

Mouse disabled (mDab1): a Src binding protein implicated in neuronal development

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Here, we identify a mouse homolog of the *Drosophila* Disabled (Dab) protein, mDab1, and show it is an adaptor molecule functioning in neural development. We find that mDab1 is expressed in certain neuronal and hematopoietic cell lines, and is localized to the growing nerves of embryonic mice. During mouse embryogenesis, mDab1 is tyrosine phosphorylated when the nervous system is undergoing dramatic expansion. However, when nerve tracts are established, mDab1 lacks detectable phosphotyrosine. Tyrosine-phosphorylated mDab1 associates with the SH2 domains of Src, Fyn and Abl. An interaction between mDab1 and Src is observed when P19 embryonal carcinoma (EC) cells undergo differentiation into neuronal cell types. mDab1 can also form complexes with cellular phosphotyrosyl proteins through a domain that is related to the phosphotyrosine binding (PTB) domains of the Shc family of adaptor proteins. The mDab1 PTB domain binds to phosphotyrosine-containing proteins of 200, 120 and 40 kDa from extracts of embryonic mouse heads. The properties of mDab1 and genetic analysis of Dab in *Drosophila* suggest that these molecules function in key signal transduction pathways involved in the formation of neural networks.

Keywords: Grb2/mDab1/PTB/Shc/Src

Introduction

Numerous developmental processes are regulated by signaling cascades that alter protein phosphotyrosine levels. Many extracellular cues are linked to cellular responses via transmembrane receptor protein tyrosine kinases (PTKs) and phosphatases (PTPs). In the nervous system, transmembrane kinases and phosphatases are required for neuronal differentiation and survival, neurite extension, the directed growth of the neuronal growth cone and the fasciculation of nerve bundles (Snider, 1994; Callahan *et al.*, 1995; Tessier-Lavigne, 1995; Desai *et al.*, 1996; Krueger *et al.*, 1996). These transmembrane receptors are regulated directly by specific ligands. Cytoplasmic PTKs are also involved in the development of the nervous system, although the ligands which induce their activation are less well understood (Gertler *et al.*, 1989; Grant *et al.*, 1992; Umemori *et al.*, 1994). There is growing evidence that these kinases are regulated in pathways responding

to components of the extracellular milieu and may function to regulate axonal growth downstream of receptors which lack intrinsic kinase activity (Bixby and Harris, 1991).

The non-receptor PTK Src is highly expressed in the developing mammalian nervous system (Maness *et al.*, 1990; Maness, 1992). During neurogenesis, Src kinase activity increases and Src becomes concentrated in growth cones of neurons. Growth cones migrate by extending actin-rich filopodia and lamellipodia, and tyrosine phosphorylation is important for the formation of these actin structures (Wu and Goldberg, 1993; Goldberg and Wu, 1995). Neurons cultured from mice that lack Src extend neurites less well than wild-type neurons, when plated on surfaces coated with the neural cell adhesion molecule L1 (Ignelzi *et al.*, 1994). This defect is specific, since neurons lacking the Src relatives Fyn or Yes extend neurites normally (Beggs *et al.*, 1994). Moreover, neurons from mice lacking Fyn extend only short neurites on the neural cell adhesion molecule NCAM-140 but extend long neurites on L1 (Beggs *et al.*, 1994). Src and Yes are not needed for neurite extension on NCAM-140. These specific defects point to the existence of adhesion-stimulated, Src- and Fyn-dependent, regulatory processes required for neurite extension. Signals from neurotrophin receptor PTKs, such as TrkA, may also be relayed through Src. Nerve growth factor (NGF)-induced neurite extension is Src dependent in PC12 pheochromocytoma cells (Kremer *et al.*, 1991; Vaillancourt *et al.*, 1995).

The non-receptor tyrosine kinase Abl participates in nervous system development in *Drosophila*. The *Drosophila* Abl (dAbl) protein is found in many cell types in the developing embryo, but expression is highest in the cell bodies and axons of neurons of the developing central nervous system (CNS) (Gertler *et al.*, 1989; Bennett and Hoffmann, 1992). Flies lacking the *dAbl* gene develop past metamorphosis but die as adults before or soon after eclosion (Henkemeyer *et al.*, 1987). Five genes were identified in screens for dominant second site mutations that exacerbate the *dAbl*⁻ phenotype and have been dubbed HDA (haploinsufficient, dependent upon *dAbl*) genes (Gertler *et al.*, 1989; Hill *et al.*, 1995). When heterozygous for a mutation in a HDA gene, *dAbl*⁻ but not *dAbl*⁺ embryos die as embryos, with a characteristic terminal phenotype. The neurons of the CNS are present in normal number and extend axons, but gaps are apparent in the commissural and longitudinal axon bundles (Gertler *et al.*, 1989, 1993; Hill *et al.*, 1995). Three of the HDA genes, *disabled* (*dab*), *prospero* and *fax* have been cloned and have distinct properties (Vaessin *et al.*, 1991; Gertler *et al.*, 1993; Hill *et al.*, 1995). Homozygous mutations in the HDA genes *dab* and *fax* in a *dAbl* mutant background result in almost complete loss of CNS axonal tracts. The *dab* and *fax* genes also show dosage-sensitive interactions with each other (Gertler, 1992; Hill *et al.*, 1995) as well as with *dAbl* and, therefore, may have related functions.

