A PP2A regulatory subunit positively regulates Ras-mediated signaling during *Caenorhabditis elegans* vulval induction

Derek S. Sieburth,[1] Meera Sundaram,[2] Robyn M. Howard,[2] and Min Han[1,3]

[1]Howard Hughes Medical Institute, Department of Molecular, Cellular, and Developmental Biology, University of Colorado at Boulder, Boulder, Colorado 80309-0347 USA; [2]Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6145 USA

We describe evidence that a regulatory B subunit of protein phosphatase 2A (PP2A) positively regulates an RTK–Ras–MAP kinase signaling cascade during *Caenorhabditis elegans* vulval induction. Although reduction of *sur-6* PP2A-B function causes few vulval induction defects in an otherwise wild-type background, *sur-6* PP2A-B mutations suppress the Multivulva phenotype of an activated *ras* mutation and enhance the Vulvaless phenotype of mutations in *lin-45 raf*, *sur-8*, or *mpk-1*. Double mutant analysis suggests that *sur-6* PP2A-B acts downstream or in parallel to *ras*, but likely upstream of *raf*, and functions with *ksr-1* in a common pathway to positively regulate Ras signaling.

[*Key Words*: Suppressor of *ras*; phosphatase 2A; vulval induction; KSR; Raf; SUR-8]

Received June 24, 1999; revised version accepted August 11, 1999.

The protein phosphatase 2A (PP2A) enzymes, one of four families of serine/threonine protein phosphatases, are key regulators of many cellular events controlled by protein phosphorylation (Shenolikar 1994; Millward et al. 1999). PP2A is a heterotrimer, composed of a catalytic (C) subunit, an associated (A) subunit, and a regulatory (B) subunit. The C and A subunits form a core complex to which one of several classes of B subunits can bind. This differential binding of regulatory B subunits can influence the catalytic activity and substrate specificity of the catalytic core in vitro (Mayer-Jaekel and Hemmings 1994). However, although in vitro studies have identified numerous potential PP2A substrates, the broad substrate specificity has limited the ability to identify physiological targets or to understand normal PP2A regulation (Shenolikar 1994).

Recent studies have shown that PP2A can regulate signal transduction pathways. For example, PP2A stably interacts with and inactivates $Ca2^{+}$-calmodulin-dependent protein kinase IV (CaMKIV) (Westphal et al. 1998). PP2A has also been proposed to inactivate MEK and MAP kinase, because PP2A can dephosphorylate MEK and MAP kinase in vitro (for review, see Millward et al. 1999), and interference of PP2A activity by SV40 small t antigen results in activation of MAP kinase in vivo (Sontag et al. 1993). Genetic studies in *Drosophila* suggested that PP2A both positively and negatively affects Ras pathway signaling during R7 photoreceptor cell fate specification (Wassarman et al. 1996).

Here we demonstrate that a B regulatory subunit of PP2A promotes Ras signaling during *Caenorhabditis elegans* vulval development. In *C. elegans*, a *ras*-mediated signal transduction pathway in part controls the fates of six cells, the vulval precursor cells (VPCs). An inductive signal from the anchor cell activates an RTK–Ras–MAP kinase signal-transduction pathway to induce the three neighboring VPCs (P5.p, P6.p, and P7.p) to adopt vulval cell fates. These cells undergo three rounds of division followed by morphogenesis to form the vulval structure. The remaining three VPCs (P3.p, P4.p, and P8.p) adopt nonvulval cell fates and, instead, divide only once before fusing with the surrounding hypodermis (Horvitz and Sternberg 1991). Mutations that result in the mis-specification of vulval cell fates have defined many of the genes necessary for normal vulval differentiation (Kornfeld 1997; Sternberg and Han 1998). Loss-of-function mutations in positively acting components of this pathway can cause fewer than three VPCs to adopt vulval cell fates, leading to a Vulvaless (Vul) phenotype. A gain-of-function mutation in *let-60 ras* (*n1046gf* or G13E), causes more than three VPCs to adopt vulval cell fates, leading to a Multivulva (Muv) phenotype.

To identify genes that act downstream of *let-60 ras*, screens have been conducted for mutations that suppress the Muv phenotype of *let-60 ras(n1046gf)* mutants.

[3]Corresponding author.
E-MAIL mhan@colorado.edu; FAX (303) 492-7744.

Table 1. sur-6 *mutations enhance the vulval defects of* lin-45 *Raf,* mpk-1 *MAP kinase, or* sur-8 *but not* ksr-1 *mutants*

Genotype[a]	Percent Vul[b]	Average percent induction[c]	(No.)	Percent larval lethal[d] (*n*)
sur-6(ku123)	0	100	(26)	0 (474)
sur-6(ku123)/qDf8	0	100	(28)	0 (531)[e]
sur-6(cs24)	2	99	(48)	0 (195)
sur-6(cs24)/qDf8	8	97	(26)	0 (386)[e]
+/qDf8	0	100	(16)	N.D.
sur-6(cs24)/+	0	100	(35)	N.D.
lin-45(sy96)	58	53	(38)	86 (290)
sur-6(ku123); lin-45(sy96)	100	5	(17)	94 (228)
lin-45(ku112)	0	100	(26)	<1 (66)
sur-6(cs24); lin-45(ku112)	87	50	(24)	80 (360)
mpk-1(ku1)	17	97	(29)	7 (229)
sur-6(ku123); mpk-1(ku1)	82	76	(28)	77 (263)
sur-8(ku167)	0	100	(18)	<1 (271)
sur-6(ku123); sur-8(ku167)[f]	71	56	(24)	<1 (263)
sur-6(cs24); sur-8(ku167)	65	67	(20)	3 (198)
ksr-1(ku68)	0	100	(23)	24 (257)
sur-6(ku123); ksr-1(ku68)	3	99	(40)	17 (282)
sur-8(ku167); ksr-1(ku68)[g]	100	4	(19)	85 (164)
ksr-1(n2526)	1	99	(68)	2 (607)
sur-6(cs24); ksr-1(n2526)	3	99	(62)	1 (88)
sur-8(ku167); ksr-1(n2526)	67	64	(24)	54 (132)

