Specific Contributions of the Small GTPases Rho, Rac, and Cdc42 to Dbl Transformation*

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DbI is a representative prototype of a growing family of oncogene products that contain the Dbl homology/pleckstrin homology elements in their primary structures and are associated with a variety of neoplastic pathologies. Members of the Dbl family have been shown to function as physiological activators (guanine nucleotide exchange factors) of the Rho-like small GTPases. Although the expression of GTPase-defective versions of Rho proteins has been shown to induce a transformed phenotype under different conditions, their transformation capacity has been typically weak and incomplete relative to that exhibited by dbl-like oncogenes. Moreover, in some cases (e.g. NIH3T3 fibroblasts), expression of GTPase-defective Cdc42 results in growth inhibition. Thus, in attempting to reconstitute dbl-induced transformation of NIH3T3 fibroblasts, we have generated spontaneously activated (“fast-cycling”) mutants of Cdc42, Rac1, and RhoA that mimic the functional effects of activation by the Dbl oncoprotein. When stably expressed in NIH3T3 cells, all three mutants caused the loss of serum dependence and showed increased saturation density. Furthermore, all three stable cell lines were tumorigenic when injected into nude mice. Our data demonstrate that all three Dbl targets need to be activated to promote the full complement of Dbl effects. More importantly, activation of each of these GTP-binding proteins contributes to a different and distinct facet of cellular transformation.

The dbl oncogene was first identified by transfection of fibroblasts with DNA from a human diffuse-B-cell lymphoma (1, 2). Since then, over 15 different oncogene products have been described that bear strong sequence and functional homology to the original Dbl protein (3, 4). Operationally, Dbl family members have been defined as proteins that contain the tandem arrangement of a pleckstrin homology domain adjacent to a unique domain (approximately 180 amino acids) found only in members of this family, and hence termed the Dbl homology domain. Many of these proteins possess high oncogenic activity, and indeed, most of the Dbl family members were initially found in gene transfer experiments through their ability to potently transform fibroblasts. Oncogenic activation of these cellular proto-oncogenes often occurs by a specific mutation or a chromosomal rearrangement event, which results in continuous, unregulated activity of the mutated proteins.

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To date, most Dbl family members have been shown to serve as activators, or guanine nucleotide exchange factors (GEFs), for Rho-like proteins (i.e. Cdc42, Rac, and Rho) (3). Like all GTP-binding proteins, members of the Rho subfamily function as binary molecular switches that are “on” in the GTP-bound state and “off” in the GDP-bound state (5–7). Deactivation (transition from the GTP to the GDP state) is achieved by their intrinsic GTP hydrolytic capability, which is further stimulated by GTPase activating proteins (GAPs). Activation of the GTP-binding proteins occurs in response to a variety of stimuli (such as cell cycle progression and growth factor/cytokine stimulation) and is mediated by GEFs, which stimulate the dissociation of bound GDP. GTP then rebinds, thus triggering the conformational change that leads to the activated state of the molecule.

Because nucleotide exchange is the only biochemical activity demonstrated by Dbl proteins, and because transformation and exchange activities share common structure/function features (8), it has been assumed that the activation of Rho proteins is the basis for the oncogenic activity demonstrated by Dbl proteins. A logical extension of this reasoning is that activated alleles of Rho proteins should be transforming when introduced into cells. Such dominant-positive reagents are typically generated by mutations of residues that are critical for GTP hydrolysis, thus rendering the protein GTPase-defective. When introduced into a cell, the GTPase-defective GTP-binding protein elicits a persistent stimulation of its signaling cascade, resulting in an exaggerated phenotype that directly demonstrates its involvement in a particular pathway. This is exemplified in the case of Ras, in which expression of either the Ras(G12V) or Ras(Q61L) GTPase-defective mutant is oncogenic (9), and indeed, such mutations are found in a significant fraction of human tumors (10).

For members of the Dbl family, elucidation of their transformation mechanism has not been straightforward. Some oncogenic activity has been observed upon expression of the GTPase-defective proteins RhoA(Q63L), Rac1(G12V), and Cdc42(G12V) in fibroblasts and in immunocompromised mice (11–17). Furthermore, dominant-negative mutants of these proteins were shown to block Ras-induced transformation, indicating their critical role in proliferative signaling pathways (12–15). However, the oncogenic capacity of these proteins has been typically incomplete and weak. Moreover, stable overexpression of GTPase-defective Rho proteins has tended to be difficult. In particular, we have consistently found that significant overexpression of the GTPase-defective alleles (i.e. G12V or Q61L) of Cdc42 in NIH3T3 cells actually has detrimental effects on cell growth. This has prompted us to consider the
idea that for proper signaling, Cdc42 must undergo a complete cycle of GTP binding and hydrolysis.

