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# Activation of the Guanine Nucleotide Exchange Factor Dbl Following ACK1-Dependent Tyrosine Phosphorylation[1]

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**Signals triggered by diverse receptors modulate the activity of Rho family proteins, although the regulatory mechanism remains largely unknown. On the basis of their biochemical activity as guanine nucleotide exchange factors (GEFs), Dbl family proteins are believed to be implicated in the regulation of Rho family GTP-binding proteins in response to a variety of extracellular stimuli. Here we show that GEF activity of full-length proto-Dbl is enhanced upon tyrosine phosphorylation. When transiently coexpressed with the activated form of the non-receptor tyrosine kinase ACK1, a downstream target of Cdc42, Dbl became tyrosine-phosphorylated. *In vitro* GEF activity of Dbl toward Rho and Cdc42 was augmented following tyrosine phosphorylation. Moreover, accumulation of the GTP-bound form of Rho and Rac within the cell paralleled ACK-1-dependent tyrosine phosphorylation of Dbl. Consistently, activation of c-Jun N-terminal kinase downstream of Rho family GTP-binding proteins was also enhanced when Dbl was tyrosine-phosphorylated. Collectively, these findings suggest that the tyrosine kinase ACK1 may act as a regulator of Dbl, which in turn activates Rho family proteins.** 

Receptor-mediated signals trigger the activation of low molecular weight GTP-binding proteins mostly through the action of guanine nucleotide exchange factors (GEFs). For Rho family GTP-binding proteins including Rho, Rac, and Cdc42 (1), a range of GEFs have been identified composing the Dbl family (2). All members of this family possess the Dbl homology (DH) and pleckstrin homology (PH) domains in tandem, which are important for the catalysis of guanine nucleotide exchange. The proto-oncogene product Dbl (from here on referred to as Dbl) is a prototype of the Dbl family, which acts on all Rho family members *in vitro* (3–5). DH/PH domains locate within the C-terminal portion, while the N-terminal portion contains a coiled-coil domain. Truncation of N-terminal half renders Dbl oncogenic, implying that the N-terminal portion is responsible for the regulation of Dbl functions (3).

The involvement of specific members of the Dbl family in the regulation of Rho family proteins has been clarified, for instance, in G protein-coupled receptor-mediated signaling. Both G protein $\alpha$ (G$\alpha$) and $\beta\gamma$ (G$\beta\gamma$) subunits have been implicated in the activation of Rho family proteins (6–8). G$\alpha$13 directly binds to p115-RhoGEF enhancing its GEF activity, while G$\alpha$12 inhibits G$\alpha$13 stimulation of p115-RhoGEF activity (9, 10). Thus, p115-RhoGEF can directly link G$\alpha$ to Rho. On the other hand, Ras-GRF1/CDC25$^{Mm}$ exerts stimulation of GDP/GTP exchange of Rac in G$\beta\gamma$-dependent manner (11). However, the Ras-GRF1/CDC25$^{Mm}$ pathway does not account for G$\beta\gamma$ stimulation of the Rho family in many types of cells because Ras-GRF1/CDC25$^{Mm}$ is expressed only in brain. Recently, the association of G$\beta\gamma$ with an N-terminal portion of Dbl was reported (12), which may provide a link between G$\beta\gamma$ and Rho family members in non-neuronal cells. In contrast to G protein-coupled receptors, regulatory mechanisms whereby other types of receptors trigger the activation of Rho family proteins remain largely unknown.

The non-receptor tyrosine kinase ACK1 was originally characterized as a downstream target of the Rho

Abbreviations used: CRIB, Cdc42/Rac-interactive-binding; DH, Dbl homology; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; G$\alpha$, G protein $\alpha$ subunit; G$\beta\gamma$, G protein $\beta\gamma$ subunits; GEF, guanine nucleotide exchange factor; GST, glutathione-*S*-transferase; HA, hemagglutinin; HEK, human embryonic kidney; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; PAK, p21-activated kinase; PH, pleckstrin homology; PIX, PAK-interacting exchange protein; SH3, Src homology 3.