[a]*ksr-1(ku68)* (R531H) is a strong loss-of-function, possibly dominant-negative allele (Sundaram and Han 1995). *ksr-1(n2526)* (W255stop) is a putative null allele (Kornfeld et al. 1995). *lin-45(sy96)* and *lin-45(ku112)* are hypomorphic (partial loss-of-function) alleles (Han et al. 1993; Sundaram and Han 1995); *lin-45(sy96)* contains a splice site mutation, and the *lin-45(ku112)* lesion is unknown. *sur-8(ku167)* (E430K) is a strong hypomorphic allele (Sieburth et al. 1998). *mpk-1(ku1)* (A38V) is a hypomorphic allele (Wu and Han 1994). *qDf8* removes the flanking genes *mec-8* and *fog-3* and thus should remove *sur-6* (R. Ellis, pers. comm.). For deficiency strains, *sur-6* was marked with *unc-13,* and *qDf8* was marked with *ces-1.* No other defects were observed in *sur-6/qDf8* strains. *lin-45(sy96)* was linked to *dpy-20, ksr-1(ku68)* was linked to *lon-2. lin-45(ku112)* was linked to *dpy-20* for double mutants with *ksr-1. sur-6(ku123)* was linked to *unc-29* for double mutants with *lin-45, ksr-1;* and *sur-8.* All other strains were unmarked. The *dpy-20, unc-29,* and *lon-2* markers were also tested in the control strains to show that they have no effects on vulval induction.
[b]Percent of animals in which <3 VPCs adopted vulval fates, as scored under Nomarski optics (Sieburth et al. 1998). In some *sur-6* strains, VPCs were occasionally undivided or absent and may have adopted 4° fates (Clark et al. 1993). Nondivision of VPC P5.p, P6.p, or P7.p occurs infrequently (4/24 *ku123; ku167* animals, 3/23 *cs24;ku167* animals, 3/65 *cs24;n2526* animals). %Vul is calculated only for those animals in which P5.p, P6.p, and P7.p were present and divided at least once.
[c]Average percent of VPCs adopting a vulval cell fate, as scored under Nomarski optics (Sieburth et al. 1998; 100% for wild type). Induction is calculated only for those animals in which P5.p, P6.p, and P7.p were present and divided at least once.
[d]Percent of animals arresting in early larval stages with a clear, rod-like phenotype (Sieburth et al. 1998). (N.D.) Not determined.
[e]For *qDf8* experiments, *n* corresponds to the entire brood of *sur-6/qDf8* mothers, half of which should also have been *sur-6/qDf8* hemizygotes. No more than 25% of such broods arrested as embryos, suggesting that *sur-6/qDf8* (like *sur-6/sur-6*) has no significant embryonic lethal phenotype.
[f]*sur-6(ku123); sur-8* double mutants were 37% embryonic lethal with an average brood size (live worms plus dead embryos) of 26 (*n* = 263).
[g]Data from Sieburth et al. (1998).

These screens have identified mutations in components acting downstream of Ras (such as *lin-45 raf*, *mek-2* MEK, and *mpk-1* MAP kinase), as well as in some new components such as *ksr-1* and *sur-8* (Sternberg and Han 1998). KSR-1 is a conserved putative kinase (Kornfeld et al. 1995; Sundaram and Han 1995; Therrien et al. 1995), and SUR-8 is a conserved Ras-binding protein with leucine-rich repeats (Selfors et al. 1998; Sieburth et al. 1998). Both KSR-1 and SUR-8 appear to stimulate signaling at the level of Ras or Raf (Therrien et al. 1995; Sieburth et al. 1998; this work). Here we describe the identification and characterization of another positive regulator of Ras-mediated signaling, *sur-6*, which encodes a regulatory PR55/B subunit of PP2A (PP2A-B).

Results

We screened for mutations that suppress the Muv phenotype caused by an activated *let-60 ras* mutation, *n1046gf* (Sundaram and Han 1995) or that enhance the Vul phenotype caused by a hypomorphic *lin-45 raf* mutation, *ku112* (M. Sundaram, unpubl.). These screens identified two mutations, *ku123* and *cs24*, that define the gene *sur-6* (suppressor of ras). These *sur-6* mutations cause few or no vulval defects in an otherwise wild-type background; *ku123* and *cs24* mutants display an average vulval induction of 100% or 99%, respectively (Table 1). However, both *sur-6* mutations suppress the Muv phenotype of *let-60(n1046gf)* animals (Table 2), and enhance

Table 2. *Epistasis analysis of* sur-6 *and* ksr-1 *with Muv mutations*

sur-6 or *ksr-1* mutation[a]	Muv mutation[b]	Percent Muv[c] (*n*)	Percent average induction[d] (*n*)
+/+	+/+	0 (many)	100 (many)
+/+	*let-60(n1046gf)*	87 (276)	154 (27)
sur-6(ku123)	*let-60(n1046gf)*	6 (240)	103 (26)
sur-6(cs24)	*let-60(n1046gf)*	19 (107)	104 (33)
sur-6(ku123)/sur-6(cs24)	*let-60(n1046gf)*	3 (31)	N.D.
sur-6(ku123)/+	*let-60(n1046gf)*	22 (148)	115 (31)
sur-6(cs24)/+	*let-60(n1046gf)*	40 (53)	N.D.
sur-6(ku123)/qDf8	*let-60(n1046gf)*	1 (209)	N.D.
+/qDf8	*let-60(n1046gf)*	5 (281)	N.D.
+/+	*HSP–raf(gf)*	47 (30)	119 (30)
sur-6(ku123)	*HSP–raf(gf)*	47 (17)	125 (17)
+/+	*HSP–raf(gf)*	47 (17)	118 (17)
ksr-1(ku68)	*HSP–raf(gf)*	46 (28)	117 (28)
+/+	*lin-1(e1275)*	99 (68)	N.D.
sur-6(ku123)	*lin-1(e1275)*	100 (102)	N.D.
+/+	*lin-15(n765)*	100 (59)	157 (28)
sur-6(ku123)	*lin-15(n765)*	6 (351)	102 (20)

