p120 Ras GTPase-activating protein associates with fibroblast growth factor receptors in *Drosophila*

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Btl (breathless) and Htl (heartless), the two FGFRs (fibroblast growth factor receptors) in *Drosophila melanogaster*, control cell migration and differentiation in the developing embryo. These receptors signal through the conserved Ras/mitogen-activated protein kinase pathway, but how they regulate Ras activity is not known. The present study shows that there is a direct interaction between p120 RasGAP (Ras GTPase-activating protein), a negative regulator of Ras, and activated FGFRs in *Drosophila*. The interaction is dependent on the SH2 (Src homology 2) domains of RasGAP, which have been shown to interact with a phosphotyrosine residue within the consensus sequence (phospho)YXXPXD. A potential binding site that matches this consensus is found in both Btl and Htl, located between the transmembrane and kinase domains of each receptor. A peptide corresponding to this region was capable of binding RasGAP only when the tyrosine residue was phosphorylated. This tyrosine

residue appears to be conserved in human FGFR-1 and mediates the association with the adapter protein CrkII, but no association between dCrk (*Drosophila* homologue of CrkII) and the activated FGFRs was detected. RasGAP was a substrate of the activated FGFR kinase domain, and mutation of the tyrosine residue within the potential binding site on the receptor prevented tyrosine phosphorylation of RasGAP. RasGAP attenuated FGFR signalling *in vivo* and this ability was dependent on both its SH2 domains and its GAP activity. On the basis of these results, we propose that RasGAP is directly recruited into activated FGFRs in *Drosophila* and plays a role in regulating the strength of signalling through Ras and the mitogen-activated protein kinase pathway.

Key words: Btl (breathless), fibroblast growth factor receptor (FGFR), GTPase-activating protein (GAP), Htl (heartless), Ras, tyrosine phosphorylation.

INTRODUCTION

FGF (fibroblast growth factor) receptors (FGFRs) belong to a family of transmembrane receptors that possess a cytoplasmic tyrosine kinase domain [1,2]. After binding their ligand, these RTKs (receptor tyrosine kinases) undergo dimerization, which activates the intracellular tyrosine kinase domain. This leads to the autophosphorylation of the receptor intracellular region and also the *trans*-phosphorylation of exogenous substrates on tyrosine residues. These phosphotyrosine residues provide specific docking sites for cytosolic proteins that contain SH2 (Src homology 2) or PTB (phosphotyrosine-binding) domains, which can then elicit a wide range of downstream signalling pathways. A key pathway is the Ras/MAPK (mitogen-activated protein kinase) pathway that is activated in response to the activation of many RTKs. The small GTPase, Ras, cycles between an active GTP-bound state and an inactive GDP-bound state. The active form of Ras can bind and activate the kinase Raf, which then triggers a kinase cascade, culminating in the activation of MAPK. The activation of Ras at the plasma membrane is brought about by the recruitment of a complex containing the adapter protein Grb2 (growth-factor-receptor-bound protein 2) and the Ras guanine-nucleotide exchange factor Sos (son of sevenless) to the site of receptor activation. In certain cases, such as EGFR (epidermal growth factor receptor), the recruitment of Grb2–Sos can be via a direct interaction between the SH2 domain of Grb2 and the phosphotyrosine residues of the receptor [3,4]. However, for

FGFR, Grb2 cannot bind directly to the receptor [5]. Hence, the recruitment of Grb2–Sos in response to FGFR activation is indirect, via receptor substrates such as FRS2 (fibroblast growth factor receptor substrate 2) [6,7].

Similar to Ras activators, Ras deactivators can also be recruited to the site of receptor activation to ensure proper regulation of the signal. One such deactivator is p120 RasGAP (Ras GTPase-activating protein), a cytoplasmic protein that stimulates the intrinsic GTPase activity of Ras through its C-terminal GAP domain [8,9]. p120 RasGAP is recruited to activated RTKs via its N-terminal adapter region that contains two SH2 domains. It has been shown that this recruitment is directly to the receptor with vertebrate EGFR and platelet-derived growth factor receptor β [10,11] and also in *Drosophila* with the Torso receptor [12]. In some cases, this recruitment leads to tyrosine phosphorylation of p120 RasGAP [13,14]. There have been conflicting reports regarding the interaction between p120 RasGAP and FGFR. In *Xenopus*, p120 RasGAP has been shown to be present and tyrosine-phosphorylated in an FGFR-1 complex [15,16], whereas in other reports on cell lines, this interaction remains unclear [17].

There are two FGFR homologues in *Drosophila*, Btl (breathless) and Htl (heartless), which share 54 and 52 % identity with human FGFR-1 over their cytoplasmic domains. Btl is required for the migration of embryonic tracheal and glial cells in response to external guidance cues [18]. A ligand for Btl has been identified to be the FGF-like protein Branchless, which directs

Abbreviations used: Btl, breathless; dCrk, *Drosophila* homologue of CrkII; EGFP, enhanced green fluorescent protein; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; FGFR, FGF receptor; FRS2, fibroblast growth factor receptor substrate 2; GAP, GTPase-activating protein; Grb2, growth-factor-receptor-bound protein 2; GST, glutathione S-transferase; Htl, heartless; MAPK, mitogen-activated protein kinase; pTyr peptide, phosphorylated peptide; RasGAP, Ras GAP; RTK, receptor tyrosine kinase; SH2, Src homology 2; Sos, son of sevenless; Tyr peptide, non-phosphorylated peptide; UAS, upstream activator sequence; WB, Western blotting.

