

Downstream-of-FGFR Is a Fibroblast Growth Factor-Specific Scaffolding Protein and Recruits Corkscrew upon Receptor Activation

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Fibroblast growth factor (FGF) receptor (FGFR) signaling controls the migration of glial, mesodermal, and tracheal cells in *Drosophila melanogaster*. Little is known about the molecular events linking receptor activation to cytoskeletal rearrangements during cell migration. We have performed a functional characterization of Downstream-of-FGFR (Dof), a putative adapter protein that acts specifically in FGFR signal transduction in *Drosophila*. By combining reverse genetic, cell culture, and biochemical approaches, we demonstrate that Dof is a specific substrate for the two *Drosophila* FGFRs. After defining a minimal Dof rescue protein, we identify two regions important for Dof function in mesodermal and tracheal cell migration. The N-terminal 484 amino acids are strictly required for the interaction of Dof with the FGFRs. Upon receptor activation, tyrosine residue 515 becomes phosphorylated and recruits the phosphatase Corkscrew (Csw). Csw recruitment represents an essential step in FGF-induced cell migration and in the activation of the Ras/MAPK pathway. However, our results also indicate that the activation of Ras is not sufficient to activate the migration machinery in tracheal and mesodermal cells. Additional proteins binding either to the FGFRs, to Dof, or to Csw appear to be crucial for a chemotactic response.

Fibroblast growth factor (FGF) receptors (FGFRs) control diverse cellular processes, including cell proliferation, differentiation, migration, and survival during metazoan development (for reviews, see references 29 and 31). FGFRs belong to a large family of transmembrane receptor tyrosine kinases (RTK) and activate a variety of downstream signaling molecules. The transcriptional responses to FGFR activation are elicited, at least in part, via the Ras/mitogen-activated protein kinase (MAPK) pathway and the phosphorylation of Ets-domain-containing transcriptional regulators (25). Specific responses in the nucleus are then most likely determined by interactions with preexisting nuclear components on genomic enhancers or repressor elements (1, 20, 40). Much less is known about how RTK signaling or FGFR signaling in particular regulates cytoplasmic events in the control of cell movement.

In *Drosophila melanogaster*, FGFR signaling has been implicated in the migration of several tissues, notably, in the spreading of mesoderm, the migration of tracheal cells and glial cells in the embryo, and the recruitment of mesodermal cells into the genital disk and in the formation of air sacs during larval stages (2, 4, 11, 12, 18, 36). In vivo imaging in live *Drosophila* embryos has shown that FGFR signaling indeed leads to dynamic cytoskeletal reorganizations during migration, resulting in the formation of filopodial extensions in tip cells (33, 36). Despite the importance of FGFR signaling in cell migration in *Drosophila*, the molecular events linking signal reception to these cytoskeletal events remain elusive.

Similar to other RTKs, FGFRs are activated by receptor

dimerization upon ligand binding, which then leads to activation of the intracellular kinase domain and autophosphorylation of the RTK on specific tyrosine residues (for a review, see reference 38). Some of these phosphorylated tyrosines provide docking sites for Src-homology 2 (SH2) domain-containing proteins that propagate the signal inside the cell.

In contrast to most other RTKs, FGFRs lack a Grb2 binding site and thus need to recruit an intermediate docking molecule to activate the MAPK pathway. In vertebrates, many of the cellular responses of FGFs, including the activation of the MAPK pathway, are mediated through the recruitment of the membrane-linked docking protein SNT/FRS2 (19, 26, 43). SNT/FRS2 constitutively interacts with the juxtamembrane domain of the FGFR through its phosphotyrosine binding domain (27, 44) and recruits other signaling molecules upon tyrosine phosphorylation. Recruitment of the Grb2-Sos complex or the phosphatase SHP2 on SNT/FRS2 leads to activation of the MAPK pathway, whereas recruitment of Grb2-Gab1 leads to activation of the cell survival pathway via phosphatidylinositol 3-kinase (PI3K) interaction (14, 19, 28).

In *Drosophila*, genetic studies have identified a potential, FGFR-specific signaling mediator, Downstream-of-FGFR (Dof) (also known as Heartbroken or Stumps [16, 21, 42]). Dof is expressed exclusively in the tissues expressing the two FGFRs Heartless (Htl) and Breathless (Btl), i.e., in mesodermal, tracheal, and a subset of glial cells (42). Dof encodes an ankyrin-repeat- and coiled-coil-containing protein and represents the founding member of a small family of proteins including BCAP and BANK, two vertebrate proteins that have recently been identified and shown to regulate B-cell-receptor-specific PI3K activation and calcium mobilization, respectively (24, 45). Genetic epistasis experiments in *Drosophila* have shown that Dof functions downstream of the activated FGFR and upstream of Ras (16, 21, 42), but neither the role of Dof

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in the interpretation of the chemotactic response to FGFR signaling has been addressed so far, nor has Dof been analyzed at the molecular level. Since the FGFR signaling system plays such a dominant role in cell migration in several tissues in *Drosophila*, a first step towards understanding the complex events linking receptor activation to cytoskeletal rearrangements is the elucidation of the role of Dof.

In this work, we first show that Dof is a cellular substrate of the *Drosophila* FGFRs, becomes phosphorylated upon receptor activation, and recruits the tyrosine phosphatase Corkscrew (Csw), an event essential for Ras/MAPK activation. Using Dof variant constructs and in vivo rescue assays, we find that although Ras activation is required for chemoattraction, Ras activation is not sufficient to induce tracheal cell migration. Our studies incorporate the Dof protein into the chemotactic response downstream of FGF and demonstrate that both Ras-dependent and Ras-independent events contribute to efficient cytoskeletal reorganizations, leading to cell migration.

MATERIALS AND METHODS

Drosophila strains and genetics. The generation of the transgenic UAS*dof* flies was described previously (42). The *dof* mutant *dof*⁹³², identified in a mutant collection generated in the laboratory of C. Klämbt (15), contains a stop codon at position 225 of the Dof amino acid sequence. The UAS*Stor*⁴⁰²¹-*btl* (UAS*btl*⁴⁰²¹) flies were provided by S. Leever and E. Hafen (derived from the *hs-torso*⁴⁰²¹-*FGFR1* construct described in reference 32). The *btl*-Gal4 line (39) was used to drive upstream activated sequence (UAS) transgenes in tracheal cells, the *twist*-Gal4 line (obtained from M. Akam) was used for studies of mesodermal cells, and the *pannier*-Gal4 line was used for studies of ectodermal cells. The mutated UAS*dof* transgenes were introduced into the *Drosophila* genome by P-element-mediated germ line transformation (35). At least three independent transformant lines were analyzed for each construct. The expression of each deletion mutant protein was assayed with anti-Dof antibodies, except for transgenes 1 to 368, 1 to 484, and 485 to 600, in which expression was checked at the level of RNA.

The rescue assay was described previously (42). Briefly, UAS*dof*-transgenes/+; *dof*⁹³²/+ flies were crossed to either *btl*-Gal4/+; *dof*⁹³²/+ or *twi*-Gal4/+; *dof*⁹³²/+ flies and embryos were collected overnight at 25°C. Homozygous *dof* mutant embryos were identified either by the lack of Eve-expressing cells at the dorsal midline, which indicate defects in mesoderm spreading (*btl*-Gal4 crosses), or by the lack of tracheal cell migration (*twi*-Gal4 crosses). Rescue of mesodermal cell migration was scored with the Evenskipped antibody (see Results).

Embryo stainings. Embryo fixation, staining, and light and confocal fluorescence microscopy were performed as previously described (6). We used the following primary antibodies: monoclonal antibody 2A12, which stains the lumen of the tracheal tree (kindly provided by N. Patel), anti-Evenskipped (kindly provided by M. Frasch), mouse monoclonal anti-DSRF (13), and anti-double-phosphorylated extracellular signal-regulated kinase (anti-dpERK) (Sigma). Weak fluorescent signals were amplified by the use of biotinylated tyramide (NEN Life Science Product) followed by streptavidin-Texas Red or streptavidin-fluorescein secondary antibodies. Fluorescent images were captured by confocal microscopy (Leica TCS) and processed by use of Photoshop software (Adobe) and represent projections of sections on one focal plane. For the other stainings, we used biotinylated anti-mouse immunoglobulin G and biotinylated anti-mouse immunoglobulin M secondary antibodies, which were revealed by using the biotinylated horseradish peroxidase ABC kit (Vectastain).