The *dab* gene encodes a 2412 residue protein (Dab) that co-localizes with dAbl to the cell bodies and axons of embryonic CNS neurons (Gertler *et al.*, 1993). Dab is essential for normal CNS development, even in the presence of dAbl. Dab is tyrosine phosphorylated in insect cells and, given the co-localization with dAbl in the CNS, it has been suggested that Dab may be a physiological substrate of dAbl (Gertler *et al.*, 1993). However, the role of tyrosine phosphorylation in regulating Dab function, and the identities of the PTKs that phosphorylate Dab, remain unclear. The kinase activity of dAbl is dispensable for normal embryonic development, unless the levels of Dab or other HDA gene products are reduced by heterozygous mutations (Henkemeyer *et al.*, 1990). Using a temperature-sensitive mutant, dAbl kinase activity was shown to be required in *dab* heterozygotes after the time of cell fate specification and during the time of axonogenesis in the embryonic CNS (Henkemeyer *et al.*, 1990). Despite the loss of nerve bundles in the CNS, the total number of neurons is unaffected (Gertler *et al.*, 1990). These results suggest that dAbl has kinase-dependent and -independent roles in development. Other PTKs that are expressed in the fly CNS, such as *Drosophila* Src (dSrc) (Simon *et al.*, 1985), may substitute for the dAbl kinase requirement in CNS development, provided the levels of Dab are normal.

Here we report the identification of mDab1, a mammalian homolog of Dab. mDab1 was cloned based upon its interaction with Src in a yeast two-hybrid screen. The *mdab1* gene is expressed as a variety of spliced mRNAs in the nervous system and in some cell lines, and mDab1 proteins are differentially expressed and tyrosine phosphorylated during neural development. When phosphorylated on tyrosine, mDab1 binds to the SH2 domains of Src, Fyn and Abl. mDab1 also forms complexes with cellular phosphotyrosyl proteins, through a phosphotyrosine binding (PTB) domain. We suggest that mDab1 is an adaptor protein that participates in development of the nervous system.

Results

Identification of a murine homolog of Dab

To find Src-interacting proteins that are involved in mouse embryonic development, we used the yeast two-hybrid system. A library of mouse embryo cDNAs expressed as VP16 transactivation domain fusion proteins was screened for interaction with a fusion protein containing Src and the LexA DNA binding domain. The individual fusion proteins alone are unable to activate transcription, but stable interaction between them results in the transcription of the yeast *HIS3* gene and the bacterial *lacZ* gene (Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995; Vojtek and Hollenberg, 1995). Of 5×10^{-6} clones analyzed, 200 scored positive for both reporters with wild-type Src and 70% of these were dependent upon the catalytic activity of Src. Two known Src-interacting proteins, Fak (Cobb *et al.*, 1994; Schaller *et al.*, 1994) and Sam68 (Taylor and Shalloway, 1994; Fumagalli *et al.*, 1995; M.Brown, personal communication), were identified in addition to a number of cDNAs encoding novel proteins. Two identical cDNA clones, B3 and C46, had significant homology with the *Drosophila dab* gene and were analyzed further.

Complete cDNA clones for mDab1 were isolated by screening an embryonic day (E) 15–17 mouse brain cDNA library with the B3 probe (Figure 1A). These clones represented at least three different mRNAs, encoding mDab1 isoforms of 555, 217 and 271 residues (Figure 1A). The mDab217 mRNA diverges from mDab555 at a consensus splice donor sequence at codon 199, encodes a further 18 residues before a termination codon, and terminates with a 3'-untranslated sequence distinct from mDab555. The mDab271 mRNA contains an additional exon of 270 nucleotides inserted between codons 241 and 242 of mDab555. This exon encodes 30 residues before a stop codon. We also identified a fragment of a potential fourth cDNA using RT-PCR, inserted in the mDab555 mRNA at another consensus splice donor sequence, between residues 239 and 242. The B3 and C46 clones isolated in the two-hybrid screen include residues 106–274 of mDab555 (Figure 1A, broken underline). The common mDab1 initiation codon is preceded by an in-frame termination codon and is in a good consensus for translational initiation (Kozak, 1991).