[a]*ku123* was marked with *unc-29* in all strains except those with *n1046.* The full genotype of *ku123/cs24* was *n1046/sy130 dpy-20; ku123/cs24.* The full genotype of *ku123*/+ was *ku123/unc-29; n1046; him-5*/+. The full genotype for *cs24*/+ was *n1046/sy130 dpy-20; cs24*/+. *sy130* encodes the same *let-60 ras(G13E)* substitution as *n1046.* Full genotypes for deficiency analysis: *ces-1 qDf8/unc-13 ku123; n1046* and *ces1 qDf8/ unc-13 dyp-24; n1046. HSP–raf(gf); ksr-1(ku68)* animals (and their paired controls) were marked with *unc-24.*

[b]For *HSP–raf(gf)* description, see text and Sieburth et al. (1998). Also see Sieburth et al. (1998) for positive control results (suppression of *HSP–raf(gf)* by *mek-2* and *mpk-1* alleles). *HSP–raf(gf)* strains were heat shocked during the early-mid L3 stage for 80 min at 36°C. *lin-15* strains were grown at 18°C.

[c]Percent Muv was determined by examining adult hermaphrodites with a dissecting microscope for the presence of ectopic ventral protrusions [for *let-60(gf), lin-1* and *lin-15* experiments], or by examining L4 larvae under Nomarski optics [for *HSP–raf(gf)* experiments].

[d]See Table 1 footnote. In 1/34 *cs24; n1046gf* animals, P4.p and P8.p were undivided and may have adopted 4° fates. Induction is calculated here only for animals in which P(4-8).p divided at least once.

the vulval induction defects and larval lethality caused by weak alleles of *lin-45 raf* (Table 1). These strong genetic interactions suggest that the *sur-6* mutations reduce signaling by the Ras pathway at a point downstream or in parallel to *let-60 ras*.

sur-6(ku123) and *sur-6(cs24)* appear to strongly reduce (but not eliminate) *sur-6* gene function. These *sur-6* alleles and a deficiency of the *sur-6* locus each semidominantly suppress the Muv phenotype of *let-60(n1046gf)* mutants (Table 2), suggesting the *sur-6* locus is haplo insufficient. RNA-mediated inhibition of *sur-6* also suppresses the *let-60(n1046gf)* Muv phenotype and causes a partial Vul phenotype in a wild-type background (Table 3), arguing that these are loss-of-function phenotypes. *qDf8* fails to complement the weak Vul phenotype of *sur-6(cs24)* mutants, and *sur-6/sur-6* homozygotes and *sur-6/qDf8* hemizygotes display similar phenotypes (Tables 1 and 2), consistent with the *sur-6* mutations strongly reducing *sur-6* function. This notion is further supported by the fact that the suppressor phenotype of *sur-6(ku123)* can be rescued by injecting wild-type DNA containing the *sur-6* gene (see below and Materials and Methods). Nevertheless, the *sur-6* mutations are likely non-null, because RNA inhibition suggests that the *sur-6* null phenotype is embryonic lethal (Table 3; see below). Because reducing *sur-6* function reduces vulval induction in sensitized genetic backgrounds, we conclude that *sur-6* normally plays a positive role in regulating Ras pathway signaling during vulval development.

sur-8 and *ksr-1* are two other genes that display genetic interactions similar to those of *sur-6*. Strong loss-of-function mutations in *sur-8* or *ksr-1* cause few defects on their own, but strongly modify the phenotypes of other Ras pathway mutants (Kornfeld et al. 1995; Sundaram and Han 1995; Sieburth et al. 1998). We found that *sur-6;sur-8* double mutants display a synthetic Vul phenotype (Table 1), consistent with these mutations potentially affecting different aspects of Ras pathway regulation. Interestingly, however, *sur-6;ksr-1* double mutants resemble the *sur-6* or *ksr-1* single mutants (Table 1). Because reducing the function of either *ksr-1* or *sur-6* has the same effect as reducing the function of both, it is likely that *sur-6* acts together with *ksr-1* in a common signaling pathway to regulate Ras signaling.

raf genetically acts downstream of *ras* in *C. elegans* and in *Drosophila* (Dickson et al. 1992; Han et al. 1993), and mammalian Raf is a direct Ras effector (for review, see Katz and McCormick 1997). *Drosophila ksr* has been shown previously to function genetically downstream or in parallel to *ras* and upstream of *raf* (Therrien et al. 1995). We tested whether this was also true for *C. elegans ksr-1* and *sur-6*. An activated *raf* transgene under

Table 3. *RNA interference of* sur-6 *PP2A-B, PP2A-C, or PP2A-A*

Genotype	dsRNA[a] or transgenes[b]	Percent Vul or Muv (*n*)	Percent avg. induction (*n*)
+	no dsRNA	0 (many)	100 (many)
+	*sur-6* PP2A-B	19 Vul (21)	94 (21)
+	PP2A-C	5 Vul (22)	99 (22)
let-60(n1046gf)	no dsRNA	95 Muv (19)	173 (19)
let-60(n1046gf)	*sur-6* PP2A-B	3 Muv (29)	101 (29)
let-60(n1046gf)	PP2A-C	70 Muv (23)	132 (23)
let-60(n1046gf)	*sur-8*	15 Muv (33)	106 (33)
let-60(n1046gf)	no dsRNA	93 Muv (59)	143 (59)
let-60(n1046gf)	*sur-6* PP2A-B	7 Muv (15)	103 (15)
let-60(n1046gf)	PP2A-A	57 Muv (14)	125 (14)
let-60(n1046gf)	no transgenes	82 Muv (242)	175 (23)
let-60(n1046gf)	*sur-6* PP2A-B	20 Muv (172)	110 (48)
let-60(n1046gf)	PP2A-C	65 Muv (82)	N.D.

