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A Unique Autophosphorylation Site on Tie2/Tek Mediates Dok-R Phosphotyrosine Binding Domain Binding and Function

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Tie2/Tek is an endothelial cell receptor tyrosine kinase that induces signal transduction pathways involved in cell migration upon angiopoietin-1 (Ang1) stimulation. To address the importance of the various tyrosine residues of Tie2 in signal transduction, we generated a series of Tie2 mutants and examined their signaling properties. Using this approach in conjunction with a phosphorylation state-specific antibody, we identified tyrosine residue 1106 on Tie2 as an Ang1-dependent autophosphorylation site that mediates binding and phosphorylation of the downstream-of-kinase-related (Dok-R) docking protein. This tyrosine residue is contained within a unique interaction motif for the phosphotyrosine binding domain of Dok-R, and the pleckstrin homology domain of Dok-R further contributes to Tie2 binding in a phosphatidylinositol 3′-kinase-dependent manner. Introduction of a Tie2 mutant lacking tyrosine residue 1106 into endothelial cells interferes with Dok-R phosphorylation in response to Ang1. Furthermore, this mutant is unable to restore the migration potential of endothelial cells derived from mice lacking Tie2. Together, these findings demonstrate that tyrosine residue 1106 on Tie2 is critical for coupling downstream cell migration signal transduction pathways with Ang1 stimulation in endothelial cells.

Development of a functional cardiovascular system is dependent on the regulated proliferation, migration, and differentiation of endothelial cells in two discrete processes known as vasculogenesis and angiogenesis (47). Vasculogenesis occurs principally during embryonic development to establish the early vessel network, which is subsequently remodeled through angiogenesis. Here, new capillaries arise from preexisting larger vessels to give rise to a more complex vascular network with a hierarchy of both large and small vessels. Periendothelial support cells are then recruited to the nascent vessels to surround the endothelial tubes and stabilize the vessel (9).

Cellular events in vascular development are controlled by molecular signal transduction pathways that are often mediated by cell surface growth factor receptors known as receptor tyrosine kinases. A number of these receptors, including those from the vascular endothelial growth factor (VEGF) receptor and TIE receptor subfamilies, have been identified on the surfaces of endothelial cells (65). Such receptors are membrane-spanning proteins comprising an extracellular ligand binding domain and an intracellular catalytic tyrosine kinase domain, followed by a carboxy-terminal tail. Ligand-mediated receptor oligomerization triggers activation of the kinase and autophosphorylation at a specific set of tyrosine residues, which serve as docking sites for intracellular signaling molecules containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (45). Functional differences between the VEGF and TIE receptors within the endothelial cell lineage may be explained in part by the unique series of signaling molecules associated with each receptor (55).

Growth factors acting on the vascular endothelium presently include five members of the VEGF family and four members of the angiopoietin family (65). Although the VEGF family possesses overlapping receptor specificity, the angiopoietins isolated to date appear to bind exclusively to the Tie2/Tek receptor tyrosine kinase and the ligand for the closely related Tie1 receptor remains elusive. Interestingly, these ligands can dynamically regulate receptor activation as angiopoietin-1 (Ang1) and Ang4 stimulate tyrosine phosphorylation of the receptor while Ang2 and Ang3 can inhibit this phosphorylation in certain cellular contexts (10, 35, 48, 57, 58). Identification of a family of natural agonists and competitive antagonists for Tie2 implies that there is exquisite control over the signal transduction pathways mediated by this receptor.

Coordinated expression of the angiopoietins and Tie2 is required for the angiogenic remodeling and vessel stabilization processes that occur subsequent to the initial vasculogenic actions of VEGF receptors 1 and 2. Gene-targeting studies have revealed that mice deficient in Tie2 or Tie2 kinase activity do not undergo sufficient sprouting and remodeling of the primary capillary plexus, leading to incomplete development of the heart and head regions (13, 49). There is also a dramatic reduction in the number of endothelial cells in these mice (13), owing to impaired survival of the endothelium in the absence of Tie2 (25, 46). Disruption of the Tie2 agonistic ligand, Ang1, results in embryonic lethality,

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with defects in angiogenesis that are strikingly similar to those seen upon disruption of Tie2 (54). Interestingly, however, the defects observed in these mice are less severe than those observed in mice lacking Tie2, implicating the additional angiopoietins in Tie2 function. Transgenic overexpression of Ang2 in endothelial cells results in vascular defects that resemble those seen in the absence of Ang1 or Tie2 (35), demonstrating that Ang2 can potentially regulate Ang1 function in vivo by antagonizing the effects of Ang1 on Tie2.

Underdevelopment of the vasculature in mice lacking Tie2 signaling pathways has been attributed to defects in both sprouting angiogenesis and intussusceptive microvascular growth (44). During sprouting angiogenesis, activated endothelial cells secrete matrix-degrading proteinases and migrate through the basement membrane into the surrounding tissue. A role for Tie2 signaling in endothelial cell migration is supported by numerous studies showing that activation of Tie2 by Ang1 results in the stimulation of cell motility, including sprout and tubule formation (19, 28, 31, 43, 57, 61), and that a modified form of Ang1 known as Ang1* synergizes with VEGF during sprouting angiogenesis in vivo (1). Ang2 can also stimulate tyrosine phosphorylation of Tie2 in several endothelial cell types, leading to tubule formation on fibrin and collagen matrices (38, 57), and Ang2 augments fibroblast growth factor 2-induced chemotaxis (38). Endothelial cell migration in response to Ang1 is contingent upon changes in the intracellular architecture of the cell, which may depend in part on the activity of phosphatidylinositol (PI) 3′ kinase. Upon Ang1 stimulation, the SH2 domain-containing p85 subunit of PI 3′ kinase is recruited to Tie2 via tyrosine residue 1100 in the carboxy-terminal tail of the receptor, leading to activation of the enzyme (24, 29). Addition of PI 3′ kinase inhibitors in cell motility assays blocks Ang1-stimulated migration of both endothelial and nonendothelial cells expressing Tie2 (15, 24) as well as Ang2-stimulated chemotaxis of endothelial cells (38). Interestingly, however, inhibition of PI 3′ kinase activity can only partially inhibit the chemotactic effect of Ang1 on endothelial cells (24), thereby implying that additional Tie2 binding partners may also contribute to Ang1-mediated endothelial cell migration.

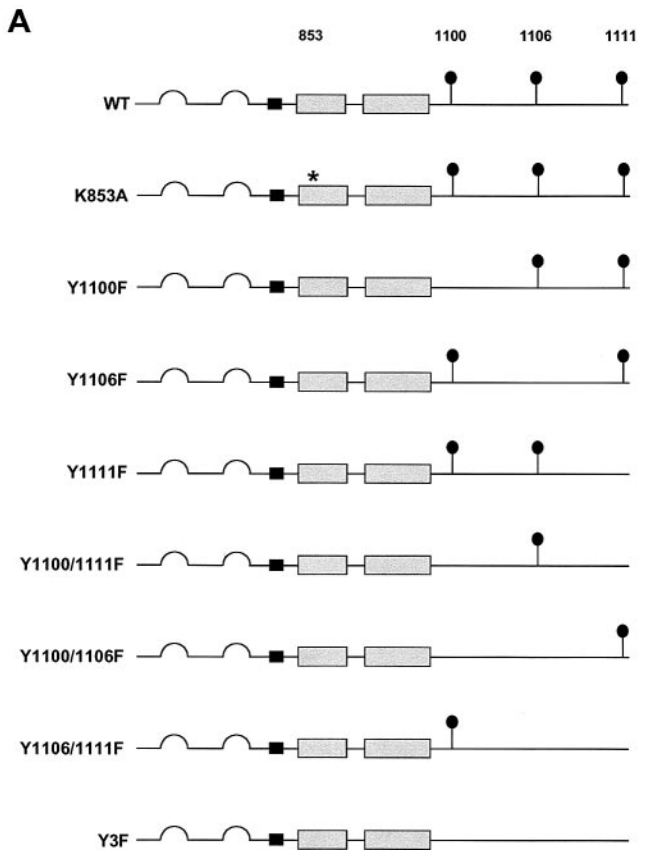
Phosphorylation of Tie2 further results in its association with a docking protein related to downstream of kinase (Dok), known as Dok-R (also referred to as p56^{Dok2} and FRIP) (22). Five Dok family proteins that are characterized by an amino-terminal pleckstrin homology (PH) domain, a central PTB domain, and a carboxy-terminal region rich in tyrosine and proline residues have been isolated (5, 8, 12, 17, 22, 32, 40, 64). Recruitment of Dok-R to the activated Tie2 receptor via its PTB domain results in the phosphorylation of Dok-R on multiple tyrosine residues. This phosphorylation establishes binding sites for the Ras GTPase-activating protein and the adaptor protein Nck, both of which have been implicated in cell motility and actin rearrangement (16, 30, 37). An equivalent Nck binding site on Dok (also known as p62^{Dok1}) mediates cell migration in response to insulin (41), and overexpression of a mutant form of Dok lacking the carboxy-terminal region (which includes the Nck binding site) inhibits the migration potential of metastatic melanoma cells (21). We have recently demonstrated that the expression of Dok-R can potentiate Ang1-induced cell migration (36). This effect is dependent on the association of Dok-R with an Nck/p21 activated kinase (Pak) complex, and the localization of Pak with Tie2 at the membrane surface is required for Pak kinase activation (36). Enzyme activation is essential for Pak function (20), which underscores the importance of recruiting this molecular complex to the phosphorylated Tie2 receptor to mediate Ang1-stimulated cell motility.

