Competition between SOCS36E and Drk modulates Sevenless receptor tyrosine kinase activity

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Summary

Modulation of signalling pathways can trigger different cellular responses, including differences in cell fate. This modulation can be achieved by controlling the pathway activity with high precision to ensure robustness and reproducibility of the specification of cell fate. The development of the photoreceptor R7 in the Drosophila melanogaster retina has become a model in which to investigate the control of cell signalling. During R7 specification, a burst of Ras small GTPase (Ras) and mitogen-activated protein kinase (MAPK) controlled by Sevenless receptor tyrosine kinase (Sev) is required. Several cells in each ommatidium express sev. However, the spatiotemporal expression of the boss ligand and the action of negative regulators of the Sev pathway will restrict the R7 fate to a single cell. The Drosophila suppressor of cytokine signalling 36E (SOCS36E) protein contains an SH2 domain and acts as a Sev signalling attenuator. By contrast, downstream of receptor kinase (Drk), the fly homolog of the mammalian Grb2 adaptor protein, which also contains an SH2 domain, acts as a positive activator of the pathway. Here, we apply the Förster resonance energy transfer (FRET) assay to transfected Drosophila S2 cells and demonstrate that Sev binds directly to either the suppressor protein SOCS36E or the adaptor protein Drk. We propose a mechanistic model in which the competition between these two proteins for binding to the same docking site results in either attenuation of the Sev transduction in cells that should not develop R7 photoreceptors or amplification of the Ras–MAPK signal only in the R7 precursor.

Key words: SOCS36E, Sevenless, Receptor regulation

Introduction

Signalling through the activation of receptor tyrosine kinases (RTKs) is one of the most critical mechanisms used by metazoans to control growth and development. Upon ligand binding, autophosphorylation of RTK cytosine residues provides the docking sites for a variety of phosphotyrosine-binding proteins. The specific recruitment of these proteins, which harbour various catalytic and/or scaffolding domains, determines the signalling output. Perturbation of this signalling, by mutations or other genetic alterations, results in deregulated kinase activity and cancer (Blume-Jensen and Hunter, 2001). A major deactivation pathway for RTKs is receptor downregulation, which involves ligand-induced internalisation by means of endocytosis, followed by degradation in lysosomes (Bache et al., 2004).

The photoreceptor specification of the Drosophila melanogaster retina is a model system with which to study RTK signalling. The compound eye of Drosophila is formed by an array of well-organised ommatidia in which clusters of cells will specify the photoreceptors and cone cells. The photoreceptor R7 is specified by high activity of the mitogen-activated protein kinase (MAPK) through the activation of the Sevenless (Sev) RTK (Banerjee et al., 1987; Hafen et al., 1987; Tomlinson and Ready, 1986), whereas cells that express Sev, but do not activate it, are specified as other photoreceptors or cone cells (Freeman, 1996; Simon et al., 1991; Tomlinson et al., 1988; Tomlinson and Ready, 1987). Sev is temporarily activated in the R7 by the action of the Boss ligand expressed in the adjacent R8 photoreceptor (Cagan et al., 1992; Hart et al., 1990; Reinke and Zipursky, 1988). It is not yet clear how the inactivation of Sev in cells that should not become R7 photoreceptors is maintained. The presence of negative regulators of Sev in those cells will provide a fail-safe mechanism in case of perturbations that could result in the wrong cell fates.

