Tyrosine Phosphorylation of Dbl Regulates GTPase Signaling*

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Background: Dbl is a GEF that activates the small GTPases RhoA, Cdc42, and Rac1. The events that regulate Dbl activity are unknown.

Results: Growth factors lead to phosphorylation of Dbl Tyr^{510} and stimulate its GEF activity.

Conclusion: Dbl activation cycle is tightly regulated by the activity of tyrosine kinases and phosphatases.

Significance: Understanding signaling pathways enhances our understanding of their roles in health and disease states.

Rho GTPases are molecular "switches" that cycle between "on" (GTP-bound) and "off" (GDP-bound) states and regulate numerous cellular activities such as gene expression, protein synthesis, cytoskeletal rearrangements, and metabolic responses. Dysregulation of GTPases is a key feature of many diseases, especially cancers. Guanine nucleotide exchange factors (GEFs) of the Dbl family are activated by mitogenic cell surface receptors and activate the Rho family GTPases Cdc42, Rac1, and RhoA. The molecular mechanisms that regulate GEFs from the Dbl family are poorly understood. Our studies reveal that Dbl is phosphorylated on tyrosine residues upon stimulation by growth factors and that this event is critical for the regulated activation of the GEF. These findings uncover a novel layer of complexity in the physiological regulation of this protein.

The Dbl family of guanine nucleotide exchange factors (GEFs)^2 comprises >80 members that facilitate the activation of the small GTPases Rac1, Cdc42, and/or RhoA. Dbl was the first identified mammalian GEF and as such is considered to be the prototypic member of the family (1). Two highly conserved domains characterize Dbl family GEFs: a unique Dbl homology domain and a pleckstrin homology domain. The Dbl homology domain is the minimal unit necessary for GEF activity that binds the substrate GTPase and catalyzes the exchange of GDP for GTP in its binding pocket (2). Following GEF-mediated activation, the bound GTP is hydrolyzed, and the GTPase returns to its quiescent state. The pleckstrin homology domain is thought to mediate proper localization of the GEF to specific subcellular compartments (3–5). In response to growth factor stimulation, Dbl-mediated GTPase signaling regulates numerous cellular activities such as cytoskeletal rearrangements, gene expression, and vesicular trafficking, thereby promoting cell proliferation (6–10).

Because GTPases control multiple and diverse aspects of cellular physiology, proper regulation of GEF activities is crucial for normal cellular function. Indeed, mutations that cause GEF overexpression or gain of function result in persistent activation of downstream pathways and lead to aberrant cell growth that manifest in developmental disorders and cancers. For example, mutations in Fgd1 cause the X-linked developmental disorder, Aarskog-Scott syndrome (11), and chromosomal rearrangements of the Bcr gene contribute to leukemogenesis (12). Mutations in Plekhg4 are associated with heritable autosomal spinocerebellar ataxia (13, 14). Overexpression of P-rex1, Ect2, and Tiam1 has been noted in cancers of the breast, lung, and colon, respectively (15–20), and overexpression of Dbl was reported in sarcomas and tumors of neuroectodermal origins (21–24). As our appreciation for the involvement of Dbl family GEFs in disease grows, the need for understanding the mechanisms that regulate these proteins increases.

The upstream events that regulate the GEF activity of Dbl are poorly understood. Dbl is thought to exist in an inhibited state, which is activated by growth factor receptors; however, the molecular mechanisms that underlie the inhibited state and its release are incompletely understood. Some studies proposed an intramolecular autoinhibited state brought about by interactions between the amino and carboxyl termini of Dbl (25). Other studies demonstrated that association with the molecular chaperones Hsc70 and Hsp90 regulate Dbl activity by controlling its degradation by the proteasome (26, 27). The mechanisms by which growth factor stimulation leads to release of Dbl inhibition are completely unknown.

In this study, we aimed to understand the upstream events that relieve Dbl autoinhibition and stimulate its GEF activity. We show that a key feature of the activation cycle of Dbl involves reversible, growth factor-sensitive, tyrosine phosphorylation.

MATERIALS AND METHODS

Cell Culture—COS7 cells and HEK293T cells were cultured in DMEM supplemented with 10% FBS (Hyclone) in 5% CO$_2$ at 37 °C. NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum (Hyclone). Parental and EGFR-expressing CHO cells were a generous gift from Dr. Cathleen Carlin, Case Western Reserve University School of Medicine and were cultured in minimum essential medium α supplemented with 10% FBS (Hyclone).

Molecular Constructs—Dbl constructs encoded the reading frame encoding the full-length protein (residues 1–925) in the

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^{2} The abbreviations used are: GEF, guanine nucleotide exchange factor; EGFR, EGF receptor; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]-pyrimidine.

pCMV6 vector, or the pCEFL-GST vector (for focus formation experiments). Recombinant GTPases were expressed in *Escherichia coli* as glutathione *S*-transferase fusions and purified as described previously (28). Grb2 cDNA (HA-tagged in the pCGN vector) was a generous gift from Dr. Dafna Bar-Sagi, New York University School of Medicine. All transfections were done using PolyFect (Qiagen).

Chemicals—EGF (Sigma) was dissolved in serum-free DMEM and used at a final concentration of 50 nM. 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2, EMD Millipore) was dissolved in dimethyl sulfoxide and used at a final concentration of 1 or 10 μM, as was Iressa (gefitnib, Selleck Biochemicals). The phosphatase inhibitor-activated sodium orthovanadate (Sigma) was added during the last 10 min to a final concentration of 1 mM.

