The Chaperone-like Properties of Mammalian Inhibitor-2 Are Conserved in a *Drosophila* Homologue[†,‡]

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ABSTRACT: Phosphatase inhibitor-2 (I-2) is a mammalian phosphoprotein that binds to the catalytic subunit of type 1 serine/threonine phosphoprotein phosphatase (PP1c) and inhibits its activity in vitro. Recombinant PP1c differs from native PP1c in several biochemical criteria, including the requirement for Mn^{2+}, sensitivity to vanadate, and *p*-nitrophenyl phosphate (*p*NPP) phosphatase activity. I-2 can convert recombinant PP1c into a native-like activity in vitro. It has therefore been suggested that I-2 may act as a molecular chaperone for PP1 in vivo. We have identified a *Drosophila* homologue (I-2Dm) in a two-hybrid screen for PP1c-binding proteins. The sequence of I-2Dm is 35% identical with that of I-2, whereas the catalytic subunits themselves are >85% identical in flies and humans; however, we show that many biochemical properties of I-2 are conserved. Like I-2, I-2Dm can convert recombinant PP1c to a native-like activity. This strongly suggests that this ability is an essential, conserved role of I-2 and I-2Dm.

Reversible protein phosphorylation is a ubiquitous regulatory mechanism in eukaryotic cells. While earlier studies focused on protein kinases, it is now clear that protein phosphatases play an equally tightly regulated and important role in the control of cellular phosphoproteins (reviewed in refs *1*−*3*). Two structurally related protein phosphatase families, PP1[1] and PP2A, contribute more than 90% of the serine/threonine phosphatase activity in mammalian cells. PP1 is involved in cell cycle control and the regulation of glycogen metabolism, gene expression, muscle contraction, in memory and learning, and in a host of other processes. Both PP1 and PP2A are controlled by regulatory proteins that modify their substrate specificity and subcellular location (*4*).

First identified in rabbit skeletal muscle more than 20 years ago (*5*), mammalian I-2 is a heat-stable PP1c binding protein present in all mammalian tissues that have been examined (*6*, *7*; reviewed in ref *8*). I-2−PP1c complexes isolated from tissue are inactive [though this may depend on the purification method that is used (*9*)], but the phosphatase can be reactivated by incubation with MgATP (*10*). Activation of the latent complex is accompanied by the phosphorylation of I-2 on Thr-72 by GSK3. In freshly prepared rabbit skeletal muscle extract, ∼30% of the soluble phosphorylase phosphatase can be attributed to the MgATP-dependent form. I-2 is also phosphorylated on three serine residues (Ser-86, Ser-120, and Ser-121) by CKII. This CKII phosphorylation does not alter I-2 activity, but facilitates phosphorylation by GSK3α (*11*). The activation cycle of MgATP-dependent phosphatase is complicated and somewhat controversial. Association of I-2 with PP1c rapidly inhibits the catalytic activity of the enzyme, and seems more slowly to change its conformation to an inactive state (reviewed in refs *2* and *8*). Phosphorylation of I-2 by GSK3 is thought to induce a conformational change in PP1c, which then slowly recovers activity. This recovery may correlate not with the GSK3-induced conformational change, but with subsequent autodephosphorylation of I-2 Thr-72 by PP1c. Over a longer time frame, the complex reverts to an inactive state. Various regions of I-2 have been implicated in different parts of this cycle (*12*).

I-2 can interact with denatured PP1c to promote refolding, yielding an active enzyme (*13*). Furthermore, recombinant PP1c, which differs from native PP1c in particular biochemical characteristics, behaves much more like native PP1c after incubation with I-2 and reactivation by GSK3 (*13*, *14*). This has led to the proposal that I-2 is a molecular chaperone for PP1.

I-2 levels and the subcellular location both vary through the cell cycle. I-2 protein levels peak during the S phase and mitosis (*15*), and an I-2−GFP fusion protein was found to be cytosolic during the G1 phase and nuclear during the S phase (*16*). Mutation to Ala of any of the four CKII or GSK3 phosphorylation sites prevented nuclear accumulation in the S phase, suggesting that phosphorylation at these sites may have consequences not revealed by analyzing I-2−PP1 complexes in vitro. The same study identified a putative nuclear localization signal (NLS) and showed that mutation to alanine of two lysines in this sequence also prevented nuclear accumulation. Comparison with the SV40 NLS suggested that CKII phosphorylation at Ser-120 and Ser-

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[‡] The I-2Dm sequences reported in this paper have been deposited at the EMBL database with accession numbers AJ249461 (genomic) and AJ249462 (cDNA).

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[1] Abbreviations: PP1, type 1 serine/threonine protein phosphate; PP1c, catalytic subunit of PP1; I-2, inhibitor-2; *p*NPP, *p*-nitrophenyl phosphate; CKII, casein kinase-2; GSK3, glycogen synthase kinase-3.