We have therefore used an alternative scheme for activation of ectopically expressed GTPases; rather than a mutation that blocks GTP hydrolysis, we have generated mutants that possess enhanced intrinsic GTP→GDP exchange rate but maintain normal GTP hydrolytic activity. Thus, in vivo, these mutated (“fast-cycling”) GTP-binding proteins become activated spontaneously, and more closely reflect their in vitro activation by the Dbl oncoprotein. Indeed, we have previously shown that Cdc42(F28L) is activated in vivo, and that its stable overexpression in NIH3T3 cells is accompanied by a few hallmarks of malignant transformation (18). Here, we use the fast-cycling versions of Cdc42, Rac1, and RhoA (i.e. the primary GTP-binding protein targets of Dbl) to assess their relative contributions to the total phenotype exhibited by Dbl-transformed cells.

EXPERIMENTAL PROCEDURES

Molecular Constructs—Rac1(F28L) and RhoA(F30L) mutations were made using a polymerase chain reaction strategy identical to that used earlier for generating the Cdc42(F28L) mutant (18). The reaction included two internal primers harboring the Phe→Leu mutation, two external pET15b primers, and a template of the wild-type gene in pET15b.

Expression of recombinant proteins in Escherichia coli was performed exactly as described previously (18, 19). For transient expression in COS cells, the cDNAs encoding the GTP-binding proteins were subcloned into the (HA-tagged) pKH3 vector or the (Myc-tagged) pcDNA3 vector, using the BamHI-EcoRI restriction sites. For stable expression in NIH3T3 cells, constructs were subcloned into the (HA-tagged) pJ4H vector using the same restriction sites. For focus formation assays, a 3′ BamHI site was added to all constructs by polymerase chain reaction, and the BamHI-BamHI fragments were subcloned into the BamHI-digested pZipNeo vector, where correct orientation was verified by restriction digestion.

Cell Culture—Stable cell lines were generated by co-transfection of NIH3T3 cells with the indicated genes in the pJ4H vector, together with pcDNA3-Neo using the LipofectAMINE method (Life Technologies, Inc.). Neomycin-resistant colonies were selected by two consecutive culturing steps in DMEM supplemented with 10% calf serum and neomycin (418; 600 μg/ml; Life Technologies, Inc.). Resistant colonies were screened for expression of the desired protein by Western blotting the total lysates with anti-HA antibodies (HA.11; Berkely Antibody Co.). The Dbl-expressing cell line was generated by transformation of NIH3T3 cells with pZip-onco-Dbl (8), followed by the isolation of a stable cell line expressing the recombinant protein targets of Dbl.

For primary focus formation assays, the indicated constructs in the pZip-onco-Dbl vector were used to transfect subconfluent NIH3T3 cells in 6-well plates using the LipofectAMINE method. After 2 days, each well was split into two 100-mm plates and cultured in DMEM supplemented with 10% calf serum. Two weeks after transfection, cells were fixed with formaldehyde and stained with crystal violet, and foci were scored under a microscope. For secondary focus formation assays, 1000 cells were transfected with the indicated constructs and were mixed with 2 × 103 NIH3T33 cells and cultured in DMEM supplemented with 10% calf serum. After 10 days, foci larger then 3 mm were scored from fixed and stained plates.

Transfection protocols, cell culture and lysis, immunoprecipitation, kinase assays, and soft-agar growth assays were described in detail earlier (18, 20, 21).

Biochemical Assays—Nucleotide exchange was monitored using the mant-GDP fluorescence assay (22) or the binding of [35S]GTPyS as described (23). For measurements of GTP hydrolysis, 1 μM purified protein was incubated with 20 μM Tris-HCl (pH 8.0). 100 mM NaCl, 1 mM diithiothreitol, 0.5 mM bovine serum albumin, 1 μM GTP, 100 μM [γ-32P]GTP (30 Ci/mmol, NEN Life Science Products) in the presence of 15 μM EDTA for wild-type protein or 5 μM EDTA for fast-cycling protein at room temperature for 20 min. Hydrolysis was initiated by dilution with 20 μM Tris (pH 8.0), 100 mM NaCl, 1 mM diithiothreitol, 0.5 mM bovine serum albumin, 20 μM MgCl2, with or without 0.01 μM Cdc42-GAP purified as described previously (23). GTP hydrolysis was measured at room temperature for Cdc42 and Rac1 and at 37 °C for RhoA.

