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Essential, Overlapping and Redundant Roles of the Drosophila Protein Phosphatase 1α and 1β Genes

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ABSTRACT

Protein serine/threonine phosphatase type 1 (PP1) has been found in all eukaryotes examined to date and is involved in the regulation of many cellular functions, including glycogen metabolism, muscle contraction, and mitosis. In Drosophila, four genes code for the catalytic subunit of PP1 (PP1c), three of which belong to the PP1α subtype. *PP1β9C* (*flapwing*) encodes the fourth PP1c gene and has a specific and nonredundant function as a nonmuscle myosin phosphatase. *PP1α87B* is the major form and contributes ~80% of the total PP1 activity. We describe the first mutant alleles of *PP1α96A* and show that *PP1α96A* is not an essential gene, but seems to have a function in the regulation of nonmuscle myosin. We show that overexpression of the PP1α isozymes does not rescue semilethal *PP1β9C* mutants, whereas overexpression of either PP1α96A or PP1β9C does rescue a lethal *PP1α87B* mutant combination, showing that the lethality is due to a quantitative reduction in the level of PP1c. Overexpression of PP1β9C does not rescue a *PP1α87B, PP1α96A* double mutant, suggesting an essential PP1α-specific function in Drosophila.

ONE of the most widespread mechanisms of post-translational regulation of proteins is the addition of phosphate by protein kinases; this phosphorylation is antagonized by protein phosphatases. Reversible phosphorylation of proteins can regulate their activity, cellular location, or binding affinity. The antagonistic actions of protein kinases and protein phosphatases are of equal importance in determining the degree of phosphorylation of each substrate protein. Among the serine/threonine protein phosphatases, serine threonine protein phosphatase type 1 (PP1) forms a major class and is highly conserved among all eukaryotes examined to date (Lin *et al.* 1999). PP1 is involved in the regulation of many cellular functions, including glycogen metabolism, muscle contraction, and mitosis (reviewed in Bollen 2001; Cohen 2002; Ceulemans and Bollen 2004).

In *Drosophila melanogaster*, as in mammals, two PP1 subtypes exist: PP1α (homologous to mammalian PP1α and PP1γ) and PP1β (homologous to mammalian PP1δ, also known as PP1β). Three genes code for the PP1α isozyme and are named after their respective chromosomal location: *PP1α13C* (FlyBase: *Pp1-13C*), *PP1α87B* (*Pp1-87B*), and *PP1α96A* (*Pp1α-96A*). Only one gene, *PP1β9C* (*flapwing*, *flw*), encodes the PP1β type. The protein sequences of PP1α and PP1β are extremely similar (88% identity over the first 300 amino acids), yet the PP1α/β subtype difference is conserved in mammals (Dombrádi *et al.* 1993).

In vitro, the catalytic subunit of PP1 (PP1c) dephosphorylates a wide variety of substrates, but *in vivo* it is found complexed to a number of different proteins that modify its substrate activity and specificity and target it to specific locations. Such targeting and regulatory proteins are therefore key to investigating the role of PP1 in specific subcellular processes [*e.g.*, glycogen metabolism through PTG (Printen *et al.* 1997) or Dpp receptor type 1 deactivation through Sara (Bennett and Alphey 2002)]. In a yeast two-hybrid assay for PP1c-binding proteins in Drosophila, we found that the large majority of PP1c-binding proteins bind all four PP1c isozymes (Bennett *et al.* 2006). Very few proteins have been identified that specifically bind some PP1c isozymes but not others. These include mammalian Neurabin I and Neurabin II/Spinophilin, which bind PP1α and PP1γ, but not PP1δ (MacMillan *et al.* 1999; Terry-Lorenzo *et al.* 2002), and Drosophila MYPT-75D, which binds PP1β, but not PP1α (Vereshchagina *et al.* 2004).

PP1α87B is the most abundant PP1c isozyme in Drosophila. In third instar larvae, PP1α87B contributes ~80% of the total PP1 activity (Dombrádi *et al.* 1990). In *PP1α87B* heterozygous mutants, the total PP1 activity is decreased by 40% but viability is not affected (Dombrádi *et al.* 1990). *PP1α87B* homozygotes are lethal and show suppression of position-effect variegation,

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PP1α87B        293 ILKPADKRKK                                  302
PP1α13C        293 ILKPVEKRKK                                  302
PP1α96A        293 ILKPADKRRFVYPNFGSSGRPLTPPRGAN--NKNKKK 327
PP1β9C         294 ILKPSEKKAKYLYSGMNSSRPTTPQRSAPMLATNKKK 330
hPP1β          294 ILKPSEKKAKYQYGGLNSGRPVTPPRTA---NPPKKR 327
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Figure 1.—Multiple-sequence alignment of the C termini of Drosophila PP1c and human PP1β (hPP1β). The amino acid sequences for PP1c are extremely conserved between the Drosophila PP1c proteins as well as Drosophila PP1c compared to human PP1 (Dombrádi *et al.* 1993). PP1α87B and PP1α13C have lost the C terminus, which contains a Cdk phosphorylation site (T P P/Q R).

chromosome hypercondensation, and abnormal spindle structure (Axton *et al.* 1990; Dombrádi *et al.* 1990; Baksa *et al.* 1993).

PP1α13C is located within intron 4 of the gene *abnormal chemosensory jump 6* (*acj6*). A loss-of-function deletion of part of *acj6*, which also removes *PP1α13C*, is homozygous viable. *PP1α13C* is therefore not an essential gene and has no unique essential function that is not redundant with the other PP1c genes. Deletion of *acj6* affects the sensilla, maxillary palp sense organs, and the laminar plexus, causing chemosensitive behavior defects. These visual and behavioral defects of the *acj6* deletion are not complemented by overexpression of *PP1α13C* cDNA (Clyne *et al.* 1999) and may therefore be entirely due to the deletion of *acj6*.