Plasmid construction. The constructs prepared for the rescue assay were generated by PCR using the appropriate oligonucleotides (sequences of oligonucleotides on request) and subcloned into the pUAS vector. For expression in Schneider cells, cDNA were cloned in the pMT/V5-HisA vector (Invitrogen) containing the heavy metal methalothionein promoter. All constructs were verified by sequencing before embryo injection or before transfection of S2 cells.

Cell transfection, cell lysates, and immunoprecipitation. *Drosophila* Schneider (S2) cells were cultured at 25°C in Schneider's *Drosophila* medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml) (complete medium). For transfection experiments, 3 × 10⁶ S2 cells were plated per 35-mm-diameter dish in complete medium. Cells were transiently transfected with different combina-

tions of plasmids (0.6 µg total) using the Effectene reagent following the manufacturer's instructions (Qiagen). Protein expression was induced 24 h after transfection by addition of 0.6 mM CuSO₄ for the indicated times. Cells were lysed in modified RIPA buffer (50 mM Tris [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.4 mM EDTA, 10% glycerol, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, Protease Inhibitors Cocktail [Roche]). For immunoprecipitation assays, cell lysates were precleared with protein G-Sepharose beads (Amersham Pharmacia) for 3 h at 4°C on a rocking platform and then incubated with the primary antibody overnight at 4°C on a rocking platform. Protein-antibody complexes were recovered by incubation with protein G-Sepharose beads for 1 h at 4°C. Bead-bound complexes were washed four times with cold lysis buffer and boiled with 2× SDS sample buffer. For Western blot analysis of total cell lysates, equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, transferred on nitrocellulose or polyvinylidene difluoride Immobilon (Millipore) membranes, and probed using appropriate antibodies in 5% dry milk in TBS-T (20 mM Tris-HCl [pH 7.6]; 150 mM NaCl, 0.1% Tween 20). Proteins were visualized using enhanced chemiluminescence (ECL kit; Amersham Corp.) following the manufacturer's instructions. The following primary antibodies were used for immunoprecipitation and Western blot experiments: anti-Myc 9B11 (Cell Signaling), anti-V5 (Invitrogen), anti-dpERK (Sigma), anti-PY20 (Transduction Laboratories), and anti-Csw (kindly provided by L. A. Perkins).

RESULTS

Functional dissection of the Dof protein in vivo. Expression of a *dof* cDNA under the indirect control of tissue-specific enhancers is sufficient to rescue migration defects of tracheal and mesodermal cells in a *dof* mutant embryo (42). We used this rescue assay for a functional analysis of the Dof protein. Modified UAS-*dof* transgenes were expressed with either the *btl*-Gal4 or the *twi*-Gal4 drivers in *dof* mutant embryos in order to identify regions of Dof that are important for its function in tracheal and mesodermal cell migration, respectively (Fig. 1; Table 1). For each construct, several transgenic lines were established and tested (see Materials and Methods). The capacity of each transgene to rescue tracheal cell migration was scored by counting the formation of different tracheal branches in rescued embryos (Table 1).

Expression of a full-length *dof* transgene in the tracheal system of *dof* mutant embryos rescues most aspects of tracheal patterning, except for the visceral branches, which do not extend properly (42) (Fig. 2A, subpanel c). This is most likely due to the fact that the underlying visceral mesoderm is not correctly specified in *dof* mutant embryos and tracheal cells lack the appropriate support along their migratory route (5, 42). In addition, since the mesoderm is used by most tracheal branches as a migratory substrate (8), the tracheal network of rescued mutant embryos has a less well organized appearance than that of a wild-type embryo (42) (Fig. 2A, subpanels a and c).

Deletion from the C terminus showed that transgenes coding for Dof proteins containing at least the N-terminal 600 amino acids were able to substitute for the lack of endogenous Dof function in vivo and allowed for a significant rescue of tracheal cell migration, albeit with somewhat different quantitative efficiencies (Fig. 2A; Table 1). One of the major defects observed in these rescued tracheal trees was a discontinuity of the dorsal trunk (DT), with an average of two to three DT breaks for the Dof850 transgene and of four breaks for the Dof600 transgene (Fig. 2A; Table 1). In addition, the transgenes displayed various efficiencies in rescuing the formation of dorsal branches (DB) and ganglionic branches (GB) (Table

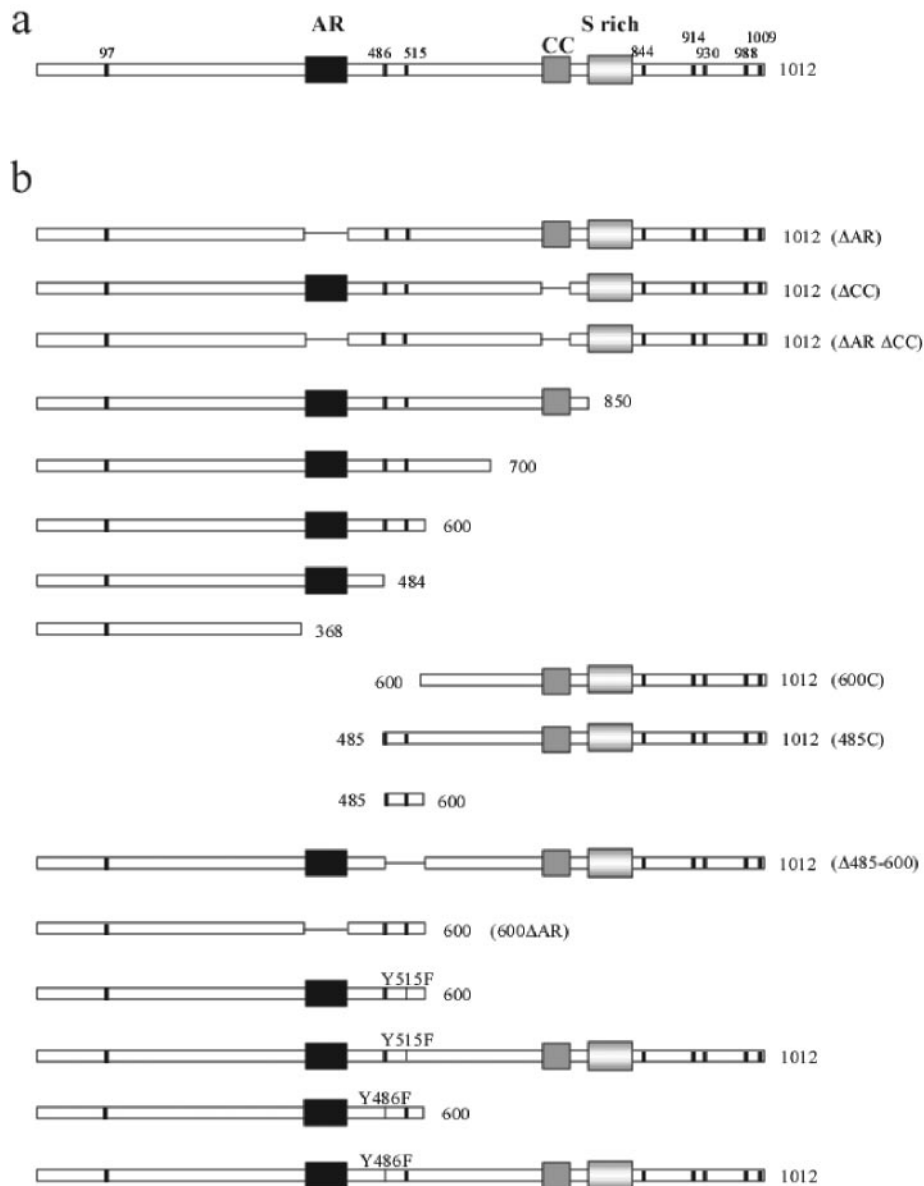


FIG. 1. (a) Schematic representation of the Dof protein. Black box, ankyrin repeats (amino acids 373 to 431); dark grey box, coiled-coil domain (amino acids 703 to 741); light grey box, serine-rich region (amino acids 768 to 828). Tyrosine residues that could serve as SH2 domain binding sites are shown as thick vertical black lines. (b) Schematic representation of the different constructs used in the rescue assays. Each construct was placed downstream of UAS sequences.

