The Ca\textsuperscript{2+}-Calmodulin-Activated Protein Phosphatase Calcineurin Negatively Regulates Egf Receptor Signaling in Drosophila Development

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ABSTRACT
Calcineurin is a Ca\textsuperscript{2+}-calmodulin-activated, Ser-Thr protein phosphatase that is essential for the translation of Ca\textsuperscript{2+} signals into changes in cell function and development. We carried out a dominant modifier screen in the Drosophila eye using an activated form of the catalytic subunit to identify new targets, regulators, and functions of calcineurin. An examination of 70,000 mutagenized flies yielded nine specific complementation groups, four that enhanced and five that suppressed the activated calcineurin phenotype. The gene canB2, which encodes the essential regulatory subunit of calcineurin, was identified as a suppressor group, demonstrating that the screen was capable of identifying genes relevant to calcineurin function. We demonstrated that a second suppressor group was sprouty, a negative regulator of receptor tyrosine kinase signaling. Wing and eye phenotypes of ectopic activated calcineurin and genetic interactions with components of signaling pathways suggested a role for calcineurin in repressing Egf receptor/Ras signal transduction. On the basis of our results, we propose that calcineurin, upon activation by Ca\textsuperscript{2+}-calmodulin, cooperates with other factors to negatively regulate Egf receptor signaling at the level of sprouty and the GTPase-activating protein Gap1.

The only protein phosphatase regulated by both Ca\textsuperscript{2+} and calmodulin, calcineurin is a key player in Ca\textsuperscript{2+} signal transduction from yeast to humans and has been implicated in a wide array of processes, from disease progression to development (for a general review, see Rusnak and Mertz 2000). In the immune system, calcineurin is essential for T-cell activation and is the target of immunosuppressant drugs such as cyclosporin (Liu et al. 1991; Clipstone and Crabtree 1992). An abundant neuronal protein, calcineurin has been implicated in various forms of synaptic plasticity (reviewed in Yakel 1997). In addition, calcineurin is involved both in the development of cardiac valves (Ranger et al. 1998) and in hypertrophy of cardiac muscle following disease or injury (Molkentin et al. 1998).

The enzyme consists of an \~60-kD catalytic subunit, calcineurin A (canA), bound to the regulatory subunit, calcineurin B (canB), a 19-kD EF-hand Ca\textsuperscript{2+}-binding protein (reviewed in Klee et al. 1998). CanB is essential for phosphatase activity and can be dissociated from canA only by denaturants. CanA has two variable regions at the N and C termini, a highly conserved catalytic domain, and a regulatory region. The regulatory region consists of a binding site for Ca\textsuperscript{2+}-calmodulin and a short autoinhibitory domain that blocks substrate access to the active site in the absence of Ca\textsuperscript{2+}-calmodulin.

A Ca\textsuperscript{2+}-calmodulin-independent, constitutively active phosphatase is made by deleting the canA regulatory region (O’Keeffe et al. 1992). Studies from a number of different organisms indicate that, aside from a small degree of Ca\textsuperscript{2+} sensitivity mediated by canB, activated calcineurin functions identically to the full-length, Ca\textsuperscript{2+}-calmodulin-activated form (Mendoza et al. 1996; Shibasaki et al. 1996; Winder et al. 1998).

Calcineurin is activated by a sustained increase in intracellular Ca\textsuperscript{2+} levels that can result from the opening of intracellular Ca\textsuperscript{2+} channels in response to phosphoinositide (PI) signaling (reviewed in Berridge 1993). PI signaling is initiated by the activation of a phosphatidylinositol-specific phospholipase C, either PLC\textbeta\textsuperscript{1} by G-protein-coupled receptors (GPCR) or PLC\gamma by receptor tyrosine kinases (RTK). PI-PLCs cleave phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) to yield inositol 1,4,5-trisphosphate (InsP\textsubscript{3}), which then activates the InsP\textsubscript{3} receptor Ca\textsuperscript{2+} channel.

