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Opposing Actions of CSW and RasGAP Modulate the Strength of Torso RTK Signaling in the *Drosophila* Terminal Pathway

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Summary

In *Drosophila*, specification of embryonic terminal cells is controlled by the Torso receptor tyrosine kinase. Here, we analyze the molecular basis of positive (Y630) and negative (Y918) phosphotyrosine (pY) signaling sites on Torso. We find that the *Drosophila* homolog of RasGAP associates with pY918 and is a negative effector of Torso signaling. Further, we show that the tyrosine phosphatase Corkscrew (CSW), which associates with pY630, specifically dephosphorylates the negative pY918 Torso signaling site, thus identifying Torso to be a substrate of CSW in the terminal pathway. CSW also serves as an adaptor protein for DRK binding, physically linking Torso to Ras activation. The opposing actions of CSW and RasGAP modulate the strength of the Torso signal, contributing to the establishment of precise boundaries for terminal structure development.

Introduction

Receptor tyrosine kinases (RTKs) regulate a diverse array of biological processes ranging from cellular metabolism to cell growth, differentiation, and development. Numerous studies have defined the following paradigm for RTK action (reviewed in Fantl et al., 1993; van der Geer et al., 1994; Heldin, 1995). Binding of a cognate ligand to the extracellular domain of an RTK results in receptor dimerization/oligomerization, activation of the intracellular kinase domain, and autophosphorylation of the receptor on tyrosine residues. The tyrosine phosphorylation of the receptor generates specific binding sites for a variety of cytosolic signaling molecules, many of which contain phosphotyrosine (pY) recognition modules such as Src homology 2 (SH2) or pY-binding (PTB) domains. The proteins bound to the activated RTK then initiate distinct and overlapping signaling pathways that ultimately induce the appropriate cellular response. Although much is known about RTK signal transduction, there are still fundamental questions that remain to be resolved. In particular, identifying receptor pY sites and their effector molecules, as well as determining the molecular basis behind which various combinations of signaling molecules regulate the strength and duration of an RTK signal, is critical.

One model system that has been particularly useful in determining the molecular mechanisms of RTK signal transduction is that involving the *Drosophila* RTK Torso. Early in *Drosophila* embryogenesis, signaling through Torso leads to the formation of specialized anterior and posterior terminal structures termed the acron and telson, respectively (reviewed by Duffy and Perrimon, 1994). Although Torso is expressed uniformly on the surface of the embryo, only receptors in the terminal regions are activated, due to the spatially restricted delivery of a diffusible ligand (Sprenger and Nusslein-Volhard, 1992; Casanova and Struhl, 1993). After ligand binding and receptor activation, the Torso signal is transduced from the membrane to the nucleus by a conserved set of positive effector molecules, including corkscrew (CSW), kinase suppresser of Ras (KSR), daughter of sevenless (DOS), 14–3–3, DRK (the *Drosophila* homolog of Grb2), son of sevenless (SOS), Ras, D-Raf, D-MEK, and Rolled MAPK (Ambrosio et al., 1989; Perkins et al., 1992; Lu et al., 1993; Hou et al., 1995; Therrien et al., 1995; Herbst et al., 1996; Raabe et al., 1996; Li et al., 1997). Ultimately, the maternally derived Torso pathway results in the zygotic expression of *tailless* (*tll*) and *huckebein* (*hkb*) in defined terminal regions of the embryo. In the posterior end, *tll* and *hkb* are regulated solely by the Torso signal; therefore, monitoring the expression pattern of these genes provides an excellent "readout" of the strength of the Torso signal.

To further define the molecular basis of Torso action, we previously identified the two major sites of Torso that become phosphorylated upon receptor activation (Cleghon et al., 1996). By establishing transgenic fly lines expressing only mutant Torso receptors, we found that both sites are crucial for the correct specification of terminal cell fate in developing embryos. One site, Y630, serves to positively transduce the Torso signal, whereas the second site, Y918, functions as a negative regulator. Mutation of Y630 reduced *tll* expression in the posterior region and resulted in embryos with a partial loss-of-function (lof) phenotype; in contrast, mutation of Y918 expanded *tll* expression and generated embryos with a gain-of-function (gof) phenotype. Surprisingly, the simultaneous mutation of both Y630 and Y918 restored signaling to wild-type (WT) levels under certain conditions, suggesting that both sites function in a compensatory manner to modulate the strength of the Torso signal.

The Y630 and Y918 sites are located in noncatalytic regions of Torso and are likely to function as binding sites for downstream signaling proteins. In fact, we have found that CSW associates with the pY630 site (Cleghon

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et al., 1996). CSW is a nonreceptor protein tyrosine phosphatase containing tandem N-terminal SH2 domains (Perkins et al., 1992). By genetic criteria, CSW has been identified as a positive effector of Torso (Perkins et al., 1992) and other RTK signaling pathways in *Drosophila* (Allard et al., 1996; Perkins et al., 1996). Likewise, the vertebrate homolog of CSW, SHP-2, functions as a positive transducer of RTK signaling in *Xenopus laevis* (Tang et al., 1995; O'Reilly and Neel, 1998) and in mammals (Streuli, 1996; Neel and Tonks, 1997; Saxton et al., 1997; Hadari et al., 1998). Although CSW was one of the earliest effectors of Torso signaling to be identified, elucidating CSW function has not been an easy task. Numerous studies, however, have demonstrated that catalytic activity is essential for both CSW and SHP-2 to transduce an RTK signal (Tang et al., 1995; Allard et al., 1996; Perkins et al., 1996; Allard et al., 1998; Hadari et al., 1998; O'Reilly and Neel, 1998). Thus, a complete understanding of CSW and SHP-2 action requires the identification and characterization of biologically relevant substrates. The *Drosophila* protein DOS has recently been identified as a substrate of CSW in the Sevenless (Sev) signaling pathway (Herbst et al., 1996). In addition, the platelet-derived growth factor (PDGF) receptor (Klinghoffer and Kazlauskas, 1995) and SHPS1/SIRP1a (Fujioka et al., 1996) have been suggested to be SHP-2 substrates. However, the way in which the dephosphorylation of these putative substrates translates into the positive transmission of an RTK signal remains unclear.