Despite intense investigation into the signal transduction pathways initiated by Tie2, it is presently not well understood which tyrosine residues are phosphorylated on the activated receptor to bridge interactions with downstream signaling molecules. A recent mass spectrometry-based approach to identify phosphorylation sites on the autophosphorylated Tie2 intracellular domain revealed tyrosine residue 1106 but not 1100 as an autophosphorylation site (39). Using mutant forms of Tie2 in conjunction with a phosphorylation-state-specific antibody, we have identified tyrosine residue 1106 on Tie2 as an Ang1-dependent autophosphorylation site that is required to mediate the binding and phosphorylation of Dok-R. Binding of Dok-R to Tie2 is mediated through a unique PTB domain binding motif flanking tyrosine residue 1106, and the PH domain of Dok-R also contributes to Tie2 binding in a PI 3′-kinase-dependent manner. Mutation in tyrosine residue 1106 cannot restore the migratory potential of endothelial cells derived from Tie2-null mice, thereby demonstrating the importance of this tyrosine residue in linking downstream cell migration signal transduction pathways with Ang1 stimulation in endothelial cells.

MATERIALS AND METHODS

Expression vectors and mutagenesis. The cDNA encoding full-length Tie2 (22) was used as a template to generate tyrosine-to-phenylalanine point mutations with the QuikChange site-directed mutagenesis kit (Stratagene). Following the mutagenesis reactions, each mutant DNA was again subjected to PCR with primers suitable for the isolation of a 535-nucleotide *Xma*I-*Xho*I fragment corresponding to the last 178 amino acids of mouse Tie2. The mutant fragments were then used to replace the corresponding *Xma*I-*Xho*I region of wild-type (WT) Tie2 in pBluescript (Stratagene). Full-length mutant cDNAs were then subcloned from pBluescript into pcDNA3.1 (Invitrogen) as 3,388-nucleotide *Bam*HI-*Xho*I fragments, and all mutations were verified by sequencing. The cDNA representing kinase-inactive Tie2 (K853A) in pcDNA3.1 has been described previously (22). The cDNAs encoding full-length Dok-R or Dok-R^{PTB*} (23) were cloned into pFLAG-CMV2 (Sigma) and used as templates to produce Dok-R^{PH*} and Dok-R$^{PH*/PTB*}$, respectively. Arginine residues 27 and 28 in the PH domain of Dok-R were altered to alanine by using the QuikChange site-directed mutagenesis kit (Stratagene), and all mutations were confirmed by sequencing.

Cell culture, transfection, and stimulation. Human embryonic kidney line 293T (HEK293T), COS1, and EA.hy926 endothelial cells (a gift of Cora Edgell, Chapel Hill, N.C.) were maintained on 10-cm-diameter plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin, and 200 mM L-glutamine (all Gibco BRL) in a 5% CO_2 incubator at 37°C. EA.hy926 cells were further supplemented with hypoxyanthine-aminopterin-thymidine (Sigma). Indicated cDNAs were introduced into HEK293T, COS1, or EA.hy926 cells with Lipofectin transfection reagent (Gibco BRL) according to the manufacturer's instructions. For Ang1 stimulations on EA.hy926 cells, cells were serum starved for 18 h, pretreated with either 0.1 or 1 mM sodium orthovanadate, pH 8.0, for 10 min at 37°C, and stimulated with 5 ml of conditioned medium in the presence of sodium orthovanadate for 10 min. Production of Mock and Myc epitope-tagged Ang1 (Ang1-MH) conditioned media has been described elsewhere (24). For LY294002 treatment of HEK293T cells, stock LY294002 (Sigma) was resuspended in ethanol and cells were incubated in 5 ml of DMEM containing either 10 μl of LY294002 (40 μM final concentration) or 10 μl of ethanol alone for 1 h at 37°C. Immunoprecipitations and Western blotting were performed as described previously (22).



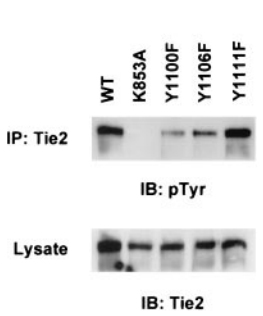


FIG. 1. Tyrosine residues 1100 and 1106 are important phosphorylation sites on Tie2. (A) Schematic representation of mouse Tie2 receptor mutants. The structure of WT Tie2 is shown depicting the two immunoglobulin homology loops, the juxtamembrane region (black boxes), the split tyrosine kinase domain (grey boxes), and three tyrosine residues at positions 1100, 1106, and 1111 in the carboxy-terminal tail (black circles). Tyrosine residues were altered to phenylalanine alone (Y1100F, Y1106F, and Y1111F) or in combination (Y1100/1111F, Y1100/1106F, and Y1106/1111F) as indicated by the deleted circles. All three tyrosine residues have been altered to phenylalanine in Y3F. The asterisk indicates the position of lysine residue 853 that has been altered to alanine (K853A) to generate a kinase-inactive Tie2. (B) Lysates from 293T cells expressing WT Tie2 or Tie2 mutants were immunoprecipitated (IP) with anti-Tie2 antibodies and immunoblotted (IB) with anti-phosphotyrosine (pTyr) antibodies. Tyrosine phosphorylation of Y1100F and Y1106F was reduced compared to that in WT Tie2, and K853A remained unphosphorylated. Nonimmunoprecipitated lysates demonstrate the equal expression of WT Tie2 and Tie2 mutants in the initial lysates upon immunoblotting with anti-Tie2 antibodies.

Phosphopeptide blocking. Phosphopeptides used as competitors of polyclonal anti-phospho-Tie2 (Ab-1) antibody (Oncogene Research Products) were as follows and have been described previously (24): Y814, NPDPTIpYPVLDWN; Y1100, MLEERKTpYVNTTLYE; and Y1106, VNTTLpYEKFTY. Peptide (0.2 μg/μl) was added to a 1:5,000 working dilution of polyclonal antiserum in phosphate-buffered saline and incubated for 2 h at 4°C with rocking prior to Western blotting.

Generation of endothelioma cells. Primary endothelial cells derived from early gestation embryos homozygous for the $Tie2^{\Delta sp}$-null allele (13) or WT littermates were immortalized by infection with retrovirus coding for the polyoma virus middle T antigen (60). Endotheliomas were subsequently maintained in DMEM supplemented with 11% FBS, 3 mM L-glutamine, 100 U of penicillin-streptomycin/ml, 5 μM β-mercaptoethanol, 1 mM sodium pyruvate, 1× nonessential amino acids, and 5 ng of human VEGF/ml. For Ang1 stimulations, cells were serum starved for 12 h and stimulated in the presence of 1 mM sodium orthovanadate, pH 8.0, as described above for EA.hy926 cells. $Tie2^{\Delta sp/\Delta sp}$ endothelial cells were transfected with Lipofectamine 2000 (Gibco BRL) according to the manufacturer's instructions. Transfected cells were identified by fluorescence-activated cell sorter (FACS) analysis with monoclonal anti-Tie2 antibody (Pharmingen).

Antibodies used for immunoprecipitation and Western blotting. The commercially available antibodies used were as follows: polyclonal anti-Tie2 C-20 antibody (Santa Cruz), monoclonal anti-Tie2 antibody (Pharmingen), monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc.), and monoclonal anti-Flag M2 antibody (Sigma). The polyclonal anti-phospho-Tie2 (Ab-1) antibody (Oncogene Research Products) was raised against a phosphopeptide corresponding to amino acids 1095 through 1108 on mouse Tie2 [1095- EERRT pYVNTTLpYEK-1108-(C), in which the lysine at amino acid 1098 was altered to arginine for immunization]. Monoclonal antibodies specific to the extracellular domain of Tie2 (MTE32) and polyclonal anti-Dok-R antibodies have been described previously (22). Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase were purchased from Bio-Rad.

Cell migration assay. $Tie2^{wt/wt}$ or $Tie2^{\Delta sp/\Delta sp}$ endothelioma 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-size modified Boyden chamber (Falcon). Serum-starved cells were allowed to migrate for 4 h in 250 μl of conditioned medium or DMEM alone, and membranes were fixed and counted as described previously (24). Student's *t* test was used to test the statistical significance with a 95% confidence interval. All experiments were performed with two replicates each, and 10 random counts were taken per replicate.

RESULTS

Identification of phosphorylated tyrosine residues on Tie2. To address the importance of the various tyrosine residues of Tie2 in signal transduction, we generated a series of Tie2 mutants in which tyrosine residues have been altered to phenylalanine to interfere with their ability to become tyrosine phosphorylated. It was previously demonstrated that tyrosine residue 1100 in the carboxy-terminal tail region of Tie2 mediates signaling pathways downstream of PI 3′ kinase and also serves as a binding site for the adaptor proteins Grb2 and Grb7 (24). Since the signaling potential of the other tyrosine residues outside the catalytic domain of Tie2 has yet to be described, mutations were introduced in tyrosine residues 1106 and 1111 in the carboxy-terminal tail region of Tie2 (Fig. 1A). Mutations in tyrosine residues 1100, 1106, or 1111 are herein referred to as Y1100F, Y1106F, and Y1111F, respectively (Fig. 1A). Compound mutations harboring alterations in two of the three carboxy-terminal tyrosine residues (Y1100/1111F, Y1100/1106F, and Y1106/1111F) or all three tyrosine residues (Y3F) were also generated (Fig. 1A). Transient expression of full-length WT Tie2 in HEK293T cells resulted in its tyrosine phosphorylation independent of Ang1 stimulation, while a kinase-inactive mutant of Tie2 (K853A) remained unphosphorylated (Fig. 1B) (22). Expression of the various point mutants

