Protein phosphatase 1 activity in *Drosophila* mutants with abnormalities in mitosis and chromosome condensation

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A *Drosophila* gene encoding a protein phosphatase 1 (PP1) has been sequenced, and lethal mutations in this locus (87B) analysed. Two mutants ($ck19^{e211}$ and $ck19^{hs46}$), which disrupt mitosis, lack the 87B isoenzyme and express only ~20% of wild type PP1 activity. The promoter region of the gene is deleted in the $ck19^{e211}$ mutant. A third mutant ($ck19^{e078}$), which shows suppression of position effect variegation, but has little effect on mitosis, possesses ~35% of wild type PP1 activity. The results indicate that the PP1 87B isoenzyme is involved in regulation of chromosome condensation at interphase as well as mitosis.

Protein phosphatase; *Drosophila*; Gene sequence; Mitosis; Position effect variegation

1. INTRODUCTION

A key enzyme in the regulation of the cell cycle is the product of the cell division cycle 2 (*cdc2*) gene, which encodes a protein serine/threonine kinase that becomes dephosphorylated and activated at the G2-M boundary (reviewed in [1]). The protein phosphatases which regulate the activation of cdc2 and/or dephosphorylate its substrates are only beginning to be identified. Four principal protein serine/threonine phosphatase catalytic subunits have been identified in eukaryotic cells, namely protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B) and protein phosphatase 2C (PP2C) and shown to regulate a variety of cellular processes [2,3]. cDNA cloning studies have revealed that PP1 and PP2A are remarkably conserved through evolution [4,5]. Recent work in *Xenopus* egg extracts has demonstrated that concentrations of the toxin okadaic acid which inhibit PP2A (but not PP1, PP2B or PP2C) stimulate the dephosphorylation and activation of cdc2, whereas specific inhibition of PP1 by inhibitors 1 and 2 does not [6]. Although PP1 does not appear to be involved in cdc2 activation, there is evidence that it nevertheless plays an important role in mitosis. Mutants in the fungus *Aspergillus nidulans* [7] and fission yeast *Schizosaccharomyces pombe* [8] that are defective in anaphase, are affected in genes that encode proteins with a strikingly high level of sequence identity to mammalian PP1 [9]. However, protein phosphatase activities in the mutant cells were not investigated.

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In *Drosophila*, 3 isoforms of PP1 have been identified by cDNA cloning, with the possible existence of another inferred from hybridisation of cDNA to a fourth gene locus [10,11]. The locus encoding one of these isoforms, PP1 87B, lies within a region of chromosome 3 which has previously been dissected by saturation mutagenesis [12]. Mutants were therefore available which could be examined for protein phosphatase activity and phenotype. We show elsewhere that two of these lethal mutants ($ck19^{e211}$ and $ck19^{hs46}$) have defects in mitosis [13]. A third mutant ($ck19^{e078}$) in the same complementation group [12] also dies at the late larval stage, but shows little abnormality in cell division [13]. Here, we have analysed the structure of wild type and mutant genes and measured PP1 activity at the late larval stage.

2. MATERIALS AND METHODS

2.1. *Isolation and sequencing of genomic clones*

A wild type (OregonR) genomic library in the CosPer cosmid vector (kindly provided by Dr John Tamkun, University of Colorado, Bolder, USA), was screened with the 3′ non-coding 160 bp fragment of PP1 87B cDNA [10], which was labelled by random hexanucleotide priming using [α^{32}P] dCTP [14]. Two positive clones with ~43 kb inserts were isolated and digested with *Eco*RI. An 8 kb fragment, positive with the PP1 87B cDNA, was subcloned into the *Eco*RI site of the Bluescript pKS$^+$ vector and 1.9 kb of it was sequenced by methods described previously [10].

A library of genomic DNA from $ck19^{e211}$/TM3 flies, partially cleaved with *Bam*HI, was constructed in the vector λEMBL4 [15]. Two clones positive with a 5′ non-coding 172 bp fragment of PP1 87B cDNA, with insert sizes of 18 and 14 kb, were isolated. After digestion with *Bam*HI, identical 11 kb positive fragments from each clone were subcloned into the *Bam*HI site of Bluescript and 1.6 kb encompassing the mutant gene was sequenced.