Several mDab1 relatives were identified in a database search (Figure 1B) including p96 (Xu *et al.*, 1995, now referred to as mDab2; DDBJ/EMBL/GenBank accession No. U18869) and its human homolog, DOC2 (Mok *et al.*, 1994; Albertsen *et al.*, 1996; U39050), that are widely expressed proteins. A mDab-related gene, M110.5, has also been identified in the *Caenorhabditis elegans* genome sequencing project (Wilson *et al.*, 1994). The alignment of these proteins with mDab1 and *Drosophila* Dab shows greatest sequence conservation in an amino-terminal region of 136 residues (Figure 1A, overline). Throughout this region, Dab and mDab1 are 52% identical (72% similar) (Figure 1B). The mDab1 and the *Drosophila* Dab proteins are related in two other areas (alignments not shown). One stretch of 73 amino acids from residue 400 in mDab555 and 1913 in *Drosophila* Dab is 20.5% identical (41% similar) and has an unusually high concentration of hydroxy amino acids (19%), glutamine (12%) and proline (12%). Another stretch of 33 amino acids, from residue 508 in mDab555 and 2082 in Dab, is 36% identical (45% similar) and is also rich in hydroxy amino acids (19%). The two similar hydroxy amino acid-rich regions may represent conserved sites of phosphorylation. The *Drosophila* Dab protein is known to be phosphorylated to high stoichiometry on serine (Gertler, 1992), which may be important for its function.

Bork and Margolis (1995) pointed out that the amino-terminal conserved region of Dab is distantly related in sequence to the ShcA PTB domain (also known as PI domain) (Kavanaugh and Williams, 1994). The ShcA PTB is composed of a β -sandwich that is capped with a charged α -helix (Zhou *et al.*, 1995). A specific phosphopeptide can bind antiparallel to the $\beta 5$ strand of the PTB, stabilized by contacts with several residues in the PTB (asterisks, Figure 1C). The phosphotyrosine on the peptide interacts with several hydrophilic and positively charged residues at one end of the PTB, including Arg67, Ser151, Lys169 and Arg175. These residues are conserved across the Shc group of PTBs (Kavanaugh and Williams, 1994; Lai *et al.*, 1995; O'Bryan *et al.*, 1996). Mutation of Arg67 or Arg175 to Gln reduced binding of the ShcA PTB to phosphorylated targets by 36 and 100%, respectively (Zhou *et al.*, 1995).

lines SY5Y and PC12, or in fibroblast lines Rat1, 10T1/2 or NIH3T3 (data not shown). P19 cells are pluripotent and can be induced to differentiate into neural ectoderm when grown in aggregates in the presence of all-*trans*-retinoic acid (RA) (McBurney *et al.*, 1982; Jones-Villeneuve *et al.*, 1983). Three to five days after addition of RA, P19 cultures are composed of glioblasts and neuroblasts. By 7 days, >50% of the cells are axon-bearing embryonic neurons, and the remainder are glia (Rudnicki and McBurney, 1987).

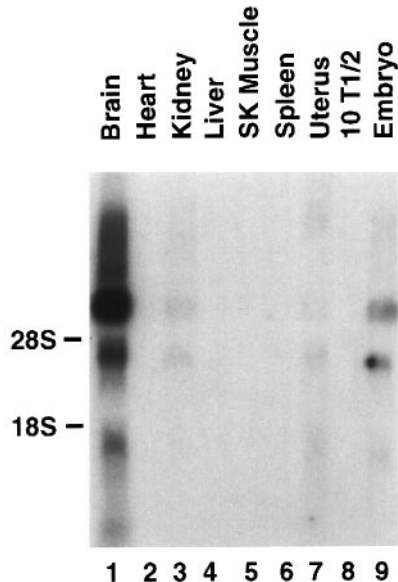


Fig. 2. mDab1 RNA expression. Northern blot analysis with the mDab1 B3 clone cDNA probe on adult mouse total RNA from indicated tissues (40 µg, lanes 1–7), 10T1/2 mouse fibroblasts and mouse embryo E10.5 RNA (10 µg, lanes 8 and 9).