All mammalian PP1c proteins have a 25- to 27-amino acid C-terminal region that contains a cdk phosphorylation motif (T P P/Q R) (Ishii *et al.* 1996), the threonine residue of which (T320 in human PP1α) has been shown to be important for G_1 progression in cell cycle (Berndt *et al.* 1997). In Drosophila PP1α, this region is retained in PP1α96A, but lost from PP1α87B and PP1α13C (Figure 1). This is the first study that genetically characterizes *PP1α96A* by mutational analysis, revealing a function in nonmuscle myosin regulation (see results).

Strong alleles of *PP1β9C* (*PP1β9C*^{6} and *PP1β9C*^{7}) show failure in maintaining larval muscle attachment, have crumpled or blistered wings, and lack indirect flight muscles (IFMs). The weak and viable allele *PP1β9C*^{1} is flightless due to disorganized IFMs, but otherwise appears normal (Raghavan *et al.* 2000). The nonmuscle myosin phosphatase-targeting subunit MYPT-75D binds PP1β and targets it to its substrate, nonmuscle myosin regulatory light chain (Spaghetti squash, Sqh). Dephosphorylation of Sqh inhibits contraction mediated by the nonmuscle myosin heavy chain (nmmHC; Zipper, Zip). *PP1β9C*^{6} mutant clones have elevated levels of phospho-Sqh and presumably hyperactivated nmmHC. Mutations in genes that lead to downregulation of nmmHC activity, *e.g.*, *nmmHC*, *Rho*, and *RhoGEF2*, rescue the semilethality of *PP1β9C*^{6}, showing that regulation of nonmuscle myosin activity is the single essential function of *PP1β9C* (Vereshchagina *et al.* 2004). In contrast to most PP1c-binding proteins, MYPT-75D binds specifically to PP1β and not to PP1α (Vereshchagina *et al.* 2004), which may account for the specificity of the *PP1β9C* phenotypes and the genetic interactions between *PP1β9C* and genes involved in the regulation and function of cytoplasmic myosin.

In this study, we describe the first mutant alleles of *PP1α96A* and show that the gene is not essential. However, *PP1α96A* mutants enhance the weak, viable allele *PP1β9C*^{1} through nonmuscle myosin heavy chain, indicating that *PP1α96A* has a role in the regulation of nonmuscle myosin. We tested for redundancy between PP1α and PP1β by ubiquitously expressing PP1c in *PP1β9C* and *PP1α87B* mutant backgrounds, respectively. We show that overexpression of PP1α does not rescue the semilethality of *PP1β9C*^{6}, whereas overexpression of PP1β does rescue the lethality of a *PP1α87B* mutant, showing that the lethality of *PP1α87B* is due to a quantitative reduction in the level of PP1c, rather than a *PP1α87B*-specific function in the development of Drosophila. However, expression of PP1β does not rescue a *PP1α87B, PP1α96A* mutant combination, which suggests that at least one essential process, possibly late metamorphosis, depends on PP1α function specifically.

MATERIALS AND METHODS

Fly strains and genetics: pUAS-HA-PP1c constructs were as previously described (Bennett *et al.* 2003). UAS-HA-PP1c insertions on the third chromosome were recombined with either *arm-Gal4* or *PP1α87B*$^{87Bg\text{-}3}$. *w; arm-Gal4, UAS-HA-PP1c* males were crossed to *w, PP1β9C*^{6}*/FM7c* virgin females to test for complementation of *PP1β9C*. *w; PP1α87B*$^{87Bg\text{-}3}$*, UAS-HA-PP1c/TM6B* males were crossed to *w; arm-Gal4; PP1α87B*^{1}*/TM6B* virgin females to test for complementation of *PP1α87B*. The *PP1α87B*^{1} chromosome is marked with *e*. *PP1α96A*^{1} was generated by the Drosophila Gene Search Project (Toba *et al.* 1999) and has a P{GSV6} insertion in the 5′-UTR of *PP1α96A* (line GS11179). The *PP1α96A*^{2} null mutant was generated by imprecise excision of *PP1α96A*^{1} with *P{Δ2-3}* transposase (Laski *et al.* 1986). The original *PP1α96A*^{1} chromosome was found to have at least one lethal mutation outside of the 96A region, which was removed by recombining it to *ru, h, st, ry, e* prior to the excision experiments, so the complete genotypes for *PP1α96A*^{1} and *PP1α96A*^{2} are *ru, h, st, ry, e, PP1α96A*^{1} and *ru, h, st, ry, e, PP1α96A*^{2}, respectively.

Fly extracts and immunoblotting: Flies were taken up and homogenized in 2× SDS sample buffer (100 mm Tris–HCl, pH 6.8, 200 mm dithiothreitol, 4% SDS, 20% glycerol, bromophenol blue) and the proteins were separated by SDS–polyacrylamide gel electrophoresis (Sambrook *et al.* 1989). Separated proteins were transferred onto an Immobilon-P PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with 5% nonfat powdered milk in PBST (137 mm NaCl, 3 mm KCl, 10 mm Na_2HPO_4, 2 mm KH_2PO_4, 0.1% Tween 20) and incubated with 1:1000 anti-HA 12CA5 (F. Hoffmann La-Roche) or 1:2000 anti-α-tubulin T9026 (Sigma, St. Louis) as a primary and 1:10,000 horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma) as a secondary antibody. HRP was detected with Supersignal West Pico (Pierce, Rockford, IL) and blue-sensitive X-ray film (GRI).

