Dok-R plays a pivotal role in angiopoietin-1-dependent cell migration through recruitment and activation of Pak

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Tek/Tie-2 is an endothelial cell (EC)-specific receptor tyrosine kinase that plays a critical role in angiogenesis via its regulation by the angiopoietin family of growth factor ligands. Angiopoietin-1 (Ang1) can promote EC migration; however, the signaling mechanisms underlying this process remain elusive. Here we demonstrate that Dok-R/Dok-2 can associate with Tek in ECs following Ang1 stimulation, resulting in tyrosine phosphorylation of Dok-R and the subsequent recruitment of Nck and the p21-activating kinase (Pak/Pak1) to the activated receptor. Ang1-mediated migration is increased upon Dok-R overexpression and this requires a functional Nck binding site on Dok-R. Localization of this Dok-R–Nck–Pak complex to the activated Tek receptor at the cellular membrane is coincident with activation of Pak kinase. The ability of Dok-R to bind Nck is required for maximal activation of Pak and overexpression of Pak results in increased Ang1-mediated cell motility. Our study outlines a novel signaling pathway underlying Ang1-driven cell migration that involves Dok-R and its recruitment of Nck and the subsequent activation of Pak.

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Introduction

Angiogenesis, the process by which new blood vessels arise from the pre-existing vasculature, occurs during embryonic vascular development as well as in normal physiological and pathological growth in adults. In the adult, sprouting angiogenesis is the most common type of vascular growth and it is characterized by the activation of vascular endothelial cells (ECs), which are induced by

various angiogenic stimuli to undergo several distinct cellular processes such as proliferation, migration and cytoskeletal reorganization. Collectively, these events are crucial for proper establishment of a vascular network (Klagsbrun and Moses, 1999).

The organization and remodeling of blood vessels is a tightly regulated process controlled in part by paracrine signals, many of which are initiated by binding of growth factor ligands to their cognate transmembrane receptor tyrosine kinases (RTKs) expressed on the surface of ECs. Ligand binding leads to RTK activation and autophosphorylation. The vascular endothelial growth factor (VEGF) receptors (VEGFRs) and the TIE receptors are two such subfamilies of RTKs that are expressed almost exclusively on ECs (Mustonen and Alitalo, 1995). Much of our understanding of the importance of these RTKs in angiogenesis has stemmed from genetic experiments, which have clearly demonstrated a role for signaling pathways mediated through these receptors in discrete phases of mouse vascular development. While the ligand VEGF and its receptors, VEGFR-1 and VEGFR-2, are required for the establishment of a primitive vascular network, the angiopoietins and the TIE receptors, Tie/Tie-1 and Tek/Tie-2, play a subsequent role in the remodeling and stabilization of the vasculature once it has been specified and patterned (reviewed in Jones *et al.*, 2001).

The angiopoietins are a new family of growth factor ligands that bind specifically to the Tek receptor. To date, four angiopoietins (Ang1–4) bind Tek and behave as either agonists (Ang1 and Ang4) or context-dependent antagonists (Ang2 and Ang3) of Tek kinase activity (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997; Valenzuela *et al.*, 1999). Mice engineered to lack Ang1 or Tek or to overexpress the Tek antagonistic ligand, Ang2, die *in utero* due to vascular hemorrhaging, defects in cardiac development, capillary sprouting and the proper patterning of vessels (Dumont *et al.*, 1994; Sato *et al.*, 1995; Suri *et al.*, 1996; Maisonpierre *et al.*, 1997). Such genetic studies of Ang1/Tek signaling have been further complemented by *in vitro* experiments, which have demonstrated that Ang1 can induce EC sprout formation, motility and can stabilize tubule structures formed in collagen matrices (Koblizek *et al.*, 1998; Witzenbichler *et al.*, 1998; Fujikawa *et al.*, 1999; Hayes *et al.*, 1999; Jones *et al.*, 1999). These reports have clearly established the necessity of Tek signaling for proper formation and maturation of the vasculature during embryogenesis.

Although these findings suggest a role for Ang1 signaling in EC migration and angiogenic sprout formation, the molecular mechanisms by which these processes occur have yet to be defined. Ang1 binding to Tek results in tyrosine phosphorylation of the receptor and thus provides high affinity binding sites for downstream

adapter and docking proteins that are subsequently recruited to the receptor via their Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Two such proteins that are recruited to the Tek receptor in a phosphotyrosine-dependent manner include the regulatory subunit of phosphatidylinositol (PI) 3-kinase, p85, and the docking protein Dok-R (Jones and Dumont, 1998; Kontos *et al.*, 1998; Jones *et al.*, 1999). Recruitment of p85 to tyrosine phosphorylated Tek has been shown to cause activation of PI3-kinase, which regulates both EC survival and motility (Kontos *et al.*, 1998; Witzenbichler *et al.*, 1998; Fujikawa *et al.*, 1999; Jones *et al.*, 1999; Papapetropoulos *et al.*, 2000). Interestingly, inhibition of PI3-kinase activity by pharmacological agents only partially decreases Ang1-mediated cell migration, suggesting that an alternative PI3-kinase-independent pathway may regulate motility downstream of the Tek receptor (Jones *et al.*, 1999).