Differentiated P19 cultures were found to express 60, 80 and 120 kDa mDab1 isoforms by immunoblot analysis of anti-mDab1 immunoprecipitations from lysates of these cells (Figure 3A). However, in undifferentiated P19 cells, only p80 was observed. The abundance of this isoform increased ~5-fold during neuronal differentiation, then declined after day 5. The p60 and p120 isoforms were not detected in undifferentiated cells, and their expression peaked at day 3 of differentiation. Blotting the same immunoprecipitates with anti-phosphotyrosine antibodies revealed that p60, p80 and p120 are tyrosine phosphorylated during differentiation, with the maximal phosphorylation of all proteins occurring at day 5 (Figure 3A). No change in mDab1 expression or tyrosine phosphorylation was detected when P19 cells were induced to differentiate into muscle lineages (Edwards *et al.*, 1983; data not shown). Various isoforms were detected in the hematopoietic cell lines LSTRA (120 and 36 kDa), Jurkat (120 and 36 kDa), K562 (36 kDa) and 32D (120 and 45 kDa) (data not shown).

Using anti-peptide antibodies, we found that the p80 and p60 forms of mDab1 contain the common amino-terminal sequence encoded by all of the cloned cDNAs, and that the p120 and p80 forms contain the C-terminal sequence specific to the mDab555 mRNA. *In vitro* translated mDab555 has an apparent M_r of 75 kDa. When expressed in fibroblasts, mDab555 migrates at 80 kDa (see below), possibly due to phosphorylation, and comigrates with p80 detected in P19 cells (data not shown). The p45 and p36 forms detected in hematopoietic cells react with the anti-mDab(B3) and anti-mDab(N) antibodies, and appear to correspond to the products of the mDab271 and mDab217 mRNAs, respectively (data not shown). The mRNAs encoding the p60 and p120 forms have not yet been cloned, but we assume that these

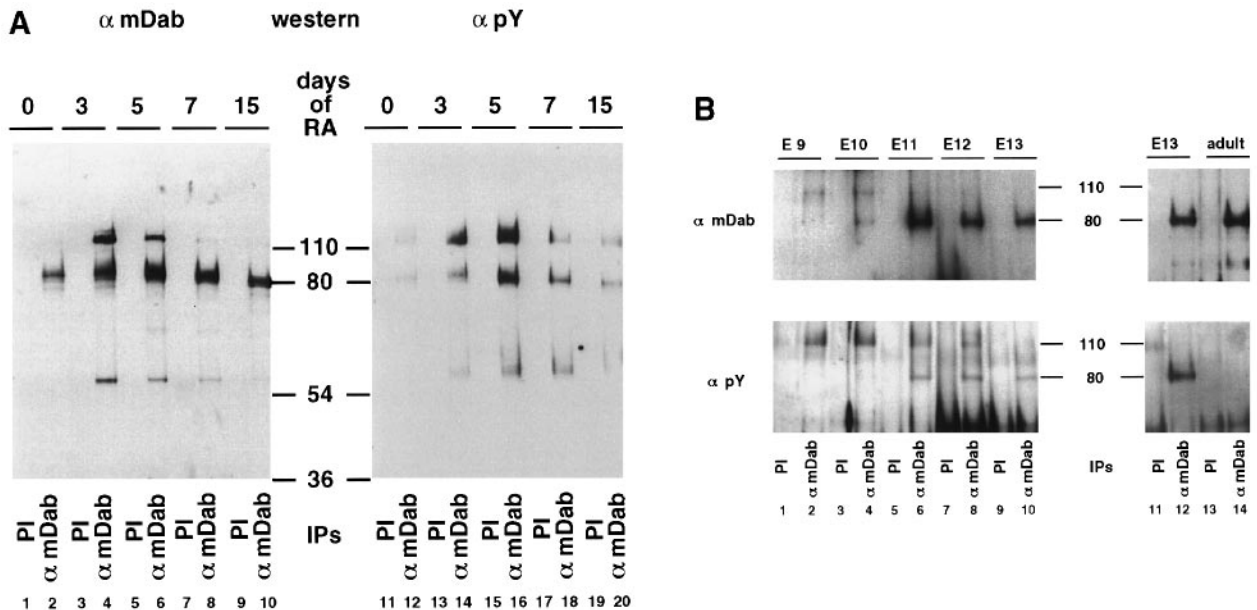


Fig. 3. Expression and tyrosine phosphorylation of the mDab1 isoforms during P19 cell differentiation and neural development. (A) P19 cells were induced to differentiate along the neural lineage by treatment with RA for the indicated times, and cell lysates were immunoprecipitated with anti-mDab(B3) (even lanes) or pre-immune (PI, odd lanes) antibodies. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted to detect mDab1 [lanes 1–10; anti-mDab(B3) antibody] and phosphotyrosine (lanes 11–20; 4G10 antibody). (B) Lysates from mouse embryo heads (left panels 0.5 mg/ml, right panels 5 mg/ml in total protein) at the indicated stages of development were immunoprecipitated with anti-mDab(B3) (even lanes) or pre-immune sera (odd lanes), then immunoblotted with anti-mDab(B3) (upper) or anti-phosphotyrosine (lower) antibodies.

proteins contain the PTB, since they react with anti-mDab(B3) antibody.