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family GTP-binding protein Cdc42, and belongs to the focal adhesion kinase family (13). However, physiological roles of ACK1 remain largely unknown. For ACK1/Cdc42 interaction, the Cdc42/Rac-interactive-binding (CRIB) domain of ACK1 plays a pivotal role (13, 14). In addition to kinase catalytic and CRIB domains, a Src homology 3 (SH3) domain and multiple proline-rich SH3 domain-binding motifs are identified within ACK1 (13, 15). Although the binding partner of the SH3 domain remains unknown, the proline-rich motifs are responsible for the binding to the adaptor protein Grb2 (15). Thus, ACK1 may also act downstream of receptor tyrosine kinases, such as the epidermal growth factor (EGF) receptor. In fact, transient increase in the tyrosine phosphorylation level of ACK1 was observed upon EGF treatment (15).

ACK2 is a second member of the Cdc42-associated tyrosine kinase family, whose overall primary structure is closely related to that of ACK1 (16). Tyrosine phosphorylation of ACK2 was stimulated by integrin $\beta$1-mediated cell adhesion in a Cdc42-dependent manner (17).

Herein, we show that Dbl is tyrosine-phosphorylated upon coexpression of activated ACK1. Following this, GEF activity of Dbl is augmented, implicating tyrosine phosphorylation as a critical event for Dbl regulation. Evidence for the activation of the Rho family within the cell is also presented.

## MATERIALS AND METHODS

*Cell culture and DNA transfection.* Human embryonic kidney (HEK) 293 and COSm6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. Plasmid DNAs were introduced into subconfluent cells by a standard calcium phosphate · DNA coprecipitation method. Following 20 h culture, cells were washed and incubated in DMEM supplemented with 1 mg/ml of bovine serum albumin for 20 h.

*Antibodies.* Mouse monoclonal antibodies against c-Myc (9E10), Flag (M2), glutathione-*S*-transferase (GST) (sc-138), and hemagglutinin (HA) (12CA5) epitopes were purchased from BAbCO, Eastman Kodak, Santa Cruz Biotechnology, and Boehringer, respectively. Rabbit antibodies against mouse IgG (55480) was purchased from Cappel. Mouse monoclonal antibodies against phosphotyrosine (PY99), and RhoA (sc-418), and a rabbit polyclonal antibody against ACK (sc-323) were purchased from Santa Cruz Biotechnology. A mouse monoclonal antibody against Rac1 (R56220) was purchased from Transduction Laboratories.

*Plasmid construction.* The expression plasmid pCMV5-ACK was described elsewhere (15). Expression plasmids for two ACK1 mutants (catalytically inactive ACK1 (K214R) and constitutively active ACK1 (L543F)) were constructed by polymerase chain reaction-based mutagenesis procedures using a site-directed mutagenesis kit (STRATAGENE). pCMV5-Myc-Dbl (12), pCMV5-Flag-Cdc42(G12V) (18), and pCMV5-Flag-RhoA(T19N) (11) were described elsewhere. pCMV5-Flag-Cdc42(G12V/T35A) was generated by mutagenizing pCMV5-Flag-Cdc42(G12V) using a site-directed mutagenesis kit (Takara). pGEX-RhoBD (an expression plasmid for the Rho-binding domain (amino acids 7–89) of mouse Rhotekin fused to GST) (19) and pGEX-PAK-CRIB (an expression plasmid for the c-Myc-tagged CRIB domain (amino acids 67–150) of rat $\alpha$ p21-activated kinase (PAK) fused to GST) (20) were kindly provided by Junji Yamauchi and Kenji Tago, respectively. All mutated nucleotides were confirmed by DNA sequencing.

*Immunoprecipitation and immunoblotting.* Immunoprecipitation and immunoblotting were performed as described elsewhere (15).

*ACK1 kinase assay.* HEK293 cells were lysed in IP buffer (50 mM Hepes-NaOH (pH 7.3), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 2 mM $MgCl_2$, 1 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 20 mM $\beta$-glycerophosphate, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin), and centrifuged at 20,000*g* for 10 min at 4°C. ACK1 was immunoprecipitated with an anti-ACK antibody conjugated to protein A-Sepharose (Amersham Pharmacia). Immunoprecipitates were washed four times with IP buffer followed by incubation in kinase reaction buffer (50 mM Hepes-NaOH (pH 7.3), 5 mM $MgCl_2$, 5 mM $MnCl_2$) containing 7.5 $\mu$g of myelin basic protein (MBP) (Sigma) and 10 $\mu$M [$\gamma$-$^{32}$P]ATP (1,850 TBq/mol) for 10 min at 30°C. Reaction was stopped by addition of 5 × Laemmli sample buffer. Proteins were separated by SDS-PAGE, and radioactivity incorporated into the substrate was quantitated.