Dok-R (also known as Dok-2 and FRIP) is a member of a new class of docking proteins, termed the DOK family, that includes p62 (Dok-1), Dok-L (Dok-3) and possibly IRS-3 (Carpino *et al.*, 1997; Lavan *et al.*, 1997; Yamanashi and Baltimore, 1997; Nelms *et al.*, 1998; Cong *et al.*, 1999; Lemay *et al.*, 2000). Structurally, the DOK family members are characterized by an N-terminal pleckstrin homology (PH) domain followed by a central PTB domain and a proline- and tyrosine-rich C-terminal tail. Dok-R is recruited to activated Tek via its PTB domain, which results in its subsequent tyrosine phosphorylation, thereby establishing binding sites for the small GTPase-activating protein for Ras, p120^{RasGAP} (RasGAP) and the adapter protein Nck (Jones and Dumont, 1998). In addition, Dok-R can constitutively interact with the adapter protein Crk (Jones and Dumont, 1998). Both RasGAP and Nck play a role in cell adhesion, motility and actin polymerization (McGlade *et al.*, 1993; Ren *et al.*, 1993; Noguchi *et al.*, 1999; Chen *et al.*, 2000; Kulkarni *et al.*, 2000; Howe, 2001), which implies that Dok-R may serve a scaffolding role for proteins that modulate migration. Recently, a role for Dok in cellular migration had been proposed by Noguchi *et al.* (1999), as overexpression of Dok potentiates cellular motility in response to insulin in an Nck-dependent fashion. Furthermore, Nck associates with a host of effector proteins involved in cytoskeletal rearrangement and motility such as the serine/threonine kinase Pak (Bokoch *et al.*, 1996; Kiosses *et al.*, 1999; Sells *et al.*, 1999). Interestingly, VEGF-mediated assembly of focal adhesion complexes and migration has been shown to require the interaction of Nck with Pak and subsequent Pak activation (Stoletov *et al.*, 2001). An equivalent Nck binding site to that on Dok has also been identified on Dok-R, suggesting a potential role for Dok-R in cell migration mediated through Nck.

We wanted to examine whether Dok-R could potentially modulate an alternative, or PI3-kinase-independent, pathway for Ang1-mediated migration downstream of the Tek receptor (Jones *et al.*, 1999). We demonstrate that Ang1 stimulation of ECs causes the phosphotyrosine-dependent binding of Dok-R to Tek *in vivo* and the subsequent tyrosine phosphorylation of Dok-R. Once phosphorylated, Dok-R is able to recruit Nck in ECs, which leads to tyrosine phosphorylation of Nck. To investigate the role of Dok-R in Ang1-mediated migration,

we engineered several point mutations in Dok-R, which abrogate its ability to associate with either Nck or RasGAP. Overexpression of wild-type Dok-R potentiates Ang1-mediated cell motility, which is dependent upon phosphorylation of tyrosine (Y) 351 of Dok-R since mutation of Y351 to phenylalanine abolishes the ability of Dok-R to interact with Nck (Jones and Dumont, 1999). We further demonstrate that the Nck binding protein, Pak, is recruited to Tek at the cell membrane and that this recruitment results in an Ang1-dependent Pak kinase activation in ECs. Furthermore, we demonstrate that Pak kinase activity is dependent on phosphorylation of Y351 on Dok-R. Moreover, we substantiate a role for Pak in Ang1-driven migration by demonstrating that its overexpression potentiates Ang1-mediated motility and requires phosphorylation of Dok-R on Y351. Our results demonstrate a novel pathway underlying Ang1/Tek-mediated migration, which requires Dok-R phosphorylation and recruitment of an Nck-Pak complex at the membrane, which leads to Pak activation in ECs.

Results

Angiopoietin-1 stimulation induces Dok-R tyrosine phosphorylation and migration of ECs

SVR ECs express relatively high levels of Tek and Dok-R (Jones and Dumont, 1998 and data not shown). To assess whether Dok-R could associate with Tek and become tyrosine phosphorylated in response to Ang1 stimulation of ECs, we stimulated SVRs with conditioned medium containing Ang1 (Jones *et al.*, 1999; Teichert-Kuliszewska *et al.*, 2001). Briefly, conditioned medium collected from HEK 293T cells stably expressing a form of Ang1 fused to the Myc and His epitope tags, herein referred to as Ang1-MH, was used as stimulant while conditioned medium harvested from the untransfected parental cell line, referred to as Mock, was used as the control stimulant. Treatment of SVR ECs with Ang1-MH stimulated the tyrosine phosphorylation of Tek and its co-immunoprecipitation with Dok-R (Figure 1A), which is the first documentation that this interaction occurs in ECs. Furthermore, Ang1-MH stimulation of SVRs also resulted in the tyrosine phosphorylation of Nck and its co-immunoprecipitation with phosphorylated Dok-R (Figure 1B). The increased amount of phosphorylated Nck found in Dok-R immunoprecipitates relative to Nck immunoprecipitates suggests that most of the Nck associated with Dok-R is tyrosine phosphorylated.

We have shown previously that Ang1-MH functions as a potent chemoattractant for Py4-1 and human umbilical vein ECs (Jones *et al.*, 1999). Thus, we set out to determine whether SVRs would also respond in a similar fashion. Using a modified Boyden chamber motility assay, SVR cells migrated as robustly with Ang1-MH treatment as that seen with VEGF when compared with Mock-treated cells (Figure 1B). This result was not anticipated since most other EC types tested generally display a 2-fold increase in Ang1-MH-mediated migration as compared with a 5-fold increase seen in VEGF-induced cell migration (Jones *et al.*, 1999). This enhanced migration of SVRs may reflect the relatively high levels of Tek expression seen in SVRs (data not shown). SVR ECs respond to Ang1-MH stimulation by activating Tek, resulting in tyrosine phosphorylation of

