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corkscrew Encodes a Putative Protein Tyrosine Phosphatase That Functions to Transduce the Terminal Signal from the Receptor Tyrosine Kinase torso

Lizabeth A. Perkins,* Inger Larsen,*† and Norbert Perrimon*†
*Department of Genetics
†Howard Hughes Medical Institute
Harvard Medical School
Boston, Massachusetts 02115

Summary

We describe the characterization of the Drosophila gene, *corkscrew* (*csw*), which is maternally required for normal determination of cell fates at the termini of the embryo. Determination of terminal cell fates is mediated by a signal transduction pathway that involves a receptor tyrosine kinase, torso, a serine/threonine kinase, D-raf, and the transcription factors, tailless and huckebein. Double mutant and cellular analyses between *csw, torso, D-raf*, and *tailless* indicate that *csw* acts downstream of *torso* and in concert with *D-raf* to positively transduce the *torso* signal via *tailless*, to downstream terminal genes. The *csw* gene encodes a putative nonreceptor protein tyrosine phosphatase covalently linked to two N-terminal SH2 domains, which is similar to the mammalian PTP1C protein.

Introduction

The establishment of cellular identities along the anterior–posterior axis of the Drosophila embryo is under the control of three groups of maternally expressed genes (Nüsslein-Volhard et al., 1987; St. Johnston and Nüsslein-Volhard, 1992). Patterning of the gnathal and thoracic regions is under the control of the "anterior" genes, which establish a morphogenetic gradient of bicoid protein (Driever and Nüsslein-Volhard, 1988a, 1988b). Abdominal segmentation is under the control of the "posterior" genes, in which localization of nanos activity defines a region where posterior body pattern can be specified by other morphogens (Hulskamp et al., 1989; Irish et al., 1989; Struhl, 1989). An activity encoded by the "terminal" genes is required for the formation of the head (acron) and tail (telson) (reviewed by Nüsslein-Volhard et al., 1987; St. Johnston and Nüsslein-Volhard, 1992).

The terminal genes contain six maternal members, *Nasrat* (Degelmann et al., 1986), *D-raf* or *l(1)pole hole* (Perrimon et al., 1985; Ambrosio et al., 1989b), *fs(1)pole hole* (Perrimon et al., 1986), *trunk, torso* (Schupbach and Wieschaus, 1986), and *torso-like* (Nüsslein-Volhard et al., 1987). When any one of these gene products are absent in the female germline, similar maternal-effect defects on embryonic development are observed; i.e., embryos with both anterior and posterior terminal deletions. Posteriorly, these deletions encompass from 0%–20% egg length or all structures posterior to abdominal segment 7. Anteriorly, deletions are harder to analyze because of interactions between terminal and anterior genes, but missing are regions of the head skeleton, supraesophageal ganglia, and optic lobes. Two of the maternal terminal genes have been molecularly characterized. *torso* encodes a putative transmembrane receptor tyrosine kinase (Casanova and Struhl, 1989; Sprenger et al., 1989) and *D-raf* encodes a putative serine/threonine kinase homologous to the human *raf-1* proto-oncogene (Mark et al., 1987; Nishida et al., 1988). Neither *torso* RNA or protein nor *D-raf* RNA are spatially restricted to the embryonic poles (Ambrosio et al., 1989a; Casanova and Struhl, 1989; Sprenger et al., 1989).

Dominant *torso* alleles exist that give rise to embryos that lack thoracic and abdominal structures, but retain terminal structures (Klingler et al., 1988; Strecker et al., 1989). Genetic epistasis experiments using the *torso*^{Dominant} alleles have allowed the maternal terminal genes to be positioned within a developmental pathway of action where all maternal genes except *D-raf* function upstream of *torso*. The *torso-like* gene product has been shown by mosaic analysis to function in the follicle cell epithelium during oogenesis and has been postulated to be involved in the production of the putative torso ligand (Stevens et al., 1990) that is believed to trigger localized activation of torso at the egg poles.

Localized activation of torso ultimately controls the transcriptional regulation of the zygotic gap genes *tailless* (*tll*) and *huckebein* (*hkb*). *tll* is expressed in cells at both embryonic poles and encodes a putative transcription factor of the steroid receptor superfamily (Pignoni et al., 1990). *hkb* encodes a putative transcription factor with multiple zinc fingers and is also expressed at the embryonic poles (Bronner and Jäckle, 1991; H. Jäckle, personal communication). Localized expression of tll and hkb activate and/or repress further downstream terminal-specific genes. For example, the posterior expression patterns of the gap gene *hunchback* (*hb*), the homeotic gene *forkhead* (*fkh*), and the pair-rule gene *fushi tarazu* (*ftz*) have been shown to be mediated by both *tll* and *hkb* gene activities (Mlodzik et al., 1987; Tautz, 1988; Weigel et al., 1990).

A current model (reviewed by Klingler, 1990; Siegfried et al., 1990; Perkins and Perrimon, 1991; St. Johnston and Nüsslein-Volhard, 1992) suggests that the maternal genes act in a signal transduction pathway such that activation of the torso receptor tyrosine kinase at the termini would initiate a phosphorylation cascade mediated through D-raf and ultimately affecting the transcription of the zygotic transcription factors tll and hkb.

The establishment of cell fates at the termini of the embryo provides a unique genetic system in which to dissect a signal transduction pathway. Here, we provide genetic and molecular evidence that the gene *corkscrew* (*csw*) is a member of the terminal class signal transduction pathway. Our genetic and developmental analyses suggest that *csw* acts in concert with *D-raf* and functions as a positive transducer of the *torso* terminal signal. The *csw* gene encodes a putative nonreceptor protein tyrosine phosphatase covalently linked to two N-terminal Src homology (SH) 2 do-

Table 1. Origin of *csw* Mutations

Allele	Origin	Reference
csw^{VA199}	ethylmethane sulfonate	Perrimon et al. (1985)
csw^{C114}	X-ray	Perrimon et al. (1985)
csw^{13-87}	Spontaneous	This report
$csw^{e13d.3}$	ethylmethane sulfonate	This report
csw^{19-106}	Spontaneous	This report
csw^{6}	ethylmethane sulfonate	This report

mains. While the tyrosine phosphatase domain implies an enzymatic function for csw in transducing the torso signal, the presence of the Src homology 2 (SH2) domains suggests that csw effects its role by mediating heteromeric protein interactions.

Results

csw Is a Terminal Class Gene

csw is an X-linked locus that maps to chromosomal bands 2D3–4 (Perrimon et al., 1985). All six *csw* mutations (Table 1) characterized to date are required for zygotic viability, since individuals hemizygous or homozygous for all *csw* alleles die during pupal stages. In addition to its zygotic role, *csw* is required maternally, since embryos derived from females lacking *csw* activity during oogenesis die during embryogenesis. Externally, these embryos, which we will refer to as *csw* mutant embryos, though twisted or U-shaped appear like wild type (Figure 1B). However, *csw* mutant embryos show abnormal development of their internal terminal structures that include disruption anteriorly of the cephalopharyngeal skeleton and dorsal bridge and posteriorly of the posterior midgut and malpighian tubules (data not shown). Four of the *csw* alleles behave genetically as nulls; while two others have residual activity (see Experimental Procedures). In this paper, all the analyses were performed using the genetically null alleles.

Since *csw* mutant embryos show defects at the termini of the embryo, we examined the expression of the downstream zygotic genes required for tail development, *tll*, *hb*, *ftz*, and *fkh*, in *csw* mutant embryos. At the syncytial blastoderm stage in wild-type embryos, *tll* is expressed symmetrically from 0%–20% and 80%–100% egg lengths. This pattern evolves by the cellular blastoderm stage into smaller domains with the posterior domain extending from 0%–15% egg length (Pignoni et al., 1990) (see Figure 3A). In *csw* mutant embryos (see Figure 3B), at both the syncytial and cellular blastoderm stages, the posterior *tll* domain is severely reduced relative to wild type.