PBD Assay—This assay has been described in detail (24, 25). Briefly, COS-7 cells were transiently transfected with the cDNA for the indicated GTP-binding protein in the pKHi vector, with or without oncogenic Dbl in the pcMV vector. Twenty-four hours posttransfection, cells from 60-mm plates were lysed in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 20 μM β-glycerol-phosphate, 20 μM GTP, 1 μM sodium vanadate, and 10 μg/ml each of leupeptin and aproitin, and incubated with 50 μM of recombinant glutathione S-transferase (GST-PBD) (20). GST-PBD was then precipitated with glutathione-agarose beads, washed three times with lysis buffer, and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using the indicated antibodies.

Mant-GDP fluorescence assay—Stable cell lines were cultured on dual-chamber microscope slides (Nunc) for 2 days in normal media, and then serum-starved for 12 h and fixed with 3.7% formaldehyde. Slides were then sequentially incubated with anti-vinculin antibodies (Sigma), Oregon Green-conjugated goat anti-mouse antibodies, Texas Red phallolidin, and Hoechst-33342 (all from Molecular Probes). The slides were visualized and photographed on a Nikon Eclipse 600 fluorescence microscope.

RESULTS

Biochemical Characterization of the Fast-cycling Mutants of Cdc42, Rac1, and RhoA

A phenylalanine residue corresponding to position 28 in Ras is highly conserved in the Ras superfamily of small GTPases, in which it has been shown to interact with the guanine base of the nucleotide (26–29). Conservative substitution of this residue to a leucine resulted in a reduced affinity of the protein to guanine nucleotides in Ras (30) and Cdc42 (18), leading to spontaneous activation (i.e. GTP binding) of the mutated protein when expressed in cultured cells. We have generated the corresponding mutations in Rac1 and RhoA (i.e. Rac1(F28L) and RhoA(F30L)) and expressed and purified these mutants to homogeneity from E. coli. The ability of these purified proteins to bind GTP·S was compared, and is shown in Fig. 1A. As is typical for all GTP-binding proteins, the wild-type versions of Rac1, Cdc42, and RhoA show only negligible levels (<10%) of [35S]GTPyS binding activity in the presence of 15 mM MgCl2. The addition of EDTA, which chelates the tightly bound Mg2+ ion (31), leads to complete exchange of the bound GDP for GTP·S (defined as 100% in Fig. 1A). The Cdc42, Rac1, and RhoA point mutants, on the other hand, exhibit significant [35S]GTPyS binding activity, even in the presence of high Mg2+ (i.e. 63, 76, and 53% of the maximal binding, respectively), indicating a significantly higher basal nucleotide exchange activity. To fully assess the biochemical properties of the mutated GTP-binding proteins, we have also measured their ability to hydrolyze GTP in the presence and absence of the Cdc42-GAP (32). The Cdc42-GAP was purified as described (18). The Cdc42, Rac1, and RhoA point mutants were assayed with both [γ-32P]GTP, and GTP hydrolysis was initiated by the addition of Mg2+ (33). As can be seen from Fig. 1B, both the point-mutated Cdc42 and Rac proteins showed intrinsic GTP hydrolytic rates that were comparable to their wild-type counterparts. The intrinsic GTP hydrolytic activity of RhoA has been shown to be consistently observed to be lower than the corresponding activities for Rac and Cdc42, and this activity is slightly reduced (30–50%) in the fast-cycling RhoA mutant. A similar effect has been seen when examining the analogous point mutation in Ras (30). More importantly, each of the point mutants is fully responsive to GAP stimulation, yielding turnover numbers for GTP hydrolysis that are virtually indistinguishable from those for the wild-type proteins. Taken together, our in vitro results indicate that Cdc42(F28L), Rac1(F28L), and RhoA(F30L) all
with 0.1 mM B, protein-bound radioactivity was measured using nitrocellulose filtration. Proteins were incubated with 10 μM [35S]GTPγS (0.3 Ci/mmol) in the absence (empty bars) or presence (hatched bars) of 20 μM EDTA, and protein-bound radioactivity was measured using nitrocellulose filtration. The half-life for GTP hydrolysis was obtained by fitting the data to a single exponential process. Data shown are the means of two duplicate measurements (S.E., <10%).

Cdc42(F28L), Rac1(F28L), and RhoA(F30L) Are fast-cycling in vitro. A, [35S]GTPγS binding. One μM purified wild-type or mutated proteins were incubated with 10 μM [35S]GTPγS (0.3 Ci/mmol) in the absence (empty bars) or presence (hatched bars) of 0.01 μM Cdc42-GAP. Aliquots were removed after 2, 5, 10, 20, and 30 min, and protein-bound radioactivity was measured by nitrocellulose filtration. The half-life for GTP hydrolysis was obtained by setting the data to a single exponential process. Data shown are the means of two duplicate measurements (S.E., <10%).