Immunoprecipitations from Cell Lysates—Cells were lysed in 1 ml of 20 mM Hepes, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Igepal, 20 mM NaF, 20 mM β-glycerophosphate, 1 mM vanadate, 200 μm phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotinin. Lysates were spun at 14,000 rpm for 20 min, and cleared supernatant was combined with anti-Dbl antibody (Santa Cruz Biotechnology, sc-89, 1:100 dilution) or anti-HA antibody (Covance, MMS-101R, 1:100). The lysate-antibody mixture was mixed at 4 °C for 1 h before the addition of 50 μl of protein A beads (Millipore) and mixing at 4 °C for 2.5 h. Beads were washed three times with lysis buffer and spun at 3,500 rpm for 3 min. Precipitated beads were boiled in 2.5× Lamelli buffer, and the supernatant was resolved by 8–12% SDS-PAGE. Tyrosine phosphorylation was visualized using anti-phosphotyrosine antibody (4G10-platinum, Millipore, 1:1000 dilution).

Immunoprecipitations from Mouse Brain—Whole brain extracts from 10-day-old mice were homogenized manually in 1 ml of 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 150 mM NaCl, 25 mM Tris-HCl (pH 8.0), 0.25 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotinin. The homogenate was cleared by centrifugation at 14,000 rpm for 45 min at 4 °C, followed by immunoprecipitation with Dbl antibody as described above.

Site-directed Mutagenesis—Site-directed mutagenesis was performed according to the manufacturer's instructions (QuikChange XL II, Agilent).

Starvation and Stimulation of CHO Cells—Five hours prior to lysis, cells were switched to MEMα containing 0.5% FBS. After 3 h, cells were placed on ice for 2 h and treated as indicated (EGF, Iressa, or PP2). Cells were then incubated at 37 °C for 15 min, lysed, and subjected to immunoprecipitations as described above.

Grb2-Dbl Association—COS7 cells were co-transfected with HA-Grb2 and Dbl constructs, harvested in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1 mM vanadate, 200 μm phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin and aprotinin, and centrifuged at 14,000 rpm for 20 min. Association was visualized by anti-HA immunoblotting of anti-Dbl immunoprecipitates.

GEF-GTPase Association Assays—These were designed based on the established high affinity of GEFs to their nucleotide-free form of their cognate GTPases (29–31). Wild-type GTPases and their respective nucleotide-free mutants (Cdc42(T17N) or RhoA(T19N)) were expressed in *E. coli* as GST fusions and purified as described previously (32). The purified GTPases were immobilized on glutathione agarose and washed extensively with cell lysis buffer supplemented with 50 μM GDP, 10 mM MgCl$_2$ (wild-type GTPase), or 10 mM EDTA (Asn[17]/Asn[19] GTPase). Dbl-transfected cells were lysed in the same lysis buffer, incubated with immobilized GTPases at 4 °C for 2 h, and washed three times in the respective buffers, prior to SDS-PAGE and anti-Dbl immunoblotting.

GTPase Activation Assays—To measure GEF activity, the fraction of endogenous GTPase that is in the active (GTP-bound) state was determined using an established selective precipitation approach (33–35). Briefly, HEK293T cells were transfected with Dbl cDNA, serum-starved for 31 h, and lysed in 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM MgCl$_2$, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotinin. Lysates were spun at 14,000 rpm for 20 min, and cleared supernatant was combined with ~20 μg of immobilized GST-fused GTPase-binding domain of rhotekin and rotated at 4 °C for 2 h (34). Beads were washed three times in lysis buffer and resolved on SDS-PAGE. The amount of activated (bead-associated) GTPase was visualized with anti-RhoA (Cytoskeleton, 1:200 dilution) immunoblotting and compared with the total GTPase levels as determined by anti-RhoA immunoblotting of whole cell lysates.

NFκB Transcriptional Activation Assays—NIH3T3 cells were plated in triplicate in a 24-well plate and co-transfected with the NFκB response element fused to the luciferase reporter construct (pGL3B-HIV1-luc), the β-galactosidase-expressing pCH110 (Pharmacia), and Dbl cDNA. Thirty-six hours after transfection, cells were washed in serum-free medium and starved for 17 h prior to harvesting cell lysates and measuring luciferase and β-galactosidase activities. Luciferase expression was normalized to β-galactosidase activity to account for differences in transfection efficiency.

Focus Formation Assays—The indicated constructs were used to transfect subconfluent NIH3T3 cells in 10-cm plates. After 48 h, media was replaced, and cells were refed every other day. Approximately 2 weeks after transfection, cells were fixed with methanol and stained with crystal violet, and foci were manually scored.