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the migration of tracheal cells [19]. Htl is initially required for the migration of the mesoderm over the ectoderm and, later on, for the specification of certain heart and somatic muscle precursors and the ensheathing of axons in the embryonic central nervous system [20–22]. Ligands for Htl have not yet been identified. Both *Drosophila* FGFRs have been shown to signal through the Ras pathway [21,23]; however, the link between their activation and the activation of Ras is unknown, since there is no obvious homologue of FRS2 in *Drosophila*. The only intracellular protein known to bind FGFRs is Dof (also known as Heartbroken or Stumps), which has been shown, in a yeast two-hybrid assay, to bind the C-terminal region of FGFRs [24]. Dof is essential for the transmission of the FGFR signal to Ras [25–27], although the mechanism for this is still not known. Negative regulation of the FGFR signal in *Drosophila* has been shown only by the general RTK down-regulator Sprouty [28].

In the present study, we demonstrate association between RasGAP [29], the *Drosophila* p120 RasGAP orthologue, and the activated *Drosophila* FGFRs Btl and Htl. We present evidence that the SH2 domains of RasGAP mediate binding to an evolutionarily conserved juxtamembrane phosphotyrosine in the activated *Drosophila* FGFRs. This is the first evidence for direct recruitment of p120 RasGAP to the autophosphorylation sites of activated FGFRs. Our results suggest a role for RasGAP in the regulation of FGFR signalling in *Drosophila* by down-regulating Ras at its site of activation.

EXPERIMENTAL

Plasmid construction

Full-length RasGAP cDNA with a C-terminal myc epitope tag in the *Drosophila* expression vector pUAST has been described previously [29]. myc-tagged RasGAP cDNA was subcloned from pUAST as an *Sma*I fragment into the *Msc*I polylinker site of the *Schizosaccharomyces pombe* expression vector pREP4 [30]. A plasmid carrying the SH2–SH3–SH2 region (amino acids 83–343) in pGEX-KG has been described previously [12]. Missense mutations were introduced into these plasmids by PCR-mediated mutagenesis as follows: for the SH2 domains, a highly conserved arginine residue was replaced with leucine (amino acid 110 in the N-terminal domain and amino acid 278 in the C-terminal domain); for the SH3 domain, the highly conserved tryptophan residue was replaced with alanine (amino acid 219 in the SH3 domain); for the GAP domain, the arginine residue corresponding to the arginine finger was replaced with lysine (amino acid 695 in the GAP domain). Tor-Htl was subcloned from the pUAST vector (a gift from Dr S. Leever, Cancer Research UK London Research Unit, London, U.K.) into pBS as a *Kpn*I–*Xba*I fragment. Missense mutations were introduced by PCR-mediated mutagenesis as follows: the juxtamembrane tyrosine residue was replaced with phenylalanine (amino acid 402 of Htl). Both the original Tor⁴⁰²¹-Htl and the mutant Tor-Htl^{Y402F} were cut out of pBS as *Kpn*I–*Xba*I fragments, blunt-ended and cloned into the *Sma*I polylinker sites of the *S. pombe* expression vector pREPCD1, a derivative of pREP1 [30]. pGEX-Drk^{WT} and pGEX-Drk^{SH3*} have been described previously [31]. Wild-type dCrk (*Drosophila* homologue of the adapter protein CrkII; amino acids 12–271) was subcloned from pBS (a gift from Dr S. Abmayr, Pennsylvania State University) as a *Pvu*II fragment into the *Sma*I polylinker site of pGEX-KG. Missense mutations were introduced by PCR-mediated mutagenesis as follows: for the SH2 domain, a highly conserved arginine residue was replaced with leucine (amino acid 38 in the dCrk SH2 domain). All plasmids were constructed in

Escherichia coli DH5 α and sequenced to check for the presence of correct mutations.

Purification of GST (glutathione S-transferase) fusion proteins

GST fusion proteins were expressed in the *E. coli* DH5 α strain by induction with 0.2 mM isopropyl β -D-thiogalactoside for 2 h at 37 °C. Cells were harvested by centrifugation and lysed by sonication in the following solution: 50 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0), 150 mM NaCl, 5 mM 2-mercaptoethanol and 1 mM PMSF. Triton X-100 (1%, v/v) was added after sonication, and proteins were allowed to solubilize at 4 °C for 20 min with gentle agitation. The lysates were centrifuged at 10 000 g and the supernatants were incubated with glutathione–Sephadex 4B (Amersham Biosciences) to immobilize the GST fusion proteins, washed four times in lysis buffer + 1% Triton X-100, aliquoted in the presence of 50% (v/v) glycerol and stored at –80 °C until use.