*Nucleotide exchange assay.* HEK293 cells were lysed in IP buffer, and centrifuged at 20,000*g* for 10 min at 4°C. Myc-tagged Dbl was immunoprecipitated with the anti-Myc antibody 9E10 conjugated to protein A-Sepharose. After washing twice with IP buffer, immunoprecipitates were subjected to nucleotide exchange assays. Nucleotide exchange assays were performed essentially as described elsewhere (11) except that the reaction mixture was incubated on ice.

*Affinity precipitation of RhoA using GST-RhoBD.* Affinity precipitation of RhoA using GST-RhoBD was performed essentially as described by Ren *et al.* (19). *Escherichia coli* BL21 DE3 cells transformed with pGEX-RhoBD were grown at 30°C to early logarithmic phase. Expression of GST-RhoBD was induced by 0.5 mM isopropyl $\beta$-D-thiogalactoside for 4 h. Harvested cells were washed with wash buffer (50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl), resuspended in lysis buffer A (50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 20 $\mu$g/ml aprotinin, 1 mM $Na_3VO_4$), and disrupted by sonication. Disrupted cells were centrifuged at 25,000*g* for 20 min at 4°C, and the supernatant was stored at −80°C. Glutathione-Sepharose beads (Amersham Pharmacia) were mixed with an aliquot of the supernatant containing 20 $\mu$g of GST-RhoBD for 30 min at 4°C, and washed three times with wash buffer. COSm6 cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 $\mu$g/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 20,000*g* for 10 min at 4°C. Supernatants were incubated for 45 min at 4°C with GST-RhoBD conjugated to glutathione-Sepharose beads. Subsequently, Sepharose beads were washed four times with buffer containing 50 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 $\mu$g/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Precipitated RhoA was detected by immunoblotting using a monoclonal antibody against RhoA.

*Affinity precipitation of Rac1 using GST-PAK-CRIB.* Affinity precipitation of Rac1 using GST-PAK-CRIB was performed essentially as described by Benard *et al.* (20). Bacterially produced GST-PAK-CRIB was prepared in a similar way as described for GST-RhoBD. Glutathione-Sepharose beads were mixed with the lysate containing 10 $\mu$g of GST-PAK-CRIB for 30 min at 4°C, and washed three times with wash buffer. COSm6 cells were lysed in lysis buffer B (50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 10 mM $MgCl_2$, 200 mM NaCl, 2% (v/v) Nonidet P-40, 10% (v/v) glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 $\mu$g/ml each of leupeptin and aprotinin, and 2 mM $Na_3VO_4$), and centrifuged at 20,000 × *g* for 10 min at 4°C. Superna-

