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Recruitment of the protein tyrosine phosphatase CSW by DOS is an essential step during signaling by the Sevenless receptor tyrosine kinase

Ronald Herbst[1,2], Xiaolong Zhang[3,4], Jun Qin[3,4] and Michael A.Simon[1,5]

[1]Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020, [2]DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304 and [3]Laboratory of Biophysical Chemistry NHLBI National Institutes of Health, Bethesda, MD 20892, USA

[4]Present address: Department of Biochemistry and Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

[5]Corresponding author
e-mail: msimon@leland.stanford.edu

The pleckstrin homology (PH) domain-containing protein Daughter of Sevenless (DOS) is an essential component of the Sevenless receptor tyrosine kinase (SEV) signaling cascade, which specifies R7 photoreceptor development in the *Drosophila* eye. Previous results have suggested that DOS becomes tyrosine phosphorylated during SEV signaling and collaborates with the protein tyrosine phosphatase CSW. We have investigated this possibility by identifying tyrosine residues 801 and 854 of DOS as the phosphorylated binding sites for the CSW SH2 domains. We show that these sites become phosphorylated in response to SEV activation and that phosphorylation of both sites is required to allow CSW to bind DOS. Mutant DOS proteins in which either Y801 or Y854 of DOS has been changed to phenylalanine are unable to function during signaling by SEV and other receptor tyrosine kinases. In contrast, we find that a mutant DOS protein in which all tyrosine phosphorylation sites except Y801 and Y854 have been removed is able effectively to provide DOS function during SEV signaling and to rescue the lethality associated with *dos* loss-of-function mutations. These results indicate that a primary role for DOS during signaling by SEV and other receptor tyrosine kinases is to become phosphorylated at Y801 and Y854 and then recruit CSW.

Keywords: Corkscrew/Daughter of Sevenless/protein tyrosine phosphatase/Sevenless/signal transduction

Introduction

Receptor tyrosine kinases (RTKs) regulate cellular growth, differentiation and metabolism in response to extracellular signals. The binding of a specific ligand to the extracellular domain of an RTK results in receptor dimerization, activation of the intracellular kinase domain and autophosphorylation of the RTK on specific tyrosine residues (Schlessinger and Ullrich, 1992). In many RTKs, the phosphorylation of these tyrosine residues can then stimulate the activity of intracellular signal transduction pathways by providing docking sites for src homology (SH) 2 domain-containing proteins. Among the signal transduction proteins known to be recruited directly to activated RTKs are phospholipase Cγ (PLCγ), phosphatidylinositol 3′ kinase (PI3K), the protein tyrosine phosphatase SHP-2 and the adaptor molecule GRB2, which is itself bound to the Ras activator SOS (Cantley *et al*., 1991; Pawson, 1995).

In recent years, it has become evident that RTKs can also regulate the activity of intracellular signaling pathways in a more indirect fashion. Rather than binding directly to SH2 domain-containing proteins, many RTKs phosphorylate other proteins, called scaffold or adaptor proteins, that reside at the plasma membrane (Pawson and Scott, 1997). The phosphorylation of these scaffold proteins stimulates intracellular signaling pathways by providing docking sites for many of the same SH2 domain-containing proteins previously shown to bind directly to activated RTKs. One well-studied example of the use of phosphorylated scaffold proteins during RTK signaling involves the insulin receptor (IR). IR activation leads to the rapid phosphorylation of several closely related scaffold proteins, called insulin receptor substrates (IRSs), which then provide multiple binding sites for signaling molecules such as PI3K and SHP-2 (Yenush and White, 1997). Other examples of phosphorylated scaffold proteins used in mammalian cells include Shc, Gab1, Gab2, p62^{dok} and FRS2 (reviewed in Pawson and Scott, 1997; Gu *et al*., 1998). Similarly to IR, many other RTKs appear to phosphorylate several scaffold proteins. Given this multitude of scaffold proteins, a key step in understanding the detailed action of a particular RTK is to identify which scaffold proteins become phosphorylated and which of the SH2 domain-containing signaling proteins must be recruited to each scaffold protein.

One of the most extensively studied examples of RTK signaling is the role of the Sevenless RTK (SEV) during R7 photoreceptor development in the *Drosophila* compound eye (Zipursky and Rubin, 1994). Each subunit of the eye, called an ommatidium, contains a central core of eight photoreceptor cells (R1–R8) that can be distinguished by their position and morphology. The decision of the R7 precursor cell to become a photoreceptor is absolutely dependent on the activation of SEV by its ligand, Bride of Sevenless (BOSS), which is expressed on the surface of the neighboring R8 photoreceptor. In the absence of either SEV or BOSS function, the R7 precursor cell fails to become a photoreceptor and instead differentiates as a lens-secreting cone cell.

Once bound to BOSS, SEV stimulates the activation of the RAS1/MAPK intracellular signaling pathway by direct recruitment of DRK, the *Drosophila* homolog of the mammalian GRB2 protein, and its binding partner, the RAS1-activating protein SOS, to a phosphorylation site in the C-terminal tail of SEV (Olivier *et al*., 1993; Simon *et al*., 1993). However, a mutant SEV protein lacking this

putative phosphorylation site retains considerable function (Raabe *et al*., 1995). These results suggest that SEV also regulates RAS1 function through other mechanisms including the use of phosphorylated scaffold proteins to recruit DRK–SOS. Once activated by SOS, RAS1 stimulates a protein kinase cascade leading to the activation of Rolled, the *Drosophila* MAPK homolog, which then regulates the function of several transcription factors including the ETS-type transcription factors PNT^{P2} and YAN (Wasserman *et al*., 1995).

In addition to the core components of the RAS1/MAPK pathway, SEV signaling also requires the action of the Corkscrew protein tyrosine phosphatase (CSW) (Allard *et al*., 1996). CSW and its mammalian homolog SHP-2 are characterized by the presence of two N-terminal SH2 domains as well as a single protein tyrosine phosphatase (PTP) catalytic domain (reviewed in Feng and Pawson, 1994). SHP-2 has been implicated in signaling by many mammalian RTKs including the receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors and insulin. In *Drosophila*, CSW has been shown to be important for signaling by the EGF receptor homolog, the Torso RTK (TORSO) as well as for SEV (Perkins *et al*., 1992, 1996; Allard *et al*., 1996). In several cases, inhibition of CSW/SHP-2 function has been shown to reduce RTK-induced MAPK activation (reviewed in Matozaki and Kasuga, 1996). These observations have led to the suggestion that one important function of CSW/SHP-2 is to modulate RAS activation. Studies of signaling by the PDGF receptor and the closely related TORSO RTK, which is required to specify cell fates at the termini of the early *Drosophila* embryo, have supported two models for how such regulation of RAS might be achieved. In both models, CSW/SHP-2 uses its SH2 domains to bind to a phosphorylation site on the activated RTK. In one model, CSW/SHP-2 then stimulates RAS activity by becoming phosphorylated in its C-teminal tail region and providing a docking site for GRB2 (Bennet *et al*., 1994; Li *et al*., 1994). In the second model, CSW acts by dephosphorylating a $p120^{GAP}$-binding site on the RTK and thus preventing the inactivation of RAS (Cleghon *et al*., 1998).

In contrast, the analysis of the role of CSW during R7 development has indicated that CSW has either different or additional roles during signaling by SEV (Allard *et al*., 1996). For example, while the expression of activated versions of RAS or RAF can drive R7 photoreceptor development even in the absence of functional SEV, signaling by these activated proteins still requires CSW function. This suggests that a crucial portion of CSW function occurs either downstream of RAS/RAF activation in the MAPK pathway or in a separate signaling pathway. Furthermore, the activation of SEV does not lead to detectable phosphorylation of CSW. This failure of CSW to become phosphorylated by SEV is similar to the lack of SHP-2 phosphorylation in response to IR activation. Furthermore, the C-terminal region of CSW, which contains the putative GRB2-binding site, is dispensable during SEV signaling (Allard *et al*., 1998). These results suggest that the role of CSW during SEV signaling might be quite different from during signaling by either TORSO or PDGF receptor.

A key step in understanding how CSW functions during SEV signaling is identifying the important regulators of CSW activity in the R7 cell. One protein that has been suggested for this role is the product of the *daughter of sevenless* (*dos*) gene (Raabe *et al*., 1996). Genetic analysis has indicated that DOS is a positively acting component of RTK pathways and has suggested that CSW and DOS might act together during SEV signaling (Herbst *et al*., 1996; Raabe *et al*., 1996). *dos* encodes a putative phosphorylated scaffold protein containing an N-terminal pleckstrin homology (PH) domain in addition to multiple tyrosine residues that are potential binding sites for CSW as well as other SH2 domain-containing proteins including GRB2, SHC, PLCγ, PI3K and NCK (Raabe *et al*., 1996). Consistent with a role for DOS as a phosphorylated binding partner of CSW, tyrosine-phosphorylated DOS and a catalytically inactive CSW protein stably associate *in vivo* in a CSW SH2 domain-dependent manner, and isolated CSW SH2 domains can precipitate tyrosine-phosphorylated DOS from cell extracts (Herbst *et al*., 1996).

While these observations are consistent with the idea that the crucial role of DOS during SEV signaling is to become phosphorylated and bind CSW, the ability of DOS to interact with many other signaling molecules raised the question of which of the possible actions of DOS are actually required for signaling by SEV and other RTKs. In this report, we have addressed this question by identifying phosphorylated Y801 and Y854 of DOS as the binding sites for the CSW SH2 domains. We show that these sites become phosphorylated in response to SEV activation and that mutation of either residue to phenylalanine inactivates DOS function and generates a DOS protein that dominantly interferes with the action of wild-type DOS. Furthermore, a DOS protein that can only be phosphorylated at these two tyrosine residues supports signaling by SEV and other RTKs. These results support a model in which a crucial role for DOS during RTK signaling is to become phosphorylated at Y801 and Y854 and provide binding sites for CSW.