Fluorescence Microscopy—NIH3T3 cells were seeded on glass coverslips and transfected with the indicated constructs (opr empty pCMV6 vector as control) using PolyFect according to the manufacturer's instructions (Qiagen). Thirty-one hours post-transfection, cells were serum-starved for 17 h, fixed with 3.7% paraformaldehyde, permeabilized with 0.2% Triton X-100, stained with the indicated reagents, and imaged using the Leica TCS SP2 microscope. Dbl proteins were visualized using Santa Cruz Biotechnology sc-89 antibody and a secondary Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) antibody; the actin cytoskeleton was visualized with Texas Red-conjugated phalloidin (Invitrogen).
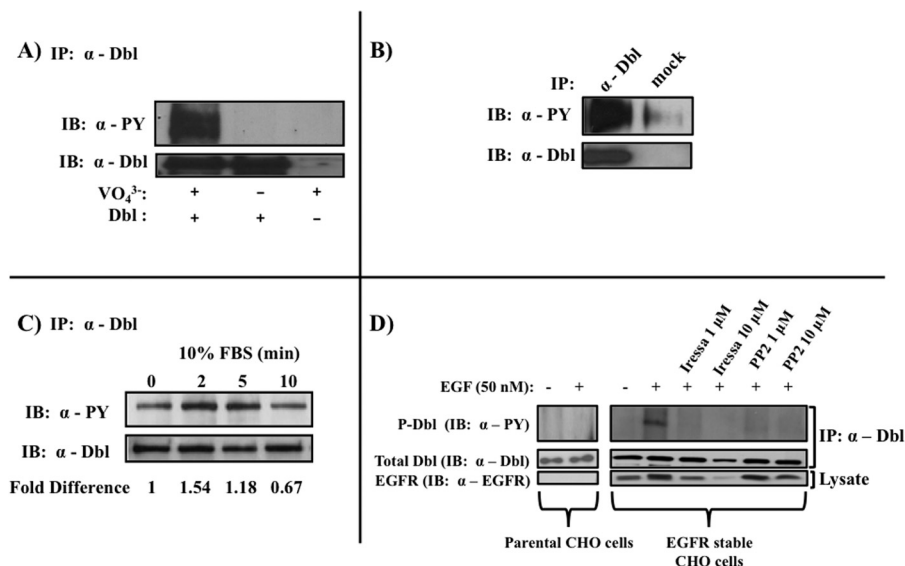
FIGURE 1. **Tyrosine phosphorylation of Dbl.** Anti-Dbl immunoprecipitates from transfected COS7 cells (*A*) or whole mouse brains (*B*) were immunoblotted (*IB*) with anti-phosphotyrosine (*PY*) antibodies. *C*, regulation of Dbl phosphorylation by serum. Serum-starved Dbl-transfected COS7 cells were stimulated with 10% FBS for the indicated duration, and tyrosine phosphorylation was examined as described in *A*. *D*, regulation of Dbl phosphorylation by EGF receptor and Src family kinases. Dbl-transfected EGFR expressing and parental CHO cells were starved, stimulated with EGF (50 nM), and treated with 1 or 10 μM Iressa or 1 or 10 μM PP2. Starvation and stimulation conditions are as described under "Materials and Methods." All blots are representative of at least three independent experiments.

RESULTS

Tyrosine Phosphorylation of Dbl—In a search for post-translational events that may regulate the GEF activity of Dbl, we found that anti-Dbl immunoprecipitates give a strong signal on anti-phosphotyrosine immunoblots. Importantly, the signal was only visible in the presence of the tyrosine phosphatase inhibitor sodium orthovanadate (Fig. 1*A*). The physiological relevance of this finding is evident by the observation that tyrosine phosphorylation of Dbl occurs in intact tissue where the protein is expressed, such as mouse brain (Fig. 1*B*). Moreover, we found that when serum-deprived COS7 cells are treated with serum, Dbl phosphorylation transiently increases after 2–5 min and then tapers off (Fig. 1*C*). These observations show that Dbl is phosphorylated on tyrosine residue(s) and that this modification is a regulated event, dependent on growth factors. To begin to delineate the upstream events that regulate Dbl phosphorylation, we used pharmacological inhibitors of two major intracellular tyrosine kinases: Iressa (gefitinib), which inhibits the EGF receptor tyrosine kinase, and the inhibitor of Src family tyrosine kinases, PP2. To confirm these findings we examined whether expression of the EGFR kinase affects the phosphorylation of Dbl. In CHO cells that stably overexpress the EGFR kinase, stimulation with EGF caused a marked phosphorylation of Dbl (Fig. 1*D*). Moreover, modification was inhibited by treatment with either Iressa or PP2. Importantly, Dbl expression, but not phosphorylation, was observed in the parental CHO cell line, which does not express EGFR (Fig. 1*D*). Taken together, these findings indicate that phosphorylation of Dbl is mediated by mitogenic stimulation through the activated EGF receptor and a Src family kinase. Furthermore, the data suggest that in this pathway, the Src family kinase is downstream of EGFR.

Site(s) of Tyrosine Phosphorylation in Dbl—We utilized a bioinformatics approach to identify the tyrosine residue(s) that
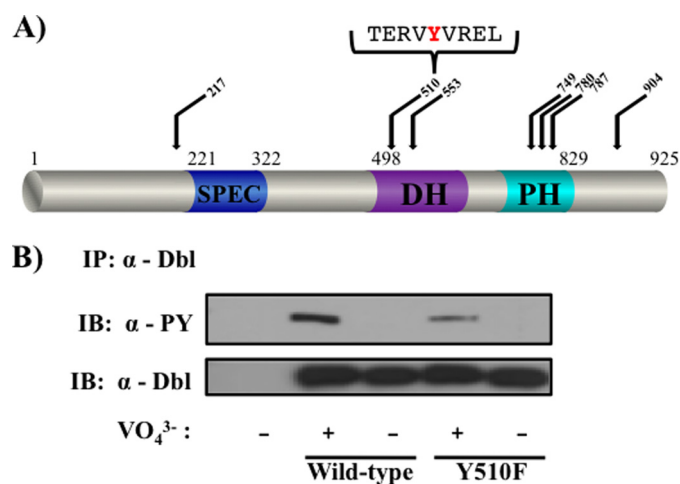