We also examined the expression patterns of the genes *hb, fkh*, and *ftz* (Figure 2 and Figures 3E and 3F), which have been shown to be under the control of both of the zygotic transcription factors tll and hkb (Casanova, 1990; Weigel et al., 1990). At blastoderm stages, wild-type embryos zygotically express hb in anterior and posterior domains, with the posterior expression beginning as a cap at the posterior pole. This cap subsequently disappears from the pole, due to repression from the *hkb* gene (Casanova, 1990), leaving a stripe from 10%–20% egg length

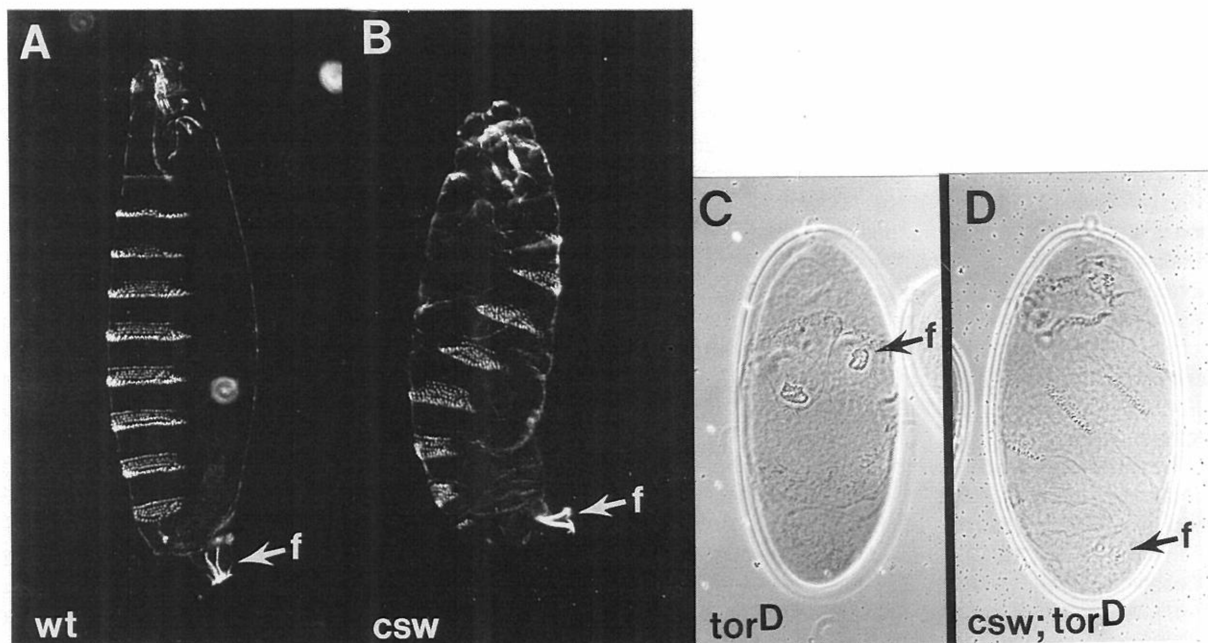


Figure 1. csw Suppresses the *torso*^{Dominant} Phenotype

(A and B) are dark field photographs of cuticular preparations of wild type (wt) (A); and embryos derived from csw^{C114} germline clones (csw) (B). (C and D) are phase contrast photographs of embryos derived from homozygous tor^{RL3} females (tor^{D}) at 25°C (C) and homozygous csw^{C114}; tor^{RL3} female germ cells (csw;tor^{D}) at 25°C (D). The embryos in (B) and (D) show the csw phenotype, and in (C) the tor^{D} or spliced phenotype is observed. Abbreviation: f, filzkorper.

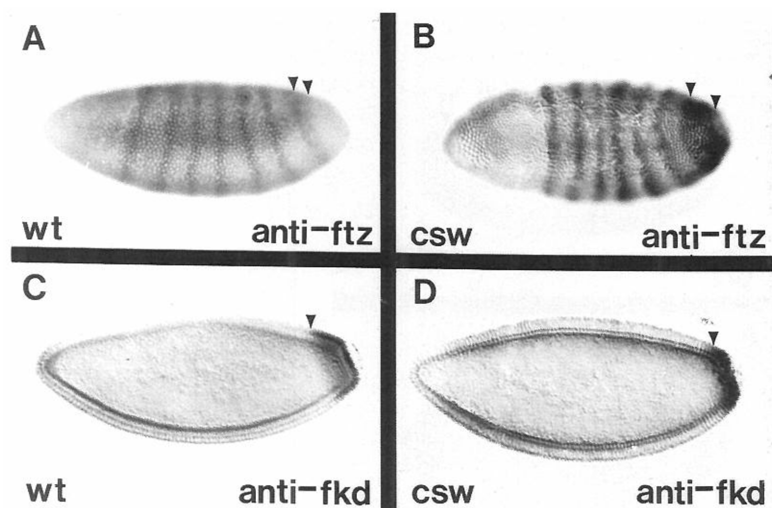


Figure 2. Zygotic Genes *ftz* and *fkh* Are Misexpressed in *csw* Mutant Embryos

Blastoderm stage embryos with *ftz* and *fkh* expression patterns revealed in wild-type embryos (A and C) and *csw* mutant embryos derived from females bearing germline clones (B and D). (A and B) ftz expression pattern; note that in the *csw* mutant embryo, the seventh ftz stripe is expanded posteriorly. (C and D) fkh protein expression pattern; note that in the *csw* mutant embryo, fkh expression is reduced posteriorly. Arrowheads indicate the anterior and posterior limits of staining at the posterior end of the embryos.

(Lehmann and Nüsslein-Volhard, 1987; Tautz et al., 1987; Tautz, 1988) (Figure 3E). In *csw* mutant embryos, hb does not retract from the pole, but rather remains as a terminal cap (Figure 3F). This altered pattern of hb expression is like that observed in *hkb* mutant embryos where hb continues to be expressed at the pole due to lack of the *hkb* repressing activity (Casanova, 1990). In wild-type blastoderm embryos, fkh is expressed from both 0%–13% and 94%–100% egg lengths (Weigel et al., 1989) (see Figure 2C). However, and again as in *hkb* mutant embryos (Weigel et al., 1990), in *csw* mutant embryos, the posterior *fkh* domain is reduced posteriorly (see Figure 2D). In wild-type blastoderm embryos, ftz is expressed in seven evenly spaced stripes of cells along the anterior–posterior axis, with the seventh stripe positioned between 10% and 20% egg length (Hafen et al., 1984; Figure 2A). In *csw* mutant embryos, the seventh ftz stripe, while present, is expanded posteriorly (Figure 2B). This effect, though similar to that of *hkb* mutant embryos (Weigel et al., 1990), is more extreme; i.e., the seventh stripe appears expanded further posterior than in *hkb* mutant embryos.