In this study, we have investigated the molecular mechanisms by which the positive (pY630) and negative (pY918) phosphotyrosine signaling sites of Torso modulate the strength of the terminal pathway signal. We find that the recently characterized *Drosophila* homolog of RasGAP (P. Feldmann et al., submitted) associates with Torso pY918 to transduce the negative effects of this site. In addition, we demonstrate that CSW, the positive effector of the Torso pY630 site, is phosphorylated by Torso on Y666 and that pY666 serves as a binding site for DRK. Further, we find that Torso is a substrate of CSW both in vitro and in vivo, that CSW specifically dephosphorylates Torso at the pY918 negative signaling site, and that this dephosphorylation event requires the binding of CSW to pY630 of Torso. Thus, this study identifies Torso to be a biologically relevant target of CSW and indicates that by dephosphorylating the pY918 site, CSW prevents the negative regulator RasGAP from associating with Torso. In the context of the *Drosophila* embryo, the competition between positive and negative signaling sites on Torso modulates the strength of the Torso signal, thereby defining the precise spatial expression pattern of the terminal zygotic genes *tll* and *hkb*.

Results

D-RasGAP Functions as a Negative Effector of Torso Signaling

Recently, a *Drosophila* homolog of mammalian p120-RasGAP was identified (P. Feldmann et al., submitted). Like its vertebrate counterpart, D-RasGAP contains a tandem array of SH2-SH3-SH2 (SHS) domains in the N terminus and a GAP catalytic domain in the C terminus. To date, no lof mutations in D-RasGAP have been identified; therefore, to determine whether D-RasGAP functions in the Torso pathway, we examined the effect of a dominant-negative (DN) D-RasGAP protein on the establishment of terminal cell fates in the developing embryo. Based on the observation that expression of the N terminus of mammalian RasGAP dominantly interferes with the function of the full-length endogenous protein (Medema et al., 1992; DeClue et al., 1993; Clark et al., 1997), we generated a truncated form of D-RasGAP containing only the N-terminal SHS domains (designated DN D-RasGAP). We then used the Gal4-UAS system (Brand and Perrimon, 1993) to express either the DN D-RasGAP construct or one encoding the full-length D-RasGAP in early embryos. The effect of the DN and full-length D-RasGAP transgenes on the terminal pathway was subsequently monitored by measuring *tll* expression in the posterior region of embryos at late syncytial blastoderm stages. In WT embryos, *tll* is expressed from 0%–15% egg length (EL) (average of 14.5% with 30 embryos; Figure 1A). Expression of the UAS full-length D-RasGAP transgene had no significant effect on *tll* expression (average 14.7% with 30 embryos, data not shown). However, expression of UAS-DN D-RasGAP expanded the domain of *tll* expression to 0%–18% EL (average 18.3% with 30 embryos; Figure 1B). Because *tll* expression is controlled exclusively by the Torso pathway in the posterior region of embryos, the ability of the DN D-RasGAP transgene to expand *tll* expression provides evidence that D-RasGAP functions in the terminal pathway and further indicates that D-RasGAP is a negative effector of Torso signaling.

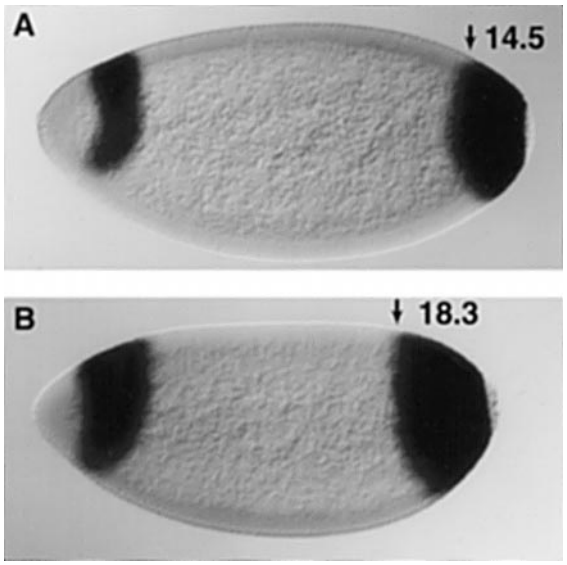

Figure 1. Expression of a Dominant-Negative Form of D-RasGAP Expands the Domain of *tll* Expression in *Drosophila* Embryos

In situ hybridization patterns of *tll* are shown in WT (A) or in *P[w+, GAL4-VP16]mata4/P[w+, UAS-DN D-RasGAP]* embryos (B). The posterior domain of *tll* expression is indicated as percent egg length (EL), with 0% corresponding to the posterior pole.

D-RasGAP Binds to the Negative pY918 Signaling Site of Torso

Mammalian RasGAP has been shown to associate with a variety of activated RTKs (Kazlauskas et al., 1990; Margolis et al., 1990; Medema et al., 1996), to be a negative effector of the PDGF receptor (Valius et al., 1995),

and to downregulate Ras signaling (Boguski and McCormick, 1993). Interestingly, the expansion of *tll* expression that we observed in embryos expressing UAS-DN D-RasGAP was similar to that previously observed in embryos expressing Torso^{Y918F} (Figure 1; Cleghon et al., 1996). Therefore, we investigated whether D-RasGAP is the signaling molecule that transduces the negative effects of the Torso pY918 site. Because the mammalian RasGAP/RTK interaction is mediated by the binding of the RasGAP SH2 domains to specific pY residues on the RTK, we examined whether the isolated SH2 domains of D-RasGAP would associate with Torso in a pY-dependent manner. For this analysis, the SHS domains of D-RasGAP were expressed in bacteria as a glutathione S-transferase (GST) fusion protein. The GST-SHS fusion protein was then immobilized on glutathione Sepharose beads and incubated with Sf9 insect cell lysates containing constitutively active Torso (4021), kinase-inactive Torso (TKM), or pY mutant 4021-Torso proteins (Y630F, Y918F, or YY630/918FF). Following extensive washing, the GST-SHS complexes were examined for the presence of Torso by immunoblot analysis. As shown in Figure 2A, 4021 and Y630F Torso proteins associated with the D-RasGAP SHS domains, whereas unphosphorylated TKM or mutant proteins containing the Y918F substitution did not. These results indicate that D-RasGAP associates with Torso in a pY-specific manner requiring the Y918 site.