[a]In the case of *sur-6* PP2A-B, PP2A-C or PP2A-A (but not *sur-8*), nearly complete embryonic lethality was observed in progeny laid 8–24 hr after double-stranded (ds) RNA injection. Most surviving *sur-6(RNAi)* animals displayed a weakly Uncoordinated phenotype. Vulval induction was scored in surviving progeny laid at least 6 hr postinjection (for the wild-type background), or in progeny laid 6–8 hr postinjection, before the lethal period began [for the *let-60(gf)* background]. In the wild-type background, *sur-6 (RNAi)* caused variable vulval defects, including defects in vulval induction (2° to 3° or hybrid fate transformation; 5/31 animals), VPC generation (absence of P5.p, P6.p, or P7.p, or failure of those cells to divide; 10/31 animals), and vulval lineage execution (failure of P5.pxx to complete last round of vulval division; 1/31 animals). The VPC generation defects were almost never seen in the *let-60(n1046gf)* background. In the wild-type background, PP2A-C RNAi also caused occasional defects in vulval induction (P5.p executed a "hybrid" lineage, generating only 3 vulval descendants and 1 nonvulval descendant; 1/23 animals), VPC generation (absence of P5.p; 1/23 animals), and vulval lineage execution (failure of P5.pxx to complete last round of vulval division; 1/23 animals). %Vul (and induction) are calculated only for animals in which P5.p, P6.p, and P7.p were present and divided at least once. %Muv (and induction) are calculated only for animals in which P(4-8).p were present and divided at least once.
[b]We used the *col-10* promoter to coexpress both sense and antisense RNA fragments in the hypodermis (see Material and Methods).

the control of a heat shock promoter [*HSP–raf(gf)*] causes a Muv phenotype that can be suppressed by mutations in the downstream Ras pathway components *mek-2* MEK or *mpk-1* MAP kinase (Sieburth et al. 1998). In contrast, this *HSP–raf(gf)* Muv phenotype is not suppressed by *ksr-1* or *sur-6* mutations (Table 2). Therefore, *ksr-1* and *sur-6* likely act upstream of *lin-45 raf*. Consistent with this, *sur-6(ku123)* also fails to suppress the Muv phenotype caused by a relatively weak mutation of *lin-1*, which encodes an ETS domain transcription factor acting downstream of *mpk-1* MAP kinase (Jacobs et al. 1998; Tan et al. 1998). The genetic placement of *ksr-1* and *sur-6* suggests that *ksr-1* and *sur-6* may act in a regulatory branch that modifies or cooperates with Ras/Raf.

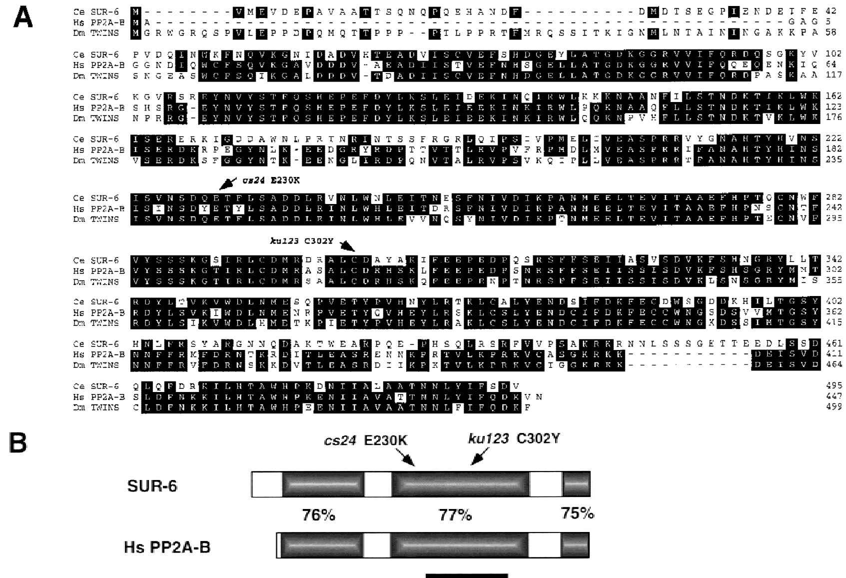
We cloned *sur-6* by genetic mapping followed by transformation rescue (see Materials and Methods). An 11-kb fragment, pDS89, which contains *sur-6*(+) rescuing activity was predicted by the *C. elegans* genome sequencing consortium to contain a single gene (F26E4.1) that encodes a PR55 family regulatory B subunit of PP2A (PP2A-B, Fig. 1). The *sur-6* alleles *ku123* and *cs24* each contain single G → A substitutions that introduce amino acid substitutions at highly conserved positions within PP2A-B (Fig. 1). The PP2A-B coding region, when expressed under the control of a heat shock promoter in transgenic animals, can rescue the *sur-6(ku123)* mutant (see Materials and Methods), and RNA interference of PP2A-B phenocopies the *sur-6* suppression and partial Vul phenotypes (Table 3). Thus, we conclude that *sur-6* encodes PP2A-B. SUR-6 shares >59% overall amino acid identity with PP2A-B from human or *Drosophila*, with three large stretches of at least 75% identity (Fig. 1B). In mammals there are three PR55/B isoforms that differ in spatial and temporal expression (Mayer-Jaekel and Hemmings 1994). SUR-6 is most similar to the mammalian Bα subtype.