A genetic screen for negative regulators of the Sev pathway (Sese et al., 2006) identified Suppressor of cytokine signalling 36E (Socs36E) (Almudi et al., 2009). Socs36E is expressed in those cells that express sev, but do not become R7 photoreceptors, and is not expressed in the presumptive R7 (Almudi et al., 2009). SOCS proteins are conserved from flies to mammals and were initially identified as repressors of cytokine signalling through a negative-feedback loop that inhibits the cytoplasmic TK Janus (JAK–STAT signalling pathway) (Endo et al., 1997; Hilton et al., 1998; Starr et al., 1997; Yoshimura et al., 1995). Socs36E encodes the Drosophila homolog to the mammalian SOCS3 and SOCS4 (Callus and Mathey-Prevot, 2002; Karsten et al., 2002) and, similarly to other members of the SOCS family, contains an SH2 domain flanked by a variable N-terminal domain and a conserved C-terminal domain, termed the SOCS-box. The SH2 binds phosphorylated tyrosine residues (Tyr-P), whereas the SOCS-box participates in a ubiquitin ligase complex to promote the degradation of target proteins (Zhang et al., 1999). In addition to the JAK–STAT pathway, SOCS proteins also regulate other signalling pathways, including RTKs (Almudi et al., 2009; Baetz et al., 2004; Callus and Mathey-Prevot, 2002; Kario et al., 2005; Krebs and Hilton, 2003; Rawlings et al., 2004). Genetic analyses demonstrated that Socs36E negatively regulates Sev and epidermal growth factor receptor (EGFR) pathways (Almudi et al., 2009; Callus and Mathey-Prevot, 2002; Rawlings et al., 2004). Two pieces of evidence suggest a direct interaction...
between SOCS36E and Sev. First, it was demonstrated by co-
immunoprecipitation assays that the mammalian orthologs of
Socs36E bind directly to EGFR through their SH2 domains (Kario
et al., 2005; Nicholson et al., 2005). Second, the sev gain-of-
function phenotype, which results in extra R7 cells in the retina, is
suppressed by overexpression of Socs36E, and this suppression is
impaired by mutations in the SH2 domain (Almudi et al., 2009).
Moreover, overexpression of the SH2-containing protein Drk
enhances Sev signalling except in the presence of Socs36E (Almudi
et al., 2009). Drk, the fly homolog to the mammalian Grb2, is the
adaptor protein that links Sev to Sos and its downstream effectors
Ras and MAPK (Olivier et al., 1993; Simon et al., 1993). These
results suggest a mechanism for Sev modulation in which the SH2-
containing proteins SOCS36E and Drk would compete for the
phosphorylated Sev receptor.

To characterise the molecular mechanism of the interactions
between Sev, Drk and SOCS36E, we explored whether these
proteins are able to come into physical contact with one another.
To this aim, we took advantage of Förster resonance energy
transfer (FRET) analysis, which allows us to determine the
proximity of proteins beyond the resolution of conventional
optical microscopy and, therefore, monitor protein–protein
interactions in living cells (Ciruela, 2008; Gordon et al., 1998).
Because FRET is based on photon energy resolution, the
maximum distance between proteins of interest to detect energy
transfer is typically 5–10 nm. Here, we demonstrate that both
Drk and SOCS36E physically interact with Sev, thus supporting
a model in which SOCS36E represses the Sev pathway by
competing with Drk to bind to the activated receptor.

Results and Discussion
Activated Sev stimulates tyrosine phosphorylation in
transfected S2 cells
The sevS11 allele is a constitutively activated form that consists of
the C-terminal Sev protein, which includes the intracellular tyrosine
kinase domain, bound to the signal peptide of the Drosophila
cuticle protein CP3 and the 10 amino acid Myc epitope (Basler et
al., 1991). sevS11 transgenic flies exhibit a striking rough eye
phenotype because of the transformation of non-neural cone cells
into R7 photoreceptors (Fig. 1A,B) (Basler et al., 1991).

We generated new ligand-independent sev constructs containing
cyan fluorescent protein (CFP) (SevS11-CFP; Fig. 1C). S2 cells
transfected with SevS11-CFP showed Sev localised mainly in
cytoplasmic and vesicular accumulations, probably owing to the
constant turnover of the protein because of its constitutive activity
(Fig. 1D,E). To confirm that this construct functions in a ligand-
independent manner, similarly to sevS11 in transgenic flies, we used
an antibody against Tyr-P to visualise tyrosine phosphorylation as
a marker of constitutive activation of Sev and subsequent kinase
activity. We found that cells transfected with SevS11-CFP had a
higher Tyr-P signal in comparison with non-transfected cells (Fig.
1D–D’,E–E’). Moreover, the SevS11-CFP fusion protein
colocalised with Tyr-P (Fig. 1D’,E’). Furthermore, a transgene of
the SevS11-CFP construct resulted in the formation of
supernumerary R7 cells when expressed in the developing eye
(Fig. 1F–H). These results indicated that SevS11-CFP is functional
and that SevS11 activity is not affected by the addition of a
fluorochrome. Thus, transfection of SevS11–CFP in S2 cells provides
a new and fast assay with which to study RTK interactions in vivo.