Preparation of cDNA and RT–PCR: Total RNA from third instar larvae or adult flies was extracted using Trizol (Invitrogen, San Diego) according to the manufacturer's instructions.

cDNA was prepared using the SuperScript first-strand synthesis system for RT–PCR kit (Invitrogen) according to the manufacturer's instructions. The *PP1α96A* RT–PCR primers (CTGCGGGGAATTCGACAACG and TGTGTGTGTGGCCG TTTGTAG) anneal at the C terminus of *PP1α96A*, which is not deleted in *PP1α96A²*.

RESULTS

Mutagenesis and analysis of *PP1α96A*: *PP1α96A is not an essential gene:* The role of *PP1α96A* has remained unclear because no mutants have been described. Therefore, we decided to generate mutants for this gene and found that one of the P{GSV6} elements from the Drosophila Gene Search Project (Toba *et al.* 1999) was inserted in the 5′-UTR of *PP1α96A* (Figure 2A). We named this allele *PP1α96A¹*. The original *PP1α96A¹* chromosome was homozygous lethal; however, *PP1α96A¹/Df(3R)crb87-5* flies were viable and without phenotype even though *Df(3R)crb87-5* completely deletes *PP1α96A* (Wustmann *et al.* 1989; Kellerman and Miller 1992; our own molecular analysis, not shown). This suggested that the *PP1α96A¹* chromosome contained at least one additional and lethal mutation, but that *PP1α96A¹* itself might be viable. We exchanged the majority of the chromosome by generating a *ru, h, st, ry, e, PP1α96A¹* recombinant chromosome. This recombinant was homozygous viable and exhibited no obvious mutant phenotype apart from the markers, showing that we had removed a lethal mutation unrelated to *PP1α96A¹*. P insertions in 5′-UTRs often reduce the transcription level of the respective gene. We therefore used RT–PCR to assess the transcript levels of *PP1α96A* in *PP1α96A¹* homozygous and heterozygous third instar larvae and found that the level of *PP1α96A* transcript was greatly reduced in *PP1α96A¹* homozygotes (Figure 2B).

We generated several deletion derivatives by imprecise excision of *PP1α96A¹* and molecularly analyzed their breakpoints. We found that one of these, *PP1α96A²*, was a deletion of 1.7 kb, which removed the proximal promoter region, transcription start, and exons 1–3 of *PP1α96A*, but not the adjacent gene *CG13617* (Figure 2A). RT–PCR on extracts from *PP1α96A²* homozygous and heterozygous flies failed to detect *PP1α96A* transcript in the homozygotes (Figure 2C); taking this with the molecular analysis of the deletion, we concluded that *PP1α96A²* is a null allele for *PP1α96A*. *PP1α96A²* is homozygous viable, fertile, and without any obvious phenotype, showing that *PP1α96A* is not an essential gene. Unlike *PP1α87B*, *PP1α96A* is not a suppressor of position-effect variegation (not shown).

Genetic interactions between PP1α96A and PP1α87B mutants: We assume that PP1α96A contributes at most 10% of the total PP1 activity in Drosophila, as PP1α87B and PP1β9C contribute ~80 and 10%, respectively (Dombrádi *et al.* 1990; Vereshchagina *et al.* 2004). Therefore, we wondered whether *PP1α96A²* homozygotes might show

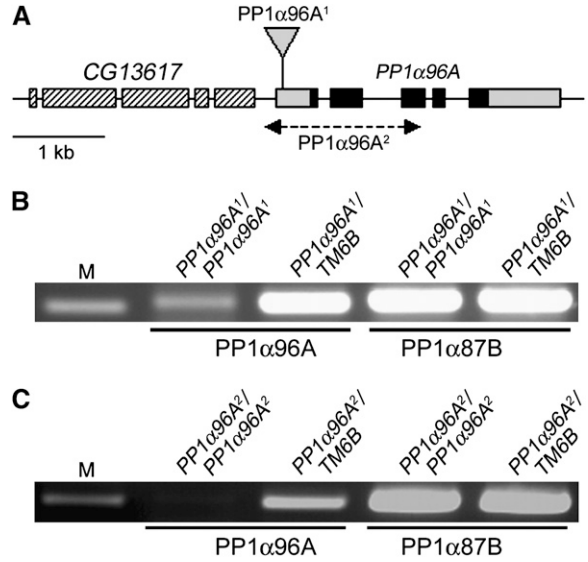

Figure 2.—(A) *PP1α96A* gene structure and mutants. Coding regions (black) and untranslated regions are indicated. The direction of transcription for *CG13617* and *PP1α96A* is from left to right. *PP1α96A¹* is due to an insertion in the 5′-UTR of *PP1α96A*. *PP1α96A²* is a 1.7-kb deletion that deletes the proximal promoter region, transcription start, and exons 1–3 of *PP1α96A*, but not the adjacent gene *CG13617*. (B) The level of *PP1α96A* transcript is reduced in *PP1α96A¹* homozygotes. (C) No *PP1α96A* transcript can be detected in *PP1α96A²* homozygotes. M, marker (200-bp band).

