Protein phosphatase 2A positively and negatively regulates Ras1-mediated photoreceptor development in *Drosophila*

David A. Wassarman, Noah M. Solomon, Henry C. Chang, Felix D. Karim, Marc Therrien, and Gerald M. Rubin¹

Howard Hughes Medical Institute and Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3200 USA

Protein phosphatase 2A (PP2A), a heterotrimeric serine/threonine phosphatase present in most tissues and cell types, has been implicated in the regulation of cell cycle progression, DNA replication, transcription, and translation. Here we present genetic evidence suggesting that PP2A functions downstream of Ras1 in the Sevenless receptor tyrosine kinase (RTK) signal transduction pathway that specifies R7 photoreceptor cell fate in the developing *Drosophila* eye. Ras1 and downstream cytoplasmic kinases, Raf, MEK, and MAPK, comprise an evolutionarily conserved cascade that mediates the transmission of signals from RTKs at the plasma membrane to specific factors in the nucleus. Using transgenic flies expressing constitutively activated Ras1 or Raf proteins that function independently of upstream signaling events, we show that a reduction in the dose of the gene encoding the catalytic subunit of PP2A stimulates signaling from Ras1 but impairs signaling from Raf. This suggests that PP2A both negatively and positively regulates the Ras1 cascade by dephosphorylating factors that function at different steps in the cascade.

[**Key Words:** *Drosophila* eye development; PP2A; Raf; Ras; signal transduction; R7 photoreceptor]

Received November 3, 1995; revised version accepted December 13, 1995.

Reversible protein phosphorylation, mediated by kinases and phosphatases, is a common mechanism utilized to regulate basic cellular processes in eukaryotes. Protein phosphatase 2A (PP2A) is one of the four major classes of protein serine/threonine¹ phosphatases (Cohen 1989; Mumby and Walter 1993; Walter and Mumby 1993). The heterotrimeric PP2A enzyme consists of a core complex, a 36-kD catalytic subunit bound to a 65-kD regulatory subunit (PR65 or A-subunit), and one of a number of regulatory subunits (B-subunits) that range in size from 54 to 74 kD. The activity and substrate specificity of the core complex is modulated by association of a B-subunit.

In *Drosophila*, genes for the catalytic and PR65 subunits of PP2A were cloned by sequence similarity to their mammalian counterparts (Orgad et al. 1990; Mayer-Jaekel et al. 1992). Whereas mutations affecting these genes have not been identified, mutations in a B-subunit (PR55) were isolated in two independent screens. The PR55 pupal-lethal allele *twins^P* was isolated because of a partial pattern duplication that occurs in the wing imaginal disc (Uemura et al. 1993), and the late pupal/early adult-lethal allele *aar¹* was isolated be-

cause of mitotic abnormalities in the larval brain (Mayer-Jaekel et al. 1993). Weaker adult-lethal alleles generated by mobilization of the *twins^P* P element have duplicated bristles or missing photoreceptors (Shiomi et al. 1994). Therefore, cell cycle regulation and cell fate determination are affected in a cell-type-specific manner by PR55 mutations that differentially modulate PP2A phosphatase activity (Mayer-Jaekel and Hemmings 1994). Cell cycle and morphological defects are also observed in yeast upon mutation of the catalytic or regulatory subunits of PP2A (Healy et al. 1991; Ronne et al. 1991; van Zyl et al. 1992; Kinoshita et al. 1993; Lin and Arndt 1995). Furthermore, in vitro, PP2A dephosphorylates factors that regulate progression through the cell cycle (Coleman and Dunphy 1994) and mitogen-activated protein (MAP) kinases that function in signal transduction pathways controlling cell differentiation and proliferation (Hunter 1995).

Here, we present analysis of mutations in the catalytic subunit of *Drosophila* PP2A that affect a Ras1 signaling pathway controlling cell fate determination in the eye. The *Drosophila* compound eye is composed of ~800 identical units called ommatidia (Wolff and Ready 1993). Each ommatidium is comprised of eight photoreceptor cells (R1–R8), four non-neuronal cone cells, and eight

¹Corresponding author.

accessory cells. The identity and position of cells in the adult eye is established in the eye imaginal disc during larval development. Undifferentiated cells in the eye disc are sequentially recruited to adopt a particular developmental fate based on temporally and spatially restricted signals from neighboring cells.

A cytoplasmic Ras1 signal transduction cascade induces R7 neuronal differentiation and appears to be required for the determination of all photoreceptors (Zipursky and Rubin 1994; Wassarman et al. 1995). The cascade is triggered by binding of the Sevenless (Sev) receptor tyrosine kinase (RTK), expressed in the R7 precursor cell, to the Bride of Sevenless (Boss) ligand, expressed in the neighboring R8 cell. Autophosphorylated Sev is bound by the adaptor protein Downstream of receptor kinases (Drks), which in turn bind the guanine nucleotide exchange factor Son of Sevenless (Sos), localizing it to the plasma membrane where it can convert Ras1 into the active GTP-bound form (Simon et al. 1991, 1993; Olivier et al. 1993). Subsequently, Raf serine/threonine kinase is targeted to the plasma membrane by binding to Ras1 and is thought to be activated through phosphorylation by an unknown kinase (Dickson et al. 1992; Dent and Sturgill 1994). Activated Raf phosphorylates and activates the tyrosine/threonine kinase MEK [Downstream suppressor of Raf (Dsor1; Tsuda et al. 1993)], which finally activates the serine/threonine kinase MAPK [Rolled (Rl; Biggs et al. 1994)].