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121 might regulate the I-2 NLS and so control the subcellular distribution of I-2.

One physiological role proposed for I-2 is in the control of sperm motility. Nonmotile immature sperm have elevated GSK3 activity, which may activate I-2−PP1c complexes, particularly those containing the testis-specific PP1 isoform PP1γ1 (*17*). Incubation of these sperm with PP1 inhibitors induced motility, suggesting that I-2 inhibits PP1 activity in mature sperm to permit their motility.

Identification of I-2 homologues in nonmammalian species would permit the identification of functionally and structurally conserved domains, and potentially facilitate a genetic approach with which to complement previous biochemical studies. It has been suggested that *Saccharomyces cerevisiae* Glc8p, another heat-stable inhibitor of PP1c, may be homologous to I-2, though its sequence is only 24% identical with that of mammalian I-2 (*18*, *19*). Recently, an I-2-related sequence from *Drosophila melanogaster* has been described (*19*). This is not the fly homologue of I-2 as it is testis-specific while I-2 activity has been detected in *D. melanogaster* head extracts, as well as in the whole organism (*20*). It was therefore named inhibitor-t (I-t). We report here the identification and biochemical characterization of an authentic *D. melanogaster* homologue of mammalian I-2 which we name I-2Dm.

EXPERIMENTAL PROCEDURES

Two-Hybrid System. Two-hybrid screening for proteins capable of binding with PP1β9C was carried out essentially as described in refs *21* and *22*. A cDNA encoding full-length PP1β9C was isolated from a 0−4 h embryonic cDNA library (*23*). The start codon of PP1β9C was modified to a *Nde*I site by PCR and subcloned into the *Nde*I site of pAS2 to create pAS2-PP1β9C. A *Drosophila* third instar larval cDNA library in pACT was screened for cDNAs that interact with pAS2-PP1β9C. I-2Dm clones were screened against the other *Drosophila* PP1 isoforms by transformation into Y190 carrying pAS2-PP1α87B, pAS2-PP1α96A, or pAS2-PP1α13C. pAS2-PP1α87B has been described previously (*22*), and pAS2-PP1α13C and pAS2-PP1α96A were constructed essentially like pAS2-PP1β9C was.

Sequence Analysis. The sequence of the cDNA was determined using cycle sequencing with fluorescent terminators and an automated sequencer (model ABI 377 from Perkin-Elmer Corp.) according to the manufacturer's instructions. Oligonucleotide primers were synthesized by MWG Biotech. Sequence analysis and searches of sequence databases were performed using Sequencher (Gene Codes Inc.) and GCG (*24*).

I-2Dm cDNA and Cosmid Isolation. A cDNA containing the complete I-2Dm ORF was obtained by hybridization using the screening procedure described in ref *25*. Screening of 5×10^5 clones from a 0−4 h embryonic library (*23*) using the two-hybrid cDNA as a probe identified 16 I-2Dm cDNAs, the longest of which was purified.

A gridded filter from the HGMP Resource Centre, representing approximately 18 000 cosmid clones, was screened by hybridization with a I-2Dm cDNA probe. Construction of this library is described in refs *26* and *27*. Eight positives, 53F1, 51G6, 69D3, 111F2, 112F2, 116G2, 118A12, and 135C7, were identified and purified. The position of introns in the I-2Dm open reading frame was determined from sequencing DNA derived from cosmid 135C7. Three of these cosmids (51G6, 69D3, and 135C7) have previously been mapped to 67C1-11 (European *Drosophila* Genome Project, unpublished; see http://edgp.ebi.ac.uk).

RNA Analysis. Total RNA was purified from whole adults and dissected heads using Trizol (Life Technologies Inc.). Poly(A)$^+$ RNA was purchased from Clontech. One microgram of poly(A)$^+$ or 10 μg of total RNA was used per lane for Northern blots. RNA in situ hybridization was performed as described in ref *28*.

Protein Expression. The start codon of the full-length I-2Dm and PP1β9C cDNAs were mutagenized to *Nde*I sites, subcloned into pET28a (Novagen), and expressed in *Escherichia coli*. Purified PP1β9C and I-2Dm were dialyzed against 20 mM Tris-HCl (pH 7.5), 0.1% (v/v) 2-mercaptoethanol, and 60% (v/v) glycerol. Protein concentrations were measured using a protein assay kit (Bio-Rad).