a phenotype in a background of reduced total PP1c. We introduced $PP1\alpha87B^{87Bg\text{-}3}/+$, which lowers the total PP1 activity by 40%, into a *PP1α96A²* background, but found that adult flies heterozygous for *PP1α87B* and homozygous for *PP1α96A* ($PP1\alpha87B^{87Bg\text{-}3}$, $PP1\alpha96A^{2}/PP1\alpha87B^{+}$, *PP1α96A²*) have normal viability and appearance (not shown). $PP1\alpha87B^{87Bg\text{-}3}/PP1\alpha87B^{1}$ *trans*-heterozygotes (which lack ~80% of total PP1 activity) die during pupariation and, until pupariation, do not lag behind in development compared to their heterozygous siblings. We also examined flies lacking wild-type copies of both *PP1α87B* and *PP1α96A* ($PP1\alpha87B^{87Bg\text{-}3}$, $PP1\alpha96A^{2}/PP1\alpha87B^{1}$, and *PP1α96A²*). These mutants, which might lack as much as 90% of total PP1 activity, died slightly earlier than $PP1\alpha87B^{87Bg\text{-}3}/PP1\alpha87B^{1}$ and pupariated 1–2 days later than their siblings. This suggests a weak enhancement of *PP1α87B* by *PP1α96A*, although a possible effect of genetic background cannot be ruled out. The pupal lethality of *PP1α87B* mutants, with or without *PP1α96A²*, could indicate a high requirement for total PP1 or PP1α during metamorphosis.

PP1α96A enhances PP1β9C through zip: Of the three PP1α genes in Drosophila, *PP1α96A* is the only one that has the conserved final 25–27 amino acids at its C terminus (Figure 1), but a related sequence is also present in *PP1β9C*. We therefore wondered whether a mutation in *PP1α96A* would genetically interact with *PP1β9C*. To test this, we used the weak allele *PP1β9C¹*,

TABLE 1

***PP1α96A* is an enhancer of *PP1β9C*^{1} (from cross *PP1β9C*^{1}/*FM7* ; *Df(3R)crb87-5/TM6B* × *PP1α96A*^{1}/*PP1α96A*^{1})**

F_1 genotype	
PP1β9C^{1}/*Y* ; *PP1α96A*^{1}/*Df(3R)crb87-5*	0
PP1β9C^{1}/*Y* ; *PP1α96A*^{1}/*TM6B*	29
FM7/*Y* ; *PP1α96A*^{1}/*Df(3R)crb87-5*	45
FM7/*Y* ; *PP1α96A*^{1}/*TM6B*	44
PP1β9C^{1}/+ ; *PP1α96A*^{1}/*Df(3R)crb87-5*	55
PP1β9C^{1}/+ ; *PP1α96A*^{1}/*TM6B*	51
FM7/+ ; *PP1α96A*^{1}/*Df(3R)crb87-5*	65
FM7/+ ; *PP1α96A*^{1}/*TM6B*	37
Total	326

The viable *trans*-heterozygous allelic combination *PP1α96A*^{1}/*Df(3R)crb87-5* is lethal in a *PP1β9C*^{1} mutant background. *Df(3R)crb87-5* completely removes *PP1α96A*. This shows that *PP1α96A* has an essential function in a *PP1β9C*^{1} mutant background.

TABLE 2

***nmmHC* (*zip*) suppresses the enhancement of *PP1β9C*^{1} by *PP1α96A* (from cross *PP1β9C*^{1}/*FM7* ; *Df(3R)crb87-5/TM6B* × *zip*^{1}/CyO ; *PP1α96A*^{1}/*PP1α96A*^{1})**

F_1 genotype	
PP1β9C^{1}/Y ; *zip*^{1}/+ ; *PP1A96A*^{1}/*Df(3R)crb87-5*	10
PP1β9C^{1}/Y ; CyO/+ ; *PP1α96A*^{1}/*Df(3R)crb87-5*	0
PP1β9C^{1}/Y ; *zip*^{1}/+ ; *PP1α96A*^{1}/*TM6B*	12
PP1β9C^{1}/Y ; CyO/+ ; *PP1α96A*^{1}/*TM6B*	16
FM7/Y ; *zip*^{1}/+ ; *PP1α96A*^{1}/*Df(3R)crb87-5*	18
FM7/Y ; CyO/+ ; *PP1α96A*^{1}/*Df(3R)crb87-5*	3
FM7/Y ; *zip*^{1}/+ ; *PP1α96A*^{1}/*TM6B*	3
FM7/Y ; CyO/+ ; *PP1α96A*^{1}/*TM6B*	5
PP1β9C^{1}/+ ; *zip*^{1}/+ ; *PP1α96A*^{1}/*Df(3R)crb87-5*	10
PP1β9C^{1}/+ ; CyO/+ ; *PP1α96A*^{1}/*Df(3R)crb87-5*	20
PP1β9C^{1}/+ ; *zip*^{1}/+ ; *PP1α96A*^{1}/*TM6B*	10
PP1β9C^{1}/+ ; CyO/+ ; *PP1α96A*^{1}/*TM6B*	23
FM7/+ ; *zip*^{1}/+ ; *PP1α96A*^{1}/*Df(3R)crb87-5*	16
FM7/+ ; CyO/+ ; *PP1α96A*^{1}/*Df(3R)crb87-5*	16
FM7/+ ; *zip*^{1}/+ ; *PP1α96A*^{1}/*TM6B*	7
FM7/+ ; CyO/+ ; *PP1α96A*^{1}/*TM6B*	22
Total	191

A mutant copy of the nonmuscle myosin heavy chain *zipper* suppresses the lethality of *PP1β9C*^{1}/Y ; *PP1α96A*^{1}/*Df(3R)crb87-5*, suggesting that *PP1α96A* enhances *PP1β9C*^{1} through the known role of *PP1β9C* in nonmuscle myosin regulation.

which is viable but flightless due to defects in the indirect flight muscles (Raghavan *et al.* 2000). *PP1β9C*^{1}/*Y; PP1α96A*^{1}/+ and *PP1β9C*^{1}/*Y; Df(3R)crb87-5*/+ were viable and do not exhibit any phenotype apart from rare defects in the posterior part of the wing (not shown). *PP1β9C*^{1}/*Y; PP1α96A*^{1}/*Df(3R)crb87-5*, however, was completely lethal (Table 1), showing that *PP1α96A* indeed interacts genetically with *PP1β9C*. The enhancement of *PP1β9C*^{1} by *PP1α96A* is specific to *PP1α96A* and not due to an overall reduction of PP1 activity, because *PP1β9C*^{1}/Y; *PP1α87B*$^{87Bg\text{-}3}$/+ is viable (not shown).