Results

Identification of CSW-binding sites in DOS

As a first step towards evaluating the importance of the recruitment of CSW by DOS during RTK signaling, we identified the phosphorylation sites in DOS that mediate the interaction with the SH2 domains of CSW. We made use of our previous observation that expression of a catalytically inactive CSW (CSW^{CS}) in *Drosophila* SL2 cells leads to the formation of a stable complex between CSW^{CS} and tyrosine-phosphorylated DOS, which is protected from dephosphorylation by wild-type CSW and other endogenous PTPs (Herbst *et al*., 1996). This same interaction can also be observed between DOS and wild-type CSW when cells are treated with the PTP inhibitor pervanadate, thus suggesting that the primary effect of the CSW^{CS} change is to prevent CSW from dephosporylating DOS. Since the interaction of CSW^{CS} and tyrosine-phosphorylated DOS depends on CSW SH2 domain function, we reasoned that DOS purified from these complexes would be phosphorylated at the CSW SH2 domain-binding sites and perhaps at other sites as well. To examine this possibility, extracts of *Drosophila* SL2 cells producing

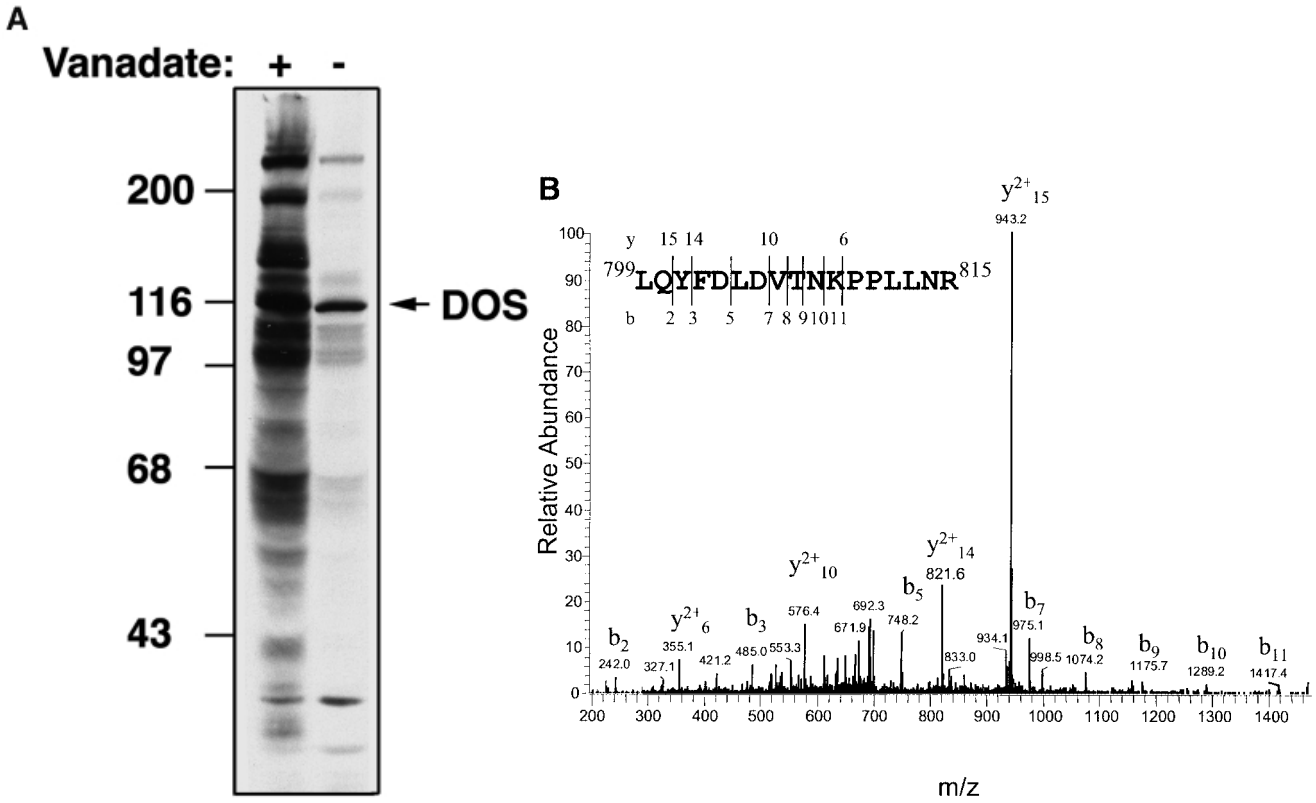


Fig. 1. Identification of CSW-binding sites in DOS. (**A**) Inactive CSW^{CS} protects DOS from dephosphorylation by endogenous PTPs. SL2-CSW^{CS} cells were lysed in the presence (+) or absence (–) of the PTP inhibitor vanadate and the lysates were incubated for 1 h at room temperature. Insoluble material was then removed by centrifugation and the cleared lysates were analyzed by SDS–PAGE and anti-phosphotyrosine immunoblots. In the absence of vanadate, virtually all proteins became dephosphorylated, except for DOS, which was in a stable complex with CSW^{CS}. (**B**) Mass spectrometric identification of the tyrosine phosphorylation sites in DOS. For the identification of CSW-binding sites, tyrosine-phosphorylated DOS was purified from SL2-CSW^{CS}-DOS cells as described in Materials and methods. Under the conditions used, only tyrosine residues which are involved in the formation of a CSW^{CS}–DOS complex are phosphorylated. Shown is the LC/MS/MS spectrum of the +3 charged phosphopeptide 799-LQYFDLDVTNKPPLLNR-815. Y801, but not T807, was identified as phosphorylated. N- and C-terminal fragment ions were labeled as b and y, respectively, according to the Biemann nomenclature (Biemann, 1988).

CSW^{CS} (SL2-CSW^{CS} cells) were prepared in the absence of PTP inhibitors and incubated at room temperature to allow for dephosphorylation of cellular proteins by endogenous phosphatases. Under these conditions, DOS is the major phosphotyrosine-containing protein in the extract (Figure 1A). The proteins in the extract were then denatured by treatment with SDS. After dilution of the SDS, tyrosine-phosphorylated proteins were purified by anti-phosphotyrosine affinity column chromatography. The eluted proteins were then analyzed by SDS–PAGE. Coomassie Blue staining of the gel revealed a major band that was the expected size for DOS (115 kDa).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was then used to analyze an in-gel trypsin digestion of the 115 kDa band. Two phosphopeptides were identified as judged by the disappearance of two peaks and the appearance of two new peaks with a mass of 80 Da less upon treatment of the trypsin digest with a calf intestine phosphatase. Liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) was used to sequence these two phosphopeptides and identify the phosphorylation sites. As shown in Figure 1B, the LC/MS/MS spectrum of a +3 charged peptide confirms the phosphopeptide as 799-LQYFDLDVTNKPPLLNR-815. The observation of the b2 (m/z 242.0) and b3 (m/z 485.0) ions (Biemann, 1988) unambiguously identified Y801 rather than T807 as the phosphorylated residue, and this was confirmed further by the y2 + 14 and y2 + 15 ions. Similarly, Y854 was identified as phosphorylated in the other phosphopeptide (data not shown).

The phosphorylation of DOS Y801 and Y854 in SL2-CSW^{CS} cells and the dependence of the CSW^{CS}–DOS interaction on CSW SH2 domain function suggested that these phosphorylated tyrosines might be the binding sites for the CSW SH2 domains. Further support for this hypothesis came from the analysis of the amino acid sequences surrounding Y801 and Y854. Each region showed a high degree of similarity to sequences found in the IRS1 protein and other phosphorylated scaffold proteins such as GAB-1, GAB-2, FRS-2, other IRS family members and the receptor-type SHP-2 substrate SHPS. The region around Y801 is particularly well conserved among these proteins and is characterized by hydrophobic residues at the –2, +1, +3 and +5 positions relative to the tyrosine residue. In IRS-1, the tyrosine residues corresponding to DOS Y801 and Y854, Y1172 and Y1222, have been identified as the binding sites for the SHP-2 SH2 domains (Sun *et al.*, 1993; Rocchi *et al.*, 1995).

The ability of the Y801 and Y854 sites to serve as CSW SH2 domain-binding sites was tested directly in a series of *in vitro* binding experiments. Tyrosine-phosphorylated peptides representing the Y801 and Y854 sites of DOS

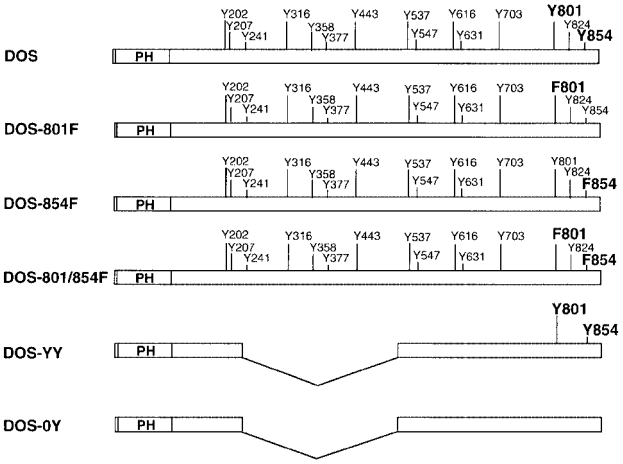
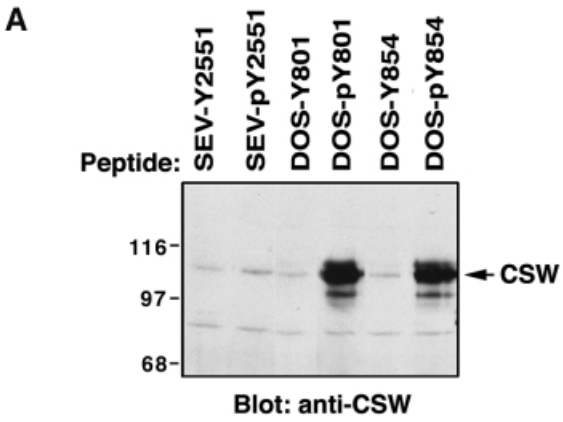


Fig. 2. Schematic presentation of the DOS constructs used.