GPCRs and RTKs activate an integrated signaling network that includes the Ras/mitogen-activated protein (MAP) kinase cascade, PI3-kinase, and the small GTPase Rho. Depending upon the cellular context, these pathways can antagonize or cooperate with each other and with PI signaling. For example, T-cell activation requires the activation of both NFAT, which is transduced to the nucleus upon dephosphorylation by calcineurin, and AP1, which acts downstream of Ras and MAP kinase (Crabtree 1999).

Conversely, PI signaling has been found to antagonize the Ras pathway in Drosophila. The Egf receptor and Ras/MAP kinase cascade are essential for formation of...
wing veins and photoreceptor (R) cells in the eye. Mutations in the single phospholipase Cγ gene, small wing (sl), cause the formation of extra R7 cells and wing vein material (Thackeray et al. 1998) and also genetically interact with Egfr-receptor-signaling components (Thackeray et al. 1998; Powe et al. 1999). A recently proposed model for sl-mediated repression of Egf receptor signaling was based on the identification of the GTPase-activating protein Gap1 as an InsP3 receptor. PLCγ-generated InsP3 is converted to InsP4, which then activates Gap (Powe et al. 1999). Gap converts the active form of Ras, Ras-GTP, to the inactive form, Ras-GDP.

The Drosophila genome contains three canA genes and two canB genes that are 75 and 88% similar to the vertebrate genes, respectively. To date, no mutants have been described for any of the five genes. To study calcineurin function in Drosophila, we expressed a constitutively active form of canA during imaginal development and examined the resulting phenotypes. The activated calcineurin rough eye phenotype was used to perform a genetic modifier screen. We were able to successfully isolate and characterize specific enhancers and suppressors and identified two suppressors as canB2 and sprouty. The activated calcineurin rough eye was also tested extensively for genetic interactions with an array of signaling cascades. Taken together, the genetic evidence is consistent with calcineurin functioning as a negative regulator of Egf receptor/Ras signaling during imaginal development, possibly in the same pathway as PLCγ.

MATERIALS AND METHODS

Stocks: The following published stocks were used in this study: Egfr-G (Nusslein-Volhard et al. 1984); Egfr-G1 (Baker and Rubin 1989); dhk-G, sos-G, Ras85D-G, and Ras85D-G (Simon et al. 1991); Gap1-G and Gap1-G (Gaul et al. 1992); sty-G and sty-G (Hacohen et al. 1998); sl-G and sl-G (Thackeray et al. 1998); pm-Gp2057 and pm-Gp4062 (O’Neill et al. 1994);psi1 (Klambt et al. 1991); psi-G (Perrimon et al. 1996); argo-G (Schmucker et al. 1997); clb-G (Pat et al. 2000); dos-G and dos-G (Herbst et al. 1996); sos-G (Yagi et al. 1998); ksr-Gp27, pkl-Gp27, Dsor-Gp220, et-G30, phl-Gp221, and aop-Gp221 (Karim et al. 1996);ina1 and ina1 (Carthew and Rubin 1990); phl-Gp225 (Ghang et al. 1995); aop1 (Rogge et al. 1995); and Rho-Gp220 and Rho-Gp220 (Strutt et al. 1997).

Calcineurin constructs: The constitutively active form of canA, canA-G, was made from the canA gene Pp2B-14D (Brown et al. 1994) by inserting a stop codon at amino acid residue 456 (out of 570). PCR was carried out using primers that incorporated a 3′ stop codon and flanking restriction sites. A full-length Pp2B-14D construct was made by using the same 5′ primer and a 3′ primer at the end of the open reading frame (ORF). The Pp2B-14D cDNA L04578 (Rubin et al. 2000) was used as a template for both. Full-length canB was cloned by PCR using wild-type genomic DNA as a template, as the ORF of canB-G is contained within a single exon. All constructs were confirmed by sequencing. For expression in transgenic animals, the constructs were inserted into pUAST and pGMR, which contains glass response elements (Moses and Rubin 1991).