Genetic interaction between different mutants often indicates that the genes act in the same pathway. Since *PP1β9C* has a single essential and nonredundant function as a nonmuscle myosin phosphatase, we wondered whether the lethality of *PP1β9C*^{1}/Y; *PP1α96A*^{1}/*Df(3R)crb87-5* is due to a failure in nonmuscle myosin regulation and possibly to hyperactivation of nmmHC. To test this, we introduced the mutant *zip*^{1} into a *PP1β9C*^{1}/Y; *PP1α96A*^{1}/*Df(3R)crb87-5* background and found that this rescued the lethality (Table 2), suggesting that the genetic interaction between *PP1β9C* and *PP1α96A* is indeed related to the role of *PP1β9C* in the regulation of nonmuscle myosin.

Redundancy between PP1α and PP1β: *Overexpression of PP1α87B or PP1α96A does not complement PP1β9C:* Although the protein sequences of PP1α and PP1β are extremely similar (Dombrádi *et al.* 1993), they show structural differences that are also conserved in humans. This suggests that the two subtypes might have differential, nonredundant functions. To test for redundancy of PP1β, we expressed PP1α using the UAS/Gal4 system (Brand and Perrimon 1993) to see whether this would rescue the semilethal missense mutant *PP1β9C*^{6}. The altered amino acid of *PP1β9C*^{6}, Y^{133}, is thought to form a hydrogen bond to the peptide backbone of the substrate (Egloff *et al.* 1997); PP1β9C^{6} protein is therefore likely to bind its substrates with lower affinity.

w PP1β9C^{6}/*FM7c* virgin females were crossed to males of the genotype *w; arm-Gal4, UAS-HA-PP1c/TM6B*. The presence of *arm-Gal4* induces expression of *UAS-HA-PP1c* constructs throughout the fly and does not cause a phenotype. The expression level of *UAS-HA-PP1α13C* with *arm-Gal4* was extremely low (<40 times lower than *arm-Gal4, UAS-HA-PP1α87B*, not shown); therefore, *UAS-HA-PP1α13C* was not included in the complementation analysis. As expected, expressing HA-PP1β9C completely restored the viability (Table 3) as well as the wing phenotype (Raghavan *et al.* 2000) of *PP1β9C*^{6}/Y males. However, *PP1β9C*^{6}/Y could not be rescued by expression of HA-PP1α87B or HA-PP1α96A (Table 3, summarized in Table 5). Western Blots with α-HA showed clear expression of HA-PP1β9C and slightly lower levels of HA-PP1α87B and HA-PP1α96A (Figure 3). We subsequently found that the expression levels of HA-PP1α96A and HA-PP1β9C with *arm-Gal4* were sufficient to complement a *PP1α87B* mutant, which showed that the expressed proteins are functional (see below).

Overexpression of PP1α96A and PP1β9C can rescue PP1α87B: PP1α87B is the major PP1c isozyme in Drosophila and contributes ~80% of the total PP1 activity (Dombrádi *et al.* 1990). We wondered whether *PP1α87B* has a specific and nonredundant role in the development of Drosophila, which cannot be performed by another PP1c. We tested this by expressing PP1α96A

TABLE 3

Expressing HA-PP1c in a PP1β9C (*PP1β9C^{6}*) mutant background (from cross *w, PP1β9C^{6}/FM7c* × *w* ; *arm-Gal4, UAS-HA-PP1c*/TM6B)

F_1 genotype	UAS-HA-PP1β9C	UAS-HA-PP1α87B	UAS-HA-PP1α96A
w, PP1β9C^{6}/Y ; *arm-Gal4, UAS-HA-PP1c*/+	47	0	1
w, PP1β9C^{6}/Y ; *TM6B*/+	0	1	1
FM7c/Y ; *arm-Gal4, UAS-HA-PP1c*/+	28	40	35
FM7c/Y ; *TM6B*/+	9	17	30
w, PP1β9C^{6}/+ ; *arm-Gal4, UAS-HA-PP1c*/+	46	37	37
w, PP1β9C^{6}/+ ; *TM6B*/+	20	33	44
FM7c/+ ; *arm-Gal4, UAS-HA-PP1c*/+	48	48	35
FM7c/+ ; *TM6B*/+	27	30	35
Total	225	206	218

PP1β9C^{6} is a strong, semilethal allele of *PP1β9C*. Overexpression of HA-PP1β9C rescues the semilethality of *PP1β9C^{6}*, while overexpression of HA-PP1α does not, showing that PP1β9C is not functionally redundant with PP1α.

and PP1β9C in a *PP1α87B* mutant background, using the alleles *PP1α87B^{1}* and *PP1α87B$^{87Bg\text{-}3}$*. *PP1α87B^{1}* is an EMS-induced hypomorphic point mutant with a single amino acid replacement (G220S) in a residue that is conserved in all protein serine/threonine phosphatases of the PP1/PP2A/PP2B family (Dombrádi and Cohen 1992). *PP1α87B$^{87Bg\text{-}3}$* is an amorphic mutant resulting from DNA rearrangement in the 5′-end of *PP1α87B* (Axton *et al.* 1990). *PP1α87B^{1}/PP1α87B$^{87Bg\text{-}3}$ trans*-heterozygotes are lethal. *w; arm-Gal4; PP1α87B^{1}/TM6B* female virgins were crossed to *w; PP1α87B$^{87Bg\text{-}3}$, UAS-HA-PP1c/TM6B* males to generate flies that express HA-PP1c in a *PP1α87B* mutant background.

