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Membrane Binding and Enzymatic Activation of a Dbl Homology Domain Require the Neighboring Pleckstrin Homology Domain

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Dbl-homology (DH) domains are invariably located immediately N-terminal to a pleckstrin homology (PH) domain. To understand the functional relationship between these two domains we expressed the DH domain alone, the PH domain alone, and the DH-PH combination of the invasion inducing protein Tiam-1 fused to glutathione-S-transferase (GST) or green fluorescent protein (GFP). We found that the GST-DH-PH and the GST-PH constructs bind to preparations of brain membranes and to the $\beta\gamma$ subunits of trimeric G proteins *in vitro,* while the GST-DH and GST control do not. The GFP-DH-PH and GFP-PH constructs are localized to peripheral membranes of COS-7 cells *in vivo,* while GFP and GFP-DH domain constructs are found diffusely in the cytoplasm. The DH-PH domain combination activates Jun N-terminal kinase (JNK) strongly, but the DH domain alone and the PH domain alone have little effect. We conclude that membrane localization and enzymatic activation of the DH domain require the adjacent PH domain.

Pleckstrin homology (PH) domains are ~100 amino acid modules found in numerous eukaryotic proteins which are thought to have a pivotal role in cellular signal transduction [1-4]. A large family of such proteins, which includes the proto-oncogenes Dbl, Vav, Ost/Dbs and Bcr, also contain a Dbl homology (DH) domain, a ~180 amino acid module which is invariably located immediately N-terminal to a PH domain [5]. DH domains function as guanine nucleotide exchange factors (GEFs) for small G proteins of the Rho family. These small G proteins heavily influence the actin cytoskeleton [6] and can also, apparently independently, activate specific transcription and other responses by means of the Jun N-terminal kinase (JNK) pathway [7-10]. The various members of the Rho protein family are also required for normal cell cycle progression [11]. Previous studies have shown that transformation by oncoproteins containing the DH-PH combination requires both an intact DH and an intact PH domain, but that other parts of these molecules are not essential [12-14]. These findings naturally raise questions as to the role of the PH domain in the DH-PH combination. Previous experiments have shown that PH domains bind to at least two different classes of ligand. The N-terminal regions of certain PH domains binds with variable affinity to various phosphatidylinositol compounds [15-19]. The C-terminal peptide of certain PH domains binds with variable affinity to $\beta\gamma$ subunits of trimeric G proteins ($G\beta\gamma$) [21-23]. Binding of certain PH domains to protein kinase C isotypes and other proteins has also been reported [23-26]. Recent studies suggest that Ptb domains, which bind to specific tyrosine phosphorylated peptides, are members of the PH domain family, and also bind to proteins by means of interactions involving the C-terminal region [27,28]. These findings suggest that PH domains allow proteins to bind to cellular membranes and form specific protein complexes in a signal dependent manner [29,4]. It therefore seems plausible that the DH-PH domain combination is anchored to the membrane by the neighboring PH domain, that the PH domain may also bind to and be regulated by PH domain binding proteins, and that DH domain activity may require membrane localization. To begin to test these hypotheses we have expressed various constructs including the DH and PH domains of the invasion and metastasis inducing gene, Tiam-1 [30], together and separately, for use in a variety of *in vitro* and *in vivo* assay systems. Proviral insertion into the Tiam-1 gene resulted in increased metastasis and invasiveness in T-lymphoma cells [30]. Tiam-1 was shown to contain a DH-PH domain segment as well as a second more N-terminal PH domain

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and to be well conserved in protein sequence across vertebrate species [31]. Homologues of Tiam-1 have been identified in *Drosophila*, which intriguingly have a role in synaptic transmission [32]. Tiam-1 is a powerful activator of Rac 1 [33], a member of the Rho family of small G proteins. Proteins of this family in turn can activated JNK, suggesting that JNK activity should reflect the enzymatic activity of an appropriate DH domain. Our findings suggest that the C-terminal Tiam-1 PH domain is involved in both membrane localization and activation of the neighboring DH domain and that efficient activation of the JNK pathway also requires the combination of the DH and PH domains.

