# Transforming Potential of Dbl Family Proteins Correlates with Transcription from the Cyclin D1 Promoter but Not with Activation of Jun NH$_2$-terminal Kinase, p38/Mpk2, Serum Response Factor, or c-Jun*

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The *dbl* family of oncogenes encodes a large, structurally related, family of growth-regulatory molecules that possess guanine nucleotide exchange factor activity for specific members of the Rho family of Ras-related GTPases. We have evaluated matched sets of weakly and strongly transforming versions of five Dbl family proteins (Lfc, Lsc, Ect2, Dbl, and Dbs) to determine their ability to stimulate signaling pathways that are activated by Rho family proteins. We found that the transforming potential of this panel did not correlate directly with their ability to activate Jun NH$_2$-terminal kinase, p38/Mpk2, serum response factor, or c-Jun. In contrast, transient stimulation of transcription from the cyclin D1 promoter provided a strong correlation with transforming potential, and we found constitutive up-regulation of cyclin D1 protein in Dbl family protein-transformed cells. In addition, we observed that at least two Dbl family members (Lfc and Ect2) induced changes in the actin cytoskeleton and exhibited nuclear signaling profiles that are consistent with a broader range of *in vivo* substrate utilization than is predicted from their *in vitro* exchange specificities. In summary, although Dbl family proteins exhibit signaling profiles that are consistent with their *in vivo* activation of Rho proteins, stimulation of cyclin D1 transcription is the only activity that correlates with transforming potential, thus suggesting that deregulated cell cycle progression may be important for Dbl family protein transformation.

The Dbl-related proteins are a large family of growth-regulatory molecules that function as guanine nucleotide exchange factors (GEFs)[1] and activators of specific members of the Rho family of proteins (reviewed in Refs. 1 and 2). Mammalian members of the Dbl family include: Tiam1, a protein involved in T cell lymphoma invasiveness (3); Bcr, the translocation partner of the Abl tyrosine kinase in Philadelphia chromosome positive human leukemias (4); Fgd1, a gene product associated with the development of Aarskog-Scott syndrome (5); Sos1/2 and RasGRF, two known activators of Ras proteins (6–8); and the products of the *dbl* (9), *vav* (10), *ect2* (11), *lbc* (12), *lfc* (13), *lsc* (14), *ost* (15), *dbs* (16), *net1* (17), and *tim* (18) oncogenes. Typically, the deregulated expression of Dbl family members in NIH 3T3 fibroblasts results in changes in the morphological and proliferative properties of these cells, conferring upon them a highly transformed phenotype (1, 2).

The region of sequence similarity that defines a Dbl family member consists of an approximately 200 amino acid Dbl homology (DH) domain, which is novel to this family of proteins, followed immediately by a pleckstrin homology (PH) domain. Because most members of the Dbl family share little structural similarity outside of the tandem DH and PH domains, it is presumed that these structural motifs contain sequences that are responsible for the transforming activity. Indeed, recent studies have shown that the transforming activity of several Dbl family members is dependent upon the structural integrity of their DH/PH domain modules (9, 11, 13–16).

Although the mechanism by which the deregulated expression of a Dbl family member induces a transformed phenotype has not yet been resolved, there is some evidence that they may do so through interaction with members of the Rho family of Ras-related GTPases (19–23). The Rho family of proteins comprises at least 12 distinct members including RhoA, RhoB, RhoC, RhoD, RhoE, RhoG, Rac1, Rac2, CDC42Hs, TC10, and TTF (24). Rho proteins function as regulated molecular switches, cycling between a biologically active GTP-bound and an inactive GDP-bound form (19). They are activated by specific Rho GEFs that promote the formation of GTP-bound complexes and are inactivated by specific GTPase-activating proteins that stimulate their intrinsic rate of GTP hydrolysis. Dbl family members have been shown to have GEF activity specific for Rho family members (15, 25–30), and, in several instances, this activity has been mapped to the DH/PH domains (30, 31). Because the constitutive activation of several Rho family mem-

[1] The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; JNK, Jun NH$_2$-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SRF, serum response factor; HA, hemagglutinin; PAE, porcine aortic endothelial; GST, glutathione *S*-transferase; CMV, cytomegalovirus; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.

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bers has been shown to be transforming (32–35), Dbl-related proteins may exert their transforming activity via constitutive activation of Rho function.

Until recently, Rho family members were thought to be primarily involved in the organization of actin-based cytoskeletal structures (36–39), and it was generally assumed that these biological activities were, at least in part, mediating the oncogenicity of these proteins. CDC42Hs stimulates the formation of actin microspikes and filopodia, whereas Rac1 causes membrane ruffling and lamellipodia formation, and RhoA regulates the formation of actin stress fibers. More recently, evidence has emerged linking Rho family proteins, and by extension the exchange factors that activate them, to the regulation of gene expression. First, constitutively activated Rac1 and CDC42Hs are activators of JNKs (also known as stress-activated protein kinases) and p38/Mpk2, but not the p42 and p44 ERKs (40–43). ERKs, JNKs, and p38 proteins represent distinct subsets of the mitogen-activated protein kinase (MAPK) family. Upon activation, JNK in turn activates the ATF-2 and Jun nuclear transcription factors, which can dimerize with other transcription factors to stimulate transcription from promoters containing AP-1 and related DNA sequences (*e.g.* the c-*jun* promoter) (44). Stimulation of p38 activity results in activation of the ATF-2 transcription factor (45). Second, RhoA, Rac1, and CDC42Hs have been shown to activate SRF (46), which cooperates with ternary complex factors (Elk-1 and SAP1) and the DNA elements found in certain promoters such as the c-*fos* promoter (47). Finally, a direct link between RhoA, Rac1, and CDC42Hs and cell cycle progression has been made (42), and this may be mediated in part by regulating the expression of cyclin D1 (48).

Although the repertoire of signaling pathways that are responsive to activation by Rho family members is rapidly expanding, relatively little is known of the signaling activities of their putative regulators, the Dbl-related proteins. Not only is it unclear whether or not Dbl and Rho proteins can activate the same signaling pathways, it remains uncertain whether they transform cells via the same mechanism. The focus-forming activity of Dbl family members often far exceeds that of activated mutants of their putative GTPase targets, suggesting that the biological pathways that are mediated by these two families of proteins may not precisely overlap (1). However, the recent determination that several Dbl family members (Fgd1, Dbl, Ost, and Vav) are potent activators of JNK in transient assays suggests that this family of oncoproteins, like their putative GTPase targets, may regulate multiple nuclear signaling pathways (40, 49, 50).

In the present study, we have examined a panel of five Dbl family members (Ect2, Lfc, Lsc, Dbs, and Dbl) for their ability to regulate the transduction of signals to the nucleus as measured by (*a*) stimulation of JNK and p38/Mpk2 MAPK activity, (*b*) stimulation of c-Jun and SRF transcriptional activation, and (*c*) regulation of cyclin D1 expression. By utilizing matched pairs of strongly and weakly transforming mutant derivatives, we were able to assess whether the strength of the signaling activity correlated with transforming potential. We observed that Dbl family proteins typically exhibited a broad range of signaling activities consistent with their activation of Rho family proteins *in vivo*. Of the different signaling activities analyzed, stimulation of cyclin D1 transcription was the only activity that correlated well with transforming potential, thus suggesting that deregulated cell cycle progression may be important for transformation. Finally, we observed that two of the panel members (Ect2 and Lfc) induced changes in the actin cytoskeleton, and exhibited signaling profiles that were consistent with a broader range of *in vivo* substrate utilization than was predicted from their *in vitro* GEF specificity.

## EXPERIMENTAL PROCEDURES

*Expression Vectors and Reporter Plasmids*—The pCTV3H, pCTV3HA, and pAX142 mammalian expression vectors have been described previously (13). Initially, constructs that place an in-frame epitope from the hemagglutinin (HA) protein of influenza virus at the $NH_2$ termini of the various Dbl family members were made by subcloning into the *Hpa*I site of pCTV3HA as described below. All constructs were then digested with *Mlu*I/*Sun*I and shuttled into corresponding sites in the pAX142 mammalian expression vector. Unless otherwise indicated, all assays were performed using the pAX142 derivatives where expression is controlled by the EF-1α promoter. All fragments that were synthesized by polymerase chain reaction were sequenced in their entirety to ensure that only specified mutations had occurred.