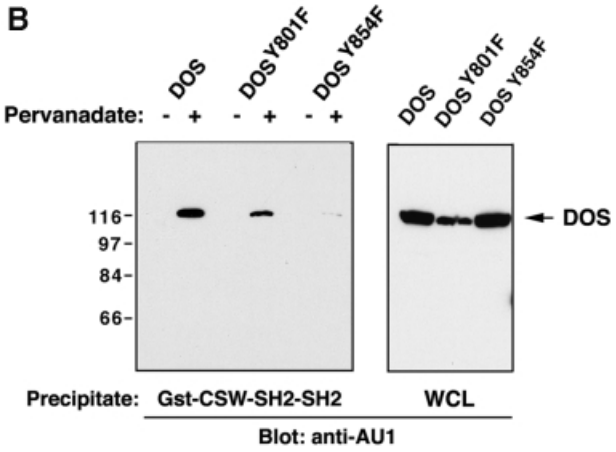


Fig. 3. Binding of CSW to DOS residues Y801 and Y854. (**A**) Binding of CSW to DOS phosphopeptides. For each sample, the lysate of 1×10^7 SL2 cells was incubated with unphosphorylated or phosphorylated peptides, cross-linked to Affigel-15, as indicated. The binding of CSW was analyzed by SDS–PAGE followed by immunoblotting with the anti-CSW antiserum CT1. CSW only bound to the tyrosine-phosphorylated peptides DOS-pY801 and DOS-pY854. CSW did not bind to the peptide SEV-pY2551, which corresponds to the autophosphorylation site in the C-terminus of SEV. (**B**) Binding of DOS and DOS mutants to CSW SH2 domains. SL2 cells expressing AUI-tagged versions of either wild-type DOS, DOS^{Y801F} or DOS^{Y854F} were left untreated or were incubated with pervanadate for 10 min in order to increase DOS tyrosine phosphorylation. The cells were lysed and the lysates incubated with the fusion protein GST–CSW-SH2-SH2, which contains both CSW SH2 domains. Binding of DOS and DOS mutants to the CSW SH2 domains was analyzed by SDS–PAGE followed by immunoblotting with mAb AUI (left panel). To control for expression levels, an anti-AUI immunoblot was performed on whole-cell lysates (WCL) from the indicated cell lines (right panel).

were coupled to agarose beads and assayed for their ability to precipitate endogenous CSW from SL2 cell lysates. As shown in Figure 3A, the tyrosine-phosphorylated peptides representing the DOS phosphorylation sites Y801 and Y854 were able to precipitate endogenous CSW from SL2 cell lysates. In contrast, corresponding unphosphorylated peptides as well as an unrelated tyrosine-phosphorylated peptide representing a putative C-terminal autophosphorylation site of SEV failed to bind CSW. To verify further Y801 and Y854 as CSW SH2 domain-binding sites, we mutated the Y801 or Y854 sites (DOS^{Y801F} and DOS^{Y854F}; see Figure 2 for a schematic presentation of DOS constructs) and assayed the effect of these mutations on the binding of DOS to a GST fusion protein containing both CSW SH2 domains (GST–CSW-SH2-SH2). Stable SL2 cell lines expressing AU1-tagged versions of either wild-type DOS, DOS^{Y801F} or DOS^{Y854F} were treated with pervanadate to induce DOS tyrosine phosphorylation, lysed and subjected to precipitation by glutathione–agarose beads to which the GST–CSW-SH2-SH2 was bound (Figure 3B). While the binding of the wild-type DOS protein to GST–CSW-SH2-SH2 could be detected easily, mutation of Y854 significantly reduced the interaction of DOS with the CSW SH2 domains. The mutation of DOS Y801 to phenylalanine had a less pronounced effect on binding to GST–CSW-SH2-SH2, but the interaction was still reduced. Taken together, these data confirm that phosphorylation of DOS Y801 and Y854 generates binding sites for the SH2 domains of CSW.

DOS tyrosine residues Y801 and Y854 are each required for stable binding to CSW

Our analysis of the sites of phosphorylation in CSW^{CS}-bound DOS and of the binding of DOS to CSW-containing fusion proteins *in vitro* supports a model in which both SH2 domains of CSW are bound simultaneously to phosphorylated Y801 and Y854 of DOS. In order to determine the importance of each of these interactions to the DOS–CSW interaction, we assayed the effects of mutating either Y801 or Y854 to phenylalanine residues. For these experiments, stable SL2 cell lines were established that expressed both CSW^{CS} and AU1 epitope-tagged versions of either wild-type DOS, DOS^{Y801F} or DOS^{Y854F}. In addition, we generated an AU1 epitope version of DOS (DOS^{YY}) in which all tyrosines outside of the DOS PH domain except Y801 and Y854 were either removed by deletion or changed to phenylalanine (see Figure 2). The ability of the mutant DOS proteins to form a complex with CSW^{CS} was monitored by their ability to be co-precipitated with CSW^{CS} and to be protected from dephosphorylation. As shown in Figure 4, changing either Y801 or Y854 to phenylalanine markedly affects the

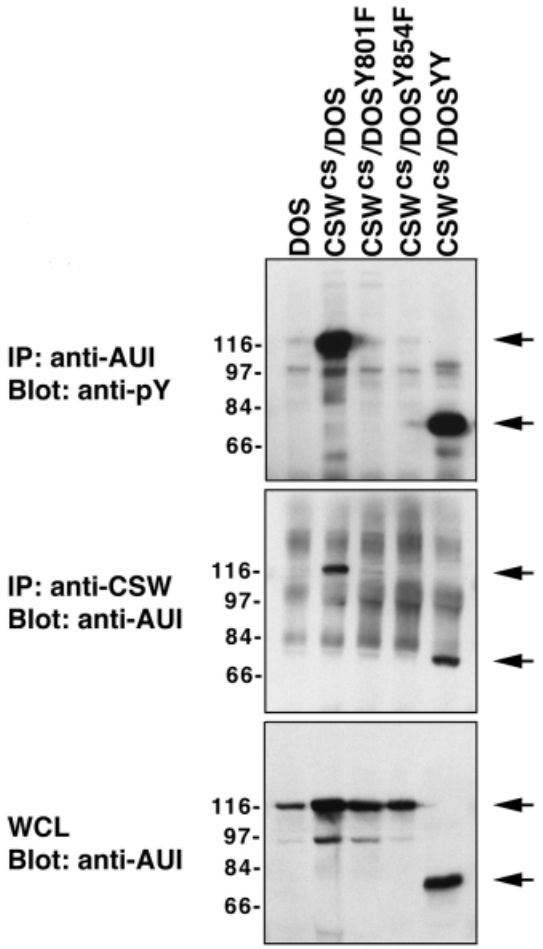


Fig. 4. DOS residues Y801 and Y854 are both required for efficient complex formation with CSW in intact cells. SL2 cells co-expressing CSW^{CS} together with AUI-tagged versions of wild-type DOS or DOS mutants were lysed and the lysates subjected to immunoprecipitation with mAb AUI. Cells which only express wild-type DOS were used as a control. Constitutive tyrosine phosphorylation of individual DOS constructs was analyzed by anti-phosphotyrosine immunoblotting (upper panel). Complex formation of DOS and DOS mutants with CSW^{CS} was determined by anti-CSW immunoprecipitation followed by anti-AUI immunoblotting (middle panel). To control for expression levels of DOS constructs in the different cell lines, whole-cell lysates (WCL) were blotted with mAb AUI (bottom panel). In contrast to wild-type DOS and DOS^{YY}, DOS^{Y801F} and DOS^{Y854F} are not constitutively phosphorylated in the presence of CSW^{CS} and do not form a stable complex with the phosphatase. The arrows indicate the position of the full-length and internally deleted (DOS^{YY}) forms of DOS.

interaction of DOS with CSW^{CS}. Compared with wild-type DOS, co-precipitation of DOS^{Y801F} with CSW^{CS} was substantially reduced, while DOS^{Y854F} co-precipitation could not be detected at all (Figure 4). Similarly, the ability of CSW^{CS} expression to induce DOS tyrosine phosphorylation was eliminated by each mutation. In contrast, the ability of the DOS^{YY} protein to interact with CSW^{CS} was not impaired. When combined with the phosphorylation site mapping and *in vitro* binding studies, these results demonstrate that the phosphorylation of both Y801 and Y854 of DOS is essential for binding to CSW. These results also suggest that interaction of the CSW SH2 domains with DOS residue Y854 is the major contributor to the overall binding affinity.

DOS tyrosine residues Y801 and Y854 are phosphorylated in response to SEV activation

Our previous studies have demonstrated that the expression in SL2 cells of an activated form of SEV, SEV^{S11} (Basler *et al.*, 1991), leads to a significant induction of DOS tyrosine phosphorylation (Herbst *et al.*, 1996). When combined with the ability of DOS to bind CSW upon phosphorylation at Y801 and Y854, this observation suggests that an important function of SEV might be to phosphorylate DOS Y801 and Y854 and thus generate the CSW-binding sites. To provide evidence for this model, we further examined the effect of expressing SEV^{S11} on the state of tyrosine phosphorylation of the DOS^{YY} protein. SL2 cells were generated that expressed SEV^{S11} under the control of an inducible heat shock promoter and also constitutively expressed either AU1-tagged wild-type DOS, DOS^{YY} or a mutated version of DOS^{YY}, called DOS^{0Y}, in which the Y801 and Y854 residues were changed to phenylalanine. As shown in Figure 5, heat shock induction resulted in rapid synthesis of SEV^{S11} and led to increased tyrosine phosphorylation of both wild-type DOS and DOS^{YY}. In contrast, induction of an inactive version of SEV^{S11}, SEV^{S11KM}, had no effect on DOS tyrosine phosphorylation. Unlike wild-type DOS and DOS^{YY}, DOS^{0Y} was not phosphorylated to detectable levels upon SEV^{S11} induction. These results demonstrate that SEV can mediate the tyrosine phosphorylation of at least one, and possibly both, of the CSW-binding sites in DOS. They also support a model in which activated SEV either directly or indirectly phosphorylates DOS and thus initiates the recruitment of CSW to DOS.