MATERIAL AND METHODS

Expression of Tiam1-GST constructs in E. coli. A cDNA encoding the C-terminal region of Tiam-1 was obtained from Dr. John Collard. PCR primers were designed to amplify out selected cDNAs and add 5′ *EcoRI* and a 3′ *SalI* sites to allow directional cloning into appropriate expression vectors [34]. Primers were designed to amplify out segments encoding the DH-PH combination, the DH domain alone and the PH domain alone (see figure 1). The DH domain contains three structurally conserved regions (SCR) numbered 1 to 3, each consisting of a relatively well conserved peptide sequence [35]. The functional DH domain is believed to require all three of these SCRs and about 20 amino acids C terminal to SCR3. Our constructs start a amino acid 1024, the first amino acid of SCR1 being at 1041. The last amino acid of the DH alignment is 1233, and our DH domain construct ends at 1255. The PH domain constructs are also flanked by at least 20 amino acids at both N and C-termini. PCR products were first cloned into pGEM-T vector (Promega), verified by nucleic acid sequencing and correct size inserts were cut out with *EcoRI* and *SalI*, purified by agarose gel electrophoresis, and inserted into pGEX4T-1 (Pharmacia). The correctness of the various constructs was confirmed by expression of fusion proteins of the expected SDS-PAGE molecular weight and immunoreactivity (figure 1). GST fusion proteins were expressed and purified with glutathione-agarose affinity resin (Sigma) as described previously [21].

Transient expression of Tiam1 constructs in COS-7 cells and microscopic observation. The cDNAs encoding NPH, DH-CPH, DH and CPH of Tiam-1 were also cloned into *EcoRI* and *SalI* sites in the pCI-Neo-GFP vector described previously [34]. Briefly, this vector is based on the pCI-Neo vector of Promega but is engineered to express a Ser65>Thr mutant form of *Aequoria victoria* Green Fluorescent Protein (GFP). The cDNA encoding the GFP was codon optimized to maximize expression in mammalian cells [36]. We altered the original pCI-Neo polylinker to accomodate this construct with an appropriate 5′ Kozak sequence and added a 3′ polylinker allowing insertion of the same PCR constructs used to make GST fusion proteins. Transfection of pCI-Neo-GFP plasmids into COS-7 cells was performed with Lipofectamin (Gibco/BRL) using the protocol suggested by the manufacturer. 5μg of plasmid DNA and 5μl of lipofectamin were used for each transfection on each 60mm dish seeded with 5×10^5 cells 24 hours before transfection. The GFP transfected cells were viewed on a Zeiss Axiophot fitted with epifluorescence equipment and filters for the visualization of fluorescein flourescence after treatment with −20°C methanol for 5min as described [34].

Brain membrane, G$\beta\gamma$ preparations and binding assays in vitro. Crude bovine brain membrane (cBBM), NaOH-extracted cBBM (NaBBM) and NaOH extracted and chymotrypsin-treated cBBM (ChyBBM) were made as described previously [37,19]. Briefly 2μl of these concentrated membrane preps, containing 20μg of protein in the case of the cBBM material, were incubated with ~2μg of each fusion protein on ice for 30 minutes. The preparations were then made up to a volume of 20μl and spun at r_{max} 436,000g for 40 minutes through a 10% sucrose layer in a Beckman TL100 centrifuge. The membrane pellets were collected and run out on SDS-PAGE, and protein bands quantitated using a digital gel documentation system and GelBase Pro software (UV Products Ltd. Cambridge U.K.). Results were presented as relative optical density units. G$\beta\gamma$ was prepared from bovine brain by the method of Sternweis [38] as described previously [21]. Preparations of G$\beta\gamma$ were run out on SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were probed with GST fusion proteins and binding was detected using anti-GST monoclonal antibody (Santa Cruz) and goat anti-mouse alkaline phosphatase conjugate (Sigma) and the reaction was developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate chromagens (Sigma) as described [21].