Modifier screen: The canA-G transgene was inserted onto TM3 (TCAG) and CyO (CCAG) by inserting a stop codon at amino acid residue 456 (out of 570). PCR was carried out using primers that incorporated a 3′ stop codon and flanking restriction sites. A full-length Pp2B-14D construct was made by using the same 5′ primer and a 3′ primer at the end of the open reading frame (ORF). The Pp2B-14D cDNA L04578 (Rubin et al. 2000) was used as a template for both. Full-length canB was cloned by PCR using wild-type genomic DNA as a template, as the ORF of canB-G is contained within a single exon. All constructs were confirmed by sequencing. For expression in transgenic animals, the constructs were inserted into pUAST and pGMR, which contains glass response elements (Moses and Rubin 1991).

Figure 1.—The canA-G rough eye is sensitive to the dose of glass and canB-AF. SEMs are from flies of the following genotypes: (A) w1118; (B and D) TCAG; (C) TCAG/gfl-G (E) Df(1)J70; F; TCAG; and (F) TCAGB. (A, B, C, and F) Eyes from males; (D and E) eyes from females. Compared to a normal eye (A), the presence of canA-G resulted in a rough eye phenotype (B and D). The rough eye in B is from a line in which the canA-G transgene is inserted on the TM3 balancer (TCAG). The TCAG rough eye was significantly suppressed by removing one copy of glass (C). One copy of canB-AF was removed by introducing the deficiency Df(1)J70, which resulted in suppression of the TCAG rough eye (E). Increasing canB levels by inserting a canB-G transgene onto TCAG (TCAGB) increased the severity of the rough eye (F). The whitish patch near the center of the eye in F is necrotic tissue.
mentation groups were carried out by using standard procedures (GreenSPAN 1997). During meiotic mapping the complementation group alleles were detected in each generation on the basis of their ability to modify TCAG.

**Adult eye microscopy:** Scanning electron microscopy (SEM) was carried out on adult flies with an environmental SEM in wet mode. Samples were prepared as described (WOLF 2000), except that the flies were not fixed before dehydration and critical point drying. All micrographs are displayed at the same magnification (×150). Fixing, embedding, and sectioning of adult eyes were done as described (WOLF 2000).

**Characterization of the canB2 suppressor group:** Genomic DNA was made from chromosome 2, suppressor group 1 allele 87 by isolating mutant first instar larvae from a CS2-1t/+
CyO-GFP<sup>res</sup> line (SULLIVAN et al. 2000) and from the parent isogenic line. To map the inversion breakpoint by PCR, primers were made at base pair positions −426, +60, and +1582 relative to the start of canB2 transcription.

**Sequencing sprouty:** Primers were designed to amplify 700- to 800-bp overlapping fragments that spanned the sty ORF, which is contained within a single exon. Genomic DNA was isolated from the isogenic parent line and from embryos homozygous for the CS3-3 EMS allele ECE(3)518 by using an ECE(3)518/CyO-GFP<sup>res</sup> line. After PCR amplification, the fragments were purified by using the QIAquick PCR purification kit and then directly sequenced. The sty ORF from the isogenic line was identical to the sequence reported in ADAMS et al. (2000), which differs slightly in the predicted amino acid sequence from that previously published (HACOHEN et al. 1998). The EMS allele contained a C-to-T mutation at position 748 relative to the start of the ORF sequence.

**RESULTS**

**Activated calcineurin constructs:** We selected the canA gene Pp2B-14D for our experiments because it is expressed throughout development, including in eye discs (BROWN et al. 1994; K. SULLIVAN, unpublished results). The adjacent canA gene at 14F1 (gene designation CG9819; ADAMS et al. 2000) encodes a protein that is 83% identical to Pp2B-14D; however, canA-14F is not represented in the expressed sequence tag collection and the expression pattern has not been characterized. The canA gene at 100B4, which was incorrectly localized to 21B, is undetectable by Northern analysis (GUERNINI et al. 1992) and appears to have a highly restricted expression pattern (K. SULLIVAN, unpublished results).