Lfc-D13HA, Lfc-P3HA, Lfc-D6, and Lfc-D7 have been described previously (13). Lfc-D7HA was made by isolating the *Mlu*I/*Sun*I fragment from Lfc-D7, filling in the *Mlu*I site with T4 DNA polymerase, and inserting the fragment into pCTV3HA digested with *Hpa*I/*Sun*I. Lfc-D6HA was made by replacing the *Fsp*I/*Sun*I fragment of Lfc-D13HA with the *Fsp*I/*Sun*I fragment of Lfc-D6.

Dbs-HA1 was made by shuttling the *Fsp*I/*Sun*I fragment of the TL19–10c2 cDNA (16) into pCTV3HA cut with *Hpa*I/*Sun*I. Dbs-HA2 was made by isolating the *Nsi*I fragment from TL19–10c2, blunting the fragment with T4 DNA polymerase, and ligating it to pCTV3HA digested with *Hpa*I. Dbs-HA6 was made by replacing the *Fsp*I/*Sun*I fragment of Dbs-HA2 with the *Fsp*I/*Sun*1 fragment of Dbs-HA1.

Ect2-HA1 and Ect2-HA2 were made by isolating the *Xba*I (HA1) and *Xba*I/*Xho*I (HA2) fragments from the TL17–5cA3 cDNA (51), filling in the ends of these fragments with T4 DNA polymerase, and ligating them to pCTV3HA cut *Hpa*I. Ect2-HA3 was made by replacing the *Pfl*MI/*Sun*I fragment of Ect2-HA1 with the *Pfl*MI/*Sun*I fragment from TL17–5cA3.

As an initial step in constructing HA-tagged Dbl constructs, the *Bam*HI fragment was isolated from pZIP-proto-*dbl* (52), blunted with T4 DNA polymerase and ligated to pCTV3H digested with *Hpa*I (pCTV3H-proto-*dbl*). In order to make Dbl-HA1, primers Dbl1 and Dbl2 (Dbl1: ACG GAT CCA CGC GTC CCG GGC CAC CAT GGA CAA TGG CAA TAG CTT G; Dbl2: ACG GAT CCA TGC ATA GAA TCA TTA AC) were used to amplify a 609-base pair *dbl* fragment using pCTV3H-proto-*dbl* as template. This fragment was ligated into the *Eco*RI site of pBS-SK+ (Stratagene). After sequencing, the fragment was isolated from the vector using *Sma*I and ligated to pCTV3HA digested with *Hpa*I (Dbl-HA3). The *Fsp*I fragment from pCTV3-proto-*dbl* was then isolated and ligated into the *Fsp*I site of Dbl-HA3. In order to make Dbl-HA2, primers Dbl2 and Dbl3 (Dbl3: ACG AAT TCA CGC GTC CCG GGC CAC CAT GGC AGA AGC AAA TCC CCG GAG A) were used to amplify a 2064-base pair fragment using pCTV3H-proto-*dbl* as template. This fragment was ligated into the *Eco*RI site of pBS-SK+. Following sequencing, the fragment was isolated using *Eco*RV/*Sma*I and ligated to pCTV3HA digested with *Hpa*I (Dbl-HA4). Dbl-HA2 was made by replacing the *Psp*1406I/*Sun*I fragment of Dbl-HA4 with the *Psp*1406I/*Sun*I fragment from pCTV3H-proto-*dbl*.

Lsc-D7 and Lsc-D3 have been described previously (14). Lsc-D7HA was made by isolating the *Hpa*I/*Sun*I fragment from Lsc-D7 and ligating into pCTV3HA cut *Hpa*I/*Sun*I. This construct was then cut with *Mlu*I/*Hpa*I, and a linker was inserted to remove the termination codon within the *Hpa*I site. Lsc-D3HA was made by replacing the *Fsp*I/*Sun*I fragment of Lsc-D7HA with the *Fsp*I/*Sun*I fragment from Lsc-D3.

Reporters for the analysis of cyclin D1 activity (CD1(-963)-Luc), c-Jun activity (5XGal-Luciferase and Gal4-Jun-(1–254)), and SRF activity ((SREm)₂-Luc) have been described previously (48, 53, 54). FLAG epitope-tagged expression vectors for JNK1 and p38/Mpk2, and the bacterial expression vectors encoding GST-ATF2 and GST-Jun-(1–79) were provided by M. Karin. pCMVnlac encodes the sequences for the β-galactosidase gene under the control of the CMV promoter (provided by J. Samulski).

*Cell Culture, Transfection, and Transformation Assays*—COS-7 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose) supplemented with 10% fetal bovine or newborn calf serum, respectively. Transfection of COS-7 cells was achieved with the LipofectAMINE reagent (Life Technologies, Inc.) as described by the manufacturer. Thirty h after transfection, the medium was changed to DMEM containing 0.5% fetal bovine serum, and after 14 h of incubation lysates were prepared as described previously (45). NIH 3T3 cells were transfected by calcium phosphate co-precipitation as described previously, allowed to recover for 30 h, and starved in DMEM with 0.5% newborn calf serum for 14 h before lysate preparation (55–57). Focus-formation assays were performed in NIH 3T3 cells exactly as described

(55). Cognate empty vectors of each plasmid were employed as controls.

NIH 3T3 cell lines that stably express the pCTV3HA derivatives of Dbl-HA1, Dbs-HA6, Ect2-HA3, and Lsc-D7HA were generated by calcium phosphate co-precipitation, followed by selection for 14 days in growth medium supplemented with hygromycin B (200 $\mu$g/ml).

*Transient Expression Reporter Gene Assays*—Analysis of luciferase expression in transiently transfected NIH 3T3 cells was performed as described using enhanced chemiluminescent reagents and a Monolight 2010 luminometer (Analytical Luminescence, San Diego CA) (56). $\beta$-Galactosidase activity in transiently transfected NIH 3T3 cells was determined exactly as described (58). All assays were performed in duplicate. Data shown are from one experiment performed in duplicate ($\pm$ S.E.), and are representative of at least three independent experiments.

*Immunoprecipitation and in Vitro Kinase Assays*—JNK1 and p38/Mpk2 activity was analyzed in COS-7 cells following transfection of FLAG-tagged JNK1 or p38/Mpk2 and the various tester constructs. Cells were transfected in 100-mm plates and starved for 14 h, and then lysates were collected in 1 ml of lysis buffer containing protease and phosphatase inhibitors (45). JNK1 or p38/Mpk2 was immunoprecipitated with anti-FLAG (M2; Kodak/IBI) antibody as indicated, and the kinase activity was measured using 2 $\mu$g of GST-Jun-(1–79) or GST-ATF2-(1–254), respectively, as substrate. *In vitro* kinase reactions were carried out for 20 min at 30 °C, and stopped with 2× SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins were fractionated on 12.5% SDS-PAGE and blotted to Immobilon (Millipore). Blots were dried and exposed to film for 1–3 h and then subjected to quantitation on a PhosphorImager (Molecular Dynamics). Blots were subsequently probed with anti-FLAG antibody (M2; Kodak/IBI) to visualize expression of the p38/Mpk2 or JNK1 construct. A 1/10 volume of the lysate used in the immunoprecipitations was fractionated on 12.5% SDS-PAGE and probed with anti-HA antibody (BAbCO) to visualize Dbl family protein expression levels in the lysate. Following incubation with horseradish peroxidase-labeled anti-mouse secondary antibodies, Western blots were developed with ECL reagents (Amersham Pharmacia Biotech).

The abundance of cyclin D1 protein in stably selected cell lines was determined by Western blot analysis as described previously (53) using a monoclonal cyclin D1 antibody (HD11; Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase-labeled anti-mouse secondary antibodies, and protein was visualized with ECL (Amersham Pharmacia Biotech). Blots were subsequently probed with an anti-$\alpha$-tubulin antibody (5H1; Ref. 59) to establish an internal control for protein levels.

*Analysis of Actin Stress Fiber and Lamellipodia Formation*—Analysis of actin stress fibers and lamellipodia was performed as described previously (60). Briefly, porcine aortic endothelial (PAE) cells were injected in the nucleus with pAX142-*lfc-D7HA*, pAX142-*lfc-D13HA*, pAX142-*ect2-HA3*, or pAX142-*ect2-HA2* (25 $\mu$g/ml). Subsequently, cells were starved in serum-free growth medium for 12–14 h and fixed in 4% formaldehyde as described previously (60). Expressed HA-tagged Dbl proteins were visualized by indirect immunofluorescence using anti-HA antibodies, and polymerized actin was stained with phalloidin.