Loss-of-function mutations in *boss*, *sev*, or *seven in absentia* (*sina*), which encodes a nuclear protein of unknown function, specifically block induction of the cascade in the R7 precursor cell, causing it to develop as a cone cell (Zipursky and Rubin 1994; Wassarman et al. 1995). Conversely, activation of the cascade in cone cells, caused by loss-of-function mutations in negative regulators or overexpression of positive regulators of the cascade, leads to transformation to the R7 fate. Therefore, R7 and cone cell precursors have equivalent developmental potential but different developmental fates because of the on/off state of the Ras1 cascade.

To identify additional components of the Sev signal transduction pathway functioning downstream of Ras1 we conducted a genetic screen for mutations that would dominantly increase or decrease the number of cone cell to R7 cell transformation events caused by expression of

constitutively activated Ras1 in the eye (Karim et al. 1996). As expected, several loci identified in this screen encode components of the Ras1 cascade: *raf*, *Dsor1*, and *rl*. In addition, mutations were isolated in *kinase suppressor of ras* (*ksr*), which encodes a novel kinase that functions upstream of or in parallel to *raf* (Therrien et al. 1995), *phyllopod* (*phyl*), which encodes a novel nuclear protein that promotes neuronal differentiation in a subset of photoreceptor precursor cells (Chang et al. 1995; Dickson et al. 1995), and *yan*, which encodes a transcription factor that negatively regulates differentiation of all photoreceptors (Lai and Rubin 1992).

Mutations in the catalytic subunit of PP2A were also isolated in this screen. Mutating one of the two copies of *PP2A* increases the number of cone cell to R7 cell transformation events caused by constitutively activated Ras1. Conversely, *PP2A* mutations decrease the number of transformation events caused by constitutively activated Raf. This suggests that PP2A dephosphorylates multiple components in the Ras1 cascade and functions as both a negative and positive regulator of the Sev signal transduction pathway.

Results

ER2-6 is a dominant modifier of the activated Ras1 rough eye phenotype

To identify genes that function downstream of Ras1 in the Sev signal transduction pathway, we conducted a screen using transgenic flies that carry a constitutively activated form of Ras1 (*Ras1^{Val12}*) expressed in the eye under control of the *sev* enhancer and promoter (*sev-Ras1^{Val12}*; Fortini et al. 1992; Karim et al. 1996). Expression of the *sev-Ras1^{Val12}* transgene results in the transformation of non-neuronal cone cells to R7 photoreceptor cells, in addition to other defects that alter the regular array of ommatidia, producing an externally visible rough eye (Fig. 1A,B). The severity of the rough eye phenotype is dependent on the strength of the signaling pathway, because flies with two copies of the transgene have rougher eyes than those with only one copy (Karim et al. 1996).

Based on these observations, we scored the rough eye

Figure 1. *PP2A* dominantly enhances the rough eye of *sev-Ras1^{Val12}*. Scanning electron micrographs of wild-type (A), +/CyO P[ry, *sev-Ras1^{Val12}*]CR2 (B), and *PP2A²²⁵⁸*/CyO P[ry, *sev-Ras1^{Val12}*]CR2 (C) adult eyes.

phenotype of ~850,000 F₁ progeny from crosses between isogenic males mutagenized with ethylmethane sulfonate (EMS) or X rays and transgenic *sev-Ras1^{Val12}* females (described in detail in Karim et al. 1996). Loss-of-function mutations in positive regulators of the Sev pathway, such as *ksr*, *raf*, *Dsor1* (MEK), *rl* (MAPK), or *phyl*, suppress the rough eye phenotype, whereas mutations in negative regulators, such as *yan*, enhance the phenotype.

Nineteen X-ray-induced *ER2-6* (*Enhancer of Ras 2-6*) alleles were isolated as dominant enhancers of *sev-Ras1^{Val12}* (i.e., *ER2-6/sev-Ras1^{Val12}* flies have rougher eyes than +/*sev-Ras1^{Val12}* flies) (Table 1; Fig. 1B,C). Although *ER2-6* alleles dominantly modify the *sev-Ras1^{Val12}* rough eye phenotype, they are homozygous recessive embryonic lethal, suggesting that the *ER2-6* locus has additional functions outside of eye development.