1). Expression of the *Drosophila Serum Response Factor/blistered (DSRF/bs)* gene, a transcriptional target of Bnl/FGF signaling in terminal tracheal cells (13, 22, 23), was also recovered in the tracheal trees of rescued embryos (Fig. 2A; Table 1). Although expression of the first 600 amino acids of the protein (Dof600) allowed only for a partial rescue of tracheal morphogenesis, all six types of primary branches were formed, and branch fusion occurred in many segments (Fig. 2A, subpanel e), suggesting that this part of the protein retains the capacity to read out the activation state of the FGFR and to relay the signal to the migration machinery. Thus, Dof600 will be considered as a minimal Dof protein. Deletion of an additional 116 amino acids from the C terminus of Dof600 abolished the

function of this minimal Dof600 protein (construct *dof1-484* [Fig. 2A; Table 1]).

The loss of rescue activity upon the deletion of amino acids 485 to 600 in Dof600 suggests that this region is crucial for the function of Dof in tracheal cell migration. However, this region was found to be sufficient neither to rescue tracheal cell migration of *dof* mutant embryos by itself (construct *dof485-600*) nor when associated with the C-terminal part of the protein (construct *dof485-1012*) (Fig. 2; Table 1). These results demonstrate that the first 484 amino acids of the Dof protein are essential for its function. A full-length Dof protein, in which amino acids 485 to 600 were deleted (construct *dofΔ485-600*), also rescued tracheal cell migration (Table 1). This demon-

TABLE 1. Rescue capacities of the transgenes analyzed in this study^a

Construct	Rescue in trachea	Efficiency (arbitrary) ^b	Analysis of rescued embryos			DSRF expression	Rescue in mesoderm ^d
			DT breaks ^c	DB (<i>n</i> = 10)	GB (<i>n</i> = 10)		
Full dof	+	+++	0–1 (<i>n</i> = 24)	10	9–10	+	+
1–850	+	++	2–3 (<i>n</i> = 25)	8	6–7	+	+
1–700	+	+	3 (<i>n</i> = 29)	8	7	+	+
1–600	+	+	4 (<i>n</i> = 32)	6	5–6	+	+
1–484	–					–	–
1–368	–					–	–
600–1012	–					–	–
485–1012	–					–	–
485–600	–					ND	–
dof Δ485–600	+	++	1–2 (<i>n</i> = 21)	9	8–9	+	+
ΔAR	+	++	1–2 (<i>n</i> = 41)	9–10	9	+	+
ΔCC	+	++	1 (<i>n</i> = 15)	9–10	9	+	+
ΔARΔCC	+	++	1–2 (<i>n</i> = 36)	9–10	9	+	+
600ΔAR	–					+	–
600Y515F	–					–	–
Y515F	+	++	1–2 (<i>n</i> = 27)	9	8	+	+
600Y486F	+	+	5 (<i>n</i> = 40)	4	3	+	+/-
Y486F	+	++	1–2 (<i>n</i> = 21)	9–10	9–10	+	+

^a The capacity of each transgene to rescue tracheal cell migration was scored by counting the formation of different tracheal branches per side of rescued embryos. Mesodermal rescue was scored by counting Evenskipped-positive cells on the dorsal side of the embryo. Abbreviations: DT, dorsal trunk; DB, dorsal branch; GB, ganglionic branch; ND, not done.

^b Symbols: +, positive; ++, high; +++, very high.

^c *n*, number of rescued embryos.

^d Symbols: +, more than 75% of cells positive for Evenskipped compared to wild-type embryos; +/-, between 20 and 75% of cells positive for Evenskipped; –, less than 10% of cells positive for Evenskipped.

strates that although the region of amino acids 485 to 600 plays a crucial role in the minimal Dof600 protein, its function might be redundantly contained in the C terminus of the Dof protein.

Somewhat surprisingly, expression of transgenes lacking the two recognizable domains of Dof, the coiled-coil domain and the ankyrin repeats, allowed for an efficient rescue of tracheal cell migration, suggesting either that these domains are not necessary for Dof function or, alternatively, that they might carry out functions that are redundant with other parts of the molecule. We favor the second hypothesis since deletion of the ankyrin repeats in the Dof600 protein completely abolished the function of the minimal Dof600 protein in the migration rescue assay (construct *dof600*ΔAR [Table 1] [also see below and Fig. 6A, subpanel e]).

As already mentioned, Dof is also essential for mesodermal cell spreading and migration, and this requirement can also be rescued by expression of a full-length *dof* transgene in the mesoderm of *dof* mutant embryos with the *twi*-Gal4 driver (42) (Fig. 2B, subpanel c). Using the same strategy as for the tracheal system, we tested the capacity of the different *dof* transgenes to rescue mesodermal cell migration of *dof* mutant embryos, monitoring the development of pericardial cells that express Evenskipped in the dorsal region of the embryo (Fig. 2B; Table 1). Similar to the results observed for tracheal cell migration, the first 600 amino acids of Dof are sufficient to rescue mesoderm migration and pericardial development of *dof* mutant embryos; deletion of either the region containing amino acids 485 to 600 or the first 484 amino acids from the minimal Dof600 protein completely abolished mesodermal rescue activity (Fig. 2B; Table 1). The coiled-coil region and the ankyrin repeats were also dispensable when deleted in the context of full-length Dof (Table 1); however, deletion of the

ankyrin repeat in the context of the minimal Dof600 protein abolished activity *in vivo*.

Based on these observations, we conclude that the first 484 amino acids of Dof are absolutely essential for its function in tracheal and mesodermal cell migration. When fused to these N-terminal sequences, the region between residues 485 and 600 of Dof (or alternatively the much longer region between 600 and the C terminus) can mediate the migratory response in tracheal and mesodermal cells upon FGFR activation. Based on these results, we concentrated our efforts on the identification of the role of the N-terminal sequences (amino acids 1 to 484) and the C-terminal sequences (amino acids 485 to 600) of the minimal Dof600 protein in the chemotactic response.

Dof is specifically phosphorylated upon FGF signaling in Schneider cells. In order to address molecular aspects of the function of Dof in FGF signaling, we activated the FGF signaling pathway in *Drosophila* Schneider cells (S2) by expressing constitutive active versions of either of the two FGFRs (Btl or Htl) together with Dof under the control of a heavy metal-inducible promoter (see Materials and Methods).

It has previously been argued that Dof is an essential signaling component for the FGF pathway since in its absence, FGF-dependent ERK phosphorylation and hence activation of the Ras/MAPK pathway are not observed (16, 21, 42). Furthermore, Dof was found to be exclusively needed for FGF-mediated signal transduction since other RTK signaling systems, such as Torso or EGF, can activate the MAPK cascade in *dof* mutant embryos and do not rely on Dof for efficient signaling (6, 21, 42). To characterize our reconstituted cell culture system, we first investigated the requirement for Dof to activate the MAPK pathway in S2 cells upon FGF induction. FGF signaling was induced in S2 cells by expressing constitu-