Immunoprecipitation and assay of JNK activity. COS-7 and NIH-3T3 cells were transiently transfected with the GFP-DH-CPH, GFP-DH, GFP-CPH and GFP control as described above and harvested 48 hours after transfection. The cells were lysed in 50mM Hepes-KOH (pH=7.3), 300mM NaCl, 5mM EDTA, 0.1% NP40, 1mM DTT, 1mM p-nitrophenylphosphate (PNPP), 10mM β-glycerophosphate and 100μM Vanadate in the presence of a cocktail of protease inhibitors. Cell debris were spun down and supernatants were precleared twice with normal rabbit serum plus Pansorbin (Calbiochem). The JNK protein was immunoprecipated with the rabbit anti-JNK polyclonal antibody 247, a kind gift of Dr. Michael Karin. Immunoprecipitated JNK was used in a kinase assay using recombinant human c-Jun N-terminus as the substrate as described [39]. Phosphorylated c-Jun was resolved by SDS-PAGE, c-Jun radioincorporation was visualized autoradiographically and digitally quantitated using our gel documentation system as described above.

RESULTS

Expression and in vitro binding of Tiam1 constructs. The segments of Tiam-1 used to produce the various DH-PH, DH and the PH domain constructs are shown diagrammatically in the left of figure 1. The right of figure 1 shows SDS-PAGE gels of these purified glutathione-S-transferase (GST) fusion proteins. Protein bands of apparent molecular weight about 44kDa (GST-NPH), 68kDa (GST-DH-CPH), 51kDa (GST-DH), 43kDa (GST-CPH), and 26kDa (GST) are seen. These are as expected and show that the protein expression is efficient and produces proteins of the expected size which are with the exception of the GST-NPH construct not subjected to significant degradation. The various GST fusion proteins were assessed for binding to preparations of crude bovine brain membranes (cBBM), cBBM preparations which had been extracted with 0.1M NaOH (NaBBM), and NaBBM preparations which had been then further extracted with chymotrypsin (ChyBBM) made using a protocol based on that of Steiner and Bennett [37]. Previous work showed that cBBM preparations contain a large amount of associated proteins, but that very little protein is found in either NaBBM or ChyBBM preparations [19]. The Tiam-1 GST-DH-CPH and GST-CPH fusion proteins bind to cBBM and show somewhat weaker but still apprecia-

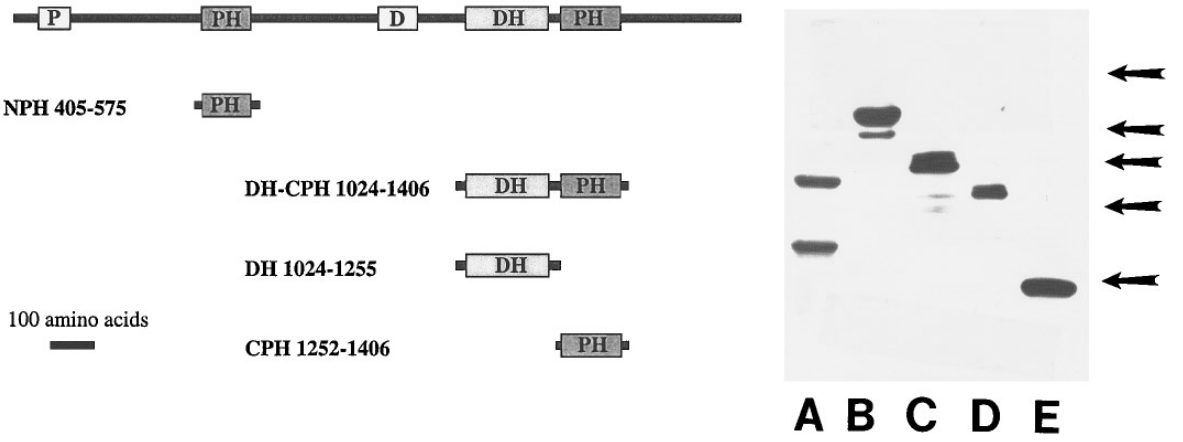

FIG. 1. Left: diagram of cDNAs used. Tiam-1 includes a putative PEST region (P), a second PH domain closer to the N-terminus, and a DHR domain (D) [30,47]. Right: Western-blot of Tiam-1-GST fusion proteins and GST control with antibody to GST. Lane A: GST-NPH; B: GST-DH-CPH; C: GST-DH; D: GST-CPH; and E: GST control. Arrows show positions of SDS-PAGE standards of molecular weight from top to bottom of 97.4, 66.2, 55, 42.7, and 31 kDa, respectively.