Taken together, these data suggest that maternally provided *csw* positively affects the activity of *tll*, as well as further downstream genes of the terminal system. We conclude that *csw* is required maternally and functions in the terminal system upstream of the zygotic gap gene *tll*.

csw Acts Downstream of *torso*

To analyze whether *csw* activity is required upstream or downstream of the membrane-bound *torso* activity, we tested whether the effect of an hyperactive *torso* allele can be negated by mutations in csw. If *csw* is required to transduce the torso signal, then absence of *csw* should suppress a torso gain-of-function phenotype. We utilized the dominant, temperature-sensitive *torso* allele, tor^{RL3}, where two copies are required to produce the $torso^{Dominant}$, or "spliced," phenotype (Klingler et al., 1988; Strecker et al., 1989) (see Figure 1C). tor^{RL3} homozygous females that carry *csw* germline clones produced only eggs that failed to hatch. Cuticle preparations of these embryos revealed only cuticles with the *csw* phenotype (see Figure 1D), indicating that lack of *csw* activity suppresses the tor^{RL3} dominant phenotype. We conclude that *csw* is a member of the terminal class signal transduction pathway and functions downstream of the receptor tyrosine kinase, torso.

csw Acts in Concert with *D-raf*

Like *csw*, only one other maternally required terminal gene, *D-raf*, has been shown by genetic epistasis experiments to act downstream of *torso* (Ambrosio et al., 1989b). In *D-raf* null alleles, unlike *csw* null alleles, *tll* expression is entirely missing posteriorly (data not shown). There exist *D-raf* hypomorphic alleles (e.g., $D\text{-}raf^{PB26}$) where *tll* expression is severely reduced (Figure 3C). To determine whether all terminal activity in *csw* null alleles could be deleted by reducing *D-raf* activity, we examined a variety of single and double mutant combinations of *csw* and *D-raf* alleles. A complete description of these experiments will be presented elsewhere (L. A. P. and N. P., unpublished data); however, for all allelic combinations tested, results paralleling those shown in Figure 3 are observed. In embryos derived from germlines mutant for both the *csw* null allele, csw^{VA199}, and the hypomorphic *D-raf* allele, $D\text{-}raf^{PB26}$, *tll* is entirely missing posteriorly (Figure 3D). This observation in the double mutant, where *tll* expression is less than in either single mutant alone, suggests that *csw* and *D-raf* act in concert to regulate *tll* expression (see Discussion for possible models). The same effect is observed when other terminal downstream genes, *hb* (Figures 3E–3H), *fkh*, and *ftz* (data not shown) are used as markers of terminal activity.

Cloning of the csw Locus

The *csw* locus resides in a genetically defined region next to the gene *polyhomeotic* (*phm*) (Perrimon et al., 1985). Two deficiencies, *Df(1)JA52* and $Df(1)pn^{38}$, delimit *csw* and *phm* from adjacent complementation groups and further mapping experiments (data not shown) indicate that *csw* is located distally to *phm*. A 235 kb chromosomal walk of overlapping phage (Dura et al., 1987) was generated in

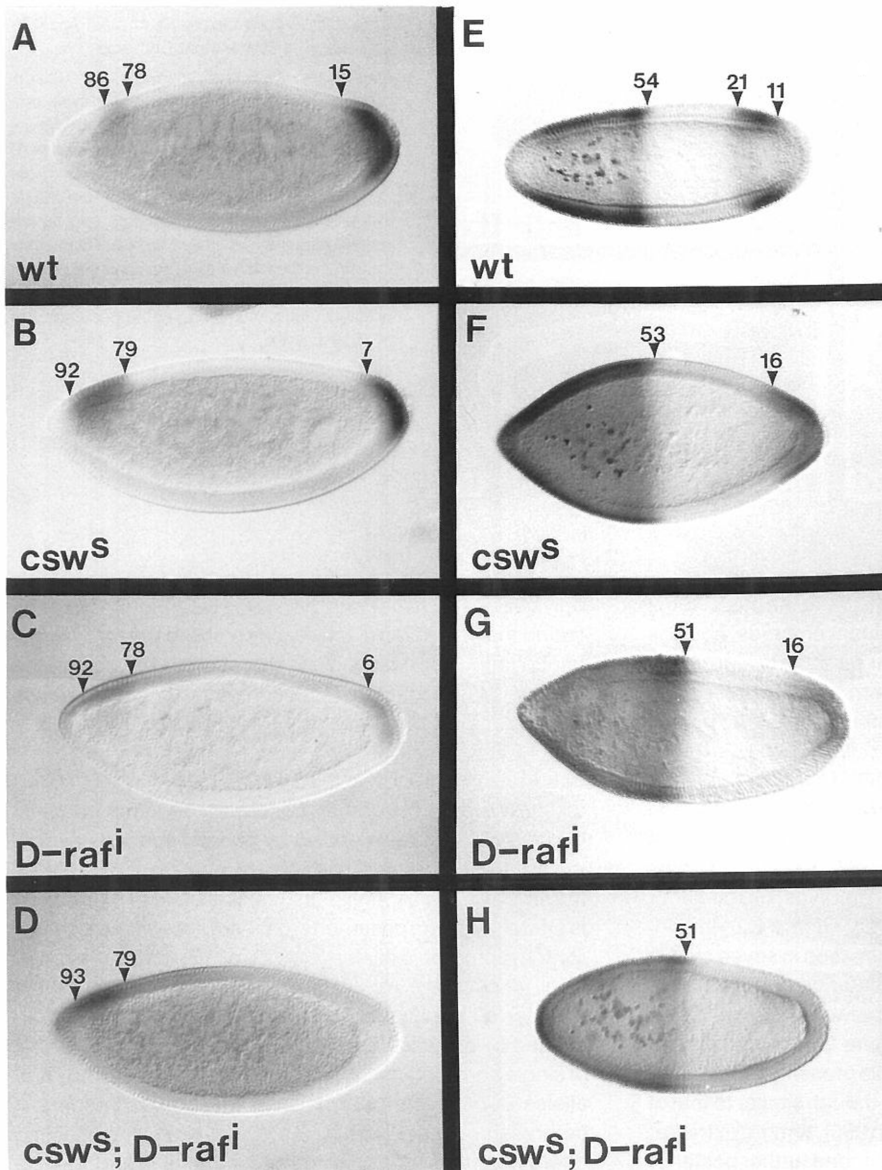


Figure 3. *csw* and *D-raf* Act in Concert to Transduce the Terminal Signal

Shown are blastoderm stage embryos that reveal the *tll* RNA (A, B, C, and D) and hb protein (E, F, G, and H) expression patterns. The *csw*^{s} allele is *csw*^{VA199} and the *D-raf*^{i} allele is *D-raf*^{PB26}. Numbers refer to percent egg length. (A) In wild type at the cellular blastoderm stage, *tll* is expressed, at the posterior, from 0%–15% egg length. (B and C) In either single mutant, posterior *tll* expression is reduced. (D) In the double mutant combination, posterior *tll* expression is completely deleted. Note that anteriorly in the double mutant combination, like each single mutant, *tll* expression is expanded anteriorly (a complete description of this anterior expansion will be presented elsewhere (L. A. P. and N. P., unpublished data). A similar synergistic or additive effect is observed when hb is used as the reporter for terminal activity. (E) In wild type at the cellular blastoderm stage hb is initially expressed, at the posterior, as a polar cap that resolves itself into a posterior stripe from 10%–20% egg length. (F and G) In either single mutant, posterior hb expression remains a polar cap that does not resolve into a posterior stripe; furthermore, the anterior border of this posterior hb domain is shifted posteriorly. (H) In the double mutant combination, posterior hb expression is completely deleted. Note that no single or double mutant combination affects the anterior domain of hb expression, whose posterior border of expression remains between 51% and 54% egg length.

the effort to clone the *phm* locus. From this we obtained DNA (kindly provided by Dr. H. Brock) from the genomic region in which *csw* was believed to reside. We utilized this DNA to isolate further overlapping λ EMBL3 clones encompassing the approximately 45 kb of genomic DNA distal to the *phm* locus (Figure 4).

We generated Southern blots of genomic DNA from wild type, as well as each of the *csw* alleles and the deficiences in the region. From these, we identified along our walk restriction fragment-length polymorphisms for one *csw* allele, *csw*$^{19-106}$ and confirmed the location of the distal *Df(1)pn*^{38} breakpoint (Dura et al., 1987) (Figure 4).