Expression and tyrosine phosphorylation of mDab1 during embryogenesis

To determine the expression pattern of mDab1 during mouse embryogenesis, extracts were prepared from heads and trunks of embryos between E9 and E13. By immunoprecipitation and Western blotting procedures, both mDab1 p80 and p120 were detected in lysates from heads of embryonic mice, whereas expression in the trunk lysates was much lower (Figure 3B, and data not shown). mDab1 p120 was detected at E9, which corresponds to early stages of neural development (Stainier and Gilbert, 1990) and at E10. The expression of p120 decreased after E10 and was not detected in adult brain (Figure 3B). In contrast, the expression of mDab1 p80 increased between E10 and E11 and remained high in adult brain. The tyrosine phosphorylation of mDab1 p80 was maximal on E10 and E11, declined thereafter and was undetectable in the adult brain.

In order to determine which kinases might phosphorylate mDab1 during mouse brain development, we examined mDab1 tyrosine phosphorylation at E13 in mice homozygous for mutations in the *src*, *fyn* or *abl* genes (Soriano *et al.*, 1991; Tybulewicz *et al.*, 1991; Stein *et al.*, 1992). mDab1 was immunoprecipitated from lysates of mutant embryos and wild-type littermates, and phosphotyrosine levels were determined by Western blotting. In all cases, the levels of mDab1 isoforms and their levels of phosphorylation were the same in mutant and wild-type animals (data not shown), suggesting that none of these kinases alone is responsible for mDab1 phosphorylation. mDab1 is therefore phosphorylated either by a number of redundant kinases or by an as yet untested kinase.

To establish which cell types in the embryonic head were expressing mDab1, mDab1 was localized by whole mount immunodetection in E10.5 embryos. Nerve tracts were identified by double label immunostaining with antibody to a general axonal marker (Placzek *et al.*, 1990). mDab1 expression was observed in the head in neural tracts corresponding to the developing cranial nerves, such as the oculomotor and the trochlear nerves (Figure 4, left). In the body, mDab1 expression in the spinal accessory nerve and dorsal root ganglia was apparent (Figure 4, right). At day E13, mDab1 expression was observed in sensory nerves that innervate the vibrissae, and in developing bone in the extremities (data not shown). All nerves identified at these times by neurofilament antibody also expressed mDab.

These results show that mDab1 is localized in nerves and is tyrosine phosphorylated at times when the nervous system is undergoing rapid expansion and axonal networks are developing. mDab1 is also expressed in the adult brain, but is not tyrosine phosphorylated detectably. This suggests that mDab1 interacts with PTKs during the development of the nervous system, and may act to transduce signals at this time.

mDab1 binds to tyrosine kinases

The identification of mDab1 in a yeast two-hybrid screen suggested that mDab1 would interact with PTKs. To determine the nature of the interaction between mDab1

and Src, we first analyzed β -galactosidase expression in yeast expressing mDab1 clone B3 and various Src mutants (Table I). Src mutants with either a deleted SH3 domain or Tyr416 and 527 mutated to Phe interacted with mDab1 as strongly as did wild-type Src. However, neither a Src mutant with a defective SH2 domain nor a catalytically inactive Src interacted with mDab. The interaction of mDab1 with Src, therefore, requires tyrosine phosphorylation of mDab1 but not Src, and the Src SH2 but not SH3 domain. This would be consistent with phosphorylation of mDab1, in the region encoded by B3 (Figure 1A), providing a binding site for the SH2 domain of Src.

The two-hybrid results suggested that the B3 fragment of mDab555 is a substrate for Src. To test whether the mDab555 product, p80, is phosphorylated by Src in mammalian cells, p80 was expressed alone or together with activated Src (Src527F) in 293T fibroblasts. mDab1 was recovered by immunoprecipitation from cell lysates and detected by immunoblotting with antibodies to mDab1 or phosphotyrosine (Figure 5A, middle and lower panels). Expression of Src527F induced the tyrosine phosphorylation of mDab1 p80, suggesting that mDab1 p80 is a substrate for Src or a Src-activated tyrosine kinase.

We next examined whether the phosphorylation of mDab1 generates binding sites for the SH2 domain of Src or other PTKs. For these experiments, lysates containing tyrosine-phosphorylated or unphosphorylated mDab1 p80 were mixed with the Src, Fyn, Abl or Csk SH2 domain, presented as GST fusion proteins immobilized on glutathione beads. After washing, mDab1 bound to the immobilized SH2 domains was detected by immunoblotting (Figure 5A, top panel). Tyrosine-phosphorylated but not control mDab1 associated with the Src and Fyn SH2 domains *in vitro*. mDab1 also interacted with the Abl SH2 domain, but less well than with Src or Fyn, and did not interact with the Csk SH2 domain. The Abl and Csk SH2 domains, however, do form high affinity complexes with other tyrosine-phosphorylated molecules (Sabe *et al.*, 1994; Duyster *et al.*, 1995).