PP2A-B subunits modulate the activity and/or substrate specificity of the PP2A-A/C catalytic core (Mayer-Jaekel and Hemmings 1994). SUR-6 is the only predicted PR55/B family member encoded by the *C. elegans* genome, although other types of regulatory B subunits (such as PR56/B′) are also present. The *C. elegans* genome is predicted to encode a single PP2A-A subunit (F48E8.5) and a single PP2A-C subunit (F38H4.9), which share >90% amino acid identity with their mammalian counterparts. As expected, the PP2A-A subunit can bind to both SUR-6 PP2A-B and the PP2A-C subunit, as assayed by the yeast two-hybrid system (data not shown). Given the positive role of *sur-6* PP2A-B defined by genetic analysis, *sur-6* PP2A-B could either function to activate the catalytic core, which in turn would activate Ras pathway signaling, or it could function to relieve inhibition of Ras signaling by the core complex (Fig. 2A).

To determine the requirements for PP2A during *C. elegans* development, we used RNA interference (RNAi) (Fire et al. 1998) to block *sur-6* PP2A-B, PP2A-A, or PP2A-C expression. For each PP2A gene (but not for *sur-8* or *ksr-1*), RNAi caused highly penetrant embryonic lethality in both wild-type and *let-60(n1046gf)* backgrounds. Embryos arrested at ~100 cell stage, with widely variable cell sizes (data not shown). Thus, unlike *ksr-1* and *sur-8*, PP2A appears to be absolutely required during embryonic development in addition to functioning later during vulval induction. Because the *sur-6(ku123)* and *sur-6(cs24)* mutations caused little or no embryonic lethality, even when hemizygous (Table 2), the two functions of *sur-6* PP2A-B appear separable, with these *sur-6* point mutations primarily affecting *sur-6* PP2A-B function in vulval development but not in embryogenesis.

To avoid the PP2A(RNAi) lethality and test its effects on vulval development, we examined the last surviving progeny of RNA-injected mothers (Table 3). In this assay, *sur-6(RNAi)* caused a partial Vul phenotype in a wild-

Figure 1. Sequence comparison between SUR-6 PP2AB and human PP2A-Bα. (*A*) Amino acid alignment of *C. elegans* SUR-6 PP2A-B (as predicted by cDNA analyses; see Materials and Methods, GenBank accession no. AF174643) with a human PP2A-Bα isoform (GenBank accession no. 231445; Mayer et al. 1991) and *Drosophila Twins* PP2A-B (GenBank accession no. 543716; Uemura et al. 1993). Identical amino acids are shaded. The position of the C302Y and E230K missense mutations identified in *sur-6(ku123)* and *sur-6(cs24)* mutants, respectively, are denoted. C302 and E230 are conserved in PP2A-B from all species in which it has been identified, from human to yeast. (*B*) Structural comparison of *sur-6* PP2A-B and human PP2A-Bα non-neuronal isoform. Percent amino acid identity is shown for shaded regions. Nonshaded regions share 20% or less amino acid identity. The overall amino acid identity is 59%. The bar denotes the region with similarity to the c-Abl proposed substrate-binding domain (amino acids 257–378).



type background and efficiently suppressed the *let-60(n1046gf)* Muv phenotype. However, PP2A-C(RNAi) caused few vulval defects and only weakly suppressed the *let-60(n1046gf)* Muv phenotype. Similar results were obtained by use of a hypodermal-specific promoter to drive expression of *sur-6* PP2A-B or PP2A-C RNAs (Table 3). Thus, it is still unclear whether PP2A catalytic activity promotes and/or inhibits Ras-mediated vulval induction.

Discussion

We have shown for the first time a positive and specific regulatory role for a PP2A regulatory PR55/B subunit in Ras-mediated signal transduction. We propose that *sur-6* PP2A-B influences the catalytic activity of PP2A toward specific Ras pathway substrate(s), such as Raf or KSR, leading to the enhancement of Ras pathway signaling.

Genetic analysis shows that *sur-6* PP2A-B positively regulates Ras pathway signaling during vulval induction. First, reducing *sur-6* PP2A-B function by mutation or RNA inhibition suppresses the excess vulval cell fate specification caused by an activated *let-60 ras* allele (but not that caused by an activated *raf* transgene). Second, *sur-6* PP2A-B mutations enhance defects in vulval cell-fate specification caused by weak mutations in the Ras pathway components *lin-45 raf* or *mpk-1* MAP kinase. However, reducing *sur-6* PP2A-B function by mutation or RNA inhibition only mildly reduces vulval induction in an otherwise wild-type background, suggesting that *sur-6* PP2A-B activity might not be essential for vulval induction when other components are functioning normally.

sur-6 PP2A-B appears to regulate Ras pathway signaling in multiple tissues besides vulval precursor cells. For example, a *sur-6* PP2A-B mutation suppresses the male mating defect caused by an activated *ras* mutation (data not shown) and enhances the rod-like larval lethality of *lin-45 raf* or *mpk-1* MAP kinase mutants. However, *sur-6* PP2A-B also appears to have functions that are currently not known to involve the Ras pathway. RNA inhibition of *sur-6* PP2A-B function reveals an absolute requirement for *sur-6* during embryogenesis. Additional roles of *sur-6* PP2A-B in the generation or survival of

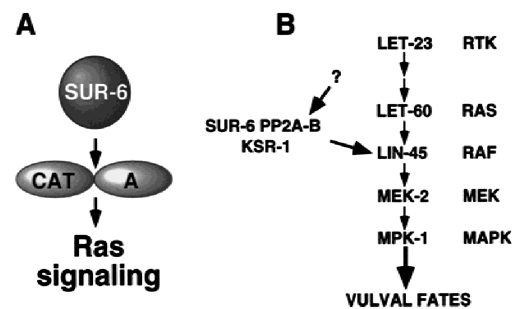


Figure 2. Models for SUR-6 PP2A-B function in the vulval induction pathway. (*A*) SUR-6 PP2A-B is likely to regulate the activity of a PP2A core complex composed of a C and A subunit to influence Ras signaling. SUR-6 PP2A-B is a positive regulator of Ras signaling, so SUR-6 could function either to activate the PP2A core, which in turn activates Ras signaling, or to inhibit the PP2A core that normally inhibits Ras signaling. Potential targets for SUR-6 PP2A-B-regulated PP2A activity include LIN-45 Raf and KSR-1. (*B*) Model for the signal transduction pathway regulated by SUR-6. The receptor tyrosine kinase LET-23 is activated by an inductive signal that leads to the activation of LET-60 Ras. Ras–GTP recruits Raf kinase to the membrane and Raf phosphorylates and activates the MEK-2 MEK/MPK-1 MAPK kinase cascade. We showed that SUR-6 PP2A-B positively regulates signaling at a branch point at the level of Ras or Raf. KSR-1 and SUR-6 may work together to strengthen Ras pathway output during vulval cell fate specification.