Immunoprecipitations and GST pull-downs from *Drosophila* and *S. pombe* extracts

For immunoprecipitations, polyclonal anti-RasGAP was immobilized on Protein G–Sephadex beads (Sigma) by incubating for 1 h at 4 °C before washing twice with lysis buffer. An extract equivalent to ten flies or 50 μ g of *S. pombe* protein was added to these beads and incubated for 2 h at 4 °C. The beads were washed four times with lysis buffer before analysing by SDS/PAGE and WB (Western blotting). For pull-downs with GST fusion proteins, a *Drosophila* extract equivalent to ten flies was incubated with 5 μ g of the fusion proteins bound to glutathione–Sephadex 4B for 2 h at 4 °C. The beads were washed four times with lysis buffer before analysing by SDS/PAGE and WB.

Peptide pull-downs

Peptides corresponding to the conserved juxtamembrane sequence FNEYEFPLDSNWE from Btl and Htl were synthesized in either tyrosine-phosphorylated or non-phosphorylated form and biotinylated at the N-terminus. Streptavidin–agarose beads (Sigma) were blocked with 5% (w/v) BSA in lysis buffer for 30 min at 4 °C before adding an aqueous solution containing 2 mM biotinylated peptides and incubating for a further 1 h. The beads were washed three times with lysis buffer before adding a *Drosophila* extract equivalent to ten flies and incubating for 2 h at 4 °C. The beads were then washed four times with lysis buffer before being analysed by SDS/PAGE and WB.

General biochemical and immunological techniques

Monoclonal and polyclonal anti-RasGAP antibodies have been described previously [29]. Anti-myc monoclonal antibody 9E10 was a gift from Dr C. J. Marshall (The Institute of Cancer Research, London, U.K.) and anti-phosphotyrosine antibody PY100 was obtained from Cell Signaling Technology. Protein samples were separated by SDS/PAGE and electroblotted on to PVDF membranes. Blots of monoclonal anti-RasGAP antibody and anti-myc were blocked in 5% (w/v) skimmed milk-TBST (25 mM Tris/HCl, pH 7.5/150 mM NaCl/0.1% Tween 20) for 30 min before adding the antibody at 1:100 dilution in 5% skimmed milk-TBST and further incubating for 1–2 h; washes were performed with TBST. Anti-phosphotyrosine blots were blocked in 5% BSA-TBST for 1 h before adding the antibody at 1:10 000 dilution for a further 1–2 h; washes were performed with TBST. Horseradish peroxidase-conjugated secondary antibodies

and enhanced chemiluminescence substrates were used according to the manufacturer's instructions (Pierce).

Methods for *Drosophila*

Flies were raised and crossed at 25 °C according to standard procedures. Transgenic lines were made by a P-element-mediated transformation into a y^w stock. The *GAL4* line MS1096 (chromosome 1) was used for expression in the dorsal pouch of the wing disc. The *GAL4* line *hsp70-GAL4* (chromosome 2) was used for the low-level ubiquitous expression of UAS (upstream activator sequence) constructs at 25 °C (obtained from the Bloomington Stock Center). *UAS-EGFP* (where EGFP stands for enhanced green fluorescent protein; chromosome 2) line was obtained from the Bloomington Stock Center. The *UAS-tor⁴⁰²¹-btl* and *UAS-tor⁴⁰²¹-htl* lines were as described previously [29]. Wings were dissected, dehydrated in ethanol and mounted in Euparal. Digital photographs of the wings were processed in Openlab software (Improvision, Coventry, U.K.). Soluble extracts from *Drosophila* adults were prepared in the following lysis buffer: 20 mM Tris/HCl (pH 7.0), 2 mM EDTA, 150 mM NaCl, 1 % Triton X-100, 10 % glycerol, 5 mM sodium vanadate, 5 mM 2-mercaptoethanol and protease inhibitors (Complete mix; Boehringer Mannheim) plus 1 mM PMSF.

Methods for *S. pombe*

Methods for the molecular genetics and biochemistry of *S. pombe* and media [YES (yeast extract with supplements) and EMM (Edinburgh minimal medium)] for growth of *S. pombe* cultures have been described previously [32]. pREP plasmids were transformed into the *S. pombe* strain CB14 (*h⁻ ade6-M210 leu1-32 ura4-D18*) by the lithium acetate method. *S. pombe* cell extracts were prepared by vortex-mixing with glass beads in *Drosophila* lysis buffer + protease inhibitors, followed by centrifugation at 10 000 g for 10 min.

RESULTS

RasGAP associates with activated Btl and Htl in vitro

Activated versions of FGFRs, composed of the intracellular part of Btl or Htl fused to the extracellular and transmembrane domains of a gain-of-function *torso* mutant *tor⁴⁰²¹* [23,29], were expressed in adult *Drosophila*. Extracts of these flies were incubated with immobilized GST fusions of the SH2–SH3–SH2 region of RasGAP, with either wild-type (SH232^{WT}) or mutant (SH2*32*) SH2 domains. The myc-tagged Tor-Btl and Tor-Htl proteins were precipitated by wild-type (SH232^{WT}) fusion protein, but not by the double SH2 mutant (SH2*32*) fusion protein (Figure 1A). Tyrosine-phosphorylated Tor-Htl, detected in the extract by a phosphotyrosine-specific antibody (PY100), was depleted in the unbound fraction, since it associated with the wild-type SH232 fusion protein during incubation (Figure 1B). Although most of the tyrosine-phosphorylated Tor-Htl associates with the SH232 fusion protein, less than 5 % of the total Tor-Htl does so, indicating that the stoichiometry of tyrosine phosphorylation on the activated receptor is low, as reported for the Tor-Btl fusion protein [33]. The small fraction of Tor-Btl and Tor-Htl pulled down by the SH232 fusion protein migrates faster after SDS/PAGE compared with the bulk of the receptor protein. Although we consistently observed association of both Tor-Btl and Tor-Htl with the SH232 fusion protein in several independent experiments, the binding of Tor-Btl appeared to be weaker; this could reflect a lower stoichiometry of phosphorylation of Tor-Btl compared with Tor-Htl or a weaker binding affinity for the RasGAP SH2 domains.