PP1α87B^{1}/PP1α87B$^{87Bg\text{-}3}$ was rescued by overexpression of *UAS-HA-PP1α87B* with *arm-Gal4*, as expected, but also by overexpression of HA-PP1α96A or HA-PP1β9C (Table 4, summarized in Table 5). The rescued flies had a normal appearance (not shown). This indicated that *PP1α96A* and *PP1β9C* can substitute for *PP1α87B* in its absence and suggests that the lethality of *PP1α87B* mutants is due to a reduction of overall PP1 activity and not to an essential and nonredundant function of *PP1α87B*.

Overexpression of PP1β9C does not rescue PP1α87B, PP1α96A double mutants: As shown above, expression of PP1β9C rescues a *PP1α87B* mutant. However, the presence of wild-type *PP1α96A* (and/or *PP1α13C*) in this background could mask an essential requirement for PP1α that PP1β could not perform. Therefore, we expressed PP1β9C in a *PP1α87B, PP1α96A* double-mutant background, by crossing *w; arm-Gal4, PP1α87B$^{87Bg\text{-}3}$, PP1α96A^{2}/TM6B* to *w; UAS-HA-PP1β9C, PP1α87B^{1}, PP1α96A^{2}/TM6B*. We found that overexpression of PP1β9C did not rescue the lethality of *PP1α87B, PP1α96A* double mutants (Table 6), indicating that Drosophila PP1α has at least one essential function that PP1β9C cannot perform, possibly during late metamorphosis, as *PP1α87B, PP1α96A* double mutants with *arm-Gal4, PP1β9C* die as late pupae.

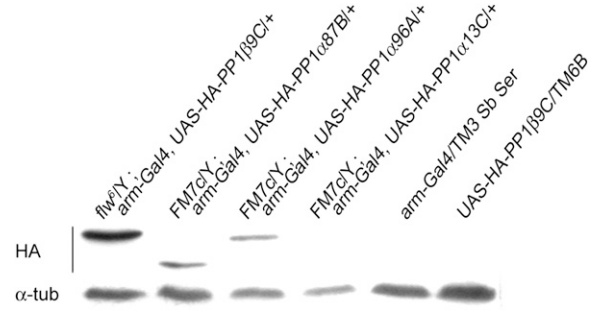

Figure 3.—Expression levels of UAS-HA-PP1c with *arm-Gal4* in the progeny of the crosses *PP1β9C^{6}/FM7c* × *arm-Gal4, UAS-HA-PP1c*. Expressed levels of HA-PP1c were detected with anti-HA. The amount of expressed HA-PP1α13C is below detection levels. HA-PP1α87B has slightly higher mobility because of its shortened C terminus (see Figure 1). α-tub, α-tubulin.

DISCUSSION

Protein phosphatase 1, as well as the PP1α/PP1β subtype difference, is highly conserved between Drosophila and mammals. Humans have three PP1 genes: PP1α and PP1γ are homologs of the Drosophila PP1α genes, and PP1δ (also known as PP1β) corresponds to PP1β.

We show here that expression of HA-PP1α87B and HA-PP1α96A does not rescue a semilethal allele of PP1β, which further supports our previous conclusion that *PP1β9C* has an essential function that cannot be performed by *PP1α* (Vereshchagina *et al.* 2004). It also allows us to rule out an alternative explanation for the *PP1β9C* mutant phenotype—that *PP1β9C*, but none of the PP1α forms, is expressed in a specific subset of cells during fly development. The data above rule out this

TABLE 4

Expressing HA-PP1c in a *PP1α87B* (*PP1α87B^{1}/PP1α87B^{87Bg-3}*) mutant background (from cross *w* ; *arm-Gal4* ; *PP1α87B^{1}/TM6B* × *w* ; *PP1α87B^{87Bg-3}*, *[UAS-HA-PP1c]/TM6B*)

F_1 genotype	No UAS-HA-PP1c	UAS-HA-PP1α87B	UAS-HA-PP1α96A	UAS-HA-PP1β9C
w/w ; *arm-Gal4/+* ; *PP1α87B^{1}/PP1α87B^{87Bg-3}*, *[UAS-HA-PP1c]*	0	42	18	27
w/Y ; *arm-Gal4/+* ; *PP1α87B^{1}/PP1α87B^{87Bg-3}*, *[UAS-HA-PP1c]*	0	49	24	26
w/w ; *arm-Gal4/+* ; *PP1α87B^{1}/TM6B*	34	40	38	49
w/Y ; *arm-Gal4/+* ; *PP1α87B^{1}/TM6B*	21	52	22	44
w/w ; *arm-Gal4/+* ; *PP1α87B^{87Bg-3}*, *[UAS-HA-PP1c]/TM6B*	20	51	39	33
w/Y ; *arm-Gal4/+* ; *PP1α87B^{87Bg-3}*, *[UAS-HA-PP1c]/TM6B*	34	39	34	36
w/w ; *arm-Gal4/+* ; *TM6B/TM6B*	0	0	0	0
w/Y ; *arm-Gal4/+* ; *TM6B/TM6B*	0	0	0	0
Total	109	273	175	215