ble binding to NaBBM and ChyBBM *in vitro* (figure 2A). The Tiam-1 GST-NPH construct showed comparable binding to all membrane preparations, and the GST-DH domain alone and GST control showed very weak binding to cBBM and undetectable binding to the extracted membranes. PH domains of numerous proteins have been shown to bind G$\beta\gamma$ *in vitro* [20] and evidence has been presented suggesting an *in*

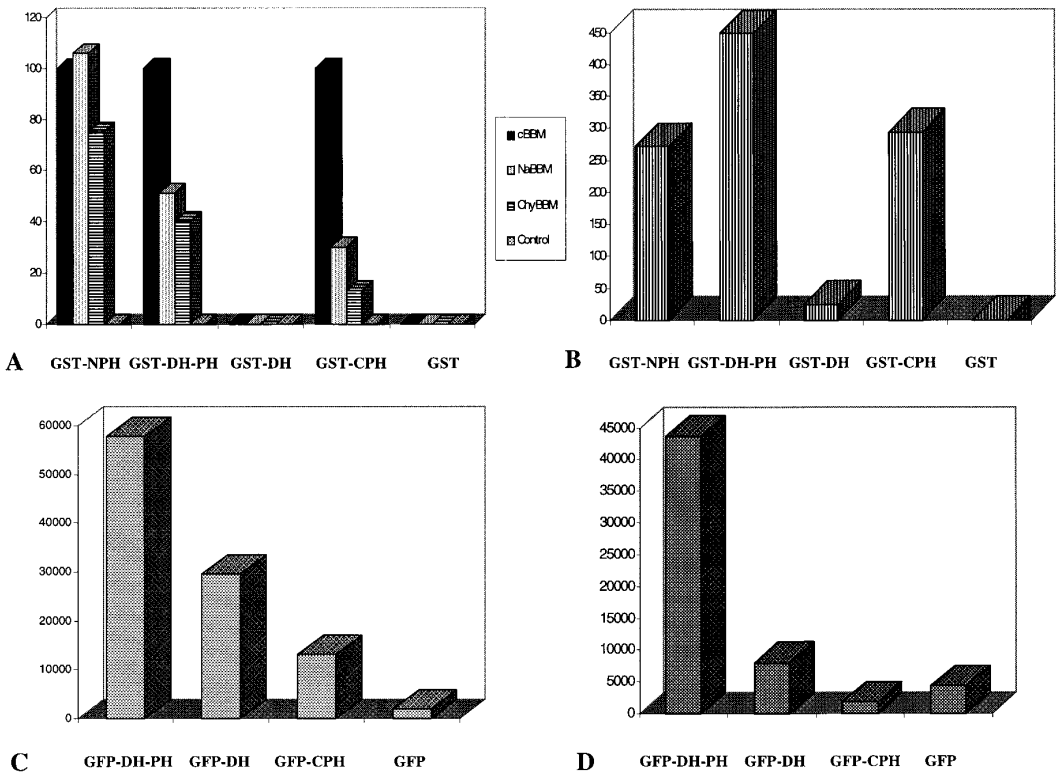

FIG. 2. A: Binding of GST-Tiam-1 constructs and GST control to various brain membrane preparations as outlined in text. The results are mean values of two independent experiments. B: binding of GST-Tiam-1 constructs to purified G$\beta\gamma$ subunits (average of two experiments). C: the activation of JNK by Tiam-1 GFP-DH-CPH, GFP-DH, GFP-CPH, and GFP control constructs transfected into COS-7 cells. D: the same experiment as C performed in NIH-3T3 cells.

vivo interaction also [40,22]. Bovine brain G$\beta\gamma$ preparations were run out on SDS-PAGE and binding of GST fusion proteins to the Gβ band was detected using a GST antibody as described previously [21]. The GST-CPH, GST-DH-CPH and GST-NPH fusion proteins showed strong binding in this assay, while the GST and GST-DH showed only background binding (figure 2B).