Fig. 1. SevS11–CFP construct promotes tyrosine phosphorylation. (A) Wild-type eye. (B) SevS11 eye.
(C) SevS11–CFP construct scheme. (D–D’) Transfected cell with SevS11–CFP (cyan) shows an increase in Tyr-P
(p-Tyr; red) levels (arrowhead in bright field) compared with non-transfected cells (arrow). (E–E’) SevS11–CFP
and Tyr-P (red) colocalisation in a zoom of a transfected cell. (F) Semi-thin section from a GMR–Gal4 eye.
(G) Semi-thin section from a GMR-Gal4/UAS-SevS11-CFP eye. Some ommatidia exhibit extra R7 cells (green).
(H) Histogram showing distribution profiles of number of R7 cells per ommatidium in each genotype represented.
Scale bars: 10 μm.
**Direct interaction between Sev and Drk**

It has been previously reported that Drk interacts specifically with the phosphorylated tyrosine residue 2546 (Tyr2546) of activated-Sev RTK through its SH2 domain (Raabe et al., 1995). A direct interaction between Drk/Grb2 and another RTK, EGFR, has also been described in mammals (Sorkin et al., 2000). We used FRET analysis to monitor this interaction in vivo and demonstrate that Drk binds directly to Sev under our experimental conditions.

Cells were transfected with SevS11–CFP and red fluorescent protein (RFP)–Drk constructs (Fig. 2A–C). RFP–Drk was evenly distributed in the cytoplasm after a single transfection (Fig. 2C). However, in co-transfected cells, the distribution of RFP–Drk protein shifted to colocalisation with SevS11–CFP (Fig. 2D–D*). In addition, colocalisation depends on SevS11–CFP concentration, because cells transfected with high SevS11–CFP concentration showed better colocalisation than cells with low SevS11–CFP (supplementary material Fig. S1). This colocalisation was due to a direct binding between Drk and Sev, because FRET analysis showed an efficiency of 14.6±2% in 95% of the examined transfected cells (n=20 cells; Fig. 2D*). These results confirmed that Drk functions through a direct binding to Sev, as in vertebrates, where Grb2 interacts physically with EGFR (Sorkin et al., 2000).

**Direct interaction between Sev and SOCS36E**

Although the interaction between SOCS36E and Sev has been suggested to be highly specific by genetic analysis (Almudi et al., 2009), no direct evidence for physical binding has been reported. To understand the molecular mechanism of SOCS36E as an attenuator of Sev, we first analysed the physical interaction between SevS11–CFP and haemagglutinin-tagged SOCS36E (HA–SOCS36E) using immunoprecipitation. S2 cells transfected with SevS11–CFP and HA–SOCS36E demonstrated the capability of the SH2 domain of SOCS36E to bind (Fig. 3A). To examine the domains involved in this interaction, we hypothesised that Drk functions through a direct binding to Sev, as in vertebrates, where Grb2 interacts physically with EGFR (Sorkin et al., 2000).

Cells co-transfected with SevS11–CFP and RFP–Drk constructs (Fig. 2A–C) showed an efficiency of 14.6±2% in 95% of the examined transfected cells (n=20 cells; Fig. 2D*). These results confirmed that Drk functions through a direct binding to Sev, as in vertebrates, where Grb2 interacts physically with EGFR (Sorkin et al., 2000).

**SOCS36E competes with Drk to bind to Sev**

As Drk and SOCS36E bind directly to Sev, we hypothesised that the intermediate phenotype observed in UAS-Socs36E–UAS-drk; sev-Gal4 sevS11 eyes of adult flies (Fig. 4A) (Almudi et al., 2009) could be due to competition between SOCS36E and Drk for the same docking site. Thus, direct binding of SOCS36E to Sev would impede the physical interaction between Sev and Drk to trigger Sev signalling.