An activated form of Pp2B-14D, canA<sup>act</sup>, was made by deleting the autoinhibitory and calmodulin-binding domains (O’KEEFE et al. 1992; MENDOZA et al. 1996). The canA<sup>act</sup> construct was expressed in Drosophila under the control of glass response elements, which induce transcription uniformly in cells posterior to the morphogenetic furrow in the eye imaginal disc (MOSES and RUBIN 1991).

Flies carrying one copy of the canA<sup>act</sup> transgene had mild rough eyes compared to wild type (Figure 1, A and B), and the eyes of flies carrying two copies exhibited a stronger phenotype (data not shown). Consistent with observations in other systems, both full-length canA and activated canA without a functional canB-binding domain did not cause any detectable phenotypes when expressed throughout development (data not shown).
Removing one copy of glass by introducing the null allele gl^60J (Moses and Rubin 1991) strongly suppressed the canA^mt6 rough eye phenotype (cf. Figure 1C to 1B). This demonstrates that the canA^mt6 rough eye phenotype is dependent on glass and is not caused by the insertion site of the transgene or by some other factor.

Reducing the dosage of canB-4F or canB2 by introducing deficiencies that uncover the 4F or 43E genomic region resulted in suppression of the canA^mt6 rough eye (Figure 1E and data not shown). Western blots confirmed that canB protein is present in the eye disc (data not shown); however, it is not known whether the protein is derived from one or both canB genes. Consistent with the effect of reduced canB levels, canB4F^a, which alone has no phenotype (data not shown), increased the severity of the canA^mt6 rough eye (cf. Figure 1F to 1B).

Because expression occurs throughout the later stages of eye development, glass-dependent transgenes can affect many different processes. On the basis of our observations, activated calcineurin may have multiple effects.

### Table 2

Map location and classification of canA^mt6 modifier groups

<table>
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</tr>
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<tr>
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<td>66D–67E</td>
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</table>

The genetic interactions were scored as N for no effect, S for suppression, and E for enhancement. —, the mapping or cross was not done.

**Figure 3**—Rough eye phenotypes of specific modifier groups isolated in the canA^mt6 screen. SEMs and thin sections are from male flies of the following genotypes: (A and E) w^1118, (B and F) TCAG; (C and G) TCAG/CE3-3^w66, (D and H) TCAG/CS3-1^w61. For comparison, eyes from the parent line are displayed in A and E. The TCAG rough eye (B) had aberrant ommatidial morphology and organization in sections (F). CE3-3 significantly increased the exterior roughness (C) and disruption of ommatidia (G) over TCAG. CS3-1 suppressed both the outer rough eye (D) and the ommatidial defects (H) of TGCA.
on the differentiation and morphology of photoreceptor and other cell types (data not shown; see also Figure 3F). However, we did not observe an effect of *canA<sup><seq_pk></seq_pk></sup> on cell proliferation or cell death (data not shown).

**Dominant modifier screen:** The *canA<sup><seq_pk></seq_pk></sup>* rough eye phenotype was modifiable; i.e., it was sensitive to transgene dose and was specifically modified by *canB*, which is essential for *canA* function. Thus, the rough eye was a good candidate for a dominant modifier screen. We prepared an isogenic wild-type stock and inserted *canA<sup><seq_pk></seq_pk></sup>* onto the chromosome 3 balancer TM3 (Figure 1, B and D), which carries the dominant visible marker *Sk*. We screened 70,000 progeny of TM3-*canA<sup><seq_pk></seq_pk></sup>* (TCAG) females and EMS- or X-ray-treated males (Figure 2). Each individual *F<sub>1</sub>* with an enhanced or suppressed TCAG rough eye was backcrossed to TCAG to confirm the modification and to determine the chromosomal location. About 21% of the modifiers initially isolated bred true, and stable lines of the confirmed modifiers were established over either TCAG or the chromosome 2 balancer CCAG (CyO-*canA<sup><seq_pk></seq_pk></sup>*). Because chromosome 2 balancers, including CyO, harbor one or more suppressors of the TCAG phenotype (data not shown), chromosome 2 suppressors were difficult to balance and are thus underrepresented in the final tally. Modifiers on chromosome 1 were also underrepresented, in part because the balancers carry a dominant eye mutation, *Bar*, that significantly interfered with scoring TCAG modification. A total of 5 viable and 123 lethal modifiers were isolated in the screen (Table 1).