ER2-6 is allelic to the catalytic subunit of PP2A

The *ER2-6* locus was placed within the polytene interval 28C2-D1, based on overlapping deletions present in 13 alleles (Table 1). A lethal P-element line, l(2)02496

(Karpen and Spradling 1992), inserted at 28D1-2 (Berkeley Drosophila Genome Project, pers. comm.) failed to complement the lethality of *ER2-6* alleles. Sequencing of genomic DNA adjacent to the P element revealed that it is located 320 bp upstream of the translation start site for the gene encoding the catalytic subunit of PP2A (Orgad et al. 1990). Quantitative DNA blot analysis of *PP2A* alleles, using a genomic probe covering the *PP2A* coding region, indicated that the *PP2A* gene is deleted in all but two alleles, *PP2A²²⁰²* and *PP2A²²⁵⁸* (Table 1; data not shown).

The *PP2A* gene was isolated and sequenced from libraries prepared from *PP2A²²⁰²* or *PP2A²²⁵⁸* genomic DNA (Fig. 2). *PP2A²²⁰²* contains a deletion in the second exon of bases 202–206 and an insertion of the sequence GGCAAGTGAAG at the same location, which results in a frameshift. If translated, the mutant gene would encode a 73-amino-acid protein, containing 67 of 309 amino acids of PP2A and 6 amino acids of extra sequence. *PP2A²²⁵⁸* has a 16-bp deletion, bases –7 to 9, that spans the translation start site. The next in-frame methionine does not occur for another 66 amino acids. *PP2A* alleles that contain large chromosomal deletions are stronger enhancers of the *sev-Ras1^{Val12}* rough eye phenotype than *PP2A²²⁰²* or *PP2A²²⁵⁸*, suggesting that there is at least one additional enhancer of *sev-Ras1^{Val12}* contained within the 28B-D region (Table 1).

Table 1. *PP2A* alleles

<i>PP2A</i> allele	<i>sev-Ras1^{Val12}</i>	Cytology
<i>XE-2202</i>	weak	insertion—exon 2
<i>XE-2258</i>	weak	deletion—exon 1
<i>XE-52</i>	med	Df
<i>XE-2267</i>	strong	Df
<i>XE-2269</i>	med	Df
<i>XE-3781</i>	strong	Df
<i>XE-715</i>	med-strong	Df(2L) 27E;28D1
<i>XE-2387</i>	strong	Df(2L) 27E2;28D1
<i>XE-2794</i>	med-strong	Df(2L) 27E2;28D1
<i>XE-2860</i>	strong	Df(2L) 27E2;28D1
<i>XE-3801</i>	med-strong	Df(2L) 27E2;28D1
<i>XE-3595</i>	med-strong	Df(2L) 27F2;28D1
<i>XE-2519</i>	med-strong	Df(2L) 28A2;D1
<i>XE-2668</i>	strong	Df(2L) 28A4;D1
<i>XE-3494</i>	med-strong	Df(2L) 28A4;D1
<i>XE-3841</i>	strong	Df(2L) 28A4;D1
<i>XE-2750</i>	strong	Df(2L) 28B2;D3
<i>XE-3103</i>	strong	Df(2L) 28B2;D3
<i>XE-3696</i>	med	Df(2L) 28C2;D1
<i>l(2)02496</i>	none	28D1-2
<i>l(2)05559</i>	none	28D1-2; 27A1-2
<i>l(2)K06009</i>	none	28D1-2
<i>l(2)K12502</i>	none	28D1-2; 60F1-5
<i>l(2)K16501</i>	none	28D1-2; 25A6-7

PP2A alleles isolated in the *sev-Ras1^{Val12}* screen or P-element-induced alleles. (XE) An X-ray-induced allele isolated in the *sev-Ras1^{Val12}* screen; [l(2)] a P-element-induced allele [l(2)02496 and l(2)05559; Karpen and Spradling (1992); l(2)K06009, l(2)K12502, and l(2)K16501, Torok et al. (1993)] with the P-element insertion site indicated in the third column (Berkeley Drosophila Genome Project, pers. comm.). The level of enhancement of the rough eye phenotype caused by *sev-Ras1^{Val12}* is indicated in the second column. Analysis of quantitative DNA blots revealed that the *PP2A* coding region is deleted in alleles marked Df.

PP2A is required during multiple stages of development

All 19 *PP2A* alleles are recessive embryonic lethal, as are all of the allelic combinations. Homozygous clones of *PP2A²²⁰²* or *PP2A²²⁵⁸* generated in the eye using the FLP–FRT system (Xu and Rubin 1993), consisting of the yeast FLP recombinase and its target FRT sequence, were not recovered, indicating that *PP2A* is required for cell proliferation or viability (data not shown). Furthermore, germ-line clones of *PP2A²²⁰²* or *PP2A²²⁵⁸* generated using the FLP–DFS (Dominant Female Sterile) system (Hou et al. 1995) were not recovered because oogenesis is blocked at approximately stage 5 (data not shown). Therefore, as has been suggested by its broad substrate specificity in vitro, *PP2A* has a critical role in multiple developmental processes in vivo.