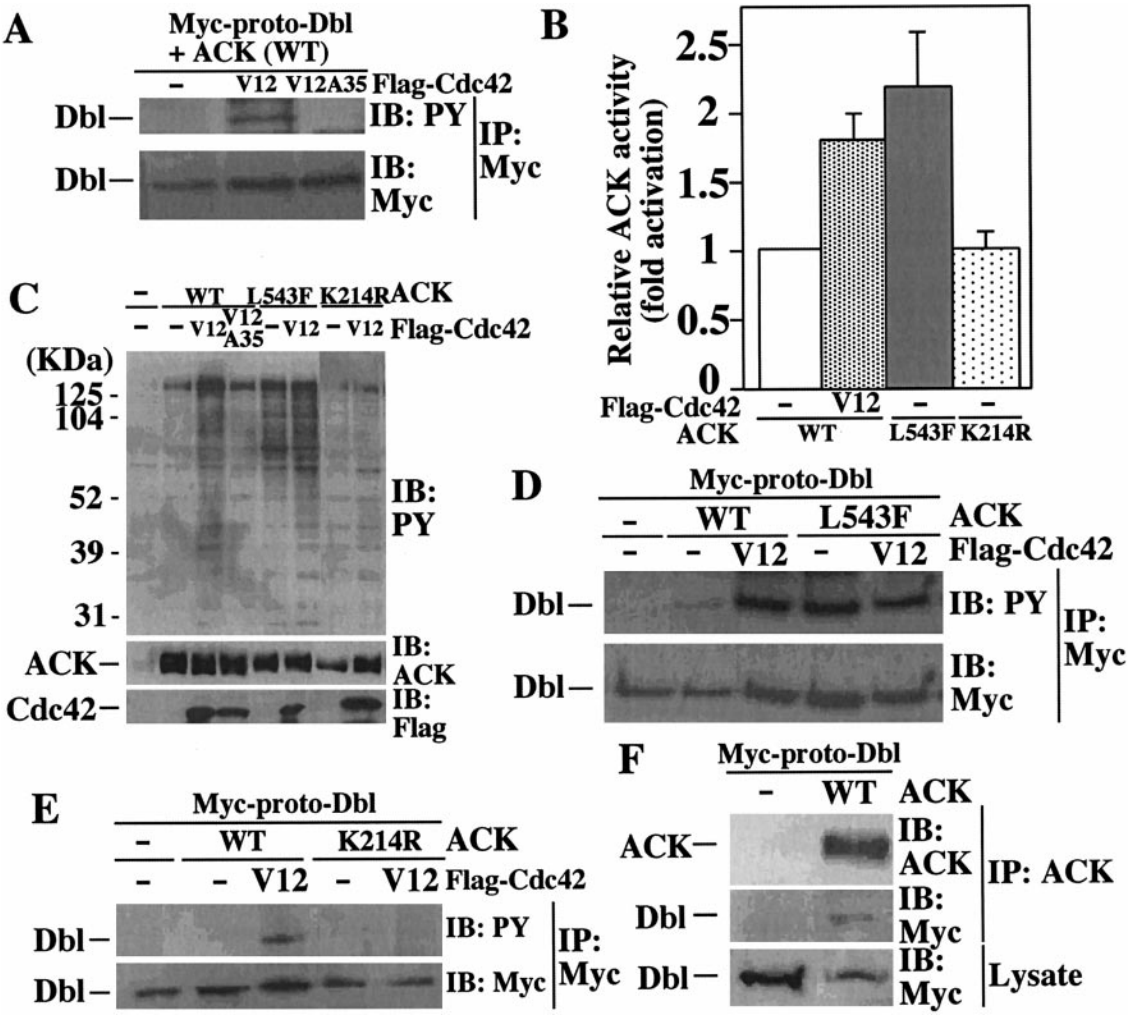


**FIG. 1.** Activated ACK1-induced tyrosine phosphorylation of Dbl. (A) Tyrosine phosphorylation of Dbl by coexpression of ACK1(WT) and Cdc42(G12V). Myc-tagged Dbl and ACK1(WT) were expressed with or without Flag-tagged Cdc42 mutants in HEK293 cells as indicated. Anti-Myc immunoprecipitates were probed with an anti-phosphotyrosine antibody (upper), and then reprobed with an anti-Myc antibody (lower). The expression level of ACK1 in each sample was virtually identical. (B) *In vitro* kinase activity of ACK1. Wild-type and mutant ACK1 proteins were expressed with or without Flag-tagged Cdc42(G12V) in HEK293 cells as indicated. Anti-ACK1 immunoprecipitates were subjected to *in vitro* kinase assays. Values are shown as means ± SE (n = 9–12). (C) Tyrosine phosphorylation of cellular proteins. Wild-type and mutant ACK1 proteins were expressed with or without Flag-tagged Cdc42 mutants in HEK293 cells as indicated. Cellular proteins soluble in IP buffer were separated by SDS-PAGE, and subjected to immunoblotting as indicated. (D) Tyrosine phosphorylation of Dbl by coexpression of constitutively active ACK1(L543F). Myc-tagged Dbl, ACK1, and Flag-tagged Cdc42 were expressed in HEK293 cells as indicated. Anti-Myc immunoprecipitates were subjected to immunoblotting as in A. (E) Inability of kinase-deficient ACK1(K214R) to tyrosine phosphorylate Dbl. Myc-tagged Dbl, ACK1, and Flag-tagged Cdc42 were expressed in HEK293 cells as indicated. Anti-Myc immunoprecipitates were subjected to immunoblotting as in A. (F) Association of Dbl with ACK1. Myc-tagged Dbl was expressed with or without ACK1(WT) in HEK293 cells. Anti-ACK immunoprecipitates were probed with an anti-Myc antibody (lower), and then reprobed with an anti-ACK antibody (upper). Representative results of at least three independent experiments are shown.