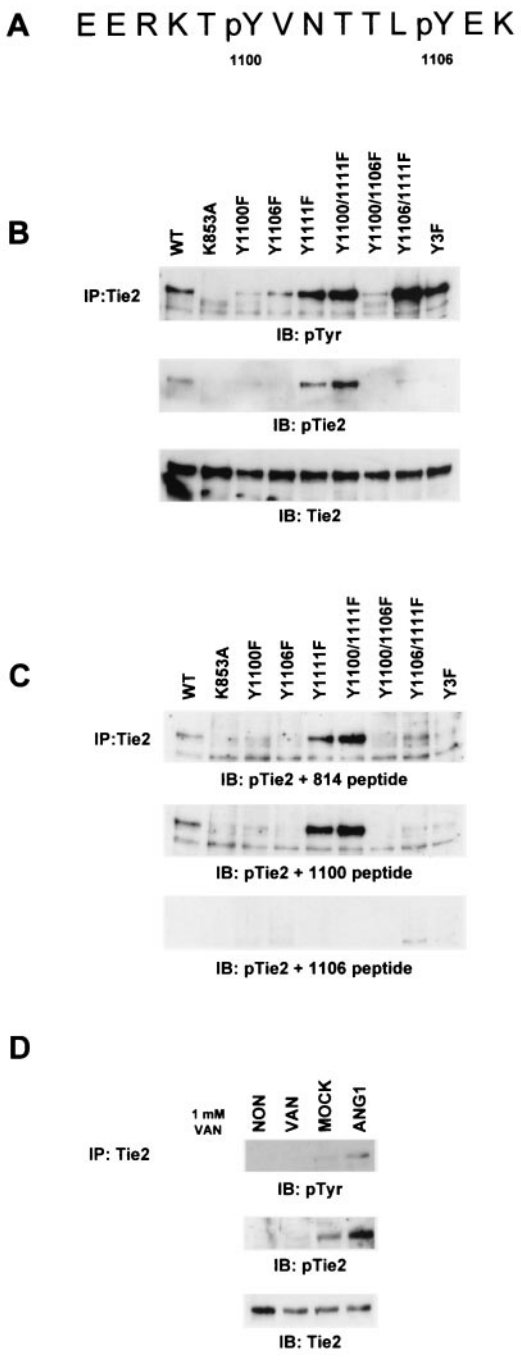


FIG. 2. Tyrosine residue 1106 on Tie2 is phosphorylated in endothelial cells upon Ang1 stimulation. (A) Amino acid sequence of Tie2 encompassing tyrosine residues 1100 and 1106 that corresponds to the phosphopeptide used to generate phospho-specific Tie2 antibodies. (B) Lysates prepared from COS1 cells expressing WT Tie2 and Tie2 mutants were immunoprecipitated (IP) with anti-Tie2 antibodies and immunoblotted (IB) with either anti-phosphotyrosine (pTyr) or anti-phospho-Tie2 (pTie2) antibodies. Mutants lacking tyrosine residue 1106 were poorly recognized by anti-phospho-Tie2 antibodies despite their overall phosphorylation, as indicated by anti-phosphotyrosine antibodies. The anti-phospho-Tie2 immunoblot was reprobed with anti-Tie2 antibodies to confirm the expression of all mutants. (C) Synthetic phosphopeptides encompassing tyrosine residues 814, 1100, and 1106 on Tie2 were incubated with anti-phospho-Tie2 antibodies prior to immunoblotting as described above. Competition of antibody recognition occurred with the residue 1106 peptide but not the 814 or 1100 peptides. (D) Lysates from EA.hy926 endothelial cells left untreated (NON) or treated with Mock or Ang1 conditioned medium or sodium orthovanadate alone (VAN) were immunoprecipitated with anti-Tie2 antibodies and immunoblotted with either anti-phosphotyrosine or anti-phospho-Tie2 antibodies. Phosphorylation of Tie2 in response to Ang1 was detected by the phospho-specific Tie2 antibody. The anti-phospho-Tie2 immunoblot was reprobed with anti-Tie2 antibodies to confirm the presence of Tie2 in all immunoprecipitates.

of Tie2 revealed that tyrosine phosphorylation of both Y1100F and Y1106F is greatly reduced while Y1111F remains highly tyrosine phosphorylated when expressed in 293T cells (Fig. 1B). Importantly, the Y1100F and Y1106F mutants retained their kinase activity as downstream signaling partners of Tie2 became tyrosine phosphorylated when coexpressed with these mutants (see Fig. 3 and unpublished observations). Collectively, these findings imply that tyrosine residues 1100 and 1106 on Tie2 may represent in vivo autophosphorylation sites.

Tyrosine 1106 is phosphorylated in Ang-1-stimulated endothelial cells. To further examine the possibility that tyrosine residues 1100 and 1106 on Tie2 may represent in vivo phosphorylation sites, we utilized a commercially available antibody raised against a phosphopeptide encompassing tyrosine residues 1100 and 1106 (Fig. 2A). Specificity of this phospho-specific Tie2 antibody was confirmed through use of the panel of Tie2 mutants described in Fig. 1A. Tie2 mutants were expressed in COS1 cells as described above, and their phosphorylation patterns were examined. Immunoblotting of Tie2 immunoprecipitates with antibodies recognizing phosphotyrosine demonstrated that Y1100F and Y1106F were only weakly phosphorylated compared to WT Tie2 and that a compound mutant lacking both tyrosine residues 1100 and 1106 (Y1100/1106F) was also weakly phosphorylated (Fig. 2B). All other compound mutants were phosphorylated at a level equivalent to or greater than that seen with WT Tie2 (Fig. 2B). Immunoblotting with phospho-specific Tie2 antibodies revealed that the antibody could not detect the phosphorylation of any mutants lacking tyrosine residue 1106, although it could still recognize the relative phosphorylation of the Y1100F and Y1100/1111F mutants lacking tyrosine residue 1100 (Fig. 2B). This reduced signal was particularly noticeable in the Y1106F, Y1106/1111F, and Y3F mutants compared with the level of phosphorylation seen with anti-phosphotyrosine antibodies (Fig. 2B). Since these mutants all lack tyrosine residue 1106 on Tie2, these findings suggest that the phospho-specific Tie2 antibody might be highly specific for phosphorylation on this tyrosine residue.

To determine whether phosphorylated tyrosine residue 1106 is the major epitope for this antibody, synthetic phosphopeptides representing putative tyrosine autophosphorylation sites on Tie2 were used as competitors of the phospho-specific Tie2 antibody. Phosphopeptides encompassing tyrosine residues 1100 and 1106 in the carboxy-terminal tail region and residue 814 in the juxtamembrane region (24) were incubated with the antibody prior to Western blotting of mutant Tie2 immunoprecipitates. Figure 2C demonstrates that neither the residue 814 nor residue 1100 phosphopeptides had any effect on the antibody recognition of phosphorylated Tie2, while the residue 1106 phosphopeptide completely blocked the antibody. Taken

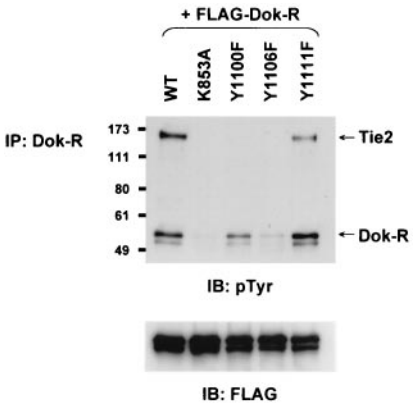


FIG. 3. Tyrosine residue 1106 on Tie2 is required for Dok-R phosphorylation. WT Tie2 or Tie2 mutants were transiently coexpressed with FLAG-tagged Dok-R in 293T cells, and lysates were immunoprecipitated (IP) with anti-Dok-R antibodies. Immunoblotting (IB) with anti-phosphotyrosine (pTyr) antibodies demonstrated that tyrosine phosphorylation of Dok-R was greatly reduced when coexpressed with Y1106F and compared with WT Tie2. Immunoblotting with anti-FLAG antibodies indicated the presence of Dok-R in all immunoprecipitates.

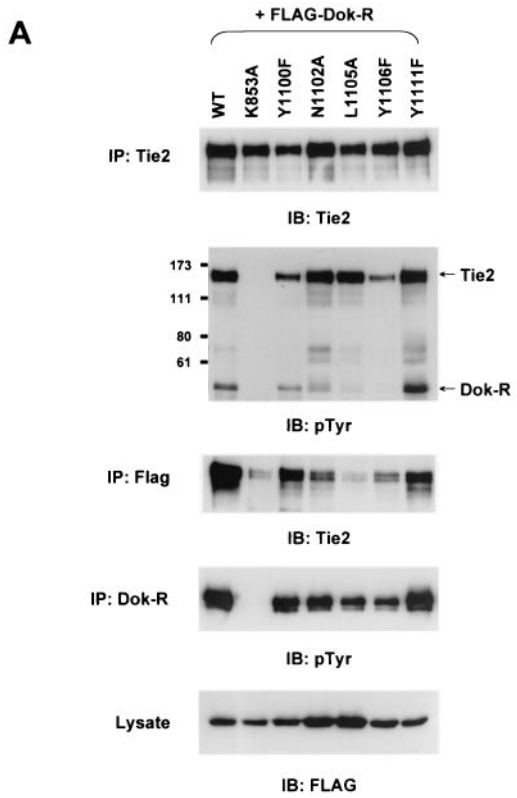
together, these results indicate that the phospho-specific Tie2 antibody recognizes tyrosine phosphorylated residue 1106 on Tie2.

The availability of an antibody highly specific to phosphorylated tyrosine residue 1106 on Tie2 prompted us to examine whether this site might be phosphorylated in endothelial cells upon Ang1 stimulation. EA.hy926 endothelial cells were stimulated with conditioned medium from 293T cells stably transfected with Ang1-MH or conditioned medium from nontransfected control 293T cells (Mock), and stimulations were performed in the presence of sodium orthovanadate to reduce phosphatase activity (24). Lysates from treated cells were immunoprecipitated with anti-Tie2 antibodies and immunoblotted with either anti-phosphotyrosine or anti-phospho-specific Tie2 antibodies. Conditioned medium containing Ang1-MH stimulated the tyrosine phosphorylation of Tie2, and the phosphorylation pattern seen with the phospho-specific Tie2 antibody was almost identical to that seen with anti-phosphotyrosine antibodies (Fig. 2D). The low levels of phosphorylation seen with Mock conditioned medium reflect the production of endogenous Ang1 by 293T cells (36). These findings strongly suggest that tyrosine residue 1106 on Tie2 represents an in vivo autophosphorylation site of the receptor.