Fig. 1. Ang1-MH stimulation induces EC migration and tyrosine phosphorylation and association of a Tek/Dok-R-Nck complex. (A) DMEM alone (–) or Ang1-MH (+)-conditioned medium was used to stimulate SVR ECs for 10 min in the absence of phosphatase inhibitors. Proteins from Tek immunoprecipitates were separated by SDS–PAGE and immunoblotted with antibodies recognizing phosphotyrosine (pY), Tek and Dok-R, which revealed that Ang1-MH can stimulate the tyrosine phosphorylation of Tek and the co-immunoprecipitation of tyrosine phosphorylated Dok-R. Both lysates contained equal concentrations of Tek receptor. (B) Ang1-MH (+) stimulation of SVR ECs results in the tyrosine phosphorylation of Dok-R and co-immunoprecipitation of tyrosine phosphorylated Tek and Nck when compared with DMEM-stimulated lysates (–). Immunoprecipitations of Dok-R (first two lanes, anti-Dok-R blot) and Nck (last two lanes, anti-Nck blot) with their respective antibodies revealed that equal amounts of protein were immunoprecipitated from both stimulated (+) and unstimulated (–) lysates. Immunoprecipitation of tyrosine phosphorylated Nck from Ang1-MH-stimulated lysates could co-immunoprecipitate more tyrosine phosphorylated Dok-R than unstimulated (–) lysates (last two lanes). (C) SVR ECs were seeded in the top chamber of a modified Boyden chamber while chemoattractants (Mock, Ang1-MH or 50 ng/ml VEGF) were placed in the bottom well. Stimulation with Ang1-MH and VEGF resulted in a 5-fold increase in migration over Mock-stimulated cells. Data points are represented as the number of migrated cells per field. All experiments were performed in triplicate and differences between Mock-stimulated and ligand-stimulated migration values were statistically significant ($P < 0.05$).

Dok-R and Nck and thus, serve as a model to further investigate the role of Dok-R and Nck in Ang1-mediated EC migration.

Phosphorylation of Y351 on Dok-R is required for interaction with Nck

We have shown that Ang1-MH stimulation results in the phosphorylation of Dok-R and its association with Nck. The SH2 domains of Nck and RasGAP are known to interact with phosphorylated tyrosine within the context YxxP (Songyang *et al.*, 1993). We have previously generated point mutations in Dok-R that alter tyrosine

Fig. 2. Dok-R recruits Nck through tyrosine phosphorylation of Y351. (A) The positions of three tyrosine residues (276, 304 and 351) in Dok-R found within the context YxxP are indicated. Double mutations in Y276 and Y304 to phenylalanine (F) abolish Dok-R binding to RasGAP (Dok-R^{DM}) while mutation of Y351 to F abrogates binding to Nck (Dok-R^{Y351F}). (B) Overexpression of Tek with Dok-R, or its mutants, in HEK 293T cells resulted in the tyrosine phosphorylation of Dok-R, Dok-R^{DM} and Dok-R^{Y351F} and their co-immunoprecipitation with tyrosine phosphorylated Tek. Nck could co-immunoprecipitate with Dok-R or Dok-R^{DM}, but this interaction was abrogated in Dok-R^{Y351F}-transfected cells. Equal amounts of Tek and Dok-R/mutant were observed in transfected cell lysates (CL). (C) Lysates prepared from Tek-Dok-R and Tek-Dok-R^{Y351F} cell lines stimulated with Mock or Ang1-MH for 1 h in the absence of phosphatase inhibitors were immunoprecipitated with anti-Tek antibodies. Ang1-MH can stimulate the tyrosine phosphorylation of Tek in stable cell lines and the co-immunoprecipitation of tyrosine phosphorylated Dok-R or its mutants. Mock stimulation of these cells resulted in basal levels of Tek tyrosine phosphorylation. Immunoprecipitation of Tek also resulted in the co-immunoprecipitation of Nck in Ang1-MH-stimulated lysates of Tek-Dok-R cells, but not Tek-Dok-R^{Y351F} mutant cells. Immunoblotting with anti-Tek antibodies revealed equal amounts of Tek protein expression in all lysates.

residues that mediate binding to Nck (Y351F) and RasGAP (Y276,304F–DM) to phenylalanine (Jones and Dumont, 1999; see Figure 2A). To determine whether these point mutations in Dok-R affected its recruitment to activated Tek, we introduced Tek and either Dok-R, or one of its mutants, into HEK 293T cells. As reported previously, overexpression of Tek in HEK 293T cells results in activation of the receptor in an Ang1-independent context (Jones and Dumont, 1998; Jones *et al.*, 1999). Immunoprecipitation of Tek results in its tyrosine phosphorylation and co-immunoprecipitation of Dok-R and its mutants (Figure 2B). Dok-R binding to activated Tek also results in tyrosine phosphorylation of Dok-R and mutant forms. Furthermore, co-immunoprecipitation experiments also revealed that Dok-R and Dok-R^{DM} could interact with Nck, but that this binding was abolished in Dok-R^{Y351F}-transfected cells (Figure 2B). These results demonstrate that the differently mutagenized Dok-R polypeptides are efficiently recruited to Tek and that Y351 on Dok-R is required for downstream recruitment of Nck.

In order to easily manipulate Dok-R signaling pathways, we utilized a 293Tek cell line that responds to Ang1-MH stimulation in a manner virtually identical to SVR and other ECs (Jones *et al.*, 1999). We generated 293Tek cell lines stably expressing vector alone (Tek-Vec

Fig. 3. Nck recruitment to Dok-R is required for Ang1-mediated migration. (A) Lysates prepared from Tek-Dok-R or mutant cell lines were subjected to immunoblot analysis using anti-HA antibodies that recognized transfected Dok-R/mutant (inset). Equal Dok-R-expressing clones of Tek-Vec, Tek-Dok-R, Tek-Dok-R^{DM} and Tek-Dok-R^{Y351F} stable cell lines (inset) were placed in a migration assay as described previously using Mock, Ang1-MH or 30 ng/ml bFGF in Mock media as chemoattractants. Ang1-MH stimulation of Tek-Vec cells resulted in a 3-fold increase in migration over Mock, and expression of Dok-R or Dok-R^{DM} potentiated this Ang1-dependent motility. However, Tek-Dok-R^{Y351F} cells displayed a dramatic reduction in migration. Stimulation of cells with bFGF resulted in relatively equal migration levels. All experiments were performed in triplicate using three different clones (data not shown). Migration differences between Mock and Ang1-MH stimulations of all cell lines, and of Ang1-MH stimulations of Tek-Dok-R^{Y351F} compared with Tek-Vec/Tek-Dok-R/Tek-Dok-R^{DM} were all found to be statistically significant ($P < 0.05$). (B) Tek-Dok-R and Tek-Dok-R^{Y351F} stable cell lines seeded on coverslips were stimulated with Mock- or Ang1-MH-conditioned media for 4 h and then immunostained using antibodies against actin. Mock-stimulated cells displayed a diffused dispersion of actin at the cell membrane with no membrane protrusions. However, cells stimulated with Ang1-MH displayed localized regions of actin and Tek-Dok-R cells displayed a greater frequency and extent of membrane projections (arrowheads) than did Tek-Dok-R^{Y351F} cells.