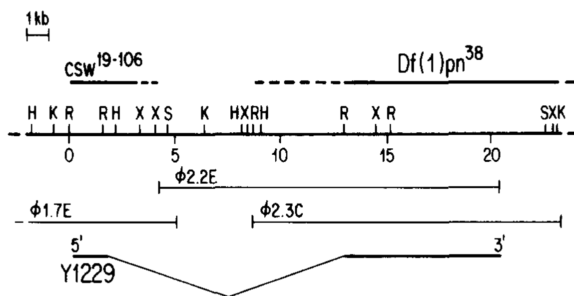


Figure 4. The Molecular Organization of the *csw* Gene and Known DNA Rearrangements

Shown is a molecular map of the genomic DNA known to encompass the *csw* gene. Indicated above the map are the locations of rearranged DNA in the *csw* allele, $csw^{19\text{-}106}$, and the deficiency $Df(1)pn^{38}$. For $csw^{19\text{-}106}$, an approximately 3.2 kb deletion, the solid line indicates the genomic fragments known to be partially or completely deleted, and the stippled line indicates uncertainty. For $Df(1)pn^{38}$, the stippled line indicates the fragment bearing the distal breakpoint, and the solid line represents DNA known to be missing. Individual phage used to establish the genomic map are indicated below the molecular map. The *csw* cDNA *Y1229* hybridizes to the genomic fragments indicated by the horizontal line and the direction of transcription is indicated. The enzymes used to establish the restriction map are EcoRI, R; HindIII, H; KpnI, K; SalI, S; and XhoI, X.

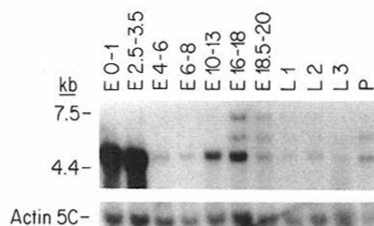


Figure 5. Developmental Northern Analysis of *csw* Expression

Ten micrograms of poly(A)+ RNA, prepared from various developmental stages, was loaded in each lane, electrophoresed, transferred to nitrocellulose, and probed with a ^{32}P-labeled *csw* cDNA. As shown, the amount of RNA loaded per lane is essentially equal, as calibrated by hybridization with a probe from the *actin 5C* gene (Fyrberg et al., 1983). Lanes are marked according to the specific stage: numbers during embryonic stages (E) refer to hours of development after fertilization; larval stages L1, L2, and L3 refer to first, second, and third instar larvae, respectively; and pupae are 0–24 hr after pupation. The most abundant transcript, approximately 4.7 kb, while observed throughout development, is most strongly observed from 0 to 3.5 hr postfertilization. Two less abundant transcripts, of approximately 6.0 and 7.2 kb, appear most prominently during late embryogenesis, but upon longer exposures are observed throughout all larval and pupal developmental stages. Not shown are two low abundance, developmentally regulated transcripts at about 2.8 kb and 1.6 kb.

Reasoning that these molecular lesions were close to, or within, the *csw* gene, we utilized fragments from the region to generate radiolabeled probes to screen for cDNAs from a 0–4 hr embryonic cDNA library (see Experimental Procedures). The longest of the cDNA clones we isolated, *Y1229*, is 4.6 kb in length and hybridized to genomic DNA spanning approximately 20 kb. To test whether *Y1229* represented a cDNA from the *csw* locus, we inserted *Y1229* into a transformation vector 3′ to the inducible *hsp-70* promoter (C. Thummel, personal communication). Germ-line transformation of this construct allowed us to test for rescue of both the zygotic pupal lethality, as well as the maternal effect embryonic lethality. When *csw* mutant flies carrying the P element *P[w,hs-Y1229]* were heat shocked daily for 1 hr at 37°C, starting at early larval stages, the zygotic pupal lethality was rescued (data not shown). Rescue of the maternal effect embryonic lethality was also observed following heat shock of 0–5 hr *csw;P[w,hs-Y1229]* embryos derived from *csw* mutant germ lines (data not shown). These results indicate that cDNA *Y1229* encodes functions sufficient to rescue both the zygotic pupal lethality, as well as the maternal effect embryonic lethality of *csw* mutations.

csw Is Expressed throughout Development

The expression of the *csw* cDNA *Y1229* was examined throughout development by Northern blot analysis and during embryogenesis by in situ hybridization to whole-mount embryos. Developmental Northern blots hybridized with probes from *Y1229* (Figure 5) reveal three major developmentally regulated mRNA species, the most abundant of which, at 4.7 kb, is expressed throughout embryonic, larval, pupal, and adult stages. Two larger transcripts, at 6.0 and 7.2 kb, are first observed during late embryogenesis (Figure 5), but upon longer exposures are also observed throughout larval, pupal, and adult stages (data not shown). *Y1229* likely represents a close to full-length message, since it is similar in size to the major 4.7 kb transcript observed on Northern blots and is also capable of rescuing both maternal and zygotic *csw* functions.

Whole-mount in situ hybridization of embryos with single-stranded sense and antisense *Y1229* probes reveals that *csw* is expressed uniformly throughout all tissues during embryogenesis; i.e., no terminal-specific expression is observed either before, during, or after the blastoderm stages (data not shown).

The *csw* Gene Encodes a Putative Cytoplasmic Protein Tyrosine Phosphatase with Two SH2 Domains

The nucleotide and amino acid sequences (Figure 6) and a schematic representation of the major features of the csw protein (Figure 7A) are shown. The 4,543 bp *csw* cDNA *Y1229* defines an open reading frame encoding an 841 aa protein with a molecular weight of 92.4 kd. The csw protein shows striking homologies with the catalytic domains of known protein tyrosine phosphatases (PTPase proteins) (Krueger et al., 1990; Figure 7C), as well as the noncatalytic SH2 domains (Koch et al., 1991; Figure 7B).

Protein tyrosine phosphatases exist as a diverse family of receptor and nonreceptor enzymes that function to dephosphorylate tyrosyl residues (reviewed by Fischer et al., 1991). All PTPase proteins share a segment of approximately 250 aa, referred to as the core PTPase domain (Figure 7C), within which is a stretch of 8 highly conserved aa (consensus: [I/V]HCXAGXXR[S/T]G) that uniquely identifies the PTPase domain. These conserved amino acids are thought to form the catalytic center, since at