Of the 123 lethal modifiers, 62 fell into 11 complementation groups and the rest (61) were single hits (Table 2). Examples of the suppression and enhancement of *canA<sup><seq_pk></seq_pk></seq_pk>* by the complementation groups are illustrated with SEMs and sagittal eye sections (Figure 3). Compared to TCAG alone (Figure 3, B and F), *CE3-3* (calcineurin enhancer, chromosome 3, group 3)/TCAGB eyes had increased exterior roughness (Figure 3C) and increased disruption of the number and organization of the photoreceptor and support cells (Figure 3G). *CS3-1* (calcineurin suppressor, chromosome 3, group 1) was the largest suppressor group, and both SEMs and eye sections of *CS3-1*/TCAG alleles (Figure 3, D and H and data not shown) revealed that, as expected, it reverted the TCAG phenotype toward wild type (Figure 3, A and E). Other suppressor groups had similar effects on the TCAG rough eye (Figure 5 and data not shown).

Modifier groups that act directly on the glass enhancer do not specifically modify *canA<sup><seq_pk></seq_pk></seq_pk>*. Nonspecific groups were identified by testing whether any of the complementation groups modified rough eyes caused by unrelated, glass-dependent transgenes. Two of the enhancer groups, *CE3-1* and *CE2-1*, modified rough eye phenotypes caused by other glass-dependent transgenes, such as *sina<sup>el</sup>* or *yam<sup>el</sup>* and *reaper glass* (data not shown), but only *CE3-1* and *CE2-1* modified the rough eye phenotypes caused by these transgenes.

We further separated the specific modifier groups into two classes by determining whether they modified the *canA<sup><seq_pk></seq_pk></seq_pk>* rough eye phenotype caused by TCAGB (TM3-*canA<sup><seq_pk></seq_pk></seq_pk>, *canB<sup><seq_pk></seq_pk></seq_pk>*). Class I genes, such as Ca<sup>++</sup>* signaling components or dephosphorylation targets, act downstream of calcineurin and will modify the rough eye phenotype of TCAG and TCAGB. However, class II groups, which act at the level of *canB*, such as *canB* factors that regulate its expression, will modify TCAG but not TCAGB. Only two groups, *CE3-3* and *CS2-1*, failed to modify TCAGB (Table 2). Class I and class II modifier groups were mapped by meiotic recombination and by failure to complement deficiencies. The results from both methods were used to estimate the cytological map position of each group (Table 2).

*Suppressor group CS2-1 is canB2:* Meiotic mapping localized the class II group *CS2-1* to 44A;50B, and deficiencies refined the region to 42B3;43E18. Polytenic chromosome analysis revealed a large deficiency in *CS2-1* that uncovered 43E6;44B1 (Figure 4). *CS2-1* was an inversion with breakpoints at 43A1-2 and 43E13-18 (Figure 4). The left breakpoint of *CS2-1* fails to complement two independent alleles of *pk*, a gene in 43A1 that is required for tissue polarity in the wing, haltere, and notum (Gubb et al. 1999). However, the other *CS2-1* alleles complemented *pk* alleles, the *pk* mutation in *CS2-1* was viable, and *pk* did not modify TCAG (data not shown). The right inversion breakpoint, 43E13-18, was lethal when uncovered by deficiencies in the 43E region, and these deficiencies also failed to complement other...
CS2-1 alleles. Thus, the right breakpoint of the CS2-1° inversion corresponds to the CS2-1 TCAG suppressor.

One of the canB genes, canB2, maps to 43E16 and was a strong candidate for CS2-1. The CS2-1° canB2 deficiency uncovered canB2, since CS2-1°/CyO, cn flies were cn°, indicating that the deficiency breaks to the left of cn. PCR on genomic DNA from homozygous CS2-1° flies revealed that the right breakpoint of the insertion occurred between base pair positions −452 and +60, relative to the canB2 start of transcription (Figure 4, data not shown). The gene on the left side of the breakpoint, cn, was not disrupted in CS2-1°, because CS2-1°/CyO, cn flies were cn°. Additionally, Western blots of homozygous CS2-1°, CS2-1°, and CS2-1° larvae that were probed with canB antibodies revealed that, compared to similarly staged controls, total canB protein levels were reduced in CS2-1° alleles (data not shown).