Figure 1 *Drosophila* RasGAP associates with activated Btl and Htl in vitro

(A) GST fusion proteins containing WT (wild-type) SH232 (lanes 2 and 5) or mutant SH2*32* domains (lanes 3 and 6) of RasGAP were incubated with an extract from *Drosophila* expressing either Tor-Htl or Tor-Btl proteins. Associated Tor-Htl or Tor-Btl was detected by WB with anti-myc antibodies (9E10; α -myc) raised against the myc tag of the receptors (upper panel). An extract equivalent to 5 % of that incubated with the fusion proteins was loaded in lanes 1 and 4. Equal loading of the GST fusion proteins was shown by staining the membrane with Coomassie Brilliant Blue (lower panel). (B) A GST fusion protein containing the wild-type SH232 domains of RasGAP was incubated with an extract from *Drosophila* expressing Tor-Htl protein. Htl was visualized by WB with a phosphotyrosine-specific antibody (PY100; upper panel, α -pTyr) or anti-myc antibodies (lower panel).

Identification of putative RasGAP-binding sites on the FGFR

Alignments made between the cytoplasmic regions of mammalian FGFR-1 and the *Drosophila* FGFRs revealed that out of the seven known autophosphorylation sites on mammalian FGFR-1 [34], five are conserved in Btl and Htl. Analysis of the sequence surrounding these tyrosine residues revealed that the conserved juxtamembrane tyrosine residue in Btl (Tyr⁶⁹⁷) and Htl (Tyr⁴⁰²) fits the consensus for binding to mammalian p120 RasGAP SH2 domains, (phospho)YXXPXD [35]. A peptide corresponding to this consensus region, spanning 13 amino acids that are identical in both Btl and Htl, was tested for its ability to bind RasGAP. myc-tagged RasGAP proteins, some with adapter domain mutations, were expressed in *Drosophila* and incubated with immobilized peptides in either the non-phosphorylated (referred to as Tyr peptide hereafter) or tyrosine-phosphorylated form (referred to as pTyr peptide hereafter; Figure 2A). Immobilized pTyr peptide was capable of precipitating wild-type RasGAP expressed in adult *Drosophila*, whereas immobilized Tyr peptide was not. The double SH2 and single N-terminal SH2 mutants of RasGAP did not associate with the pTyr peptide either. The single C-terminal SH2 mutant of RasGAP showed a much reduced ability to associate with the pTyr peptide, whereas the single SH3 mutant showed no decrease in affinity for the pTyr peptide.

To validate further that the juxtamembrane tyrosine residue of Btl and Htl is a binding site for RasGAP, the phosphorylated and non-phosphorylated peptides were tested for their ability to compete with activated FGFRs in pull-down experiments similar to those already shown with the adapter region of RasGAP (Figure 2B). Tor-Htl expressed in adult *Drosophila* was precipitated by immobilized SH232^{WT} in the presence of the highest concentration of Tyr peptide (200 μ M), whereas there was a large decrease in the amount of activated FGFR precipitated in the presence of even the lowest concentration of pTyr peptide (25 μ M). Taken together, these results show that RasGAP can associate with the conserved juxtamembrane tyrosine residue of FGFR when it is phosphorylated. This interaction requires both of the SH2 domains of RasGAP for maximal binding.

Figure 2 RasGAP associates with a conserved region of FGFRs in a phosphotyrosine-dependent manner

(A) Biotinylated peptides, corresponding to the conserved sequence FNEYFPLDSNWE from Btl and Htl, either phosphorylated on tyrosine (upper panel) or not (middle panel) were immobilized on streptavidin–agarose beads. Wild-type RasGAP (lane 1), double SH2 mutant RasGAP^{SH2*32*} (lane 2), single N-terminal SH2 mutant RasGAP^{SH2*32} (lane 3), SH3 mutant RasGAP^{SH23*2} (lane 4) or single C-terminal SH2 mutant RasGAP^{SH232*} (lane 5) proteins were expressed in *Drosophila*. Extracts from these flies (lower panel) were incubated with the immobilized peptides. Associated RasGAP was detected by WB with α -myc antibodies. (B) Immobilized GST fusion proteins containing wild-type SH232 domains (lanes 2–10) of RasGAP were incubated with an extract from *Drosophila* expressing Tor-Htl in the presence (lanes 3–10) or absence (lane 2) of an additional peptide. An extract equivalent to 5% of that incubated with the fusion proteins was loaded in lane 1. Increasing concentrations (25, 50, 100 or 200 μ M) of either the phosphotyrosine peptide pTyr (lanes 3–6) or the non-phosphorylated peptide Tyr (lanes 7–10) were added to the extract before incubation. Associated Tor-Htl that still precipitated with the fusion protein was detected by WB with α -myc antibodies.