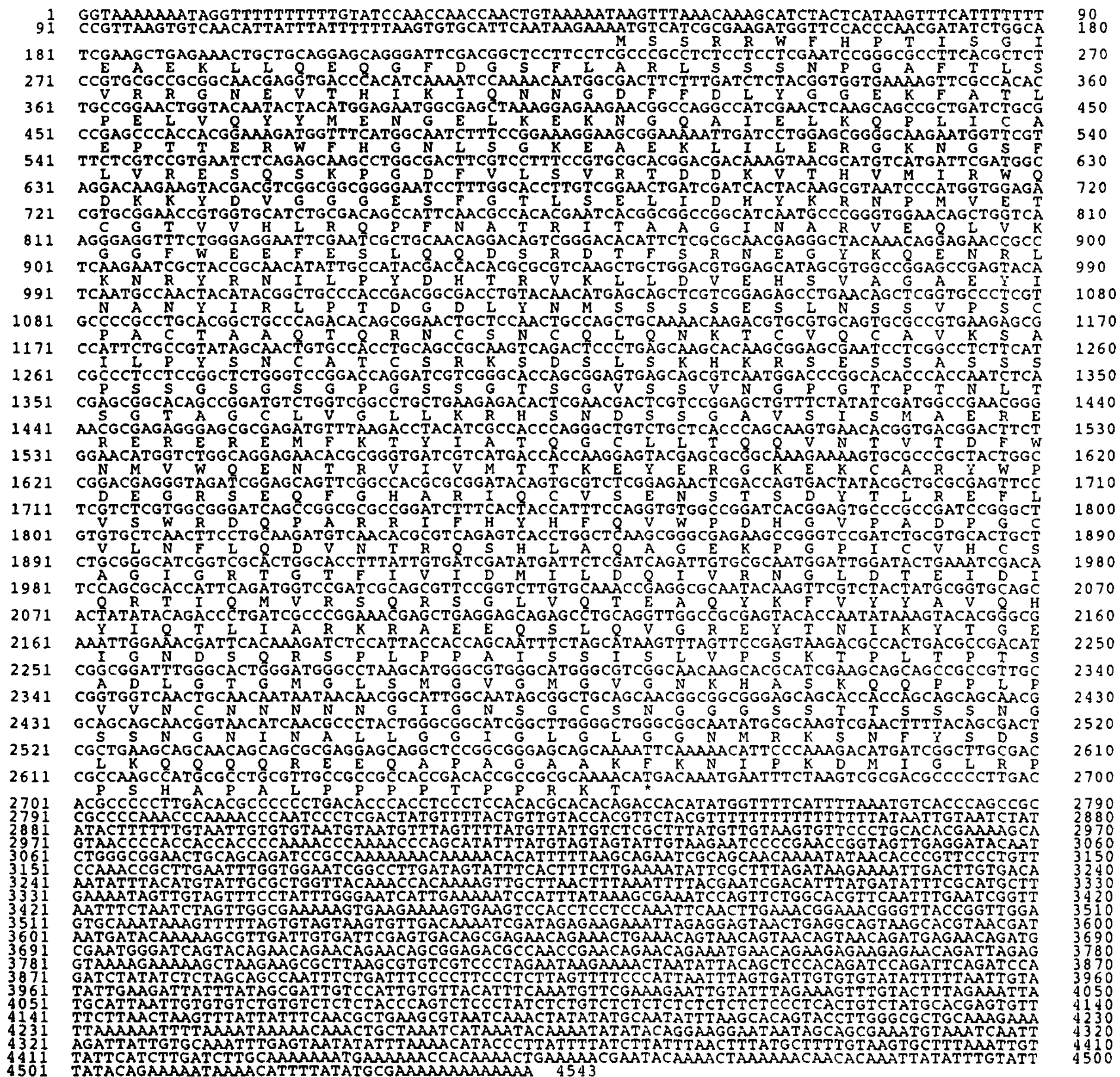


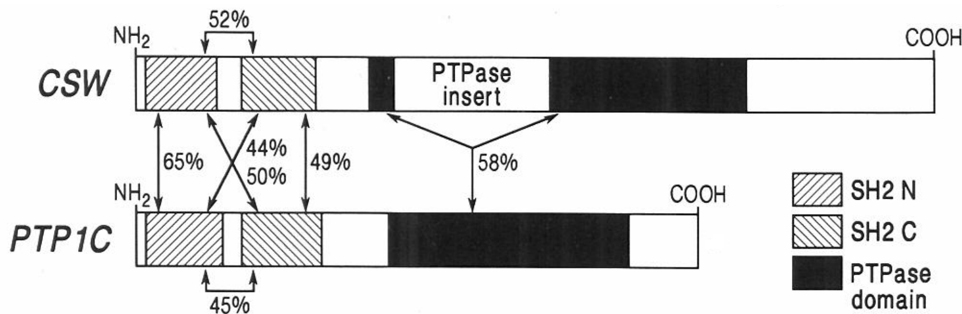
Figure 6. The Nucleotide and Amino Acid Sequences for *csw* cDNA *Y1229*

The nucleotides are numbered from the 5′ end of the cDNA. The nucleotides preceding the initiating methionine are a good match to the consensus for translation initiation (Cavener, 1987). Good Drosophila codon usage was observed throughout the length of the open reading frame. Amino acid sequences are indicated under the nucleotide sequence using the standard one letter code.

most of these positions, any substitution severely reduces enzymatic activity; furthermore, two residues, cysteine (C) and arginine (R), were found to be absolutely essential for enzyme activity (Streuli et al., 1990). All residues in the putative catalytic center of the csw protein are conserved. The sequence and hydropathy of the csw protein suggests it is a nonreceptor PTPase.

The csw PTPase domain is most similar to the human nonreceptor PTPase PTP1C, with which it shares 58% identity. The next most closely related PTPase proteins are the receptor forms LCA, or CD45, (Matthews et al., 1990), PTPα and PTPγ (Krueger et al., 1990), which share with csw identity at 47%, 47%, and 46% of amino acid residues in the core PTPase domain, respectively. The csw PTPase domain is unusual among the known PTPase proteins, since it is interrupted by a hydrophilic serine- and cysteine-rich stretch of approximately 150 aa, the PTPase insert (Figures 7A and 7C), which shares no homologies with other PTPase proteins. The spacing of cysteine residues within the PTPase insert is reminiscent of the cysteine-rich domains of Raf and protein kinase C family members as well as human estrogen and glucocorticoid receptors (Bruder et al., 1992). The conservation between the csw core PTPase domain and PTPase domains from

A. Schematic representation of csw and PTP1C proteins



B. Alignment of SH2 domains

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csw N      6 WFHPTISGIEAEKLLQEQG.FDGSFLARLSSSNPGAFTLSVRRGN.........EVTHIKIQN.NGDFFDLYGGEKFATLPELVQYYMEN
csw C    111 WFHGNLSGKEAEKLILERG.KNGSFLVRESQSKPGDFVLSVRTDD.........KVTHVMIRW.QDKKYDVGGGESFGTLSELIDHYKRN
PTP1C N    6 WFHRDLSGLDAETLLKGRG.VHGSFLARPSRKNQGDFSLSVRVGD.........QVTHIRIQN.SGDFYDLYGGEKFATLTELVEYYTQQ
PTP1C C  112 WYHGHMSGGQAETLLQAKG.EPWTFLVRESLSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMC.EGGRYTVGGLETFDSLTDLVEHFKKT
lck      127 WFFKNLSRKDAERQLLAPGNTHGSFLIRESESTAGSFSLSVRDFDQNQGEV....VKHYKIRNLDNGGFYISPRITFPGLHELVRHYTNA
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C. Alignment of core PTPase domains

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                                                          289       444
                                                            PTPase insert
csw    247 GYKQENRLKNRYRNILPYDHTRVKLLDVEH.SVAGAEYINANYI..  ..YIATQGCLLTQQVNTVTDFWNMVWQENTRVIVMTT
PTP1C  267 GQRPENKGKNRYKNILPFDHSRVILQGRDS.NIPGSDYINANYIKNQLLGPDENAKTYIASQGCLEA....TVNDFWQMAWQENSRVIVMTT
PTPα   251 ASKEENKEKNRYVNILPYDHSRVHLTPVEG..VPDSDYINASFI.....NGYQEKNKFIAAQGPKEE....TVNDFWRMIWEQNTATIVMVT
PTPγ    34 SNHPENKHKNRYINILAYDHSRVKLRPLPGKDSKHSDYINANYV.....DGYNKAKAYIATQGPLKS....TFEDFWRMIWEQNTGIIVMIT