Rho is accompanied by diverse biological phenotypes accompanied through interactions with different target proteins. To assess whether the Phe → Leu mutation indeed results in the spontaneous activation of the different GTP-binding proteins in vivo, we have utilized a modification of an assay developed to assess the activation level of Ras (24, 25). This assay is based on the GTP-specific high affinity interaction between the tested GTP-binding protein and the binding domain of its target, PAK-3 (20), which is fused to GST to enable affinity precipitation. Thus, a recombinant GST fusion protein containing the p21-binding domain (PBD, also known as the CRIB (Cdc42/Rac-interaction binding) domain) (35) of PAK-3 immobilized on glutathione-agarose was incubated with lysates from COS-7 cells transfected with the different forms of Cdc42 and Rac1. Following extensive washes, the lysates (Fig. 2A, middle panel) and the precipitated GST-PBD beads (Fig. 2A, top panel) were electrophoresed and blotted for the (HA-tagged) GTP-binding proteins. Fig. 2A, middle panel, shows that each of the GTP-binding proteins were expressed to significant and similar levels. As expected, the co-expression of Cdc42 and Dbl (compare lanes 1 and 5), as well as Rac1 and Dbl (compare lanes 3 and 6), resulted in the enhanced precipitation of the GTP-binding protein, indicating that Dbl activates each of these proteins in cells. More importantly, the amounts of the fast-cycling versions of Rac1 and Cdc42 precipitated with GST-PBD were markedly higher than those of the wild-type GTP-binding proteins (compare lanes 1 and 2 or lanes 3 and 4). We have used this assay also to compare the activation level of the fast-cycling version (F28L) with that of the GTPase-defective version (Q61L) of Cdc42 and Rac1. Under essentially identical experimental conditions, 20% of the expressed Cdc42(Q61L) precipitated with GST-PBD, versus 18% of the fast-cycling, Cdc42(F28L) mutant (%; data not shown). This verifies that a significantly larger fraction of each Phe → Leu mutant is in the GTP-bound state, compared with the corresponding wild-type protein, and is consistent with the idea that the fast-cycling versions of Cdc42 and Rac1 are spontaneously activated when ectopically expressed in cultured cells.

Another well characterized signaling end point for Cdc42 and Rac1 is a nuclear transcriptional activator, the e-Jun kinase (JNK1) (21, 36, 37). We have previously reported that the expression of the Cdc42(F28L) mutant in COS-7 cells leads to activation of JNK1 (18). We show here that this is also true for the Rac1(F28L) mutant. Fig. 2B shows the results of an experiment in which wild-type Rac1, the GTPase-defective Rac1(Q61L) mutant, and the fast-cycling Rac1(F28L) mutant were co-transfected into COS-7 cells together with flag-tagged JNK1, and then immunoprecipitated kinase assays were performed following anti-flag immunoprecipitation. The JNK1 precipitated from cells expressing the Rac1(F28L) mutant exhibited levels of protein kinase activity (measured by the phosphorylation of e-Jun) that were comparable to those measured in cells expressing the Rac1(Q61L) mutant and significantly higher than the activity precipitated from cells expressing wild-type Rac1 or vector alone.

We have also established NIH3T3 cell lines that stably express HA-tagged forms of Cdc42/F28L, Rac1/F28L, and RhoA/F30L. Taking advantage of the high affinity interaction between JNK and e-Jun, we examined the endogenous JNK activity in these stable cell lines as well as from cells expressing the oncogenic Dbl protein. Lysates from the different cell lines were incubated with recombinant GST-e-Jun immobilized on glutathione beads. The precipitated GST-Jun-JNK1 complexes were washed, incubated with MgCl2 and [γ-32P]ATP, electro-
phoresed, and autoradiographed. JNK activity, visualized as $^{32}$P incorporation into the GST-Jun protein, is shown in Fig. 2C. It is clear from these data that JNK activity is stimulated 3–5-fold in cell lines expressing fast-cycling Cdc42, Rac1, or oncogenic Dbl, relative to mock-transfected cells. Stimulation of JNK activity by fast-cycling RhoA, can be detected only in cells expressing relatively high levels of RhoA(F30L), in accord with previous reports (36, 37).