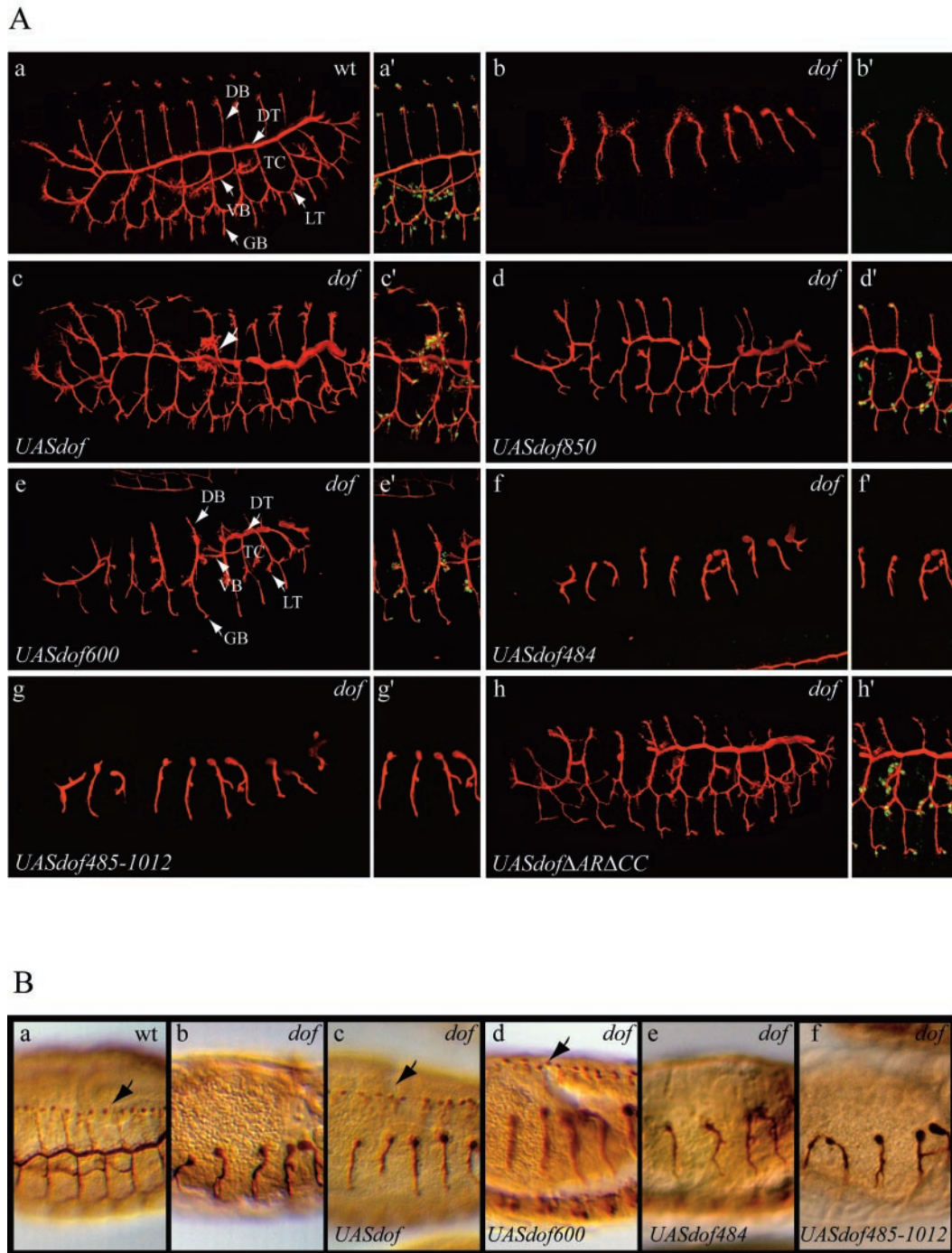


FIG. 2. (A) Rescue of tracheal cell migration by altered versions of the Dof protein. The lumen of the tracheal system of embryos was visualized with the 2A12 antibody (red) (subpanels a to h and a' to h'), and terminal cells were visualized with the anti-DSRF antibody (green) (subpanels a' to h'). Confocal projections of a representative embryo are shown for a wt embryo (subpanels a and a'), for a *dof*^{-/-} embryo (subpanels b and b'), and for rescued embryos expressing the following transgenes in a *dof*^{-/-} background under the control of the *bt*Gal4 driver: *UASdof* (subpanels c and c'), *UASdof850* (subpanels d and d'), *UASdof600* (subpanels e and e'), *UASdof484* (subpanels f and f'), *UASdof485-1012* (subpanels g and g'), and *UASdofΔARΔCC* (subpanels h and h'). Abbreviations: DB, dorsal branch; TC, transverse connective; VB, visceral branch; LT, lateral trunk; GB, ganglionic branch. The arrow in subpanel c points to the misrouted VB. (B) Rescue of mesodermal cell migration by altered versions of the Dof protein. The lumen of the tracheal system of embryos was visualized with the 2A12 antibody and the pericardial cells with anti-Evenskipped antibodies (subpanels a to f). Sections of representative embryos are shown for a wt embryo (subpanel a), for a *dof*^{-/-} embryo (subpanel b), and for rescued embryos expressing the following transgenes in a *dof*^{-/-} background under the control of the *twi*Gal4 driver: *UASdof* (subpanel c), *UASdof600* (subpanel d), *UASdof484* (subpanel e), and *UASdof485-1012* (subpanel f). Arrows point to the presence of Eve-positive cells (subpanels a, c, and d).

tive active versions of Btl or Htl, which consisted of fusions between the transmembrane and the extracellular domain of the Torso⁴⁰²¹ protein and the intracellular domain of each FGFR (32). Compared with an activated form of Btl (Torso⁴⁰²¹-Btl; Btl^{act}) expressed alone in S2 cells, coexpression of Btl^{act} with Dof significantly increased MAPK activity as revealed by anti-dpERK antibody detection on Western blot (Fig. 3A) (9, 10). These results suggest that similar to the situation observed in vivo, Dof is important to fully activate ERK upon FGFR activation in S2 cells.

We then asked whether Dof was a specific substrate of FGF signaling and investigated its phosphorylation level upon FGFR or Torso signaling in S2 cells. As shown in Fig. 3B, expression of constitutive active Btl or Htl led to strong tyrosine phosphorylation of Dof, demonstrating that Dof is indeed a phosphotarget, either a direct or an indirect one, of both *Drosophila* FGFRs (see below). Interestingly, Dof remained unphosphorylated upon signaling from the Torso RTK (Fig. 3B), demonstrating that Dof is not a target of all RTKs.

The first 600 amino acids of the protein, defined through the in vivo rescue experiments as the minimal Dof600 protein, were phosphorylated on tyrosine residues in response to Btl/FGFR activation to a similar extent as the full-length protein (Fig. 3C). In contrast, no tyrosine phosphorylation was detected on the shorter Dof484 protein under the same conditions of induction (Fig. 3C). These observations link our results obtained with the in vivo rescue assays to the state of phosphorylation of Dof in S2 cells, demonstrating that the region between amino acids 485 and 600 is necessary for Dof to be recognized or used as a substrate for FGFR-dependent phosphorylation. Interestingly, Dof derivatives (Dof485C and Dof600C) comprising only the C-terminal part of Dof (thus missing the first 484 amino acids) were found to be phosphorylated to a much lesser extent (Fig. 3C and data not shown; see also below).

Dof interacts with both FGF receptors. Since Dof becomes phosphorylated upon FGF signaling (above) and genetically acts downstream of both FGFRs (21, 42), we investigated whether Dof interacted with the FGFRs Btl and Htl.

S2 cells were cotransfected with myc-tagged versions of *btl^{act}* or *htl^{act}* together with V5-tagged wild-type *dof* or deletion mutant forms of *dof*. To investigate the ability of Dof to form a complex with the receptor, we analyzed the capacity of these two proteins to coimmunoprecipitate from S2 cell lysates. As shown in Fig. 4, full-length Dof or the minimal Dof600 protein formed a complex with the activated version of both *Drosophila* FGFRs upon induction. With the aim to determine whether this interaction was dependent on the constitutive kinase activity of Btl^{act}, we generated a wild-type *btl* construct. However, expression of this construct in S2 cells led to ligand-independent kinase activation, possibly due to dimer formation caused by overexpression of the receptor (data not shown). Therefore, we could not assay the interaction of Dof with a nonactivated form of the receptor. However, Dof has recently been shown to directly interact with the kinase domain of both Htl and Btl in yeast two-hybrid assays (3), suggesting that this interaction might be constitutive and not dependent on the activation state of the FGFRs. More recently, we have now shown that a kinase-dead version of Btl still interacts with Dof, despite its