Protein Phosphatase and Inhibitor Assays. Phosphorylase phosphatase assays were performed using a commercial kit (Life Technologies Inc.). I-2 was assayed as described in ref *12*. One unit of I-2 is the amount that gives 50% inhibition of 0.01 milliunit of PP1. GSK3β and CKII were purchased from New England Biolabs. Kinase reactions were performed as described in ref *29*. Partially purified I-2Dm (2 μg) was incubated in 20 μL of reaction mixture [20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 50 mM KCl, 10 mM $MgCl_2$, and 0.2 mM [γ-^{32}P]ATP (200 Ci/mol)] in the presence of CKII (80 milliunits), GSK3β (20 milliunits), or both. After incubation at 30 °C for 90 min, the reaction was terminated by the addition of a 5× SDS sample buffer. Half of each reaction mixture was separated by SDS−PAGE. Incorporation of labeled phosphate was visualized by autoradiography and quantified by scintillation counting of excised bands.

Gel Filtration of PP1β9C and I-2Dm−PP1β9C. PP1β9C and I-2Dm were preincubated in 20 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, and 0.1% (v/v) 2-mercaptoethanol for 60 min at 30 °C. In preliminary experiments, I-2Dm was titrated against PP1β9C to identify a ratio at which no free PP1β9C came off the column. Gel filtration was performed on a 10 mm × 300 mm Superose 12 HR column (Pharmacia) in 25 mM triethanolamine-HCl (pH 7.5), 1 mM $MnCl_2$, 0.1% (v/v) 2-mercaptoethanol, 0.03% (w/v) Brij-35, 5% (v/v) glycerol, and 200 mM NaCl. The column was developed at a flow rate of 0.5 mL/min. Fractions (0.25 mL) were collected and dialyzed as described above. Pharmacia molecular mass standards were used for column calibration.

RESULTS

Isolation of Drosophila Inhibitor-2 cDNA. The key shared characteristic of PP1 regulatory proteins is that they bind PP1c. We therefore used the yeast two-hybrid system to identify *Drosophila* proteins capable of binding *Drosophila* PP1c (see Experimental Procedures). Two hundred forty-one partial cDNAs were isolated from 5×10^6 clones as strong *HIS*$^+$ *lacZ*$^+$ positives in combination with pAS2-PP1β9C but not control plasmids. These were found to correspond to 36 genes by cross hybridization and sequence analysis, with 24 genes being represented more than once (unpublished data). Several of these exhibited a significant degree of sequence homology with known mammalian PP1