Cytoskeletal Evidence—Another well established end point for the activation of Cdc42, Rac, and Rho is cytoskeletal reorganization (38). The controlled dynamic rearrangements of actin-based cytoskeletal elements have been shown to play important roles in motility (39–43), differentiation (44–46), the establishment of cell polarity (47, 48), and growth factor-induced cell shape changes (49–51). We have therefore undertaken a systematic investigation of the cytoskeletal changes associated with the stable overexpression of Cdc42(F28L), Rac1(F28L), or RhoA(F30L) in the cell lines described above. Standard optical microscopy revealed that each cell line displayed distinct morphological characteristics that were especially evident in low density (30% confluence) cultures (Fig. 3). Cells stably expressing Cdc42(F28L) were elongated with multiple extensions (Fig. 3, Cdc42(F28L), right panel). Cells that expressed RhoA(F30L) exhibited pronounced extensions, similar to those observed in Lbc-transformed cells (52), whereas cells that expressed Rac1(F28L) lacked any visible extensions from the cell surface but exhibited thickened cell borders, possibly reflecting lamellipodia (Fig. 3, Rac1(F28L), right panels). These morphological phenotypes are in agreement with those observed upon microinjection of the GTPase-defective versions of these GTP-binding proteins into Swiss-3T3 fibroblasts, (53). In addition, we found that approximately 3% of the Cdc42(F28L)-expressing cells exhibit a giant cell, multinucleate morphology, similar to Dbl-transformed fibroblasts (Fig. 3, Cdc42(F28L), left panel; see also Refs. 18 and 1).
We further investigated these morphological phenotypes using specific optical staining coupled to fluorescence microscopy as shown in Fig. 4. The various NIH3T3 stable cell lines were cultured on microscope chambers and fixed with 3.7% formaldehyde. Filamentous actin structures were visualized using Texas Red-conjugated phalloidin, focal adhesion complexes were visualized with anti-vinculin antibodies followed by Oregon Green-conjugated anti-mouse IgG (Molecular Probes), and Hoechst 33342 stain was used for nuclear staining. Treated slides were observed under a Nikon Eclipse 600 fluorescence microscope (magnification, × 400). The top panels (triple) show the combined fluorescence from all three stains, obtained by a multichromatic filter. Control cells (left panels) represent NIH3T3 cells mock-transfected with empty vector and selected for antibiotic resistance as the other cell lines. Bottom two panels show actin staining pattern of mononucleated Dbl and Cdc42(F28L) cells (magnification, × 600). Control NIH3T3 cells (i.e. transfected with empty vector and selected for antibiotic resistance like the other cell lines) showed the typical extended cell shape with a single nucleus, well oriented stress fibers, and a relatively small number of focal adhesion complexes. Dbl-transformed cells showed an enhanced actin staining (although the stress fibers appeared disorganized), with a significant fraction (8–12%) of the cells being large and multinucleated. The cells expressing oncogenic Dbl also exhibited cortical actin structures along the cell periphery, with a pronounced "crown-like" array of vinculin-containing focal adhesion complexes along the cell border.

Cell lines expressing the fast-cycling mutants displayed very characteristic morphological changes that confirmed and extended the phenotypes observed by regular microscopy described above. A fraction of the cells expressing the Cdc42(F28L) mutant are giant and multinucleated, similar to the phenotype observed for cells expressing oncogenic Dbl. However, the F-actin staining pattern was very unique to the Cdc42(F28L)-expressing cells, as characterized by a vast array of actin microspikes extending outward from the cell surface, each of which has a single focal adhesion complex at its tip. RhoA(F30L)-expressing fibroblasts displayed a dramatic increase in the number of well oriented stress fibers, accompanied by numerous, enlarged focal adhesion complexes. Neither the Cdc42(F28L)- nor RhoA(F30L)-expressing cells showed significant cortical actin structures. This was markedly different from the Rac1(F28L)-expressing cells, which exhibited significantly fewer stress fibers (those detected were relatively short and disorganized) but displayed a prominent arrangement of cortical actin at the leading edge of the cell (lamellipodia).

A few conclusions can be drawn from the comparative morphologies of the different cell lines. First, when stably expressed in fibroblasts, the fast-cycling mutants of Cdc42, Rac1, and RhoA give rise to unique morphological characteristics (the formation of filopodia, lamellipodia, and actin stress fibers, respectively), lending additional support to the notion that these mutants are spontaneously activated in vivo. Secondly, the morphological characteristics of Dbl-transformed cells possess features that can be observed in each of the individual fast-cycling cell lines, i.e. some of the cells are large and multinucleated, and all cells exhibit enhanced stress fibers, cortical actin, and focal adhesion complexes. We interpret this to indicate that in Dbl-transformed cells, all three GTP-binding proteins are activated.
proteins are activated, with each protein contributing a unique characteristic to the overall morphology of Dbl-expressing cells.