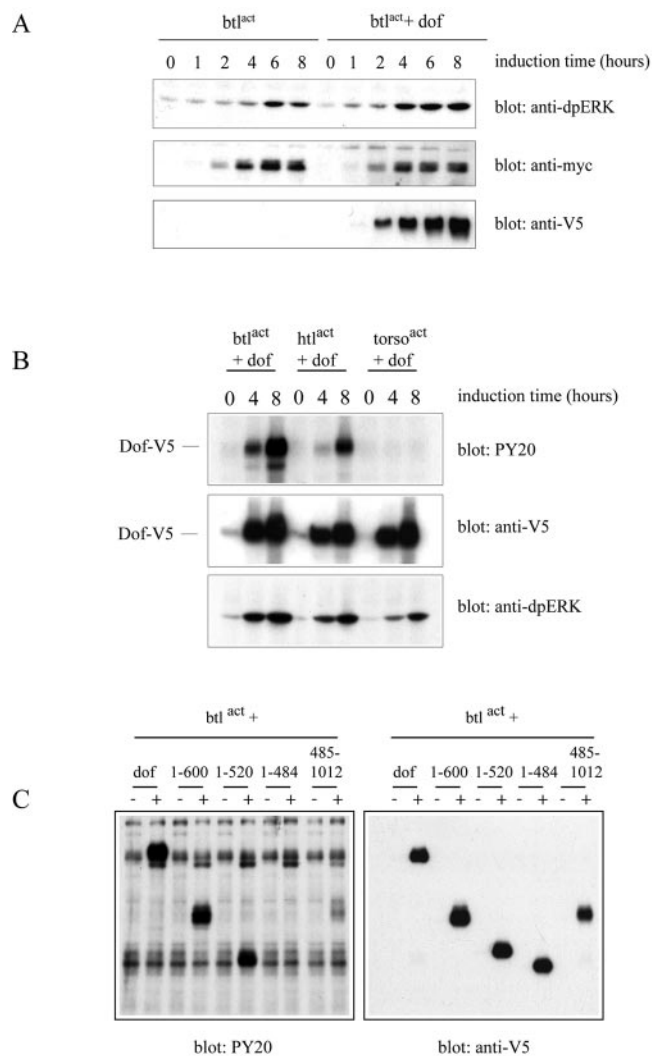


FIG. 3. (A) Dof is important to activate ERK upon FGF signaling. S2 cells transfected with *myc-btl^{act}* alone or in combination with *dof-V5* were lysed after induction of expression for the indicated times with 0.6 mM CuSO₄. Whole-cell lysates were subjected to SDS-PAGE, and ERK phosphorylation levels were examined by immunoblot analysis with anti-dpERK antibodies. Protein levels were evaluated for myc-Btl^{act} and Dof-V5. (B) Dof is specifically phosphorylated upon FGF signaling. S2 cells were transfected with the indicated combination of constructs and lysed after induction of expression for the indicated times. Tyrosine phosphorylation levels were investigated on whole cell extracts by antiphosphotyrosine immunoblotting (upper panel), Dof-V5 expression levels by anti-V5 immunodetection (middle panel) and activation of ERK by anti-dpERK immunoblotting (bottom panel). (C) The different Dof proteins are not phosphorylated to the same extent. S2 cells were transfected with the indicated combination of expression plasmids and lysed after 8 h of induction (+). Transfected cells left untreated for the same time were used as controls (-). Tyrosine phosphorylation of the different constructs was assayed with antiphosphotyrosine antibodies (left panel) and protein expression levels by anti-V5 immunoblotting (right panel).

inability to phosphorylate itself or the associated Dof protein (data not shown).

The shorter Dof484 protein was also coimmunoprecipitated with Btl (Fig. 4), although it is not phosphorylated upon FGF signaling (Fig. 3). No interaction with Btl was detected when

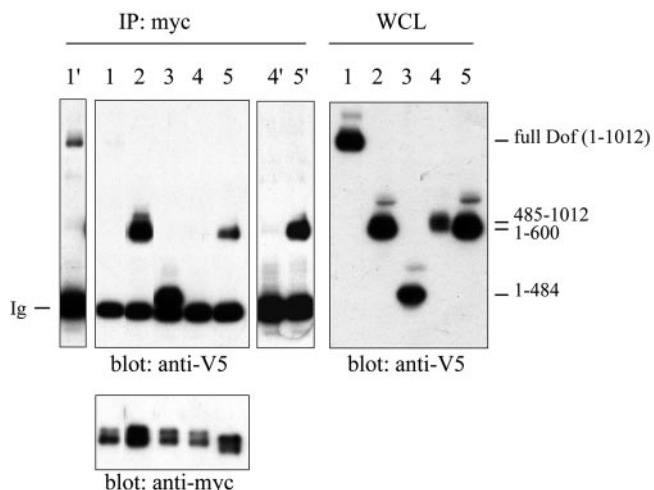


FIG. 4. Dof interacts with both *Drosophila* FGFRs. S2 cells transfected with various plasmids (lane 1, *myc-btl^{act}* + *dof-V5*; lane 2, *myc-btl^{act}* + *dof600-V5*; lane 3, *myc-btl^{act}* + *dof485-V5*; lane 4, *myc-btl^{act}* + *dof485-1012-V5*; lane 5, *myc-hil^{act}* + *dof600-V5*) were lysed after 8 h of induction with 0.6 mM CuSO_4 . Whole-cell lysates (WCL) were immunoprecipitated (IP) with anti-myc antibodies, subjected to SDS-PAGE and immunoblotted with anti-V5 antibodies (left top panel) or anti-myc antibodies (left bottom panel). Protein expression levels were assayed by anti-V5 immunoblotting (right panel). Overexpression of the anti-V5 immunoblot is shown for lane 1 (lane 1') and lanes 4 and 5 (lanes 4' and 5'). The weaker interaction of Dof compared to Dof600 might be due to an intramolecular interaction of the N and C terminus of Dof (3). Ig, immunoglobulins.

the Dof600C or the Dof485C proteins, consisting of the C-terminal parts of the Dof protein, were coexpressed with the receptor in S2 cells (Fig. 4 and data not shown). These results indicate that the region of Dof required to form an efficient complex with the receptor resides in the most N-terminal part of the protein, in the first 484 amino acids. These observations also suggest that the inability of the C-terminal part of Dof (lacking the first 484 amino acids) to rescue tracheal or mesodermal migration might be due to the incapacity of these proteins to form a complex with the FGFRs and to become efficiently phosphorylated.

In order to fine-map the interaction domain between Dof and the FGFRs, a series of deletion constructs were generated in the context of the minimal Dof600 protein, coexpressed with *Btl^{act}* in S2 cells and tested for their ability to coimmunoprecipitate with the FGFR. The following deletion constructs were generated: *dof120-600*, *dof600* Δ 120-240, *dof600* Δ 217-288, *dof600* Δ 240-373, *dof600* Δ 373-436 (corresponding to *dof600* Δ AR), and *dof600* Δ 436-486. All these deletion mutant versions of Dof600 retained the ability to form a complex with *Btl* (data not shown), suggesting that Dof600 might contain several redundant receptor interaction domains.

Tyrosine 515 is essential for the function of the Dof600 protein in vivo and serves as a Csw binding site. We have shown above that the region between amino acids 485 and 600 of the minimal Dof protein is important to mediate mesodermal and tracheal cell migration in vivo and that this region is essential for Dof to become a phosphoprotein upon FGF signaling. These observations suggest that this region either becomes phosphorylated on tyrosine residues upon FGF signal-

ing or is important for Dof to be recognized by the activated receptor as a substrate.

Dof contains five tyrosine residues in the region 485 to 600 (Y486, Y515, Y563, Y564, and Y592 [42]). One of these tyrosines is embedded in an environment mimicking a putative consensus binding site for the SH2 domain of PI3K (Y486, pYMEM) and one represents a potential binding site for the SH2 domain of the nonreceptor tyrosine phosphatase Csw (Y515, LNpYISV). To find out whether these putative SH2 binding sites are critical for Dof function when phosphorylated, we replaced each of the two tyrosines with the nonphosphorylatable residue phenylalanine and tested the capacity of the altered proteins to mediate tracheal and mesodermal cell migration in our in vivo rescue assay.