The small adapter proteins dCrk and Drk do not associate with FGFRs

The juxtamembrane tyrosine residue of vertebrate FGFR-1 (Tyr⁴⁶³) is a docking site for the adapter protein CrkII [36]. *Drosophila* has a single Crk family member, dCrk [37], which is 49% identical with CrkII, and the ability of this small adapter to bind to *Drosophila* FGFRs was investigated. GST fusion proteins of dCrk with either a wild-type or mutant SH2 domain were expressed in *E. coli* and tested for their ability to associate with myc-tagged Tor-Htl expressed in adult *Drosophila* (Fig-

Figure 3 The small adapter proteins dCrk and Drk do not associate with the FGFRs in *Drosophila*

Immobilized GST fusion proteins of Drk with a wild-type (lane 2) or mutant (lane 3) SH3 domain and immobilized GST fusion proteins of dCrk with a wild-type (lane 4) or mutant (lane 5) SH2 domain were incubated with an extract from *Drosophila* expressing Tor-Htl. An extract equivalent to 5% of that incubated with the fusion proteins was loaded in lane 1. Activated Htl that precipitated with the fusion proteins was detected by WB with α -myc antibodies.

ure 3). Neither the wild-type nor the SH2 mutant dCrk fusion protein was capable of associating with myc-tagged Tor-Htl. To confirm that the SH2 domain was functional, the blot was reprobed with a phosphotyrosine-specific antibody (PY100). This result confirmed that wild-type dCrk, but not the SH2-domain mutant, was capable of precipitating a number of unidentified phosphotyrosine-containing proteins (results not shown). This shows that the interaction between the Crk family of adapter proteins and the FGFRs is not conserved in *Drosophila*.

Drosophila Ras is required for FGFR signalling, but how the receptors are linked to Ras activation is not known. *Drosophila* lacks a homologue of FRS2, which links mammalian FGFRs to the Grb2–Sos complex; therefore we tested the ability of Drk, the *Drosophila* homologue of Grb2, to bind directly to FGFRs. GST fusion proteins of Drk with either wild-type or mutant SH3 domains were not capable of associating with myc-tagged Tor-Htl expressed in adult *Drosophila* (Figure 3). This result shows that the lack of an FRS2 homologue in *Drosophila* is not compensated by the direct recruitment of Drk to FGFRs, suggesting that, similar to vertebrates, there is an FGFR substrate that recruits the Drk–Sos complex to the receptors.

RasGAP is tyrosine-phosphorylated in response to FGFR signalling in *Drosophila*

Wild-type RasGAP and Tor-Htl or Tor-Btl were either expressed by themselves or co-expressed together in adult *Drosophila*. RasGAP was immunoprecipitated from extracts of these flies and its tyrosine phosphorylation state was assessed by WB using a phosphotyrosine-specific antibody (Figure 4A). Tyrosine phosphorylation of RasGAP could only be detected when it was co-expressed with activated Htl or Btl. To assess the role of the adapter region and the catalytic activity of RasGAP in this process, various adapter domain mutants and a catalytically inactive form of RasGAP were co-expressed with Tor-Htl in *Drosophila* and immunoprecipitated as before (Figure 4B). The double SH2-domain mutant RasGAP was not phosphorylated on tyrosine, whereas the N- and C-terminal single SH2 mutant

Figure 4 Tyrosine phosphorylation of RasGAP in response to FGFR signalling in *Drosophila* requires both the SH2 domains

(A) RasGAP was immunoprecipitated (IP) with a polyclonal antibody from extracts of adult *Drosophila* expressing wild-type RasGAP only (lane 1), Tor-Htl only (lane 2), wild-type RasGAP co-expressed with Tor-Htl (lane 3) or wild-type RasGAP co-expressed with Tor-Btl (lane 4). The tyrosine phosphorylation state of the immunoprecipitated RasGAP was assessed by WB with an α -pTyr antibody (upper panel). The blot was reprobbed with an anti-RasGAP monoclonal antibody to confirm the successful immunoprecipitation of RasGAP (middle panel). An extract equivalent to 5% of that incubated with the antibody was subjected to WB with α -myc antibodies against the myc tags of the activated receptors and RasGAP (lower panel). (B) RasGAP was immunoprecipitated with a polyclonal antibody from extracts of adult *Drosophila* co-expressing Tor-Htl with either wild-type RasGAP (lane 1), double SH2-domain mutant RasGAP^{SH2*32} (lane 2), single N-terminal SH2-domain mutant RasGAP^{SH2*32} (lane 3), SH3 domain mutant RasGAP^{SH2*32} (lane 4), single C-terminal SH2-domain mutant RasGAP^{SH2*32} (lane 5) or catalytically inactive RasGAP^{R695K} (lane 6). The tyrosine phosphorylation state of the immunoprecipitated RasGAP was assessed by WB with an α -pTyr antibody (upper panel). The blot was reprobbed with an anti-RasGAP monoclonal antibody to confirm the successful immunoprecipitation of RasGAP (middle panel). An extract equivalent to 5% of that incubated with the antibody was subjected to WB with α -myc antibodies against the myc tags of activated Htl and RasGAP (lower panel).

forms of RasGAP showed a much reduced state of tyrosine phosphorylation. The SH3 domain mutant and the catalytically inactive RasGAP mutant did not show a decrease in tyrosine phosphorylation compared with wild-type RasGAP. These results show that RasGAP is tyrosine-phosphorylated in the presence of activated FGFR. Maximal phosphorylation of RasGAP requires both the SH2 domains to be intact, whereas it does not require the SH3 domain or the GAP activity.