csw    479 KEYERGKEKCARYWPDEGRSEQFGHARIQCVSENSTSDYTLREF..........LVSWRDQPA..RRIFHYHFQVWPDHGVPADPGCVLNFL
PTP1C  354 REVEKGRNKCVPYWPEVGMQRAYGPYSVTNCGEHDTTEYKLRTL..........QVSPLDNGDLIREIWHYQYLSWPDHGVPSEPGGVLSFL
PTPα   332 NLKERKECKCAQYWPDQG.CWTYGNIRVSVEDVTVLVDYTVRKFCIQ.......QVGDMTNRKPQRLITQFHFTSWPDFGVPFTPIGMLKFL
PTPγ   117 NLVEKGRRKCDQYWPTEN.SEEYGNIIVTLKSTKIHACYTVRRFSIRNTKVKKGQKGNPKGRQNERVVIQYHYTQWPDMGVPEYALPVLTFV

csw    559 QDVNTRQSHLAQAGEKPGPICVHCSAGIGRTGTFIVIDMILDQIVRNGLDTEIDIQRTIQMVRSQRSGLVQTEAQYKFVYYAVQHYIQTLIA
PTP1C  436 DQINQRQESLPHA....GPIIVHCSAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQMVRAQRSGMVQTEAQYKFIYVAIAQFIETTKK
PTPα   416 KKVKACNPQYA......GAIVVHCSAGVGRTGTFVVIDAMLDMMHT...ERKVDVYGFVSRIRAQRCQMVQTDMQYVFIYQALLEHYLYGDT
PTPγ   208 RRSSAARMPET......GPVLVHCSAGVGRTGTYIVIDSMLQQIKD...KSTVNVLGFLKHIRTQRNYLVQTEEQYIFIHDALLEAILGKET
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Figure 7. Comparison of the csw Protein and Its SH2 and Core PTPase Domains with PTP1C and Other Related Proteins

(A) Schematic representation of csw and PTP1C proteins. The relative positions of the most N-terminal (SH2 N) and C-terminal (SH2 C) SH2 domains, as well as the PTPase core domains are indicated. Note that the PTPase core domain of the csw protein is interrupted by an approximately 150 aa region (PTPase insert) bearing no homology with known PTPase domains. The percent identities between the various regions are shown.

(B) Alignment of SH2 domains. Shown are the amino acid sequences of the SH2 domains from csw, PTP1C, and the next most closely related SH2 domain from the mammalian protein tyrosine kinase lck (Rouer et al., 1989). Residues conserved between csw and either of the other two proteins are in bold type. Invariant residues are indicated by an arrowhead (note in csw SH2 C, residue 125 is not conserved). Basic residues conserved and thought to participate in phosphotyrosine interactions (Koch et al., 1991) are indicated by a diamond. However, this conservation is not maintained in csw or PTP1C at the position marked with an open circle.

(C) Alignment of core PTPase domains. Shown are the amino acid sequences of the PTPase domain from csw, PTP1C, and the next two most closely related PTPase domains from the PTPα and PTPγ proteins (Krueger et al., 1990). Residues shared by csw and at least two other PTPase proteins are shown in bold type. Residues highly conserved and/or invariant among the known PTPase proteins are indicated by an arrowhead. Residues known to be essential for catalytic activity are indicated by a star. The position of the PTPase insert in the csw protein is shown.

organisms as evolutionarily distant as humans suggests that the csw protein functions as a protein tyrosine phosphatase.

The csw protein contains, 5′ to its PTPase core domain, two noncatalytic SH2 domains, which have been found in a number of different proteins that associate with the activated PDGF and/or EGF receptors, including phospholipase C(PLC)-γ1, $p21^{ras}$GTPase-activating protein (GAP), phosphatidylinositol 3′-kinase (PI3K), and Src and Src-like tyrosine kinases (for reviews, see Koch et al., 1991; Cantley et al., 1991). The N-terminal SH2 domains of the csw protein share between 45% and 65% identity with the next most closely related SH2 domains, those from the mammalian phosphatase PTP1C and the mam-

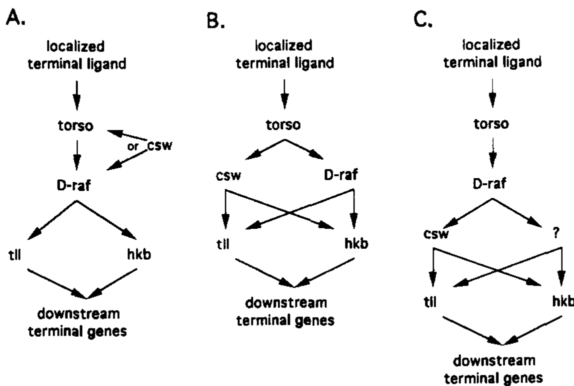

Figure 8. Models for the Role(s) of csw in the Terminal Signal Transduction Pathway

These models of *csw* function include only those components presently known to act in the terminal system, which is undoubtedly far from saturation, so the arrows shown are not meant to imply a direct effect, but simply an epistatic relationship between the terminal class genes. See text for details.

malian protein tyrosine kinase lck (Rouer et al., 1989) (Figures 7A and 7B).

The overall structure of the csw protein is like that of the mammalian PTPase PTP1C (Shen et al., 1991; Plutzky et al., 1992; Yi et al., 1992) (Figure 7). The two proteins are 52% identical, and when conserved amino acids are taken into account they become 69% similar. One major difference between the two proteins is the presence in the csw protein of the PTPase insert, which is not observed in PTP1C. The amino acids 5′ to the first SH2 domain of PTP1C differ among the published reports (MLSRG in Shen et al., 1991 and MVR in Plutzky et al., 1992 and Yi et al., 1992). The same residues in the csw protein (MSSRR) (see Figure 6) are more similar to those reported by Shen and his colleagues. Several possibilities could explain these differences; however, genomic sequencing of the *csw* region has revealed that an intron is positioned after the nucleotides encoding the amino acid at position 4 (Perkins and Larsen, unpublished). It therefore seems likely that these differences arise by use of different 5′ exons.

Discussion

The csw protein is a new member of the growing family of protein tyrosine phosphatases that have been described. csw is the first Drosophila PTPase family member whose gene mutations are providing a means for genetic analyses of function. We report that *csw* is a member of the terminal class signal transduction pathway, whose other members are essential for determination of terminal cell fates. Specifically, *csw* functions downstream of the receptor tyrosine kinase torso and in concert with the serine/threonine kinase, D-raf.

Functions of the csw Protein

Unlike the other reported Drosophila PTPases that encode receptor forms (Streuli et al., 1989; Hariharan et al., 1991; Tian et al., 1991; Yang et al., 1991), *csw* encodes a nonreceptor protein tyrosine phosphatase. Further, the PTPase core domain of csw is covalently linked to two N-terminal SH2 domains. While the presence of the tyrosine phosphatase domain implies an enzymatic function for csw in transducing the torso signal, the presence of the SH2 domains suggests that csw effects its role by mediating heteromeric protein interactions.

The protein encoded by the *csw* gene shares 52% identity with the human protein tyrosine phosphatase, PTP1C (Shen et al., 1991; Plutzky et al., 1992; Yi et al., 1992) (Figure 7A). The overall structural similarity between the two proteins is remarkable in that both contain two N-terminal SH2 domains and a single C-terminal core PTPase domain. Besides PTP1C, three other proteins have been described that encode two SH2 domains each, (GAP, PLC-γ1, and the p85 subunit of PI3K), and each of these has been shown to interact physically with and transduce the signals generated from the activated EGF and/or PDGF receptors (reviewed by Cantley et al., 1991). Also, the PTP1C protein has been shown to bind physically to the activated EGF receptor in vitro (Shen et al., 1991).

SH2 domains are thought to regulate heteromeric protein interactions by recognizing peptide sequences within which lies a tyrosine phosphorylation site, such that when the tyrosine becomes phosphorylated, it then acts as the "ligand" and binds with high affinity to an SH2 domain. Not all SH2 domains recognize all phosphorylated tyrosines. Specificity is introduced in at least two ways: first, SH2 domains fall into distinct families, based on their amino acid sequences, and second, the amino acids that surround the phosphorylated tyrosines are recognized by specific SH2 domains (reviewed by Koch et al., 1991).