Tyrosine residue 1106 on Tie2 is required for Dok-R phosphorylation. Since the signaling output from tyrosine residue 1106 on Tie2 has yet to be defined, we examined whether this site might be required for the association of the docking protein Dok-R with Tie2. Recruitment of Dok-R to the phosphorylated receptor through its PTB domain allows Dok-R to serve as a substrate of Tie2 and thereby become tyrosine phosphorylated (22). To determine whether tyrosine residue 1106 may comprise a binding site for Dok-R on Tie2, Dok-R was coexpressed with Tie2 mutants in 293T cells and its phosphorylation status was examined. Dok-R immunoprecipitates revealed a reduction in Dok-R phosphorylation when Dok-R was coexpressed with Y1106F and coimmunoprecipitation of Y1106F with Dok-R could not be observed (Fig. 3). In contrast, both WT Tie2 and Y1111F associate with phosphorylated Dok-R. Despite the reduced phosphorylation of Y1100F and Y1106F, Dok-R was tyrosine phosphorylated to some extent when coexpressed with Y1100F (Fig. 3) and coimmunoprecipitation of the weakly phosphorylated receptor could be seen in longer exposures of the immunoblot (data not shown). Mutation of tyrosine residue 1106 therefore affects Dok-R binding and phosphorylation and suggests that this region may comprise a binding target for the PTB domain of Dok-R.

Definition of a consensus motif for Dok-R PTB domain binding. PTB domains often recognize and interact with phosphotyrosine residues contained within asparagine-proline-*X*-phosphotyrosine (NP*X*pY; in the single-letter amino acid code, where X is any amino acid) motifs in target proteins (63). Interestingly, however, this sequence was not present in Tie2. In an attempt to further define the target motif required for Dok-R binding to phosphorylated Tie2, additional mutations were introduced in Tie2 in amino acids immediately upstream of tyrosine residue 1106. Asparagine residue 1102 (located at the −4 position relative to the phosphotyrosine) and leucine residue 1105 (located at the −1 position relative to the phosphotyrosine) were altered to alanine to yield N1102A and L1105A mutants, respectively. Dok-R was coexpressed with these Tie2 mutants in 293T cells, and the phosphorylation status of both Tie2 and Dok-R was examined. Immunoblotting of Tie2 immunoprecipitates with anti-phosphotyrosine antibodies demonstrated that the kinase activity of the N1102A and L1105A mutants is not impaired, as both mutant receptors appear to be autophosphorylated to a level comparable to that observed in WT Tie2 (Fig. 4A). Analysis of Dok-R phosphorylation in the same immunoprecipitates indicated that, while some phosphorylated Dok-R remains associated with the N1102A mutant, reduced amounts of phosphorylated Dok-R are associated with the L1105A mutant as well as the Y1106F mutant (Fig. 4A). Similarly, Dok-R immunoprecipitates also reveal a reduction in Dok-R phosphorylation when Dok-R is coexpressed with L1105A and Y1106F and a moderate reduction in Dok-R tyrosine phosphorylation was also seen upon coexpression with Y1100F and N1102A compared to that in WT Tie2 (Fig. 4A). Accordingly, reduced association of these mutants with Dok-R was also observed upon immunoblotting for Tie2 (Fig. 4A). These findings imply that the presence of a leucine residue at the −1 position in addition to the phosphotyrosine is critical for Dok-R binding to Tie2 and that additional residues amino terminal to the phosphotyrosine further contribute to binding.

While these studies were under way, a consensus binding motif for the PTB domain of Dok was defined using a combinatorial peptide library approach as Y/M*XX*N*X*LpY (Fig. 4B) (52). The PTB domain of Dok was shown to strongly select the hydrophobic residues tyrosine and methionine at the −6 position relative to the phosphotyrosine, and leucine was exclusively selected at the −1 position. Asparagine was fixed in all phosphopeptides at the −3 position. Comparison of the consensus target motif of the Dok PTB domain with that of Dok-R on Tie2 revealed a number of similarities (Fig. 4B). Notably, the presence of leucine at the −1 position in addition to the phosphotyrosine appears to comprise a strict requirement for Dok-R binding to Tie2 and the presence of asparagine within



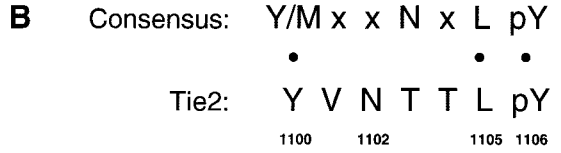


FIG. 4. A unique phosphotyrosine-containing motif on Tie2 mediates Dok-R binding. (A) Amino acid residues upstream of tyrosine residue 1106 were altered to alanine and coexpressed in 293T cells with Dok-R. Lysates were immunoprecipitated (IP) with anti-Tie2, anti-FLAG, or anti-Dok-R antibodies and immunoblotted (IB) with anti-phosphotyrosine (pTyr) antibodies to determine the phosphorylation states of both Tie2 and Dok-R. Although N1102A and L1105A remained tyrosine phosphorylated, reduced amounts of phosphorylated Dok-R were associated with the mutant receptors. Phosphorylation of Dok-R upon coexpression with L1105A was similar to that observed upon coexpression with Y1106F. Nonimmunoprecipitated lysates demonstrated the equal expression of Dok-R in the initial lysates upon immunoblotting with anti-FLAG antibodies. (B) Comparison of the amino acid sequence surrounding tyrosine residue 1106 on Tie2 with the consensus motif defined for Dok PTB domain binding. Note the conservation of the leucine and tyrosine residues at the −1 and −6 positions, respectively.

the target motif (position −4 versus −3) is also important. Tyrosine residue 1100 at the −6 position further contributed to Dok-R binding, although mutation of this residue to phenylalanine was likely a conserved substitution tolerated by the PTB domain (52). Taken together, these findings support previous observations that PTB domains are not strictly limited to binding NP*X*pY phosphopeptides (63).

The PH and PTB domains of Dok-R mediate membrane localization. The trace amounts of Dok-R phosphorylation seen upon coexpression with L1105A and Y1106F but not with the K853A kinase-inactive mutant of Tie2 suggested that a PTB domain-independent mechanism might contribute to Dok-R recruitment near the activated receptor. As Dok family proteins also contain a PH domain, we examined the possibility that the PH domain may also be required for Dok-R localization. Point mutations were introduced in the PH and PTB domains of Dok-R to interfere with the ability of key arginine residues to coordinate binding to phosphate groups in phosphoinositide- and phosphotyrosine-containing target sequences, respectively (14, 67). Dok-R and mutant forms were introduced into 293T cells with either WT Tie2 or the kinase-inactive (K853A) or Y1106F Tie2 mutants, and the ability of the mutants to associate with Tie2 and undergo tyrosine phosphorylation was examined. Disruption of the PTB domain appears to have a more dramatic effect on Dok-R phosphorylation than disruption of the PH domain, although mutation of both domains completely abolishes Dok-R phosphorylation (Fig. 5A). Notably, although phosphorylation of the PH domain mutant was reduced compared with intact Dok-R when coexpressed with WT Tie2, this mutant could still associate with the receptor to some extent while the PTB domain mutant could not. Mutation of the PTB domain binding site on Tie2 (Y1106F) was required to interfere with the ability of the PH domain mutant to undergo tyrosine phosphorylation and receptor binding (Fig. 5A). These results support previous findings that tyrosine residue 1106 on Tie2 is the binding site for the PTB domain of Dok-R and that both the PH and PTB domains are important for the targeting of Dok-R near the receptor.

Accumulation of PH domain-containing proteins at the plasma membrane is dependent on the generation of phosphoinositide lipid products by activated PI 3′ kinase (4). Since the PH domain of Dok-R is important for Dok-R phosphorylation, we next wanted to determine whether PI 3′ kinase activity is required for this phosphorylation. Previous studies have established that the Tie2-mediated recruitment and activation of PI 3′ kinase is dependent on the presence of tyrosine residue 1100 (24, 29). However, as mutation of this tyrosine residue only partially interferes with Dok-R phosphorylation (Fig. 3), we examined whether mutation of this site in conjunction with tyrosine residue 1106 would preclude phosphorylation of Dok-R in a manner similar to that observed when both the PH and PTB domains were mutated. The compound mutant Y1100/1106F was coexpressed with Dok-R in 293T cells, and phosphorylation of Dok-R was compared to that observed upon coexpression with either WT Tie2 or Y1106F. Tie2 immunoprecipitates revealed that, although some phosphorylated Dok-R remains associated with Y1106F, phosphorylated Dok-R cannot be detected in complex with the double-mutant receptor (Fig. 5B). To further support the possibility that PI 3′ kinase activity may aid in the recruitment and phosphorylation of Dok-R, parallel transfectants were treated with the PI 3′ kinase inhibitor LY294002. In Tie2 immunoprecipitates, the amount of phosphorylated Dok-R precipitated with the WT Tie2 receptor was moderately reduced and the level of Dok-R phosphorylation seen upon coexpression with Y1106F in the presence of LY294002 (Fig. 5B) was comparable to that seen with the mutant PH domain (Fig. 5A). Despite the reduction in Dok-R phosphorylation seen with WT Tie2 here, addition of