Mutation Y801 or Y854 of DOS blocks function in vivo

In order to determine the importance of the recruitment of CSW by DOS during RTK signaling, we assayed the *in vivo* function of DOS^{Y801F}, DOS^{Y854F} and a version of DOS ($DOS^{Y801F, Y854F}$) in which both sites were mutated. Each mutation was placed into a construct that contained a *dos* cDNA expressed under the control of a hybrid *sevenless* enhancer/heat shock promoter transcriptional control element. This cassette (*sE*) directs constitutive expression in a subset of cells in the *Drosophila* eye including the photoreceptor R7, R3 and R4 precursor cells as well as all four of the cone cell precursors (Basler *et al.*, 1989). In addition, the *sE* cassette can also provide pulses of ubiquitous expression when the flies are placed at 37°C for short periods.

The mutated *sE-dos* constructs were introduced into the genome by P element-mediated germline transformation and assayed in two ways for their ability to function during eye development. The first assay took advantage of our previous observation that flies expressing the inhibiting CSW^{CS} protein driven by the *sE* cassette have roughened eyes. This rough-eye phenotype results from the frequent failure of the R3, R4 and R7 cells to differentiate as photoreceptor cells due to insufficient CSW function (Herbst *et al.*, 1996). Since this phenotype is significantly enhanced by the loss or inactivation of one allele of *dos* (Figure 6F and G), we asked if the expression of the mutant DOS proteins could suppress the phenotype of flies that both carried the *sE-csw*^{CS} transgene and were heterozygous for a *dos* loss-of-function allele. The second

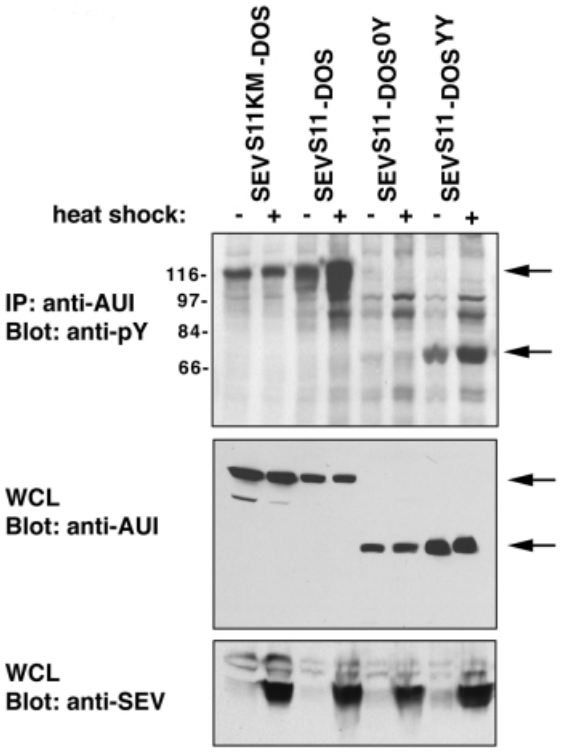


Fig. 5. DOS residues Y801 and Y854 are phosphorylated in response to SEV activation. SL2 cells which constitutively express AUI-tagged versions of DOS, DOS^{0Y} or DOS^{YY}, together with SEV^{S11}, which is under the control of a heat shock promotor, were incubated at 37°C for 30 min and then returned to 23°C for 6 h to allow for expression of SEV^{S11}. Cells that express a kinase-inactive version of SEV^{S11}, SEV^{S11KM}, were used as a control. Cells were lysed and tyrosine phosphorylation of DOS and DOS mutants was analyzed by anti-AUI immunoprecipitation, followed by anti-phosphotyrosine immunoblotting (upper panel). Amido black staining of the filter revealed that equal amounts of DOS were present in each pair of untreated and heat-shocked samples (data not shown). To control for expression of the DOS and SEV^{S11} constructs, whole-cell lysates (WCL) were probed with monoclonal AUI (middle panel) or the anti-SEV mAb 78C10 (bottom panel). Upon heat shock induction of SEV^{S11}, both wild-type DOS and DOS^{YY}, which only retains tyrosine residues Y801 and Y854, showed increased phosphorylation of tyrosine residues. DOS^{0Y}, in which all tyrosine residues outside the PH domain are removed, did not become phosphorylated. The arrows indicate the position of the full-length and internally deleted (DOS^{0Y} and DOS^{YY}) forms of DOS.

assay took advantage of our observation that DOS function is a limiting factor during signaling by the activated Sev^{S11} allele. Sev^{S11} flies have rough eyes that result from the commitment of cone cell precursors, which express but normally fail to activate SEV due to their lack of contact with the BOSS-producing R8 cell, to R7 cell fate. Since expression of additional wild-type DOS under *sE* control (*sE-dos*) can substantially enhance the rough-eye phenotype of Sev^{S11} flies (Figure 6B and C), the function of mutant DOS proteins was tested by examining their ability to enhance the Sev^{S11} phenotype.

In both of these assays, the mutant DOS proteins failed to function normally (Figure 6). While the expression of wild-type DOS strongly suppressed the rough-eye phenotype of *sE-csw*^{CS}; *dos*/+ flies, expression of either DOS^{Y801F}, DOS^{Y854F} or $DOS^{Y801F,Y854F}$ led instead to markedly enhanced eye roughness. Similarly, expression of the mutant DOS proteins led to a significant suppression of the Sev^{S11} phenotype rather than the enhancement caused by the expression of wild-type DOS. These results suggest that mutation of Y801 or Y854 yields DOS proteins that inhibit rather than contribute to signaling by SEV. Consistent with this idea, otherwise wild-type flies that carry two copies of either the *sE-dos*^{Y801F} or *sE-dos*^{Y854F} transgenes had either slightly or moderately rough eyes, respectively. Sectioning of the eyes of these flies showed that their ommatidia frequently lacked R7 cells and occasionally lacked one or two of the R1–R6 class photoreceptors (Figure 7). This phenotype is similar to that previously observed for *sE*-driven expression of dominantly inhibiting forms of CSW and RAS1. These results indicate that the presence of tyrosines, and presumably tyrosine phosphorylation, at positions 801 and 854 is crucial for DOS function and strongly support the model that the binding of CSW to these sites is crucial for signaling by SEV. In contrast, flies carrying two copies of the *sE-dos* transgene generally had eyes with wild-type morphology. However, we did note that certain insertions of the *sE-dos* transgene did result in slightly rough eyes when present in two copies (Figure 7A). Sectioning of these eyes revealed the presence of some ommatidia in which the R7 photoreceptor cell appeared to be morphologically transformed into an R1–R6 class photoreceptor (Figure 7D).

The mutant proteins were also examined for their ability to function in tissues other than the eye. Since animals that lack zygotic DOS function die prior to pupation, heat shock promoter-driven expression of DOS was used to test whether expression of the mutant proteins could rescue the defects of animals heterozygous for two *dos* loss-of-function alleles, *dos*$^{1.46}$ and *dos*$^{2.42}$ (Herbst *et al*., 1996). In these experiments, animals were incubated at 37°C for 1.5 h each day throughout their development. Upon expression of wild-type DOS, *dos*$^{1.46}$/*dos*$^{2.42}$ individuals were recovered at the expected Mendelian ratios and were normal in appearance. Sectioning of these eyes showed that most of the ommatidia were constructed normally. However, some ommatidia were observed that lacked either R7 or the normal number of outer photoreceptors. In contrast, expression of either DOS^{Y801F} or DOS^{Y854F} failed to yield any viable *dos*$^{1.46}$/*dos*$^{2.42}$ adults. Since SEV is not required for viability, these results suggest that the binding of CSW to DOS is also crucial for signaling by RTKs besides SEV. Consistent with this idea, both DOS and CSW appear to be important contributors to signaling downstream of TORSO and the *Drosophila* EGF receptor homolog, DER (Perkins *et al*., 1992, 1996; Raabe *et al*., 1996).