PP1α87B^{1}/PP1α87B^{87Bg-3} is a lethal allelic combination of *PP1α87B*. Overexpression of HA-PP1α87B, HA-PP1α96A, and HA-PP1β9C rescues the lethality of *PP1α87B^{1}/PP1α87B^{87Bg-3}*, showing that HA-PP1α96A and HA-PP1β9C can substitute for the function of *PP1α87B* in a *PP1α87B* mutant background.

possibility, as overexpression of PP1α87B and PP1α96A did not rescue strong *PP1β9C* alleles, while expression of PP1β9C, under the control of the same driver, fully rescued the phenotype of *PP1β9C^{6}*. The specific, nonredundant function of PP1β has been identified as regulation of nonmuscle myosin activity, possibly through the PP1β-specific interactor MYPT-75D (Vereshchagina *et al.* 2004). *MYPT-75D* is the fly homolog of mammalian MYPT3 (myosin phosphatase-targeting subunit) and is related to *Mbs* (myosin-binding subunit), which is the homolog of mammalian MYPT1 and MYPT2. Mbs regulates myosin activity by targeting PP1c to its substrate myosin regulatory light chain (Mizuno *et al.* 1999). Recently, it was shown that human MYPT3 co-immunoprecipitated with PP1δ, and phosphorylation of MYPT3 by protein kinase A activated PP1δ toward cytoplasmic myosin regulatory light chain (Yong *et al.* 2006).

Of the four Drosophila PP1c genes, *PP1α87B*, *PP1α13C*, and *PP1β9C* have been previously characterized by mutational analysis (Axton *et al.* 1990; Clyne *et al.* 1999; Raghavan *et al.* 2000). By generating a viable and fertile null allele of *PP1α96A*, we found that *PP1α96A* is not an essential gene. It does, however, have an essential function in the *PP1β9C^{1}* mutant background, and this function probably relates to the regulation of nonmuscle myosin. PP1α96A, but not PP1α87B or PP1α13C, was able to bind to a fragment of MYPT-75D that lacked the N terminus in the yeast two-hybrid system (L. Alphey, unpublished results) although it did not bind to the whole MYPT-75D protein, whereas PP1β did (Vereshchagina *et al.* 2004). Thus, it is possible that PP1α96A may have a weak affinity to bind MYPT-75D and enhances PP1β by being partly redundant with it. Alternatively, PP1α96A could be partially redundant with *PP1β9C* through binding to Mbs. Studies on the crystal structure of the vertebrate PP1δ-MYPT1 complex suggest that the tyrosine residues Y305 and Y307 at the C terminus of chicken PP1δ are particularly important for its binding to the second ankyrin repeat of MYPT1 (Terrak *et al.* 2004). Y305 and Y307 are in the C-terminal part that PP1α87B

TABLE 5

Summary of overexpression experiments

	Rescued by overexpression of *arm-Gal4, UAS-HA-*			
PP1 mutant	*PP1β9C*	*PP1α87B*	*PP1α96A*	*PP1α13C*
PP1β9C^{6}/Y	+	−	−	−
PP1α87B^{87Bg-3}/PP1α87B^{1}	+	+	+	(−)

The semilethal *PP1β9C^{6}* mutant was rescued by overexpression of PP1β9C (+) but not by overexpression of PP1α87B or PP1α96A (−). A lethal *trans*-heterozygous *PP1α87B* mutant was rescued by overexpression of PP1β9C, as well as PP1α87B and PP1α96A. Since the expression level of UAS-HA-PP1α13C was extremely low (see Figure 3), it was not included in the overexpression experiments in a *PP1α87B* mutant background. ND, not determined.

TABLE 6

Expressing HA-PP1β in a *PP1α87B, PP1α96A* mutant background (from cross *w* ; *arm-Gal4* ; *PP1α87B*$^{87Bg-3}$, *PP1α96A*^{2}/*TM6B* × *w* ; *UAS-HA-PP1β9C, PP1α87B*^{1}, *PP1α96A*^{2}/*TM6B*)

F_1 genotype	
w/*w* ; *arm-Gal4*/+ ; *PP1α87B*$^{87Bg-3}$, *PP1α96A*^{2}/*UAS-HA-PP1β9C, PP1α87B*^{1}, *PP1α96A*^{2}	0
w/*Y* ; *arm-Gal4*/+ ; *PP1α87B*$^{87Bg-3}$, *PP1α96A*^{2}/*UAS-HA-PP1β9C, PP1α87B*^{1}, *PP1α96A*^{2}	0
w/*w* ; *arm-Gal4*/+ ; *PP1α87B*$^{87Bg-3}$, *PP1α96A*^{2}/*TM6B*	46
w/*Y* ; *arm-Gal4*/+ ; *PP1α87B*$^{87Bg-3}$, *PP1α96A*^{2}/*TM6B*	48
w/*w* ; *arm-Gal4*/+ ; *UAS-HA-PP1β9C, PP1α87B*^{1}, *PP1α96A*^{2}/*TM6B*	48
w/*Y* ; *arm-Gal4*/+ ; *UAS-HA-PP1β9C, PP1α87B*^{1}, *PP1α96A*^{2}/*TM6B*	48
w/*w* ; *arm-Gal4*/+ ; *TM6B*/*TM6B*	0
w/*Y* ; *arm-Gal4*/+ ; *TM6B*/*TM6B*	0
Total	190

Overexpression of HA-PP1β9C does not rescue the lethality of *PP1α87B*$^{87Bg-3}$, *PP1α96A*^{2}/*PP1α87B*^{1}, or *PP1α96A*^{2}. This suggests that HA-PP1β9C cannot substitute for the function of PP1α and that therefore there is an essential requirement for PP1α in Drosophila development.