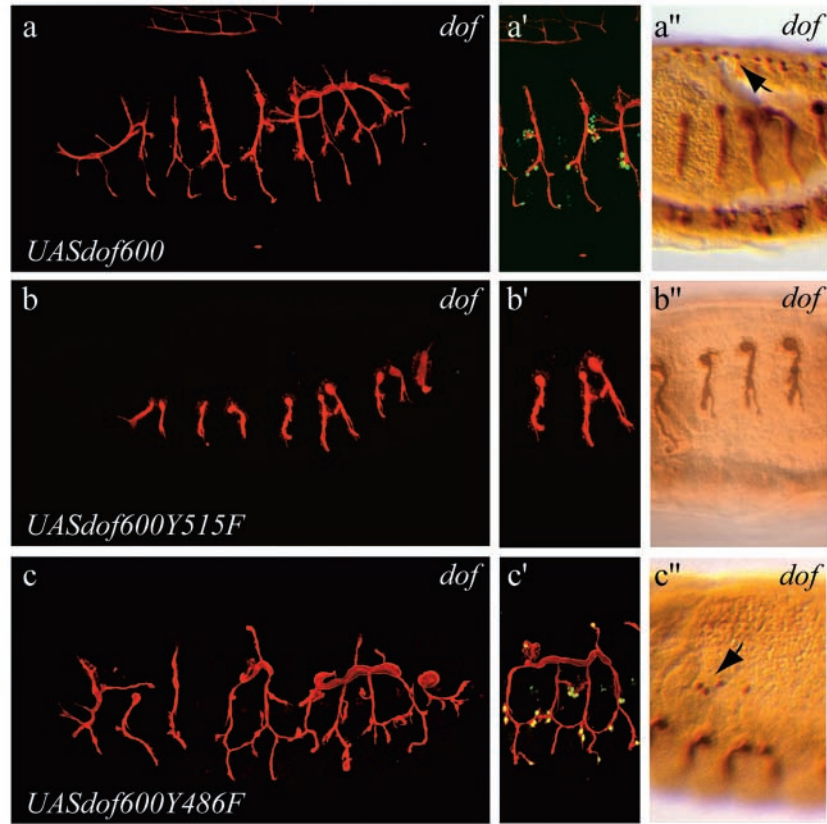
Expression of a transgene coding for the minimal Dof600 protein, in which tyrosine 515 is replaced by phenylalanine, did not allow rescue of tracheal cell migration in *dof* mutant embryos, and no activation of DSRF was observed (Fig. 5A, subpanels b and b'). This demonstrates that residue 515 plays an important role for the function of the minimal Dof protein in vivo. In contrast, mutation of tyrosine 486 only slightly affected the function of the minimal Dof protein, and rescue of tracheal cell migration and DSRF expression was still readily observed (Fig. 5A, subpanels c and c'; Table 1). The same transgenes were also tested for their ability to restore mesodermal cell migration, and consistent with our finding in the trachea, we observed that *dof600Y515F* did not rescue mesodermal spreading in *dof* mutant embryos (Fig. 5A, subpanels b", and c"; Table 1). In contrast, the Dof600Y486F protein, although somewhat less efficient than the Dof600 protein, retained the ability to rescue mesodermal cell migration. These observations demonstrate that tyrosine 515 of Dof600 plays a crucial role in both tracheal and mesodermal cell migration.

Since residue 515 is part of a putative consensus binding site for SH2 domains of the phosphatase Csw, we investigated the capacity of Dof and Csw to form a protein complex in S2 cells. No constitutive interaction was detected between Csw and Dof600 in S2 cell extracts in the absence of FGF signaling (Fig. 5B, lane 1). However, Csw and Dof600 formed a complex upon FGF stimulation (Fig. 5B, lane 2). Substitution of the tyrosine 515 with phenylalanine significantly weakened the formation of a Csw/Dof complex upon FGF stimulation, demonstrating that tyrosine 515 of Dof plays an important role in the recruitment of Csw. Mutation of tyrosine 486 had no effect on the recruitment of Csw (Fig. 5B, lane 6).

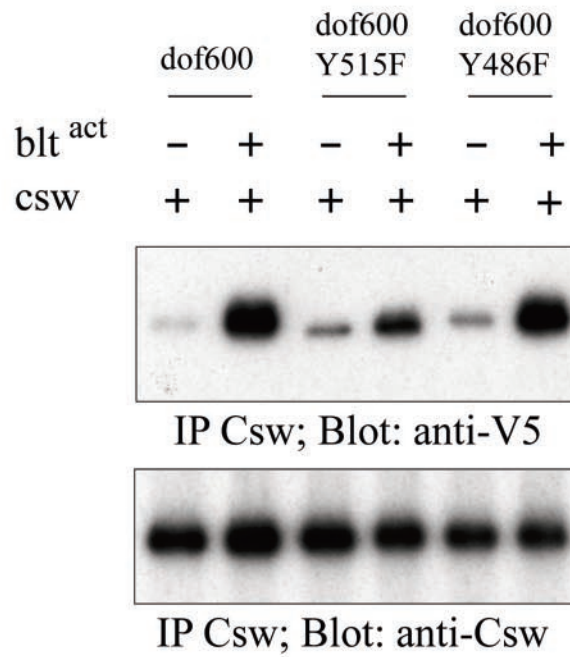
Based on these observations and the in vivo rescue experiments, we conclude that the recruitment of Csw by Dof upon FGF signaling is important for tracheal and mesodermal cell migration. These results are in line with genetic experiments showing that mutations in *csw* produce a phenotype identical to *bnl*, *btl*, and *dof*; i.e., tracheal and mesodermal cells fail to migrate (17, 30) (see Discussion).

Recruitment of Csw via Dof is essential for the activation of the MAPK pathway. The cellular responses to FGF signaling in tracheal and mesodermal cells include the formation of dynamic filopodial extensions (33, 36), the activation of the Ras/MAPK pathway (9, 10), and the induction of nuclear gene expression—for example, the activation of *DSRF/bs* in terminal tracheal cells (13, 41). In order to find out whether each of the deletion constructs we generated affected these responses in a

A



B



similar manner, we assayed their capacity to activate the Ras/MAPK pathway and the *DSRF/bs* gene (Fig. 6; Table 1).

To measure the *in vivo* activation of the Ras/MAPK pathway, we made use of the observation (Alain Jung, unpublished data) that simultaneous expression of the activated form of the *btl* receptor and a *dof* transgene in dorsal ectodermal cells under the indirect control of the *pannier*-Gal4 driver is sufficient to activate the MAPK pathway, as revealed with anti-dpERK immunostaining (Fig. 6A, subpanel b). Expression of either construct alone was not sufficient to lead to an accumulation of dpERK (Fig. 6A, subpanel a). Using this assay, we tested all the transgenes described above (Table 1) for dpERK staining in the dorsal ectoderm. As shown in Fig. 6, we observed a rather strict correlation between the transgenes rescuing tracheal cell migration and the ones that activated the MAPK cascade upon FGF signaling, with the exception of *dof600ΔAR* (see below). The failure of the *dof600Y515F* transgene to activate the Ras/MAPK pathway, combined with the previous observations that tyrosine 515 is essential for rescue function *in vivo* and interaction with Csw in S2 cells, suggest that the recruitment of Csw to the activated receptor complex is a crucial step in the FGFR-dependent activation of Ras and the MAPK pathway, and that Ras signaling might play a crucial role in FGF-guided cell migration.

Activation of the MAPK cascade is not sufficient to direct cell migration. Surprisingly, coexpression of the *dof600ΔAR* transgene with *btl^{act}* resulted in the phosphorylation of ERK, despite the failure of this transgene to rescue tracheal cell migration (Fig. 6A, subpanels c and e). When we assayed the tracheal system in *dof* mutant embryos expressing *Dof600ΔAR*, we indeed found that despite the lack of migration, *DSRF/Bs* was expressed in a subset of terminal tracheal cells (Fig. 6A, subpanels c and e). These results suggest that local activation of Ras/MAPK pathway is sufficient to activate transcriptional target genes of FGFR signaling, but not tracheal cell migration. A number of other observations support this interpretation; these will be outlined in detail in the Discussion.

DISCUSSION

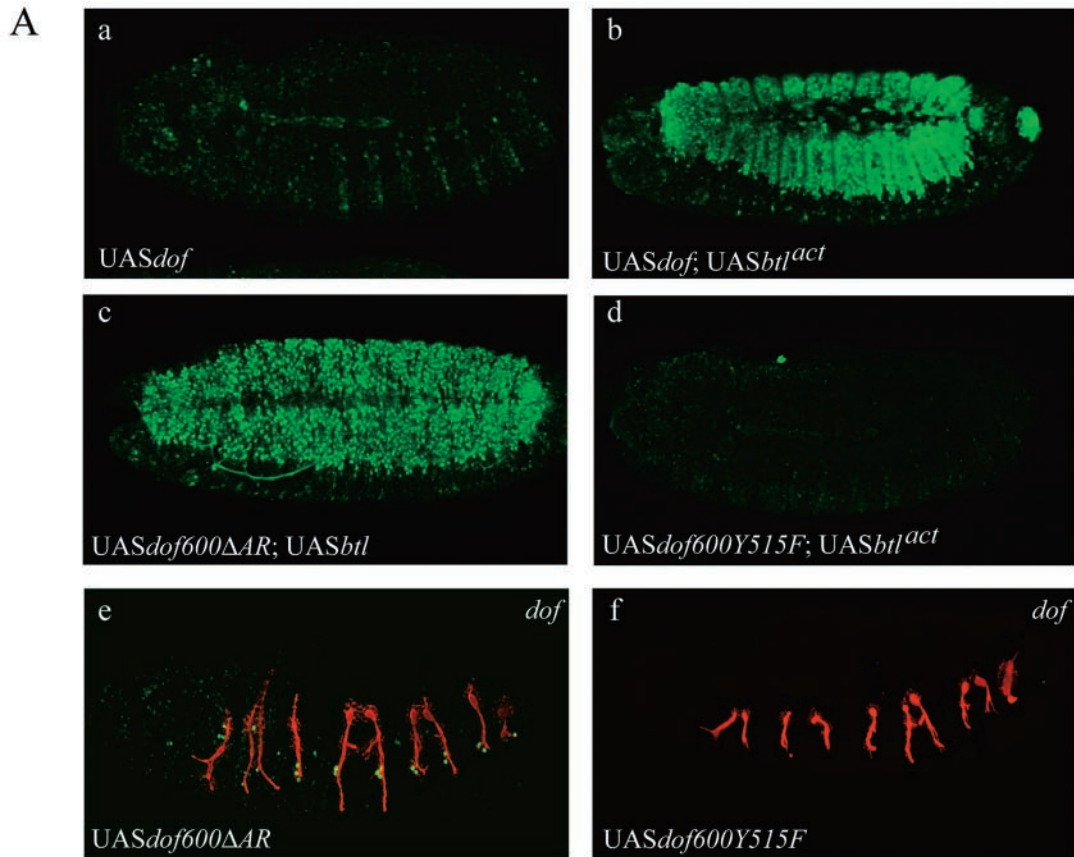
We have performed a functional characterization of Dof, a putative adapter protein that functions specifically in FGF signal transduction in *Drosophila*. By combining reverse genetics, cell culture assays, and biochemical approaches we have demonstrated that Dof is indeed a substrate for the *Drosophila* FGFRs. After defining a minimal Dof rescue protein, we identified two regions of Dof important for its function in meso-