To confirm that the association with Torso was mediated by the SH2 domains of D-RasGAP, we mutated the Arg residue in the highly conserved FLVRES sequence of each D-RasGAP SH2 domain and then tested the mutant SHS protein for its ablility to bind Torso. As shown in Figure 2B, the mutant SHS protein was unable to bind activated 4021 Torso, demonstrating that functional SH2 domains are required for the interaction with Torso pY918.

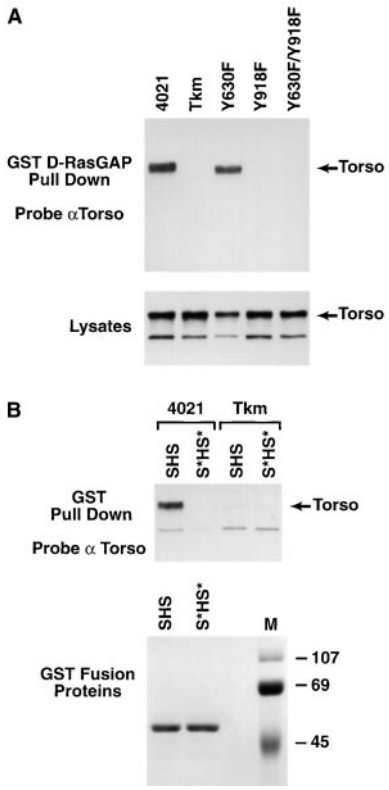

Figure 2. D-RasGAP Associates with pY918 of Torso

(A) A GST fusion protein containing the SH2-SH3-SH2 (SHS) domains of D-RasGAP was incubated with Sf9 cell lysates containing constitutively active 4021, kinase-inactive TKM, 4021^{Y630F}, 4021^{Y918F}, or 4021$^{Y630F/Y918F}$ Torso proteins. Torso proteins associating with the GST-SHS fusion protein were visualized by immunoblot analysis using Torso antibodies (αTorso; top). Aliquots of the Sf9 cell lysates used in this experiment were probed with αTorso to demonstrate equivalent expression levels (bottom).

(B) GST fusion proteins containing WT (SHS) or mutant SH2 domains (S*HS*) of D-RasGAP were incubated with lysates containing 4021 or TKM Torso proteins. Proteins associating with the GST-SHS complexes were visualized by immunoblot analysis using αTorso (top). Aliquots of the GST fusion proteins were examined by SDS-PAGE and Coomassie blue staining (bottom). The apparent molecular weights of the protein standards in lane M are indicated.

Torso Phosphorylates CSW at Y666, Generating a Consensus Binding Site for DRK

Previously, we showed that CSW associates with Torso pY630 via its SH2 domains and that this interaction results in the in vivo tyrosine phosphorylation of CSW in Sf9 cells and in *Drosophila* embryos (Cleghon et al., 1996). To address the functional significance of this phosphorylation, as well as to further understand the role of CSW as a positive effector of Torso signaling, we initiated experiments to identify the residue of CSW that is phosphorylated by Torso. For this study, CSW was coexpressed with Torso in Sf9 cells and phosphorylated by Torso in immune complex kinase assays. ^{32}P-labeled CSW was then resolved by SDS-PAGE, extracted from the gel matrix, and digested with trypsin. The resulting tryptic phosphopeptides were separated and eluted from a reversed-phase HPLC C-18 column. When the radioactivity released from the column was quantitated, a major peak in fraction 14 was detected (Figure 3A). Phosphoamino acid analysis and N-terminal sequencing of the peptide isolated in fraction 14 revealed that it contained phosphotyrosine and was phosphorylated on the second residue after the trypsin cleavage site (Figure 3B). By examining the amino acid sequence of CSW, we found that the only tyrosine located two residues downstream from a trypsin cleavage site is Y666, suggesting that this is the residue phosphorylated. To confirm the identification of Y666, we generated a mutant CSW protein containing a Tyr to Phe substitution at amino acid 666 (i.e., Y666F). CSW^{Y666F} was coexpressed with Torso in Sf9 cells and analyzed as described above. As shown in Figure 3C, the peak of radioactivity eluting in fraction 14 was absent from CSW^{Y666F}, confirming that Y666 is the site of Torso-dependent tyrosine phosphorylation.

The sequence context of the CSW Y666 site, pY-T-N-I, represents a consensus binding site for DRK (i.e., pY-X-N-X; Songyang et al., 1993). By genetic analysis, DRK has been shown to be a positive effector of the terminal pathway (Hou et al., 1995); however, Torso does not contain a consensus DRK-binding site, nor has it been found to directly associate with DRK (Cleghon et al., 1996). Therefore, to determine whether DRK indirectly couples to the Torso receptor by interacting with CSW at pY666, we performed binding experiments using