DOS function does not require tyrosine phosphorylation at sites other than Y801 and Y854

We have demonstrated previously that the induction of SEV^{S11} expression in cells that also express CSW^{CS} leads to a marked increase in DOS tyrosine phosphorylation (Herbst *et al*., 1996). Since the DOS in these cells is already bound to CSW^{CS} and therefore phosphorylated at Y801 and Y854, this increase in DOS tyrosine phosphorylation is likely to result from the phosphorylation of DOS at additional tyrosine residues. Consistent with this idea, the induction of activated SEV results in a decrease in the electrophoretic mobility of DOS in SDS–acrylamide

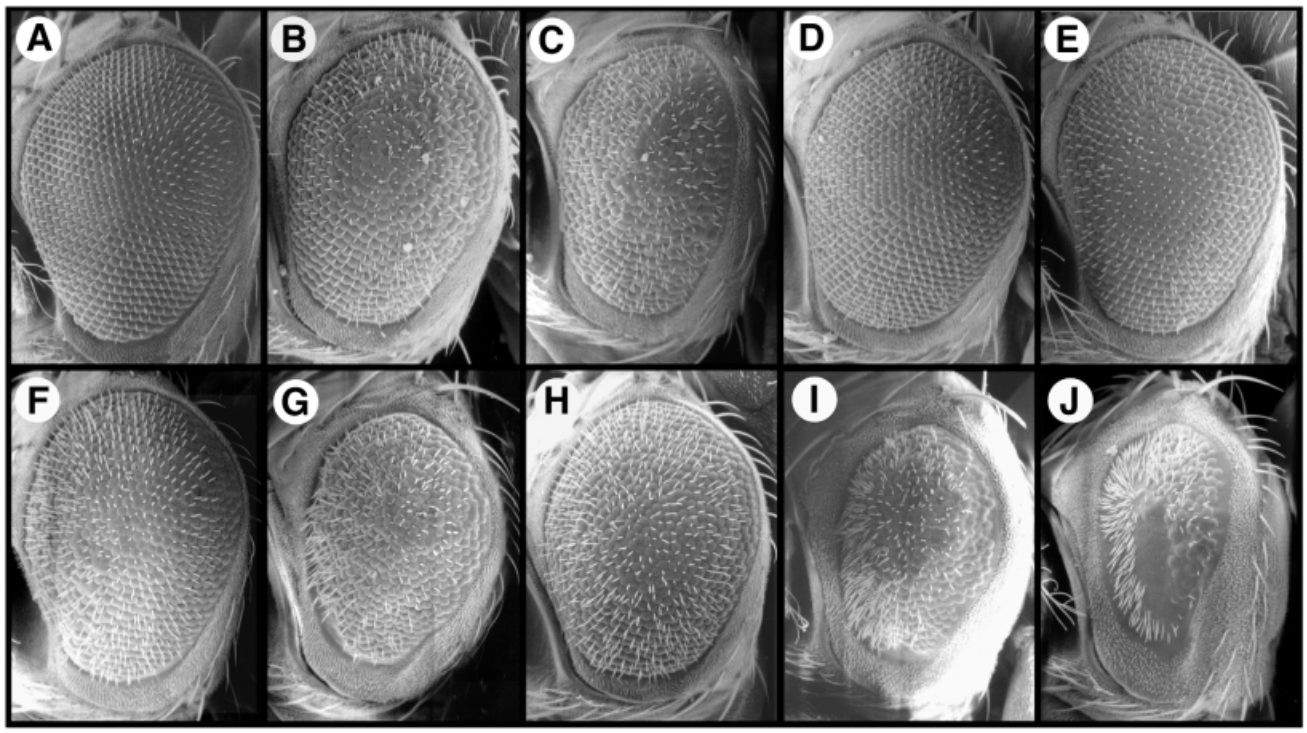


Fig. 6. DOS^{Y801F} and DOS^{Y854F} fail to function during eye development. (**A**) A scanning electron micrograph (SEM) of a phenotypically wild-type eye (w^{1118}) characterized by the regular pattern of the ommatidia that comprise the compound eye. (**B**) An SEM of a male fly hemizygous for Sev^{S11}. The eye is rough in appearance due to the presence of multiple R7 cells. (**C**) An SEM of a male fly hemizygous for Sev^{S11} and heterozygous for *P[sE-dos]*. The expression of wild-type DOS enhances the Sev^{S11} phenotype. (D and E) SEMs of male flies hemizygous for Sev^{S11} and heterozygous for either (**D**) $P[sE\text{-}dos^{Y801F}]E$ or (**E**) $P[sE\text{-}dos^{Y854F}]B$. In contrast to wild-type DOS, expression of either DOS^{Y801F} or DOS^{Y854F} suppresses the Sev^{S11} phenotype and the resulting eyes are only slightly rough. (**F**) An SEM of a fly heterozygous for $P[sE\text{-}csw^{CS}]1\text{-}1$ (Herbst *et al*., 1996). The eye is roughened due to the frequent absence of photoreceptors R7, R3 and R4. (**G**) An SEM of a fly heterozygous for $P[sE\text{-}csw^{CS}]1\text{-}1$ and $dos^{1.46}$. Due to the loss of one functional *dos* allele, the phenotype caused by $P[sE\text{-}csw^{CS}]1\text{-}1$ is more pronounced, resulting in a smaller and rougher eye. (**H**) An SEM of a fly heterozygous for both $P[sE\text{-}csw^{cs}]1\text{-}1$, $dos^{1.46}$ and *P[sE-dos]10*. Increased expression of wild-type DOS suppresses the enhancing effect of the $dos^{1.46}$ allele on the $P[sE\text{-}csw^{cs}]1\text{-}1$ phenotype. (I and J) SEMs of male flies heterozygous for $P[sE\text{-}csw^{cs}]1\text{-}1$, $dos^{1.46}$ and either (**I**) $P[sE\text{-}dos^{Y801F}]E$ or (**J**) $P[sE\text{-}dos^{Y854F}]B$. In contrast to wild-type DOS, expression of either DOS^{Y801F} or DOS^{Y854F} enhances the $P[sE\text{-}csw^{cs}]1\text{-}1$, $dos^{1.46}$ phenotype and the resulting eyes are much rougher.

gels. These results, combined with the presence of putative binding sites in DOS for several SH2 domain-containing signaling molecules, raised the possibility that DOS might contribute to RTK signaling by providing docking sites for the SH2 domains of important signaling molecules besides CSW. In order to investigate this possibility, we assayed the ability of the DOS^{YY} protein, which lacks all tyrosine residues outside of the PH domain except for Y801 and Y854, to provide DOS function. Our first step was to assay the ability of DOS^{YY} to provide DOS function under conditions of limiting DOS function. Like wild-type DOS, *sE*-driven DOS^{YY} expression was able to suppress the rough-eye phenotype of $sE\text{-}csw^{CS}$, *dos*/+ flies and enhance the rough-eye phenotype of Sev^{S11} flies (Figure 8). While direct quantitative comparison between the function of DOS^{YY} and wild-type DOS was difficult because of possible differences in the expression of transgenes inserted at different chromosomal sites, both of the $sE\text{-}dos^{YY}$ transgenes that were tested appeared to provide a level of DOS function that was similar to or greater than that provided by wild-type DOS transgenes.

The ability of the DOS^{YY} protein to mediate signaling in these two assays suggests that tyrosine phosphorylation of DOS at sites other than Y801 and Y854 is not crucial for DOS function. However, since neither of these assays was conducted in the absence of a functional wild-type *dos* allele, the possibility remained that even though the limiting feature of DOS in these assays was the ability to recruit CSW to Y801 and Y854, the recruitment of SH2 domain-containing signaling molecules to other sites on the remaining wild-type DOS protein might still have contributed to DOS function. In order to address this issue, the ability of DOS^{YY} to act as the sole source of DOS function was tested. Similarly to wild-type DOS, we found that heat shock-driven expression of DOS^{YY} was sufficient to restore fully the viability of either $dos^{1.46}$/$dos^{2.42}$ animals or animals homozygous for the dos^{P115} allele. The dos^{P115} allele results from the insertion of a P element into the first intron of *dos*. Since the translation starts site of *dos* lies in the first exon, dos^{P115} is likely to lack all *dos* function (Raabe *et al*., 1996). Sectioning of the eyes of the rescued flies revealed that the majority of their ommatidia contained an R7 photoreceptor cell (Figure 8I). These results indicate that DOS^{YY} is capable of providing all of the essential DOS functions needed during signaling by both SEV and other RTKs, such as DER, that are required for viability.

Discussion

During *Drosophila* development, the PH domain-containing scaffold protein DOS functions downstream of