Subcellular localization of Tiam-1 GFP fusion proteins in vivo in COS-7 cells. To test if the Tiam-1 PH domain would bind to membranes in living cells we generated fusion proteins with a codon optimized and Ser65>Thr mutant version of the *Aequoria victoria* green fluorescent protein (GFP) using the pCI-Neo-GFP vector described previously [34]. These constructs express well when transfected into COS-7 cells, and we were able to detect strong and essentially undegraded protein bands reactive with GFP antibody at the expected size in cell homogenates (not shown). When viewed with epifluorescence microscopy the GFP-DH-CPH (figure 3a-d) and GFP-CPH (figure 3e-f) domain constructs were clearly localized to the plasma membrane region when transfected into COS-7 cells. Live COS-7 cells expressing either the GFP-PH domain or the GFP-DH-CPH domain showed a marginal band of GFP fluorescence which was not visible with the GFP control. We found that fixation of such cells in −20°C methanol made the plasma membrane binding results easier to see, as we had found previously with the βIϵII-spectrin PH domain fused to GFP [34] (figure 3a-f). For comparision purposes we include a previously published micrograph of a COS-7 cell transfected with GFP-βIϵII-spectrin PH domain (figure 3h). The GFP localization at the cell margin seen with the DH-PH and DH constructs was qualitatively similar, but clearly less intense than that seen previously with the $\beta\epsilon$II-spectrin construct. The GFP-DH construct showed only diffuse labeling in live cells, and showed some localization to currently unidentified internal cellular components following fixation in −20°C methanol (figure 3g). The GFP-NPH construct showed very strong membrane localization comparable to that seen with the spectrin construct (not shown). GFP when expressed alone, as reported previously, showed only diffuse cytoplasmic staining in live cells and was completely removed by fixation in −20°C methanol (shown in reference 34).

Activation of Jun N-terminal kinase (JNK) in COS-7 and NIH-3T3 cells. Previous studies have shown that certain Rho family proteins can activate JNK by means of an enzyme cascade homologous to the MAP kinase pathway [7]. Other workers have shown that Dbl and Ost/Dbs, which contain DH-PH domain combinations, can activate JNK in COS-7, NIH-3T3 and Hela cells [12,42]. Since DH domains activate Rac/Rho family members, it seems likely that activation of the Tiam-1 DH domain should also result in activation of JNK. If this is the case, JNK activity could then be used as a simple assay for DH domain enzymatic activity. As shown in figures 2c and 2d the Tiam-1 DH-PH domain combination activated JNK strongly in both COS-7 cells (2c) and in NIH-3T3 cells (2d). In contrast the PH domain alone and the DH domain alone had a much weaker activity, a difference particularly apparent in NIH-3T3 cells. We also performed these experiments with the pCI-Neo-Mod vector described previously [34] which does not contain GFP cDNA and expresses the cloned inserts without a fusion partner. Similar results were obtained (not shown).

DISCUSSION

These experiments provide several lines of evidence consistent with the hypothesis that the C-terminal PH domain in the DH-PH domain combination of Tiam-1 binds the neighboring DH domain to the cellular membranes both *in vitro* and *in vivo*. Furthermore the PH domain is required for the efficient enzymatic activation of the DH domain as assayed by JNK activity. The experiments also show that the DH domain on its own does not bind preparations of brain membranes or of G$\beta\gamma$ *in vitro* nor does it have the ability to localize to membranes *in vivo*. These findings make a strong case that the function of the PH domain in the DH-PH combination is to allow this protein complex to become localized to cellular membranes. The activation of the enzymatic properties of the DH domain presumably involves the localization of the GEF activity in proximity to the appropriate small G-proteins, which are also normally membrane localized by means of C-terminal isoprenylation modifications [6]. This mechanism is analagous to that used by several other signal transduction pathways in which enzymes such as phospholipases, lipid kinases and other regulators of small G proteins are brought into contact with their membrane localized lipid and protein substrates. Presumably the DH domains and PH domains of other members of the large and continuously growing Dbl protein family will function in a similar way.