PP2A negatively regulates R7 cell differentiation induced by activated Ras1

PP2A alleles dominantly enhance the rough eye phenotype of *sev-Ras1^{Val12}* by increasing the number of supernumerary R7 cells (Table 2). Two approaches were employed to measure the number of R7 cells per ommatidium. First, the activity of a transgene that expresses chloramphenicol acetyl transferase (CAT) under control of the R7-specific *Rh4* promoter (*Rh4CAT*) was measured (Table 2A) (Fortini and Rubin 1990). Several studies have shown that the CAT activity of adult fly head extracts is proportional to the number of R7 cells (O'Neill et al. 1994; Chang et al. 1995). Second, the num-

Figure 2. Mutations within the *PP2A* genomic locus. A map of the *PP2A* locus is shown with 5' and 3' untranslated regions depicted as open boxes and coding exons as solid boxes. Arrows denote the position of mutations within the P-element induced allele l(2)02496 and the X-ray-induced alleles, *PP2A*²²⁰² and *PP2A*²²⁵⁸.

ber of R7 cells in apical tangential sections through adult retinæ was counted (Table 2B).

The enhanced rough eye phenotype of *PP2A*²²⁵⁸/*sev-Ras1*^{Val12} flies relative to *+ / sev-Ras1*^{Val12} flies is reflected by a 48% increase in the CAT activity of head extracts (Table 2A) and a 55% increase in the number of R7 cells per ommatidium in sections (Table 2B). In addition, CAT assays reveal that *PP2A*²²⁵⁸ causes a 48% increase in the number of supernumerary R7 cells that result from overexpression of an activated form of Sev, *Sev*^{S11} (Table 2A) (Basler et al. 1991). Therefore, the Sev

pathway is negatively regulated by the activity of PP2A on a factor functioning downstream of Ras1 (Fig. 3).

PP2A positively regulates R7 cell differentiation induced by activated Raf

The Raf serine/threonine kinase functions downstream of Ras1 in the Sev pathway, and, like *Ras1*^{Val12}, expression of constitutively activated Raf under control of the *sev* enhancer (*sE-raf*^{tor4021}) results in recruitment of cone cell precursors as R7 cells, producing a rough eye (Dickson et al. 1992). *PP2A*²²⁵⁸ weakly suppresses the rough eye phenotype of *sE-raf*^{tor4021} (data not shown), which is reflected by a 36% decrease in the CAT activity of head extracts (Table 2A) and a 30% decrease in the number of R7 cells per ommatidium in sections (Table 2B).

To determine whether PP2A also functions as a positive regulator of signaling from endogenous Raf during R7 development, genetic interactions were tested with a

Table 2. R7 cells per ommatidium

	<i>PP2A</i> ⁺ / <i>PP2A</i> ⁺	<i>PP2A</i> ⁻ / <i>PP2A</i> ⁺
A. Normalized CAT activity		
Wild type	1.0	1.1 (±0.4)
<i>sev</i> ^{S11}	2.9 (±0.4)	4.3 (±0.4)
<i>sev-Ras1</i> ^{Val12}	2.3 (±0.4)	3.4 (±0.2)
<i>sE-raf</i> ^{tor4021}	4.2 (±0.3)	2.7 (±0.3)
B. R7 cells per ommatidium in sections		
<i>sev-Ras1</i> ^{Val12}	1.32 (±0.05)	2.05 (±0.11)
<i>sE-raf</i> ^{tor4021}	2.95 (±0.1)	2.06 (±0.1)
<i>raf</i> ^{HM7} R7	0.62 (±0.02)	0.44 (±0.02)
Outer	5.78 (±0.03)	5.27 (±0.02)

Analysis of the number of R7 photoreceptors per ommatidium. The BT97 allele of *raf*^{tor4021} (Dickson et al. 1992) and the CR1 allele of *sev-Ras1*^{Val12} (H. Chang and G. Rubin, unpubl.) were used in conjunction with the *PP2A*²²⁵⁸ allele in these assays. (A) Rh4CAT activity of extracts prepared from the heads of adult flies of the indicated genotypes. The CAT activity of extracts prepared from wild-type flies was arbitrarily set at 1.0. The standard deviation, within parentheses, is the average of the standard deviations for at least three experiments (see Materials and methods). *PP2A*²²⁰² does not significantly enhance the CAT activity of *sev-Ras1*^{Val12} but does suppress *sE-raf*^{tor4021} to the same extent as *PP2A*²²⁵⁸ (data not shown). In addition, *PP2A*²²⁰² and *PP2A*²²⁵⁸ do not affect the development of extra R7 cells that result from overexpression of *phyl* (data not shown) (Chang et al. 1995; Dickson et al. 1995). *PP2A* may be a weak enhancer of the *sev-Ras1*^{Val12} rough eye, relative to other negative regulators of the pathway like *yan*, because its opposing functions downstream of Ras and Raf partially cancel each other. (B) Tangential sections of adult retinæ of the indicated genotype were scored for photoreceptors of the R7 or outer (R1–R6) class. Wild-type ommatidia contain one R7 and six outer photoreceptor cells. For each genotype, ~500 ommatidia from at least four eyes were scored. The standard deviation represents the variation from eye to eye in the number of R7 or outer cells per ommatidium.