SH2 domains may also regulate intramolecular associations. The SH2 domains of members of the Src family of nonreceptor protein tyrosine kinases are thought to bind intramolecularly to a carboxy-terminal phosphotyrosine residue, and in this way render the enzyme "inactive" (reviewed by Cantley et al., 1991). Dephosphorylation of the tyrosine would allow the enzyme to assume an "open" conformation, and the now "activated" enzyme would be available to interact with other cellular phosphotyrosine-containing proteins. The csw core PTPase domain is unique among the reported PTPase core domains in that it is interrupted by a hydrophilic serine- and cysteine-rich stretch of approximately 150 aa. This "PTPase insert" is reminiscent of the "kinase insert" within the kinase catalytic domains of several receptor tyrosine kinases (e.g., PDGF receptor, CSF-1 receptor, and FGF receptor) (for review, see Ullrich and Schlessinger, 1990). These kinase inserts are highly conserved between species and may play a role in receptor function. Within the PDGF receptor kinase insert is a tyrosine residue that mutational analysis suggests regulates interactions with cellular substrates, specifically PI3K (Kazlauskas and Cooper, 1989). It is interesting that the csw protein contains two tyrosine residues, one within the PTPase insert (Tyr298) and the other within the SH2 C domain (Tyr168), whose neighboring residues suggest they are likely targets for phosphorylation. These potential tyrosine phosphorylation sites may be used to regulate the activity of the csw protein, as do family

members of the Src tyrosine kinases, and/or these sites may be recognized by specific substrates, as observed for receptor tyrosine kinases with "kinase insert" regions.

csw Functions in the Terminal Class Signal Transduction Pathway

Our results suggest that in the wild type, csw functions downstream of torso to positively transduce the terminal signal, that csw alone is not sufficient to transduce all of the terminal signal, and that csw acts in concert with D-raf to transduce the terminal signal.

Since the most severe *csw* mutant phenotype deletes only a subset of the posterior information deleted by a null allele of *torso*, csw alone is not sufficient to transduce all of the terminal information generated by torso. Further, our genetic results suggest that csw functions in concert with the serine/threonine kinase, D-raf, as a positive transducer of the terminal signal generated by the receptor tyrosine kinase, torso. These experiments, which included both single and double mutant combinations of *csw* and *D-raf*, utilized null alleles of *csw*, as well as hypomorphic alleles of *D-raf*. Since in null alleles of *D-raf*, all terminal activity is missing (i.e., posterior *tll* and *hb* expression is entirely missing) we utilized the *D-raf* hypomorphic alleles, with residual posterior *tll* and *hb* expression, to visualize probable interactions between *csw* and *D-raf*. When embryos are made doubly mutant for both null *csw* and hypomorphic *D-raf* alleles, the terminal activity is reduced to an extent greater than in either single mutant alone. This synergistic or additive effect, which does not imply a direct interaction between the csw and D-raf proteins, suggests that both loci are required to maintain the "strength" or "quality" of the torso signal, and mutations at either locus decrease the level or amount of torso signal transduced to the nucleus.

Much work is needed to clarify our understanding of how a PTPase might function during signal transduction. Clearly, the family of PTPase enzymes appears to be as large and structurally diverse as the family of protein tyrosine kinases and, like the protein tyrosine kinases, we might logically expect PTPases to regulate protein function by controlling phosphorylation. Presently the most well characterized PTPase is CD45, a receptor form found on the surface of T cells and other nucleated hematopoietic cells (reviewed by Klausner and Samelson, 1991; Veillette and Davidson, 1992). In T cells, CD45 is associated with the T cell receptor protein complex and when inactivated, by mutation or antibody-induced coaggregation, it inhibits T cell activation, the hallmark of which is tyrosine phosphorylation. A current model suggests that CD45 dephosphorylates, thereby activating two members of the Src family of protein tyrosine kinases, lck and fyn. In addition, though distantly related to the PTPase family, string, the Drosophila homolog of cdc25, which has been implicated in cell cycle regulation (Edgar and O'Farrell, 1989; 1990), has been shown to have an endogenous tyrosine phosphatase activity (Gautier et al., 1991). Biochemical studies have shown that cdc25-mediated dephosphorylation of the serine/threonine kinase cdc2 triggers initiation of mitosis (reviewed by Nurse, 1990).

How and where does csw function in the terminal class signal transduction pathway? We favor a model whereby the csw protein functions directly upon a member of the terminal system (e.g., torso, D-raf or an unidentified terminal protein) to "up-regulate" its activity (Figure 8A). When this up-regulation is missing, by mutation at the *csw* locus, then the quality or level of terminal signal is reduced. For example, we can envision at least two ways that csw could up-regulate torso. First, csw may function to "recycle" the torso receptor. During early embryonic stages the torso protein is evenly distributed along the anterior–posterior axis, yet at the posterior pole, there is a gradient of activity, that is highest at the pole (Casanova and Struhl, 1989), presumably due to a higher concentration of a locally produced ligand. It is believed that following ligand binding, receptor tyrosine kinases become activated by first dimerizing and then autophosphorylating specific tyrosine residues. If the torso protein is limiting where the terminal gradient is highest, csw may function to recycle the torso receptor by SH2-mediated binding and dephosphorylation of the receptor.

Second, csw may function at the level of the torso receptor to remove a negatively regulating phosphotyrosine. The activated torso tyrosine kinase may autophosphorylate tyrosines that facilitate dimerization but prevent torso from achieving l00% of its activity. In this case, following dimerization, csw would act to dephosphorylate inhibiting phosphotyrosines and thereby allow full generation of the torso activity.

Although the models outlined above are appealing, at this point, our data cannot rule out other equally likely models. Taking the mammalian EGF and PDGF receptor tyrosine kinases as examples, we cannot assume that all genes operating between the torso receptor and the zygotic transcription factors have been identified. csw may function on an unidentified component that lies on an alternate, partially redundant, pathway as D-raf (Figure 8B). Alternatively, csw may act downstream of D-raf, in concert with another transducer, to ultimately activate the transcription factors tll and hkb (Figure 8C). In this model, a second transducer is required, since csw alone is not sufficient to transduce all of the terminal signal. A test of this model awaits a means to test genetically for epistasis between csw and D-raf.

Whatever specific role csw plays to transduce the torso-generated terminal signal, it is significant that in Drosophila, as for the mammalian PTPase, PTP1C, a protein tyrosine phosphatase can be implicated in the transduction of a signal generated from a receptor tyrosine kinase. Since it is evident that different receptor tyrosine kinases utilize overlapping sets of downstream proteins to transduce their signals, it will be interesting to determine whether a PTPase functions downstream of all receptor tyrosine kinases. If so, is each receptor tyrosine kinase coupled to its own PTPase or is there a ubiquitous PTPase that functions as a general transducer downstream of all receptor tyrosine kinases?

Experimental Procedures

Genetic Strains

Dominant female sterile stocks: two different strains that carry the

X-linked dominant female sterile mutation ovo^{D1} (Busson et al., 1983) were used: *C(1)DX, y f/Y* females crossed to ovo^{D1} v^{24}/Y males and *C(1)DX, y f/Y; F38/F38* females crossed to *w* ovo^{D1} FRT^{101}/Y; *F38/F38* males.

csw: six *csw* alleles, all of which are required for viability were used in this study. The origin of these mutations is shown in Table 1. *VA199, C114, e13d.3* and *13–87* behave genetically as null mutations (amorphs), while *19–106* and *6* have residual activity (hypomorphs).

torso: the dominant temperature-sensitive *torso* allele, tor^{RL3}, where two copies are required to produce the $torso^{Dominant}$ or "spliced" phenotype (Klinger et al., 1988; Strecker et al., 1989), was used in this study. It is kept using the *CyO* balancer. At 18°C, embryos homozygous for tor^{RL3} form head and tail structures with variably deleted and/or defective thoracic and abdominal segments. At 25°C, central thoracic and abdominal segments are deleted, and terminal head and tail structures are expanded centrally.