In this experiment, we observed a tyrosine-phosphorylated protein (Figure 4A, lane 4, filled arrowhead) in the RasGAP immunoprecipitate from flies expressing activated Btl. This phosphoprotein co-migrated with the faster migrating form of Tor-Btl (Figure 4A, lane 4, open arrowhead), suggesting that it is probably a tyrosine-phosphorylated form of the receptor that can be co-immunoprecipitated with RasGAP. However, because of the similarity in molecular mass of RasGAP and Tor-Btl and the fact that both proteins are myc-tagged, we were unable to confirm by anti-myc WB that Tor-Btl can be co-immunoprecipitated with RasGAP. We did not observe a band corresponding to Tor-Htl in the RasGAP immunoprecipitates.

Tyrosine phosphorylation of RasGAP in a heterologous system requires its intact SH2 domains and the juxtamembrane tyrosine residue of Htl

From the results shown in Figure 4, RasGAP could be either a direct substrate of the receptor or a substrate of a cytoplasmic tyrosine kinase activated by FGFR signalling. To investigate these possibilities, we co-expressed RasGAP and activated Htl in a heterologous system, namely the fission yeast *S. pombe*. In *S.*

pombe, there is very little endogenous tyrosine kinase activity and there are no downstream cytoplasmic tyrosine kinases that may become activated in response to FGFR signalling. Furthermore, since *S. pombe* does not have homologues of FGFRs, it is unlikely to have adapter proteins that could mediate indirect association between RasGAP and the activated FGFR. Tor-Htl was co-expressed with either wild-type RasGAP or the double SH2-domain mutant RasGAP. RasGAP was immunoprecipitated from yeast cell extracts, and its tyrosine phosphorylation state was assessed with a phosphotyrosine-specific antibody (Figure 5). Wild-type RasGAP, but not the double SH2-domain mutant RasGAP, was phosphorylated on tyrosine when co-expressed with Tor-Htl in *S. pombe*.

To assess the role of the juxtamembrane Tyr⁴⁰² of Htl in this process, it was mutated to phenylalanine in the activated version of the receptor (Tor-Htl^{Y402F}), and expressed with wild-type RasGAP or the double SH2-domain mutant RasGAP in *S. pombe*. RasGAP was immunoprecipitated from these extracts, and its tyrosine phosphorylation state was assessed using a phosphotyrosine-specific antibody (Figure 5). Mutating the juxtamembrane tyrosine of Tor-Htl to phenylalanine abolished tyrosine phosphorylation of RasGAP in this heterologous system. The Tor-Htl^{Y402F} receptor was still autophosphorylated, although to a lesser extent, showing that it still has kinase activity (results not shown).

Taken together, these results provide strong evidence that RasGAP is a direct substrate of the activated FGFR kinase domain. Requirement of the SH2 domains of RasGAP was confirmed, suggesting that RasGAP interacts directly with autophosphorylation sites on FGFR without the need for any adapter

Figure 5 RasGAP is tyrosine-phosphorylated directly by activated Htl in *S. pombe*

Wild-type RasGAP (lanes 1 and 3) or double SH2-domain mutant RasGAP^{SH2*32*} (lanes 2 and 4) were co-expressed in *S. pombe* with wild-type Tor-Htl (lanes 1 and 2) or Tor-Htl^{Y402F} (lanes 3 and 4). RasGAP was immunoprecipitated with a polyclonal antibody from extracts of these *S. pombe* cells and its tyrosine phosphorylation state was assessed by WB with an α -pTyr antibody (upper panel). The blot was reprobed with an anti-RasGAP monoclonal antibody to confirm the successful immunoprecipitation of RasGAP (lower panel).

proteins. Since the phosphorylation of RasGAP by activated Htl requires the conserved juxtamembrane tyrosine of the receptor, this is probably the major binding site for the SH2 domains of RasGAP.

GAP activity and both SH2 domains of RasGAP are required to attenuate FGFR signalling *in vivo*

The results presented above show that the SH2 domains of RasGAP can bind to FGFRs *in vitro* and the tyrosine phosphorylation of RasGAP when co-expressed with activated FGFRs indicates that the interaction can occur *in vivo*. A possible function of this interaction *in vivo* could be to down-regulate the activity of Ras at the site of receptor activation by recruiting RasGAP. To examine this hypothesis, the effects of ectopically expressing activated FGFR together with RasGAP in the wing imaginal disc were investigated. Ras signalling is required for wing vein formation: decreasing Ras signalling limits vein formation, whereas increasing Ras signalling produces extra wing vein material. We have shown previously that expression of activated FGFRs in the wing imaginal disc produces extra wing vein material in a Ras-dependent manner and co-expression of RasGAP suppresses the formation of extra wing veins [29]. Tor-Htl was co-expressed in the dorsal pouch of the wing disc with various RasGAP single-domain mutants (Figure 6). As controls, Tor-Htl was co-expressed with EGFP, wild-type RasGAP and the double SH2-domain mutant RasGAP, which was shown previously to be unable to suppress FGFR signalling. The extra wing vein material induced by expressing Tor-Htl was not suppressed by the co-expression of EGFP. This level of extra wing vein material was compared with the level seen when various RasGAP proteins were co-expressed with Tor-Htl. Wild-type RasGAP gave strong, although not complete, suppression of the extra wing vein material, whereas the double SH2-domain mutant RasGAP did not, as shown previously [29]. The single N- and C-terminal SH2-domain mutant RasGAP proteins were also not capable of suppressing the extra wing vein material, whereas the