dermal and tracheal cell migration. The N-terminal 484 amino acids are required for the recruitment of Dof to the FGFRs, while tyrosine 515 becomes phosphorylated upon signaling and recruits the phosphatase Csw. Although Csw recruitment represents an essential step towards the activation of the Ras/MAPK pathway, the activation of the latter does not appear to be sufficient to signal to the migration machinery in tracheal and mesodermal cells. Additional proteins binding to the activated receptor, to Dof, or unidentified substrates for Csw appear to be crucial for a chemotactic response (schematically represented in Fig. 7).

Dof is an FGFR-specific scaffolding protein. Genetic epistasis experiments have shown that Dof functions downstream of the activated FGFRs and upstream or in parallel to Ras (16, 21, 42). However, the biochemical function of Dof in the interpretation of the chemotactic response to FGFR signaling has not been addressed so far. Using *in vivo* rescue assays, we first identified a minimal Dof protein containing the first 600 amino acids of Dof that allows rescue of both mesodermal and tracheal cell migration. Although the rescue in the tracheal system is not as efficient as the rescue observed with the wild-type construct, all six branches can migrate out, demonstrating that the first 600 amino acids of Dof retains the capacity to read out the local activation state of the FGFRs and to relay the signal to the migration machinery, albeit with somewhat reduced efficiency. Deletion from the C terminus of this *dof* minigene, as well as internal deletions, resulted in loss of rescue capacity, thus identifying regions of functional importance.

We have built on the results obtained in this *in vivo* assay and analyzed all of the constructs in a *Drosophila* S2 cell culture assay, in which we activated either the FGFR or the Torso signaling system. We found that both full-length Dof and Dof600 are phosphorylated on tyrosine residues upon FGF signaling, but that Torso cannot use Dof as a substrate. These results are consistent with *in vivo* data showing that Dof is exclusively needed for FGF-mediated signal transduction and that Torso is able to activate the MAPK cascade in the absence of Dof in *dof* mutant embryos (6, 21, 42). Using coimmunoprecipitation experiments, we show that Dof forms a complex with both FGFRs and that the first 484 amino acids, although not phosphorylated upon FGF signaling, are required and sufficient for the association with the FGFRs, demonstrating that phosphorylation of Dof is not necessary for complex formation. Our cell culture analysis is in line with a recent report showing that the N-terminal part of Dof directly interacts with the kinase domains of Btl and Htl in yeast two hybrid assays (3). In addition, we have observed that both the juxtamembrane and the C terminus of Btl can be deleted without affect-

FIG. 5. (A) Rescue of tracheal and mesodermal cell migration by altered versions of the Dof600 protein. The lumen of the tracheal system of embryos was visualized with the 2A12 antibody in red (subpanels a to c and a' to c') or in brown (subpanels a'' to c''), terminal cells were visualized with the anti-DSRF antibody in green (subpanels a' to c') and the pericardial cells with anti-Evenskipped antibodies in brown (subpanels a'' to c''). Confocal projections are shown for a representative rescued embryo expressing the following transgenes in a *dof^{-/-}* background under the control of the *btGal4* driver: UAS*dof600* (subpanels a and a'), UAS*dof600Y515F* (b and b') and UAS*dof600Y486F* (subpanels c and c'). Sections are shown for representative rescued embryos expressing the following transgenes in a *dof^{-/-}* background under the control of the *twiGal4* driver: UAS*dof600* (subpanel a''), UAS*dof600Y515F* (subpanel b''), and UAS*dof600Y486F* (subpanel c''). (B) Tyrosine 515 serves as a Csw binding site. S2 cells cotransfected with *csw* and the different forms of V5-tagged *dof600* as indicated on the figure, with or without *myc-btl^{act}*, were lysed after 8 h of induction with 0.6 mM CuSO₄. Whole-cell lysates were immunoprecipitated with anti-Csw antibodies, subjected to SDS-PAGE and immunoblotted with anti-V5 antibodies (upper panel) and anti-Csw antibodies (bottom panel).



B

constructs	dpERK expression	Migration Rescue
Full dof	-	
<i>btl^{act}</i>	-	
<i>btl^{act}</i> + Full dof	+	+
<i>btl^{act}</i> + 1-850	+	+
<i>btl^{act}</i> + 1-700	+	+
<i>btl^{act}</i> + 1-600	+	+
<i>btl^{act}</i> + 1-484	-	-
<i>btl^{act}</i> + 1-368	-	-
<i>btl^{act}</i> + 600-1012	-	-
<i>btl^{act}</i> + 485-1012	-	-
<i>btl^{act}</i> + 485-600	-	-
<i>btl^{act}</i> + dof Δ485-600	+	+
<i>btl^{act}</i> + ΔAR	+	+
<i>btl^{act}</i> + ΔCC	+	+
<i>btl^{act}</i> + ΔARΔCC	+	+
<i>btl^{act}</i> + 600ΔAR	+	-
<i>btl^{act}</i> + 600Y515F	-	-
<i>btl^{act}</i> + Y515F	+	+
<i>btl^{act}</i> + 600Y486F	+	+
<i>btl^{act}</i> + Y486F	+	+