Figure 3. Model for the role of PP2A in the Sev signal transduction pathway. Activation of the Sev RTK leads to an increase in the amount of active GTP-bound Ras1. Raf acts downstream of Ras1 and is activated by an unknown mechanism possibly involving Ksr, which has been shown to function either between Ras1 and Raf or in a parallel pathway. Activated Raf phosphorylates and activates MEK, which in turn activates MAPK. Here, we show that PP2A negatively regulates signaling between Ras1 and Raf and positively regulates signaling downstream of Raf. In vitro studies have shown that PP2A dephosphorylates MEK and MAPK inhibiting their kinase activity, but this has not yet been demonstrated for the Sev pathway.

hypomorphic *raf* allele, *raf^{HM7}* [Melnick et al. 1993]. *raf^{HM7}* is a temperature-sensitive allele that produces a normal Raf protein but is associated with lower levels of *raf* transcription resulting in a reduction in the number of photoreceptors of both the R7 and R1–R6 classes (Table 2B). *PP2A²²⁵⁸* dominantly enhances the *raf^{HM7}* phenotype, further reducing both the number of outer and R7 cells per ommatidium (Table 2B). Therefore, in contrast to its affect on signaling from Ras1, PP2A positively regulates signaling initiated by Raf during R7 and outer photoreceptor development (Fig. 3).

Discussion

The isolation of mutations in the catalytic subunit of PP2A in a genetic screen for genes that function in the Sev RTK pathway provides the first genetic evidence that PP2A is a key regulator of Ras-mediated signal transduction pathways. Using constitutively activated forms of Ras1 and Raf that function independently of upstream signaling events, we have been able to study the role of PP2A at two different points in the pathway. Interestingly, PP2A serves dual functions, acting as a negative regulator of signaling downstream of Ras1 and a positive regulator of signaling downstream of Raf.

Potential targets of PP2A in the Ras1 pathway

What are the substrates for PP2A in the Ras1 pathway? Phosphorylation of Raf at different sites can either activate or inhibit its kinase activity, making it a possible substrate for negative or positive regulation by PP2A [Heidecker et al. 1992; Daum et al. 1994; Bugering and Bos 1995]. In vitro studies suggest that PP2A is not the major enzyme responsible for dephosphorylation and inactivation of Raf [Sontag et al. 1993; Dent et al. 1995], but these studies have not addressed the role of PP2A in reversing inhibitory phosphorylation events that have been shown to occur at at least three sites [Heidecker et al. 1992]. Ksr kinase is also a potential target for negative regulation by PP2A; it appears to function between Ras1 and Raf and contains four consensus phosphorylation sites for MAPK, but it is not known whether phosphorylation of Ksr modulates its activity [Therrien et al. 1995]. PP2A has been shown to dephosphorylate and inactivate both MEK and MAPK in vitro [Sontag et al. 1993; Alessi et al. 1995] (Fig. 3). However, *PP2A* mutant alleles do not alter the external rough eye phenotype caused by a hypomorphic allele of *Dsor1*/MEK (*Dsor1^{XS-520}*; Karim et al. 1996) or a gain-of-function allele of *rl*/MAPK (*rl^{SEM}*; Brunner et al. 1994) (data not shown), but *PP2A* gene dose may not be rate-limiting in the context of this part of the pathway.

Because PP2A exhibits broad substrate specificity in vitro and at high concentrations displays little substrate selectivity, it has been difficult to assess physiological targets of PP2A [Cohen 1989; Mumby and Walter 1993; Walter and Mumby 1993]. However, it may be possible to examine potential substrates for PP2A using the system described in this paper. For example, we can ask

whether *PP2A* mutants suppress the supernumerary R7 phenotype of transgenic flies expressing mutant forms of *raf^{tor4021}* that cannot be negatively regulated by phosphorylation (i.e., Raf proteins that have mutations or deletions of residues that when phosphorylated inhibit Raf kinase activity).

PP2A has opposing functions in the Ras1 pathway

How does PP2A perform opposite functions in the same pathway? Different forms of PP2A may be responsible for its multiple action in the Sev pathway. This is not unreasonable because both the activity and substrate specificity of the PP2A core complex, the catalytic subunit bound to the regulatory A-subunit, is modulated differently by different B-subunits [Cohen 1989; Mumby and Walter 1993; Walter and Mumby 1993]. At least two B-subunits, PR54 and PR55 [D. Glover, unpubl.], are present in *Drosophila*. Mutations in *PR55* have been shown to disrupt photoreceptor development [Shiomi et al. 1994], but the P alleles *twins^P* and *aar¹* do not alter the rough eye phenotypes caused by *sev-Ras1^{Val12}* or *sE-raf^{tor4021}* (data not shown). It is possible that other enhancer groups isolated in the *sev-Ras1^{Val12}* screen correspond to the *PR54* locus or as yet unidentified B-subunits [Karim et al. 1996].

Different forms of PP2A may also have opposing functions at different stages of mitotic initiation, analogous to PP2A function downstream of Ras1 versus downstream of Raf in the Sev pathway. Studies in *Saccharomyces cerevisiae* suggest that PP2A has a positive role in entry into mitosis [Lin and Arndt 1995], whereas in *Schizosaccharomyces pombe* [Kinoshita et al. 1993] and *Xenopus* [Lee et al. 1994] PP2A appear to negatively regulate this process.