tants were incubated for 1 h at 4°C with GST-PAK-CRIB conjugated to glutathione-Sepharose beads. Subsequently, Sepharose beads were washed three times with buffer containing 25 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 30 mM $MgCl_2$, 40 mM NaCl, and 1% (v/v) Nonidet P-40, and twice with the same buffer without Nonidet P-40. Precipitated Rac1 was detected by immunoblotting using a monoclonal antibody against Rac1.

*c-Jun N-terminal kinase (JNK) 1 kinase assay.* JNK1 kinase assays were performed as described elsewhere (12).

## RESULTS

Figure 1 shows tyrosine phosphorylation of Dbl in response to activated AKC1. When activated by Cdc42(G12V), ACK1 induced tyrosine phosphorylation of co-expressed Dbl (Fig. 1A). In contrast, the effector domain mutant Cdc42(G12V/T35A), which is unable to bind and activate ACK1 (data not shown, and Fig. 1C),

exerted no effect. The upper tyrosine phosphorylated band in the second lane represents phosphorylated ACK1 that non-specifically bound to protein A-Sepharose (data not shown). We generated a constitutively active ACK1 by replacement of leucine-543 with phenylalanine on the basis of the observation that a homologous mutant (L107F) of human PAK1 exhibited constitutively enhanced kinase activity (21). Leucine-543 of ACK1, which locates adjacent to the CRIB domain, participates in the interaction with the C-terminal portion of the Switch II region of Cdc42 (14), suggesting that the L543F mutation may mimic Cdc42-dependent conformational change that is prerequisite for the induction of kinase activity. *In vitro* kinase activity of wild-type and mutant ACK1 proteins immunoprecipitated from transfected cells is shown in Fig. 1B. We used MBP as a substrate because the catalytic domain of ACK1 (13) fused to GST was able to phosphorylate MBP *in vitro* (data not shown). Eisenmann *et al.* (22) as well recently employed MBP in their ACK1 kinase assays. The kinase activity of ACK1 (L543F) was comparable to that of wild-type ACK1 activated by co-expression of Cdc42(G12V). Apparently small (only 2-fold) increase of the kinase activity may be due to a possible association with Dbl of a serine/threonine kinase(s) that phosphorylates MBP, which may cause a high basal phosphorylation level. For precise comparison of the kinase activity of wild-type and mutant ACK1 proteins, recombinant full length molecules are needed, yet we have been unsuccessful for obtaining them so far by using *E. coli* and Sf9 expression systems. The tyrosine phosphorylation pattern of cellular proteins is shown in Fig. 1C. Consistent with *in vitro* kinase activity, ACK1 activated by Cdc42(G12V) or the L543F mutation induced tyrosine phosphorylation of a wide variety of cellular proteins. The constitutively active mutant ACK1(L543F) stimulated Dbl tyrosine phosphorylation even in the absence of Cdc42(G12V) (Fig. 1D), whereas kinase-deficient ACK1 (K214R) did not (Fig. 1E). Furthermore, co-immunoprecipitation of ACK1 with Dbl was observed (Fig. 1F), implying that ACK1 may directly phosphorylate Dbl. Yet, we are currently unable to detect *in vitro* phosphorylation of Dbl by the ACK1 kinase catalytic domain (13) because of insolubility of the recombinant Dbl protein.