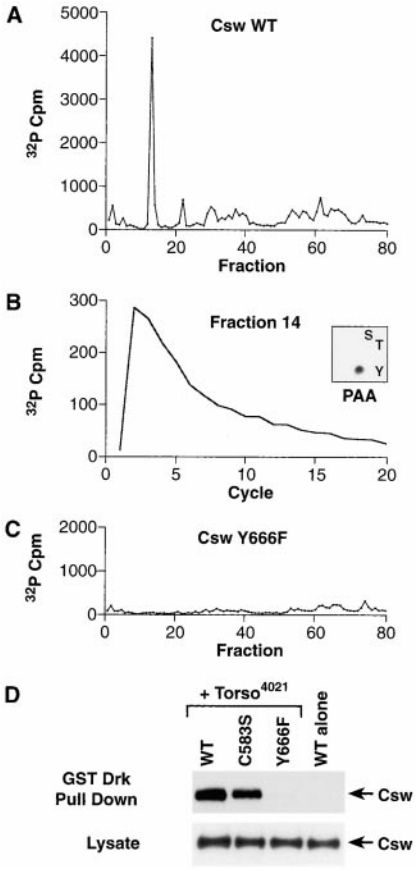

Figure 3. Identification of Y666 as a Site of CSW Phosphorylation and DRK Binding

(A) CSW was coexpressed with Torso in Sf9 cells and phosphorylated by Torso in immune complex kinase assays. Isolated ^{32}P-labeled CSW proteins were digested with trypsin, and the tryptic phosphopeptides were separated and eluted from a reversed-phase HPLC C-18 column. The profile of the ^{32}P radioactivity released from the column is shown.

(B) The peptide isolated in fraction 14 was subjected to automated Edman degradation in a spinning-cup sequenator. The ^{32}P radioactivity released in each cycle is depicted. Phosphoamino acid analysis of fraction 14 is shown as an insert. The relative positions of free serine (S), threonine (T), and tyrosine (Y) are indicated.

(C) CSW^{Y666F} was analyzed as described in (A), and the profile of radioactivity released from the reversed-phase HPLC column is shown.

(D) A GST-DRK fusion protein was incubated with lysates from Sf9 cells expressing CSW^{WT} alone or coexpressing Torso with CSW^{WT}, CSW^{C583S}, or CSW^{Y666F}. CSW proteins associating with the GST-DRK fusion protein were visualized by immunoblot analysis using CSW antibodies (αCSW; top). Aliquots of the Sf9 cell lysates were probed with αCSW (bottom).

a GST-DRK fusion protein and Sf9 cell lysates containing either non-tyrosine-phosphorylated CSW proteins (expressed alone in Sf9 cells) or tyrosine-phosphorylated CSW proteins (coexpressed with Torso). As shown in Figure 3D, tyrosine-phosphorylated CSW^{WT} and CSW^{C583S} associated with the DRK fusion protein. However, neither the non-tyrosine-phosphorylated CSW^{WT} nor the CSW^{Y666F} mutant that had been coexpressed with Torso were precipitated by the DRK fusion protein, demonstrating that DRK associates with CSW in a pY-dependent manner that requires the pY666 site. Further, these findings establish the first physical link between the activated Torso receptor and Ras activation via the binding of CSW to Torso, DRK to CSW, and SOS to DRK.

pY918 of Torso Is a Specific Substrate of CSW Activity In Vitro

If the sole function of CSW is to serve as an adaptor protein linking DRK to Torso, then a catalytically inactive CSW molecule should be sufficient to fulfill this role. Indeed, the inactive CSW^{C583S} mutant does bind Torso at pY630, is subsequently tyrosine phosphorylated on Y666 (data not shown), and is capable of binding DRK (Figure 3D). However, the enzymatic activity of CSW is required for CSW to transduce the Torso signal. Thus, the identification of CSW substrates is critical. Since the cellular colocalization of enzymes and their targets is an emerging theme in signal transduction and since CSW directly interacts with Torso, we examined whether the Torso receptor itself might be a substrate of CSW. For these experiments, we developed an in vitro phosphatase assay using soluble CSW and autophosphorylated ^{32}P-labeled Torso. CSW^{WT} and phosphatase-inactive CSW^{C583S} proteins were expressed in Sf9 cells and purified from crude lysates using ion exchange chromatography (Figure 4A). ^{32}P-labeled Torso was then incubated with various amounts of purified CSW^{WT} and CSW^{C583S}, and the radioactivity released from Torso was monitored. We found that CSW^{C583S} displayed no detectable activity, whereas CSW^{WT} was active as a phosphatase and was able to dephosphorylate Torso in vitro (Figure 4B). Using a phosphotryptic/HPLC assay (Cleghon et al., 1996), we next investigated whether CSW displays preferential activity toward a particular site of Torso phosphorylation. Torso was incubated with purified CSW^{WT} or CSW^{C583S}, and the dephosphorylation of individual sites on Torso was monitored by measuring the levels of HPLC-isolated ^{32}P-labeled tryptic phosphopeptides. As demonstrated in Figure 4C, CSW^{WT} efficiently dephosphorylated the pY918 site of Torso in a dose-dependent manner but displayed little activity toward the pY630 site. In similar experiments, CSW^{C583S} exhibited no detectable activity toward either the pY630 or pY918 sites of Torso (data not shown). Thus, Torso pY918 is a specific target of CSW activity in vitro.

CSW Specifically Dephosphorylates pY918 of Torso In Vivo

To investigate whether Torso pY918 is a biologically relevant substrate of CSW, we examined the effect of CSW on the in vivo tyrosine phosphorylation state of Torso. Torso proteins were immunoprecipitated from Sf9 cells expressing Torso alone or coexpressing Torso with CSW^{WT} or inactive CSW^{C583S}. By immunoblot analysis using an anti-pY antibody, we found that the level of tyrosine-phosphorylated Torso was reduced approximately 50% in Sf9 cells coexpressing Torso and CSW^{WT} (Figure 5A). To determine whether the observed reduction in tyrosine phosphorylation was due to the specific dephosphorylation of the pY918 site, we used the isolated SHS domains of D-RasGAP as an affinity reagent to measure the level of Torso pY918, since this fusion protein specifically interacts with the pY918 site (Figure