In conclusion, mutations in the gene encoding the *Drosophila* PP2A catalytic subunit will be useful for deciphering the pleiotropic roles of PP2A in vivo. Furthermore, the genetic placement of PP2A in the Ras1 cascade and the unexpected finding that PP2A both positively and negatively regulates the cascade should lead to the identification of PP2A substrates.

Materials and methods

Drosophila stocks and genetics

Fly culture and crosses were performed according to standard procedures. The screen for dominant modifiers of *sev-Ras1^{Val12}* is described in detail in Karim et al. (1996). Flies carrying the *raf^{HM7}* allele were raised at 18°C; all other crosses were performed at 25°C.

Molecular analysis of PP2A mutants

To sequence the *PP2A* locus, a 9-kb genomic DNA fragment was isolated from λZAP (Stratagene) libraries prepared from *PP2A²²⁰²* or *PP2A²²⁵⁸* *Bam*HI-digested DNA. The *PP2A* genomic region was sequenced on both strands, and molecular lesions were determined by comparison to the wild-type sequence [Orgad et al. 1990]. Putative splice junctions were determined by comparison of *PP2A* cDNA and genomic sequences. All

splice sites conform to the consensus sequences for *Drosophila*. DNA sequences were obtained using a Pharmacia ALF automated sequencer and analyzed using Staden (R. Staden, Medical Research Council of Molecular Biology, Cambridge, UK) and Genetics Computer Group (University of Wisconsin, Madison) software. Genomic DNA flanking the P-element insertion in l(2)02496 was cloned by plasmid rescue [Mlodzik et al. 1990] and sequenced using a primer complementary to the P-element inverted repeat [Karpen and Spradling 1992].

Sectioning and scanning electron microscopy

Scanning electron microscopy of adult eyes was performed as described in Kimmel et al. (1990). Fixation and sectioning of adult eyes were performed as described by Tomlinson and Ready (1987).

CAT assays

CAT assay samples containing 10 heads from 5-day-old adult females were homogenized in 100 μ l of 250 mM Tris (pH 7.9) and freeze-thawed twice. Insoluble material was pelleted by centrifugation, and the supernatant was incubated at 65°C for 10 min. Supernatant (80 μ l) was combined with 50 μ l of 5 mM chloramphenicol, 68 μ l of 250 mM Tris (pH 7.9), and 2 μ l of 14 C-labeled acetylCoA (0.1 μ Ci). The mixture was overlaid with scintillation fluid and the rate of CAT activity was measured 8 times for 1 min over a period of 6–10 hr in a scintillation counter. For each experiment, samples were assayed in triplicate, and at least three experiments were performed for each genotype.