Figure 6 The SH2 domains and the catalytic activity of RasGAP are required *in vivo* to suppress signalling by activated Htl

All wings expressed *UAS-tor-htl* under control of the strong GAL4 driver MS1096, which drives expression in the wing imaginal disc. Misexpression of Tor-Htl induced an ectopic vein phenotype (A), with 'deltas' at the wing margin (filled arrowheads) and ectopic veins in the intervein regions (open arrowheads). The ectopic vein phenotype was efficiently suppressed by co-expression of wild-type RasGAP (B) or the SH3-domain mutant RasGAP (E), but not by co-expression of mutants of RasGAP where one SH2 domain (D, F) or both SH2 domains (C) were inactivated. Co-expression of a mutant RasGAP defective in GAP activity enhanced the ectopic vein phenotype (G), and co-expression of a different GAP protein (Gap1) did not efficiently suppress the ectopic vein phenotype (H). The genotypes are *GAL4/+; UAS-tor⁴⁰²¹-htl/UAS-EGFP* (A), *GAL4/+; UAS-tor⁴⁰²¹-htl/UAS-RasGAP^{WT}* (B), *GAL4/+; UAS-tor⁴⁰²¹-htl/+* (C), *GAL4/+; UAS-tor⁴⁰²¹-htl/UAS-RasGAP^{SH2*32}* (D), *GAL4/+; UAS-tor⁴⁰²¹-htl/UAS-RasGAP^{SH2*32*2}* (E), *GAL4/+; UAS-tor⁴⁰²¹-htl/+* (F), *GAL4/+; UAS-tor⁴⁰²¹-htl/UAS-RasGAP^{R695K}* (G) and *GAL4/+; UAS-tor⁴⁰²¹-htl/+; UAS-Gap1/+* (H). Scale bar, 250 μ m.

SH3-domain mutant RasGAP gave good suppression. The GAP domain mutant RasGAP enhanced the phenotype, indicating that it may be acting in an antimorphic or dominant-negative manner; this result excludes the possibility that RasGAP suppresses FGFR signalling by preventing a positive effector from associating with the receptor. Expression of Gap1, a related GAP protein [38], did not suppress the extra wing vein phenotype, indicating a difference in specificity between it and RasGAP. In summary, these results show that the GAP activity and both SH2 domains of RasGAP, but not its SH3 domain, are required *in vivo* to attenuate FGFR signalling.

DISCUSSION

In this report, we present evidence for a novel interaction between RasGAP and activated FGFRs in *Drosophila*. *In vitro* the N-terminal adapter region of RasGAP could associate with activated versions of the FGFRs, Btl and Htl, in an SH2-dependent manner. *In vivo*, RasGAP was tyrosine-phosphorylated when

co-expressed with activated FGFRs and this was dependent on the SH2 domains. However, in both of these experiments, it was possible that there was an adapter protein bridging the interaction between RasGAP and the receptor, and that RasGAP was phosphorylated by a tyrosine kinase activated after activation of FGFR signalling and not directly by the receptor itself. This issue was addressed by co-expression of activated Htl and RasGAP in the fission yeast *S. pombe*, which lacks FGFR homologues and is unlikely to have adapter proteins that might bridge an interaction between Htl and the RasGAP SH2 domains. Tyrosine phosphorylation of RasGAP when co-expressed with activated Htl was apparent in this heterologous system and was still dependent on the SH2 domains of RasGAP, consistent with a direct interaction of the two proteins. These results provide strong evidence that RasGAP directly associates with the activated FGFRs and is a substrate of the receptors *in vivo*. The stability and dynamics of interaction of RasGAP with FGFRs were not addressed by these experiments, but evidence was obtained for co-immunoprecipitation of RasGAP with Tor-Btl, but not with Tor-Htl.

Alignments of the mammalian FGFR-1 to the *Drosophila* FGFRs revealed a potential binding site for the RasGAP SH2 domains, conserved between species, in the form of a tyrosine residue within the consensus sequence, (phospho)YXXPD, lying between the transmembrane domain and the cytoplasmic tyrosine kinase domain of the receptors. The fact that the corresponding tyrosine residue was shown to be both an autophosphorylation site and a docking site for SH2 domains in mammalian FGFR-1 [34,36] suggested that this juxtamembrane tyrosine was a good candidate for a RasGAP-binding site on Btl and Htl. This was shown to be the case in three separate experiments. First, a peptide corresponding to this region of Btl and Htl could precipitate RasGAP expressed in *Drosophila* only when the peptide was phosphorylated on the tyrosine. Secondly, this phosphorylated peptide could readily compete with Tor-Htl for binding to immobilized RasGAP SH2 domains, whereas the non-phosphorylated peptide could not compete with this binding. Thirdly, if the juxtamembrane Tyr⁴⁰² of Htl was mutated, RasGAP was no longer tyrosine-phosphorylated when expressed in *S. pombe*. This suggests that RasGAP could not be recruited to the mutated receptor and therefore was not in a position to be phosphorylated by the active receptor kinase domain. The evidence provided indicates that the juxtamembrane tyrosine residue of Btl and Htl is both an autophosphorylation site and a binding site for the SH2 domains of RasGAP.