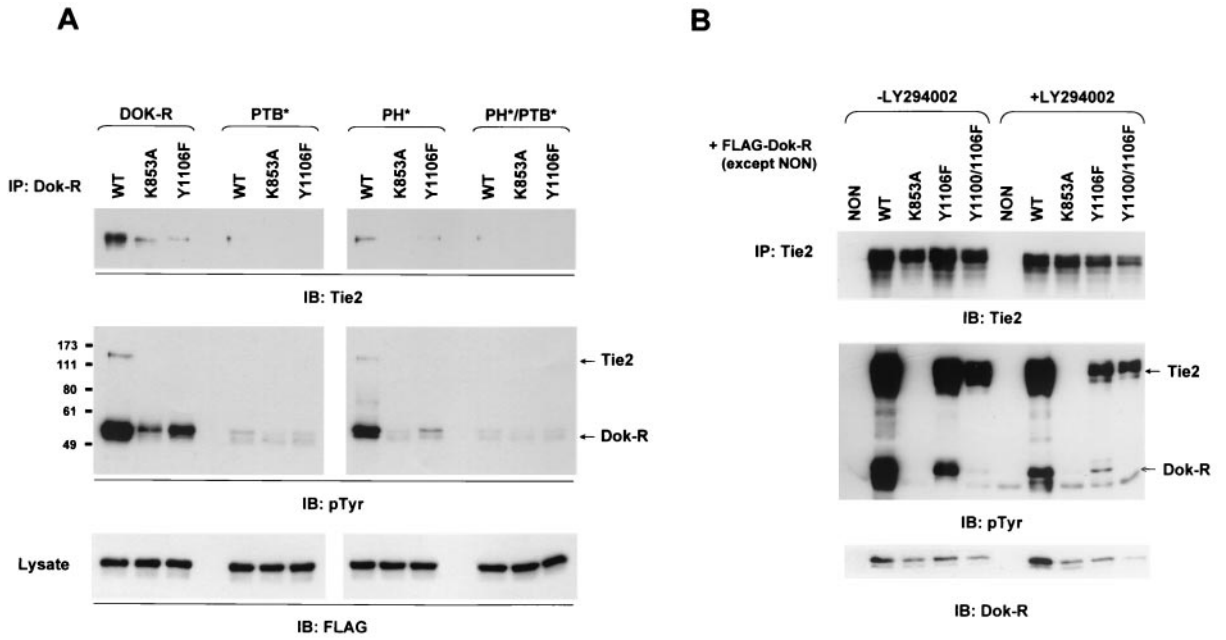


FIG. 5. The PH and PTB domains of Dok-R both contribute to membrane localization and Tie2 binding. (A) WT Tie2 or Tie2 mutants were coexpressed in 293T cells with Dok-R, Dok-R^{PTB*}, Dok-R^{PH*}, or Dok-R$^{PH*/PTB*}$, and lysates were immunoprecipitated (IP) with anti-Dok-R antibodies. Immunoblotting (IB) with anti-phosphotyrosine (pTyr) antibodies demonstrated that disruption of the PTB domain (PTB*) appears to reduce Dok-R phosphorylation more than disruption of the PH domain (PH*) and that disruption of both domains (PH*/PTB*) completely abolishes Dok-R phosphorylation when coexpressed with WT Tie2. Note the comparable levels of Dok-R phosphorylation when Dok-R was coexpressed with Y1106F and when Dok-R^{PH*} was coexpressed with WT Tie2. Coexpression of Dok-R^{PH*} with Y1106F further reduced Dok-R phosphorylation. Parallel immunoblotting with anti-Tie2 antibodies demonstrated the presence of WT Tie2 with Dok-R and Dok-R^{PH*} but not with Dok-R^{PTB*} in Dok-R immunoprecipitates. Nonimmunoprecipitated lysates demonstrated the equal expression of Dok-R in the initial lysates upon immunoblotting with anti-FLAG antibodies. (B) WT Tie2 and Tie2 mutants were coexpressed with Dok-R in 293T cells, and cells were treated with the PI 3′ kinase inhibitor LY294002 (40 μM) or vehicle alone (ethanol). Lysates were immunoprecipitated with anti-Tie2 antibodies and immunoblotted with anti-phosphotyrosine antibodies. In the presence of LY294002, Dok-R phosphorylation was markedly reduced when coexpressed with WT Tie2 and Y1106F (compare lane 2 with 7 and lane 4 with 9). Note the absence of Dok-R phosphorylation upon coexpression with Y1100/1106F (lanes 5 and 10). Parallel immunoprecipitates were immunoblotted with anti-Tie2 antibodies to confirm the expression of all mutants and with anti-Dok-R antibodies to examine the presence of coprecipitated Dok-R.

LY294002 did not abolish the interaction of WT Tie2 with Dok-R, since comparable amounts of Dok-R were coprecipitated with Tie2 in both the presence and absence of inhibitor (Fig. 5B). However, the reduced amount of WT Tie2 coprecipitated with the PH domain mutant compared to that of Dok-R (Fig. 5A) supports the contribution of the PH domain and PI 3′ kinase in the optimal localization of Dok-R near the activated receptor.

Y1106F interferes with Ang1-stimulated signal transduction in endothelial cells. We have recently demonstrated that the stimulation of endothelial cells with Ang1 causes tyrosine phosphorylation of Dok-R (36); thus, we next wanted to determine whether the mutation of tyrosine residue 1106 on Tie2 could interfere with the ability of Dok-R to become tyrosine phosphorylated in endothelial cells treated with Ang1. EA.hy926 endothelial cells transiently expressing either WT Tie2, K853A, or Y1106F with Dok-R were challenged with Mock or Ang1-MH conditioned medium in the presence of sodium orthovanadate, and immunoprecipitated lysates were examined for the presence of phosphotyrosine. Ang1-MH stimulation of endothelial cells overexpressing either WT Tie2 or Y1106F resulted in a robust increase in Tie2 phosphorylation compared to that in Mock-stimulated cells (Fig. 6). By contrast, cells expressing the kinase-inactive mutant Tie2 displayed very low levels of Tie2 phosphorylation in response to Ang1-MH stimulation, likely due to the dominant inhibitory effect of this mutant on receptor autophosphorylation. Examination of Dok-R immunoprecipitates from the same lysates revealed that, while Dok-R became tyrosine phosphorylated in response to Ang1-MH stimulation in endothelial cells expressing the WT receptor, this phosphorylation was greatly reduced in Ang1-MH-stimulated cells expressing Y1106F as well as those expressing K853A (Fig. 6). These results support the requirement for tyrosine residue 1106 on Tie2 for Dok-R phosphorylation and suggest that this site might play an important role in Dok-R-dependent cellular processes in endothelial cells.

Tyrosine phosphorylation of Dok-R in endothelial cells is required to link signaling complexes containing the adaptor protein Nck and p21-activated kinase with the activated Tie2 receptor to induce cell motility (36). Since tyrosine residue 1106 appears to play a major role in recruiting Dok-R to the phosphorylated Tie2 receptor, we examined whether the loss of this site might affect cell migration stimulated by Ang1 in endothelial cells. In order to introduce mutant forms of Tie2 into an endothelial cell line lacking functional Tie2, we utilized embryonic endothelioma cells established from the polyoma virus middle T antigen-transformed endothelial cells isolated

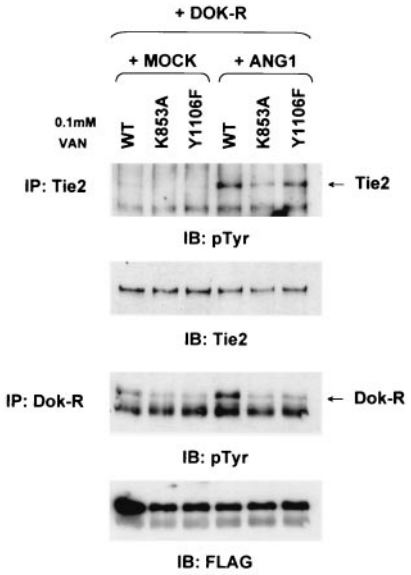


FIG. 6. Tyrosine residue 1106 on Tie2 is required for Dok-R phosphorylation following Ang1 stimulation of endothelial cells. EA.hy926 endothelial cells transiently coexpressing WT Tie2, kinase-inactive K853A, or Y1106F with FLAG-tagged Dok-R were stimulated with Mock or Ang1-MH conditioned medium in the presence of sodium orthovanadate. Lysates were divided and immunoprecipitated (IP) with either anti-Tie2 or anti-Dok-R antibodies. Immunoblotting (IB) with anti-phosphotyrosine (pTyr) antibodies indicates that while both WT Tie2 and Y1106F allow tyrosine phosphorylation of Tie2 following Ang1 stimulation when compared with K853A, Dok-R is only tyrosine phosphorylated when coexpressed with WT Tie2 following Ang1 stimulation. Membranes were reprobed with anti-Tie2 and anti-FLAG antibodies to demonstrate the presence of Tie2 mutants and Dok-R, respectively.

from embryos homozygous for the $Tie2^{\Delta sp}$-null allele (13). As expected, $Tie2^{\Delta sp/\Delta sp}$-null endothelioma cells did not express the Tie2 receptor (Fig. 7A and FACS data not shown) although $Tie2^{wt/wt}$ WT control cells expressed moderate levels of the receptor and underwent Ang1-stimulated tyrosine phosphorylation on Tie2 (Fig. 7A). To assess the chemotactic response of the endothelioma cells to conditioned medium containing Ang1-MH, $Tie2^{wt/wt}$ and $Tie2^{\Delta sp/\Delta sp}$ cells were subjected to a motility assay. Stimulation of $Tie2^{wt/wt}$ cells with Ang1 resulted in an approximately fivefold increase in cell migration compared to that in Mock-stimulated cells, while stimulation of $Tie2^{\Delta sp/\Delta sp}$ cells with Ang1 did not enhance motility (Fig. 7B). Since both $Tie2^{wt/wt}$ and $Tie2^{\Delta sp/\Delta sp}$ cells were found to express Dok-R as determined by reverse transcription-PCR and Western blot analysis (data not shown), we next wanted to determine whether expression of WT Tie2 or Tie2 mutants deficient in Dok-R binding could restore the migration defect of $Tie2^{\Delta sp/\Delta sp}$ cells. Ang1-MH stimulation of $Tie2^{\Delta sp/\Delta sp}$ endothelial cells transiently expressing WT Tie2 resulted in a consistent increase in cell motility compared to that in cells expressing the kinase-inactive Tie2 K853A mutant (Fig. 7C). By contrast, Ang1-MH-induced chemotaxis in $Tie2^{\Delta sp/\Delta sp}$ endothelial cells expressing either Y1106F or Y1100/1106F was equivalent to that seen in cells expressing K853A (Fig. 7C). All of the transfected cells responded similarly to Mock conditioned medium (Fig. 7C). Taken together, the inability of the Y1106F mutant to restore Ang1-stimulated cell migration to the level seen with WT Tie2 in $Tie2^{\Delta sp/\Delta sp}$ endothelial cells suggests that tyrosine residue 1106 plays a critical role in connecting components of the cell migration machinery with the activated Tie2 receptor.