VPCs, and in the development or function of muscles and/or motor neurons, are suggested by the fact that *sur-6(cs24)* animals and surviving *sur-6(RNAi)* animals sometimes lack one or more VPCs and are weakly uncoordinated. It would be interesting to know whether or not these defects also involve the Ras pathway.

SUR-6 PP2A-B likely functions by regulating the activity of the PP2A-A/C core complex. Mammalian PP2A-B regulatory subunits can either activate or inhibit core activity in vitro, depending on the substrate used (Mayer-Jaekel and Hemmings 1994). Genetic data in yeast support specific activating roles for PP2A regulatory subunits, because mutations in *cdc55* (a PR55/B subunit) and *rts1* (a PR56/B′ subunit) each cause a distinct subset of phenotypes associated with mutations in the PP2A catalytic core (Shu et al. 1997). If SUR-6 PP2A-B also functions to activate the PP2A catalytic core, this implies that PP2A positively regulates Ras signaling at the level of Ras or Raf (Fig. 2). Such a model would differ from previously proposed models, on the basis of experiments overexpressing SV40 small t antigen in cultured mammalian cells, that suggest PP2A inhibits MEK and MAP kinase activities (Sontag et al. 1993). However, it is likely that regulation of the Ras pathway by PP2A-C is complex, involving multiple positive and negative influences on different substrates (e.g., Wassarman et al. 1996). *sur-6* PP2A-B may stimulate PP2A core activity toward a specific subset of these substrates, such as KSR and/or Raf.

Consistent with the idea that *sur-6* PP2A-B acts by activating a PP2A core is the observation that the *sur-6(ku123)* allele affects an absolutely conserved cysteine residue within a region of PP2A-B that shares similarity to c-Abl kinase domains VI–X. This region of similarity is shared by non-kinase residues of c-Abl that may be involved in target specificity, and may thus define a region of PP2A-B involved in localization or target specificity (Mayer et al. 1991). We hypothesize that in *sur-6(ku123)* PP2A-B mutants, the PP2A core may be mislocalized or may fail to be targeted to the proper substrates. This model would be similar to the suggestion from a recent work in mammalian cells and *Xenopus* embryo explants that a PR56/B′ subunit of PP2A may interact with APC and direct PP2A to dephosphorylate specific components of the Wnt/β-catenin signaling pathway (Seeling et al. 1999).

Interestingly, our data suggest that *sur-6* PP2A-B may function together with *ksr-1*. *sur-6* PP2A-B and *ksr-1* have the same epistatic relationship with respect to *ras* and *raf*, and *sur-6; ksr-1* double mutants resemble *sur-6* or *ksr-1* single mutants. Taken together, these data are consistent with the idea that *sur-6* PP2A-B and *ksr-1* act in a common pathway to stimulate *ras*-mediated signaling at a branch point that feeds out of the pathway at the level of *ras* or into the pathway at the level of *raf* (Fig. 2B).

KSR proteins positively regulate Ras signaling in *C. elegans* and *Drosophila* (Kornfeld et al. 1995; Sundaram and Han 1995; Therrien et al. 1995), as well as in *Xenopus* oocytes and certain mammalian cells (Therrien et al. 1996). Murine KSR associates with several proteins in vivo, including Raf, MEK, and MAP kinase, and has been proposed to function as a scaffold protein involved in signal propagation through the Raf/MEK/MAP kinase cascade (Therrien et al. 1996; Stewart et al. 1999). Murine KSR is a phosphoprotein (Cacace et al. 1999); although the role of phosphorylation in KSR regulation is unclear. Thus, KSR-1 is a potential target for regulation by PP2A during vulval induction. Alternatively, KSR-1 may act to regulate PP2A-B function.

Another potential *sur-6* PP2A-B-dependent PP2A target is LIN-45 Raf. The mechanism of Raf activation is still poorly understood, but there is evidence for both inhibitory and activating phosphates on Raf (Morrison and Cutler 1997). Whereas in vitro studies suggest that PP2A can dephosphorylate Raf, it is probably not the major phosphatase to remove activating phosphates (Dent et al. 1995). However, a role for PP2A in removing inhibitory phosphates has not been ruled out. The placement of a B regulatory subunit of PP2A as a positive regulator of the Ras pathway, and the unexpected finding that it acts together with KSR-1, should lead to a better understanding of PP2A regulation and its physiological substrates.

Materials and methods

Mutants were derived from the wild-type Bristol strain, N2, and grown under standard conditions (Brenner 1974) at 20°C unless otherwise indicated. Some strains were obtained from the *Caenorhabditis* Genetics Center. The alleles and deficiencies used are described in Riddle et al. (1997) unless otherwise indicated: LGI, *unc-29(e1072, h1)*, *ces-1(n703)*, *unc-13(e1091)*, *dpy-24(s71)*, *qDf8*, *qDf5*; LGIII, *mpk-1(ku1)*, *unc-119(ed3)*; LGIV, *lin-1(e1275)*, *sur-8(ku167)* (Sieburth et al. 1998), *unc-24(e138)*, *lin-45(sy96)*, *lin-45(ku112)* (Sundaram and Han 1995), *let-60(n1046gf)*, *let-60(sy130gf)*, *dpy-20(e1282)*; LGV, *him-5(e1490)*; LGX, *ksr-1(ku68)*, *ksr-1(n2526)*, *lin-15(n765)*.