D-raf: three *D-raf* mutations were used in this study: one null, *EA75*, and two hypomorphs, *PB26* and *C110* (Perrimon et al., 1985; N. P., unpublished data).

Flies were raised on standard Drosophila media at 25°C unless indicated. Chromosomes and mutations that are not described in the text can be found in Lindsley and Grell (1968) and Lindsley and Zimm (1985, 1986, 1987, 1990).

Construction of *csw D-raf* Recombinant Chromosomes

The following chromosomes were constructed to test for interaction between *csw* and *D-raf*: csw^{VA199} $D\text{-}raf^{EA75}$ FRT^{101}; *y* csw^{6} $D\text{-}raf^{C110}$ FRT^{101}; csw^{VA199} $D\text{-}raf^{PB26}$ FRT^{101}; and, *y* csw^{6} $D\text{-}raf^{EA75}$ FRT^{101}. These recombinants were built by conventional genetic techniques using the visible markers *yellow* (*y*, 0.0) and *white* (*w*, 1.5) that flank *csw* (0.8) and *D-raf* (0.9). Recombinants between *csw* and *D-raf* were recovered over the *FM7c* balancer chromosome that carries both *yellow* and *white*. Subsequently, the FRT^{101} element (Chou and Perrimon, 1992) needed for the production of germline clones was recombined onto these chromosomes. All stocks are kept using the *FM7c* balancer chromosome.

Genetic Epistasis between *tor* and *csw*

To analyze the genetic epistasis between *tor* and *csw* the cuticle phenotype of eggs derived from csw^{C114} germline clones generated in tor^{RL3} homozygous mothers was examined. To generate such females, progeny from the cross: csw^{C114}/*FM3*; tor^{RL3}/*CyO* × ovo^{D1}/Y; tor^{RL3}/*CyO* were irradiated during larval development at a constant dose of 1000 rads (Torrex 120D X-ray machine; 100 Kv, 5 mA, 3 mm aluminum filter; Perrimon, Engstrom and Mahowald, 1984). Females with clones were crossed to Oregon R wild-type males and raised at 25°C.

Larval cuticles were prepared in Hoyers' mountant as described by van der Meer (1977). Cuticles were examined using dark-field and phase illumination.

Production of Germline Clones Using the "FLIP-DFS" Technique

Germline clones of *csw*, *D-raf*, and *csw D-raf* double mutants were generated using the "FLIP-DFS" technique (Chou and Perrimon, 1992). In brief, females carrying the X-linked lethal(s) *FM7/lethal(s)* FRT^{101} virgin females were crossed with *w* ovo^{D1} FRT^{101}/Y; *F38/F38* males. Progeny were heat shocked for 2 hr at 37°C during larval stages and females of genotypes *y w lethal* FRT^{101}/*w* ovo^{D1} FRT^{101}; *F38/+* examined for the presence of germline clones. Germline clones were identified by the presence of vitellogenic egg chambers.

In Situ Hybridizations, Immunocytochemistry, and Antibodies

In situ hybridizations on whole-mount Drosophila embryos were performed as described by Tautz and Pfeifle (1989) using the Genius kit (Boehringer Mannheim). Single-stranded sense and antisense digoxigenin-containing *csw* or *tll* DNA probes were prepared by the polymerase chain reaction labeling technique (N. Patel, personal communication) using T3 and T7 primers (Biolabs). The labeled DNA was boiled for 30 min to reduce its size. For visualization, embryos were dehydrated in ethanol and mounted in Euparal (Carolina Biologicals).

Immunocytochemistry was performed as described in Smouse et al. (1988). Embryos were dehydrated in ethanol and cleared in methyl salicylate. Antibodies, αhb (obtained from Dr. P. MacDonald), αfkh (obtained from Dr. H. Jäckle), and αβ-gal (Boehringer Mannheim), were used at dilutions ranging from 1:250 to 1:1000.

Embryos were photographed on a Zeiss Axiophot microscope using Nomarski optics.

Molecular Analyses

Genomic DNA Analyses

DNA purification, DNA cloning, Southern blot analysis, and plaque hybridizations were performed as described in Sambrook et al. (1989) and Ausubel et al. (1990). Using DNA from the putative *csw* region, generously provided by Dr. Hugh Brock, overlapping phage were isolated from a λ EMBL3 Drosophila genomic library (Blackman et al., 1987).

cDNA Isolation

cDNAs from the putative *csw* region were isolated in a series of three screens, X, Y, and Z, of a 0–4 hr embryonic cDNA library (Brown and Kafatos, 1988). Random primed probes (Feinberg and Vogelstein, 1983) were generated from genomic fragments from the region. Screen Z was probed with fragments from approximately −7 to +3 on the molecular map, screen X with fragments from approximately +3 to +13, and screen Y with fragments from approximately +13 to +19.5 (see Figure 4). Two of the screens, Z and Y, yielded cDNA clones. Clones from the Z screen failed to cross-hybridize to clones from the Y screen, and when these clones were used as probes to the region, only Y clones hybridized to DNA encompassing the *csw* rearrangements (see Figure 4). Z clones hybridized to genomic fragments from approximately −14.5 to 0 (data not shown).

RNA Analyses

Total RNA was isolated from staged embryos, larvae, and pupae by the guanidinium/cesium chloride method (Maniatis et al., 1982) and affinity-purified on oligo(dT) cellulose (Collaborative Research). Northern blot analysis was performed using standard methods (Sambrook et al., 1989). DNA probes were radiolabeled by the random primer method (Feinberg and Vogelstein, 1983).

P Element Transformation and Rescue

The P element vector pCaSpeR-hs, which carries the $white^{+}$ gene (Thummel et al., 1988; C. Thummel, personal communication) was kindly provided by C. Thummel. The *Y1229* cDNA was subcloned into pCaSpeR-hs, and, following standard protocols (Spradling, 1986), was injected into *y w/y w*; *Δ2–3,Sb/In(3)TM6* (Robertson et al. 1988) precellular embryos. This strain constitutively synthesizes an endogenous transposase that is present in the ooplasm. Six independent transformants were identified by rescue of their white eye color to near wild type. To test for rescue of the *csw* maternal effect phenotype, females with *csw* mutant germlines were mated to transformed *P[w,hs-Y1229]* males, and a 5 hr egg collection was followed by a 1 hr heat shock at 37°C. To test for rescue of the *csw* zygotic lethality, heterozygous *csw* females were mated to transformed *P[w,hs-Y1229]* males; when larvae were first observed, the vials were heat shocked daily (until eclosion) for 1 hr at 37°C.

DNA Sequencing

DNA sequencing was carried out using the dideoxy chain termination method (Sanger et al., 1977) and utilizing Sequenase (U. S. Biochemical Corp.). Sequencing templates were made on one strand by generating nested exonuclease III deletions of the entire cDNA *Y1229* using the Erase-a-Base system (Promega) and on the second strand by subcloning different restriction fragments into pBSK^{+} (Stratagene). Specific oligonucleotide primers were synthesized to extend the sequence. The coding sequence of the cDNA was determined on both strands.

DNA sequence analysis utilized the Wisconsin Genetics Computer Group (WGCG) sequence analysis package (Devereux et al., 1984). Optimal amino acid alignment between two sequences was made using the PILEUP program of WGCG. Homology searches utilized the BLAST Network Service (Altschul et al., 1990).

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