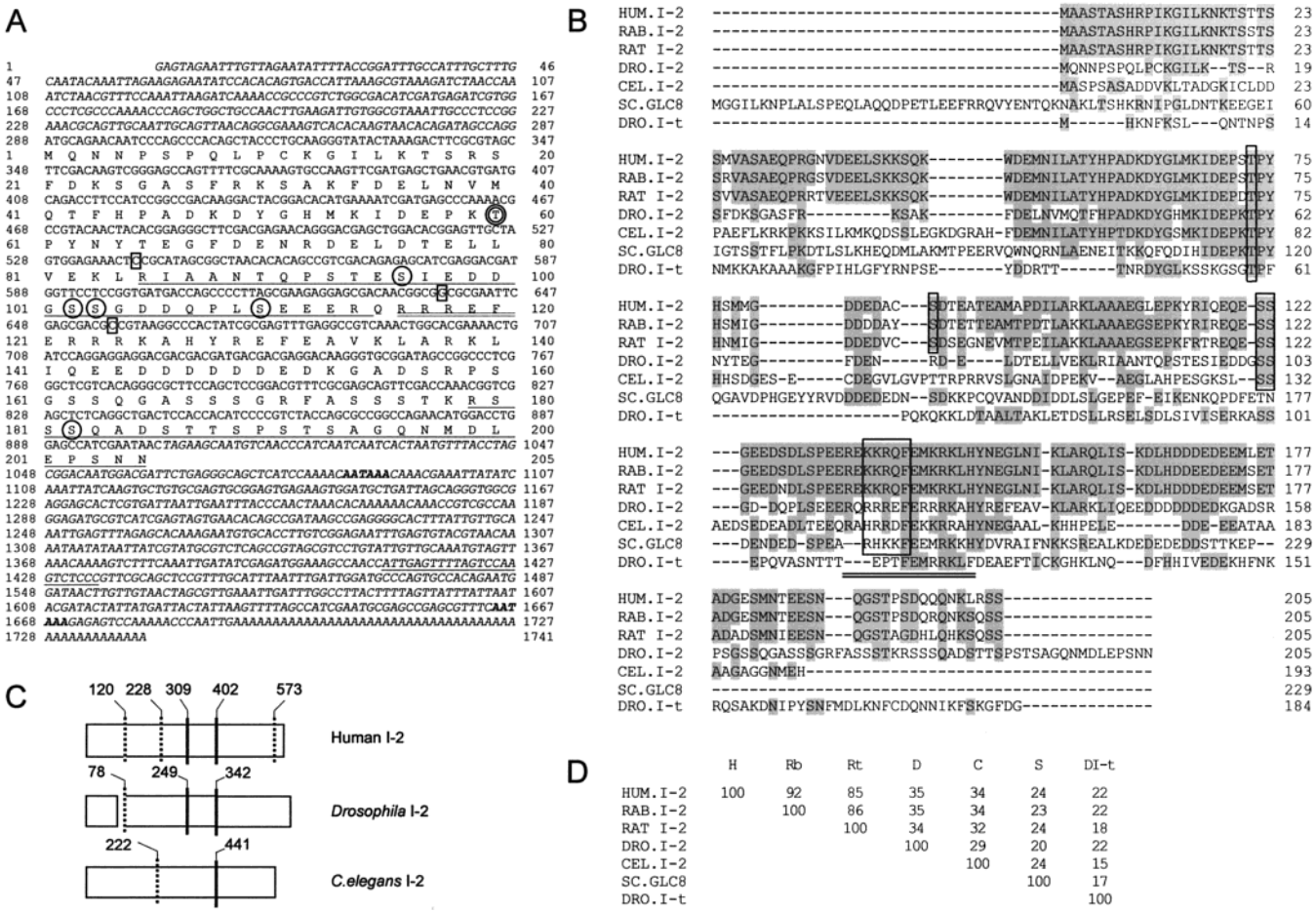

FIGURE 1: Sequence comparison of inhibitor-2 and related proteins. (A) The sequence of the coding strand of the longest I-2Dm cDNA is shown with the 205-amino acid sequence encoded by nucleotides 288−903. Untranslated regions are italicized. The predicted polyadenylation signals are bold. An insertion of 24 nucleotides found in some cDNAs is underlined. Nucleotide residues polymorphic between different cDNAs are boxed. PEST sequences are underlined. CKII sites are circled, and the GSK3 site has a double circle. A potential PP1 binding motif is marked with a double underline (see the Discussion). (B) Multiple-sequence alignment of predicted protein sequences. The rabbit I-2 protein sequence was determined directly (*34*), revealing that the initial methionine is removed. The *C. elegans* sequence is Y32H12A-69.A. For sequences that are only ~25% identical to each other, which is at the borderline of significance (*41*), several equally good alignments are usually possible. This applies here to Glc8p and I-t. Identical residues are shaded. Boxed residues are phosphorylated in rat: Thr-72 by GSK3 and Ser-86, Ser-120, and Ser-121 by CKII (*34*). A region resembling the canonical PP1 binding site [four residues in mammals, five in yeast (*38*)] is also boxed. A putative basic nuclear localization sequence (*16*), present in all of the proteins except I-t, is marked with a double underline. (C) Intron and exon structure. Position of introns in the coding regions of human, *Drosophila*, and *C. elegans* I-2. Identical intron−exon boundaries are shown with a solid line and others with a dashed line. Nucleotide numbers count from the start codon. (D) Table of % identities. Pairwise comparisons were performed using ALIGN (*42*).

regulators, including one represented by two clones encoding polypeptides that are 35% identical overall to human I-2 (*30*). The longer of the two I-2Dm cDNAs was used as a probe to isolate a cDNA encoding full-length I-2Dm. I-2Dm maps to chromosomal location 67C1-11, based on the chromosomal origin of three cosmids containing the I-2 gene.

The original I-2Dm two-hybrid cDNAs were retested with other phosphatases as bait. In this system, I-2Dm binds equally well to all four *Drosophila* PP1 isoforms, but not to PP2A (data not shown).

Sequence of Drosophila Inhibitor-2. The complete nucleotide and predicted protein sequence of I-2Dm is shown in Figure 1A. Sequence database searches revealed considerable similarity to mammalian I-2, and modest similarity to the *S. cerevisiae* protein Glc8p, whose similarity to I-2 has been described previously (*18*, *31*). Furthermore, we identified a predicted protein from *Caenorhabditis elegans* which may be a nematode homologue of I-2. Several regions that are strikingly similar are apparent between mammalian and invertebrate I-2 proteins, some of them shared by Glc8p (Figure 1). All the animal I-2 genes share one or more identical intron−exon boundaries (Figure 1C), which further indicates that they are derived from a single ancestral gene.

Expression Pattern of I-2Dm. I-2 has been identified in all mammalian tissues so far examined (*6*, *7*). However, related testis-specific genes have been identified in rats (*32*) and in *Drosophila* (*19*). Furthermore, I-2 levels have been reported to vary under cell cycle control (*15*). We therefore examined the transcription pattern of I-2Dm by Northern blotting and RNA in situ hybridization.