Why does RasGAP have tandem SH2 domains? Two possible reasons for the existence of tandem SH2 domains in the adapter region of RasGAP are (1) they may be required to bind two separate docking sites simultaneously and (2) they may stabilize the interaction with one docking site by increasing the avidity. The individual requirement of the SH2 domains in the interaction with Btl and Htl was tested in two experiments. One of them showed that the immobilized pTyr peptide of Btl/Htl was unable to precipitate RasGAP with single N- and C-terminal mutant SH2 domains to the same extent as wild-type RasGAP. This shows that the two SH2 domains in RasGAP work together to increase the strength of the interaction with the juxtamembrane phosphotyrosine of FGFRs. The second experiment showed that both SH2 domains of RasGAP were required for its maximal tyrosine phosphorylation in response to FGFR signalling in *Drosophila*. This suggests that when RasGAP has only one intact SH2 domain, it cannot form a stable enough complex with an activated tyrosine kinase, probably the FGFR in this case, to be phosphorylated efficiently. These results indicate that the tandem SH2 domains of the RasGAP adapter region are essential

for both the initial interaction with FGFRs and its subsequent phosphorylation on tyrosine. The fact that the activated FGFRs will be dimerized means that there will be two juxtamembrane phosphotyrosine-binding sites in the receptor complex, both of which may be bound to the tandem SH2 domains of RasGAP, adding to the avidity of the association and stabilizing the interaction. The requirement for both SH2 domains in the binding of RasGAP to FGFRs is consistent with the requirement of both SH2 domains for the attenuation of ectopic FGFR signalling by RasGAP in the wing.

As noted earlier, there is a corresponding juxtamembrane tyrosine residue equivalent to that of Btl and Htl in mammalian FGFR-1 that is autophosphorylated and binds the SH2 domain of mammalian CrkII [36]. However, when dCrk, the *Drosophila* homologue of CrkII, was tested for its ability to bind the FGFR homologues in *Drosophila*, no association could be detected. This suggests that the interaction between Crk and FGFRs is not conserved between mammals and *Drosophila*. Therefore it is unlikely that in *Drosophila*, RasGAP and dCrk compete *in vivo* for binding to the juxtamembrane tyrosine residues of Btl and Htl. In mammals, it is possible that CrkII and p120 RasGAP may both compete with each other for binding to the juxtamembrane tyrosine residue of FGFR-1.

Drk, the *Drosophila* homologue of mammalian Grb2, has been shown to act upstream of Ras in RTK signalling pathways and acts by recruiting the Ras activator, Sos, to the site of receptor activation [31]. Drk has been shown previously to bind directly to activated RTKs [31] or to substrates of the activated RTKs [12]. Since activation of Ras has been shown to rescue partially both *btl* [23] and *htl* [21] mutant phenotypes, the ability of Drk to interact directly with these FGFRs was tested. However, a GST fusion protein of wild-type Drk was not capable of associating with activated Btl or Htl expressed in adult *Drosophila*. This result suggests that the recruitment of Drk to the site of FGFR activation in *Drosophila* is not directly to the receptor, but requires a receptor substrate, as in the case of mammals that utilize the adapter-like protein FRS2. The lack of an FRS2 homologue in *Drosophila* suggests the involvement of a novel adapter in FGFR signalling; one candidate is the cytoplasmic protein Dof, which is essential for FGFR signalling in *Drosophila*. It acts between FGFRs and Ras, and it contains a number of tyrosine residues that lie within a consensus binding sequence for the Drk SH2 domain [25].

In conclusion, a model is proposed in which, after ligand binding, the FGFRs Btl and Htl undergo autophosphorylation on the juxtamembrane tyrosine residue, thereby providing a docking site for RasGAP. This association is stabilized by the fact that RasGAP possesses two SH2 domains, both of which are required for maximal binding to the FGFRs. Once recruited, RasGAP becomes a substrate for the active kinase domain of the receptor, potentially providing further docking sites at the location of receptor activation. Recruitment of RasGAP to the activated FGFRs would allow it to act on its substrate, namely RasGTP, thus negatively regulating the downstream signal through Ras effector pathways, such as the MAPK pathway. This model is consistent with our observations that the ability of RasGAP to attenuate FGFR signalling *in vivo* requires its GAP activity and both its SH2 domains, but not its SH3 domain, which is dispensable for FGFR binding. However, the physiological relevance of the association of RasGAP with FGFRs remains to be established. The recent identification of mutants defective in the gene encoding RasGAP, *vacuolar peduncle* (*vap*) [39], will make it possible to examine the effects of loss of RasGAP activity on FGFR signalling pathways regulating morphogenesis and differentiation in the *Drosophila* embryo.

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