Rescue was carried out by using the UAS-GAL4 system (Brand and Perrimon 1993) on CS2-1°, which had no detectable lesions aside from canB2 and had a late larval/pupal lethal stage (data not shown). CS2-1°/CyO;GAL4° flies were crossed to CS2-1°/CyO;canB-4FEx5; and the GAL4°/canB-4FEx5 progeny were screened for CS2-1° (i.e., Cy°) animals. Heat shock was not used because basal GAL4° activity at 25°C induces UAS transgenes at a low level (data not shown). In three independent crosses, the percentage of CS2-1° adults was increased from <0.2% to an average of 11% (data not shown). Thus, ectopic canB-4F successfully rescued the lethality associated with CS2-1°.

Suppressor CS3-3 is sprouty: Deficiency mapping localized CS3-3 (Figure 5) to 63C6;63E, and the X-ray allele CS3-3° had a deletion spanning 63C2;5;63E1-4 (data not shown). The EMS allele CS3-3° (Figure 5, B and E) failed to complement sty° and sty° (Hacohen et al. 1998), which are hypomorphic alleles of sprouty, a gene in 63D1. In addition, the sty alleles were able to suppress TCAG (data not shown). Sequencing the sty ORF from CS3-3° revealed that the codon corresponding to glutamine residue 250 was mutated into a stop codon (Figure 5F). This mutation removes one of the two sty homology domains and the cysteine-rich region (Hacohen et al. 1998) and is predicted to be nonfunctional.

Sprouty is a negative regulator of RTK signaling in Drosophila, including EGF receptor and EGF receptor signaling (Reich et al. 1999). Sprouty protein can bind to the E3 ligase cbl (Wong et al. 2001), the adaptor protein Drk, and Gap1 and has been proposed to facilitate Gap1 inactivation of Ras (Casci et al. 1999). A single gene in flies, sprouty has at least five homologs in mammalian genomes.

Calcineurin and EGF receptor signaling: Increased signaling through the EGF receptor, caused by either the presence of ectopic Egrf/Ras signaling components or hypomorphic mutations in negative regulators, results in the development of extra photoreceptors and wing vein material (Karim and Rubin 1998; Reich et al. 1999). Conversely, a decrease in EGF receptor signaling reduces both wing vein formation and the number of photoreceptor cells (Karim and Rubin 1998; Reich et al. 1999). Ectopic canA° caused defects in eye and wing vein development consistent with repression of Egrf/Ras signaling. Misexpression of canA° in the posterior compartment of the developing wing by using GAL4° resulted in a truncation of wing veins and a decrease in compartment size (Figure 6, A and B). GAL4° drives expression in the presumptive R3, R4, and R7 photoreceptor cells, as well as in cone cells. Sections of GAL4°/canA° Ex5 eyes revealed a decrease in the number of
Calcineurin Inhibits Egfr Signaling

Figure 6.—Ectopic expression of canA act in eye and wing discs suppresses photoreceptor and wing vein formation. The wings (A and B) and eye sections (C and D) displayed are of the following genotypes: (A) GAL4 en; (B) GAL4 en canA act UAS; (C) GAL4 sev; (D) GAL4 sev canA act UAS. Compared to the control (A), expression of canA act in the posterior wing compartment by GAL4 en (B, bracket indicates area of expression) reduced compartment size and inhibited vein formation (B, arrowheads). The expression of canA act UAS in R cells 3, 4, and 7 by GAL4 sev reduced the number of R cells per ommatidium by one or two at 8% penetrance (D). Examples of ommatidia lacking R cells are circled.