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**Fig. 2. Direct interaction between Sev RTK and Drk proteins.**

(A) SevS11–CFP and RFP–Drk constructs. (B,C) Localisation of SevS11–CFP (cyan, B) and RFP–Drk (red, C) in cells transfected separately with each construct. (D–D*) Co-transfection of RFP–Drk and SevS11–CFP. Regions where SevS11–CFP and RFP–Drk colocalised showed the highest FRET intensity (color code: blue, low intensity; red, high intensity). Scale bars: 10 μm.
To test this hypothesis, we analysed the energy transfer between SevS11–CFP and RFP–Drk in the presence of SOCS36E. As a negative control, we transfected MAPK together with SevS11–CFP and RFP–Drk, because MAPK is known to occur downstream of the pathway (Biggs et al., 1994). Double transfection of RFP–MAPK and SevS11–CFP resulted in the absence of colocalisation and energy transfer (supplementary material Fig. S2). Triple transfections showed that the presence of MAPK did not interfere with the interaction between SevS11–CFP and RFP–Drk. Every triple-transfected cell observed showed energy transfer (Fig. 4B). However, triple transfection of SevS11–CFP, RFP–Drk and SOCS36E resulted in a significant reduction in the number of cells that exhibited energy transfer between SevS11–CFP and RFP–Drk (19% of cells; Fig. 4C). Moreover, we observed a decrease in FRETeff to ~11±3% in SevS11–CFP, RFP–Drk and SOCS36E versus the ~19±5% FRETeff obtained in SevS11–CFP, RFP–Drk and MAPK cells (Fig. 4B, C).

We next checked for the ability of the SOCS36E-SH2* mutant to inhibit competition and found energy transfer between SevS11–CFP and RFP–Drk in all cells examined (n=15, FRETeff ~16±4%; Fig. 4D). This shows that the SH2 domain is necessary for docking SOCS36E to Sev.

**Conclusions**

By using FRET technology in *Drosophila* S2 cells, we provide evidence for the physical interaction of two adaptor proteins, Drk...
and SOCS36E, with activated Sev. Although both proteins share the same target, they act as positive and negative regulators, respectively. These results, together with previous genetic interaction studies (Almudi et al., 2009), demonstrate that SOCS36E directly binds to the intracellular domain of Sev and that its suppressor effect strictly depends on the presence of the SH2 domain, similarly to the interaction of SOCS-5 with EGFR in mammalian cells (Nicholson et al., 2005). In addition, recruitment of SOCS-6 by the active form of the cytoplasmic tyrosine kinase p56
\(^{lck}\) of SOCS-6 by the active form of the cytoplasmic tyrosine kinase
\(^{lck}\) mammalian cells (Nicholson et al., 2005). In addition, recruitment that its suppressor effect strictly depends on the presence of the SOCS36E directly binds to the intracellular domain of Sev and recruit SOCS36E-SH2* construct allows the energy transfer between SevS11–CFP and RFP–Drk in SevS11–CFP, RFP–Drk and SOCS36E triple-transfected cells.

Materials and Methods

Cell culture, transfection and immunoculture

S2 cells were maintained at 25°C in Drosophila Schneider’s medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Cells were transfected using Cellfectin (Invitrogen) according to manufacturer’s protocol and collected 48 hours later. Transfected cells were prepared for immunoculture as previously described (Beltran et al., 2007). We used mouse anti-p-Tyr (PY99; 1:500, Santa Cruz Biotechnology) and goat anti-mouse Rhodamine Red (1:200, Jackson ImmunoResearch Laboratories) antibodies for immunodetection.

