The Pleckstrin Homology Domain Mediates Transformation by Oncogenic Dbl through Specific Intracellular Targeting*

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The pleckstrin homology (PH) domain is an ~100 amino acid structural motif found in many cellular signaling molecules, including the Dbl oncoprotein and related, putative guanine nucleotide exchange factors (GEFs). Here we have examined the role of the Dbl PH (dPH) domain in the activities of oncogenic Dbl. We report that the dPH domain is not involved in the interaction of Dbl with small GTP-binding proteins and is incapable of transforming NIH 3T3 fibroblasts. On the other hand, co-expression of the dPH domain with oncogenic Dbl inhibits Dbl-induced transformation. A deletion mutant of Dbl that lacks a significant portion of the PH domain retains full GEF activity, but is completely inactive in transformation assays. Replacement of the PH domain by the membrane-targeting sequence of Ras is not sufficient for the recovery of transforming activity. However, subcellular fractionations of Dbl and Dbl mutants revealed that the PH domain is necessary and sufficient for the association of Dbl with the Triton X-100-insoluble cytoskeletal components. Thus, our results suggest that the dPH domain mediates cellular transformation by targeting the Dbl protein to specific cytoskeletal locations to activate Rho-type small GTP-binding proteins.

The cytoskeletal-associated Dbl oncoprotein transforms NIH 3T3 cells (1) by activation of signaling pathways involving Rho-type GTP-binding proteins (2). Proto-Dbl is a 115-kDa cytoskeletal-associated protein that is found in brain, adrenal glands, and gonads (1). Oncogenic activation occurs as an outcome of an amino-terminal truncation of proto-Dbl, where a recombination event fuses about 10 kDa of an unidentified human gene product (from chromosome 3) on to the carboxy-terminal half of Dbl (residues 498–925). The oncogenic Dbl protein contains at least two putative signaling motifs. The first is a region of 176 amino acids (residues 498–674) that was originally found to share significant homology with the Saccharomyces cerevisiae cell-division-cycle protein, Cdc24, and the breakpoint cluster region protein, Bcr (3). This region, referred to as the Dbl homology (DH) domain, has been shown to be essential both for the transformation activity of oncogenic Dbl and for its ability to act as a GEF by stimulating the guanine nucleotide exchange activity of Cdc42Hs (4, 5). The second putative signaling motif is the pleckstrin homology (PH) domain (6, 7) and includes residues 703–812. Although PH domains appear to be relatively poorly conserved, both NMR and x-ray crystallographic studies of the PH domains of pleckstrin, dynamin, and spectrin indicate that they adopt a common three-dimensional structural motif (8–11).

Over the past few years, a growing family of oncogene products and other growth regulatory proteins have been shown to contain a DH domain in tandem with a PH domain. In addition to Cdc24 and Bcr, these include the Vav, Ost, Ect-2, Lbc, Lfc, and Dbs oncoproteins (12–17) and the activators of the Ras proteins, Sos (18), and Ras-GRF (19). All indications from previous studies are that the DH domain will form a binding site and in many cases contain GEF activity for Rho-like GTP-binding proteins (8, 9, 13, 14, 20–22). However, less is known about the roles of the PH domains. In the present study, we have used the Dbl oncoprotein as a model to examine the role of the PH domain in cellular transformation and GEF activity.

EXPERIMENTAL PROCEDURES
cDNA Transfection Studies—Transfection assays were done on duplicate cultures by adding 0.001, 0.01, 0.1, and 1 μg of DNA to the recipient NIH 3T3 cells using the CaCl2-phosphate precipitation method (3). Foci (focus forming units) were scored 14 days after transfection, and the results were calculated as number of foci/pmol of DNA. The results listed in Fig. 1 and shown in Fig. 2C are the mean values of three transfection assays. Growth in soft agar was examined as described by Ron et al. (3).

Cellular Fractionation Studies—Control NIH 3T3 and different NIH 3T3 transfectants were lysed and fractionated into cytosolic (S), Triton X-100-solubilized membrane fractions (T), and Triton X-100-insoluble fractions (I) as described by Graziani et al. (2). Cells were labeled with [35S]methionine and [35S]cysteine for 3 h at 37°C. Specific Dbl products were detected by immunoprecipitation using anti-Dbl-2 antibodies (3), electrophoresed through a 12% polyacrylamide gel and autoradiographed. For the detection of the PH domains (e.g. Figs. 2B and 4D, below), cells were immunoprecipitated with anti-Flag M5 antibodies and electrophoresed through a 15% polyacrylamide gel followed by immunoblotting with anti-Flag M5 antibodies.