Northern analysis shows two I-2Dm transcripts at all stages (Figure 2A). The major transcript of 1.7 kb corresponds to the longest isolated cDNA (Figure 2A). The shorter 1.1 kb transcript probably uses an alternative polyadenylation signal (Figure 1A). Rabbit I-2 also has two transcripts generated by this mechanism (*29*). We looked for I-2Dm transcripts in adult heads, as I-2-like activity has been detected in head extracts (*20*). I-2Dm is present in heads (Figure 2A, lane 4),

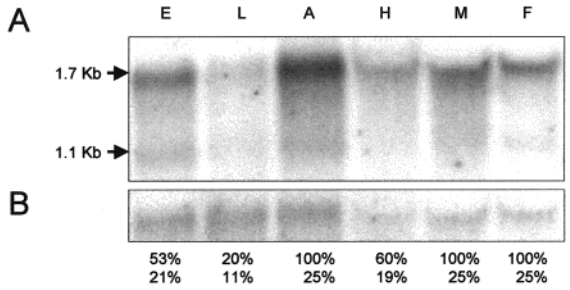

FIGURE 2: Transcription pattern of I-2Dm. (A) I-2Dm is present throughout development. Lanes 1−3 contained equal amounts of poly(A)$^+$ RNA from embryos, larvae, and adults, respectively. Lanes 4−6 contained total RNA from dissected adult heads, adult males, and adult females, respectively. I-2Dm transcripts of 1.7 and 1.1 kb were present in all lanes. (B) Filter from panel A reprobed with Dmras64B as the loading control. Relative intensities of the 1.7 and 1.1 kb bands in each lane of panel A are shown, normalized for loading based on the intensity of the Dmras64B signal.

and in equivalent levels in adult males and females (Figure 2A, lanes 5 and 6).

By RNA in situ hybridization, I-2Dm is uniformly and abundantly expressed within early embryos and more weakly but still uniformly in larval brains and imaginal disks with no apparent modulation due to cell cycle stage. We also analyzed I-2Dm expression within the testis, as I-2 has been implicated in sperm maturation in mammals. I-2Dm mRNA accumulates in primary spermatocytes during the growing stage, and is degraded shortly after meiosis (data not shown). This degradation is blocked in *aly* mutant spermatocytes, which are blocked in development before meiosis. This expression pattern is similar to that of cyclin A and other regulators of meiosis (*28*, *33*).

I-2Dm Is a Heat-Stable Inhibitor of PP1c. Bacterially expressed oligo-His-tagged I-2Dm was purified by boiling the bacterial extract for 10 min followed by Ni^{2+} affinity chromatography and gel filtration. The purified protein migrated at 31 kDa on SDS−PAGE (Figure 4), higher than its calculated molecular mass of 25 kDa (this includes the affinity tag; the predicted native molecular mass is 23 kDa). This anomalous mobility has also been observed for mammalian I-2, and indeed for I-t and Glc8p (*5*, *18*, *19*).

Purified I-2 inhibits native rabbit skeletal muscle PP1c with an IC_{50} of ∼1 nM (*5*). I-2Dm also inhibits native rabbit skeletal muscle PP1c with the same IC_{50}. To determine the effect on *Drosophila* PP1c, we expressed PP1β9C in bacteria. I-2Dm inhibited this recombinant PP1β9C with an IC_{50} of ∼0.5 nM. The inhibitory potency of I-2Dm was unaffected by boiling for 10 min, relative to that of unboiled extracts. I-2Dm was unable to inhibit PP2A (Figure 3). I-2Dm is therefore a heat-stable, specific inhibitor of PP1c.

Phosphorylation of I-2Dm. Mammalian I-2 has three CKII phosphorylation sites at Ser-86, Ser-120, and Ser-121 (*34*), of which the last two are conserved in *Drosophila* and *C. elegans* (Figure 1). Thr-72 can be phosphorylated by GSK3; with GSK3α, this phosphorylation is much more rapid following CKII phosphorylation (*11*). I-2 is a better substrate for GSK3β than for GSK3α, and GSK3β phosphorylation of I-2 is not affected by prior phosphorylation by CKII. Phosphorylation at Thr-72 is crucial for the reactivation of I-2−PP1c complexes (*11*). We therefore examined the effect of phosphorylation of I-2Dm by CKII and GSK3β (Figure 4). Phosphorylation by either kinase leads to a reduction in the electrophoretic mobility on SDS−PAGE and the extent of incorporation of radiolabeled phosphate. We analyzed the extent of phosphorylation of I-2Dm by CKII and GSK3β singly and together. No synergistic increase in the level of phosphorylation was observed (Figure 4).