To examine whether mDab1 and Src527F would form complexes in mammalian cells, both proteins were overexpressed in Rat-1 fibroblasts. Cell lysates were immunoprecipitated with antibodies to Src, and mDab1 was detected by immunoblotting (Figure 5B). mDab1 co-immunoprecipitated with Src, and was detected with both anti-mDab1 antibodies and anti-phosphotyrosine antibodies (Figure 5B and data not shown). Approximately 1% of the phosphorylated mDab1 that was present in the total cell lysate was immunoprecipitated. In addition, Src was detected in anti-mDab1 immunoprecipitates. A 60 kDa tyrosine-phosphorylated protein detected in mDab1 immunoprecipitates from 293T cells expressing mDab1 and Src527F was confirmed to be Src (Figure 5A; data not shown). Src and the mDab1 p80, therefore, formed complexes stable enough to be isolated from cells in the presence of non-ionic detergent.

Examination of the protein sequences of mDab1 revealed no consensus Src SH2 (Songyang *et al.*, 1993) or SH3 (Feng *et al.*, 1994; Yu *et al.*, 1994; Mayer and Eck, 1995) binding sites. However, the B3 region contains a motif Tyr-Gln-Tyr-Ile which is similar to the Src binding site, Tyr-Ile-Tyr-Val, on the platelet-derived growth factor receptor (Mori *et al.*, 1993; Alonso *et al.*, 1995). The first



Fig. 4. mDab1 localization during embryonic development. Mouse embryos (day E10.5) were incubated in whole mount with anti-axonal (mouse monoclonal 2H3) or anti-mDab(B3) antibodies and were visualized by indirect immunofluorescence. Anti-axonal detection (upper panel; Texas red) and anti-mDab1 signals (middle, FITC) are coincident in merged image (lower panel). Sagittal views of head (left) and body (right) reveal the major nerves developing at this time. tr, trochlear nerve; oc, oculomotor nerve; tg, trigeminal ganglion; vg, vestibular ganglion; opv, optic vesicle; an, accessory nerve; drg, dorsal root ganglia.

tyrosine in this motif, Tyr198, is a likely site to be tyrosine phosphorylated by the Src family kinases (Songyang *et al.*, 1995a). A mutant mDab555, in which Tyr198 and 200 were both changed to Phe, was phosphorylated on tyrosine to a reduced extent when co-expressed with Src527F, and bound to the Src SH2 domain *in vitro* (Figure 5A). The *in vivo* association between mDab1 and Src was reduced ~2-fold by the mutation (Figure 5A, lower panel). It seems, therefore, that Tyr198 or 200 and another of the six tyrosines in the B3 region of mDab1 might be Src binding sites.

To determine whether the interaction between mDab1 and Src could be detected under conditions where neither of the proteins was overexpressed, Src immunoprecipitates were prepared from lysates of differentiating P19 cells, and associated proteins were detected by phosphorylation

Table I. Yeast two-hybrid analysis of the mDab–Src interaction

Vector	VP16	VP16-B3
LexA–lamin	–	–
LexA–Srt(wt)	–	+
LexA–Src(FF)	–	+
LexA–Src(Δ SH3)	–	+
LexA–Src(SH2')	–	–
LexA–Src(295R)	–	–

Pairs of LexA and VP16 fusion proteins were co-expressed in yeast strain L40 and β -galactosidase activity detected by filter assay. The mDab1 B3 cDNA (VP16-B3) was tested against a panel of Src mutants and lamin (negative control). Src(wt), wild-type Src; Src(FF), Src with Tyr416 and Phe527 mutations; Src(Δ SH3), Src SH3 deletion mutant; Src(SH2'), SH2 domain mutant of Src which does not bind to phosphotyrosine; Src(295R), kinase-inactive version of Src.