cells), Dok-R (Tek-Dok-R cells) or mutant forms of Dok-R unable to bind either Nck (Tek-Dok-R^{Y351F} cells) or RasGAP (Tek-Dok-R^{DM} cells). Stimulation of these cell lines with Ang1-MH resulted in the tyrosine phosphorylation of wild-type Dok-R and its mutant forms, and their co-immunoprecipitation with activated Tek (Figure 2C and data not shown). Notably, a phosphoprotein similar to the size of Nck co-immunoprecipitates with Tek only from Ang1-MH-stimulated Tek-Dok-R lysates, but not from Tek-Dok-R^{Y351F} lysates (Figure 2C). These results illustrate that Ang1-MH can promote the tyrosine phosphorylation and association with the activated Tek receptor of Dok-R and its mutants.

Phosphorylation of Y351 on Dok-R is required for Ang1-mediated migration

Since Ang1-MH stimulation of ECs leads to the recruitment of Nck to Dok-R and both Nck and RasGAP have

been shown to influence cellular migration, we thus set out to determine whether Dok-R can mediate Ang1-dependent cell migration. We chose several independent clones of Tek-Dok-R, Tek-Dok-R^{DM} and Tek-Dok-R^{Y351F} stable cell lines that had comparable expression levels of Dok-R proteins (Figure 3A, inset). Ang1-MH stimulation of 293Tek cells stably expressing vector alone (Tek-Vec) produced a significant 3-fold increase in cell motility over stimulation with Mock (Figure 3A). Overexpression of Dok-R in Tek-Dok-R cells potentiated Ang1-mediated cell motility by almost 2-fold over Tek-Vec cells. This potentiation was not abrogated in Tek-Dok-R^{DM} cells, whereas the Tek-Dok-R^{Y351F} cells exhibited a significant reduction in Ang1-mediated migration (Figure 3A). The presence of endogenous hDok-R in 293Tek cells (data not shown) may account for the ability of Dok-R^{Y351F} to interfere with normal hDok-R function in these cells. Importantly, we also tested whether basic fibroblast

Fig. 4. Pak colocalizes with Tek through a Dok-R–Nck interaction. Antibodies recognizing Tek, Dok-R and Pak were used to detect these proteins in HEK 293T cells overexpressing Tek with either Dok-R or Dok-R^{Y351F} by confocal microscopy. Cellular distributions of Tek (red) were found at the cell membrane and were colocalized with Dok-R/ Dok-R^{Y351F} (green) as seen by the merged image (yellow). The colocalization seen between Tek and Dok-R, or its mutants, specifically required an active Tek kinase, since cells co-transfected with Tek^{K853A} and Dok-R display diffuse staining throughout the cytoplasm rather than at the membrane (inset). Similarly, in cells overexpressing activated Tek and Dok-R, Pak (green) was enriched at the cell membrane (white arrows), which colocalized with Tek, as indicated by the merged image. However, cells expressing Tek and Dok-R^{Y351F} did not have this enriched localization of Pak at the membrane and no colocalization with Tek can be seen (merge).

growth factor (bFGF) could affect cell migration and found that all of the 293Tek stable lines responded similarly to bFGF-stimulated chemotaxis (Figure 3A), which is consistent with our inability to detect Dok-R

tyrosine phosphorylation following bFGF stimulation (data not shown). The ability of these cells to undergo similar levels of bFGF-dependent migration serves to illustrate that the lack of Ang1-dependent motility observed in Tek-Dok-R^{Y351F} cells is not due to a generalized inhibition of cell migration by the Dok-R^{Y351F} protein. Taken together, these results demonstrate that Dok-R mediates Ang1-dependent cell migration and that Y351 on Dok-R is required for this activity.

Migrating cells form membrane extensions of localized actin at a focal point of the cell during directional chemotaxis, which facilitates movement by extending the cell body prior to contraction (Sheetz *et al.*, 1998). To further confirm that Dok-R plays a role in Ang1-mediated migration using another biological assay, we chose to examine actin localization in Tek-Dok-R and Tek-Dok-R^{Y351F} cells. Mock-treated Tek-Dok-R and Tek-Dok-R^{Y351F} cells displayed non-localized actin that was dispersed throughout the cell membrane (Figure 3B). However, Ang1-MH stimulation resulted in the redistribution of actin at focal points on the cell membrane. Interestingly, the Tek-Dok-R cells displayed a greater number and more extensive membrane protrusions as compared with Tek-Dok-R^{Y351F} cells, suggesting that recruitment of Nck may be required for Dok-R to mediate cellular morphological changes in response to Ang1/Tek signaling (Figure 3B). These findings implicate Dok-R and Nck in rearrangements of the actin cytoskeleton to facilitate Ang1-mediated motility.

