conserved region V is important for GSK-3 activation of PP1I [31], although the mechanism is not known.

To investigate further the interaction between PP1c and I-2, we utilised synthetic peptides from three PP1-interacting proteins. These peptides, which contain the conserved PP1 interaction motifs -(K/R)(V/I)XF- or -(K/R)X(V/I)XW-, were effective in relieving some or all of Dm I-2 inhibition of PP1-87B. This parallels previous results with mammalian PP1-interacting proteins such as G_M and M_{110} [28,34], p99 [10] and 53BP2 (Helps and Cohen, unpublished) with human PP1c. Recent studies also showed that a 20 residue peptide from NIPP-1 and a 40 residue peptide from M_{110} containing the sequence -RVXF- relieved the inhibition of human PP1c by I-2 [35,30]. We have shown here that despite the fact that Dm I-2 is only 39% identical to mammalian I-2, a site of I-2 interaction on PP1c, essential for inhibition of phosphatase activity, is clearly conserved. A peptide as small as 12 amino acids could effect this relief of inhibition. Since mutation of F or W within the motifs abrogated the effect of the peptides, we analysed the consequence of mutating F/W sites within Dm I-2 that were conserved from mammals to lower eukaryotes. Mutation of F33A in conserved region II increased the IC_{50} of Dm I-2 for PP1c approximately 30-fold, indicating that this region probably binds to PP1c. Since the sequence of Dm I-2 in this section is -RKSAKF-, and homologous sequences in other eukaryotic I-2 proteins also resemble the PP1c binding motif, it is possible that conserved region II may bind to the hydrophobic groove of PP1. The fact that the 53BP2 peptide relieved the residual inhibition of the Dm I-2(F33A) at lower concentrations than necessary to relieve the inhibition by wild type I-2, supports this hypothesis. However, a conformational change cannot be completely ruled out. Although the precise mechanism has yet to be established, alignment of I-2 sequences from lower eukaryotes with mammalian I-2 has allowed a conserved region that is important for inhibition of PP1c to be identified.

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