## RESULTS

*Functional Expression of Epitope-tagged Dbl Family Members*—Typically, NIH 3T3 cells that have been transformed by Dbl family members form foci comprising rounded, piled-up, non-refractile cells and promote tumor growth when injected subcutaneously into nude mice (1). To assess the relationship between this transforming activity and the activation of nuclear signaling pathways, matched pairs of strongly and weakly transforming derivatives of Ect2, Lfc, Lsc, Dbl, and Dbs were constructed in the mammalian expression vector pAX142. The weakly transforming derivatives were selected from large panels of mutants for each family member, and the criteria used for selection was high level of expression relative to their strongly transforming counterparts. For Dbl, we utilized the weakly transforming, full-length versions of the protein, whereas for Lfc, Lsc, Dbs, and Ect2, weakly transforming derivatives were generated by deleting portions of their respective PH domains. Additional Dbs, Lfc, and Ect2 variants were included in the panel that exhibited intermediate levels of transforming activity. All proteins tested retained intact DH domains and most retained at least some biological activity as
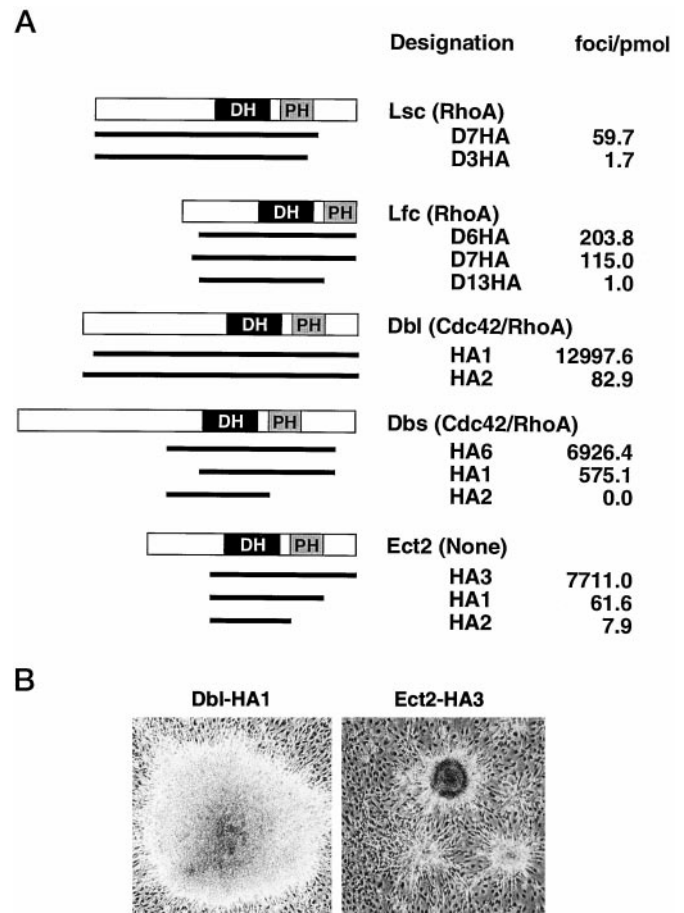


Fig. 1. **Domain structure and focus-forming activities of epitope-tagged derivatives of Dbl family members.** *A*, the domain structure of each Dbl family member is shown in the *upper line* of each matched set of derivatives (drawn approximately to scale), and the *lines below* indicate the regions of the protein included in predicted translational products of the various cDNA constructs. The tandem DH/PH domain modules are indicated by *shaded boxes*. All panel members have been tested previously for *in vitro* exchange activity for RhoA, Rac1, and CDC42Hs (1), and a summary of these results is indicated in *parentheses*. All members of the panel were assayed for transforming potential in an NIH 3T3 cell focus-formation assay. NIH 3T3 cells were transfected with 3 $\mu$g of the indicated expression vector, and cultured for 14 days before foci were counted. The data are expressed as foci per picomole of DNA and represent the average of three independent experiments. *B*, morphology of transformed foci of cells induced in NIH 3T3 cells by transfection of Dbl-HA1 and Ect2-HA3. NIH 3T3 cells were transfected with 3 $\mu$g of DNA as described above, and foci were photographed after 14 days.

evidenced by low, but reproducible focus-forming activity (Fig. 1*A*). All proteins also contained an NH$_2$-terminal HA epitope tag to verify that each exhibited detectable levels of expression in transient assays utilizing COS-7 cells (Figs. 2 and 3).

Initially, all members of the panel were assayed for transforming potential in an NIH 3T3 focus-formation assay (Fig. 1*A*). Transforming activity varied by as much as 200-fold among family members, and was due to intrinsic levels of transforming activity rather than to variability in levels of expression of the constructs (Figs. 2 and 3). For example, the strongly transforming versions of Ect2, Dbl, and Dbs showed the highest focus-forming activity ($>6 \times 10^3$ focus-forming units/pmol) and were more than 100-fold higher than their weakly transforming counterparts. Interestingly, two distinct focus morphologies were observed among panel members (Fig. 1*B*), suggesting that the transforming pathways utilized by Dbl family proteins may differ. Dbs and Dbl caused foci that were characteristically large and diffuse, whereas Ect2, Lfc, and Lsc
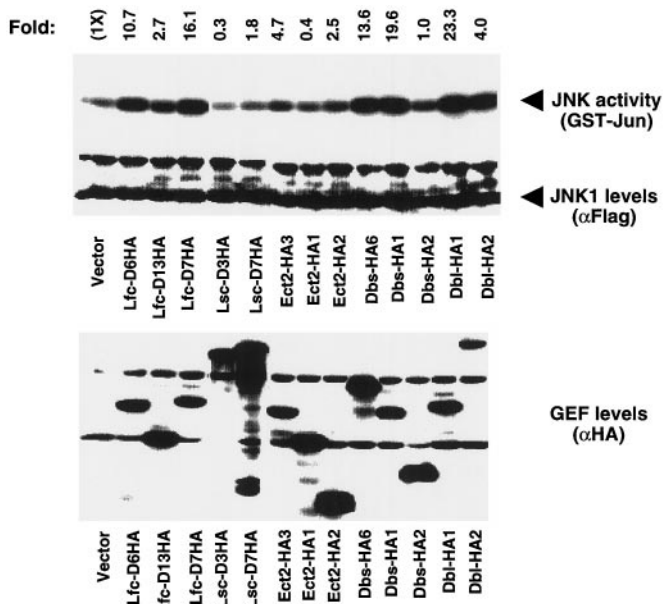
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FIG. 2. **Activation of JNK1 by Dbl family proteins.** COS-7 cells were transfected with a plasmid expressing FLAG epitope-tagged JNK1 together with the indicated HA epitope-tagged Dbl family derivative or the cognate empty vector (pAX142). Cells were serum-starved for 14 h, and JNK1 was immunoprecipitated from lysates for analysis in immune complex kinase assays with GST-Jun-(1–79) as substrate. Kinase reactions were subjected to SDS-PAGE, transferred to Immobilon filters, and exposed to film (*top panel*). Phosphorylation of GST-Jun was determined by phosphoimage analysis, and is expressed relative to the level obtained in JNK1+vector-transfected cells (*Fold*). Membranes were subsequently probed with anti-FLAG antiserum to visualize JNK1 levels in the immunoprecipitates (*middle panel*). GEF levels were determined by Western blotting and probing with antibodies directed against the HA epitope (*αHA*) (*lower panel*). Data shown are representative of three independent experiments.

foci were substantially smaller, more compact, and formed networks of interconnected foci that appeared to be of clonal origin.

*Activation of JNK1 by Dbl Family Proteins*—The JNK family of MAPKs comprises closely related enzymes that are activated by cellular stress (61). The recent observation that several members of the Rho (CDC42Hs, Rac1) and Dbl (Ost, Dbl, Fgd1, and Vav) families can activate the JNK signaling pathway prompted us to explore whether JNK activation by Dbl-related proteins correlates with their transforming potential. We examined strongly and weakly transforming derivatives of five Dbl family members (Lfc, Lsc, Dbl, Dbs, and Ect2) for their ability to activate JNK1 in an *in vitro* kinase assay (Fig. 2). This panel consisted only of Dbl family proteins that are known to be transforming in NIH 3T3 cells and for which the *in vitro* exchange specificities for RhoA, Rac1, and CDC42Hs have been determined (Fig. 1*A*).