DISCUSSION

In this report, we have identified tyrosine residue 1106 on the Tie2 receptor as an Ang1-dependent autophosphorylation site that mediates the recruitment of Dok-R and its associated cell migration machinery. Evidence to support autophosphorylation at this site has come from mass spectrometric analysis of the Tie2 kinase domain, which revealed sequential phosphorylation of a key conserved tyrosine residue in the kinase activation loop followed by phosphorylation of tyrosine residue 1106 (39). Moreover, structural analysis of the Tie2 intracellular domain indicates that tyrosine residue 1106 is found at the base of a loop formed as a result of the carboxy-terminal tail extending from and folding back to pack against the carboxy-terminal lobe of the kinase (51). In this position, the hydroxyl group of tyrosine residue 1106 would be projecting out directly into the solvent and accessible to phosphorylation. Together, these findings suggest that Ang1 stimulates Tie2 activation and autophosphorylation on tyrosine residue 1106. It is interesting to note, however, that the mass spectrometric analysis was performed in the absence of the ligand binding extracellular domain of Tie2, which raises the possibility that all angiopoietins may not induce similar autophosphorylation of Tie2 on tyrosine residue 1106. Differential phosphorylation of the receptor could underlie the requirement for multiple agonistic angiopoietins in the regulation of Tie2 signal transduction pathways.

PTB domains serve as alternates to SH2 domains for binding to tyrosine-phosphorylated proteins through recognition of amino acids amino-terminal to the phosphotyrosine (2, 18, 27). The PTB domains of the Shc and insulin receptor substrate 1 signaling proteins recognize and interact with tyrosine phosphorylated proteins containing the canonical NP*X*Y sequence with differential selectivity for hydrophobic amino acids amino-terminal to this motif (18, 26, 59). Interestingly, however, the PTB domain is not strictly limited to binding this motif, as numerous PTB domains, such as those found in the Numb proteins and FRS2α/SNT (42, 62), can bind their targets in a nonphosphotyrosine or non-NP*X*Y-motif-dependent manner (3, 11, 33) and some can also bind targets that do not contain tyrosine (6, 7). These findings demonstrate that PTB domains have evolved different requirements for the presence of phosphotyrosine in their target motifs, and this suggests that the specificity of PTB domain binding may be more dependent upon tertiary structure than on a linear sequence. Here, we demonstrate target binding diversity in a Dok family PTB domain whereby Dok-R binds the phosphorylated Tie2 receptor through a unique LpY-containing motif. In addition to binding phosphorylated receptor tyrosine kinases such as Tie2, Ret (17), and the epidermal growth factor receptor (23), Dok family PTB domains also mediate interactions with other cytoplasmic signaling molecules, including SHIP1 and Abl (8, 32, 50, 56), and Dok proteins may homodimerize through PTB domain–phosphotyrosine-mediated interactions (52). Interest-

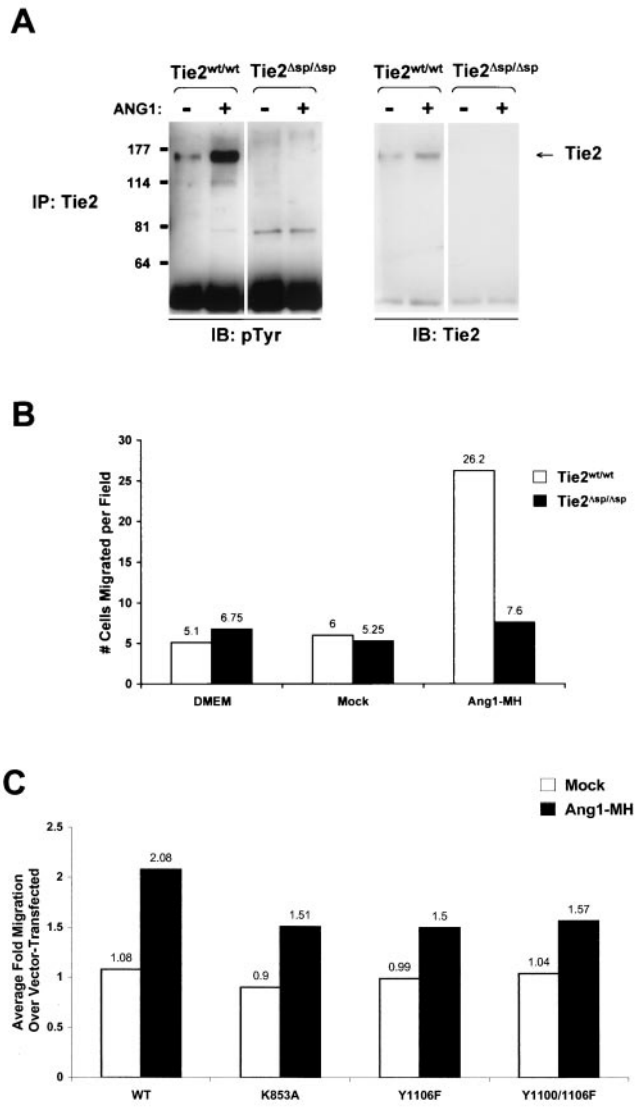


FIG. 7. Ang1-mediated endothelial cell migration requires tyrosine residue 1106. (A) Tie2$^{wt/wt}$ and Tie2$^{\Delta sp/\Delta sp}$ endothelial cells were stimulated with either Mock (−) or Ang1-MH (+) conditioned medium in the presence of sodium orthovanadate, and lysates were immunoprecipitated (IP) with anti-Tie2 antibodies. Immunoblotting (IB) with anti-phosphotyrosine (pTyr) antibodies demonstrated a marked increase in tyrosine phosphorylation in Tek$^{wt/wt}$ cells upon Ang1 stimulation that was not observed in Tie2$^{\Delta sp/\Delta sp}$ cells. Reprobing the immunoblot with anti-Tie2 antibodies confirmed the presence of Tie2 in Tie2$^{wt/wt}$ cells but not in Tie2$^{\Delta sp/\Delta sp}$ cells. (B) Tie2$^{wt/wt}$ and Tie2$^{\Delta sp/\Delta sp}$ endothelial cells were seeded in the upper chamber of a modified Boyden chamber, and conditioned medium or DMEM alone was placed in the bottom chamber. Stimulation of Tie2$^{wt/wt}$ cells with Ang1 resulted in an approximately fivefold increase in cell migration over DMEM- and Mock-stimulated cells, while stimulation of Tie2$^{\Delta sp/\Delta sp}$ cells with Ang1 did not enhance cell migration. Data are presented as the mean number of cells migrated per field. (C) WT Tie2 or Tie2 mutants were transiently expressed in Tie2$^{\Delta sp/\Delta sp}$ cells, and transfected cells were subjected to a migration assay as described above by using Mock or Ang1-MH conditioned medium. Data are presented as the fold increase in migration following Mock or Ang1-MH stimulation compared to that for the same conditions in cells transfected with vector alone. The increase in migration observed in WT-expressing Tie2$^{\Delta sp/\Delta sp}$ cells following Ang1 stimulation (standard error, ±0.41) was statistically significant ($P < 0.05$). By contrast, neither Y1106F (standard error, ±0.28) nor Y1100/1106F (standard error, ±0.30) could restore migration above the level observed for the kinase-inactive Tie2 receptor K853A (standard error, ±0.31) following Ang1 stimulation. In the presence of Mock conditioned medium, WT Tie2 (standard error, ±0.23), K853A (standard error, ±0.18), Y1106F (standard error, ±0.29), and Y1100/1106F (standard error, ±0.29) displayed comparable levels of cell migration. An equivalent percentage of Tie2$^{\Delta sp/\Delta sp}$ cells was transfected with Tie2 or Tie2 mutants as determined by FACS analysis. Shown are the results of the average fold increases from three independent experiments.

ingly, the presence of a leucine residue at the −1 position can be observed in the target binding motifs of the Ret receptor (NKLpY) and Dok proteins (M*XX*N*X*LpY between the PH and PTB domains) and the carboxy-terminal PTB domain binding motif in SHIP1 is MFENPLY. These observations suggest that this leucine residue may be an important determinant in Dok PTB domain target selectivity. Although the structure of a Dok family PTB domain in complex with a peptide ligand remains to be determined, the ability of the Dok-R PTB domain to bind distinct NP*X*pY and non-NP*X*pY sequences implies a degree of structural flexibility similar to that observed in the Numb and FRSα PTB domains.

The presence of both a PH domain and a PTB domain in the Dok family of proteins suggests that these proteins are likely recruited to the cell membrane in the vicinity of receptor binding partners during growth factor signaling. Our results demonstrate that both domains are required for an effective interaction between Dok-R and Tie2 in vivo, although the presence of an intact PTB domain appears to be critical for Tie2 binding and for ensuring high levels of the tyrosine phosphorylation of Dok-R. Similar findings have also been observed with epidermal growth factor receptor binding (23). It has recently been shown that the PH domain of Dok is necessary for the membrane localization and tyrosine phosphorylation of Dok upon insulin stimulation, and although the PTB domain of Dok was not examined, an intact NP*X*Y sequence in the insulin receptor was also required for this phosphorylation (41). Interestingly, however, deletion of the PH domain of Dok abolishes the membrane translocation and tyrosine phosphorylation of Dok following either the platelet-derived growth factor stimulation of Rat1 fibroblasts or c-Kit ligand stimulation of Mo7 hematopoietic cells (34, 66). It remains to be determined whether the PTB domain of Dok can associate with these receptors; however, phosphorylation of Dok is completely dependent on PI 3′ kinase activation. The PH domain may therefore provide the principal membrane-targeting module in the absence of a direct PTB domain binding site on an activated receptor, thereby allowing functionally related docking molecules to utilize alternate strategies for localization to cell surface receptors.

