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

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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]

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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/threonine phosphatases (Cohen 1989; Mumbery and Walter 1993; Walter and Mumbery 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* 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 *aar* was isolated because of mitotic abnormalities in the larval brain (Mayer-Jaekel et al. 1993). Weaker adult-lethal alleles generated by mobilization of the *twins* 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; Romne 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...
we conducted a genetic screen for mutations that would dominantly increase or decrease the number of cone cell transduction pathway functioning downstream of RaM regulators or overexpression of positive regulators of the known function, specifically block induction of the caspase MAPK [Rolled (R1; Biggs et al. 1994)].

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: ras, 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 ras [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 [RaslV12] expressed in the eye under control of the sev enhancer and promoter [sev–RaslV12; Fortini et al. 1992; Karim et al. 1996]. Expression of the sev–RaslV12 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
phenotype of ~850,000 F₁ progeny from crosses between isogenic males mutagenized with ethylmethane sulfonate (EMS) or X rays and transgenic sev-Rasl¹Val² females [described in detail in Karim et al. 1996]. Loss-of-function mutations in positive regulators of the Sev pathway, such as ksr, raf, Dso1 [MEK], tl [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-Rasl¹Val² [i.e., ER2-6/sev-Rasl¹Val² flies have rougher eyes than +/sev-Rasl¹Val² flies] (Table 1; Fig. 1B,C). Although ER2-6 alleles dominantly modify the sev-Rasl¹Val² 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 was used to map the locus to rough eye phenotype caused by sev-Rasl¹Val² (O’Neill et al. 1994; Chang et al. 1995). The level of enhancement of the rough eye phenotype by ER2-6 alleles (Table 1; Fig. 1B,C) indicates 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 GGCAAGTGAG 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-Rasl¹Val² rough eye phenotype than PP2A²²⁰² or PP2A²²⁵⁸, suggesting that there is at least one additional enhancer of sev-Rasl¹Val² contained within the 28B-D region (Table 1).

Table 1. PP2A alleles

| PP2A allele | sev-Rasl¹Val² | Cytology |
|-------------|---------------|----------|
| XE-2202     | weak          | insertion-exon 2 |
| XE-2258     | weak          | deletion-exon 1  |
| XE-52       | med           | Df       |
| XE-2267     | strong        | Df       |
| XE-2269     | med           | Df       |
| XE-3781     | strong        | Df       |
| XE-715      | med-strong    | Df(2L) 27E,28D1 |
| XE-2387     | strong        | Df(2L) 27E,28D1 |
| XE-2794     | med-strong    | Df(2L) 27E,28D1 |
| XE-2860     | strong        | Df(2L) 27E,28D1 |
| XE-3801     | med-strong    | Df(2L) 27E,28D1 |
| XE-3595     | med-strong    | Df(2L) 27F2,28D1 |
| XE-2519     | strong        | Df(2L) 28A2,D1 |
| XE-2668     | strong        | Df(2L) 28A4,D1 |
| XE-3494     | med-strong    | Df(2L) 28A4,D1 |
| XE-3841     | strong        | Df(2L) 28B2,D3 |
| XE-3303     | strong        | Df(2L) 28B2,D3 |
| XE-3696     | med           | Df(2L) 28C2,D1 |
| l(2)02496   | none          | 28D1-2  |
| l(2)05559   | none          | 28D1-2, 27A1-2 |
| l(2)K06009  | none          | 28D1-2  |
| l(2)K12502  | none          | 28D1-2, 60F1-5 |
| l(2)K16501  | none          | 28D1-2, 25A6-7 |

PP2A alleles isolated in the sev-Rasl¹Val² screen or P-element-induced alleles. [XE] An X-ray-induced allele isolated in the sev-Rasl¹Val² 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-Rasl¹Val² 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-Rasl¹Val² 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-

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The enhanced rough eye phenotype of PP2A^{2258} / sev-Rasl^{Val12} flies relative to + / sev-Rasl^{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).

### Table 2. R7 cells per ommatidium

|                      | PP2A^+/PP2A^+ | PP2A^-/PP2A^- |
|----------------------|---------------|---------------|
| **A. Normalized CAT activity** |               |               |
| Wild type            | 1.0           | 1.1 (±0.4)    |
| sev^{S11}            | 2.9 (±0.4)    | 4.3 (±0.4)    |
| sev-Rasl^{Val12}     | 2.3 (±0.4)    | 3.4 (±0.2)    |
| sE-raf^{tor4021}     | 4.2 (±0.3)    | 2.7 (±0.3)    |

|                      |               |               |
| **B. R7 cells per ommatidium in sections** |               |               |
| sev-Rasl^{Val12}    | 1.32 (±0.05)  | 2.05 (±0.11)  |
| sE-raf^{tor4021}    | 2.95 (±0.1)   | 2.06 (±0.1)   |
| raf^{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-Rasl^{Val12} [H. Chang and G. Rubin, unpub.] were used in conjunction with the PP2A^{2258} allele in these assays. (A) Rh4CAT activity of extracts prepared from the heads of adult 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^{2258} does not significantly enhance the CAT activity of sev-Rasl^{Val12} but does suppress sE-raf^{tor4021} to the same extent as PP2A^{2258} [data not shown]. In addition, PP2A^{2258} and PP2A^{2258} do not affect the development of extra R7 cells that result from overexpression of phy1 [data not shown] (Chang et al. 1995; Dickson et al. 1995). PP2A may be a weak enhancer of the sev-Rasl^{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 retinae 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 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^{2258} and PP2A^{2258}.

**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, rafHM7 [Melnick et al. 1993]. rafHM7 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]. PP2A2258dominantly enhances the rafHM7 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 least at 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 consensuses 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 [Dsor1XS-520; Karim et al. 1996] or a gain-of-function allele of tl/MAPK [tlSEM5; 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; Mumbly and Walter 1993; Walter and Mumbly 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 rafH710221 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; Mumbly and Walter 1993; Walter and Mumbly 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 and aar do not alter the rough eye phenotypes caused by sev–Ras1Val12 or sev–rafH710221 [data not shown]. It is possible that other enhancer groups isolated in the sev–Ras1Val12 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–Ras1Val12 is described in detail in Karim et al. [1996]. Flies carrying the rafHM7 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 ZAP (Stratagene) libraries prepared from PP2A2220 or PP2A2258 BamHI-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 [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 eye 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 µl 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 µl) was combined with 50 µl of 5 mM chloramphenicol, 68 µl of 250 mM Tris [pH 7.9], and 2 µl of 14C-labeled acetylCoA [0.1 µCi]. 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.

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