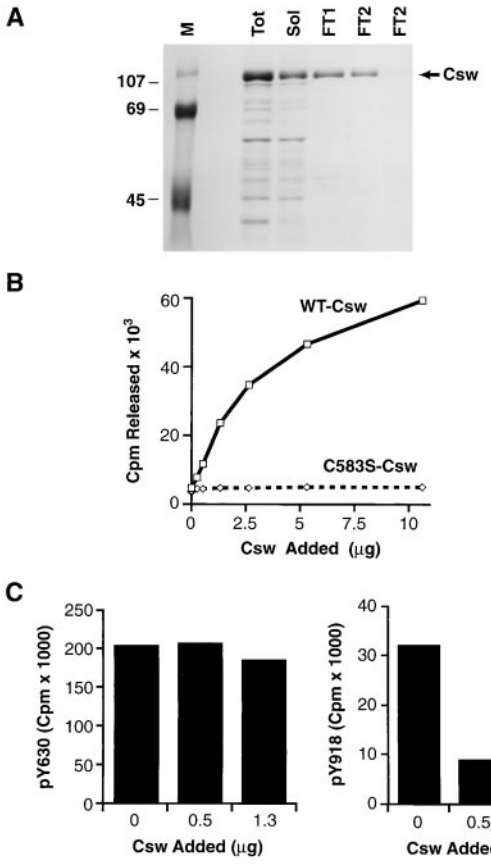

Figure 4. Torso pY918 Is a Specific Substrate of CSW In Vitro

(A) CSW was purified from Sf9 cell lysates as described in Experimental Procedures. Aliquots of the total cell sonicates (Tot), soluble supernatant (Sol), or flow-through fractions (FT1, FT2, or FT3) were analyzed for the presence and purity of CSW by SDS-PAGE and Coomassie blue staining. The position of CSW and the apparent molecular weights of protein standards in lane M are indicated.

(B) Immunocomplexes of ^{32}P-labeled Torso were incubated with various amounts of purified CSW^{C583S} or CSW^{WT}. Dephosphorylation of Torso was determined by pelleting the Torso complexes and measuring the radioactivity released into the supernatant.

(C) Torso dephosphorylated as described in (B) was digested with trypsin, and the tryptic phosphopeptides were isolated by reversed-phase HPLC as previously described (Cleghon et al., 1996). The amount of ^{32}P radioactivity present in the fractions containing pY630 and pY918 is shown.

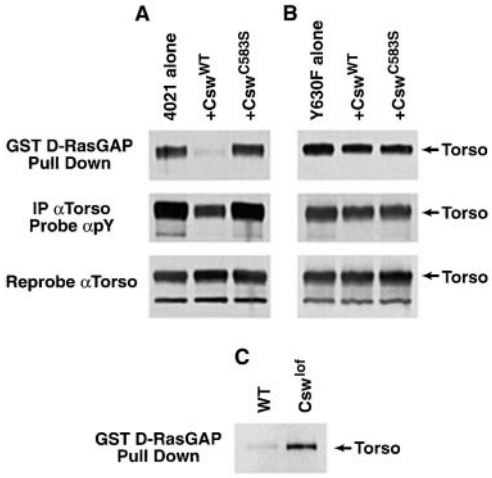

Figure 5. CSW Specifically Dephosphorylates Torso pY918 In Vivo

(A) Lysates of Sf9 cells expressing activated Torso^{4021} alone or coexpressing Torso^{4021} with either CSW^{WT}or CSW^{C583S} were incubated with the GST-SHS D-RasGAP fusion protein. Torso proteins associating with the GST-SHS fusion protein were visualized by immunoblot analysis using αTorso (top). Torso immunoprecipitates were also prepared from aliquots of the Sf9 cell lysates. The Torso immunoprecipitates were probed first with αP-Tyr (middle) and then stripped and reprobed with αTorso (bottom).

(B) As in (A), except Torso 4021^{Y630F} was used instead of Torso 4021.

(C) Embryos derived from WT or *csw*^{LE120}/*csw*^{LE120} (Csw^{lof}) females were collected from 0–3 hr after EL and lysates were then incubated with the GST-SHS D-RasGAP fusion protein, and the associated Torso proteins were visualized by immunoblot analysis using αTorso.

2). The GST-SHS D-RasGAP fusion protein was incubated with the Sf9 cell lysates, and the associated pY918-containing Torso was visualized by immunoblot analysis. As shown in Figure 5A, the level of pY918-containing Torso was readily detectable when Torso was expressed alone or coexpressed with CSW^{C583S}; however, the level was dramatically reduced when Torso was coexpressed with CSW^{WT} (Figure 5A), indicating a specific dephosphorylation of the Torso pY918 site in vivo.

We next examined whether binding to Torso at the pY630 site is required for CSW to dephosphorylate the pY918 site in vivo. For this analysis, we used the Torso^{Y630F} mutant protein, since it is phosphorylated on the pY918 site but is unable to bind CSW (Cleghon et al., 1996). Torso^{Y630F} was expressed in Sf9 cells alone or was coexpressed with either CSW^{WT} or CSW^{C583S}. By immunoblot analysis using an anti-pY antibody, we found that all samples contained approximately equal levels of tyrosine-phosphorylated Torso^{Y630F} (Figure 5B). Similarly, when the GST-SHS D-RasGAP fusion protein was used as an affinity reagent to detect pY918, we found that the level of pY918-containing Torso^{Y630F} was equivalent in all samples and was readily detectable in cells coexpressing Torso^{Y630F} and CSW^{WT}. Therefore, we conclude that CSW must bind to the pY630 site of Torso in order to specifically dephosphorylate the pY918 site.

Finally, if Torso is a physiological substrate of CSW, then we would expect that *Drosophila* embryos lacking CSW activity would contain higher levels of pY918-containing Torso than would WT embryos. To address this question, embryos from WT or *csw*^{LE120} females were collected at 0–3 hr after egg laying (a time where Torso is known to function) and examined for the level of Torso pY918 using the isolated SHS domains of D-RasGAP. As shown in Figure 5C, the levels of pY918-containing Torso were clearly elevated in embryos lacking CSW activity, indicating that Torso pY918 is a biologically relevant CSW target.