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2.2. *Preparation of* Drosophila *extracts*

Drosophila, at the third instar larval stage of development, were collected from culture medium by floating them on the top of 1.5 M NaCl, then rinsed with distilled water, dried on paper tissue and stored in liquid nitrogen or used directly. Storage of the larvae at −70°C did not affect the phosphatase activities. Two or four larvae were homogenised at 0–4°C in 0.2 ml of 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.1% 2-mercaptoethanol containing the proteinase inhibitors, benzamidine hydrochloride (5 mM), phenylmethylsulphonyl fluoride (1 mM), L-1-tosylamide-2-phenylethylchloromethyl ketone (0.1 mM) and leupeptin (4 μg/ml), which were purchased from Sigma, Poole, UK. The homogenates were centrifuged for 5 min at 6 000 × *g* (4°C) and the supernatants containing >90% of the PP1 and PP2A activities were removed for analysis.

2.3. *Measurement of protein phosphatase specific activities*

PP1 and PP2A activities were assayed in duplicate or triplicate at 15–30 fold dilutions of the larval supernatants as described in [16,17]. The release of ^{32}P-radioactivity from ^{32}P-labelled phosphorylase and casein was completely inhibited by 1 μM okadaic acid, indicating that all the activity was catalysed by PP1 and PP2A, and not caused by proteolytic cleavage to trichloroacetic acid soluble phosphopeptides. One unit of activity was that amount which catalysed the dephosphorylation of 1.0 μmol of substrate in 1 min.

3. RESULTS

3.1. *Sequence of the wild type* PP1 87B *gene*

The *PP1 87B* gene (Fig. 1) is colinear with the cDNA showing that there are no introns, and the coding region is identical to the cloned cDNA sequence reported previously [10]. In the 5′ non-coding region there is a recognisable TATA element situated at −389 to −383 and a CCAAT regulatory element at −634 to −630. A potential NFIII binding site ATTTGCAT at −288 to −281 and an putative inverted SP1 binding site