FIGURE 2. **Tyr^{510} is an important site of phosphorylation in Dbl.** *A*, domain structure of the Dbl protein. Of the 29 tyrosines of Dbl, the top-scoring putative phosphorylation sites are indicated, as well as the immediate sequence surrounding Tyr^{510}. *B*, tyrosine phosphorylation of Dbl(Y510F) was examined as in Fig. 1*A*. Data are representative of three independent experiments. *PY*, phosphotyrosine; *IB*, immunoblot; *DH*, Dbl homology domain; *PH*, pleckstrin homology domain; *SPEC*, spectrin.

are phosphorylated in Dbl. The NetPhos2.0 algorithm (36) identified seven residues in Dbl that reside within tyrosine phosphorylation consensus sequences. These putative phosphorylation sites are dispersed throughout the different domains of the protein (Tyr^{217}, Tyr^{510}, Tyr^{553}, Tyr^{749}, Tyr^{780}, Tyr^{787}, and Tyr^{904}; see Fig. 2*A*). We focused our attention to Tyr^{510}, due to its position in a high-scoring putative phosphorylation sequence in the catalytic Dbl homology domain. Using site-directed mutagenesis, we substituted Tyr^{510} of Dbl to a phenylalanine and observed a dramatic decrease (~80%) in the tyrosine phosphorylation intensity of the protein (Fig. 2*B*). The fact that some tyrosine phosphorylation persists in the Y510F mutant indicates that sites of tyrosine phosphorylation
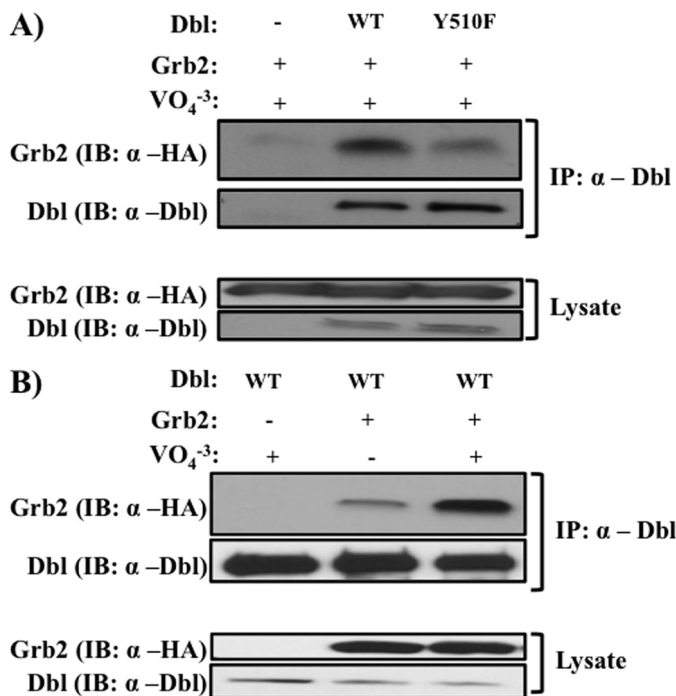
FIGURE 3. **Role of Tyr[510] in binding of Dbl to Grb2.** COS7 cells were co-transfected with indicated constructs, and association was evaluated as described under "Materials and Methods." *A*, association of Grb2 with Dbl or Dbl (Y510F). *B*, association of Grb2 with Dbl is dependent on tyrosine phosphorylation. Experiments were done as described in *A*, in the presence or absence of the tyrosine phosphate inhibitor sodium orthovanadate (1 mM). *Top panels*, presence of Grb2 and Dbl in anti-Dbl immunoprecipitates. *Bottom panels*, protein expression in cell lysates. Data are representative of three independent experiments. *IB*, immunoblot.

other than Tyr[510] exist in the protein. In attempting to identify such residues, we found that mutation of Tyr[787] did not affect the residual phosphorylation signal observed in Dbl(Y510F) (data not shown).

Regulation of Molecular Interactions of Dbl by Tyr[510] Phosphorylation—To understand the molecular consequences of tyrosine phosphorylation in Dbl, we examined its ability to associate with established effectors. Grb2 is an adapter molecule that contains an Src homology 2 domain that binds phosphorylated tyrosine residues and an Src homology 3 domain that binds proline-rich sequences (37). Due to this dual-recognition capacity, Grb2 often functions as a "bridging" adapter, allowing it to recruit multiple proteins to their cellular site of activation, as seen in the activation of Ras, where Grb2 recruits Son of Sevenless to the tyrosine-phosphorylated EGF receptor (38, 39). Because Grb2 was recently reported to be associated with Dbl (40), we sought to evaluate the role of Tyr[510] phosphorylation in this interaction. As seen in Fig. 3*A*, substitution of the Tyr[510] of Dbl with a non-phosphorylatable phenylalanine residue greatly diminished Grb2 binding. Furthermore, the association between Grb2 and Dbl required the presence of the tyrosine phosphatase inhibitor sodium orthovanadate (Fig. 3*B*). Together, these data strongly suggest that phosphorylation of the Tyr[510] of Dbl is critical for its binding to Grb2.