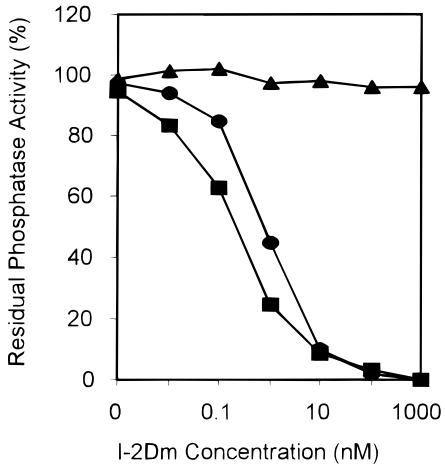

FIGURE 3: I-2Dm is a specific inhibitor of PP1c. The graph shows the phosphorylase phosphatase activity of native PP1c (●), recombinant PP1c (■), or native PP2Ac (▲) in the presence of various concentrations of recombinant I-2Dm. Phosphatase activity is shown as a percentage of the activity with no added I-2Dm. The concentration of catalytic subunits in each assay was 0.1−0.2 milliunits/mL.

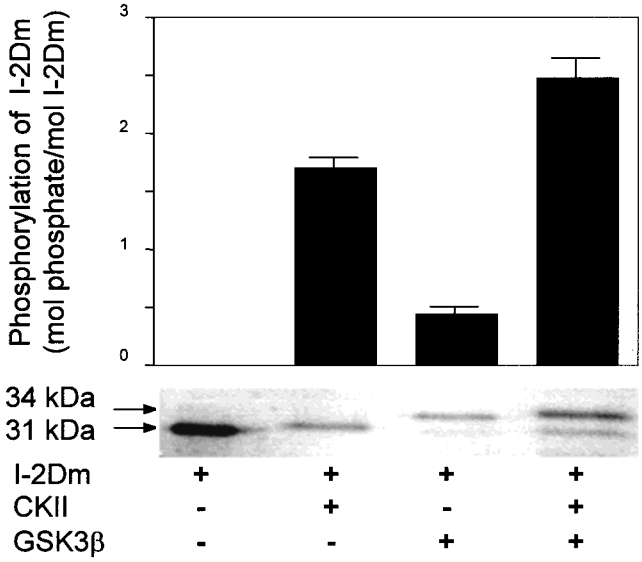

FIGURE 4: I-2Dm can be phosphorylated by CKII and GSK3. Coomassie-stained SDS−PAGE gel of 2 μg of recombinant I-2Dm (lane 1) following incubation for 90 min with [γ-^{32}P]ATP and no kinase (lane 1), 40 milliunits of CKII (lane 2), 10 milliunits of GSK3β (lane 3), or the two kinases together (lane 4). Incubation with either kinase leads to reduced mobility on SDS−PAGE. The presence of radiolabeled phosphate in the shifted bands was confirmed by autoradiography (not shown). The amount of radiolabeled phosphate incorporated in 90 min by CKII and/or GSK3β is shown above each lane. Phosphorylation of I-2Dm by CKII and GSK3 is not synergistic.

I-2Dm Can Convert PP1c to a Native-like Activity. The role of I-2 is still controversial. However, one important observation is that recombinant or denatured PP1c can be

Table 1: I-2Dm Converts Recombinant PP1c to a Native-like Activity[a]

	PP1β9C	I-2−PP1β9C	PP1c
PhP activity ratio without Mn^{2+}:with Mn^{2+}	0.17	0.65	1.00
PhP activity ratio without VO_4^{3-}:with VO_4^{3-}	0.27	0.84	1.00
*p*NPP phosphatase activity (milliunits/mg)	1682	1	36

[a] I-2Dm−PP1β9C complexes were purified by gel filtration and reactivated by incubation with MgATP and GSK3β (Figure 5). The phosphorylase phosphatase (PhP) activity of this reactivated PP1β9C was measured with and without 0.5 mM Mn^{2+} (column fractions were dialyzed, but note that all buffers up to this point contained 1 mM Mn^{2+}), and with and without 100 μM vanadate (in the presence of 0.5 mM Mn^{2+}). Finally, the *p*NPP phosphatase activity of the reactivated PP1c was measured. Untreated recombinant PP1β9C and native mammalian PP1c were assayed in the same way for comparison.

converted by I-2 to a native or native-like activity (*13*, *14*). The biochemical properties of recombinant PP1c differ in several important respects from those of PP1c purified from mammalian tissue. Recombinant PP1c is Mn^{2+}-dependent and vanadate-sensitive and exhibits potent tyrosine and *p*NPP phosphatase activity, whereas native PP1c does not require exogenous Mn^{2+}, is insensitive to 100 μM vanadate, and has little or no tyrosine or *p*NPP phosphatase activity (*13*, *14*). Incubation with I-2 followed by activation by GSK3 leads to the conversion of recombinant PP1c to native-like activity. This has led to the hypothesis that I-2 acts as a chaperone to fold PP1c into its native conformation, and that this is the key in vivo role of I-2 (*13*, *14*). We therefore explored whether I-2Dm could perform this same function.

