Transforming Potential of Dbl Family Proteins Correlates with Transcription from the Cyclin D1 Promoter but Not with Activation of Jun NH₂-terminal Kinase, p38/Mpk2, Serum Response Factor, or c-Jun*

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The Dbl-related proteins are a large 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 NH₂-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 vitro 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)†† 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 translation partner of the Abl tyrosine kinase in Philadelphia chromosome positive human leukemias (4); Fdg1, 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 dlb (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 tandem DH/PH domains (30, 31). Because the constitutive activation of several Rho family members
have been shown to activate SRF (46), which cooperates with (44). Stimulation of p38 activity results in activation of the ATF-2 transcription factors to stimulate transcription from promoters containing transcription factors, which can dimerize with other transcription activation, JNK in turn activates the ATF-2 and Jun nuclear of the mitogen-activated protein kinase (MAPK) family. Upon 43). ERKs, JNKs, and p38 proteins represent distinct subsets are activators of JNKs (also known as stress-activated protein expression. First, constitutively activated Rac1 and CDC42Hs exchange factors that activate them, to the regulation of gene biology. Rho family proteins, and by extension the membrane ruffling and lamellipodia formation, and RhoA regulates the biological activities were, at least in part, mediating the onco- structures (36–39), and it was generally assumed that these 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 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 NH2 termini of the various Dbl family members were made by subcloning into the HpaI site of pCTV3HA as described below. All constructs were then digested with MluI 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 prev- (32–35), Dbl-related proteins are activators of JNKs (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 re- sponsive to activation by Rho family members is rapidly ex- panding, 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 signal- ing pathways (49, 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/Mkp2 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 GEF specificity.
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 Dbs, weakly transforming, full-length versions of the protein, weakly transforming derivatives were selected from large panels of their respective PH domains. Additional Dbs, Lfc, and Ect2 variants were included in the panel that exhibited intermediate levels of transforming activity as evidenced by low, but reproducible focus-forming activity (Fig. 1A). All proteins also contained an NH2-terminal 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 cell focus-formation assay. NIH 3T3 cells were transfected with 3 µg of the indicated expression vector, and cultured for 14 days before foci were counted. The data are expressed as foci per micromole of DNA and represent the average of three independent experiments. The weakly transforming derivatives of Ect2, Lfc, Lsc, Dbl, and Dbs showed the highest focus-forming activity (~6 × 103 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. 1B), 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 were characteristically small and focal, as described previously (60). Briefly, porcine aortic endothelial (PAE) cells were transfected with pAX142-HA3-Ect2 and cultured for 14 days. The absolute number of foci was determined on a PhosphorImager (Molecular Dynamics). Blots were dried and exposed to film for 1–3 h and then subjected to quantitation on an ImageQuant system (Molecular Dynamics) and expressed as foci per micromole of DNA.

Expression of Actin Stress Fiber and Lamellipodia Formation—Analysis of actin stress fibers and lamellipodia was performed as described previously (25). Briefly, porcine aortic endothelial (PAE) cells were transfected with pAX142-HA3-Ect2 and cultured for 14 days. The absolute number of foci was determined on a PhosphorImager (Molecular Dynamics). Blots were dried and exposed to film for 1–3 h and then subjected to quantitation on an ImageQuant system (Molecular Dynamics) and expressed as foci per micromole of DNA.
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. 1A).

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 of 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 comparison 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. 1A). 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. Lec-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 Lec-D7HA at inducing foci. Thus, although Dbl family members share a common ability to activate p38, we have not seen a good correlation...
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 transcriptional 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 NH2-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, although transforming versions of Lfc showed moderate activation of the 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 μg), the reporter system provided a functional readout for total cellular JNK activity. Cells were co-transfected with empty expression vector or the indicated panel member (0.5 μg), cultured for 30 h, and then serum-starved (0.5% calf serum) for 14 h before extract preparation. Luciferase and β-galactosidase activity was measured and expressed as fold activation relative to the level of activation seen with the vector control. Luciferase activity was then standardized relative to β-galactosidase activity. Data shown are representative of three independent experiments for each construct performed in duplicate.

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

### Table I

| Protein | Relative focus-formation | Gal-Jun | Jnk | p38 | SRF | Cyclin D1 |
|---------|--------------------------|--------|-----|-----|-----|-----------|
| 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                    | +++    | +   | +   | +   | +/−       |

*Relative activity in NIH 3T3 focus-formation assays. Data are calculated as foci per picomole of DNA.

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

*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.

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

*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-fold.

*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.

**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 μg), the reporter vector (SGal-luciferase (2.5 μg)), and pCMV-βgal (0.5 μg) as an internal 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 μg), cultured for 30 h, and then serum-starved (0.5% calf serum) for 14 h before extract preparation. Luciferase and β-galactosidase activity was measured and expressed as fold activation relative to the level of activation seen with the vector control. Luciferase activity was then standardized relative to β-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 pCMV-βgal. Data are calculated and presented as in Fig. 4. Data shown are representative of three independent experiments performed in duplicate for each derivative.
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, pCMVlac, 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 (upper 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 α-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 G1, 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. 6A). 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 1). 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. 6B). 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 non-productive 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 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 (28, 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/Mkp2 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
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/MAPK 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 family 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 G1 (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 G1 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.
Regulated by lipid or other cofactors, as was recently shown to some Dbl family proteins may have a broader range of its expression on the actin cytoskeleton, are consistent with 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. 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 (cde42Sp) (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.

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