TABLE 3
Genetic interactions between RTK signaling components and activated calcineurin

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Two independent activated calcineurin lines were crossed to each allele, and the results were scored as N for no effect, S for suppression, and E for enhancement. The + and − marks score the relative strength of the interaction. —, the cross was not done. References for published alleles are given in MATERIALS AND METHODS.
phic mutations in Egfr, Ras, pnt, sty, Gap1, and sl did (Figure 7). TCAGB (Figure 7A) was enhanced by removing one copy of Egfr, Ras, or pnt (Figure 7, B–D) and was suppressed by Gap1 and sl (Figure 7, E and F). Both TCAGB and TCAG suppressed the rough eye caused by hypermorphic Egfr alleles: flies that have one copy of Egfr2 (Figure 7G) and TCAGB (Figure 7A) have a rough eye that closely resembles that of TCAGB alone (Figure 7H). TCAG was not detectably modified by hypomorphic Egfr, Ras, or pnt alleles (data not shown). Aside from CS3-3, none of the modifier groups corresponded to Egfr receptor/Ras signaling components that genetically interacted with TCAG (data not shown). However, it is possible that these genes are present among the 61 single hits, which have not been characterized. We also tested for genetic interactions with other signaling pathways by crossing to two Rho1 alleles, as well as mutants in components of the Notch and wingless pathways, but no convincing interactions with TCAGB or TCAG were detected (data not shown).

**DISCUSSION**

**Specificity of activated calcineurin in Drosophila development:** Several lines of evidence demonstrate that phosphatase activity is required for ectopic canAact phenotypes in Drosophila. Genetically raising or lowering the level of canB, which is essential for activity, respectively enhanced or suppressed the phenotype of canAact. Transgenic flies expressing a form of canAact that lacks an intact canB-binding site were indistinguishable from wild-type flies. Finally, the full-length phosphatase, which is inactive in the absence of Ca²⁺-calmodulin, did not have a detectable phenotype when overexpressed throughout development.

Activated calcineurin has been used reliably to identify physiologically relevant functions of calcineurin in a number of systems (Fruman et al. 1995; Mendoza et al. 1996; Molkentin et al. 1998; Winder et al. 1998). However, it remains formally possible that calcineurin does not normally function in the cells or at the stage of development in which canAact has a detectable phenotype, even though Pp2B-14D appears to be ubiquitously expressed. The role of calcineurin must be confirmed by mutational analysis of the canA and canB genes. Despite this caveat, the genetic screen presented here can still be used to identify physiological targets of calcineurin in Drosophila, as well as to provide insight into the roles calcineurin may play in development.

**Activated calcineurin A modifier screen:** The canAact screen yielded 11 complementation groups, 9 of which failed to modify rough eyes caused by other glass-induced transgenes. This demonstrates that the majority of our modifier groups do not act through the glass enhancer. We then divided the nine specific modifiers into class I genes, which act downstream of calcineurin, and class II genes, which act at the level of canB.
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Consistent with this classification, the class II group CS2-1 is canB2. The allele CS2-1\(^{100}\) had an inversion that breaks within 400 bp of the canB2 start of transcription. CS2-1\(^{97}\) and CS2-1\(^{100}\) had decreased protein levels compared to similarly staged controls. Finally, the lethality of CS2-1\(^{100}\) was partially rescued by canB-4F. The ability of canB-4F to rescue the canB2 lesion suggests that the canB-4F protein can at least partially substitute for canB2. More importantly, isolation of the calcineurin regulatory subunit in the canA\(^{alt}\) modifier screen demonstrates that the screen is capable of identifying genes that are required for calcineurin function.

The class I modifier group CS3-3 failed to complement the hypomorphic sprouty alleles sty\(^{A}\) and sty\(^{A64}\); both sty\(^{A}\) and sty\(^{A64}\) also suppressed canA\(^{alt}\); and the sty gene from CS3-3\(^{17}\) harbored a nonsense mutation (Q250Stop). Therefore, we conclude that the CS3-3 complementation group is sprouty. The fact that sty falls into the class II group suggests that sprouty functions downstream of calcineurin and/or in a parallel pathway.