Recombinant PP1β9C was run on a Superose 12 gel filtration column, from which it elutes with an apparent molecular mass of 36 kDa, as expected. This PP1β9C was mixed with I-2Dm and reapplied to the column. Fractions were assayed for phosphorylase phosphatase activity before and after phosphorylation with GSK3β. No untreated fraction showed significant phosphatase activity. After treatment with GSK3β, a peak of phosphatase activity was revealed at ~70 kDa. This peak presumably represents an I-2−PP1β9C complex which is inactive until the I-2 is phosphorylated by GSK3β. The presence of I-2 in the ~70 kDa peak was confirmed by boiling fractions from the peak and measuring their heat-stable PP1 inhibitory activity (activity of PP1 in the presence of boiled fraction, 7.4% of that of PP1 with buffer only, data not shown).

The reactivated PP1 was tested for Mn^{2+} dependence, sensitivity to 100 μM vanadate and *p*-nitrophenyl phosphate phosphatase (*p*NPPase) activity (Table 1). In each respect, reactivated I-2Dm−PP1β9C resembles native PP1c rather than the recombinant form. I-2Dm can therefore convert recombinant PP1c to a native-like activity just as mammalian I-2 can.

DISCUSSION

Like I-2, I-2Dm can convert recombinant PP1c to a native-like activity, despite the two sequences being only 35% identical. This strongly suggests that this ability is essential to the conserved role of I-2 and I-2Dm, supporting the suggestion that I-2 acts as a molecular chaperone to fold newly synthesized or denatured PP1c into its correct

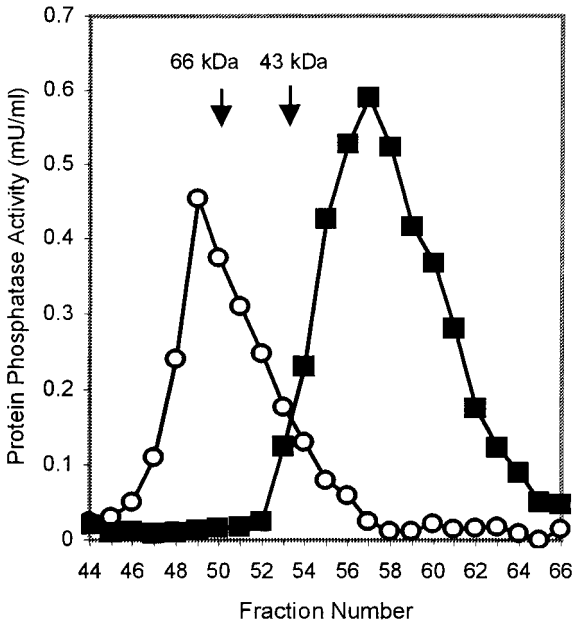

FIGURE 5: Gel filtration of the I-2Dm−PP1β9C complex. Recombinant PP1β9C (144 milliunits) was loaded onto a Superose 12 HR FPLC column (Pharmacia). The collected fractions were assayed for phosphorylase phosphatase activity (■). A single peak of activity was detected at ~36 kDa. Recombinant PP1β9C (72 milliunits) was then preincubated with an excess of I-2Dm for 60 min at 30 °C, and then loaded onto the Superose 12 HR column as before. The collected fractions were assayed for phosphorylase phosphatase activity. No fraction exhibited significant activity (not shown). Following incubation of these fractions with MgATP and GSK3β, a peak of activity was revealed at ~70 kDa (○). The arrows mark the position of protein standards BSA (66 kDa) and ovalbumin (43 kDa).

conformation. It is probably crucial to the cell that PP1 is specific for phosphoserine and phosphothreonine, and inactive against phosphotyrosine. On the other hand, this does not rule out the possibility that I-2 sequesters a pool of PP1c which can be activated by phosphorylation of the conserved Thr-72.

I-2Dm mRNA is present in all tissues and developmental stages that were examined, and I-2Dm has biochemical properties very similar to those of mammalian I-2. I-2Dm therefore probably accounts for the I-2-like activity previously detected in *Drosophila* extracts (*20*). The ubiquitous expression does not give a strong clue as to its function, but indicates that if I-2Dm activity varies through the cell cycle, as has been shown for I-2, then this is due to post-transcriptional control. Like I-2, I-2Dm has two PEST sequences predicted to destabilize the protein, and is also subject to control by reversible phosphorylation. Kakinoki et al. have shown that different I-2 kinases are present in G1 and S phase extracts from mammalian cells (*16*).