Maximal phosphorylation of Dok-R requires the presence of its PH domain, which targets the plasma membrane as a consequence of PI 3′ kinase activation via tyrosine residue 1100 (24, 29). Interestingly, although mutation of this site in conjunction with tyrosine residue 1106 is necessary to completely reduce Dok-R phosphorylation, loss of the PTB domain binding site alone is sufficient to interfere with Tie2-mediated endothelial cell migration. It was previously reported that the inhibition of PI 3′ kinase activity partially reduces cell migration potential in response to Ang1 stimulation (24). Based on

the findings reported here, it is tempting to speculate that this effect may in fact be due to impaired targeting of the PH domain of Dok-R, thereby compromising the ability of the PTB domain alone to localize Dok-R near the activated receptor. Despite evidence to support the activation of PI 3′ kinase downstream of Tie2, it remains to be determined whether tyrosine residue 1100 is an autophosphorylation site on the receptor. Structural analysis of the unliganded receptor has revealed that, while tyrosine residue 1106 projects directly out into the solvent, tyrosine residue 1100 is not solvent exposed, thereby implying that the carboxy-terminal tail must undergo a conformational change upon activation of the receptor to expose this tyrosine residue for phosphorylation (51). An alternative possibility to tyrosine residue 1100 representing an autophosphorylation site is that this residue may serve as a substrate for a tyrosine kinase associated with the activated receptor. Upon binding of the PTB domain of Dok-R to the optimal autophosphorylation site on Tie2, a Dok-R-associated kinase such as Abl or Src (Z. Master and D. J. Dumont, unpublished observations) could in turn phosphorylate tyrosine residue 1100. Such processive phosphorylation of the receptor would allow the recruitment and activation of PI 3′ kinase to further stabilize binding of Dok-R at the cell membrane through the PH domain.

Recruitment of Dok-R to the activated Tie2 receptor allows concomitant relocalization of the serine/threonine kinase Pak to the plasma membrane through its association with Nck, and formation of this complex promotes Ang1-mediated cell motility (36). In this report, we have identified the tyrosine residue on Tie2 that mediates the binding of Dok-R following Ang1 stimulation. Importantly, mutant Tie2 lacking this site is unable to restore migration in endothelial cells lacking Tie2, thereby implying that interaction of Tie2 with Dok-R is critical to link Tie2 with the migration machinery of the cell. Activation of Pak and its association with Nck is also required for VEGF-mediated endothelial cell motility downstream of VEGF receptor 2 (53). Together, these findings suggest that the signal transduction pathway initiated by Nck and Pak may be an important regulator of endothelial cell movement during blood vessel development. In contrast to the role of Tie2 in promoting endothelial cell motility, Tie1 has been proposed to counteract this effect in vivo (44). Consistent with the hypothesis that Tie1 does not stimulate cell migration, an equivalent to tyrosine residue 1106 of Tie2 is not present on Tie1 despite the conservation of tyrosine residues 1100 and 1111. Although it remains to be clearly determined whether the Tie1 intracellular domain undergoes autophosphorylation, these observations imply that Tie1 does not associate with Dok-R. The presence of a unique autophosphorylation site on Tie2 that mediates binding to Dok-R and Nck/Pak complexes underscores the importance of this receptor in modulating endothelial cell migration. Moreover, the availability of an antibody that specifically recognizes phosphorylated tyrosine residue 1106 on Tie2 may provide a new means by which to interfere with diseases characterized by altered angiogenesis.

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REFERENCES

1. **Asahara, T., D. Chen, T. Takahashi, K. Fujikawa, M. Kearney, M. Magner, G. D. Yancopoulos, and J. M. Isner.** 1998. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. Circ. Res. **83:**233–240.
2. **Blaikie, P., D. Immanuel, J. Wu, N. Li, V. Yajnik, and B. Margolis.** 1994. A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. J. Biol. Chem. **269:**32031–32034.
3. **Borg, J. P., J. Ooi, E. Levy, and B. Margolis.** 1996. The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. Mol. Cell. Biol. **16:**6229–6241.
4. **Cantley, L. C.** 2002. The phosphoinositide 3-kinase pathway. Science **296:** 1655–1657.
5. **Carpino, N., D. Wisniewski, A. Strife, D. Marshak, R. Kobayashi, B. Stillman, and B. Clarkson.** 1997. p62(dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. Cell **88:**197–204.
6. **Charest, A., J. Wagner, S. Jacob, C. J. McGlade, and M. L. Tremblay.** 1996. Phosphotyrosine-independent binding of SHC to the NPLH sequence of murine protein-tyrosine phosphatase-PEST. Evidence for extended phosphotyrosine binding/phosphotyrosine interaction domain recognition specificity. J. Biol. Chem. **271:**8424–8429.
7. **Chien, C. T., S. Wang, M. Rothenberg, L. Y. Jan, and Y. N. Jan.** 1998. Numb-associated kinase interacts with the phosphotyrosine binding domain of Numb and antagonizes the function of Numb in vivo. Mol. Cell. Biol. **18:**598–607.
8. **Cong, F., B. Yuan, and S. P. Goff.** 1999. Characterization of a novel member of the DOK family that binds and modulates Abl signaling. Mol. Cell. Biol. **19:**8314–8325.
9. **D'Amore, P. A.** 1992. Capillary growth: a two-cell system. Semin. Cancer Biol. **3:**49–56.
10. **Davis, S., T. H. Aldrich, P. F. Jones, A. Acheson, D. L. Compton, V. Jain, T. E. Ryan, J. Bruno, C. Radziejewski, P. C. Maisonpierre, and G. D. Yancopoulos.** 1996. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell **87:**1161–1169.
11. **Dho, S. E., S. Jacob, C. D. Wolting, M. B. French, L. R. Rohrschneider, and C. J. McGlade.** 1998. The mammalian numb phosphotyrosine-binding domain. Characterization of binding specificity and identification of a novel PDZ domain-containing numb binding protein, LNX. J. Biol. Chem. **273:** 9179–9187.
12. **Di Cristofano, A., N. Carpino, N. Dunant, G. Friedland, R. Kobayashi, A. Strife, D. Wisniewski, B. Clarkson, P. P. Pandolfi, and M. D. Resh.** 1998. Molecular cloning and characterization of p56dok-2 defines a new family of RasGAP-binding proteins. J. Biol. Chem. **273:**4827–4830.
13. **Dumont, D. J., G. Gradwohl, G. H. Fong, M. C. Puri, M. Gertsenstein, A. Auerbach, and M. L. Breitman.** 1994. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes. Dev. **8:**1897–1909.
14. **Ferguson, K. M., J. M. Kavran, V. G. Sankaran, E. Fournier, S. J. Isakoff, E. Y. Skolnik, and M. A. Lemmon.** 2000. Structural basis for discrimination of 3-phosphoinositides by pleckstrin homology domains. Mol. Cell **6:**373–384.
15. **Fujikawa, K., I. de Aos Scherpenseel, S. K. Jain, E. Presman, and L. Varticovski.** 1999. Role of PI 3-kinase in angiopoietin-1-mediated migration and attachment-dependent survival of endothelial cells. Exp. Cell Res. **253:**663–672.
16. **Garrity, P. A., Y. Rao, I. Salecker, J. McGlade, T. Pawson, and S. L. Zipursky.** 1996. Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. Cell **85:**639–650.
17. **Grimm, J., M. Sachs, S. Britsch, S. Di Cesare, T. Schwarz-Romond, K. Alitalo, and W. Birchmeier.** 2002. Novel p62dok family members, dok-4 and dok-5, are substrates of the c-Ret receptor tyrosine kinase and mediate neuronal differentiation. J. Cell Biol. **154:**345–354.
18. **Gustafson, T. A., W. He, A. Craparo, C. D. Schaub, and T. J. O'Neill.** 1995. Phosphotyrosine-dependent interaction of SHC and insulin receptor substrate 1 with the NPEY motif of the insulin receptor via a novel non-SH2 domain. Mol. Cell. Biol. **15:**2500–2508.
19. **Hayes, A. J., W. Q. Huang, J. Mallah, D. Yang, M. E. Lippman, and L. Y. Li.** 1999. Angiopoietin-1 and its receptor tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells. Microvasc. Res. **58:**224–237.