Work from another laboratory has shown that Tiam-1 activates the small G protein Rac1 preferentially [33,43]. Other workers have shown that Rac proteins activate JNK [7,44]. Taking these results together we would expect that activation of the DH domain of Tiam-1 should result in the activation of JNK, providing a potential assay for DH activation. The results presented here establish a direct link between the membrane localization of the Tiam-1 DH domain and the activation of JNK and show that efficient JNK activation requires the neighboring PH domain. The finding that the DH-PH domain activates JNK, a protein which

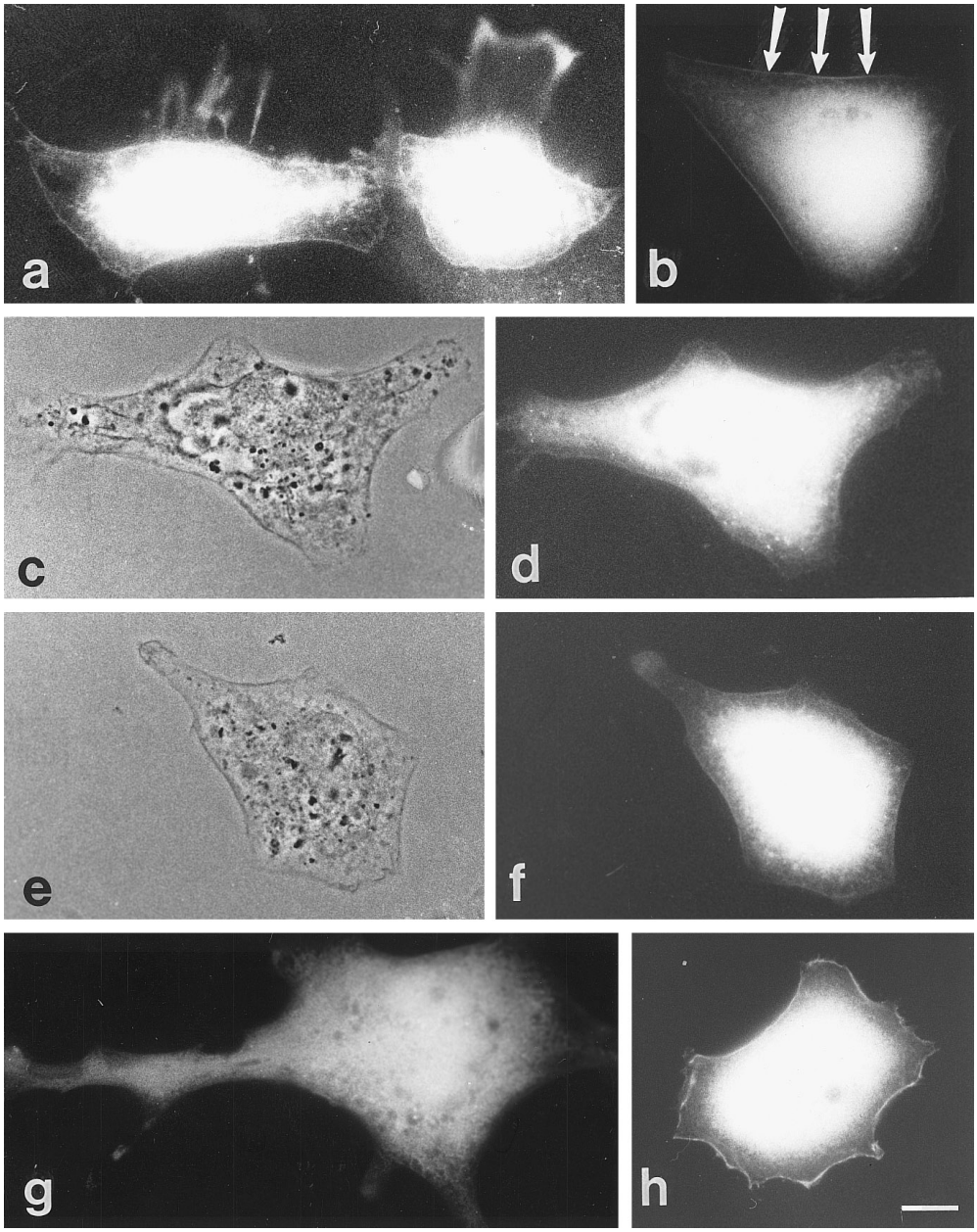

FIG. 3. COS-7 cells transfected with pCI-Neo-GFP vector with various inserts. Plates a and b and c and d were transfected with the GFP-DH-CPH domain construct. Plates a, b, and d show epifluorescence views and c shows a corresponding phase contrast view of d. Plates e and f show phase contrast (e) and epifluorescence (f) micrographs of a cell transfected with the GFP-CPH domain construct. Plate g is an epifluorescence micrograph of a cell transfected with the GFP-DH construct, while plate h is a previously published micrograph of a cell transfected with the GFP-βIϵII-spectrin construct. All magnifications are approximately equal and bar in plate h corresponds to 10 μm.

affects transcription of a variety of cellular proteins, naturally raises the question as to whether this is the primary route by which Dbl family proteins become oncogenic.

Recently Whitehead and coworkers [14] showed that the DH domain of Lfc, activated by removal of the sequence immediately N-terminal to the DH domain, was oncogenic if fused to either a CAAX box or a PH domain, but not in the absense of either. These experiments also suggest a role for the PH domain in membrane binding and activation of the neighboring DH domain and are consistent with the findings presented here. As shown here the DH-PH domain was much more effective in the JNK assay than either the PH or the DH domains alone. The relatively strong activation of JNK by the DH domain alone as seen in COS-7 cells