Rho GTP-binding Proteins Mediate Cellular Transformation

The availability of stable cell lines that express high levels of the fast-cycling mutants of Rac, Rho, and Cdc42 provides us with valuable tools to directly investigate the effects of these spontaneously activated GTP-binding proteins on various parameters of cell growth. We have first compared the saturation density and growth rate of the different cell lines in both normal and low serum conditions (5 and 0.5% calf serum, respectively), as shown in Fig. 5. A few clear differences were observed upon examining these data. Clearly, cell lines stably expressing high levels of the fast-cycling GTP-binding proteins reached saturation densities that were 3–5-fold higher than those reached by control cells (Fig. 5A). Dbl-transformed cells actually grew to a lower density under these conditions (5% serum) compared with cell lines that expressed the fast-cycling mutants. This most likely is due to a particularly large fraction (approximately 10%) of the Dbl-expressing cells being blocked in cytokinesis. At the present time, we do not know why the Dbl-transformed cells show such a striking giant-cell phenotype under conditions of high serum (even compared with Cdc42(F28L)-expressing cells). Apparently, Dbl-mediated activation causes a large percentage of the fibroblasts to uncouple an accelerated cell cycle progression and nuclear division from cytokinesis. Under low serum conditions (0.5% calf serum) (Fig. 5B), the growth of control cells was arrested during the first 24 h, followed by a progressive cell death. Cell lines expressing the fast-cycling GTP-binding proteins and Dbl-transformed cells, on the other hand, were able to steadily proliferate in low serum conditions (Fig. 5B).

Overall, the data presented in Fig. 5 show that activation of Cdc42, Rac1, and RhoA is accompanied by two hallmarks of cellular transformation: loss of contact inhibition and diminished serum dependence (54)). As indicated in Table I, constitutive expression of Cdc42(F28L) caused pronounced growth in soft agar and typically exceeded the soft agar colony formation associated with Dbl expression (see also Ref. 18). Expression of the fast-cycling Rac1 and RhoA mutants, on the other hand, was accompanied by only a weak colony formation. Thus, it appears that anchorage-independent growth exhibited by Dbl-transformed cells is mediated mainly through the activation of Cdc42.

Focus formation is widely used for assaying the loss of contact inhibition by transformed cells (55, 56) and, in fact, led to the original identification of Dbl from diffuse B-cell lymphoma DNA (1). We have utilized the stably transfected cell lines described above in a “secondary” focus formation assay (17), in which each of the cell lines was mixed with parental NIH3T3 cells at a ratio of 1:200 and plated under normal conditions (2 × 10^5 cells/100-mm plate, 5% calf serum). After 10 days, the cells were fixed and stained, and foci larger then 2 mm were scored under the microscope. As shown in Fig. 6A, only a small number of foci were consistently observed in cells expressing the fast-cycling mutants of Rac and Cdc42. However, cells expressing the RhoA(F30L) mutant showed focus forming capability that was dependent on the levels of Rho(F30L) expression (Fig. 6B).

**TABLE I**

| Growth in soft agar | Tumor formation | Tumor latency |
|---------------------|-----------------|--------------|
| %                   |                | days         |
| Cdc42(F28L)         | 34              | 4/4          | 11 ± 3       |
| Rac1(F28L)          | 7.3             | 4/4          | 10 ± 3       |
| RhoA(F30L)          | 2.7             | 4/4          | 14 ± 5       |
| Dbl                 | 23              | 4/4          | 7 ± 1        |
| Vector              | 1.0             | 0/4          |              |

* Growth in soft agar was measured as described in Ref. 18. Data are representative of three independent experiments.

* Nude mice were injected with 10^7 cells of the indicated cell line and visually inspected daily for 4 weeks.

* Tumor latency is the time between the injection and the time of detection of a tumor larger than 1.0 cm.
6, B and C), reaching 80% of the number of foci formed by Dbl-transformed cells.

We observed a similar differential potency exhibited by the different GTP-binding proteins in primary focus formation assays. Specifically, in experiments in which activated alleles of RhoA, Cdc42, or Rac1 (in the pZipNeo vector) were transfected into NIH3T3 cells, only RhoA transfections yielded a significant number of foci (data not shown). However, we have not been able to observe any synergism upon the co-transfection of the three fast-cycling mutants of Cdc42, Rac, and Rho (i.e. the number of foci observed upon transfection of activated RhoA did not change upon the addition of cDNAs encoding for activated forms of Cdc42 and Rac1 to the transfection mixture in a primary focus formation assay). We therefore conclude that the focus forming activity associated with transformation by the dbl oncogene is an outcome of its ability to specifically activate RhoA and that the other, related GTP-binding proteins do not participate significantly in promoting this specific biological activity.

It is interesting that each of these GTP-binding proteins exhibits tumorigenic activity (Table I). The subcutaneous injection of cell lines that individually express each of the fast-cycling GTP-binding proteins into immunocompromised athymic nude mice resulted in significant (>10 mm) formation of solid tumors after 2 weeks. Although no significant differences were observed between the different fast-cycling cell lines with regards to tumorigenic potency and latency, cells overexpressing oncogenic Dbl exhibited a significantly shorter latency period (Table I).