Pak colocalizes with Tek through a Dok-R–Nck interaction

Nck has previously been shown to constitutively associate with Pak whose activation has been implicated in cytoskeletal rearrangements leading to cell migration downstream of many growth factor receptors (Bokoch *et al.*, 1996; Lu *et al.*, 1997; Stoletov *et al.*, 2001). To establish whether Pak could potentially contribute to Ang1-mediated cell migration upon recruitment to Dok-R, we first examined whether Pak could colocalize with Tek at the cell membrane. HEK 293T cells grown on coverslips were transfected with Tek and either Dok-R or Dok-R^{Y351F} under conditions where Tek is activated. Indirect immunofluorescence with antibodies directed against Tek, Dok-R and Pak was used to detect the intracellular distribution of these proteins *in vivo*. As anticipated, both Tek (red) and the different forms of Dok-R (green) were colocalized at the cell membrane as indicated by the merged image (Figure 4 and data not shown). To demonstrate that Tek and Dok-R colocalization requires an active Tek kinase, Dok-R and its mutants were co-transfected with a kinase-inactive Tek (Tek^{K853A}). A requirement for an active Tek kinase was demonstrated by a dramatic lack of Dok-R localization at the membrane as Dok-R (green) was found predominantly in the cytoplasm, which was evident upon merging the two images, Tek (orange) (Figure 4, inset). Somewhat similar to the pattern of Tek and Dok-R colocalization, an enrichment of Pak was localized at the membrane (white arrowheads) in Tek + Dok-R-transfected cells, which was completely absent in Tek + Dok-R^{Y351F} cells (Figure 4). It is important to note that not every cell was transfected here (asterisk) and since our transfected Dok-R^{Y351F} must first

Fig. 5. Activated Tek promotes the formation of a Pak, Nck and Dok-R ternary complex *in vivo* and results in Pak activation. (A) SVRs were stimulated with Ang1-MH or DMEM in the presence of sodium orthovanadate. Immunoprecipitation of Dok-R resulted in co-immunoprecipitation of Pak in lysates from Ang1-MH-stimulated cells. Reprobing the membrane with anti-Dok-R antibodies revealed equal amounts of immunoprecipitated Dok-R from both stimulated and unstimulated lysates. (B) Lysates from HEK 293T cells overexpressing Pak, Tek/Tek^{K853A} and Dok-R/Dok-R^{Y351F} were immunoprecipitated with antibodies that specifically recognize Dok-R, Pak or Tek. In cells overexpressing Tek + Dok-R, both Dok-R and Pak could co-immunoprecipitate with one another. Interaction of Dok-R with Pak was not observed in cells expressing either a kinase inactive Tek (Tek^{K853A}) or Dok-R^{Y351F}. Immunoblotting of cell lysates (CL) revealed equal expression of Pak, Dok-R and Tek. (C) Pak immunoprecipitates from the above transfectants were subjected to an *in vitro* kinase assay using MBP as substrate. Tek-mediated MBP phosphorylation by Pak was 40% greater from cells that expressed Dok-R than from cells expressing Dok-R^{Y351F} or Tek^{K853A} with either Dok-R or Dok-R^{Y351F}. The graph represents MBP phosphorylation values after normalization for Pak immunoprecipitation. Immunoprecipitation of Tek and Tek^{K853A} with anti-Tek antibodies revealed that Tek, but not Tek^{K853A}, was tyrosine phosphorylated. Data shown are average of two independent experiments $\pm$ SD.

displace endogenous hDok-R, this may explain the small amount of Pak localization seen in some Tek + Dok-R^{Y351F} cells (merge).

Activated Tek promotes the formation of a Dok-R, Nck and Pak ternary complex *in vivo* and stimulates Pak kinase activity

Our immunofluorescence studies implied that following Tek activation, Pak is brought to the membrane and that

this redistribution in intracellular localization requires Dok-R and a functional Nck binding site on Dok-R. To further confirm our findings that Pak colocalizes with Tek and Dok-R at the cell membrane in ECs, we stimulated SVRs with Ang1-MH (+) or Dulbecco's modified Eagle's medium (DMEM) (–). Immunoprecipitation of Dok-R resulted in co-immunoprecipitation of Pak from Ang1-MH-stimulated lysates (Figure 5A). To further demonstrate that recruitment of Pak to the cell membrane required a functional Dok-R–Nck interaction, we over-expressed Pak and either Tek or Tek^{K853A} with either Dok-R or Dok-R^{Y351F} in HEK 293T cells. Expression of either Dok-R or Dok-R^{Y351F} with Tek results in tyrosine phosphorylation of both forms, which is not observed in cells expressing a kinase-inactive Tek mutant (see Figure 2B). In the presence of an active Tek kinase, Pak can be co-immunoprecipitated with Dok-R in a phosphotyrosine-dependent manner and disruption of the Nck binding site on Dok-R, Dok-R^{Y351F}, abrogates this interaction (Figure 5B). The inverse can also be seen, as Pak immunoprecipitates indicate the presence of Dok-R, but not Dok-R^{Y351F} (Figure 5B). These results illustrate that upon Tek activation, Pak is brought to the membrane and that this recruitment is dependent upon the ability of Dok-R to recruit Nck.

Membrane recruitment of Pak to activated growth factor receptors such as the epidermal growth factor receptor (EGFR) results in Pak kinase activation (Lu and Mayer, 1999). To determine whether recruitment of Pak to tyrosine phosphorylated Tek also increased Pak kinase activity, we subjected immunoprecipitates of Pak prepared from cells transiently transfected with Pak, Tek or Tek^{K853A} and either Dok-R or Dok-R^{Y351F} to an *in vitro* kinase assay where myelin basic protein (MBP) was used as a substrate. Increased Pak kinase activity was reflected by a 40% increase in radiolabeled MBP in Pak immunoprecipitates from cells expressing Tek/Dok-R as compared with Tek/Dok-R^{Y351F}-, Tek^{K853A}/Dok-R- or Tek^{K853A}/Dok-R^{Y351F}-expressing cells (Figure 5C). Although the difference between Dok-R and Dok-R^{Y351F} was somewhat subtle, it was highly reproducible and reflected the ability of Dok-R to recruit Pak to the membrane upon Tek activation. The expression levels of the transfected cDNAs were all comparable and the relatively high background phosphorylation seen in precipitates from Dok-R^{Y351F} co-transfectants was probably due to the residual activity of endogenous Pak from the large proportion of untransfected cells (Figure 5C). Together, these results demonstrate that recruitment of Pak through Dok-R and Nck to Tek results in an increase in Pak activity.