When transfected into COS-7 cells, all members of the panel were detectably expressed at roughly equivalent levels as judged by Western blot analysis with the anti-HA antibody (Fig. 2, *lower panel*). Immunocomplex kinase assays using GST-Jun as a substrate indicated that transforming derivatives on the panel consistently induced 1.8–23-fold increases in the *in vitro* kinase activity of JNK1 (Fig. 2, *upper panel*; summarized in Table I). Although this activity was generally impaired among the more weakly transforming variants, the degree of impairment did not always reflect relative transforming potencies. For example, Lfc-D7HA exhibited a higher level of activation of JNK1 than the more strongly transforming Lfc-D6HA, and there was no correlation between JNK1 activation and transforming activity for the three Ect2 variants. A com-
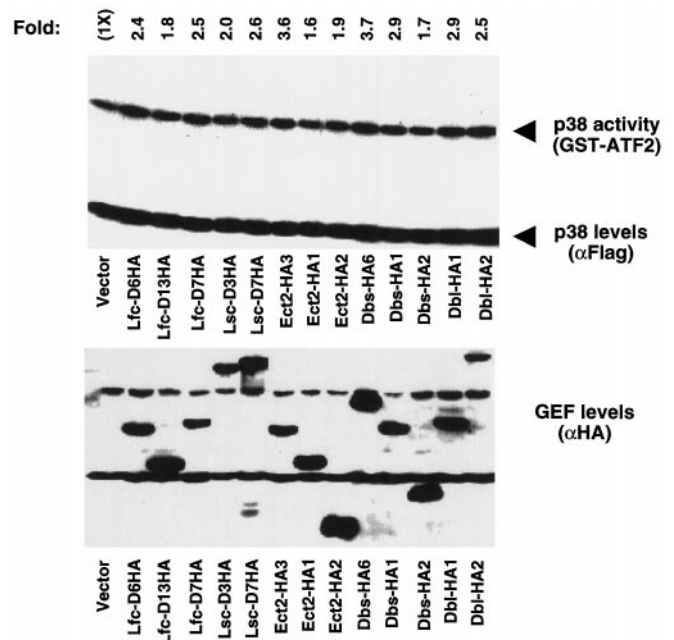


FIG. 3. **Activation of p38/Mpk2 MAPK by Dbl family proteins.** COS-7 cells were transfected with an expression vector encoding FLAG epitope-tagged p38/Mpk2 along with the indicated member of the panel. Cells were serum-starved for 14 h, and p38/Mpk2 was immunoprecipitated from lysates for analysis in complex kinase assays with GST-ATF2 as substrate (*top panel*). Phosphorylation of ATF2 was measured by phosphoimage analysis as in Fig. 2. Membranes were subsequently probed with anti-FLAG antiserum to visualize p38/Mpk2 levels in the immunoprecipitates (*middle panel*). GEF levels were determined by Western blotting as described in Fig. 2 (*lower panel*). Data shown are representative of three independent experiments.

parison between the different family members revealed similar inconsistencies. For example, Ect2-HA3 was 65-fold more efficient than Lfc-D7HA at inducing foci but showed consistently lower levels of JNK1 activity (4.7-fold *versus* 16.1 fold). We conclude that the transforming potency of a Dbl family member is not a reflection of its ability to stimulate JNK1 activity.

*Activation of p38/Mpk2 by Activated Dbl Family Proteins*— Our determination that Dbl family members consistently cause activation of JNK prompted us to examine whether Dbl family proteins could activate other MAPK-regulated signaling pathways. p38/Mpk2 forms an additional branch of the mammalian MAPK family that is regulated by many of the same upstream stimuli as JNK and for which activity can be regulated by Rho family GTPases (43). To examine the effect of Dbl family proteins on p38/Mpk2 activity, we co-transfected the panel members with a FLAG epitope-tagged p38/Mpk2 expression vector. Immunocomplex kinase assays using GST-ATF2 as a substrate indicated that transforming derivatives from the panel consistently induced 2.5–4-fold increases in the *in vitro* phosphorylating activity (Fig. 3). Although the more weakly transforming derivatives were consistently impaired in p38 activation, the degree of impairment did not always reflect the observed differences in transforming activity. For example, Dbl-HA1 and Dbl-HA2 exhibited comparable levels of p38 activation (2.9-*versus* 2.5-fold) yet differed by over 150-fold in focus-forming activity (Fig. 1*A*). As was the case with JNK1 activation, a comparison between the various family members on the panel did not reveal a good correlation between transforming potency and p38 activation. Lsc-D7HA and Dbl-HA1 showed similar levels of p38 activation (2.6- *versus* 2.9-fold); however, Dbl-HA1 is almost 200-fold more efficient than Lsc-D7HA at inducing foci. Thus, although Dbl family members share a common ability to activate p38, we have not seen a good correlation

TABLE I
*Properties of Dbl family guanine nucleotide exchange factors*

Relative activities are based on the data sets shown in the manuscript
and are representative of experiments performed in triplicate.

| Protein | Relative focus-formation$^a$ | Gal-Jun$^b$ | Jnk$^c$ | p38$^d$ | SRF$^e$ | Cyclin D1$^f$ |
|---|---|---|---|---|---|---|
| Dbs-HA6 | 6926.4 | ++ | +++ | ++ | +++ | +++ |
| Dbs-HA1 | 575.1 | + | +++ | + | +++ | ++ |
| Dbs-HA2 | 0.0 | − | − | +/− | − | +/− |
| Dbl-HA1 | 12,997.6 | +++ | +++ | + | ++++ | +++ |
| Dbl-HA2 | 82.9 | + | +/− | + | +++ | + |
| Ect2-HA3 | 7711.0 | +/− | + | ++ | ++ | +++ |
| Ect2-HA1 | 61.6 | +/− | − | +/− | ++ | ++ |
| Ect2-HA2 | 7.9 | − | +/− | +/− | + | +/− |
| Lsc-D7-HA | 59.7 | +/− | − | + | ++ | +/− |
| Lsc-D3-HA | 1.7 | − | − | + | − | − |
| Lfc-D6-HA | 203.8 | ++ | ++ | + | ++ | ++ |
| Lfc-D7-HA | 115.0 | ++ | +++ | + | + | +/− |
| Lfc-D13-HA | 1.0 | +++ | +/− | +/− | +/− | +/− |

$^a$ Relative activity in NIH 3T3 focus-formation assays. Data are cal-
culated as foci per picomole of DNA.

$^b$ *In vivo* activation of Gal-Jun transcriptional activity. Fold activa-
tion relative to vector only: −, <2-fold; +/−, 2–5-fold; +, 5–7.5-fold; ++,
7.5–15-fold; +++, 15–30-fold.

$^c$ Activation of JNK1 in transiently transfected COS-7 cells. -Fold
activation relative to vector only control: −, <2-fold; +/−, 2–4-fold; +,
5–8-fold; ++, 8–12-fold; +++, >12-fold.

$^d$ Activation of p38 in transiently transfected COS-7 cells. -Fold acti-
vation relative to vector only control: −, <1.5-fold; +/−, 1.5–2-fold; +,
2–3-fold; ++, >3-fold.

$^e$ *In vivo* activation of transcription from an SRF-dependent promoter
upstream of the luciferase gene. -Fold activation relative to vector only
control: −, <2-fold; +/−, 2–5-fold; +, 5–20-fold; ++, 20–100-fold; +++,
100–500-fold; ++++, >500-fold.

$^f$ *In vivo* activation of transcription from the cyclin D1 promoter
upstream of the luciferase gene. -Fold activation relative to vector only
control: −, <2-fold; +/−, 2–5-fold; +, 5–10-fold; ++, 10–25-fold; +++,
25–50-fold.

between p38 activation and transforming potency.