GEF activity of Dbl towards RhoA and Cdc42 is shown in Fig. 2. Transiently expressed Dbl was immunoprecipitated, and subjected to GDP binding and release assays. Expression levels of Dbl were identical among samples in each experiment as evidenced by immunoblotting (data not shown). When tyrosine phosphorylated by co-expression of ACK1(WT) plus Cdc42(G12V) or ACK1(L543F), GEF activity of Dbl towards RhoA significantly increased (Fig. 2A). This increase is likely to be ascribed to enhancement of the specific activity, but not the amount of Dbl immunoprecipitated because the amount remained unaffected upon co-expression of ACK1 and Cdc42 (Fig. 2B). Time courses of GDP exchange of RhoA are described in Figs. 2C and 2D, respectively. GEF activity toward Cdc42

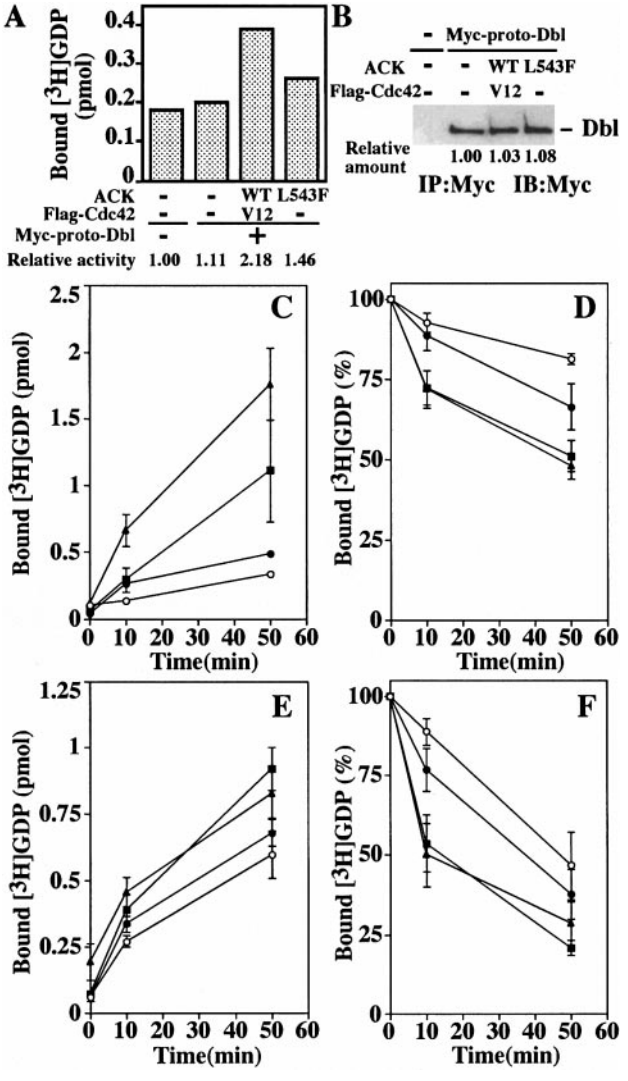


**FIG. 2.** Effect of tyrosine phosphorylation of Dbl on its GEF activity. (A) Enhanced GEF activity toward RhoA of ACK1-phosphorylated Dbl. [$^3$H]GDP binding to RhoA was measured after incubation with anti-Myc immunoprecipitates for 10 min on ice. (B) The amounts of immunoprecipitated Dbl. The amounts of immunoprecipitated Dbl from lysates used in Fig. 2A were determined by immunoblotting with an anti-Myc antibody. Densitometry analysis was performed using NIH Image. (C–F) Time courses of [$^3$H]GDP binding and release reactions. Anti-Myc immunoprecipitates from HEK293 cells transfected with empty vector (open circle), or plasmids for Myc-Dbl (filled circle), Myc-Dbl, ACK1(WT), and Flag-tagged Cdc42(G12V) (filled triangle), or Myc-Dbl and ACK1(L543F) (filled square) were subjected to [$^3$H]GDP binding (C and E) or [$^3$H]GDP release (D and F) assays for RhoA (C and D) or Cdc42 (E and F). Values are shown as means ± SE (n = 6).