is presumably related to the efficiency of the CMV promoter which produces large amounts of GFP fusion protein in these cells. The same constructs are expressed less efficiently in NIH-3T3 cells, and in these cells the activation of JNK seen by the DH domain alone is more modest.

Several studies have shown that the β-ARK PH domain binds G$\beta\gamma$ *in vitro* more strongly than the PH domain of βIϵII-spectrin [20,21]. We have shown previously that the βIϵII-spectrin PH domain binds to crude membranes, phosphatidylinositol-4,5-bisphosphate (PIP2) containing vesicles and inositol-1,4,5-trisphosphate (IP3) much more strongly *in vitro* than the β-ARK PH domain [19,34]. The robust binding of the Tiam-1 C-terminal PH domain to G$\beta\gamma$ (figure 2B) and the reduced binding to protein depleted membranes (figure 2A) more closely resemble the binding properties of the β-ARK PH domain than the βIϵII-spectrin PH domain. Other experiments show that, like the β-ARK PH domain and unlike the βIϵII-spectrin PH domain, the Tiam-1 C-terminal PH domain binds PIP2 containing vesicles poorly and does not bind IP3 in a gel filtration assay (not shown). Current studies suggest that β-ARK translocates between the cytosol and the membrane in a signal dependent manner and that activation of β-ARK is modulated both by G$\beta\gamma$ and by phospholipid binding [45,46]. It remains to be seen if membrane binding and protein binding act synergistically on the PH domain and activate Tiam-1 *in vivo* as appears to be the case with β-ARK. The full length Tiam-1 molecule contains another PH domain which binds strongly to protein depleted membranes, and also contains a potential N-terminal myristoylation sequence and a DHR domain. All of these interactions would be predicted to help anchor the N-terminal region of Tiam-1 to the membrane (see figure 1). Perhaps the N-terminus of Tiam-1 is membrane localized by a combination of these mechanisms, and the relatively weak interaction of the C-terminal PH domain regulates the local translocation of the neighboring DH domain to and from the membrane. Future studies will hopefully elucidate the mechanisms by which the membrane binding and activation of Tiam-1, β-ARK and other PH domain containing proteins are regulated.

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