Next, we asked whether tyrosine phosphorylation of Dbl is necessary for association of the GEF with its substrate GTPase. Cells expressing Dbl (or its phospho-defective mutant) were lysed and precipitated with immobilized recombinant GTPases
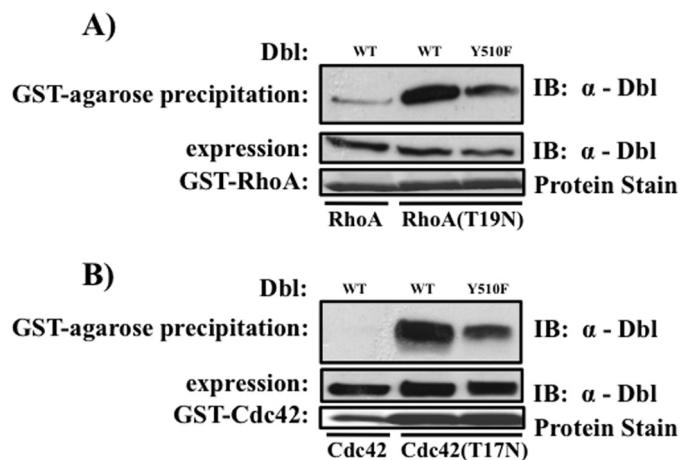
FIGURE 4. **Role of Tyr[510] in association of Dbl with substrate GTPases.** GST fusion proteins of the indicated RhoA (*A*) and Cdc42 (*B*) variants were overexpressed in *E. coli*, purified on glutathione affinity chromatography, and their nucleotide content manipulated as described under "Materials and Methods." *Top panels*, GEF presence in affinity precipitates. *Middle panels*, Dbl expression in respective cell lysates. *Lower panels*, GST-GTPases "baits" were visualized by Ponceau Red staining of the blotted membranes. Data are representative of three independent experiments. *IB*, immunoblot.
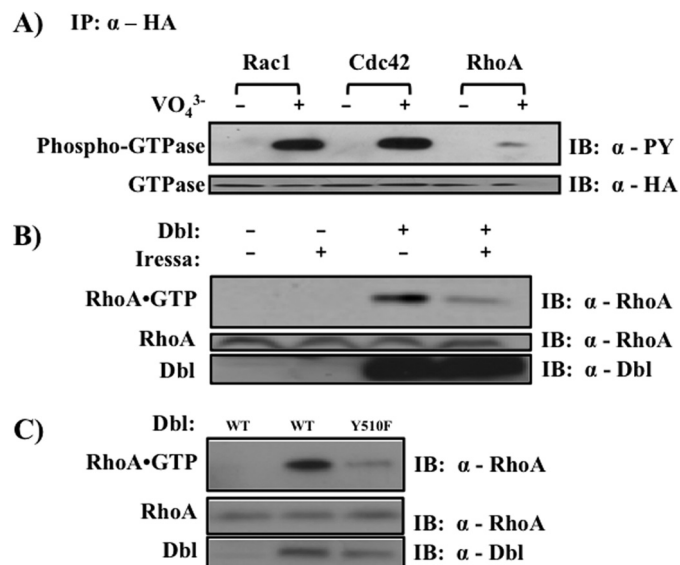
FIGURE 5. **Tyrosine phosphorylation of Dbl is necessary for proper activation of RhoA.** *A*, tyrosine phosphorylation of Cdc42, Rac1, and RhoA. COS7 cells were transiently transfected with the indicated construct and treated with orthovanadate where indicated. Tyrosine phosphorylation was examined in anti-HA immunoprecipitates. *B*, effect of tyrosine kinase inhibition on the GEF activity of Dbl. HEK293T cells were transiently transfected with the indicated constructs and serum-starved for 24 h. Thirty minutes prior to lysis, cells were treated with 10 μM Iressa, and the fraction of RhoA in the GTP-bound state was measured as described under "Materials and Methods." *Top panel*, GTP-bound RhoA visualized by anti-RhoA immunoblotting after affinity precipitation. *Lower panel*, expression levels of the indicated proteins in cell lysates. *C*, role of Tyr[510] phosphorylation in Dbl-stimulated nucleotide exchange. HEK293T cells were transiently transfected with indicated Dbl constructs, and RhoA activation was measured as described in *B*. Each panel is representative of three independent experiments. *IB*, immunoblot; *PY*, phosphotyrosine.