Measurements of GDP Dissociation from Cdc42Hs—The [3H]GDP dissociation assays were carried out as described previously (4, 5). In Fig. 3A, the amounts of GST-Dbl and GST-DH (see Fig. 1) purified from Sf9 insect cells were estimated by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis. ~200 μg of GST-Dbl or GST-DH were incubated with 1 μg of RhoA protein preloaded with [3H]GDP in 100 μl of reaction buffer at room temperature, and 16-μl aliquots were diluted into 5 ml of ice-cold termination buffer (20 mM Tris-HCl, pH 7.4, 10 mM MgCl2, and 100 mM NaCl) at various time points. In Fig. 3B, 1 μg

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domain lost the ability to grow in soft agar (data not shown). In some cases, we found that expression of a Flag-tagged PH domain of Vav caused some inhibition of Dbl-induced growth in soft agar, suggesting that the Vav PH domain (perhaps when expressed at sufficient levels) was capable of competing with the PH domain of Dbl for a cellular target. However, it is likely that the Vav PH domain is a weak competitor, since we often observed no detectable effects with either the Flag-tagged protein or when expressing the PH domain of Vav from the pKH3 vector. We also have found no detectable effects on Dbl transformation when expressing the PH domain from Cdc24 (data not shown). Mass cultures of Dbl transfectants expressing the dPH domain also displayed a less transformed phenotype compared with Dbl transfectants alone or compared with cells co-expressing Dbl and the PH domain of Vav (data not shown). Taken together, these results suggest that the PH domain of Dbl behaves as a selective antagonist of Dbl-induced transformation, possibly by binding to a saturable and specific ligand in cells.

Previously we have shown that the DH domain alone is sufficient for the GEF activity for Cdc42Hs (5). Since oncogenic Dbl also stimulates the guanine nucleotide exchange activity of Rho, we examined whether the Dbl domain is sufficient for stimulating the activation of Rho. To do this, we compared Rho-GEF activities of approximately equal amounts (~200 ng) of insect cell-expressed, purified GST-Dbl and GST-DH domain. No significant differences were observed for the abilities of the GST-Dbl and GST-DH to stimulate [3H]GDP dissociation from RhoA (Fig. 3A). These results suggest that the PH domain does not contribute to the GEF function of Dbl. This is further reinforced by the results in Fig. 3B, which show that the addition of excess Escherichia coli recombinant PH domain to GEF assay mixtures containing [3H]GDP-bound RhoA and recombinant GST-Dbl has no detectable effect on the time-course of GST-Dbl-stimulated [3H]GDP dissociation from RhoA. The GST-PH domain, alone, also shows no ability to stimulate [3H]GDP dissociation from RhoA (compared with GST alone). Similar results were also obtained with [3H]GDP-bound Cdc42Hs (data not shown). Thus, the DH domain is not involved in the interactions of Dbl with RhoA and Cdc42Hs or in the direct regulation of the GEF catalytic activity of the DH domain.

The membrane association of βARK and spectrin has been attributed to their PH domains (24, 25). The PH domains of βARK, BTK, PLCγ, IRS-1, and Ras-GRF have been shown to bind to plasma membrane-associated βγ subunits of the heterotrimeric G-proteins (26, 27), and they all behave as antigens of Gβγ-mediated signaling (28). Recent evidence also suggests that PH domains from many signaling molecules including βARK and Ras-GAP can bind to specific phospholipids, namely phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (29). These findings raised the possibility that the PH domain mediates the membrane targeting of oncogenic Dbl. It has been shown that the introduction of a membrane-targeting sequence into the Ras GEFs, Cdc25 and Sos (30, 31), was sufficient to activate Ras, and more recently, that the addition of a membrane-targeting sequence in place of the PH domain of the Lfc oncoprotein was able to restore full transformation activity (32). Thus, we examined whether the substitution of the DH domain with a membrane-targeting sequence enabled the DH domain of Dbl to induce transformation. A chimeric molecule containing the DH domain (residues 498–756) fused to the Ras membrane-targeting farnesyl ation signal sequence (designated pdHF in Fig. 1) was constructed and assayed for focus-forming activity in NIH 3T3 cells. This chimera was expressed at a comparable level to oncogenic Dbl and a percentage (10–20%) of the total chimeric molecules was targeted to the membrane...
surface (i.e. the Triton X-100 solubilized fraction (T) in Fig. 4A). However, this did not restore transforming activity to the DH domain (Fig. 1). Although, one possible explanation is that the amount of the chimera expressed at the membranesurface was not sufficient to stimulate a transforming signal, this does not seem likely based on what we have observed regarding the range of expression of oncogenic Dbl that will yield cellular transformation (34). We have reported previously that significant portions of both proto- and oncogenic Dbl are localized to the Triton X-100-insoluble fractions of transfected NIH 3T3 cells, suggesting an association with the cytoskeletal matrix (1). To address the possible role of the dPH domain in mediating this pattern of localization for the Dbl protein, stable transfectants of Dbl and Dbl deletion mutants (Fig. 1) were subjected to subcellular fractionation. The crude membrane fractions (P100) of the cells were solubilized either by 1% Triton X-100 or by treatment with 0.1% SDS and 0.25% sodium deoxycholate. Anti-Dbl immunoprecipitates revealed that a percentage of both the intact oncogenic Dbl protein and a truncation mutant pMA4 associated with the Triton X-100-insoluble fractions of cells (designated by I in Fig. 4A). However, the DH domain of Dbl, which lacks transforming ability, was localized exclusively to the cytosolic fraction (designated S in Fig. 4C). When cells expressing the Flag-tagged PH domains were subjected to similar fractionation, the PH domains were found associated with the Triton X-100-insoluble fractions (Fig. 4D). These results suggest that the dPH domain is directly responsible for the association of oncogenic Dbl with the Triton X-100-insoluble cytoskeletal fraction and thus may serve to target the catalytic DH domain to the cytoskeleton.