Discussion

To precisely define the boundaries for terminal structure development during *Drosophila* embryogenesis, it is crucial that the Torso RTK deliver a signal of appropriate strength and duration. From our studies, we have found that the magnitude of the Torso signal is modulated by

the opposing and competing actions of positive (Y630) and negative (Y918) pY signaling sites on Torso. Here, we have investigated the molecular mechanisms by which these signaling sites mediate their effect.

D-RasGAP Is the Negative Effector of Torso pY918

By combining biochemical and genetic approaches, we have found that the *Drosophila* homolog of RasGAP specifically associates with pY918 of Torso and is a negative effector of the terminal pathway. As has been observed for mammalian RasGAP/RTK interactions, D-RasGAP interacted directly with the activated Torso receptor in a pY-dependent manner requiring the pY918 site of Torso and intact SH2 domains of D-RasGAP. The localization of RasGAP to Torso pY918 suggests that D-RasGAP is the signaling molecule mediating the negative effects of this site. RasGAP proteins have been well characterized as downmodulators of Ras signaling, and RasGAP has been reported to be a negative effector of PDGF receptor signaling in vertebrate systems. Although D-RasGAP mRNA and protein have been shown to be present at high levels in precellularized embryos (i.e., 0–2 hr), a time frame coincident with Torso signaling (P. Feldmann et al., submitted), genetic analysis has not previously implicated D-RasGAP in the terminal pathway. It should be noted that another protein with GAP activity, Gap1, had been shown by genetic analysis to function in the terminal and other *Drosophila* developmental pathways (Gaul et al., 1992; Hou et al., 1995); however, the Gap1 protein does not contain any pY-binding motifs and appears to serve as a constitutive general repressor of Ras activity (Gaul et al., 1992). Here, using a dominant-negative molecule, we provide the first direct evidence that D-RasGAP functions in the terminal pathway. Expression of DN D-RasGAP increased Torso signaling and expanded *tll* expression in the posterior region of *Drosophila* embryos. Interestingly, the phenotype exhibited by DN D-RasGAP was similar to what we had previously observed for embryos expressing $Torso^{Y918F}$ mutant receptors, again consistent with the idea that D-RasGAP is the effector of the Y918 site. In both cases, we propose that the normal binding of endogenous D-RasGAP to pY918 of Torso is prevented, either by the expression of a competing dominant-inhibitory D-RasGAP or by eliminating the binding site on Torso. The inability of RasGAP to bind to pY918 would prevent downregulation of the Torso signal, effectively increasing the strength of the signal and resulting in the subsequent expansion of *tll* expression. Thus, the binding of D-RasGAP to Torso pY918, together with the ability of RasGAP to downregulate Ras signaling, provides a molecular basis for the negative effect of the pY918 signaling site.

Multiple Functions of CSW, the Positive Effector of the Y630 Site

The positive Y630 signaling site of Torso serves as a binding site for the tyrosine phosphatase CSW. Although CSW has been known to be a positive effector of Torso signaling for many years, elucidating how CSW transduces an RTK-mediated signal has been difficult. Indeed, our studies, together with previous findings (Allard et al., 1996; Herbst et al., 1996), suggest that CSW function is complex, with multiple points of action. First, as a result of the association with Torso, we have found that CSW becomes tyrosine phosphorylated on Y666 and that this phosphorylation event generates a consensus binding site for DRK (pY-X-N-X). Since Torso itself does not contain a Y-X-N-X motif, the binding of DRK to CSW establishes the first physical link from Torso to the DRK-SOS-Ras activation cascade. SHP-2 has also been found to serve as a DRK/Grb-2-binding protein in some RTK signaling pathways (Bennett et al., 1994; Li et al., 1994; Hadari et al., 1998); however, the significance of the adaptor role for both SHP-2 and CSW is unclear (Tang et al., 1995; O'Reilly and Neel, 1998). In particular, homozygous female flies expressing a truncated CSW protein that lacks the pY666 site survive and reproduce, indicating that the adaptor function of CSW is not absolutely essential for viability or female fertility (Allard et al., 1998). Thus, the function of CSW as an adaptor protein linking RTKs to DRK is likely to be redundant. Indeed, in the case of Sev signaling, the Sev receptor interacts directly with DRK (Raabe et al., 1995) and Dos has also been proposed to be a DRK-binding protein. In addition, CSW does not interact with a pY residue in the Sev receptor nor has it been found to be tyrosine phosphorylated in response to Sev activation (Allard et al., 1996); therefore, CSW would not be expected to play an adaptor role in this pathway. Nevertheless, under normal developmental conditions in the terminal pathway, the adaptor function of CSW may be significant, even though other connections between Torso and DRK/SOS/Ras may exist.

Clearly, a role as an adaptor molecule cannot be the sole function of CSW, since catalytic activity is required for CSW to transduce an RTK-mediated signal in *Drosophila*. Therefore, to fully understand the mechanisms of CSW action, biologically relevant substrates must be identified. Here, we show that Torso is a direct substrate of CSW and that CSW specifically dephosphorylates the Torso pY918 site in vitro and in vivo. In addition, we show that the dephosphorylation of pY918 requires the binding of CSW to the Torso pY630 site. This latter observation further demonstrates the specificity of CSW activity in that even when overexpressed in Sf9 cells, CSW could not recognize Torso as a substrate unless it was bound by its SH2 domains to the pY630 site. This binding localizes CSW to its target and may serve to activate CSW. Consistent with this idea, the crystal structure of SHP-2 has revealed that binding of the N-terminal SH2 domain to phosphoproteins converts SHP-2 from a closed conformation to an open, active conformation (Hof et al., 1998). In addition, the catalytic activity of SHP-2 increased dramatically when the SH2 domains were bound to appropriate pY-containing peptides (Lechleider et al., 1993; Pluskey et al., 1995).