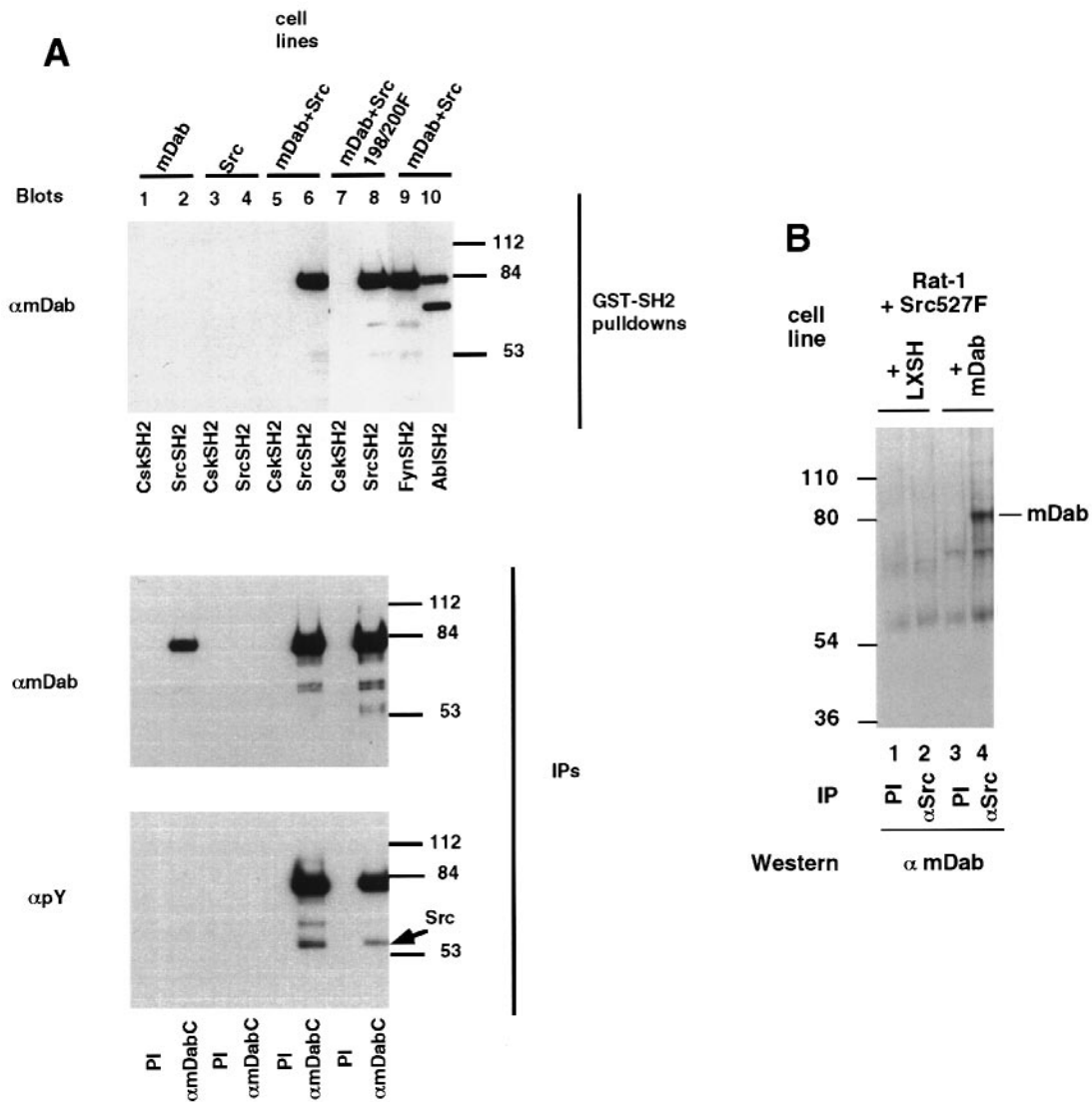


Fig. 5. mDab1 and Src interact *in vitro* and *in vivo*. (A) Lysates from 293T cells transfected with retroviral vectors encoding mDab555 alone (lanes 1 and 2); Src 527F alone (lanes 2 and 3); Src527F and mDab555 together (lanes 4, 5, 9 and 10); or Src527F and mDab555 with Tyr198 and 200 mutated to Phe (mDab198/200F; lanes 7 and 8) were either (top panel) bound to immobilized GST fusions proteins with the Src, Csk, Fyn or Abl SH2 domain or (middle and bottom panels) immunoprecipitated with anti-mDab1 (odd lanes) or pre-immune (even lanes) antibodies. Immunoblots were probed with either anti-mDab1 (top and middle panels) or anti-phosphotyrosine (bottom panel) to check for mDab1 expression and phosphotyrosine content. A 70 kDa band in the top panel is the Abl SH2 domain fusion. (B) Rat-1 cells were infected with viruses encoding Src527F and mDab1 or an empty vector. Cell lysates were immunoprecipitated with either anti-Src (lanes 2 and 4) or pre-immune serum (lanes 1 and 2) and the blot was probed with anti-mDab1 antibody.

with [γ - 32 P]ATP *in vitro*. Several labeled proteins including Src and proteins of ~60 and 80 kDa were observed (Figure 6). To test whether mDab1 proteins were present, the radiolabeled proteins were eluted from the immunoprecipitates and re-immunoprecipitated with either pre-immune serum, anti-Src antibody or anti-mDab(B3). The p60 and p80 forms of mDab1 were recovered in the second immunoprecipitation of Src kinase reactions from differentiating but not control P19 cells (Figure 6). These results suggest that active Src and mDab1 p60 and p80 associate in differentiating P19 cells at the times when these mDab1 isoforms are found to be tyrosine phosphorylated (Figure 3A) and Src specific activity is elevated (Lynch *et al.*, 1986).