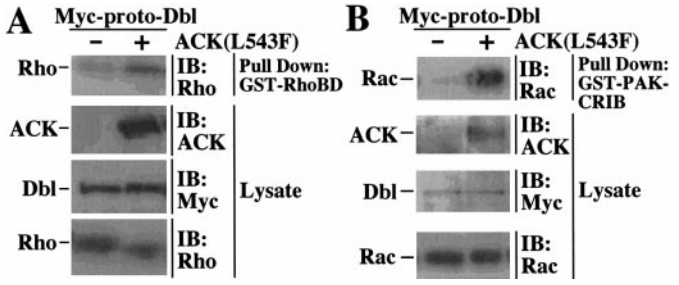


**FIG. 3.** Formation of the GTP-bound form of RhoA and Rac1 upon ACK1-induced Dbl activation. (A) Formation of RhoA · GTP. Endogenous RhoA · GTP was precipitated with GST-RhoBD from COSm6 cells expressing Myc-Dbl with or without ACK1(L543F), and detected by immunoblotting using an anti-RhoA antibody. Cell lysates were subjected to immunoblotting as indicated. (B) Formation of Rac1 · GTP. Endogenous Rac1 · GTP was precipitated with GST-PAK-CRIB from COSm6 cells expressing Myc-Dbl with or without ACK1(L543F), and detected by immunoblotting using an anti-Rac1 antibody. Cell lysates were subjected to immunoblotting as indicated. Representative results of at least three independent experiments are shown.

was also enhanced by tyrosine phosphorylation although the effect appears less remarkable in comparison with RhoA because of the high spontaneous exchange rate of Cdc42 (Figs. 2E and 2F).

To obtain further evidence for the activation of Dbl in response to ACK1-induced tyrosine phosphorylation, we employed affinity precipitation assays, which allow detecting the GTP-bound form of small GTP-binding proteins within the cell. Figure 3A shows the formation of the GTP-bound form of endogenous RhoA following co-expression of Dbl and ACK1(L543F), which parallels the increase of *in vitro* GEF activity. Likewise, the GTP-bound form of endogenous Rac1 considerably increased (Fig. 3B). In the case of Dbl alone, Rho · GTP and Rac · GTP levels were comparable to the control level in mock-transfected cells (data not shown). We were unable to measure the GTP-bound form of Cdc42 because ectopically expressed ACK1 interfered with the interaction between GST-PAK-CRIB and Cdc42 · GTP. Taken together, activated ACK1 tyrosine phosphorylates Dbl thereby stimulating its GEF activity towards all members of the Rho family.

JNK1 is a serine/threonine kinase regulated downstream of Rac and Cdc42. JNK1 activation following ACK1-dependent tyrosine phosphorylation of Dbl is illustrated in Fig. 4. Without Dbl, ACK1 did not augment JNK1 activity, while expression of Dbl alone or Dbl plus inactive ACK1 caused weak JNK1 activation. Tyrosine phosphorylation of Dbl by ACK1(L543F) lead to a significant increase in JNK1 activity as shown in Fig. 4A, consistent with enhanced GEF activity. The increased JNK1 activity was entirely sensitive to dominant-negative action of RhoA(T19N) (Fig. 4B).

## DISCUSSION

We demonstrate that GEF activity of Dbl towards Rho family proteins is enhanced upon ACK1-dependent tyrosine phosphorylation. Likewise, accumulating evidence has indicated that several Dbl family GEFs are activated following phosphorylation. GEF activity of Vav towards Rac becomes evident upon tyrosine phosphorylation by specific kinases such as Lck, whereas it is latent without stimulation (23, 24). Vav-2, a protein structurally related to Vav, exhibits GEF activity toward Rho in response to tyrosine phosphorylation (25). Also, G$\beta\gamma$ signals induce Rac-GEF activity of Ras-GRF1/CDC25$^{Mm}$ in a tyrosine phosphorylation-dependent manner (11). Furthermore, Rac-GEF activity of Tiam1 is augmented upon threonine phosphorylation by $Ca^{2+}$/calmodulin-dependent protein kinase II (26). In addition to Dbl family members, the Rap1-GEF C3G is also activated upon tyrosine phosphorylation (27). In view of these observations, GEF activity of other Dbl family members is possibly directed by phosphorylation. However, phosphorylation may not be a sole regulatory mechanism because multiple motifs responsible for protein-protein interaction are found in Dbl family GEFs. Rather, it is feasible that a variety of signaling molecules associated with