It appears that each GTP-binding protein mediates a different aspect of the transformed phenotype induced by Dbl. RhoA activation is the main contributor to the loss of contact inhibition (focus formation) and to the increase in stress fiber content and the number of focal adhesion complexes, Rac1 activation accounts for the accumulation of cortical actin at the cell periphery observed in Dbl-transformed cells, and the activation of Cdc42 provides for anchorage independence and filopodia formation and leads to the generation of large, multinucleated cells.

**DISCUSSION**

The Dbl-related proteins represent an interesting and growing family of oncogene products and cell growth regulatory factors. Members of the family were originally isolated as the transforming genes from lymphomas (1, 57, 58), osteosarcomas (59), leukemias (60, 61), and other malignancies (62), and all contain a tandem arrangement of a pleckstrin homology domain and a Dbl homology domain (for review, see Ref. 3). In the case of the prototypical member of the family, the Dbl oncoprotein, the Dbl homology/pleckstrin homology domain tandem represents the minimal unit for transformation activity (8). Thus far, the only biochemical activity that has been assigned to Dbl and other members of the family is the stimulation of the guanine nucleotide exchange activity of Rho-related GTP-binding proteins, such as Cdc42, Rac, and Rho (3). This has led to the common assumption that any protein that contains the Dbl homology/pleckstrin homology domain tandem is a GEF for a Rho-related GTP-binding protein. Similarly, it has been generally assumed that the high transformation capability exhibited by many members of the Dbl family, including Dbl itself, is the direct outcome of their GEF activity (i.e. through the activation of a Rho-related protein and its downstream signaling pathway).

A number of attempts have been made to elucidate the mechanism of Dbl transformation by overexpression of mutated Cdc42, Rac, and Rho proteins in various cell culture models. A role for Rho proteins in Ras-induced transformation has been established from studies demonstrating that dominant-negative Rho proteins block Ras transformation and from the observation of synergistic co-operativity between activated Raf and GTPase-defective versions of either RhoA, Rac1, or Cdc42 (12–15). Furthermore, cell lines expressing these GTPase-defective Rho proteins were shown to be tumorigenic and exhibited some aspects of cellular transformation in tissue culture assays (11–15, 63–65). However, these studies did not conclusively resolve the basis of Dbl-induced transformation for two reasons. First, although Dbl is potently transforming, its GTP-binding protein targets have only weak transforming activity when individually expressed in cultured fibroblasts. Second, no unified conclusion can be reached based on the different reports with regards to the proliferative outcome of a particular activated GTP-binding protein. For example, fibroblasts overexpressing GTPase-defective versions of RhoA (RhoA(Q63L) or RhoA(G14V)) were shown by some researchers (11, 15) to grow to high saturation densities, exhibit diminished serum dependence, and induce solid tumors in nude mice, whereas in other reports (13, 65, 66), they showed none of these transformation hallmarks. One possible explanation for such discrepancies is the varying levels of expression of the Rho proteins in the different cell lines studied. However, another possible explanation is that the GTPase-defective mutants of Rho-related proteins do not exactly reflect the GEF-mediated activation state of small G proteins. In support of this notion is...
our experience that constitutive expression of the Cdc42(Q61L) mutant can actually have detrimental effects on cell growth (18). Thus, in the current study, we have examined this issue in detail, with a particular emphasis on utilizing physiologically relevant activation mutants and understanding the roles of the different GTP-binding proteins in the complete transformation phenotype induced by Dbl.

Because of the difficulties that we have previously encountered in generating stable NIH3T3 cell lines that express GTPase-defective Cdc42, we set out to identify a mutation within the Cdc42, Rac, and RhoA proteins that would more closely mimic the functional effects of oncogenic Dbl. Specifically, we looked for a mutation that would allow these Dbl targets to be constitutively active through the spontaneous binding of GTP without altering their ability to cycle between the GDP- and GDP-bound states via GTP hydrolysis. We first found that a Cdc42(F28L) point mutant showed such properties (18), and in the present study, we have used this fast-cycling mutant together with the corresponding point mutants for Rac1 and RhoA to examine the contributions of these G proteins to the Dbl-transformed phenotype. As expected, the fast-cycling mutants of Cdc42, Rac1, and RhoA behave as activated G proteins in cells. The Cdc42(F28L) mutant gives rise to filopodia formation and, like Rac(F28L), stimulates JNK activity, whereas the RhoA(F30L) mutant induces actin stress fibers and the formation of focal complexes.