Cross-talk between calcineurin and Egf receptor signaling: Two lines of evidence suggest that calcineurin is a negative regulator of Egf receptor/Ras signaling. First, a negative regulator of RTK signaling, sprouty, was isolated as a suppressor of the canA\(^{alt}\) rough eye phenotype in the dominant modifier screen. Both sprouty and canA\(^{alt}\) suppressed wing vein formation and reduced the number of photoreceptor cells per ommatidium (Casci \textit{et al.} 1999). Egf receptor/Ras signaling is essential for both wing vein and R-cell formation.

A thorough examination of genetic interactions between canA\(^{alt}\) and components of RTK and other signaling pathways confirmed that canA\(^{alt}\) specifically represses the Egf receptor/Ras pathway and that it acts upstream in the pathway. The lack of convincing genetic interactions with other signaling pathways in the imaginal eye disc does not rule out a role for calcineurin in these pathways in other developmental contexts. With the exception of pnt, activated calcineurin was not modified by components downstream of Ras and was modified only by a subset of genes that act between the Egf receptor and Ras. While Gap1 and sty alleles modified activated calcineurin, drk and clb did not. Thus, calcineurin may act downstream of, or parallel to, drk and clb. The more downstream components of the Ras/MAP kinase pathway may not interact with activated calcineurin because they are too far removed from the point(s) of intersection between calcineurin and the pathway. Alternatively, these components may not be limiting, so that reduction of gene dose, which is the basis of a dominant modifier screen, would have no appreciable effect.

The hypermorphic allele Egf\(^{E1}\) inhibits Ras signaling (Baker and Rubin 1989); thus it might be expected to enhance activated calcineurin. However, low levels of inappropriate Egf receptor activity in eye development are thought to increase secretion of the Egf receptor antagonist argos (Lesokhin \textit{et al.} 1999). The argos protein inhibits subsequent Egf receptor signaling that is required for photoreceptor determination. Thus, suppression of the Egf\(^{E1}\) rough eye by canA\(^{alt}\) may be the result of activated calcineurin inhibiting inappropriate Egf receptor signaling.

Consistent with our findings, PLC\(\gamma\) is a negative regulator of Egf receptor/Ras signaling in eye and wing development (Thackeray \textit{et al.} 1998; Powe \textit{et al.} 1999). However, PLC\(\gamma\) was identified in this study as a strong suppressor of activated calcineurin, although biochemically PLC\(\gamma\) has been placed upstream of calcineurin in the PI signaling pathway. One explanation is that PLC\(\gamma\) acts on one of the other canA genes. Another possibility is that the signaling pathways activated by PLC\(\gamma\) parallel to calcineurin are required for calcineurin function.

In a recent model, PLC\(\gamma\) was proposed to inhibit Egf
receptor/Ras signaling via the activation of Gap1 by InsP3 (Powe et al. 1999). Our results suggest that PLCγ is also acting through calcineurin. The genetic evidence that we present indicates that calcineurin intersects with the Ras pathway at roughly the same point that PLCγ does, and thus we propose a modified model for the function of PI signaling in Drosophila development (Figure 8). Additionally, the fact that calcineurin can be activated by any sustained Ca²⁺ flux suggests a mechanism by which other signaling pathways, such as GPCRs acting via PLCβ, can modulate Egf receptor signaling.

In conclusion, we have demonstrated that a dominant modifier screen can be used successfully to isolate mutations in genes involved in calcineurin function. The mutations in the calcineurin B gene that we isolated in the screen will help determine the roles of calcineurin in Drosophila development. In addition, we have obtained compelling genetic evidence that calcineurin negatively regulates the Egf receptor/Ras signaling pathway at the level of Gap1 and sproutry. Calcineurin may act directly by dephosphorylating one or more signaling components, or it may target a transcription factor and act indirectly through changes in gene expression. More work will be needed to elucidate the molecular mechanism, and the modifiers isolated in the conαes8 screen should prove valuable in this endeavor. Furthermore, given the conservation of signal transduction between fruit flies and vertebrates, it is likely that the signaling network we have identified is employed in other organisms.

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