Angiopoietin-1 stimulates the activation of Pak kinase in ECs

We have shown that Tek activation can promote the formation of a Dok-R–Nck–Pak complex at the membrane and is required for cell migration. However, since Tek expression is largely restricted to ECs, we wanted to demonstrate that Ang1 could stimulate activation of endogenous Pak in ECs. SVRs were stimulated with Mock, Ang1-MH or EGF, and Pak immunoprecipitates were subjected to an *in vitro* kinase assay that measured the ability of Pak to phosphorylate MBP. Stimulation of

Fig. 6. Ang1 stimulates the activation of Pak kinase in ECs. SVRs were stimulated with Mock, Ang1-MH or 30 ng/ml EGF in Mock medium for 0, 5 or 10 min. Lysates were immunoprecipitated with anti-Pak antibodies and then subjected to an *in vitro* kinase assay using MBP. Pak immunoblots revealed relatively equal Pak immunoprecipitations. The graph represents MBP phosphorylation values after normalization for Pak immunoprecipitation. Data shown are average of two independent experiments $\pm$ SD.

SVRs with EGF as positive control was able to stimulate Pak kinase activity with a peak activity at 5 min post-stimulation, after which Pak activity was decreased (Figure 6). Previous reports have only examined the activity of Pak from EGF-stimulated cells up to 5 min (Lu *et al.*, 1997), thus whether this decrease in Pak activity at 10 min reflects a unique feature of SVR cells is unknown. In contrast, Ang1-MH stimulation of SVRs resulted in an increase in Pak activity, which continued beyond 5 min (Figure 6). Incubation of cells in Mock medium produced a modest increase in Pak kinase activity by 5 min, which plateaued by 10 min. This increase in Pak activity with Mock may be reflective of minor components in the conditioned medium that are likely to stimulate low levels of Pak activity. This is the first report of activation of Pak in ECs upon Ang1 stimulation and these findings suggest that the motility observed in SVR ECs is likely due to recruitment of this tri-molecular complex to Tek.

Angiopoietin-1-mediated cell migration is potentiated by overexpression of Pak

Pak serine/threonine kinase activity has been shown to be involved in other cellular processes in addition to its effects on the actin cytoskeleton (Tang *et al.*, 2000). To assess whether Ang1-dependent Pak activation reflected changes in cell migration, we performed motility assays using Tek-Dok-R and Tek-Dok-R^{Y351F} stable cell lines transiently overexpressing Pak. Transfected Pak led to an ~2-fold increase in Pak expression levels when compared with untransfected cells (Figure 7, inset). Pak overexpression in Mock-treated cells resulted in a modest increase in migration; however, Pak overexpression in Tek-Dok-R cells resulted in ~45% more migrating cells as compared with vector-transfected Tek-Dok-R cells upon Ang1-MH stimulation (Figure 7, graph). This potentiation of motility seen with Pak overexpression in Tek-Dok-R versus Tek-Dok-R^{Y351F} cells is probably due to Dok-R^{Y351F} behaving in a dominant-interfering manner with endogenous hDok-R. These results illustrate that Ang1/Tek-mediated Pak activation is required to initiate cell migration.

Fig. 7. Ang1-mediated cell migration is potentiated by overexpression of Pak. Tek-Dok-R and Tek-Dok-R^{Y351F} alone or transfected with Pak, were subjected to a migration assay using Mock or Ang1-MH as chemoattractant. Cells stimulated with Ang1-MH resulted in migration similar to that detected previously with Tek-Dok-R and Tek-Dok-R^{Y351F} cells. However, overexpression of Pak in Tek-Dok-R cells resulted in a potentiation of Ang1-mediated cell migration, which was not seen in Tek-Dok-R^{Y351F} cells. Cell lysates prepared from these cells revealed equal expression of Dok-R or Dok-R^{Y351F} and a 2-fold increase in Pak when overexpressed (inset).

Discussion

Activation of the Tek receptor by Ang1 has been postulated to modulate vascular maturation during angiogenesis. In this regard, Ang1 behaves as both a survival and a chemotactic agent. We and others have shown that Ang1-mediated migration is dependent in part on PI3-kinase activation (Fujikawa *et al.*, 1999; Jones *et al.*, 1999); however, it seems likely that this pathway is complemented by others since pharmacological inhibition of PI3-kinase only partially inhibited Ang1-driven motility. Here, we propose an alternative novel pathway involving the recruitment of a ternary complex to the Tek receptor composed of Dok-R, Nck and Pak. We show that following stimulation of ECs with Ang1, tyrosine phosphorylation of Tek, Dok-R and Nck results in cell migration presumably through membrane recruitment and activation of the serine/threonine kinase Pak via a Dok-R–Nck interaction (Figure 8). Our results suggest that Ang1-driven phosphorylation of Dok-R in ECs recruits Pak to the receptor through a Dok-R–Nck association, which requires phosphorylation of Y351 on Dok-R. Phosphorylation of Y351 is also required for both Ang1-dependent activation of Pak kinase and the reorganization of the actin cytoskeleton resulting in the formation of membrane protrusions, a hallmark of cell migration. Taken together, our results demonstrate that phosphorylation of Y351 on Dok-R is required for Nck-mediated recruitment of Pak and that this assembled complex orchestrates Ang1-mediated EC migration.