*Stimulation of c-Jun Transcriptional Activity by Dbl Family
Members*—The efficient activation of the JNK pathway by Dbl
family members should result in stimulation of c-Jun tran-
scriptional activity. Indeed, derivatives of Dbl family members
exhibited strong stimulation of the transcriptional activity of
Gal4-Jun-(1–254), a fusion protein that retains the c-Jun NH$_2$-
terminal activation domain (Fig. 4). Not surprisingly, strong
activation (10–20-fold) was observed among those proteins that
exhibited greatest JNK activity (Lfc, Dbs, and Dbl; Table I). We
did not see a good correlation between c-Jun activation and the
transforming activity of our panel members. For example, al-
though transforming versions of Lfc showed moderate activa-
tion of the Gal4-c-Jun reporter (10-fold), greatest activation
was observed with the non-transforming PH domain minus
derivative Lfc-D13HA (>20-fold), suggesting that transcrip-
tional activation of c-Jun is not sufficient to induce Lfc-medi-
ated transformation. Taken together, our results suggest that
JNK and c-Jun transcriptional activation is not sufficient to
account for the transforming activity associated with the var-
ious Dbl family members on the panel.

*Stimulation of SRF Transcriptional Activity by Dbl Family
Members*—Constitutively activated mutants of Rho, Rac, and
CDC42Hs have been shown to be strong transcriptional acti-
vators of SRF function, although the signaling pathways uti-
lized for this activation have not yet been determined (46). All
transforming Dbl family members on the panel exhibited mod-
erate to strong activation of SRF (50–720-fold; Fig. 5). How-
ever, SRF activation correlated poorly with focus-forming ac-
tivity. For example, although Dbl-HA2 showed a 500-fold lower
focus-forming activity than Dbl-HA1, this was not reflected in
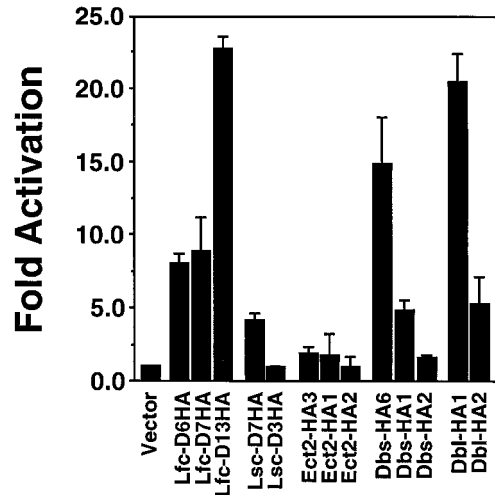a correspondingly lower activation of SRF. Similarly, the po-



FIG. 4. **Activation of c-Jun by Dbl family GEFs in NIH 3T3 cells.**
NIH 3T3 cells were transiently transfected with Gal4-c-Jun-(1–254),
composed of the yeast Gal4 DNA binding domain fused to the amino-
terminal activation domain of the c-Jun protein (0.25 $\mu$g), the reporter
construct 5XGal-luciferase (2.5 $\mu$g), and pCMVnlac (0.5 $\mu$g) as an in-
ternal control for transfection efficiency and toxicity. This reporter
system provides a functional readout for total cellular JNK activity.
Cells were co-transfected with empty expression vector or the indicated
panel member (0.5 $\mu$g), cultured for 30 h, and then serum-starved (0.5%
calf serum) for 14 h before extract preparation. Luciferase and $\beta$-galac-
tosidase activity was measured and expressed as -fold activation rela-
tive to the level of activation seen with the vector control. Luciferase
activity was then standardized relative to $\beta$-galactosidase activity. Data
shown are representative of three independent experiments for each
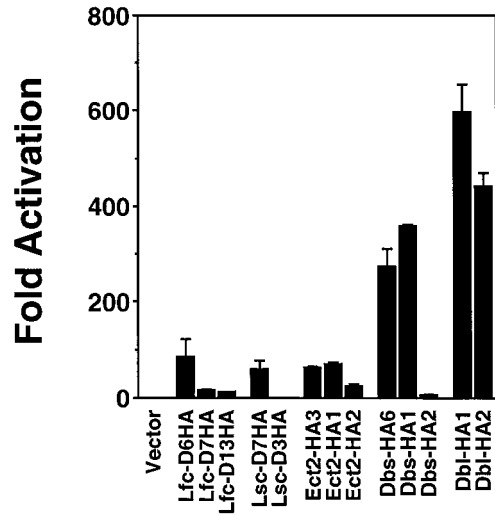construct performed in duplicate.



FIG. 5. **Activation of SRF by Dbl family proteins in NIH 3T3
cells.** Cells were co-transfected as in Fig. 4 with the reporter constructs
(SREm)$_2$-luciferase to measure GEF activation of SRF, and pCMVnlac.
Data are calculated and presented as in Fig. 4. Data shown are repre-
sentative of three independent experiments performed in duplicate for
each derivative.

tent focus-forming activity of Ect2 is not reflected in its ability
to stimulate SRF activity. Clearly, strong SRF activation alone
is not sufficient to cause potent focus-forming activity, nor is
low SRF activation predictive of weak focus-forming activity.

Interestingly, the degree of SRF activation showed a good
correlation with focus morphology and GEF specificity (Table
I). Dbl family proteins that induce large, diffuse foci, and that
have a broader range of *in vitro* substrate utilization (Dbl and
Dbs), exhibited characteristically high levels of SRF activity.
This result is in accordance with a previous report that specific
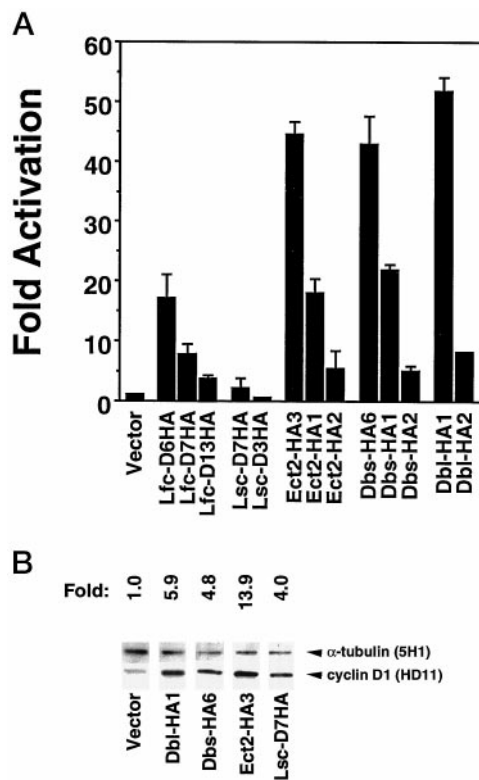Rho family proteins (including CDC42Hs and RhoA) can acti-

*Transforming Potential of Dbl Family Proteins*



FIG. 6. **Up-regulation of cyclin D1 expression by Dbl family GEFs.** *A*, NIH 3T3 cells were co-transfected with the human cyclin D1 luciferase reporter construct, pCMVnlac, and the indicated Dbl family derivative. Data are calculated and presented as in Figs. 4 and 5. Data shown represent the average of at least three independent experiments performed in duplicate. *B*, NIH 3T3 cell lines that stably express the indicated Dbl family GEFs were plated at $10^6$ cells/15-cm plate and then cultured for 24 h in growth medium supplemented with 10% calf serum. Cells were then serum-deprived (0.5% calf serum) for 24 h and lysates collected. The abundance of cyclin D1 protein (*lower panel*) was determined by Western blot analysis using a monoclonal cyclin D1 antibody (HD11; Santa Cruz Biotech), followed by phosphoimage analysis. The cyclin D1 expression levels were normalized to $\alpha$-tubulin expression levels (*upper panel*) and then expressed relative to the level observed in vector-transfected cells (*Fold*).

vate SRF via independent pathways (46). It also implies that the activation of SRF-mediated signaling pathways may contribute to the observed differences in focus morphology.

*Stimulation of Cyclin D1 Expression by Dbl Family Members*—Both Ras and Rho family proteins have an essential role in cell cycle progression through $G_1$, in part, by stimulating the expression of cyclin D1 (42, 48, 53, 62, 63). Because Dbl and Rho family members appear to activate many of the same signaling pathways, we evaluated the possibility that Dbl family proteins may also stimulate cyclin D1 expression. For these analyses, we utilized a reporter plasmid where luciferase gene expression was controlled by the cyclin D1 promoter (53). Like activated Rho proteins, all the transforming Dbl family proteins on the panel stimulated cyclin D1 promoter expression (Fig. 6*A*). Promoter stimulation correlated with transforming activity both when comparing different Dbl family members and when comparing proteins impaired in transformation with their parental derivatives (Table I). The clear correlation between cyclin D1 transcription and transforming activity implicates the deregulation of cell cycle control as a contributing mechanism to the transforming activity of Dbl family members.