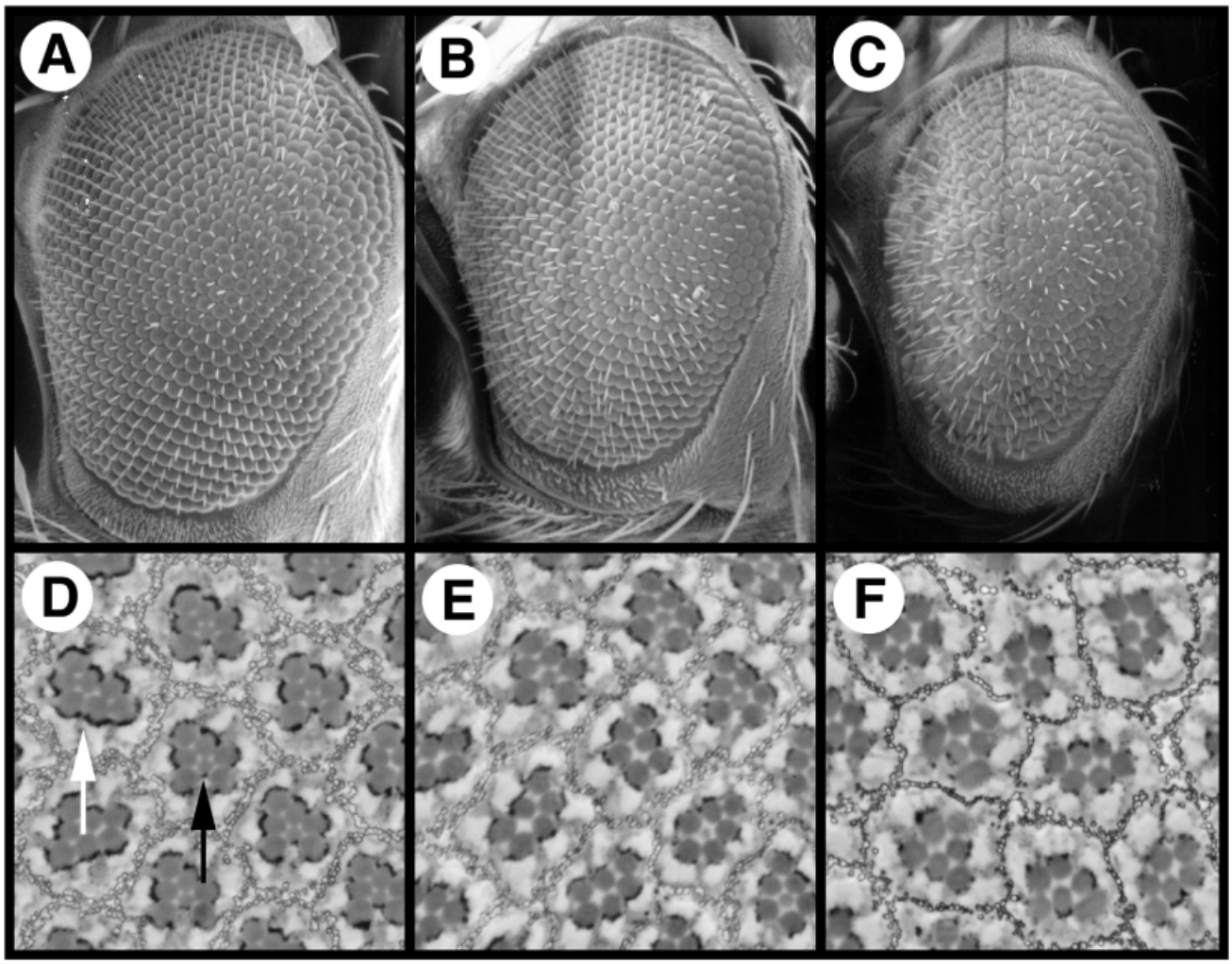


Fig. 7. Expression of either DOS^{Y801F} or DOS^{Y854F} inhibits R7 photoreceptor development. (**A** and **D**) A scanning electron micrograph (SEM) and an apical section of a *P[sE-dos]10/P[sE-dos]8* fly. The normal pattern of the seven photoreceptors (R1–R7) is revealed by the dark circular structures that represent the rhabdomeres of each photoreceptor. The R7 rhabdomere of phenotypically wild-type ommatidia (black arrow) are smaller and more centrally located than the rhabdomeres of R1–R6. The presence of two copies of *P[sE-dos]* has only a slight effect on eye development. The eyes are slightly roughened and occasional ommatidia appear simultaneously to have gained an extra R1–R6 class photoreceptor while losing their R7 class cell (white arrow). (**B** and **E**) An SEM and an apical section of eyes from a fly homozygous for *P[sE-dos^{Y801F}]E*. The presence of two copies of *P[sE-dos^{Y801F}]E* only slightly roughens the eye. However, many of the ommatidia lack an R7 cell. (**C** and **F**) An SEM and an apical section of eyes from a fly homozygous for *P[sE-dos^{Y854F}]B*. The presence of two copies of *P[sE-dos^{Y854F}]B* leads to eye roughening that is associated with the loss of R7 cells from the majority of ommatidial clusters as well as the less frequent absence of one or more R1–R6 class photoreceptors.

multiple RTKs, including the SEV RTK. We had demonstrated previously that DOS interacts with the PTP CSW in a SH2 domain-dependent manner and that DOS and CSW function in a common signaling pathway (Herbst *et al*., 1996). Our data also demonstrated that DOS was a candidate substrate of CSW, suggesting that CSW might regulate DOS by dephosphorylating certain tyrosine residues. In the present report, we have characterized in detail the DOS–CSW interaction *in vitro* and analyzed the contribution of this interaction to DOS function *in vivo*.

Recruitment of CSW to DOS is essential for DOS function

We have identified tyrosine residues Y801 and Y854 in the C-terminus of DOS as the binding sites for CSW SH2 domains. Further, we have provided evidence that these sites are phosphorylated in response to SEV activation. Together with our previous observations (Herbst *et al*., 1996) and results presented by Raabe *et al*. (1996), these data are consistent with a model in which activation of SEV by its ligand BOSS results in DOS tyrosine phosphorylation and recruitment of CSW to the plasma membrane. The importance of this recruitment step for SEV signaling is underscored by the inhibitory effect of DOS mutants that have lost the ability to bind CSW efficiently. Expression of the DOS mutants DOS^{Y801F}, DOS^{Y854F} and the double mutant DOS^{Y801F,Y854F} under control of the *sE* transcriptional cassette in transgenic flies resulted in a rough-eye phenotype and the frequent absence of R7 photoreceptor cells. In addition, expression of each of the mutant proteins suppressed, rather than enhanced, the rough-eye phenotype caused by an activated *sev* allele. These phenotypes may result from competition between wild-type and mutant DOS proteins for the ability to be phosphorylated by SEV. Thus, the expression of these mutant DOS proteins could lead to a reduction in the

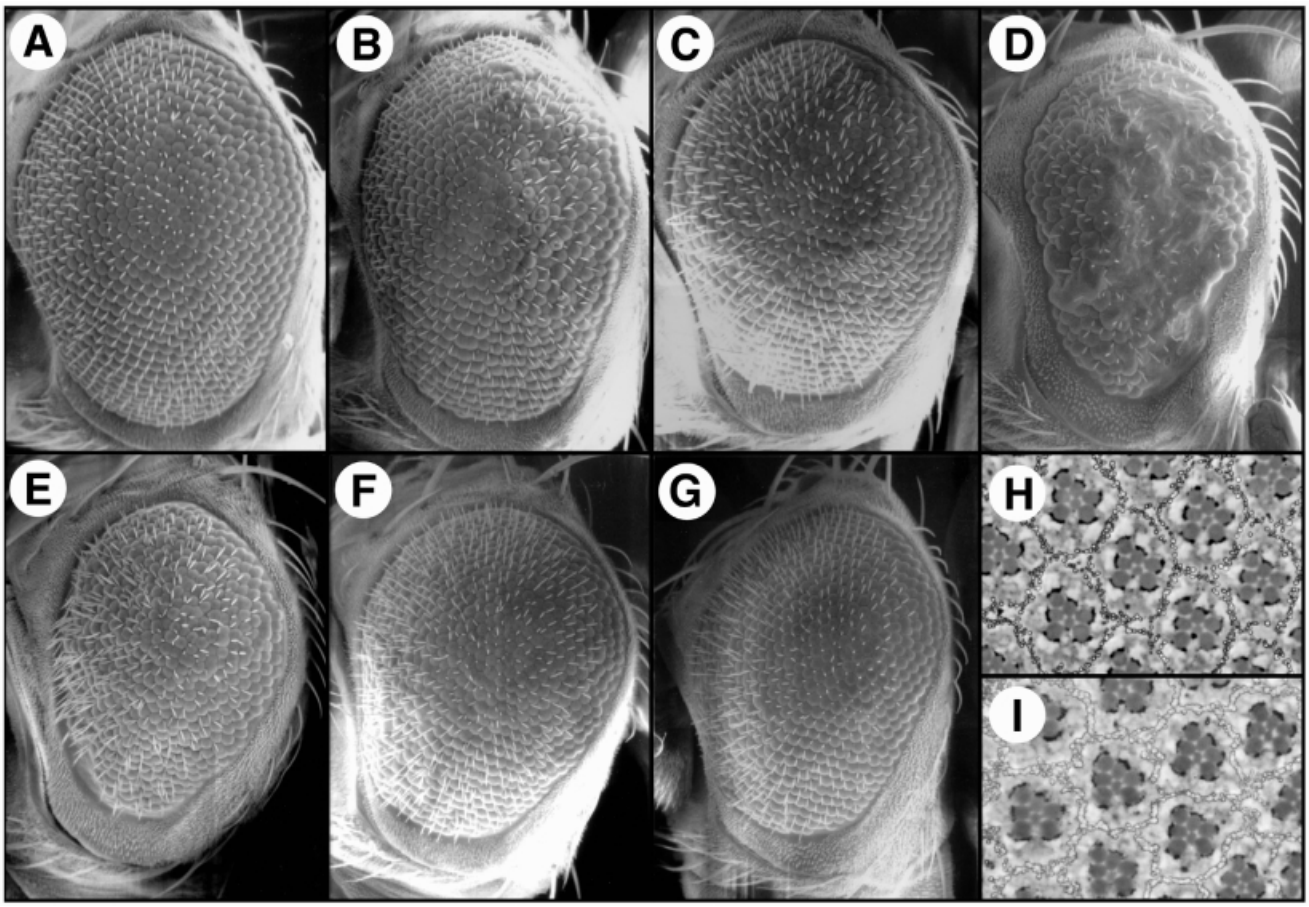


Fig. 8. The DOS^{YY} protein can provide DOS function. (A–D) Scanning electron micrographs (SEMs) of eyes of female flies heterozygous for (**A**) Sev^{S11} alone or heterozygous for both Sev^{S11} and either (**B**) *P[sE-dos]10*, (**C**) *P[sE-*dos^{YY}*]F* or (**D**) *P[sE-*dos^{YY}*]D*. Similarly to wild-type DOS, the expression of additional DOS^{YY} leads to the enhancement of SEV^{S11} signaling and results in more roughened eyes. The difference in the degree of enhancement by the *P[sE-*dos^{YY}*]F* and *P[sE-*dos^{YY}*]D* transgenes is likely to result from differences in the level of DOS^{YY} expression due to the different sites of insertion of the *P[sE-*dos^{YY}*]* element. (E–G) SEMs of eyes of flies heterozygous for (**E**) *P[sE-*csw^{cs}*]1-1*, $dos^{1.46}$ alone and heterozygous for *P[sE-*csw^{cs}*]1-1*, $dos^{1.46}$ and either (**F**) *P[sE-*dos^{YY}*]F* or (**G**) *P[sE-*dos^{YY}*]D*. Similarly to wild-type (see Figure 6G and H), increased expression of DOS^{YY} suppresses the effects of the inhibitory CSW^{CS} protein. (**H**) An apical section of an eye from a $dos^{1.46}/dos^{2.42}$ fly that was rescued by the presence of the *P[sE-dos]10* transgene. The heat shock-driven expression of wild-type DOS fully restores the viability and morphology of $dos^{1.46}/dos^{2.42}$ flies including the presence of R7 cells. (**I**) An apical section of an eye from a $dos^{1.46}/dos^{2.42}$ fly that was rescued by the presence of the *P[sE-*dos^{YY}*]F* transgene. Similarly to wild-type DOS, the expression of DOS^{YY} fully restores the viability and morphology of $dos^{1.46}/dos^{2.42}$ flies including the presence of R7 cells. However, occasional ommatidia are observed that lack either an R7 or R1–R6 class photoreceptor.

phosphorylation of wild-type DOS and a reduction in the number of available CSW-binding sites at the plasma membrane.