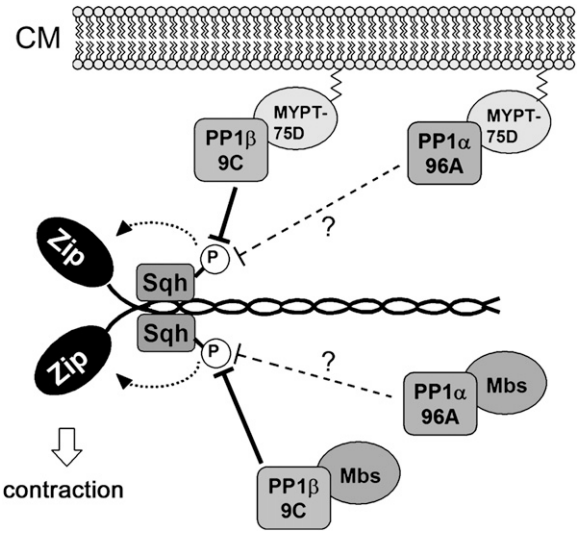

FIGURE 4.—Model for PP1α96A function in nonmuscle myosin regulation. Dephosphorylation of the nonmuscle myosin regulatory light chain (Sqh, Spaghetti Squash) inhibits the ATPase activity of nonmuscle myosin heavy chain (Zip, Zipper) and subsequent contraction. The major Sqh phosphatase PP1β9C is targeted to nonmuscle myosin by the nonmuscle myosin phosphatase-targeting subunits Mbs and MYPT-75D. MYPT-75D is prenylated and probably membrane associated. PP1α96A might have a weak affinity to Mbs and/or MYPT-75D and be partly redundant with PP1β9C. CM, cytoplasmic membrane.

and PP1α13C lack and are conserved in Drosophila PP1β (Y304 and Y306). PP1α96A has only one tyrosine residue at Y304, shifted by one amino acid compared to PP1β (Figure 1). The mammalian *Mbs* homologs MYPT1/2 seem to bind specifically to mammalian PP1β *in vivo* (HARTSHORNE 1998). Thus, even though all four Drosophila PP1c isozymes bind Mbs in the yeast two-hybrid system (VERESHCHAGINA *et al.* 2004), it may be that the majority of nonmuscle myosin phosphatase complexes *in vivo* consist of PP1β9C/Mbs and a minority of PP1α96A/Mbs (Figure 4). Both models would explain why *PP1α96A* and not, for example, *PP1α87B*, enhance *PP1β9C*^{1}: in a weak *PP1β9C* mutant background, *PP1α96A* would be able to compensate for partial loss of *PP1β9C* activity, but not in the strong and semilethal background of *PP1β9C*^{6}.

Even though PP1 is involved in the regulation of numerous cellular processes, relatively little is known about specific and nonredundant functions of the different PP1c isozymes. In mice, PP1γ (probably the testis-specific splice variant PP1γ2) has a nonredundant function in spermatogenesis, as PP1γ knockout male mice are viable but sterile with defects in spermatogenesis, while knockout female mice are viable and fertile (VARMUZA *et al.* 1999). Presumably, the somatic and female germline functions of PP1γ are redundant with PP1α and/or PP1δ. No knockout analysis exists for the other PP1c genes, although PP1α was shown to have a specific function in murine lung growth and morphogenesis (HORMI-CARVER *et al.* 2004).

The conservation of the PP1α/β difference between flies and mammals suggests an ancient qualitative difference between the two subtypes. This raises the question of whether a PP1α-specific function exists that PP1β cannot perform. We found that expression of *PP1α96A* and *PP1β9C* could complement a lethal *PP1α87B* mutant, suggesting that the role of *PP1α87B* is essential because of its high expression levels rather than structural differences with the other PP1c isozymes. However, expression of *PP1β9C* did not rescue a *PP1α87B, PP1α96A* double mutant, suggesting that at least one developmental process in Drosophila depends on PP1α specifically.

Mammalian PP1α, PP1γ1, and PP1δ localize to distinct subcellular locations in mammalian cells (ANDREASSEN *et al.* 1998; TRINKLE-MULCAHY *et al.* 2001, 2003; LESAGE *et al.* 2005). It is thought that such specific localization and function is mediated by regulators that bind the PP1c isozymes with different affinities. Neurabin I and Neurabin II/Spinophilin were identified to bind PP1γ1 and PP1α, but not PP1δ (MACMILLAN *et al.* 1999; TERRY-LORENZO *et al.* 2002), and several PP1α- and PP1γ1-specific regulators have been identified in mouse fetal lung epithelial cells (FLORES-DELGADO *et al.* 2005). In Drosophila, even though the majority of PP1c regulatory subunits seem to bind all four PP1c isozymes in the yeast two-hybrid system (BENNETT *et al.* 2006), we identified three interactors that bind PP1α specifically or with greatly increased affinity compared to PP1β (L. ALPHEY, unpublished results). One of them, *CG11416*

(*uri*), is essential for viability (J. Kirchner and L. Alphey, unpublished results) and could mediate an essential requirement for PP1α.

In this study we show that *PP1α96A* is not an essential gene, but has an essential function in nonmuscle myosin regulation in a weak *PP1β9C* mutant background. Furthermore, we demonstrate by complementation analysis that *PP1β9C* has an essential function that is nonredundant with PP1α, while *PP1α87B* is essential only because of its high expression levels. Overexpressing PP1β9C does not rescue a *PP1α87B, PP1α96A* double mutant, indicating a PP1α-specific developmental function. Together, this sheds new light on the essential, redundant, and overlapping functions of the PP1c genes in Drosophila.

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