To confirm that stimulation of the cyclin D1 promoter in a transient reporter assay reflects the constitutive up-regulation of the cyclin D1 protein in Dbl family protein transformed cells, cyclin D1 levels were measured in NIH 3T3 cell lines that had

been stably selected with transforming derivatives of several Dbl family members. In all panel members examined, 4–15-fold increases in cyclin D1 protein expression were observed when compared with vector controls (Fig. 6*B*). These differences could not be attributed to overall variation in protein expression among the various cell lines and suggest that elevated cyclin D1 expression may be required to maintain the transformed state.

*Ect2 and Lfc Induce Lamellipodia and Actin Stress Fiber Formation in PAE Cells*—Two members of the panel (Ect2 and Lfc) exhibited signaling profiles that were consistent with a broader range of substrate utilization than was predicted by their *in vitro* exchange activity. For example, the strong activation of JNK1 by Lfc-D7HA was unexpected (16.1-fold) because *in vitro* data suggest that Lfc is a specific activator of RhoA (29) and RhoA does not activate JNK1 in COS-7 cells (40, 41). The *in vitro* exchange assays that have been performed with Ect2 have failed to detect any catalytic activity and it has been proposed that Ect2 may transform cells by forming nonproductive interactions with endogenous Rho proteins (11). However, in this current study, ect2 exhibits a signaling profile (activation of JNK1 and SRF) that is more consistent with a productive interaction with Rho proteins *in vivo*. To address these apparent inconsistencies, we examined the effects of expressing Lfc and Ect2 on the organization of the actin cytoskeleton. It has been shown previously that activated derivatives of RhoA, Rac1, and CDC42Hs have profound, yet discrete effects on various actin cytoskeletal structures (36–39). CDC42Hs mediates the induction of actin microspikes and filopodia, whereas Rac1 causes membrane ruffling and lamellipodia formation, and RhoA regulates the formation of actin stress fibers (39). In mouse Swiss 3T3 cells, the assembly of these structures involves a cascade in which CDC42Hs activates Rac1, which in turn activates RhoA (39). For our analysis, we injected expression plasmids encoding strongly and weakly transforming derivatives of Ect2 and Lfc into PAE cells. The transforming derivatives of Ect2 and Lfc (Ect2-HA3 and Lfc-D7HA) induced actin stress fiber and lamellipodia formation consistent with activation of RhoA and Rac1 (Fig. 7). Interestingly, the stress fibers induced by Ect2 expression are distributed parallel to the cell membrane, which is distinct from the distribution seen in cells that express Lfc or an activated derivative of RhoA (39). The weakly transforming derivatives Lfc-D13HA and Ect-HA3 induced only marginal stress fiber formation and no lamellipodia (not shown). These results suggest that both Ect2 and Lfc can activate multiple Rho family proteins *in vivo* and is consistent with our observations on nuclear signaling by these proteins.

DISCUSSION

Dbl family proteins are one of the largest known families of transforming proteins (>20 members), yet relatively little is known of the signaling pathways that they activate (1, 2). Whereas the involvement of several family members in cytoskeletal architecture (26, 50) and in the stimulation of JNK activity has been documented (40, 49, 50), the relevance of these biological activities to cellular transformation has not yet been determined. In this current study, we have examined matched sets of weakly and strongly transforming variants of five Dbl family proteins for their ability to activate the JNK and p38/Mpk2 MAPKS, to activate the SRF and c-Jun transcription factors, and to stimulate transcription from the cyclin D1 promoter. A consistently high level of activation was observed in all assays thus establishing an important link between Dbl family protein activity and the activation of nuclear signaling pathways. Although weakly transforming derivatives of Dbl family members were often impaired in their ability to

FIG. 7. **Lfc and Ect2 induce the formation of lamellipodia and stress fibers.** PAE cells were microinjected with expression constructs encoding HA epitope-tagged Lfc-D7HA and Ect2-HA3. The actin cytoskeleton and expressed proteins were visualized, respectively, by phalloidin and indirect immunofluorescence using anti-HA antibodies. The two *side panels* show higher magnification and longer exposure micrographs to highlight the lamellipodia. The *arrow* indicates a broad lamellipodium induced by Lfc; *arrowheads* indicate the narrower lamellipodia caused by expression of Ect2. *Bars* indicate 10 nm.
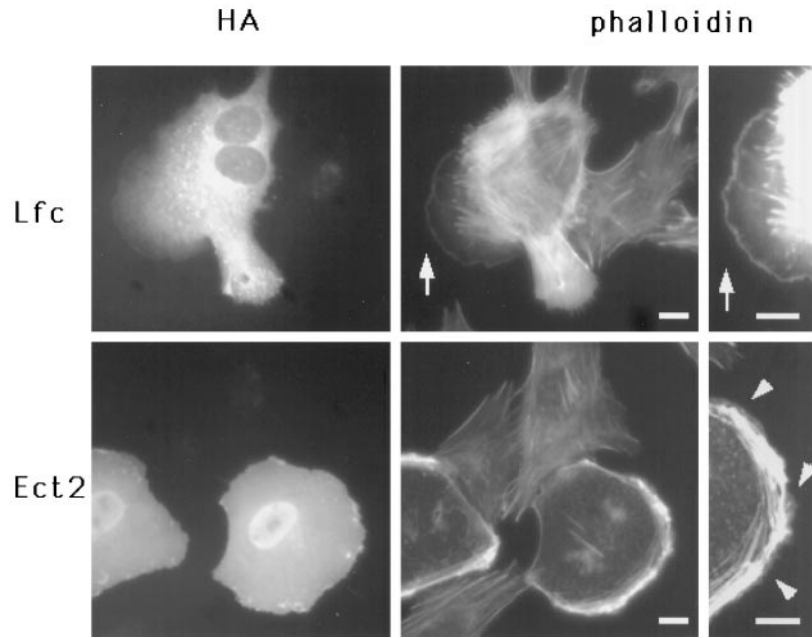
stimulate nuclear signaling pathways, only in the case of cyclin D1 activation were we able to establish a consistent correlation between a signaling event and transforming potential.

We have demonstrated a role for Dbl family proteins in the activation of MAPK signaling pathways in two ways. First, we showed that activated derivatives of most panel members stimulated the catalytic activities of JNK1 and p38/Mpk2 in transient transfection assays thus extending the repertoire of MAPKs that are responsive to Dbl proteins to include p38/Mpk2. We then established that activated derivatives of Dbl family members are able to induce c-Jun transcriptional activation. However, whereas it is now clear that many activated derivatives of Dbl family members can potently stimulate MAPK-mediated signaling pathways, the contribution of these pathways to cellular transformation remains unclear. Although several recent reports have suggested that JNK activity is required for full Bcr/Abl- and Ras-induced transformation (42, 54, 64, 65), we have observed that at least one potently transforming Dbl family member, Ect2, shows relatively weak activation of JNK and c-Jun whereas a non-transforming derivative of Lfc (Lfc-D13HA) exhibited strong activation of the c-Jun reporter. We observed similar inconsistencies when measuring p38/Mpk2 activation. Oncogenic Dbl had the highest focus-forming activity on the panel (more than 150-fold higher than its weakly transforming counterpart), yet both Dbl derivatives exhibited equivalent, weak activation of p38/Mpk2. Collectively, these observations suggest that JNK and p38/Mpk2 signaling is not always required for Dbl family protein-mediated transforming activity. These results are in accordance with our recent observation that a dominant-inhibitory version of SEK MAPK is a strong inhibitor of c-Jun transcriptional activation in NIH 3T3 cells but fails to block Dbl and Dbs transforming activity.[2]

Our demonstration that Dbl family proteins can potently stimulate transcriptional activation of SRF presents an alternative nuclear signaling pathway through which Dbl transforming activity could be mediated. However, as was the case with JNK and p38 activation, we did not observe a good correlation between transforming potency and SRF activation, thus suggesting that this does not represent a proliferative pathway that is utilized by Dbl family members. Although no Dbl family member has been shown previously to stimulate SRF activation, activated mutants of Rho, Rac, and CDC42Hs are all strong activators of SRF (41, 46). Essentially nothing is known concerning the signaling pathway through which Rho and Dbl family proteins stimulate SRF, yet the involvement of these two biochemically related families implies a common mechanism of activation. Interestingly, exchange factors for which the exchange specificities included CDC42Hs and RhoA (Dbl and Dbs) showed the highest SRF activation regardless of relative transforming potencies. These observations are in accordance with a recent report that CDC42Hs and RhoA activate SRF reporters via distinct signaling events (46) and suggest that the strong activation of SRF by Dbs and Dbl may be a consequence of the additive effects of multiple, Rho-mediated signaling pathways.