Allard *et al*. (1998) have analyzed the structural requirements for CSW function during signaling by SEV and other RTKs. In these experiments, CSW proteins with only one functional SH2 domain were found to provide sufficient CSW function to rescue the lethality associated with *csw* loss-of-function alleles. These results contrast with our data indicating that the ability of DOS to rescue the lethality of homozygous *dos* animals requires the presence of both CSW-binding sites. One possible explanation for this difference is that inactivating an SH2 domain by mutating the conserved arginine residue, as in the case of the CSW mutants, may reduce but not abolish the interaction with a tyrosine-phosphorylated target protein. However, mutating the tyrosine residue in the target might have a more severe effect on the interaction. Importantly, the inability of the single tyrosine mutants DOS^{Y801F} and DOS^{Y854F} to function *in vivo* is consistent with our *in vitro* data demonstrating that both sites are required for the formation of a high affinity DOS–CSW complex. Similarly, mutation of either of the SHP-2-binding sites in IRS-1 (Y1172 or Y1222) completely abolished the insulin-induced association of IRS-1 with SHP-2 (Rocchi *et al*., 1995).

The DOS tyrosine residues that we have identified as CSW-binding sites, together with their surrounding sequences, are highly conserved in other scaffold proteins known to interact with the CSW homolog SHP-2. In the case of IRS-1, IRS-3, GAB2 and FRS2, the corresponding residues have been confirmed as SHP-2-binding sites through mutational analysis (Rocchi *et al*., 1995; Gu *et al*., 1998; Hadari *et al*., 1998; Myers *et al*., 1998). However, Y1172 of IRS-1, the site which corresponds to DOS Y801, has also been identified as a binding site for the tyrosine kinase Fyn (Sun *et al*., 1996). This raises the possibility that the phenotypes we observed with DOS^{Y801F} and DOS^{Y854F} could result in part from a failure to recruit signaling proteins other than CSW. While we cannot

entirely rule out a possible function of other factors that may also interact with these sites, several aspects of our data suggest that CSW is a critical component. For example, the severity of the phenotypes caused by expression of DOS^{Y801F} and DOS^{Y854F} mirrored their effects on CSW binding *in vitro*. Furthermore, the expression of either DOS^{Y801F} or DOS^{Y854F} enhanced the rough-eye phenotype caused by a dominant-interfering CSW mutant (CSW^{CS}). This ability is similar to the dominant enhancement of the CSW^{CS} phenotype by *dos* loss of function. These genetic results are consistent with the idea that DOS and CSW function together in a manner that is dependent on phosphorylation of Y801 and Y854 of DOS.

The role of other sites of DOS phosphorylation

Analysis of the DOS primary structure reveals numerous potential binding sites for SH2 domain-containing signaling proteins, such as DRK, SHC and PI3K. Consistent with the phosphorylation of at least some of these sites, we have observed binding of DRK and the *Drosophila* SHC homolog to tyrosine-phosphorylated DOS in *Drosophila* SL2 cells (R.Herbst and M.A.Simon, unpublished observations). In addition, DOS contains two proline-rich sequences (PXXP) that might serve as SH3 domain-binding sites. The presence of these sites raises the possibility that recruitment of signaling proteins besides CSW might be important for DOS function. We addressed this question by analyzing the function of a DOS construct in which all tyrosine residues outside the PH domain, except for the CSW-binding sites, had been removed by point mutation or deletion (DOS^{YY}). The in-frame deletion in DOS^{YY} also removes the two PXXP motifs in the central region of the DOS primary structure. Like wild-type DOS, DOS^{YY} efficiently binds CSW and is phosphorylated on tyrosine residues (Y801 and/or Y854) in response to SEV activation. In each of our *in vivo* assays of DOS function, DOS^{YY} was fully functional. Most importantly, DOS^{YY} expression was also able to rescue the lethality of *dos* alleles with efficiency similar to wild-type DOS. The resulting rescued animals were phenotypically normal. These results suggest that a DOS protein that is only able to interact with CSW can provide all the essential zygotic functions mediated by DOS during *Drosophila* development, including the ability to function during signaling by other RTKS such as DER.

The role of DOS as a target for CSW dephosphorylation during SEV signaling

Structural and enzymatic studies of SHP-2, the mammalian homolog of CSW, have shown that binding of the N-terminal SH2 domain to a phosphorylated tyrosine-containing peptide leads to a marked increase in SHP-2 catalytic activity (Sugimoto *et al*., 1994; Hof *et al*., 1998). Thus, an expected consequence of the binding of CSW to DOS is a substantial increase in CSW catalytic activity. Given the requirement for the CSW catalytic activity during SEV signaling, a key question is the identity of the key CSW target(s) whose dephosphorylation is required for R7 photoreceptor development. In previous work, we suggested that DOS itself might be a crucial target of CSW. In particular, we proposed that DOS might contain sites of tyrosine phosphorylation that serve as docking sites for proteins inhibitory to SEV signaling and that these sites might be dephosphorylated by CSW. This idea was based both on our studies showing that DOS was a potential CSW substrate and on genetic studies indicating that reduced CSW or DOS function decreased the strength of SEV signaling. Given this model, our expectation was that mapping the sites of tyrosine phosphorylation in CSW^{CS}-bound DOS might identify both the CSW SH2 domain-binding sites and additional CSW target sites. Instead, our analysis revealed phosphorylation of only the two CSW SH2 domain-binding sites, Y801 and Y854. The failure to find additional sites of DOS tyrosine phosphorylation in CSW^{CS}-bound DOS and the ability of DOS^{YY} to provide DOS function during SEV signaling together suggest that the primary role of DOS may be to activate the catalytic activity of CSW towards other, as yet unidentified, proteins. In this model, the role of CSW's dephosphorylation of DOS would be to down-regulate signaling by eliminating its own binding sites. However, we cannot rule out that DOS contains additional CSW target sites that were not identified in our biochemical analysis.

In summary, our data demonstrate that an essential function of DOS is to recruit CSW to the plasma membrane by providing binding sites for its SH2 domains. Our results are consistent with a model in which CSW then regulates the phosphorylation of DOS as well as of other, as yet unidentified, proteins whose dephosphorylation is required for SEV signaling.

Materials and methods

Genetics and histology

Fly culture and crosses were performed using standard procedures. Fixation and sectioning (1 μm) of adult eyes was performed as described (Tomlinson and Ready, 1987). Scanning electron microscopy was performed as described (Kimmel *et al*., 1990).

For assays of the ability of the expression of the DOS^{Y801F} and DOS^{Y854F} proteins to suppress the Sev^{S11} phenotype, male flies carrying second chromosome insertions of either *P[sE-dos*^{Y801F}*]* or *P[sE-dos*^{Y854F}*]* were crossed to female flies homozygous for the Sev^{S11} P element insertion (Basler *et al*., 1989) in order to generate males hemizygous for Sev^{S11} and heterozygous for either *P[sE-dos*^{Y801F}*]* or *P[sE-dos*^{Y854F}*]*. For assays of the ability of the expression of the wild-type DOS and DOS^{YY} proteins to enhance the Sev^{S11} phenotype, female flies heterozygous for Sev^{S11} were analyzed because their Sev^{S11} phenotype is less severe and its enhancement is therefore detected more easily. Male flies carrying second chromosome insertions of either *P[sE-dos]* or *P[sE-dos*^{YY}*]* were crossed to female flies homozygous for the Sev^{S11} P element insertion in order to generate females heterozygous for both Sev^{S11} and either *P[sE-dos]* or *P[sE-dos*^{YY}*]*. For assays of the ability of the mutant DOS proteins to modify the *P[sE-csw*^{CS}*]* phenotype, the P elements carrying the *sE*-driven *dos* cDNAs were crossed to flies carrying a balanced third chromosome that carried both a *P[sE-csw*^{cs}*]* insertion (referred to as *P[sevhs-csw*^{cs}*]* in Herbst *et al*., 1996) and the $dos^{1.46}$ allele. Male flies heterozygous for both *P[sE-csw*^{cs}*]*, $dos^{1.46}$ and the *dos*-expressing transgene were analyzed. The $dos^{1.46}$ allele, *E(csw*^{CS}*)3A*$^{1.46}$ (Herbst *et al*., 1996), is a strong loss-of-function allele. In each of these assays, two independent P element insertions carrying the mutant *sE-dos* cassettes were tested and gave equivalent results.

Assays of the ability of wild-type and mutant *P[sE-dos]* transgenes to rescue flies lacking *dos* function were performed in two ways. For rescue of $dos^{1.46}/dos^{2.42}$ animals, male flies carrying second chromosome insertions of the *P[sE-dos]* transgenes were crossed to *w;* $dos^{1.46}$, *Ki/TM6B, Tb* females. The resulting *w; P[sE-dos]/+;* $dos^{1.46}$, *Ki/+* males were then crossed to *w;* $dos^{2.42}$*/TM3, Sb* females. The progeny of these crosses were raised at 25°C with a 1.5 h heat shock (37°C) each day. The ability to rescue the lethality associated with the $dos^{1.46}$ and $dos^{2.42}$ alleles was assayed by the appearance of *w; P[sE-dos]/+;* $dos^{2.42}/dos^{1.46}$, *Ki* adult flies. Rescue was considered to be complete if >75%

of the expected number ($n > 20$) of these progeny were observed. In each of these assays, two independent P element insertions carrying the mutant *sE-dos* cassettes were tested and gave equivalent results. For rescue of animals homozygous for the dos^{P115} allele, dos^{P115}/*TM6B, Tb, Hu* male flies that were heterozygous for the *P[sE-dos]* transgene were crossed to dos^{P115}, *th*, *cu*, *sr*/*TM3*, *Sb* females. The progeny of these crosses were raised at 25°C with a 1.5 h heat-shock (37°C) each day. Rescue was scored by the presence of flies that lacked both *TM3, Sb* and *TM6B, Tb, Hu* among the progeny.