Sev
\(^{S11}\) , drk, Socs36E and MAPK constructs

PCRs using primers shown in supplementary material Table S1 were carried out using genomic DNA from sev
\(^{S11}\) flies, full-length cDNA of drk and MAPK from wild-type flies, Socs36E cDNA (SD 04308 from Drosophila Gold Collection, Berkeley Drosophila Genome Project) and genomic DNA from UAS–Socs36E–SH2* flies [which carry a substitution of the conserved arginine in the SH2 domain (Arg500) for a lysine (Callus and Mathey-Prevot, 2002)]. To mutate phosphorylation in Sev, we generated Sev
\(^{S11}\) Tyr2546F–CFP construct, which is a substitution of Tyr2546 for phenylalanine. PCR products were introduced into pDONR221 (Invitrogen) via Gateway cloning to create pEntry-vectors. In the subsequent reactions, these vectors were combined with the pAWC, pARW, pAHW and pPWC vectors (T. D. Murphy, unpublished results; obtained from the Drosophila Genomics Research Center) to recombine the constructs with the CFP, RFP proteins and HA tag and express them under the Drosophila Actin5C and UAS promoters. DNA was purified using a Qiagen Midiprep Kit.

Fly transgenes and stocks

Activated Sev construct sev
\(^{S11}\) (Basler et al., 1991), sev–Gal4 sev
\(^{S11}\) –TM3 (Almudi et al., 2009) and UAS–Socs36E (Callus and Mathey-Prevot, 2002) were used for genetic interventions. drk
\(^{EP}(2)2477\) (Bloomington Stock Center) is an insertion containing upstream activation sequence (UAS) sites that drive expression of drk, hereafter called UAS–drk. UAS–GFP transgene was added to avoid titration of Gal4 when comparing with double transgene experiments. The Sev
\(^{S11}\)–CFP construct used for S2 cell transfection was cloned via Gateway in a pPWC vector to drive its expression under the Gal4/UAS system and injected in white strain Drosophila embryos. The eye-specific Glass multimer reporter GMR–Gal4 (Hay et al., 1994) was used to drive transgene expression.

Scanning electron microscopy and histology

Adult flies were prepared for scanning electron microscopy and semi-thin sections made as previously described (Almudi et al., 2009).

SOCS36E immunoprecipitation

Transfected cells were collected and lysated using standard lysis buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP40, protease inhibitor mix) and incubated for 1 hour. Immunoprecipitation was performed with Protein-G agarose bound to mouse anti-HA antibody (1:1000, Abcam) and detected with goat anti-mouse peroxidase (1:3000) secondary antibody with an EZ-ECL system (Biological Industries, Kibbutz Beit Haemek, Israel).

FRET and co-localisation analysis

For FRET analysis, 0.65 \(\mu\)g of donor molecule and 1.35 \(\mu\)g of acceptor molecules were transfected in all experiments. To check for concentration-dependent colocalisation in the Drk–Sev interaction experiment, we used additional 0.3 \(\mu\)g and 1.5 \(\mu\)g of donor Sev
\(^{S11}\)–CFP. To examine interactions between Sev, Drk and SOCS36E proteins, the FRET sensitised emission method was used. S2 cells were plated onto MatTek plates, CFP was used as donor molecule and RFP as acceptor molecule (Campbell et al., 2002). Data were acquired and analysed using a Leica TCSSP2 confocal microscope. Normalised FRET was calculated as FRET\(^{-}\) = FRET – (a \(\times\) CFP) / (b \(\times\) RFP/RFP, where FRET\(^{-}\) is corrected FRET (includes corrections for background fluorescence and crossover of donor and acceptor fluorescence through FRET values), and a and b are the fraction of bleed-through of CFP and RFP fluorescence, respectively, through the FRET filter channel (Jiang and Sorkin, 2002). FRET\(^{-}\) values are presented as FRET efficiency (FRETeff). FRETeff values were averaged from regions of interest (ROIs) observed in cells from at least three independent experiments (n=10) per condition and represented as a mean ± s.d. For
our sensors, we considered FRETeff $\geq 10\%$ as positive FRET (Galperin et al., 2004; van der Krogt et al., 2008). However, it is generally accepted that absence of FRET delivers values $<3\%$. FRET values were presented in pseudocolour mode according to a temperature-based lookup table with blue (cold) indicating low values and red (hot) indicating high values. We used the Colocalisation Finder plug-in from NIH ImageJ software to analyse the grade of colocalisation between Sev311-CEP and RFP-Drk.

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