Cell migration by Ang1 appears to be mediated through two downstream pathways: the first involving PI3-kinase activity and the second involving assembly of a Dok-R–Nck–Pak ternary complex on the Tek receptor and subsequent activation of Pak kinase (Figure 8). PI3-kinase products may also regulate Pak kinase activity (Tsakiridis *et al.*, 1996; Adam *et al.*, 1998). PI3-kinase can activate the serine/threonine protein kinase B (PKB)/Akt as a result of phosphorylation by the serine/threonine kinase, 3-phosphoinositide-dependent kinase (PDK)-1 (Alessi *et al.*, 1997; Toker and Newton, 2000). Tang *et al.* (2000) demonstrated that PKB/Akt could activate

Fig. 8. Ang1-mediated migration. A schematic representation of the signaling pathways mediated through Ang1/Tek signaling that promote cell migration. Ang1 regulates two pathways that mediated cell motility, the first being through activation of the PI3-kinase pathway and the second involving the phosphotyrosine-dependent recruitment of Dok-R, Nck and activation of Pak at the cell membrane. PI3-kinase comprises a p85 regulatory subunit, which binds the p110 catalytic subunit. Wort (wortmannin)/LY (LY294002) are pharmacological inhibitors of PI3-kinase.

Pak, consequently protecting cells against apoptosis; however, the effects on migration were not tested in their study. Furthermore, PDK-1 was also shown to activate Pak directly, but in a PI3-kinase-independent manner (King *et al.*, 2000). Although it remains to be established whether both Ang1-driven pathways converge on Pak to modulate migration, it is possible that Ang1-mediated activation of PI3-kinase may regulate Pak kinase activity and thereby promote cell motility. The ability of Tek to recruit two distinct molecular pathways to elicit a similar cellular effect underscores the importance of cell migration in Ang1/Tek signaling.

Nck can constitutively associate with Pak via an SH3-mediated interaction (Bokoch *et al.*, 1996) and the implication of Nck and Pak in cellular migration was derived from work first performed in *Drosophila* photoreceptor cells. Genetic mutation of the *Drosophila* homolog of the Nck gene, Dreadlocks (dock), disrupted axon guidance as mutant flies displayed irregular neuronal patterning perhaps as a direct consequence of a defect in cell migration (Garritty *et al.*, 1996). Interestingly, similar defects in axon projection have also recently been reported in *Drosophila* embryos lacking Pak (Hing *et al.*, 1999). Since this phenotype could be rescued with a membrane-bound form of wild-type Pak, but not with a kinase-inactive mutant, Pak activity seems to be required for normal neuronal patterning. Although it remains to be

determined whether a fly homolog to the DOK family of proteins exists, in our system, interference with the Nck binding site on Dok-R inhibits membrane localization and activation of Pak and as a consequence, dramatically reduces cell migration induced by Ang1 signaling. This conserved Nck binding consensus site is also found on Dok and IRS-3 and has further been shown to be required on Dok for insulin-mediated migration of Chinese hamster ovary cells (Noguchi *et al.*, 1999). Collectively, these experiments highlight the importance of both Nck and Pak in cell migration mediated by the DOK family proteins downstream of tyrosine kinases. Moreover, several receptors such as the Eph family and neuropilin-1 seem to function similarly in both neuronal cells and ECs (Gale and Yancopoulos, 1999), suggesting that common cellular components may regulate both sprouting angiogenesis and axon guidance.

In addition to recruiting Nck, tyrosine phosphorylation of Dok and Dok-R also creates binding sites for the SH2 domains of RasGAP (Jones *et al.*, 1999; Lock *et al.*, 1999). RasGAP has been shown to mediate changes in cell shape and adhesion (McGlade *et al.*, 1993) and more recently, cell lines isolated from RasGAP-null embryos have been reported to have a compromised ability to migrate (Kulkarni *et al.*, 2000). Despite these findings, Ang1 stimulation of Tek-Dok-R^{DM} cells, a Dok-R mutant defective for RasGAP binding, was still able to potentiate Ang1-mediated migration similarly to wild-type Dok-R, suggesting that the association between RasGAP and Dok-R may serve another function. Several recent studies have suggested that DOK proteins may serve to downregulate cellular proliferation by inhibiting the mitogen activated protein kinase (MAPK) pathway (Nelms *et al.*, 1998; Cong *et al.*, 1999; Jones and Dumont, 1999). However, the ability of Dok-R to downregulate MAPK activity does not appear to require RasGAP association (Jones and Dumont, 1999); as such, the biological effect of the association between RasGAP and Dok-R remains elusive. Interestingly, Dok-L cannot bind RasGAP as it lacks the YxxPx^D RasGAP SH2 domain target binding motif found in both Dok and Dok-R (Lemay *et al.*, 2000), and since Nck uses a similar target motif, it suggests that Dok-L may also be unable to bind Nck. Since the DOK family members are co-expressed in a number of cell types, differential binding of signaling proteins would provide a means for recruitment by the same tyrosine kinase while allowing distinct transduction of signals.

In summary, our results demonstrate that Dok-R may be involved in the regulation of cell shape and migration through control of Nck-dependent Pak relocalization and activation following Ang1 stimulation. In turn, Ang1-mediated migration through Tek and Dok-R may serve an intrinsic function of this ligand/receptor system in EC biology. Developmentally, EC precursors known as angioblasts are highly motile and have been shown to express Tek (Dumont *et al.*, 1992). Whether these cells express Dok-R remains to be determined; however, Dok-R expression can be detected by immunofluorescence in virtually all tissues in the early embryo (Jones and Dumont, 1998), suggesting that one major function of Tek signaling through Dok-R may be to direct cell motility during early angiogenic patterning. Takakura *et al.* (2000) have shown that hematopoietic stem cells *in vivo* secrete

Ang1, which can potentiate long-range migration of ECs in the developing embryo, further supporting our notion that a critical function for Ang1/Tek signaling during embryogenesis may be cell migration. Ultimately, it seems likely that a better understanding of the molecular pathways and their individual biological responses governed by Ang1/Tek signaling will allow proper assessment of the contribution of each ligand/receptor pathway to the overall process of angiogenesis.