that have been purified in either the GDP-bound form (GST-fused wild-type GTPases) or the nucleotide-free form (GST-RhoA(T19N) or GST-Cdc42(T17N) mutants). The rationale for this design is based on the notion that the highest affinity of GEFs to their GTPase is observed in the nucleotide-free form of
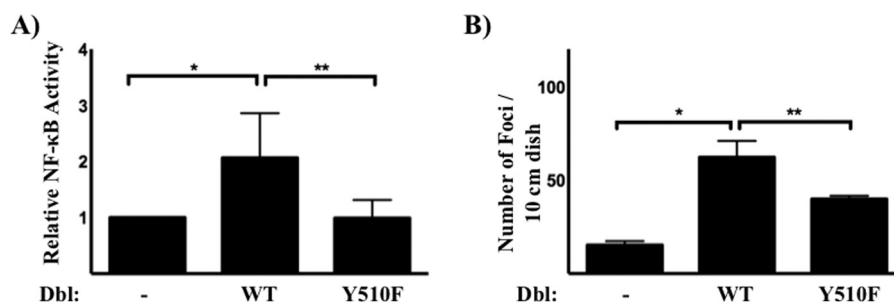
FIGURE 6. **Role of Tyr[510] phosphorylation in Dbl-induced downstream activities.** *A*, transcriptional effects. NIH3T3 cells were co-transfected with the indicated Dbl construct, an NFκB reporter construct, and a β-galactosidase expressing vector. NF-κB transcriptional activity was measured as described under "Materials and Methods." Shown are average values of three independent transfections, and statistical significance is indicated by asterix ($p < 0.05$ compared with the vector control (*) or to Dbl (**) as determined by Student's *t* test. *B*, loss of contact inhibition. Triplicate plates of NIH3T3 cells were transfected with the indicated Dbl cDNAs and monitored for foci formation as described under "Materials and Methods." *, $p < 0.05$ compared with the empty vector control; **, $p < 0.05$ compared with wild-type Dbl, as determined by a Student's *t* test (statistical significance). Data shown are representative of three independent experiments.

the latter (29–31). As expected, we observed a strong preference in the association of Dbl with the nucleotide-free forms of RhoA and Cdc42, as compared with the GDP-bound GTPases (compare *lanes 1* and *2* in Fig. 4, *A* and *B*). Importantly, we observed that the affinity of the nucleotide-free GTPase to the GEF is dramatically reduced when phosphorylation of Tyr[510] of Dbl is abolished by mutation to phenylalanine (Fig. 4, *A* and *B*). These findings indicate that that phosphorylation of Tyr[510] is required for Dbl to be able to bind substrate Rho GTPases.

Phosphorylation of Tyr[510] Is Critical for GEF Activity of Dbl— To address the role of Tyr[510] phosphorylation in Dbl activity as a GEF, we examined its ability to stimulate GTP binding by a substrate GTPase. Because both Cdc42 and Rac1 are phosphorylated on Tyr[64] (41),[3] whereas RhoA is not (Fig. 5*A*), we chose to focus on the latter GTPase. This allowed us to evaluate the effect of kinase inhibitors on Dbl actions without complication arising from the effect of the inhibitors on the substrate GTPase. We treated Dbl-transfected cells with Iressa and measured the GEF activity of Dbl by selectively precipitating GTP-bound form of RhoA ((33–35); see "Materials and Methods"). We found that treatment with Iressa all but abolished the ability of Dbl to activate RhoA (Fig. 5*B*), indicating that EGFR-mediated tyrosine phosphorylation of Dbl is essential for its activity as a GEF. Importantly, substitution of Tyr[510] to phenylalanine abolished the GEF activity of Dbl (Fig. 5*C*).

Next, we examined the role of Tyr[510] phosphorylation on further downstream signaling events. We found that the Y510F mutant of Dbl abolished transcriptional activation of NFkB (Fig. 6*A*), a downstream effector of RhoA, Rac1, and Cdc42 (42–44). These results further confirm that tyrosine phosphorylation of Dbl is necessary to activate the Rho family small GTPases. Lastly, we examined at the role of Tyr[510] phosphorylation on the signature biological activity of Dbl: transformation of cultured murine fibroblasts (45–47). We examined the outcome of Dbl overexpression on contact inhibition by quantifying the number of three-dimensional foci formed in confluent NIH3T3 cells transfected with Dbl cDNAs. Y510F mutation of Dbl caused a marked reduction in the focus-formation activity of Dbl (Fig. 6*B*).

One of the best studied outcomes of GEF-induced stimulation of Rho GTPases is rearrangement of the actin cytoskeleton.

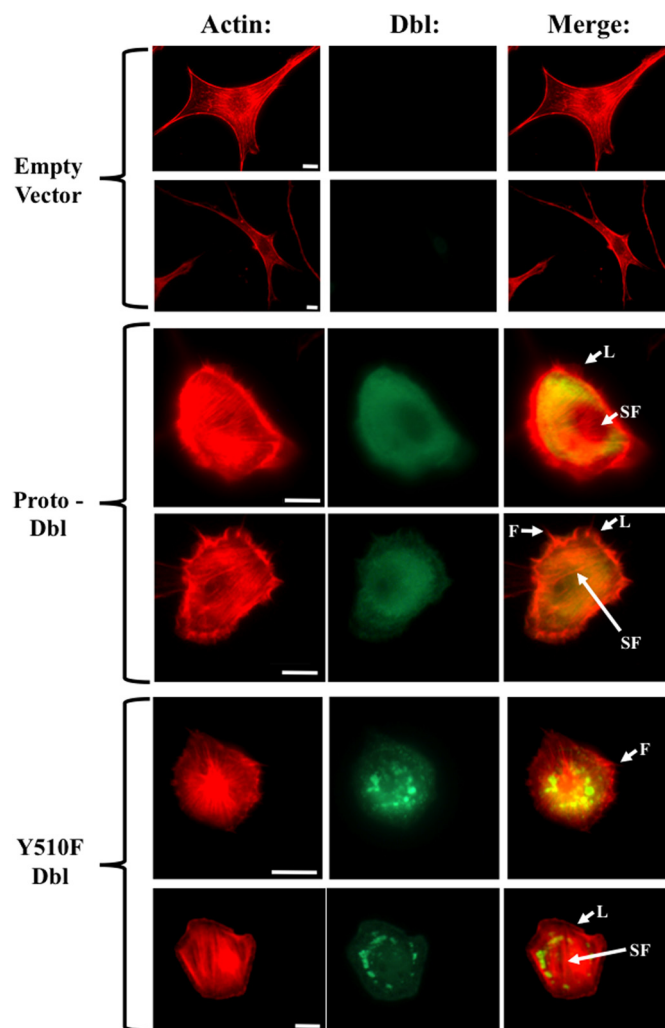[3] M. Gupta, X. Qi, V. Thakur, and D. Manor, unpublished results.