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wt                                                                  TGAATTAACCTATGTTCTGTAAACGA ATATTCTTATTGGCTTATTTGAATTTCCAA  -631
wt    TGTATTTAAATATCCCCTTGTTACCGGTAT ATCGAATAGCCTCGCCAAATGCATCAGTCT CCCATCTCTTTTCCACAGTAAGTCATGCCA  -541
wt    GGCGTGCCTGAGTGCAAGCGGGATGGCTAC TGTCCCCCTGATCAGGCTGCCACACCGCTG AAATAGAGTAACTGGACGCCATGCGTTTAA  -451
e211                                                  GGCTCGTCTTGTTCACATTGTCGCTGAG AACGTATGTCGTTTTGATTCATTGTTCTAA
wt    TCGATTACTAACAGCACTTTCGAGCCACTA TAGGGTCGAGAAGACCCGTTTCGAATGAAA ATATAAAAGTAGAGCTATTGCGGAACTCTT  -361
e211  GTCCAGATATAACAATTACCAGAACAATAT GGAATAATTATTTATTTTAGTAAATAATTA CTATAGTCAATAATTGTTAGTTGTGCAAAC
wt    GCAATGGATTGGAAATGTCCTTAATGTAAT AAGAGTAATATTACAGCAACTTCGTTATCG ATTATGCACTATATTTGCATGTTATCGATT  -271
e211  TGCACTTTACACTTTGTGTTTAACTTTCAC TATACTGTTGTAGTTTTTAGAACTTGCTAT TCGTAGCTTGAAAGAAACACGTCTCCACCC
wt    AGATAAGATGGCGTCTATTATCTGGCCATA TCCGCGACTTCCGGCAGTGTGGCAACATCA GCTAGAAGCACACTCGCCATCCCGCCCGCA  -181
e211  GAAGACTGTGGAATGC·····
wt    CTCACGCAACAGCATACGAAGAAATTTTCA TACTTGTTAGCTGTGAAAAGTATTTAGCAG AAATAGATTTCGGAAATTAAAGATTTCGAG   -91
e211  ·····
wt    GCTATTTCGGCGCTACCAACGATCGTTCGC GGTAACCTCGACACCCAACAGCAGCACTAG TGCACCAGATCCACACTTTCGCACGCAAAC    -1
e211  ·····
wt    ATGGGCGACGTGATGAATATCGACAGCATA ATATCGCGACTTCTCGAGGTGCGTGGGGCA CGGCCAGGTAAAAACGTACAGCTCTCGGAG    90
        M  G  D  V  M  N  I  D  S  I  I  S  R  L  L  E  V  R  G  A  R  P  G  K  N  V  Q  L  S  E
e211  ·····
wt    GGCGAGATCCGGGGACTTTGCTTGAAGTCG CGCGAGATCTTCCTGTCGCAGCCCATTCTG CTCGAGCTAGAGGCACCGTTGAAGATCTGC   180
        G  E  I  R  G  L  C  L  K  S  R  E  I  F  L  S  Q  P  I  L  L  E  L  E  A  P  L  K  I  C
e211  ·····
wt    GGCGACATCCATGGACAGTACTACGATCTG TTGCGTCTGTTCGAGTACGGCGGCTTTCCG CCGGAATCGAATTACCTGTTCCTCGGCGAC   270
        G  D  I  H  G  Q  Y  Y  D  L  L  R  L  F  E  Y  G  G  F  P  P  E  S  N  Y  L  F  L  G  D
e211  ·····
wt    TACGTCGATCGCGGCAAGCAATCGCTGGAG ACGATCTGCCTGCTGCTCGCCTACAAGATC AAATACTCGGAGAATTTCTTCCTGCTGCGC   360
        Y  V  D  R  G  K  Q  S  L  E  T  I  C  L  L  L  A  Y  K  I  K  Y  S  E  N  F  F  L  L  R
e211  ·····
wt    GGCAACCACGAATGCGCCAGCATTAATCGC ATATACGGATTCTACGACGAATGCAAGCGT CGCTACAGCATCAAGTTGTGGAAGACATTC   450
        G  N  H  E  C  A  S  I  N  R  I  Y  G  F  Y  D  E  C  K  R  R  Y  S  I  K  L  W  K  T  F
e211  ·····
wt    ACGGACTGCTTCAACTGCCTGCCAGTGGCG GCCATTGTCGACGAGAAGATCTTCTGCTGC CACGGTGGTCTCAGTCCCGATTTGACCTCC   540
        T  D  C  F  N  C  L  P  V  A  A  I  V  D  E  K  I  F  C  C  H  G  G  L  S  P  D  L  T  S
e211  ·····
wt    ATGGAGCAGATCCGTCGCATTATGCGGCCA ACCGATGTGCCCGACCAGGGACTGCTGTGC GATCTCCTGTGGTCCGATCCCGATAAGGAT   630
        M  E  Q  I  R  R  I  M  R  P  T  D  V  P  D  Q  G  L  L  C  D  L  L  W  S  D  P  D  K  D
e211  ·····
wt    ACCATGGGCTGGGGCGAAAACGACCGCGGC GTTAGCTTCACCTTCGGTGCCGAGGTGGTG GCCAAGTTTTTGCAGAAGCACGAGTTCGAT   720
        T  M  G  W  G  E  N  D  R  G  V  S  F  T  F  G  A  E  V  V  A  K  F  L  Q  K  H  E  F  D
e211  ·····
wt    CTCATCTGCCGAGCCCATCAAGTCGTCGAG GATGGGTACGAGTTCTTTGCCAAACGCATG CTGGTCACCCTGTTCTCGGCGCCCAACTAC   810
        L  I  C  R  A  H  Q  V  V  E  D  G  Y  E  F  F  A  K  R  M  L  V  T  L  F  S  A  P  N  Y
e211  ·····
wt    TGCGGCGAGTTCGACAATGCCGGCGCCATG ATGTCCGTGGACGATACGCTGATGTGCTCG TTCCAAATCCTCAAGCCAGCCGACAAGCGT   900
        C  G  E  F  D  N  A  G  A  M  M  S  V  D  D  T  L  M  C  S  F  Q  I  L  K  P  A  D  K  R
e211  ·····
wt    AAAAAGTAATATCACACAACTGCAGCACCA CGAGCAGTCTTTTTCTATCTAAAACAGATC AATAAGAAATCCAAACAAGATACAAAAGAT   990
        K  K
e211  ·····
wt    AAACACGACAAAAACAACCACAAACCCAAC CACACAATCAACCAGAAATAAAATCCTTTG AAACAACACATCAAATGATGCATGAAACGT  1080
e211  ·····
wt    AAGTATGGGCTGCTGACGCAGAGTAACAAA CTAGTGAATCACACTCTCACCCCAACCTTA TCTCTGTTTTTGGTCACTTTTATCATCGGC  1170
e211  ·····
wt    AGCCGGGGCGAGTCGAAATCCACAAGAAAT TGCACCATTAATTTGGTATGGAATAGC                                    1227
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Fig. 1. Comparison of the nucleotide sequences of the gene for protein phosphatase 1 87B in mutant *ck19*^{e211} (e211) and wild type (wt) *Drosophila*. Dots indicate identity of *ck19*^{e211} with wild type. The numbering of the nucleotides starts at the first base of the initiating ATG, with nucleotides preceding this, indicated by a negative sign. The CCAAT and TATAAAA boxes and putative polyadenylation signal AATAAA are indicated by double underlining. Other potential regulatory elements are underlined once. The encoded amino acid sequences of the protein phosphatase 1 87B are also shown.