Prior to this study, the best defined substrate of CSW has been DOS. DOS was originally identified to be a CSW substrate in experiments examining Sev signaling (Herbst et al., 1996; Raabe et al., 1996). Consistent with our findings, the localization of CSW to DOS via its SH2 domains is critical for CSW to recognize DOS as a substrate (Herbst et al., 1996). Genetic studies have shown

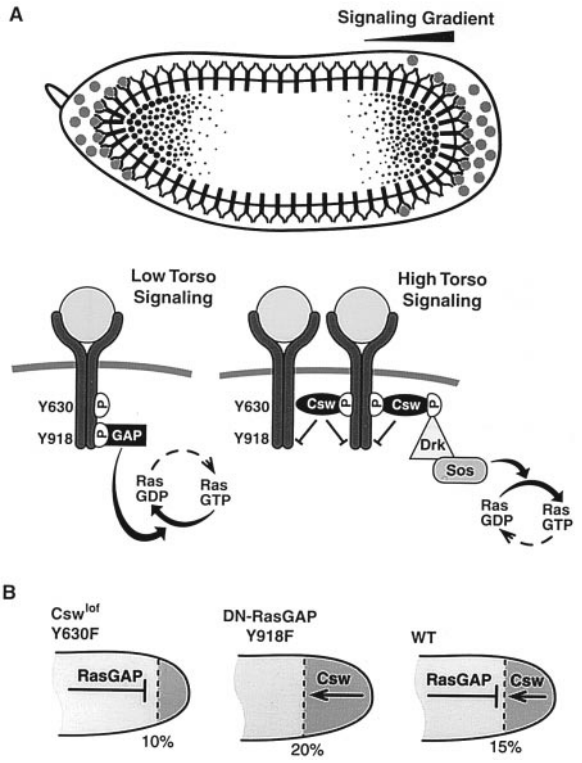

Figure 6. Torso-Dependent Signal Transduction

(A) Torso-dependent signal transduction. A model for the opposing actions of positive (pY630) and negative (pY918) signaling sites on Torso is depicted. See text of the Discussion for details.

(B) Signaling by the Torso RTK establishes an instructive gradient leading to the differential expression of the *tll* and *hkb* genes. Focusing only on *tll* expression in the posterior termini, *tll* is uniformly expressed from 0%–15% EL in WT embryos, whereas in the absence of Torso signaling, *tll* expression is undetectable (not shown). In the absence of CSW activity (Csw^{lof}) or when the pY630/CSW-binding site of Torso is mutated (Y630F), the strength of the Torso signal is diminished and *tll* expression is reduced. In the presence of DN RasGAP or when the pY918/RasGAP-binding site of Torso is mutated (Y918F), the strength of the Torso signal is increased leading to an expansion of *tll* expression. We propose that the signaling gradient, which is initiated by a diffusible ligand, is refined through the opposing actions of positive and negative signaling sites on Torso to precisely define the domain of *tll* and *hkb* expression in developing embryos.

that DOS functions in the terminal pathway, but whether CSW also dephosphorylates DOS during Torso signaling awaits further analysis. Our study, however, identifies Torso to be a biologically relevant target of CSW in the terminal pathway and indicates that by dephosphorylating the pY918 site, CSW prevents the negative regulator RasGAP from associating with Torso. These results are consistent with previous epistasis experiments indicating that CSW functions upstream or parallel to Ras in the terminal pathway (Perkins et al., 1992; Lu et al., 1993). In addition, the observation that the most severe CSW mutants cause less severe phenotypes than those produced by the loss of the RTK itself further supports the idea that CSW is involved in modulating the magnitude of RTK signaling (Perkins et al., 1996). Interestingly, the PDGF receptor has been identified as a substrate of SHP-2 (Klinghoffer and Kazlauskas, 1995). In this study, SHP-2 displayed preferential activity toward three of the five pY sites present on the PDGF receptor, including pY771, which is a binding site for mammalian RasGAP. Thus, dephosphorylation of RasGAP binding sites by CSW/SHP-2 proteins may be a conserved regulatory mechanism in RTK signaling pathways.

Based on results from this and earlier studies, we propose the following model for Torso-dependent signal transduction (Figure 6). Within the first hour after egg laying, Torso is expressed uniformly on the surface of the embryo. Shortly thereafter, Torso signaling is triggered at the termini by a localized diffusible ligand that is limiting. The diffusion of the ligand from the termini to more central regions of the embryo establishes a ligand concentration gradient that is sequentially translated into gradients of Torso, Ras, and MAPK activation (Gabay et al., 1997; Greenwood and Struhl, 1997). The immediate effect of the Torso signal is the inactivation of transcriptional repressors (e.g., NTF-1/Elf-1) and corepressors (e.g., Groucho) that are uniformly distributed throughout the embryo (Liaw et al., 1995; Paroush et al., 1997). The graded derepression of these factors then allows transcriptional activators, also uniformly present throughout the embryo, to selectively activate expression of *tll* and *hkb* in terminal regions. Due to differential sensitivity to the repressor gradient, *tll* and *hkb* are expressed in overlapping but noncontiguous terminal domains. Thus, the use of a gradient provides a mechanism whereby a single signal (the Torso signal) can regulate two separate outcomes (*tll* and *hkb* gene expression).

The internal competition between CSW and D-RasGAP binding can contribute to the establishment of the gradient in the following manner (depicted in Figure 6). In terminal areas (0% EL), high concentrations of ligand induce receptor clustering and Torso activation. Torso becomes tyrosine phosphorylated on sites including Y630. CSW binds to pY630 and stimulates Ras activation by coupling to DRK/SOS and by dephosphorylating the negative pY918 D-RasGAP-binding site on receptor dimers and oligomers. By preventing the recruitment of RasGAP to activated Torso receptors, Ras activity remains high, resulting in the delivery of a strong Torso signal near the posterior pole. In more central regions ($\sim$15% EL), a reduction in the concentration of ligand produces a region where activated receptors are further apart. Under these conditions, dephosphorylation of pY918 by CSW would be expected to be less efficient, allowing RasGAP to bind Torso and downregulate the Torso/Ras signal. The ability to modulate the strength of Torso signaling would provide several advantages. First, it could accentuate and sharpen the differences in the signaling gradient initiated by ligand concentration. In addition, the existence of positive and negative signaling sites on Torso would be more amenable to regulatory controls than would the simple diffusion of the Torso ligand. Thus, we propose that the function of the internal competition between the positive pY630 and negative Y918 signaling sites is to sharpen the gradient of MAPK activity induced by Torso, thereby defining precise boundaries for *tll* and *hkb* expression and subsequent terminal structure development.