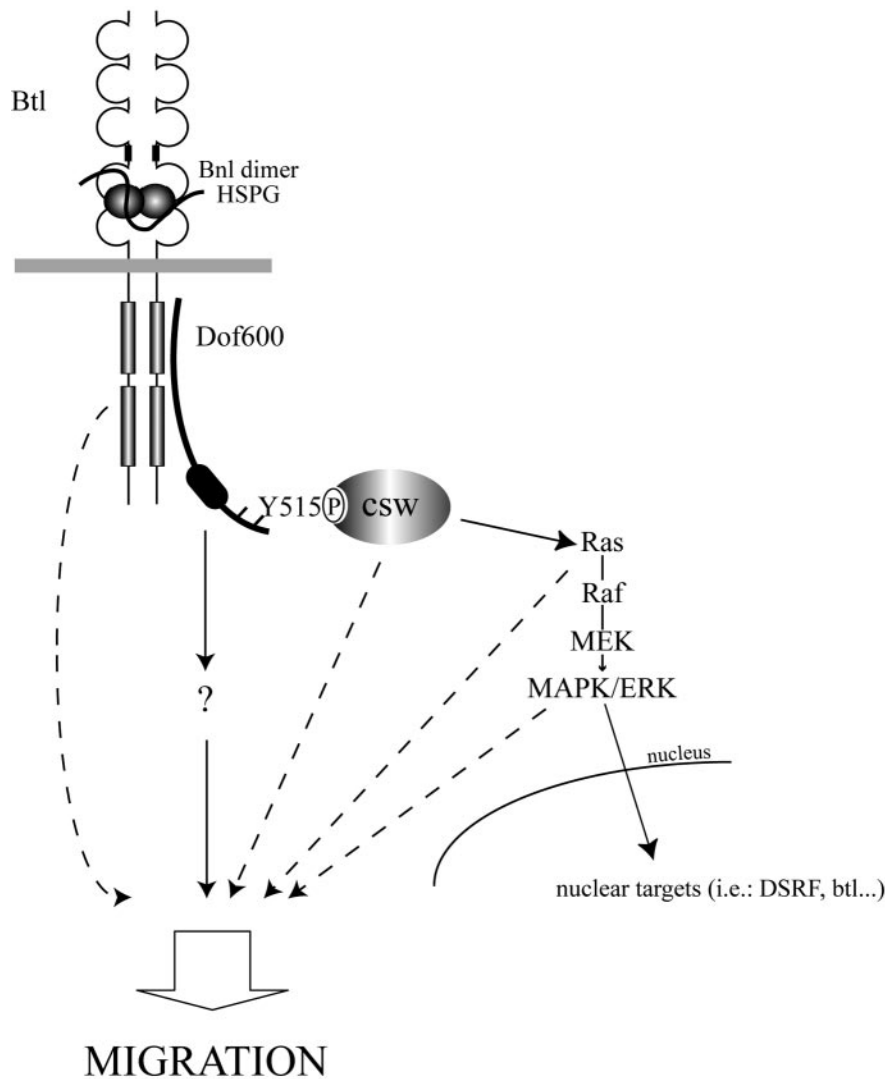


FIG. 7. Schematic model of the FGF signaling pathway in the control of cell migration in *Drosophila*. HSPG, heparan sulfate proteoglycan. See Results and Discussion for details.

ing considerably the quality of the rescue capacity of the receptor (data not shown). Thus, it appears that Dof directly docks onto the kinase domain of the FGF receptor, in contrast to the vertebrate FGFR adapter SNT/FRS2, which interacts with a sequence motif in the juxtamembrane region of the receptor (27, 44).

Csw is recruited to the FGFR signaling complex via Dof and is required for FGF guided cell migration. Since Dof becomes phosphorylated upon FGFR signaling in S2 cells, we asked

whether we could identify functionally important phosphorylation sites, the proteins recognizing these sites in the phosphorylated state, and confirm our results *in vivo* by making use of the rescue assay and genetic analysis. Two potential phosphorylation target sites were identified by sequence analysis in the essential region comprising amino acids 485 to 600. While mutation of each individual site resulted in reduced phosphorylation of Dof600 in S2 cells upon FGFR signaling (data not shown), mutation of only one of these sites, tyrosine 515, abol-

FIG. 6. (A) Activation of the MAPK pathway is important but not sufficient to direct cell migration. Activation of the MAPK pathway was visualized with anti-dpERK antibodies (subpanels a to d), the lumen of the tracheal system with the 2A12 antibody in red and terminal cells with the anti-DSRF antibody in green (subpanels e and f). Confocal projections are shown for embryos expressing the following transgenes under the control of the *pannier*Gal4 driver: UAS*dof* (subpanel a), UAS*dof* and UAS*btl^{act}* (subpanel b), UAS*dof600ΔAR* and UAS*btl^{act}* (subpanel c) and UAS*dof600Y515F* and UAS*btl^{act}* (subpanel d). (Subpanels e and f) Embryos expressing UAS*dof600ΔAR* or UAS*dof600Y515F* in a *dof^{-/-}* background under the control of the *btl*Gal4 driver are shown. (B) Summary table of the capacity of each transgene to activate dpERK in combination with an activated form of *btl*. The capacity of each *dof* transgene to rescue cell migration in *dof^{-/-}* embryos is also shown in the second column.

ished the migration rescue capacity *in vivo*. Since the functionally required tyrosine residue was part of a putative consensus binding site for the SH2 domain of the nonreceptor tyrosine phosphatase Csw/SHP-2, we tested the interaction of Csw with Dof using coimmunoprecipitation experiments and confirmed that Csw was indeed recruited to the activated signaling complex via Dof. We found in our rescue assays that both the region 485 to 600 as well as the region from 600 to the C terminus (construct *dof* Δ 485-600) were able to confer function to the signaling-deficient N terminus (residues 1 to 484). We know that the C-terminal sequences also recruit the Csw phosphatase in the absence of tyrosine 515 (data not shown), but we do not know whether they do so directly or indirectly. Further deletion analyses and biochemical studies will be required to address this question.

Genetic evidence supporting an interaction between Dof and Csw was provided some time ago by the finding that mutations in *csw* produce a phenotype identical to *bnl*, *btl*, and *dof*; i.e., tracheal and mesodermal cells fail to migrate (17, 30). The sum of these results clearly assign a crucial role for both Dof and Csw downstream of the FGFRs in the migratory response, indicating that the ligand-dependent phosphorylation of Dof leads to the recruitment of Csw to the signaling complex, ultimately triggering cell locomotion. SHP-2, the vertebrate homologue of Csw, has been shown to be required at the initial steps of gastrulation, as mesodermal cells migrate away from the primitive streak in response to chemotactic signals initiated by fibroblast growth factors (37). In addition, SHP-2 has also been found to be crucial for tubulogenesis and for the sustained stimulation of the ERK/MAPK pathway upon induction of another chemotactic factor, the hepatocyte growth factor/scatter factor (for a review, see reference 34), thus placing SHP-2/Csw as a key player in branching morphogenesis induced by diverse chemotactic factors. Therefore, it appears that both in invertebrates and vertebrates, SHP-2/Csw plays a major role in RTK signaling in the control of cell migration. The similarity of the *Drosophila* FGF signal transduction pathway to the vertebrate FGF pathway make the fly system accessible to address future issues not resolved in vertebrates, such as the targets of SHP-2/Csw involved in Ras activation and/or cell migration.

Ras/MAPK activation is required but not sufficient for FGF-guided cell migration. Using the dpERK antibody as a readout for the activation of the Ras/MAPK pathway *in vivo*, we find that abolishing the interaction between the Dof600 minimal protein and Csw abolishes the activation of the MAPK cascade upon FGFR signaling. The strong correlation we found between migration and MAPK activation when analyzing all mutant *dof* constructs in this assay (Fig. 6B) might indicate that local activation of the Ras/MAPK pathway in tracheal tip cells is sufficient to trigger the migratory response upon Btl signaling (see also (33)). However, two lines of evidence make us believe that this might not be the case.

On the one hand, we previously observed that under conditions in which all tracheal cells sustain high levels of Ras/MAPK activity (upon Ras^{V12} overexpression), tracheal cells migrate normally in wild-type embryos (data not shown; see also (16)). In sharp contrast, ectopic expression of the Bnl ligand leads to a complete disruption of directed migration (data not shown; see reference 41). Therefore, high levels of

Ras/MAPK activity do not appear to produce the same migratory response as ligand-activated FGFR signaling. Indeed, and again in contrast to ectopic Bnl (see reference 33), overexpression of Ras^{V12} in wild-type embryos does not produce significant filopodial activity in DT tracheal cells (Marc Neumann, unpublished results), confirming that the activation of Ras is not sufficient to produce cytoskeletal rearrangements by itself.

On the other hand, we also observed that while the Dof600 protein lacking the ankyrin repeats did allow FGFR-dependent activation of the Ras/MAPK pathway and downstream nuclear response genes, this protein failed to induce migration. Thus, even local Ras activation under the control of the endogenous ligand Bnl, Btl, and Dof600 Δ AR is unable to activate the migratory machinery. Interestingly, it has also been reported that Ras activation is insufficient to guide RTK-mediated border cell migration during *Drosophila* oogenesis (7).

Is Ras activation then required at all for cells to produce a cytoskeletal response and migrate directionally? Unfortunately, genetic analysis cannot be used to directly address this question in the embryo since maternal and zygotic loss of Ras activity results in embryos that do not develop far enough to analyze the tracheal system. However, when activated Ras (Ras^{V12}) is expressed in the tracheal system or in the mesoderm of *dof* mutant embryos, a certain rescue of migration can be obtained (data not shown; see also references 16, 21, and 42). This suggests that Ras signaling is essential but not sufficient for efficient FGFR-dependent cell migration; additional proteins binding to the receptor, to Dof or to Csw appear to be crucial for a chemotactic response. To analyze the role of Ras experimentally and in detail, mitotic clones lacking Ras activity should be analyzed with regard to their migration properties. Recent reports concerning the role of FGF signaling in the migration of mesodermal and tracheal cells during late larval development might provide the basis for such analyses (2, 36).

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