GGGCGG at −189 to −184 are also present (reviewed in [18]). In the 3′ non-coding region there is a putative polyadenylation site at 1037 to 1042. Assuming the addition of a poly A tail of 0.1 kb and the initiation of transcription ~25 nucleotides 3′ to −383, this would lead to an mRNA of approximately 1.5 kb, consistent with the smaller message size seen on Northern blots [10]. No open reading frames, which could encode proteins greater than 50 amino acids in length, were found in the 5′ and 3′ non-coding regions of the gene.

3.2. *Sequence of the mutation* ck19^{e211} *in the* PP1 87B *gene which causes abnormal mitosis*

The *ck19*^{e211} mutant gene (Fig. 1) diverges from wild type 165 bp 5′ to the initiating ATG of the PP1 open reading frame. The TATA box which starts at −389 in the wild type gene is therefore deleted from the *ck19*^{e211} chromosome. Deletion of this promoter region would be expected to lead to loss or reduction of transcription of the PP1 87B mRNA in the *ck19*^{e211} mutant. This finding explains why no PP1 87B mRNA is detected in this mutant by Northern blotting and is in accordance with restriction mapping studies which indicated that the deletion was larger than 6 kb [13].

3.3. *Protein phosphatase 1 activity in mutant larvae*

The activity of PP1 was examined in extracts from third instar larvae of wild type *Drosophila* and the mitotic mutant hemizygous for *ck19*^{e211}, the mutant allele being uncovered by the deletion Df(3R)E-079. Table I shows that the PP1 specific activity in this mutant was 21% of the wild type level, while PP2A activity was similar to wild type levels. The latter result establishes that the decrease in PP1 activity is specific for this enzyme and not due to deterioration of the samples. Although PP1 activity in larvae bearing one *ck19*^{e211} allele *in trans* with one wild type allele of *PP1 87B* carried on the TM6B balancer chromosome was lower than the wild type value, the difference was not significant (Table I).

The other mitotic mutant at 87B, *ck19*^{hs46} had 20% of wild type PP1 activity, PP2A activity again being normal. The result is consistent with *ck19*^{hs46} being a null mutation [13], as shown for *ck19*^{e211}. The activity of PP1 in *Drosophila* hemizygous for the lethal mutation *ck19*^{e078} was 35% of the wild type level, the level of PP2A activity being normal. PP1 activity in another mutant *ck18*^{e312}, which carries a lethal mutation in a different complementation group, was similar to wild type (Table I).