Previous studies have shown that both Ras and Rho family function are necessary for cell cycle progression through $G_1$ (42, 66, 67). Although the pathways that are utilized for this function are not yet known, the ability of Ras and Rac1 to stimulate expression of cyclin D1 would provide one such mechanism (48, 53, 62, 63, 68). cyclin D1 levels are up-regulated in cells transformed by Ras (62, 69), and Ras and Rac1 can stimulate transcription from the cyclin D1 promoter (48, 53, 68). Thus, it is possible that Ras regulates $G_1$ progression via pathways mediated by Rho proteins and/or their Dbl family regulators. Consistent with this, we have demonstrated that Dbl family proteins are also strong activators of transcription from the cyclin D1 promoter and that the extent of their activation corresponds precisely with the strength of transforming activity. In addition, we have observed elevated levels of cyclin D1 expression in stable cell lines that express transforming derivatives of Dbl family members. This is a clear demonstration that deregulated Dbl family protein activity may impinge directly on progression through the cell cycle and suggests at least one mechanism by which the growth properties of transformed cells could be altered.

The pathway by which Dbl family members are activating cyclin D1 is unclear. The promoter fragment contains an AP-1 site (at −954 of the human sequence) where activated Jun may cause stimulation of cyclin D1 expression, and a distinct region that is sensitive to stimulation by the ERK MAPKs (53). However, our current observation that Dbl family members such as

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[2] J. K. Westwick, R. J. Lee, Q. T. Lambert, M. Symons, R. G. Pestell, C. J. Der, and I. P. Whitehead, unpublished observations.

Ect2 showed low levels of JNK and Jun activation, while exhibiting maximal levels of cyclin D1 activity, argues that at least some Dbl family members may not be utilizing JNK-mediated pathways to induce cyclin D1 expression.

Our observation that Dbl family members can stimulate both p38/Mpk2 activation and expression from the cyclin D1 promoter differs from a recent report in which activation of p38/Mpk2 antagonized expression from a cyclin D1 promoter fragment (68). One possible explanation for this discrepancy is that that the promoter fragment that they used lacked the AP-1-responsive element through which p38/Mpk2 or JNK may be causing stimulation of cyclin D1 expression. In support of this, we recently demonstrated that mutations within this AP-1 site significantly impaired the responsiveness of our reporter construct to Rac1 activation (48).

There is considerable *in vitro* biochemical evidence that many of the Dbl-related proteins are regulatory molecules for the Rho family of Ras-related GTPases (1, 2). Many Dbl family proteins exhibit *in vitro* guanine nucleotide exchange activity toward Rho family members and presumably elevate the level of active GTP-bound molecules in the cell. In addition to their well characterized biochemical interaction with Dbl proteins, Rho family members oncogenically transform mammalian fibroblasts (32–35), and induce alterations in actin-based cytoskeletal structures when injected into Swiss 3T3 cells (36–39). Rho family members have also been shown to be potent stimulators of JNK and p38/Mpk2 activity (40, 41), of c-Jun and SRF transcriptional activity (41, 46), and of cyclin D1 expression (48). Our observation that Dbl family proteins stimulate the same transcriptional response elements as Rho proteins strengthens the correlation between Dbl and Rho-mediated biology and provides evidence that Dbl proteins may regulate cellular Rho functions as they relate to the activation of nuclear signaling pathways. However, it remains a persistent question why the transforming potency of Dbl family members often far exceeds that of activated derivatives of their putative GTPase targets. One possibility is that some Dbl proteins may coordinate the activity of multiple Rho-dependent signaling pathways *in vivo*, the effects of which synergize to produce the transformed phenotype. Alternatively, Dbl family proteins may possess biological activities other than guanine nucleotide exchange, and thus may coordinate the activity of multiple Rho-dependent and -independent signaling pathways.

It has also become apparent from this current study that the *in vitro* GEF specificity that is assigned to Dbl family proteins is not always predictive of *in vivo* signaling activity. For example, whereas the Ect2 protein failed to exhibit any measurable GEF activity when tested against a panel of Rho family GTPases (11), we have observed focus morphology and signaling activity associated with the Ect2 protein that indicates *in vivo* activation of Rho-related proteins. In addition, we have observed that Ect2 expression can bring about changes in the actin cytoskeleton (lamellipodia and actin stress fiber formation) that are consistent with the activation of multiple Rho family members, in particular RhoA and Rac1. Similarly, although the Lfc protein forms complexes with both the Rac1 and RhoA proteins (29), it only catalyzes exchange on RhoA in an *in vitro* assay. However, the signaling data for Lfc, and the effects of its expression on the actin cytoskeleton, are consistent with the *in vivo* activation of both RhoA and either Rac1 or a Rac1-related protein. Taken together, these results indicate that some Dbl family proteins may have a broader range of *in vivo* GEF activity than their *in vitro* specificity implies. One explanation for this finding is that *in vivo* exchange activity may be regulated by lipid or other cofactors, as was recently shown to

be the case for Tiam 1.[3] Alternatively, it is also possible that Dbl family proteins could stimulate additional Rho-regulated signaling pathways through biological activities other than guanine nucleotide exchange.

Specific Rho family proteins have been implicated as key signaling molecules that are required for oncogenic Ras transforming activity (24). Dominant negative mutants of Rho, Rac, and CDC42Hs have been shown to impair Ras transforming activity, presumably via their ability to form inactive complexes with specific Dbl family proteins, and it is generally assumed that Ras activation of Dbl family proteins will provide the link(s) with Rho family proteins (32–35, 60). This possibility is supported by genetic studies in *Schizosaccharomyces pombe* where a yeast Dbl family protein (Scd1) has been defined that links Ras with a Rho family protein (cdc42Sp) (70). Although it is still not clear whether oncogenic Ras activates Rho family proteins via distinct signaling pathways, or by stimulating cascades of Rho proteins, in either scenario, it is likely that multiple Dbl family members will be involved. The determination of which mammalian Dbl family proteins are activated by oncogenic Ras activation will establish a key component of the signaling pathways that link Ras with Rho family proteins.