We have reported previously that the DH domain is responsible for Dbl GEF function and is required for Dbl transforming activity (3, 5). Here, we demonstrate that while the dPH domain does not seem to be involved in the interactions of Rho-type small GTP-binding proteins with Dbl, it is essential for Dbl transforming activity. Thus, our present findings establish that both the DH and PH domains are required for the cellular function of Dbl. Indeed, the minimum structural unit (pMA4) of oncogenic Dbl conferring complete transforming activity just
encompasses the DH domain and the PH domain. The finding that plasma membrane-targeting of Dbl is not sufficient to confer transforming activity, coupled with the requirement of the dPH domain as the necessary and sufficient element for association of the Dbl protein with the Triton X-100-insoluble fraction of cells, suggests that function of the PH domain resides in its ability to target the catalytic DH domain to the cytoskeletal matrix. Whether this targeting function holds for other members of Dbl-related GEF family proteins remains to be seen. However, based on the observation that the PH domains of Dbl-related molecules Vav and Cdc24 do not act as effectively as inhibitors of Dbl-induced transformation, it is an attractive possibility that different members of the family of Dbl-related proteins may be targeted by their PH domains to distinct cellular locations to activate various Rho-type GTP-binding proteins, in response to different extracellular stimuli such as epidermal growth factor, platelet-derived growth factor, lysophosphatidic acid, and bradykinin. This may also explain the finding that substitution of a membrane-targeting (i.e. Ras-farnesylation) sequence for the PH domain of Lfc restored its transformation capability (32), whereas this substitution did not restore transforming activity to a Dbl protein that just contains the DH domain. It may be that Lfc needs to be targeted to the plasma membrane to optimally couple to other protein components in its signaling pathway while Dbl needs to be targeted to a cytoskeletal location.

The identity of the ligand(s) that binds to the PH domain of oncogenic Dbl will represent an important focus of future studies. It seems likely, that given the hypervariable nature of the putative ligand-binding site in the PH domains that have thus far been identified (33), a complex diversity of ligands exist that are responsible for mediating the actions of various PH domain-containing signaling molecules, including Dbl and related regulatory molecules of small GTP-binding proteins.

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Fig. 4. The PH domain mediates the cytoskeletal association of the Dbl oncoprotein. A, the membrane attachment signal from Ha-Ras targets the DH domain to the Triton X-100-soluble fraction from cell membranes. pZipneo/DHF (see Fig. 1) represents the construct encoding the DH domain of Dbl and the carboxy-terminal 16 amino acids of Ha-Ras, including the palmitylation and farnesylation sites. pZipneo/DHF* represents the construct encoding the DH domain of Dbl and the carboxy-terminal 16 amino acids of Ha-Ras (except that the cysteine which serves as the farnesylation site has been changed to serine). S represents the soluble fraction, T the Triton X-100-soluble fraction from membranes, and I is the Triton X-100-insoluble fraction. B, oncogenic Dbl is associated with the Triton X-100-insoluble fraction of cells. C, fractions of the pMA4 and DH domain transfectedants. D, fractionation of cells expressing the Dbl PH domain. The data shown in A–C were obtained by immunoprecipitating the Dbl proteins with the anti-Dbl antibody from cells that were labeled with [35S]methionine and [35S]cysteine.