Table I

Specific activities of protein phosphatases 1 and 2A in mutant and wild type *Drosphila* larvae

Strain	PP1 specific activity (mU/mg) Average ± SD (*n*)	PP2A specific activity (mU/mg) Average ± SD (*n*)
Oregon R (wild type)	7.08 ± 1.81 (17)	1.28 ± 0.38 (11)
ck19^{e211} Df(3R)E-079	1.48 ± 0.39 (16)	1.58 ± 0.55 (12)
ck19^{e211} TM6B	5.26 ± 1.26 (7)	1.56 ± 0.51 (5)
ck19^{hs46} Df(3R)E-079	1.40 ± 0.54 (7)	1.25 ± 0.31 (4)
ck19^{e078} Df(3R)E-079	2.45 ± 0.83 (10)	1.13 ± 0.22 (6)
ck18^{e312} *ck18*^{e312}	7.9 ± 2.40 (3)	not determined

PP1 and PP2A assays were carried out in the presence and absence of 2 nM okadaic acid [17] or after preincubation for 10 min in the presence and absence of 100 nM inhibitor-2 [16]. PP1 was measured by the release of ^{32}P-radioactivity from phosphorylase a (10 μM) and taken as the activity inactivated by inhibitor-2 and/or the activity not inhibited by 2 nM okadaic acid. PP1 activities measured by each procedure gave similar values. PP2A activity was measured by the release of ^{32}P-radioactivity from ^{32}P-labelled casein (^{32}P = 6 μM) after preincubation for 10 min with 100 nM inhibitor-2 to inactivate PP1. All assays were carried out 'blind', the strain being identified subsequent to the enzyme activity measurements. Several different batches of *Drosophila* larvae were examined for each strain. SD is the standard deviation and *n* is the number of extracts assayed.

4. DISCUSSION

4.1. *The* PP1 87B *gene product is the major type 1 protein phosphatase activity in larvae*

Recently, we demonstrated that *Drosophila* carrying either of two mutations (*ck19*^{e211} and *ck19*^{hs46} at the 87B locus on chromosome 3 are delayed in progress through mitosis and show defective mitotic spindle organisation, abnormal sister chromatid segregation, hyperploidy and an excessive degree of chromosome condensation [13]. Germline transformation of the mutant *ck19*^{e211} flies with the wild type *PP1 87B* gene, but not with a disrupted *PP1 87B* gene, restores normal mitosis, viability and fertility. We have now compared the sequence of the mutant *ck19*^{e211} gene with the wild type gene and have shown that a deletion is present in the promoter region of the gene (Fig. 1), explaining why no *PP1 87B* mRNA is detectable in this mutant. More importantly, we have demonstrated that this mutant possesses only 21% of wild type PP1 activity (Table I). This finding indicates that PP1 87B is the major PP1 activity measurable at the late larval stage in wild type *Drosophila*, accounting for ~80% of the activity. This conclusion was reinforced by analysis of a second mutant *ck19*^{hs46} which possessed 20% of wild type PP1 activity (Table I). This mutant makes RNA species that are 0.2 kb smaller and less abundant than wild type, while Southern blotting experiments have revealed a 0.5

kb truncation at the 5′ end of the gene [13]. The deletion would be expected to prevent translation or lead to synthesis of a truncated protein. The latter, if produced, would be inactive, since a highly conserved domain likely to be essential for catalytic activity is present in only ~200 base pairs 3′ of the initiating ATG [3,5]. The residual 20% of wild type activity seen in the mutants *ck19*^{e211} and *ck19*^{hs46} may originate from the other PP1 isoforms [11]. However, one cannot exclude the possibility that a part of the residual activity is due to the presence of residual maternal PP1 87B which may not have been degraded.