FIGURE 7. **Tyrosine phosphorylation of Tyr[510] regulates Dbl intracellular localization and activity.** Cells expressing wild-type Dbl, Dbl(Y510F), or control vector, were immunostained with anti-Dbl-antibody (*green*) and Texas Red-conjugated phalloidin (*red*) and visualized by fluorescence microscopy. *Arrowheads* indicate stress fibers (*SF*), lamellipodia (*L*), or filopodia (*F*). *Scale bar*, 10 μm.

Thus, it has been established that activation of Cdc42 stimulates filopodia formation, whereas GTP-binding by RhoA and Rac1 enhances formation of stress fibers and lamellipodia, respectively (48–50). Accordingly, ectopic expression of GEFs in cultured fibroblasts elicits the cytoskeletal pattern typical of their
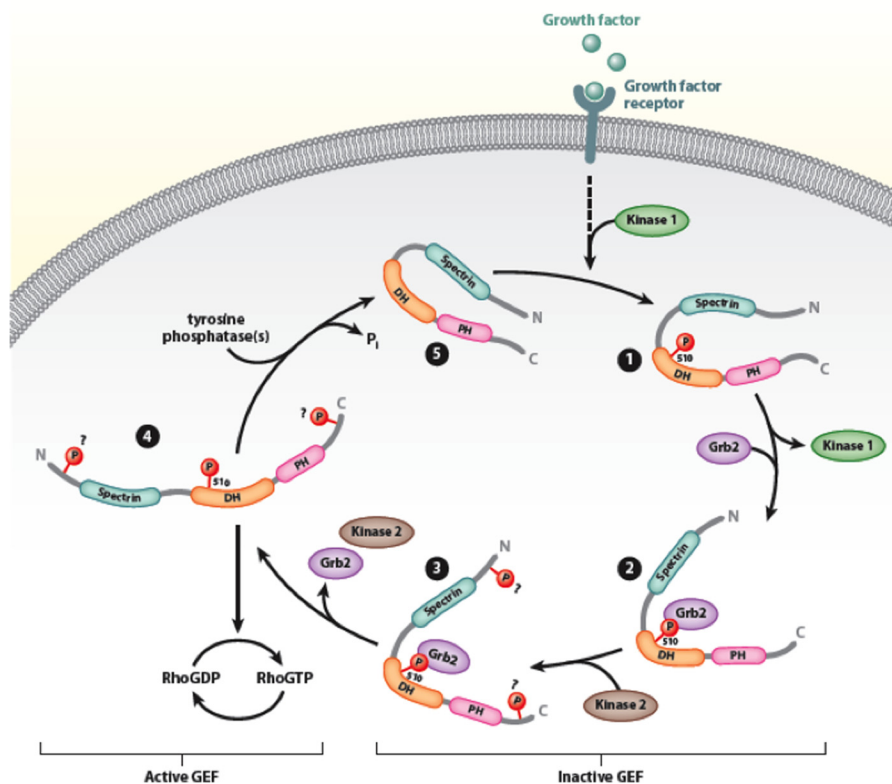
FIGURE 8. **Proposed model of Dbl regulation by tyrosine phosphorylation.** In quiescent cells, both intramolecular and intermolecular interactions maintain Dbl in an inactive state (*step 5*). Growth factor stimulation leads to phosphorylated of Tyr^{510} (*step 1*), leading to the recruitment of Grb2 (*step 2*) and other kinases (*step 3*) necessary for the phosphorylation of other phosphorylation sites. Full phosphorylation relaxes the autoinhibited structure of Dbl to one that allows high-affinity binding of substrate GTPases and facilitation of nucleotide exchange (*step 4*). *DH*, Dbl homology domain; *PH*, pleckstrin homology domain; *N*, N terminus; *C*, C terminus.

substrate GTPase and provides a convenient read-out of their signaling state (51). We therefor examined the role of Tyr^{510} phosphorylation in the ability of Dbl to induce these cytoskeletal changes in cultured NIH3T3 cells. Fig. 7 shows fluorescence micrographs of cells that express wild-type Dbl, the mutated variant Dbl(Y510F), or a control vector. We observed that expression of wild-type Dbl lead to enhanced formation of lamellipodia, filopodia, and stress fibers, as reported previously (Fig. 7) (51). The intracellular distribution pattern of the Dbl protein in these cells was diffuse throughout the cytosol with strong perinuclear and membrane localization. In contrast, the intracellular localization pattern of Dbl(Y510F) was markedly different, typified by punctate distribution throughout the cytosol. Additionally, cells that expressed the mutant GEF were smaller, rounder, and did not show the cytoskeletal reorganization induced by the wild-type protein. Rather, actin structures in mutant-expressing cells exhibited aberrant organization of the actin cytoskeleton, in a pattern that resembled "immature beginning" stress fibers, lamellopodia, and filopodia. These findings indicate that phosphorylation of Tyr^{510} is critical for proper regulation of cellular architecture. The observation that expression of the Dbl(Y510F) mutant has an evident yet altered impact on cytoskeletal reorganization suggest that other residues in Dbl are also important for this activity.