sur-6 isolation and cloning

sur-6(ku123) was isolated as a dominant suppressor in screens for suppressors of the Muv phenotype of *let-60 ras(n1046gf)* homozygotes described previously (Wu and Han 1994; Sundaram and Han 1995). *sur-6(cs24)* was isolated in screens for enhancers of the *lin-45 raf(ku112)* Vul and lethal phenotypes (M. Sundaram, unpubl.). Both alleles were obtained after ethylmethanesulfonate mutagenesis.

sur-6 was first mapped between *unc-29* and *dpy-24* of linkage group I by standard three-point mapping. *sur-6* was further mapped with *unc-29(h1) hP6 dpy-24*; *let-60(n1046)*, derived from SP1726 (gift from E. Lundquist, University of Minnesota, St. Paul, MN). Unc non-Dpy recombinants were tested for *ku123* by scoring the suppression phenotype and for the polymorphism *hP6* by PCR with primers Tc*1-1* and hP6-B (gift from D. Fitch, New York University, NY, NY). A total of 15 of 35 Unc-29 recombinants contained *ku123* and of these, one was positive for *hP6*, placing *sur-6* to the right of cosmid C03D6. *sur-6* mapped to the left of cosmid F14G10, which is the left endpoint of the complementing deficiency *qDf5* (R. Ellis, pers. comm.). Deficiency *qDf8* uncovers *sur-6* (Tables 1 and 2). *sur-6(cs24)* was mapped to an interval between *dpy-5* and *unc-101*,

and within two map units of *unc-13* by standard mapping crosses.

Cosmids in the region were tested for *sur-6*(+) activity by assaying their ability to rescue the *sur-6(ku123)* suppressor phenotype. Cosmids were injected at 5–10 µg/ml together with 40 µg/ml *unc-119*(+) transformation marker pDP#MM016 (Maduro and Pilgrim 1995) and 10 µg/ml pBluescript into *sur-6(ku123); unc-119; let-60* mutants. A single cosmid, K02A11 contained *sur-6*(+) rescuing activity, but an overlapping cosmid, F26E4, failed to rescue.

Northern analysis of mixed-stage RNA with a genomic fragment that spanned all predicted exons of F26E4.1 as a probe revealed the presence of an abundant 2.5-kb transcript and two minor transcripts of 2.3 and 1.8 kb. Approximately 1 million plaques were screened from a λgt11-mixed stage cDNA library (gift from P. Okkema, University of Illinois, Chicago, IL) with a 1.5-kb genomic probe, and 22 positive clones were isolated. One positive clone contained a partial SL1-spiced leader corresponding to base pair 19414 of cosmid K02A11. Positive clones analyzed differed in the length of the 3′ UTR because of differential use of transcription termination sites. The sequence of these clones confirmed the exon/intron structure predicted by the *C. elegans* genome sequencing consortium. A transgene (pDS54) that expresses the *sur-6* PP2A-B cDNA under the control of the Hsp16-41 promoter could rescue the suppressor phenotype of *sur-6(ku123)*. The *sur-6(ku123); let-60(n1046gf)* double mutant was used to host the transgene. When heat-shocked, 75% of the transgenic animals were Muv (average vulval induction is 138%, $n = 12$), compared with 0% Muv (100% vulval induction, $n = 20$) for non-heat-shocked controls.

The molecular lesion associated with *sur-6* mutations was identified by PCR amplifying genomic DNA from lysates of *sur-6* mutants and sequencing PCR products directly.

RNAi

RNAi with double-stranded RNA was performed essentially as described (Fire et al. 1998). PCR fragments containing >1 kb of the coding regions were used as templates for in vitro transcription reactions. RNA was injected in parallel into either *let-60(n1046gf)* or N2 hermaphrodites at a concentration of 0.5–1 mg/ml.

The *col-10* promoter was used to coexpress both sense and antisense RNA fragments in hypodermis. The *sur-6* PP2A-B transgenes are pDS94 and pDS95, which contain nucleotides 90-1488 of *sur-6* cloned in opposite orientations into a *col-10* promoter-containing vector. The PP2A-C transgenes are pDS96 and pDS97, which contain nucleotides 241-957 of PP2A-C cloned in opposite orientations into the *col-10* promoter vector. Transgenes were coinjected with the marker pTG96 (Yochem et al. 1998).

Acknowledgments

We thank Y. Han for isolating the *ku123* allele, A. Goldman for isolating the *cs24* allele, R. Ellis for information on deficiencies, D. Fitch for hP6 mapping primers, V. Ambros for the *col-10* promoter, P. Okkema for cDNA libraries, J. Brenner and C. Duffey for assistance, and K.L. Guan and G. Kao for comments on the manuscript. We thank Alan Coulson at the Sanger Centre for cosmids, and the *Caenorhabditis* Genetic Center, which is funded by the National Institutes for Health (NIH) National Center for Research Resources, for some strains used in this study. D.S.S. is funded with a predoctoral Breast Cancer Research Program Fellowship from the U.S. Army Medical Research and Material Command under DAMD17-96-6117. M.H. is a Howard Hughes Medical Institute Investigator. Supported by grants from the American Cancer Society (ACS) and NIH (GM47869) to M.H., and by grants from ACS, the McCabe Foundation, and the Penn-Hughes Developmental Biology Program to M.S.

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

Brenner, S. 1974. The genetics of *Caenorhabditis elegans. Genetics* **77:** 71–94.

Cacace, A.M., N.R. Michaud, M. Therrien, K. Mathes, C. T., G.M. Rubin, and D.K. Morrison. 1999. Identification of constitutive and ras-inducible phosphorylation sites of KSR: Implications for 14-3-3 binding, mitogen-activated protein kinase binding, and KSR overexpression. *Mol. Cell. Biol.* **19:** 229–240.

Clark, S.G., A.D. Chisholm, and R.H. Horvitz. 1993. Control of cell fates in the central body region of *C. elegans* by the homeobox gene *lin-39. Cell* **74:** 43–55.

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:** 1902–1905.