A number of interesting and surprising points have emerged when directly comparing the activities of each of the fast-cycling mutants versus oncogenic Dbl in different cell transformation assays. Perhaps foremost has been the realization that each of these Dbl targets are capable of contributing to distinct aspects of the total transformation induced by Dbl. For example, expression of Cdc42(F28L) promotes growth in soft agar with essentially identical capability as oncogenic Dbl, whereas neither the fast-cycling Rac1 nor RhoA mutants show comparable colony formation in semisolid media (Table I). Like Dbl, Cdc42(F28L) also appears to uncouple cell cycle progression and nuclear division from cell division, such that a detectable fraction (3–5%) of Cdc42(F28L)-expressing NIH3T3 cells are giant and multinucleate. On the other hand, only the fast-cycling RhoA mutant appears to induce significant focus formation activity, whereas both the Cdc42(F28L) mutant and the Rac(F28L) mutant are ineffective in these assays (Fig. 6). As a matter of fact, the focus forming activity measured in cells that express high levels of RhoA(F30L) approaches that measured in Dbl-transformed cells. Apparently, the ability of the RhoA(F30L) mutant to continuously cycle between the GDP and GTP states enables high level expression of the activated protein and, consequently, potent focus forming activity. This may explain why significant focus formation has not been detected in experiments in which the GTPase-defective RhoA mutants were used (13, 65, 66).

The observation that each GTP-binding protein mediates a distinct facet of cell transformation immediately raises the issue of identity of the specific downstream target(s) that mediates the particular signal. In this regard, inhibition of Dbl-induced focus formation was observed in the presence of a specific inhibitor of the RhoA target, p160-ROCK (66), suggesting that this effector kinase mediates the focus formation activity initiated by RhoA. Activation of Rac in neutrophils (67, 68), REF-52 cells, and COS cells (69) was shown to result in potent activation of NADPH oxidase, leading to a robust increase in intra-cellular levels of reactive oxygen species (O2·). Importantly, Rac-mediated elevation in O2 levels was shown to be a critical component of cell cycle progression (69) and Ras-induced transformation (70) in NIH3T3 cells. The Cdc42 effector, which mediates anchorage-independent growth, is more enigmatic; thus far, none of the known targets for this GTP-binding protein have been shown to potentiate such a phenotype. The availability of the fast-cycling Cdc42 should facilitate the identification of this target/effector.

It is interesting that NIH3T3 cells that express any of the three fast-cycling mutants, Cdc42(F28L), Rac(F28L), and RhoA(F30L), are able to grow to high density (i.e. lose normal contact inhibition) and to grow in low serum. For the case of Cdc42, these results differ from those reported by other groups (14, 63) when using a GTPase-defective Cdc42 mutant. We suspect that the inability of cells expressing the GTPase-defective Cdc42 to grow to high densities, or to grow in reduced serum levels, most likely reflects the difficulties that we faced when trying to generate stable cell lines overexpressing GTPase-defective Cdc42, and it argues for the advantage in studying a fast-cycling Cdc42 mutant when trying to assess the contributions that Cdc42 makes to Dbl-induced transformation. Apparently, it is the ability of the fast-cycling Rac mutant to allow cells to grow to high density and in low serum that explains how cells expressing this mutant generate tumors in nude mice.

Thus, all three of the Dbl targets, when constitutively active but still GTPase-competent, can alter different aspects of the regulation of normal cell growth and thereby initiate tumorigenic signals. The ability of Dbl to give rise to a potent malig-
nant transformation signal apparently reflects its ability to activate each of these small G protein signaling pathways (depicted schematically in Fig. 7). This would suggest that oncogene products need not necessarily be capable of exhibiting all characteristics associated with cellular transformation, although those that do will have the greatest likelihood to elicit a potent tumorigenic outcome. Although it was tempting to speculate that activated Cdc42, Rac, and RhoA would act synergistically to fully reproduce the actions of oncogenic Dbl, as might be inferred from the results of other studies (63), we have not been able to directly demonstrate such cooperation between these Dbl targets when assaying different aspects of cellular transformation. This may in part reflect the formidable problem of generating cell lines that have adequate expression of all three (fast-cycling) GTP-binding proteins and the likelihood that there exists a carefully coordinated timing in the Dbl-stimulated activation of each of these GTP-binding proteins that cannot be reproduced through their simple co-expression in cells. However, the development of fast-cycling mutants and cell lines that express each of the Dbl substrates now offers an exciting opportunity to dissect the different aspects of the total transforming signal induced by a potent oncoprotein. In the future, these tools should yield new insights into the molecular mechanisms that underlie each of the individual cellular activities that contribute to the total malignant phenotype.

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