P element transformation

For the generation of transgenic lines, wild-type and mutant *dos* cDNAs were subcloned into the transformation vector pKB267, which contains a hybrid promotor (*sE*) consisting of *sevenless* enhancers and the *hsp70* promotor (Basler *et al*., 1989). pKB267 directs expression of the transgene in photoreceptors R3, R4 and R7, as well as in the cone cells. Ubiquitous expression can be achieved by heat shock induction. Injection and transformation were done as previously described (Rubin and Spradling, 1982). All transgenes used in this study were marked by the presence of the *white* gene.

Tissue culture

The *Drosophila* Schneider cell line 2 (SL2) was maintained in Shields and Sang M3 medium (Sigma) supplemented with 10% fetal calf serum (Gibco) and 2.5 g/l bacto peptone (Difco) at 23°C. The cell lines SL2-CSW^{CS} and SL2-SEV^{S11} were described previously (Simon *et al*., 1993; Herbst *et al*., 1996). Transfections were done using Ca_2PO_4 and 10 µg of plasmid DNA (Rio and Rubin, 1985). Polyclonal cell lines were selected in G418 (Gibco; 1 mg/ml) or hygromycin B (Sigma; 200 µg/ml), or both for doubly transfected cells. For heat shock induction of SEV^{S11}, the appropriate number of cells was collected by centrifugation and resuspended in fresh medium at a density of 1.5×10^6 per ml. Cells were heat shocked for 30 min at 37°C and then returned to 23°C.

Construction of expression plasmids

For subcloning purposes, we modified the *dos* cDNA in order to introduce a *Kpn*I site 5′ of the ATG initiation codon. To this end, a 540 bp cDNA fragment covering the first 180 codons of *dos* was amplified by PCR. The forward primer was 5′-GGTACCAAGGAAATGGATCGCACTTTC-TACTAG-3′, and the reverse primer was 5′-AGGCGCTGTGGTTCCG-3′. The resulting PCR product was first subcloned into pCR™II (Invitrogen). A pBluescript-KS+ plasmid (Stratagene) that contains the complete open reading frame of *dos* was cut with *Xba*I and *Eag*I and the restriction fragment was replaced with the PCR fragment that had been isolated from pCR™II using the same enzymes. The resulting plasmid, pKS+DOS, was used for further manipulations. An AUI tag (BAbCO) was introduced at the C-terminus of DOS by site-directed mutagenesis (Kunkel, 1985), using the oligonucleotide 5′-GAGAGCAGCGGCAACAAGGAT-ACGTACCGCTACATCTGAAACTGCTGGTTCAGC-3′. Tyrosine to phenylalanine (Y/F) substitutions were done using the following oligonucleotides:

5′-GACTCGGTGTTTGTCAACACG-3′ for Y202F;
5′-AACACGGAGTTTAGCAATCGC-3′ for Y207F;
5′-GAGGAAAGCTTTGACATTCCA-3′ for Y537F;
5′-CAGCAGCCTTTTTTCAACGTCAAC-3′ for Y547/548F;
5′-GTTTTTCGATTTGACTTCATG-3′ for Y631F;
5′-CCCAATGCCTTTAAGTTGGGC-3′ for Y703F;
5′-AAGCTCCAGTTTTTTGATCTG-3′ for Y801F;
5′-GGGAATCTGTTTTCACAAGGC-3′ for Y824F; and
5′-AGCGTGGTCTTTAGATCCGTG-3′ for Y854F.

In DOS^{Y801F} and DOS^{Y854F}, only Y801 and Y854, respectively, are exchanged to phenylalanine. The double mutant $DOS^{Y801F,Y854F}$ was created by exchanging an *Xho*I restriction fragment between DOS^{Y801F} and DOS^{Y854F}. For the construction of DOS^{0Y} in which all tyrosine residues outside the PH domain are removed and DOS^{YY} that only has Y801 and Y854 (in addition to the tyrosine residues in the PH domain), we first generated the deletion construct $DOS^{\Delta NN}$ by digesting pKS+DOS with *Nru*I and religation. This results in an in-frame deletion of codons 211–507. This was followed by several rounds of mutagenesis to remove the remaining tyrosine residues. After the final round of mutagenesis, the complete *dos* cDNA was sequenced. For the generation of stable cell lines, cDNAs were subcloned as *Kpn*I fragments into the expression vector pAT-Hygro (Allard *et al*., 1996). For co-expression of DOS constructs with CSW^{CS}, an expression cassette containing a neomycin resistance marker under control of the *copia* long terminal repeat and an actin5C promotor to drive expression was placed into the *Not*I site 5′ of the *dos* cDNA (Simon *et al*., 1993).

Cell lysis and immunoprecipitation

Cells were harvested by centrifugation at 1000 r.p.m. for 5 min and lysed on ice in 200 µl of lysis buffer [50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml aprotinin, 10 mg/ml leupeptin] per 1×10^7 cells. The lysates were transferred to microfuge tubes, mixed vigorously and incubated on ice for 20 min. Lysates were pre-cleared by centrifugation at 30 000 *g* for 20 min at 4°C. For the analysis of total cellular proteins, 20 µl of cleared lysate were mixed directly with 20 µl of SDS sample buffer and boiled for 5 min before loading on an SDS–polyacrylamide gel. For immunoprecipitations, lysates were diluted with an equal volume of washing buffer (lysis buffer + 0.1% Triton X-100) and incubated with the respective antibody cross-linked to protein G–Sepharose (Sigma) for 4 h at 4°C. Precipitates were washed three times with 1 ml of washing buffer before addition of SDS sample buffer.

Immunoblotting

Proteins were separated by 9% SDS–PAGE and electrophoretically transferred to nitrocellulose filters. For immunoblotting analysis, filters were incubated in 5% milk powder solution in TBST (20 mM Tris, pH 8.0, 150 mM NaCl and 0.02% Tween-20) for 2 h at room temperature. After washing, the filters were incubated with the primary antibody overnight at 4°C, then washed four times with TBST, followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Immunoreactive proteins were visualized by using a horseradish peroxidase-catalyzed chemiluminescence reaction (ECL; Amersham).

Purification of tyrosine-phosphorylated DOS and mass spectrometric analysis

For the identification of CSW-binding sites in DOS, tyrosine-phosphorylated DOS was purified essentially as described (Herbst *et al*., 1996). Briefly, 1×10^9 SL2-CSW^{CS} cells overexpressing DOS (SL2-CSW^{CS}-DOS) were lysed in 11 ml of lysis buffer lacking PTP inhibitors. After 1 h at room temperature, insoluble material was pelleted by centrifugation at 45 000 *g* for 30 min at 4°C. SDS was added to the supernatant to a final concentration of 1% and the sample incubated at 90°C for 5 min. The sample was then diluted 1:10 with immunoprecipitation washing buffer and loaded on an anti-phosphotyrosine column (PY20). After washing the column with washing buffer, proteins were eluted with the same buffer containing 100 mM *p*-nitrophenylphosphate (Sigma). The eluate was concentrated in a Centricon concentrator (Amicon), loaded on an 8% SDS–polyacrylamide gel and the gel stained with Coomassie Blue. Based on the staining intensity, ~4 µg of DOS protein was obtained.

The procedure to identify phosphorylation sites was carried out as described before (Zhang *et al*., 1998). Briefly, a matrix-assisted laser desorption/ionization mass spectrometer (MALDI/TOF) with delayed extraction (Voyager-DE, Perseptive Biosystems, Framingham, MA) was used for the identification of phosphopeptides. An electrospray ion trap mass spectrometer (LCQ, Finnigan, San Jose, CA) coupled on-line with a microbore HPLC (Magic 2002, Auburn, CA) was used for the identification of the phosphorylation site. A monitor C18 column (5 nm particle diameter, 150 Å pore size, 0.2×50 mm dimension) was used for the on-line LC/MS/MS analysis. Phosphopeptides were generated from in-gel trypsin digestion of 25% of the DOS protein band.

Preparation of GST fusion proteins

For the generation of the GST–CSW-SH2-SH2 fusion protein, a cDNA fragment encoding CSW amino acids 1–218 was subcloned into the expression vector pGEX-2TK (Pharmacia). The purification of GST–CSW-SH2-SH2 was done as described by Frangioni and Neel (1993). For pull-down experiments, 3 µg of purified GST–CSW-SH2-SH2 were bound to glutathione–agarose before incubation with cell lysates.

Antibodies

The following monoclonal antibodies and polyclonal antisera were used: PY20, a mouse monoclonal antibody (mAb) against phosphotyrosine (Transduction Laboratories); mAb 78C10, raised against the peptide RDIYKSDYYRKEGEGLLPVR, which is localized in the catalytic domain of SEV; CT-1, a rat polyclonal antiserum raised against a GST fusion protein containing the C-terminal 159 amino acids of CSW (GST–CSW-CT); mAb AU1, which recognizes the peptide tag DTYRYI (BAbCO); and anti-GST, a rabbit polyclonal antiserum raised against GST (Upstate Biotechnology).

Peptides

The following peptides were used: DOS-Y801 (KLQYFDLDVT), corresponding to amino acids 798–807 of DOS; DOS-Y854 (SVVYRSVDFV), corresponding to amino acids 851–860 of DOS; and SEV-Y2551 (KQLYANEGVS), corresponding to amino acids 2549–2557 of SEV. In the peptides DOS-pY801, DOS-pY854 and SEV-pY2551, the tyrosine residue is phosphorylated. All peptides were obtained from Research Genetics. For binding experiments, all peptides were chemically cross-linked to Affi Gel-15, as recommended by the manufacturer (Bio-Rad).

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