Dickson, B., F. Sprenger, D. Morrison, and E. Hafen. 1992. Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. *Nature* **360:** 600–603.

Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans. Nature* **391:** 806–811.

Han, M., A. Golden, Y. Han, and P.W. Sternberg. 1993. *C. elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. *Nature* **363:** 133–140.

Horvitz, H.R. and P.W. Sternberg. 1991. Multiple intercellular signaling systems control the development of the *C. elegans* vulva. *Nature* **351:** 535–541.

Jacobs, D., G.J. Beitel, S.G. Clark, H.R. Horvitz, and K. Kornfeld. 1998. Gain-of-function mutations in the *Caenorhabditis elegans lin-1* ETS gene identify a C-terminal regulatory domain phosphorylated by ERK MAP kinase. *Genetics* **149:** 1809–1822.

Katz, M.E. and F. McCormick. 1997. Signal transduction from multiple Ras effectors. *Curr. Opin. Genet. Dev.* **7:** 75–79.

Kornfeld, K. 1997. Vulval development in *Caenorhabditis elegans. Trends Genet.* **13:** 55–61.

Kornfeld, K., D.B. Hom, and H.R. Horvitz. 1995. The *ksr-1* gene encodes a novel protein kinase involved in Ras-mediated signaling in *Caenorhabditis elegans. Cell* **83:** 902–913.

Maduro, M. and D. Pilgrim. 1995. Identification and cloning of *unc-119*, a gene expressed in the *Caenorhabditis elegans* nervous system. *Genetics* **141:** 977–988.

Mayer, R.E., P. Hendrix, P. Cron, R. Matthies, S.R. Stone, J. Goris, W. Merlevede, J. Hofsteenge, and B.A. Hemmings. 1991. Structure of the 55-kDa regulatory subunit of protein phosphatase 2A: Evidence for a neuronal-specific isoform. *Biochemistry* **30:** 3589–3597.

Mayer-Jaekel, R.E. and B.A. Hemmings. 1994. Protein phosphatase 2A—a ‘menage a trois’. *Trends Cell Biol.* **4:** 287–291.

Millward, T.A., S. Zolnierowicz, and B.A. Hemmings. 1999. Regulation of protein kinase cascades by protein phospha-

tase 2A. *Trends. Biochem. Sci.* **24:** 186–191.

Morrison, D.K. and R.E. Cutler. 1997. The complexity of Raf-1 regulation. *Curr. Opin. Cell. Biol.* **9:** 174–179.

Riddle, D.L., T. Blumenthal, B. Meyer, and J.R. Preiss. 1997. C. elegans *II*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Seeling, J.M., J.R. Miller, R. Gil, R.T. Moon, R. White, and D.M. Virshup. 1999. Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. *Science* **283:** 2089–2091.

Selfors, L.M., J.L. Schultzman, C.Z. Borland, and M.J. Stern. 1998. *soc-2* encodes a leucine-rich repeat protein implicated in fibroblast growth factor receptor signaling. *Proc. Natl. Acad. Sci.* **95:** 6903–6908.

Shenolikar, S. 1994. Protein serine/threonine phosphatases-new avenues for cell regulation. *Annu. Rev. Cell Biol.* **10:** 55–86.

Shu, Y., H. Yan, E. Hallberg, and R. Hallberg. 1997. Molecular genetic analysis of Rts1p, a B′ regulatory subunit of *Saccharomyces cerevisiae* protein phosphatase 2A. *Mol. Cell. Biol.* **17:** 3242–3253.

Sieburth, D., Q. Sun, and M. Han. 1998. SUR-8, a conserved Ras-binding protein with leucine-rich repeats, positively regulates Ras-mediated signaling in *C. elegans. Cell* **94:** 119–130.

Sontag, E., S. Fedorov, C. Kamibayashi, D. Robins, M. Cobb, and M. Mumby. 1993. The interaction of SV40 small tumor anitgen with protein phosphatase 2A stimulates the Map kinase pathway and induces cell proliferation. *Cell* **75:** 887–897.

Sternberg, P.W. and M. Han. 1998. Genetics of Ras signaling in *C. elegans. Trends Genet.* **14:** 466–472.

Stewart, S., M. Sundaram, Y. Zhang, J. Lee, M. Han, and K.-L. Guan. 1999. Kinase Suppressor of Ras (KSR) forms a multiprotein signaling complex and modulates MEK localization. *Mol. Cell. Biol.* **19:** 5523–5534.

Sundaram, M. and M. Han. 1995. The *C. elegans ksr-1* gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. *Cell* **83:** 889–901.

Tan, P.B., M.R. Lackner, and S.K. Kim. 1998. MAP kinase signaling specificity mediated by the LIN-1 Ets/LIN-31 WH transcription factor complex during *C. elegans* vulval induction. *Cell* **93:** 569–580.

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:** 879–887.

Therrien, M., N.R. Michaud, G.M. Rubin, and D.K. Morrison. 1996. KSR modulates signal propagation within the MAPK cascade. *Genes & Dev.* **10:** 2684–2695.

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 disks. *Genes & Dev.* **7:** 429–440.

Wassarman, D.A., N.M. Solomon, H.C. Chang, F.D. Karim, M. Therrien, and G.M. Rubin. 1996. Protein phosphatase 2A positively and negatively regulates Ras-mediated photoreceptor development in *Drosophila. Genes & Dev.* **10:** 272–278.

Westphal, R.S., K.A. Anderson, A.R. Means, and B.E. Waszinski. 1998. A signaling complex of Ca2+-calmodulin-dependent protein kinase IV and protein phosphatase 2A. *Science* **280:** 1258–1261.

Wu, Y. and M. Han. 1994. Suppression of activated Let-60 Ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. *Genes & Dev.* **8:** 147–159.

Yochem, J., T. Gu, and M. Han. 1998. A new marker for mosaic analysis of *Caenorhabditis elegans* indicates a fusion between hyp6 and hyp7, two major components of the hypodermis. *Genetics* **149:** 1323–1334.