The relationship between I-2Dm and I-t is particularly interesting because rats have also been shown to have a testis-specific, I-2-related gene, I-2β (*32*). Both I-t and I-2β have inhibitory potencies for PP1c 100-fold lower than I-2. It seems likely that these I-2-related proteins may inhibit or refold a PP1-related phosphatase, rather than PP1c itself. *Drosophila* has at least two testis-specific, PP1-related phosphatases, PPY55A (*35*) and PPN58A (*36*), and rats have a testis-specific PP1 isoform (*37*); however, the search for such phosphatases has by no means been exhaustive.

Structure and Function of Inhibitor-2. Sequence comparisons between I-2Dm and mammalian I-2 point to a number of conserved residues and functional domains. Several functional domains within mammalian I-2 have been identified by deletion analysis (*12*). This led to a model in which I-2 rapidly inhibits and then slowly inactivates PP1c. Phosphorylation of the inactive I-2−PP1c complex reactivates PP1, and this activity is revealed once I-2 is dephosphorylated by PP1c. Deletion of the 35 NH_2-terminal residues of I-2 increases the IC_{50} by more than 2 orders of magnitude, to ∼400 nM, but does not affect the slow inactivation of PP1c by I-2. Though necessary, this region is not sufficient for PP1c inhibition (*34*), implying that other parts of the molecule contact PP1c or are required for this region to adopt the correct conformation. Glc8p and I-t are not very similar to I-2 in this region, and have IC_{50} values for PP1c of ∼200 and 65−130 nM, respectively, with phosphorylase *a* as the substrate (*18*, *19*). Replacing the 20 amino-terminal residues of I-t with residues 1−38 of I-2 gave a fusion protein with an IC_{50} of ∼10 nM, confirming the importance of this region in PP1c inhibition. The IC_{50} of I-2Dm is ∼1 nM, as is that of wild-type mammalian I-2. Eleven of residues 2−35 are identical in I-2 and I-2Dm, of which only one is also shared by I-t (Figure 1B). It seems likely that potent inhibition of PP1c is mediated by these identical residues.

Deletion of the 59 C-terminal residues of mammalian I-2 prevents reactivation of I-2−PP1c complexes by GSK3 (*12*). GSK3 still phosphorylates the I-2 on Thr-72, suggesting that these 59 residues are required to change the conformation of PP1c, or possibly to allow the subsequent dephosphorylation of I-2. We have shown that I-2Dm can go through the same reactivation cycle as mammalian I-2, implying that the function of the C-terminus is conserved in this respect.

Thr-72 is one of the few structural features of I-2 which can be recognized in Glc8p, as well as in I-t and mammalian and invertebrate I-2 homologues. This residue can be phosphorylated by GSK3, cyclin B-cdc2, MAP kinase, and perhaps other kinases. This phosphorylation is required for reactivation of the inactive I-2−PP1c complex. Mammalian I-2 also has three CKII phosphorylation sites, of which two are conserved in I-2Dm. Phosphorylation at Ser-86 may synergistically enhance phosphorylation of I-2 by GSK3α, but this Ser is not conserved in I-2Dm. In any case, GSK3β is a better I-2 kinase, at least in vitro. Another role for these phosphorylations has also been suggested. Kakinoki et al. (*16*) reported that the fluorescent I-2−GFP fusion protein was cytoplasmic in the G1 phase but nuclear in the S phase of the cell cycle. Mutation to alanine of any one of the three CKII sites, or Thr-72, prevented this nuclear accumulation during the S phase, as did mutation of Lys-143 and -145. These two lysines are within a region (REKKRQFEMKRKLH; see Figure 1B) identified by Kakinoki et al. as a potential nuclear localization signal, whose effectiveness might be modulated by phosphorylation at Ser-120 or -121 and possibly other sites. This region is conserved in I-2Dm, but again another role has been suggested. Helps et al. (*19*) mutated to alanine Phe-115 of I-t, which corresponds to Phe-140 of I-2. This change reduces the inhibitory potency of an I-2−I-t fusion protein 200-fold, indicating that this residue is important for inhibition or binding of PP1c. Most PP1-binding proteins are thought to interact with PP1c via an R/K,V/I,X,F or R/K,X,V/I,X,F motif, or a variation thereof (*38*, *39*). Such a motif in I-2 has not yet been definitively identified. However, experiments with various I-2 or I-2-like molecules (*12*, *19*, *40*) suggest that the region around F120 in I-2Dm (boxed in Figure 1B) and extending another 10 or so residues further toward the C-terminus functions as a PP1-binding site. This may include one or more sequences that approximate the RVXF motif. Structural studies with I-2−PP1c complexes will resolve this question, and perhaps also reveal the native structure of PP1c. The crystal structure has been determined for recombinant PP1c, but as discussed above, this differs from the native protein in important biochemical characteristics such as phosphotyrosine phosphatase activity.

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