Acknowledgments

We thank T. Laverty for cytological characterization of PP2A alleles, D. Davis for assistance with the scanning electron microscopy, and T. Wolff for helpful comments on the manuscript. D.A.W. is supported by a Helen Hay Whitney fellowship. H.C.C. is supported by a training grant from the National Institutes of Health. F.D.K. is supported by an American Cancer Society fellowship. M.T. is supported by a National Cancer Institute of Canada fellowship. G.M.R. is a Howard Hughes Medical Institute Investigator.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Alessi, D.R., N. Gomez, G. Moorhead, T. Lewis, S.M. Keyse, and P. Cohen. 1995. Inactivation of p42 MAP kinase by protein phosphatase 2A and protein tyrosine phosphatase, but not CL100, in various cell lines. *Curr. Biol.* **5**: 83–295.
- Basler, K., B. Christen, and E. Hafen. 1991. Ligand-independent activation of the Sevenless receptor tyrosine kinase changes the fate of cells in the developing *Drosophila* eye. *Cell* **64**: 1069–1081.
- Biggs, W.H., K.H. Zavitz, B. Dickson, A. van der Straten, D. Brunner, E. Hafen, and S.L. Zipursky. 1994. The *Drosophila* rolled locus encodes a MAP kinase required in the Sevenless signal transduction pathway. *EMBO J.* **13**: 1628–1635.
- Brunner, D., N. Oellers, J. Szabad, W.H. Biggs, S.L. Zipursky, and E. Hafen. 1994. A gain-of-function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signalling pathways. *Cell* **76**: 875–888.
- Bugering, B.M.T. and J.L. Bos. 1995. Regulation of Ras-mediated signalling: More than one way to skin a cat. *Trends Biochem. Sci.* **20**: 18–22.
- Chang, H.C., N.M. Solomon, D.A. Wassarman, F.D. Karim, M. Therrien, G.M. Rubin, and T. Wolff. 1995. *phyllopod* functions in the fate determination of a subset of photoreceptors in *Drosophila*. *Cell* **80**: 463–472.
- Cohen, P. 1989. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58**: 485–497.
- Coleman, T.R. and W.G. Dunphy. 1994. Cdc2 regulatory factors. *Curr. Biol.* **6**: 877–882.
- Daum, G., I. Eisenmann-Tappe, H.W. Fries, J. Troppmair, and U. R. Rapp. 1994. The ins and outs of Raf kinases. *Trends Biochem. Sci.* **19**: 474–480.
- Dent, P. and T.W. Sturgill. 1994. Activation of [His]₆-Raf-1 *in vitro* by partially purified plasma membranes from v-Ras-transformed and serum-stimulated fibroblasts. *Proc. Natl. Acad. Sci.* **91**: 9544–9548.
- Dent, P., T. Jelinek, D.K. Morrison, M.J. Weber, and T.W. Sturgill. 1995. Reversal of Raf-1 activation by purified and membrane-associated protein phosphatases. *Science* **268**: 1902–1906.
- Dickson, B., F. Sprenger, D. Morrison, and E. Hafen. 1992. Raf functions downstream of Ras in the Sevenless signal transduction pathway. *Nature* **360**: 600–603.
- Dickson, B.J., M. Domínguez, A. van der Straten, and E. Hafen. 1995. Control of *Drosophila* photoreceptor cell fates by *Phyllopod*, a novel nuclear protein acting downstream of the Raf kinase. *Cell* **80**: 453–462.
- Fortini, M.E. and G.M. Rubin. 1990. Analysis of *cis*-acting requirements of the *Rh3* and *Rh4* genes reveals a bipartite organization to rhodopsin promoters in *Drosophila melanogaster*. *Genes & Dev.* **4**: 444–463.
- Fortini, M.E., M.A. Simon, and G.M. Rubin. 1992. Signalling by the Sevenless protein tyrosine kinase is mimicked by Ras1 activation. *Nature* **355**: 559–561.
- Healy, A.M., S. Zolnierowicz, A.E. Stapleton, M. Goebel, A.A. DePaoli-Roach, and J.R. Pringle. 1991. *CDC55*, a *Saccharomyces cerevisiae* gene involved in cellular morphogenesis: Identification, characterization, and homology to the B subunit of mammalian type 2A protein phosphatase. *Mol. Cell. Biol.* **11**: 5767–5780.
- Heidecker, G., W. Kolch, D.K. Morrison, and U.R. Rapp. 1992. The role of Raf-1 phosphorylation in signal transduction. *Adv. Can. Res.* **58**: 53–73.
- Hou, X.S., T.B. Chou, M.B. Melnick, and N. Perrimon. 1995. The Torso receptor tyrosine kinase can activate Raf in a Ras-independent pathway. *Cell* **81**: 63–71.
- Hunter, T. 1995. Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* **80**: 225–236.
- Karim, F.D., H.C. Chang, M. Therrien, D.A. Wassarman, T. Laverty, and G.M. Rubin. 1996. A screen for genes that function downstream of Ras1 during *Drosophila* eye development. *Genetics* (in press).
- Karpen, G.H. and A.C. Spradling. 1992. Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome Dp1187 by P element insertional mutagenesis. *Genetics* **132**: 737–753.
- Kimmel, B.E., U. Heberlein, and G.M. Rubin. 1990. The homeo-domain protein Rough is expressed in a subset of cells in the developing *Drosophila* eye where it can specify photoreceptor cell subtype. *Genes & Dev.* **4**: 712–727.
- Kinoshita, N., H. Yamano, H. Niwa, Y. Yoshida, and M. Yanagida. 1993. Negative regulation of mitosis by the fission yeast protein phosphatase ppa2. *Genes & Dev.* **7**: 1059–1071.