REFERENCES

1. Whitehead, I. P., Campbell, S., Rossman, K. L., and Der, C. J. (1997) *Biochim. Biophys. Acta* **1332,** F1–F23
2. Cerione, R. A., and Zheng, Y. (1996) *Curr. Opin. Cell Biol.* **8,** 216–222
3. Habets, G. G. M., Scholtes, E. H. M., Zuydgeest, D., van der Kammen, R. A., Stam, J. C., Berns, A., and Collard, J. G. (1994) *Cell* **77,** 537–549
4. Heisterkamp, N., Stephenson, J. R., Groffen, J., Hansen, P. F., de Klein, A., Bartram, C. R., and Grasveld, G. (1983) *Nature* **306,** 239–242
5. Pasteris, N. G., Cadle, M., Logie, L. J., Porteous, M. E., Schwartz, C. E., Stevenson, R. E., Glover, T. W., Wilroy, R. S., and Gorski, J. L. (1994) *Cell* **79,** 669–678
6. Bowtell, D., Fu, P., Simon, M., and Senior, P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6511–6515
7. Simon, M. A., Bowtell, D. D. L., Dodson, G. S., Laverty, T. R., and Rubin, G. M. (1991) *Cell* **67,** 701–716
8. Shou, C., Farnsworth, C. L., Neel, B. G., and Feig, L. A. (1992) *Nature* **358,** 351–354
9. Ron, D., Zannini, M., Lewis, M., Wickner, R. B., Hunt, L. T., Graziani, G., Tronick, S. R., Aaronson, S. A., and Eva, A. (1991) *Nat. New Biol.* **3,** 372–379
10. Katzav, S., Martin-Zanca, D., and Barbacid, M. (1989) *EMBO J.* **8,** 2283–2290
11. Miki, T., Smith, C. L., Long, J. E., Eva, A., and Fleming, T. P. (1993) *Nature* **362,** 462–465
12. Toksoz, D., and Williams, D. A. (1994) *Oncogene* **9,** 621–628
13. Whitehead, I., Kirk, H., Tognon, C., Trigo-Gonzalez, G., and Kay, R. (1995) *J. Biol. Chem.* **271,** 18388–18395
14. Whitehead, I. P., Khosravi-Far, R., Kirk, H., Trigo-Gonzalez, G., Der, C. J., and Kay, R. (1996) *J. Biol. Chem.* **271,** 18643–18650
15. Horii, Y., Beeler, J. F., Sakaguchi, K., Tachibana, M., and Miki, T. (1994) *EMBO J.* **13,** 4776–4786
16. Whitehead, I., Kirk, H., and Kay, R. (1995) *Oncogene* **10,** 713–721
17. Chan, A. M.-L., Takai, S., Yamada, K., and Miki, T. (1996) *Oncogene* **12,** 1259–1266
18. Chan, A. M.-L., McGovern, E. S., Catalano, G., Fleming, T. P., and Miki, T. (1994) *Oncogene* **9,** 1057–1063
19. Boguski, M. S., and McCormick, F. (1993) *Nature* **366,** 643–654
20. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) *Nature* **348,** 125–132
21. Valencia, A., Chardin, P., Wittinghofer, A., and Sander, C. (1991) *Biochemistry* **30,** 4637–4648
22. Kahn, R. A., Der, C. J., and Bokoch, G. M. (1992) *FASEB J.* **6,** 2512–2513
23. Hall, A. (1993) *Curr. Biol.* **5,** 265–268
24. Zohn, I. E., Campbell, S. L., Khosravi-Far, R., Rossman, K. L., and Der, C. J. (1998) *Oncogene*, in press
25. Hart, M. J., Eva, A., Evans, T., Aaronson, S. A., and Cerione, R. A. (1991) *Nature* **354,** 311–314
26. Zheng, Y., Olson, M. F., Hall, A., Cerione, R. A., and Toksoz, D. (1995) *J. Biol. Chem.* **270,** 9031–9034
27. Michiels, F., Habets, G. G., Stam, J. C., van der Kammen, R. A., and Collard, J. G. (1995) *Nature* **375,** 338–340
28. Debant, A., Serra-Pagès, C., Seipel, K., O'Brien, S., Tang, M., Park, S.-H., and Steuli, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 5466–5471

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[3] M. Symons, unpublished observations.

29. Glaven, J. A., Whitehead, I. P., Nomanbhoy, T., Kay, R., and Cerione, R. A. (1996) *J. Biol. Chem.* **271,** 27374–27381
30. Chuang, T.-H., Xu, X., Kaartinen, V., Heisterkamp, N., Groffen, J., and Bokoch, G. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10282–10286
31. Hart, M. J., Eva, A., Zangrilli, D., Aaronson, S. A., Evans, T., Cerione, R. A., and Zheng, Y. (1994) *J. Biol. Chem.* **269,** 62–65
32. Qiu, R.-G., Chen, J., Kirn, D., McCormick, F., and Symons, M. (1995) *Nature* **374,** 457–459
33. Qiu, R.-G., Chen, J., McCormick, F., and Symons, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11781–11785
34. Prendergast, G. C., Khosravi-Far, R., Solski, P. A., Kurzawa, H., Lebowitz, P. F., and Der, C. J. (1995) *Oncogene* **10,** 2289–2296
35. Khosravi-Far, R., Solski, P. A., Kinch, M. S., Burridge, K., and Der, C. J. (1995) *Mol. Cell. Biol.* **15,** 6443–6453
36. Ridley, A. J. (1994) *BioEssays* **16,** 321–327
37. Hall, A. (1994) *Annu. Rev. Cell Biol.* **10,** 31–54
38. Ridley, A. J. (1995) *Curr. Opin. Genet. Dev.* **5,** 24–30
39. Nobes, C. D., and Hall, A. (1995) *Cell* **81,** 53–62
40. Coso, O. A., Chiariello, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* **81,** 1137–1146
41. Minden, A., Lin, A., Claret, F.-X., Abo, A., and Karin, M. (1995) *Cell* **81,** 1147–1157
42. Olson, M. F., Ashworth, A., and Hall, A. (1995) *Science* **269,** 1270–1272
43. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J., and Bokoch, G. M. (1995) *J. Biol. Chem.* **270,** 23934–23936
44. Karin, M. (1995) *J. Biol. Chem.* **270,** 16483–16486
45. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) *J. Biol. Chem.* **270,** 7420–7426
46. Hill, C. S., Wynne, J., and Treisman, R. (1995) *Cell* **81,** 1159–1170
47. Marais, R., Wynne, J., and Treisman, R. (1993) *Cell* **73,** 381–393
48. Westwick, J. K., Lambert, Q. T., Clark, G. J., Symons, M., Van Aelst, L., Pestell, R. G., and Der, C. J. (1997) *Mol. Cell. Biol.* **17,** 1324–1335
49. Crespo, P., Bustelo, X. R., Aaronson, D. S., Coso, O. A., Lopez-Barahona, M., Barbacid, M., and Gutkind, J. S. (1996) *Oncogene* **13,** 455–460
50. Olson, M. F., Pasteris, N. G., Gorski, J. L., and Hall, A. (1996) *Curr. Biol.* **6,** 1628–1633
51. Whitehead, I., Kirk, H., and Kay, R. (1995) *Mol. Cell. Biol.* **15,** 704–710
52. Coppola, J., Bryant, S., Koda, T., Conway, D., and Barbacid, M. (1991) *Cell Growth Differ.* **2,** 95–105
53. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., and Pestell, R. G. (1995) *J. Biol. Chem.* **270,** 23589–23597
54. Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. (1994) *Cell* **77,** 727–736
55. Clark, G. J., Cox, A. D., Graham, S. M., and Der, C. J. (1995) *Methods Enzymol.* **255,** 395–412
56. Hauser, C. A., Westwick, J. K., and Quilliam, L. A. (1995) *Methods Enzymol.* **255,** 412–426
57. Westwick, J. K., and Brenner, D. A. (1995) *Methods Enzymol.* **255,** 342–360
58. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
59. Watanabe, G., Pena, P. P., Albanese, C., Wilsbacher, L. D., Young, J. B., and Pestell, R. G. (1997) *J. Biol. Chem.* **272,** 20063–20069
60. Qiu, R.-G., Abo, A., McCormick, F., and Symons, M. (1997) *Mol. Cell. Biol.* **17,** 3449–3458
61. Davis, R. J. (1994) *Trends Biochem. Sci.* **19,** 470–473
62. Liu, J.-J., Chao, J.-R., Jiang, M.-C., Ng, S.-Y., Yen, J. J.-Y., and Yang-Yen, H.-F. (1995) *Mol. Cell. Biol.* **15,** 3654–3663
63. Peeper, D. S., Upton, T. M., Ladha, M. H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J. A., and Ewen, M. E. (1997) *Nature* **386,** 177–181
64. Clark, G. J., Westwick, J. K., and Der, C. J. (1997) *J. Biol. Chem.* **272,** 1677–1681
65. Raitano, A. B., Halpern, J. R., Hambuch, T. M., and Sawyers, C. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11746–11750
66. Mulcahy, L. S., Smith, M. R., and Stacey, D. W. (1985) *Nature* **313,** 241–243
67. Smith, M. R., DeGudicibus, S. J., and Stacey, D. W. (1986) *Nature* **320,** 540–543
68. Lavoie, J. N., L'Allemain, G., Brunet, A., Mhller, R., and Pouysségur, J. (1996) *J. Biol. Chem.* **271,** 20608–20616
69. Bodrug, S. E., Warner, B. J., Bath, M. L., Lindeman, G. J., Harris, A. W., and Adams, J. M. (1994) *EMBO J.* **13,** 2124–2130
70. Chang, E. C., Barr, M., Wang, Y., Jung, V., Xu, H.-P., and Wigler, M. H. (1994) *Cell* **79,** 131–141