In contrast to the situation in *Drosophila*, where loss of a single isoform leads to a mitotic defect, deletion of a single PP1 gene in either fission yeast [8] or *Aspergillus* [7] has no effect on viability. Deletion of both the PP1 genes in fission yeast is required to generate a lethal phenotype. The reasons for these species differences are unknown, because the contributions of the different PP1 gene products to overall PP1 activity have not yet been assessed in either fission yeast or *Aspergillus*. The differences may, of course, lie in the multicellular nature of *Drosophila*, if the various PP1 genes are expressed in different cells.

4.2. *A threshold level of PP1 87B activity is required for the completion of mitosis*

The third mutant at 87B (*ck19*^{e078}) shows little evidence of abnormal mitosis. Nevertheless, it also dies at the late larval stage, and germline transformation with a P element bearing the wild type *PP1 87B* gene rescues the lethal phenotype [13]. The present work offers a molecular explanation for this surprising observation by demonstrating that PP1 87B activity is deficient, but not completely absent, in the *ck19*^{e078} mutant. This is consistent with Northern blotting experiments which have demonstrated that the mRNA in this mutant is the same size as wild type and present at a similar concentration [13]. Presumably, this mutant possesses a point mutation or a very small deletion/addition.

The *ck19*^{e078} mutant has 35% of wild type PP1 activity (Table I). If it is assumed that PP1 isozymes are expressed uniformly throughout larval tissues, two models can be put forward to explain why mitosis is almost normal in the *ck19*^{e078} mutant. Firstly there may be a threshold level of total PP1 activity (35%) for successful completion of cell division. This explanation assumes that at least two PP1 isoenzymes are functionally equivalent, and contribute to that threshold level. One of these is PP1 87B, since the other isoforms together supply only 20% of wild type activity, which is insufficient for normal mitotic function as shown by analysis of the *ck19*^{e211} and *ck19*^{hs46} mutants. Alternatively, the PP1 87B isoform may have a specialised role in mitosis which cannot be substituted for by other PP1 isoforms.

4.3. *Protein phosphatase 1 participates in other vital cellular processes*

The observation that the *ck19*^{e078} mutant proceeds through mitosis in a nearly normal manner, yet still dies at the larval-pupal boundary, implies that PP1 87B has another vital function at this stage, which requires greater activity of this enzyme than does mitosis. Since the lethal phenotype was rescued by germline transformation with the wild type *PP1 87B* gene, but not with a disrupted *PP1 87B* gene [13], the vital function must be supplied by *PP1 87B* or an overlapping gene. However, as no other open reading frames were found in the 5′ and 3′ noncoding regions of the *PP1 87B* gene, it would appear that PP1 is essential for a second vital process additional to its role in mitosis.

4.4. PP1 87B *mutant alleles result in suppression of heterochromatic position effect variegation*

In *Drosophila*, rearrangements which place euchromatic genes into a region of heterochromatin result in inactivation of the euchromatic genes close to heterochromatin with an accompanying condensation of chromatin structure. The transcriptional activity of the euchromatic gene varies in different cell populations leading to a 'variegated' effect. Reuter et al. [19] have reported that, in the heterozygous state, several alleles of the *ck19* lethal complementation group have the ability to suppress this heterochromatic position effect variegation. One of these, *Su var (3) 6*, has been located between 87B 5-6 and 87B 8-10 by deficiency mapping and shown to be allelic to lethals of the *ck19* complementation group, including *ck19*^{e078}. In the present work and [13], analyses of *ck19*^{e078} show that the lethality of this mutant results from deficient PP1 activity. Assuming that *ck19* is not a complex locus (no other open reading frames are present in the non-coding sequences the *PP1 87B* gene), suppression of position effect variegation must result from a mutation in the gene for PP1 87B, and not an overlapping gene. As PP1 activity is required to control condensation of the chromosomes during mitosis, it is perhaps not surprising that this enzyme also plays a role in regulating the state of interphase chromatin. It will be of considerable interest to try and identify hyperphosphorylated proteins in mutant larvae which are defective in PP1 87B.

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