- Lai, Z.-C. and G.M. Rubin. 1992. Negative control of photoreceptor development in *Drosophila* by the product of the *yan* gene, an ETS domain protein. *Cell* **70**: 609–620.
- Lee, T.H., C. Turck, and M. Kirschner. 1994. Inhibition of cdc2 activation by INH/PP2A. *Mol. Biol. Cell* **5**: 323–338.
- Lin, F.C. and K.T. Arndt. 1995. The role of *Saccharomyces cerevisiae* type 2A phosphatase in the actin cytoskeleton and in entry into mitosis. *EMBO J.* **14**: 2745–2759.
- Mayer-Jaekel, R.E. and B.A. Hemmings. 1994. Protein phosphatase 2A—A “menage a trois”. *Trends Cell Biol.* **4**: 287–291.
- Mayer-Jaekel, R.E., S. Baumgartner, G. Bilbe, H. Ohkura, D.M. Glover, and B.A. Hemmings. 1992. Molecular cloning and developmental expression of the catalytic and 65-kDa regulatory subunits of protein phosphatase 2A in *Drosophila*. *Mol. Biol. Cell* **3**: 287–298.
- Mayer-Jaekel, R.E., H. Ohkura, R. Gomes, C.E. Sunkel, S. Baumgartner, B.A. Hemmings, and D.M. Glover. 1993. The 55 kd regulatory subunit of *Drosophila* protein phosphatase 2A is required for anaphase. *Cell* **72**: 621–633.
- Melnick, M.B., L.A. Perkins, M. Lee, L. Ambrosio, and N. Perimon. 1993. Developmental and molecular characterization of mutations in the *Drosophila*-raf serine/threonine protein kinase. *Development* **118**: 127–138.
- Mlodzik, M., N.E. Baker, and G.M. Rubin. 1990. Isolation and expression of *scabrous*, a gene regulating neurogenesis in *Drosophila*. *Genes & Dev.* **4**: 1848–1861.
- Mumby, M. and G. Walter. 1993. Protein serine/threonine phosphatases: Structure, regulation, and function in cell growth. *Phys. Rev.* **73**: 673–699.
- Olivier, J.P., T. Rabbe, M. Henkemeyer, B. Dickson, G. Mbamalu, B. Margolis, J. Schlessinger, E. Hafen, and T. Pawson. 1993. A *Drosophila* SH2-SH3 adaptor protein implicated in coupling the Sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell* **81**: 857–866.
- O'Neill, E.M., I. Rebay, R. Tjian, and G.M. Rubin. 1994. The activities of two ETS-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell* **78**: 137–147.
- Orgad, S., N.D. Brewis, L. Alphey, J.M. Axton, Y. Dudai, and P.T.W. Cohen. 1990. The structure of protein phosphatase 2A is as highly conserved as that of protein phosphatase 1. *FEBS Lett.* **275**: 44–48.
- Ronne, H., M. Carlberg, G.-Z. Hu, and J.O. Nehlin. 1991. Protein phosphatase 2A in *Saccharomyces cerevisiae*: Effects on cell growth and bud morphogenesis. *Mol. Cell. Biol.* **11**: 4876–4884.
- Shiomi, K., M. Takeichi, Y. Nishida, Y. Nishi, and T. Uemura. 1994. Alternative cell fate choice induced by low-level expression of a regulator of protein phosphatase 2A in the *Drosophila* peripheral nervous system. *Development* **120**: 1591–1599.
- Simon, M.A., D.D.L. Bowtell, G.S. Dodson, T.R. Laverty, and G.M. Rubin. 1991. Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signalling by the Sevenless protein tyrosine kinase. *Cell* **67**: 701–716.
- Simon, M.A., G.S. Dodson, and G.M. Rubin. 1993. An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to Sevenless and Sos proteins *in vitro*. *Cell* **73**: 169–177.
- Sontag, E., S. Fedorov, C. Kamibayashi, D. Roberts, M. Cobb, and M. Mumby. 1993. The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the MAP kinase pathway and induces cell proliferation. *Cell* **75**: 887–897.
- Therrien, M., H.C. Chang, N.M. Solomon, F.D. Karim, D.A. Wassarman, and G.M. Rubin. 1995. Ksr, a novel protein kinase required for Ras signal transduction. *Cell* **83**: 879–888.
- Tomlinson, A. and D.F. Ready. 1987. Cell fate in the *Drosophila* ommatidium. *Dev. Biol.* **123**: 264–275.
- Torok, T., G. Tick, M. Alvarado, and I. Kiss. 1993. P-lacW insertional mutagenesis on the second chromosome of *Drosophila melanogaster*: Isolation of lethals with different overgrowth phenotypes. *Genetics* **135**: 71–80.
- Tsuda, L., Y.H. Inoue, M.-A. Yoo, M. Mizuno, M. Hata, Y.-M. Lim, T. Adachi-Yamada, H. Ryo, Y. Masamune, and Y. Nishida. 1993. A protein kinase similar to MAP kinase activator acts downstream of the Raf kinase in *Drosophila*. *Cell* **72**: 407–414.
- Uemura, T., K. Shiomi, S. Togashi, and M. Takeichi. 1993. Mutation of *twins* encoding a regulator of protein phosphatase 2A leads to pattern duplication in *Drosophila* imaginal discs. *Genes & Dev.* **7**: 429–440.
- van Zyl, W., W. Huang, A.A. Sneddon, M. Stark, S. Camier, M. Werner, C. Marck, A. Sentenac, and J.R. Broach. 1992. Inactivation of the protein phosphatase 2A regulatory subunit A results in morphological and transcriptional defects in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **12**: 4946–4959.
- Walter, G. and M. Mumby. 1993. Protein serine/threonine phosphatases and cell transformation. *Biochem. Biophys. Acta* **1155**: 207–226.
- Wassarman, D.A., M. Therrien, and G.M. Rubin. 1995. The Ras signalling pathway in *Drosophila*. *Curr. Opin. Genet. Dev.* **5**: 44–50.
- Wolff, T. and D.F. Ready. 1993. Pattern formation in the *Drosophila* retinae. In *The development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), pp. 1277–1325. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Xu, T. and G.M. Rubin. 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**: 1223–1237.
- Zipursky, S.L. and G.M. Rubin. 1994. Determination of neuronal cell fate: Lessons from the R7 neuron of *Drosophila*. *Annu. Rev. Neurosci.* **17**: 373–397.

Protein phosphatase 2A positively and negatively regulates Ras1-mediated photoreceptor development in *Drosophila*.

D A Wassarman, N M Solomon, H C Chang, et al.

Genes Dev. 1996, **10**:

Access the most recent version at doi:[10.1101/gad.10.3.272](https://doi.org/10.1101/gad.10.3.272)

References

This article cites 49 articles, 17 of which can be accessed free at:
<http://genesdev.cshlp.org/content/10/3/272.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).