DISCUSSION

Members of the Dbl family GEFs regulate signaling pathways that emanate from their downstream targets, the Rho GTPases.

As such, they control numerous intracellular processes and are critical for mediating the effects of diverse extracellular stimuli (8, 10, 52–54). Underscoring the fundamental biological importance of Dbl family members is that improper regulation of their GEF activities is associated with pathological states, including cancer, developmental and neurological disorders, and viral pathogenesis (8). To date, great progress has been made in understanding the molecular mechanisms that govern small GTPase signaling and the downstream pathways that they regulate. However, our understanding of the upstream events that control and regulate GEF actions is very limited. Especially enigmatic are the events that couple GEFs to growth factor receptors and the molecular mechanisms that control their activation/deactivation cycle.

A number of studies indicate that both intramolecular and intermolecular interactions maintain Dbl family GEFs in an inhibited basal state that is relieved upon stimulation. Members of the Vav family of GEFs are basally autoinhibited via intramolecular interactions between the amino-terminal calponin homology domain and the Dbl homology domain that hinders access of Rho GTPases to the catalytic site. Stimulation of the T cell receptor leads to the phosphorylation of amino-terminal tyrosines by a recruited kinase, structural relaxation of the autoinhibited structure, and stimulation of nucleotide exchange on substrate GTPases (55, 56). Similarly, autoinhibition in Ngef and Wgef, is maintained by the C-terminal SH3 domain and an

N-terminal polyproline region, which is released by phosphorylation of amino-terminal tyrosines (57). In the case of PDZ-RhoGEF, leukemia-associated RhoGEF, and p115, the autoinhibited state is disrupted by binding of the $G\alpha_{12/13}$ to the RGS domains of these GEFs (58–60).

Dbl was initially discovered as a potent fibroblast transforming sequence, the activity of which is stimulated upon truncation of the amino terminus of the protein (1, 7, 9, 25). Dbl has been shown to activate RhoA, Rac1, and Cdc42 and to be expressed in the central nervous system, testis, as well as in neuroectodermal cancers and Ewing sarcomas (21–24). Upstream regulation of Dbl involves signaling concepts utilized in multiple other GEFs. The protein appears to be autoinhibited by interactions between its amino-terminal proto-oncogenic sequence, and the carboxyl-terminal half of the protein (25). In addition, Dbl is regulated by interactions with the molecular chaperones Hsc70 and Hsp90 that control the rate at which the protein is degraded by the proteasome (26, 27). Here, we show that Dbl is also regulated by tyrosine phosphorylation of Tyr^{510}. The modification is stimulated by growth factors, reversible, and essential for the transient conversion of Dbl to an active GEF. Our working hypothesis regarding the role of tyrosine phosphorylation in Dbl actions is depicted in Fig. 8. According to this model, receptor stimulation leads to phosphorylation of the Tyr^{510} of Dbl by an as yet unidentified kinase, followed by association of Grb2 with the phosphorylated residue and recruitment of another kinase(s) that phosphorylates additional tyrosines. Our data strongly suggest that Dbl phosphorylation occurs through the actions of both the EGFR kinase and a member of the Src family. Our observations are in line with a model in which EGFR kinase (kinase 1 in Fig. 8) is upstream of a Src family member (kinase 2 in Fig. 8), which directly phosphorylates Dbl. Once Dbl is fully phosphorylated, it is likely to be in a conformation that allows productive interactions with the substrate GTPase. Following substrate activation, the GEF undergoes phosphatase-mediated dephosphorylation and transitions to its quiescent state.

A number of important questions remain unanswered. First, the identity of the specific kinase(s) that phosphorylate Dbl is yet to be determined. Similarly, the phosphorylation site(s) that are modified in addition to Tyr^{510} and their impact on protein function are not known presently. Perhaps most interesting is the functional relationship that may exist between the different modes of regulation of Dbl, *i.e.* whether tyrosine phosphorylation affects the proteasomal degradation of the protein or its association with cytoskeletal elements such as ERM proteins (61). Interestingly, the intracellular distribution pattern of the Dbl(Y510F) mutant bears a striking resemblance to that of wild-type Dbl upon treatment with 17-AAG, an inhibitor of the chaperone Hsp90 (55, 56). Previous studies in our laboratory have shown that Dbl associates with the molecular chaperones Hsc70 and Hsp90 and the ubiquitin ligase CHIP. These molecules regulate the levels, the localization, and the activity of Dbl. In the inactive state, association with these molecules renders Dbl a short-lived, aggregation-prone protein (26, 27). However, the mechanisms by which these inhibitory interactions are relieved have not been elucidated. The findings that the distribution pattern of Dbl(Y510F) resembles aggresomes (xxx)

raises the intriguing possibility that tyrosine phosphorylation regulates the degradation rate of Dbl.

Additionally, a stable complex between Dbl and the lipid kinase PI3KC2β was reported (40), raising the possibility that a localized change in membranous phosphoinositide levels comprises an additional level of regulation. It is possible that regulated phosphorylation is coupled to, or coordinated with, the interaction of Dbl with this lipid kinase or its catalytic products. These questions open the door for exciting future research.

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