Experimental Procedures

Generation of Transgenic Flies and Examination of Embryos

The mata4-GAL4-VP16 line was a gift from D. St Johnston (Wellcome/Cancer Research Campaign Institute, Cambridge, UK). UAS-RasGAP lines were constructed by placing cDNA constructs encoding the full-length sequence or the first 343 amino acids of D-RasGAP (P. Feldmann et al., submitted) under control of the GAL4-dependent upstream activating sequence (UAS). Transgenic lines were obtained by P element–mediated germline transformation according to Spradling (1986). In situ hybridizations on whole-mount embryos were performed according to Tautz and Pfeifle (1989). A digoxigenin-labeled antisense RNA probe was generated using a plasmid containing the *tll* cDNA (Pignoni et al., 1992). For visualization, embryos were mounted in 80% glycerol and photographed with a Zeiss Axiophot microscope equipped with Nomarski optics. The domain of *tll* expression, determined as the percent EL, was measured by scoring 30 embryos for each of the various genetic backgrounds. The Oregon R strain is WT. csw^{lof} embryos were obtained from females carrying germline clones of csw^{LE120} using the FLP-DFS technique (Chou and Perrimon 1992).

Constructs and Reagents

The construction of recombinant baculoviruses encoding Torso 4021, $Torso^{TKM}$, $Torso^{Y630F}$, $Torso^{Y918F}$, $Torso^{Y630F/Y918F}$, and CSW^{WT} has been previously described (Cleghon et al., 1996). CSW^{Y666F} and CSW^{C583S} were generated by site-directed mutagenesis using the appropriate oligonucleotides and a WT CSW cDNA clone (Perkins et al., 1992). A fragment containing the CSW sequences was then cloned into the pVL941 transfer vector for expression in Sf9 cells. To generate the GST D-RasGAP fusion proteins, sequences encoding amino acids 83–343 of D-RasGAP (GST-SHS) or these same sequences containing Arg to Leu substitutions at amino acid positions 110 and 278 (GST-S*HS*) were cloned into pGEX-KG for expression in bacteria. The GST-DRK fusion protein was a gift from T. Pawson (Samuel Lunenfeld Research Institute, Toronto). Immunoprecipitations and immunoblot analysis using Torso, CSW, and anti-pY antibodies were performed as previously described (Cleghon et al., 1996).

In Vitro Binding Assays

GST fusion proteins were expressed in bacteria and purified using glutathione sepharose as previously described (Cleghon and Morrison, 1994). Equal amounts of the immobilized fusion proteins were incubated with extracts of Sf9 cells lysed in Nonidet-40 (NP-40) buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, aprotinin [0.15 U/ml], 1 mM phenylmethylsulfonyl fluoride [PMSF], 20 μM leupeptin, 5 mM sodium vanadate) for 3 hr at 4°C. The complexes were then washed twice with NP-40 buffer and once with Tris-buffered saline (TBS) and were examined by immunoblot analysis.

Identification of CSW Phosphorylation Sites

CSW was coimmunoprecipitated with Torso from lysates of Sf9 cells coexpressing CSW and Torso, and the immune complexes were incubated in 40 μl of kinase buffer containing 25 mM HEPES (pH 7.4), 7 mM $MnCl_2$, 20 μCi [γ^{32}P]ATP at room temperature for 20 min. ^{32}P-labeled CSW proteins were isolated by SDS-PAGE and digested with trypsin. Phosphoproteins were then analyzed by reversed-phase HPLC, phosphoamino acid analysis, and Edman degradation as previously described (Cleghon et al., 1996).

Purification of CSW and In Vitro Phosphatase Assays

Sf9 cells (1×10^8) expressing CSW^{WT} or CSW^{C583S} were washed twice with TBS, and cell pellets were resuspended and sonicated in 10 mL of buffer B (25 mM Tris [pH 7.4], 2 mM EDTA, 10 mM β-mercaptoethanol, aprotinin [0.15 U/ml], 1 mM PMSF, 20 μM leupeptin). Insoluble material was removed by centrifugation, and 5 ml of the supernatant was applied to a HiTrap Q column (Pharmacia) preequilibrated with buffer B. The column was washed with buffer B, and 3 ml fractions were collected. Samples containing CSW were adjusted to a final concentration of 10% glycerol and stored at −80°C. As a substrate for in vitro CSW assays, Torso proteins were immunoprecipitated from Sf9 cells and allowed to autophosphorylate in the presence of γ-[^{32}P]ATP as described previously (Cleghon et al., 1996). After the phosphorylation reaction, the immunoprecipitates were washed extensively with NP-40 buffer to remove unincorporated radioactivity and then washed twice and resuspended in phosphatase buffer containing 50 mM HEPES [pH 7.4], 5 mM dithiothreitol, 1 mM EDTA, and 150 mM NaCl. Equal aliquots of ^{32}P-labeled Torso were incubated with various amounts of purified CSW proteins in phosphatase buffer for 15 min at 30°C. Samples were centrifuged briefly, and the radioactivity released into the supernatant was determined by scintillation counting. To examine the dephosphorylation of individual sites, phosphatase reactions were fractionated by SDS-PAGE, the ^{32}P-labeled Torso proteins were extracted from the gel and digested with trypsin, and the tryptic phosphopeptides were separated and eluted from a reversed-phase HPLC C18 column (Cleghon et al., 1996). The radioactivity present in fractions containing specific phosphopeptides was then quantitated.

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