Materials and methods

Plasmids

The plasmids encoding hemagglutinin (HA)-tagged Dok-R, Dok-R^{DM} and Dok-R^{Y351F}, Tek and Tek^{K853A} have been described previously (Jones and Dumont, 1998, 1999). HA-tagged Pak cDNA in pEBB was a kind gift from Drs B.Mayer and M.Parrini.

Cell culture

HEK 293, HEK 293T epithelial and SVR EC lines (ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin (Life Technologies). HEK 293T and 293Tek cells (Jones *et al.*, 1999) were transfected with 10 µg of Dok-R/mutant plasmids by Lipofectamine (Gibco) according to the manufacturer's instructions. Clones for the stable transfections of 293Tek cells with Dok-R/mutant forms were selected in media containing zeocin (Invitrogen). Isolated colonies were analyzed for Dok-R/mutant expression. 293Tek + Dok-R/mutant stable cell lines were maintained in DMEM + 10% FBS, plus 100 nM methotrexate (Sigma), 250 µg/ml G418 (Life Technologies) and 500 µg/ml zeocin.

Ang1 cell stimulations

Conditioned media from HEK 293T stable cell lines expressing Ang1-MH or an untransfected control (Mock) were used as stimulant throughout these studies and their preparation has been described (Jones *et al.*, 1999). For some co-immunoprecipitation experiments in SVR ECs, DMEM rather than Mock was used as an unstimulated control since trace amounts of Ang1 endogenous to HEK 293T cells (data not shown) resulted in moderate phosphorylation of the receptor and downstream proteins. For stimulation, 5 ml of Ang1-MH, Mock or DMEM, in either the presence or the absence of 1 mM sodium orthovanadate, was added to cells, incubated at 37°C for 10 min and then harvested (Jones *et al.*, 1999).

Antibodies used for immunoprecipitations, immunofluorescence and western blotting

Commercially available antibodies used were as follows: polyclonal anti-Tek C20 (Santa Cruz); monoclonal anti-actin (Sigma); monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology); monoclonal anti-HA-HRP clone 12CA5 (Boehringer Mannheim); anti-Pak antibodies (Santa Cruz); monoclonal anti-Nck (Transduction Labs). Monoclonal anti-Tek (anti-331) was provided by Dr C.Kontos and Dr K.Peters, Duke University, and antibodies specific to the extracellular domain of Tek (MTE32) were a kind gift from Fu-Kuen Lin (Amgen, Thousand Oaks, CA). The anti-Dok-R antibodies used have been described previously (Jones and Dumont, 1998). Coimmunoprecipitation and western blotting procedures have been described previously (Jones and Dumont, 1998, 1999; Jones *et al.*, 1999).

Immunofluorescence and confocal analysis

For actin staining of membrane protrusions, Tek-Dok-R and Tek-Dok-R^{Y351F} cells were seeded on glass coverslips for 18 h and then stimulated with either Mock or Ang1-MH for 4 h. For colocalization studies, HEK 293T cells were transfected with the appropriate cDNAs by lipofectamine as indicated above. All cells were fixed in 4% paraformaldehyde for 20 min and permeabilized in phosphate-buffered saline containing 1% Nonidet P40 (Sigma) for 10 min. Samples were blocked in 5% goat serum (Jackson ImmunoResearch Laboratories Inc.) and then incubated with antibodies to Tek (anti-331) (1:2000), Pak (1:2000), Dok-R (1:4000) or actin (1:200) for 2 h at room temperature. Samples were washed and incubated with secondary antibodies conjugated to fluorescein isothiocyanate (FITC) (1:100) or Texas Red (1:100) for 1 h at room temperature. Slides were mounted using Aquapolyount (Polysciences Inc.) and visualized using Zeiss

Axiovert 100M laser scanning microscope (LSM) confocal microscopy. Analyses were performed using LSM510 (version 2.3) scanning software (Zeiss). Images were captured at 630× magnification.

Cell migration assays

SVR and Tek-Vec, Tek-Dok-R, Tek-Dok-R^{DM} or Tek-Dok-R^{Y351F} cells were seeded at a density of 8.4×10^4 cells in 500 µl of DMEM + 0.1% FBS in the upper chamber of an 8 µm pore modified Boyden chamber (Falcon). Cells were allowed to migrate for 4 h in 250 µl of chemoattractant and membranes were fixed and counted as described (Jones *et al.*, 1999). A Student's *t*-test was used to test the statistical significance with a 95% confidence interval. All experiments were repeated at least twice with two or three replicates using several different clonal cell populations with each replicate sample size equaling 20 counts.

In vitro Pak kinase assays

Cell lysates were immunoprecipitated with anti-Pak antibodies for 2 h at 4°C. Immunoprecipitates were washed three times in PLC lysis buffer (without sodium fluoride or sodium pyrophosphate) (Jones and Dumont, 1998) and twice in kinase buffer (2 mM MnCl₂ + 50 mM HEPES pH 7.5 + 10 mM MgCl₂ + 0.2 mM DTT) and then incubated with 4 µg of MBP (Sigma) plus 10 µl of [γ -³²P]ATP (Amersham), 20 µM ATP (Pharmacia) for 30 min at 30°C. Kinase reactions were stopped by the addition of 2 × SDS-containing sample buffer and boiled for 10 min. Pak immunoprecipitates were electrophoresed and half the gel was used to resolve Pak loading by western analysis while the other half was dried and exposed to phosphorimager analysis and quantified (ImageQuant). The representative values of Pak kinase activity were reflected as the value of MBP phosphorylation over the amount of immunoprecipitated Pak in each of the samples. All experiments were performed twice or more with similar results. The graph represents the average of the experiments ± standard deviation (SD).

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