20. **Hing, H., J. Xiao, N. Harden, L. Lim, and S. L. Zipursky.** 1999. Pak functions downstream of Dock to regulate photoreceptor axon guidance in Drosophila. Cell **97:**853–863.
21. **Hosooka, T., T. Noguchi, H. Nagai, T. Horikawa, T. Matozaki, M. Ichihashi, and M. Kasuga.** 2002. Inhibition of the motility and growth of B16F10 mouse melanoma cells by dominant negative mutants of Dok-1. Mol. Cell. Biol. **21:**5437–5546.
22. **Jones, N., and D. J. Dumont.** 1998. The Tek/Tie2 receptor signals through a novel Dok-related docking protein, Dok-R. Oncogene **17:**1097–1108.
23. **Jones, N., and D. J. Dumont.** 1999. Recruitment of Dok-R to the EGF receptor through its PTB domain is required for attenuation of erk MAP kinase activation. Curr. Biol. **9:**1057–1060.
24. **Jones, N., Z. Master, J. Jones, D. Bouchard, Y. Gunji, H. Sasaki, R. Daly, K. Alitalo, and D. J. Dumont.** 1999. Identification of Tek/Tie2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration. J. Biol. Chem. **274:**30896–30905.
25. **Jones, N., D. Voskas, Z. Master, R. Sarao, J. Jones, and D. J. Dumont.** 2001. Rescue of the early vascular defects in Tek/Tie2 null mice reveals an essential survival function. EMBO Rep. **2:**438–445.
26. **Kavanaugh, W. M., C. W. Turck, and L. T. Williams.** 1995. PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. Science **268:**1177–1179.
27. **Kavanaugh, W. M., and L. T. Williams.** 1994. An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science **266:**1862–1865.
28. **Koblizek, T. I., C. Weiss, G. D. Yancopoulos, U. Deutsch, and W. Risau.** 1998. Angiopoietin-1 induces sprouting angiogenesis in vitro. Curr. Biol. **8:**529–532.
29. **Kontos, C. D., T. P. Stauffer, W. P. Yang, J. D. York, L. Huang, M. A. Blanar, T. Meyer, and K. G. Peters.** 1998. Tyrosine 1101 of Tie2 is the major site of association of p85 and is required for activation of phosphatidylinositol 3-kinase and Akt. Mol. Cell. Biol. **18:**4131–4140.
30. **Kulkarni, S. V., G. Gish, P. van der Geer, M. Henkemeyer, and T. Pawson.** 2000. Role of p120 Ras-GAP in directed cell movement. J. Cell Biol. **149:**457–470.
31. **Kwak, H. J., J. N. So, S. J. Lee, I. Kim, and G. Y. Koh.** 1999. Angiopoietin-1 is an apoptosis survival factor for endothelial cells. FEBS Lett. **448:**249–253.
32. **Lemay, S., D. Davidson, S. Latour, and A. Veillette.** 2000. Dok-3, a novel adapter molecule involved in the negative regulation of immunoreceptor signaling. Mol. Cell. Biol. **20:**2743–2754.
33. **Li, S. C., Z. Songyang, S. J. Vincent, C. Zwahlen, S. Wiley, L. Cantley, L. E. Kay, J. Forman-Kay, and T. Pawson.** 1997. High-affinity binding of the Drosophila Numb phosphotyrosine-binding domain to peptides containing a Gly-Pro-(p)Tyr motif. Proc. Natl. Acad. Sci. USA **94:**7204–7209.
34. **Liang, X., D. Wisniewski, A. Strife, Shivakrupa, B. Clarkson, and M. D. Resh.** 2002. Phosphatidylinositol 3-kinase and Src family kinases are required for phosphorylation and membrane recruitment of Dok-1 in c-Kit signaling. J. Biol. Chem. **277:**13732–13738.
35. **Maisonpierre, P. C., C. Suri, P. F. Jones, S. Bartunkova, S. J. Wiegand, C. Radziejewski, D. Compton, J. McClain, T. H. Aldrich, N. Papadopoulos, T. J. Daly, S. Davis, T. N. Sato, and G. D. Yancopoulos.** 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science **277:**55–60.
36. **Master, Z., N. Jones, J. Tran, J. Jones, R. S. Kerbel, and D. J. Dumont.** 2001. Dok-R plays a pivotal role in angiopoietin-1-dependent cell migration through recruitment and activation of Pak. EMBO J. **20:**5919–5928.
37. **McGlade, J., B. Brunkhorst, D. Anderson, G. Mbamalu, J. Settleman, S. Dedhar, M. Rozakis-Adcock, L. B. Chen, and T. Pawson.** 1993. The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. EMBO J. **12:**3073–3081.
38. **Mochizuki, Y., T. Nakamura, H. Kanetake, and S. Kanda.** 2002. Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn. J. Cell Sci. **115:**175–183.
39. **Murray, B. W., E. S. Padrique, C. Pinko, and M. A. McTigue.** 2002. Mechanistic effects of autophosphorylation on receptor tyrosine kinase catalysis: enzymatic characterization of Tie2 and phospho-Tie2. Biochemistry **40:**10243–10253.
40. **Nelms, K., A. L. Snow, J. Hu-Li, and W. E. Paul.** 1998. FRIP, a hematopoietic cell-specific rasGAP-interacting protein phosphorylated in response to cytokine stimulation. Immunity **9:**13–24.
41. **Noguchi, T., T. Matozaki, K. Inagaki, M. Tsuda, K. Fukunaga, Y. Kitamura, T. Kitamura, K. Shii, Y. Yamanashi, and M. Kasuga.** 1999. Tyrosine phosphorylation of p62(Dok) induced by cell adhesion and insulin: possible role in cell migration. EMBO J. **18:**1748–1760.
42. **Ong, S. H., G. R. Guy, Y. R. Hadari, S. Laks, N. Gotoh, J. Schlessinger, and I. Lax.** 2000. FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. Mol. Cell. Biol. **20:**979–989.
43. **Papapetropoulos, A., G. Garcia-Cardena, T. J. Dengler, P. C. Maisonpierre, G. D. Yancopoulos, and W. C. Sessa.** 1999. Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. Lab. Investig. **79:**213–223.
44. **Patan, S.** 1998. TIE1 and TIE2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth. Microvasc. Res. **56:**1–21.
45. **Pawson, T., and J. D. Scott.** 1997. Signaling through scaffold, anchoring, and adaptor proteins. Science **278:**2075–2080.
46. **Puri, M. C., J. Partanen, J. Rossant, and A. Bernstein.** 1999. Interaction of the TEK and TIE receptor tyrosine kinases during cardiovascular development. Development **126:**4569–4580.
47. **Risau, W., H. Sariola, H. G. Zerwes, J. Sasse, P. Ekblom, R. Kemler, and T. Doetschman.** 1988. Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. Development **102:**471–478.
48. **Sato, A., A. Iwama, N. Takakura, H. Nishio, G. D. Yancopoulos, and T. Suda.** 1998. Characterization of TEK receptor tyrosine kinase and its ligands, angiopoietins, in human hematopoietic progenitor cells. Int. Immunol. **10:**1217–1227.
49. **Sato, T. N., Y. Tozawa, U. Deutsch, K. Wolburg-Buchholz, Y. Fujiwara, M. Gendron-Maguire, T. Gridley, H. Wolburg, W. Risau, and Y. Qin.** 1995. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature **376:**70–74.
50. **Sattler, M., S. Verma, Y. B. Pride, R. Salgia, L. R. Rohrschneider, and J. D. Griffin.** 2001. SHIP1, an SH2 domain containing polyinositol-5-phosphatase, regulates migration through two critical tyrosine residues and forms a novel signaling complex with DOK1 and CRKL. J. Biol. Chem. **276:**2451–2458.
51. **Shewchuk, L. M., A. M. Hassell, B. Ellis, W. D. Holmes, R. Davis, E. L. Horne, S. H. Kadwell, D. D. McKee, and J. T. Moore.** 2000. Structure of the Tie2 RTK domain: self-inhibition by the nucleotide binding loop, activation loop, and C-terminal tail. Struct. Fold. Design **8:**1105–1113.
52. **Songyang, Z., Y. Yamanashi, D. Liu, and D. Baltimore.** 2001. Domain-dependent function of the rasGAP-binding protein p62Dok in cell signaling. J. Biol. Chem. **276:**2459–2465.
53. **Stoletov, K. V., K. E. Ratcliffe, S. C. Spring, and B. I. Terman.** 2001. NCK and PAK participate in the signaling pathway by which vascular endothelial growth factor stimulates the assembly of focal adhesions. J. Biol. Chem. **276:**22748–22755.
54. **Suri, C., P. F. Jones, S. Patan, S. Bartunkova, P. C. Maisonpierre, S. Davis, T. N. Sato, and G. D. Yancopoulos.** 1996. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell **87:**1171–1180.
55. **Tallquist, M. D., P. Soriano, and R. A. Klinghoffer.** 1999. Growth factor signaling pathways in vascular development. Oncogene **18:**7917–7932.
56. **Tamir, I., J. C. Stolpa, C. D. Helgason, K. Nakamura, P. Bruhns, M. Daeron, and J. C. Cambier.** 2000. The RasGAP-binding protein p62dok is a mediator of inhibitory FcgammaRIIB signals in B cells. Immunity **12:**347–358.
57. **Teichert-Kuliszewska, K., P. C. Maisonpierre, N. Jones, A. I. Campbell, Z. Master, M. P. Bendeck, K. Alitalo, D. J. Dumont, G. D. Yancopoulos, and D. J. Stewart.** 2001. Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. Cardiovasc. Res. **49:**659–670.
58. **Valenzuela, D. M., J. A. Griffiths, J. Rojas, T. H. Aldrich, P. F. Jones, H. Zhou, J. McClain, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, T. Huang, N. Papadopoulos, P. C. Maisonpierre, S. Davis, and G. D. Yancopoulos.** 1999. Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. Proc. Natl. Acad. Sci. USA **96:**1904–1909.
59. **van der Geer, P., S. Wiley, G. D. Gish, V. K. Lai, R. Stephens, M. F. White, D. Kaplan, and T. Pawson.** 1996. Identification of residues that control specific binding of the Shc phosphotyrosine-binding domain to phosphotyrosine sites. Proc. Natl. Acad. Sci. USA **93:**963–968.
60. **Williams, R. L., S. A. Courtneidge, and E. F. Wagner.** 1988. Embryonic lethalities and endothelial tumors in chimeric mice expressing polyoma virus middle T oncogene. Cell **52:**121–131.
61. **Witzenbichler, B., P. C. Maisonpierre, P. Jones, G. D. Yancopoulos, and J. M. Isner.** 1998. Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. J. Biol. Chem. **273:**18514–18521.
62. **Xu, H., K. W. Lee, and M. Goldfarb.** 1998. Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins. J. Biol. Chem. **273:**17987–17990.
63. **Yaffe, M. B.** 2002. Phosphotyrosine-binding domains in signal transduction. Nat. Rev. Mol. Cell Biol. **3:**177–186.
64. **Yamanashi, Y., and D. Baltimore.** 1997. Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. Cell **88:**205–211.
65. **Yancopoulos, G. D., S. Davis, N. W. Gale, J. S. Rudge, S. J. Wiegand, and J. Holash.** 2000. Vascular-specific growth factors and blood vessel formation. Nature **407:**242–248.
66. **Zhao, M., A. A. Schmitz, Y. Qin, A. Di Cristofano, P. P. Pandolfi, and L. Van Aelst.** 2001. Phosphoinositide 3-kinase-dependent membrane recruitment of p62(dok) is essential for its negative effect on mitogen-activated protein (MAP) kinase activation. J. Exp. Med. **194:**265–274.
67. **Zhou, M. M., B. Huang, E. T. Olejniczak, R. P. Meadows, S. B. Shuker, M. Miyazaki, T. Trub, S. E. Shoelson, and S. W. Fesik.** 1996. Structural basis for IL-4 receptor phosphopeptide recognition by the IRS-1 PTB domain. Nat. Struct. Biol. **3:**388–393.