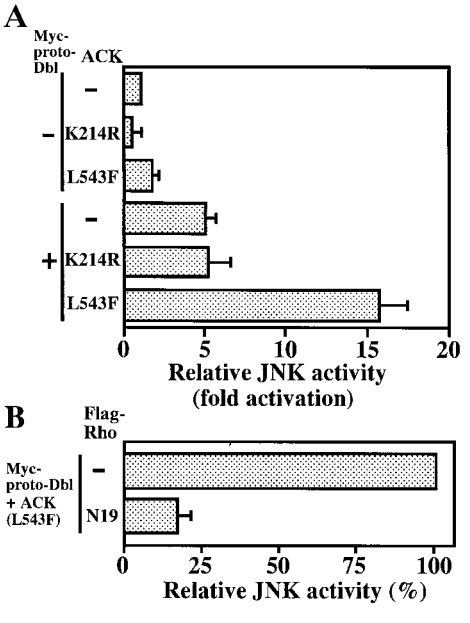


**FIG. 4.** JNK1 activation upon ACK1-induced Dbl activation. (A) Synergistic activation of JNK1 upon coexpression of Dbl and constitutively active ACK1(L543F). Kinase activities in anti-HA immunoprecipitates from HEK293 cells expressing HA-JNK1 with Dbl and ACK1 as indicated were measured. Relative activities are shown as means ± SD (n = 3–6). (B) Effect of dominant-negative RhoA(T19N) on JNK1 activation. Kinase activities were measured as in A. Relative activities are shown as means ± SD (n = 3–6).

Dbl family members also play a role in regulating the activity. Indeed, Rac-GEF activity of Vav is further modulated through the interaction with substrates and products of phosphatidylinositol 3-kinase (28). Furthermore, G$\beta\gamma$ bind to the N-terminal region of Dbl, thereby regulating its activity in the cell (12).

The activation of Dbl following ACK1-dependent tyrosine phosphorylation suggests a possible functional link between Cdc42 and other Rho family members Rho and Rac. A signaling cascade consisting of Cdc42, Rac, and Rho that regulates actin cytoskeletal structures in fibroblast cells has been proposed (1). Signal transduction downstream of Cdc42 in this cascade may explain, at least in part, by the ACK1/Dbl-mediated mechanism. Additionally, elevated GEF activity of Dbl towards Cdc42 may have implications for a positive feedback mechanism of the Cdc42 pathway. Similarly, a potential feedback loop is predicted in the PAK/PAK-interacting exchange protein (PIX) signaling system (29). PAKs have been implicated in multiple cellular processes downstream of Rac and Cdc42 (30). $\alpha$ and $\beta$ isoforms of PIX (also termed Cool-2 and -1, respectively) were isolated as a protein that binds to PAK (31, 32). PIX possesses DH/PH domains, and in fact, exhibits GEF activity towards Rho family proteins, leading to the formation of the GTP-bound form (31). PAK kinase activity was repressed upon binding of the p50 splice variant of $\beta$PIX (32), whereas $\alpha$PIX stimulates the activity (33). Conversely, PAK may modulate GEF activity of PIX within the cell by triggering conformational alteration upon binding and subsequent phosphorylation, suggesting mutual regulations.

EGF induces tyrosine phosphorylation of ACK1 presumably through Grb-2 mediated complex formation (15). Thus, it is likely that EGF receptor-mediated activation of Rho family proteins may be relayed, at least in part, by the EGF receptor/Grb2, Cdc42/ACK1/Dbl pathway. In addition to EGF receptor signaling, a variety of extracellular stimuli including temperature shift-down, high osmolarity (15), bradykinin stimulation (16), and integrin-mediated cell adhesion signals (16, 17) cause tyrosine phosphorylation of ACK1/2. Subsequently, activated ACK1 is expected to phosphorylate and activate Dbl, which in turn stimulates Rho family members. Hence, ACK1-dependent Dbl activation may be implicated in diverse signaling pathways that regulate Rho family proteins. Future studies will further reveal the biological significance of ACK1-induced Dbl activation.

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