Association of mDab1 with tyrosine-phosphorylated proteins

To examine whether the mDab1 PTB binds proteins that are tyrosine phosphorylated during neural development, the mDab1 PTB was incubated with lysates from E13 mouse heads. Associated proteins were then eluted, and detected by immunoblotting with anti-phosphotyrosine antibodies (Figure 7). The mDab1 PTB bound tyrosine-phosphorylated proteins of 200, 120, 50–65 and 40 kDa (Figure 7). These proteins did not bind to GST alone. To test whether the binding of proteins to the mDab1 PTB is phosphotyrosine dependent, binding experiments were done in the presence of the phosphotyrosine analog phenylphosphate (Figure 7). Binding of the 120 and 40 kDa

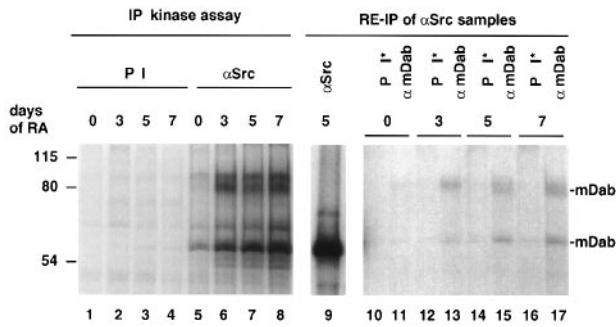


Fig. 6. Association of Src with mDab1 in differentiating P19 cells. Lysates from P19 cells induced to differentiate with RA for the times indicated were immunoprecipitated with anti-Src (lanes 5–8) or pre-immune (PI, lanes 1–4) antibodies and incubated with [γ - 32 P]ATP to allow phosphorylation (lanes 1–8). Labeled proteins were eluted and re-immunoprecipitated with either anti-Src (lane 9), anti-mDab1 (lanes 11, 13, 15 and 17) or pre-immune (PI*, lanes 10, 12, 14 and 16) antibodies, and proteins were visualized by autoradiography.

proteins was reduced significantly by phenylphosphate, and to a lesser extent by phosphoserine. However, the binding of the 50–65 kDa proteins was not affected by either competitor.

To examine whether the mDab1 PTB may bind phosphoproteins directly, a mutant mDab1 PTB containing an Arg to Glu substitution at amino acid 56 was tested. This residue is the equivalent of Arg67 in the ShcA PTB (Figure 1C), which contacts the phosphate moiety on the bound phosphopeptide (Zhou *et al.*, 1995). The mutant mDab1 PTB bound the 120 and 40 kDa embryonic head proteins less efficiently than the wild-type PTB (Figure 7), suggesting that the mDab1 PTB binds phosphorylated molecules in a similar manner to the ShcA PTB. The binding of the 55–60 kDa proteins to both the wild-type and the mutant mDab PTB, and in the presence of phosphoamino acid competitors, may suggest that the mDab1 PTB is also capable of protein–protein interactions independent of phosphorylation.

Discussion

We have identified mDab1, a mammalian homolog of *Drosophila* Dab. Dab is implicated in establishing axonal connections in the embryonic CNS, in collaboration with one or more tyrosine kinases (Gertler *et al.*, 1989). mDab1 shares several characteristics with *Drosophila* Dab. The *mdab1* gene is alternatively spliced to create several mRNAs and proteins with common amino-termini. This region is most highly conserved across the Dab family and contains a predicted PTB. mDab1 is expressed in the nervous system and some hematopoietic cell lines. There are parallels in the development of neurons and hematopoietic cells, and a number of other genes are similarly restricted in expression (Anderson, 1989). The mDab1 protein is localized in the growing nerves of embryonic mice and is tyrosine phosphorylated when the nervous system is developing, but not thereafter. Thus, mDab1 is positioned to function in mammalian CNS development. Another Dab relative, known as p96 or mDab2 in the mouse (Xu *et al.*, 1995) and DOC-2 in humans (Albertsen *et al.*, 1996), has been reported. The *mdab2* gene is also alternatively spliced, but it appears to be widely expressed and there is no evidence for tyrosine phosphorylation or

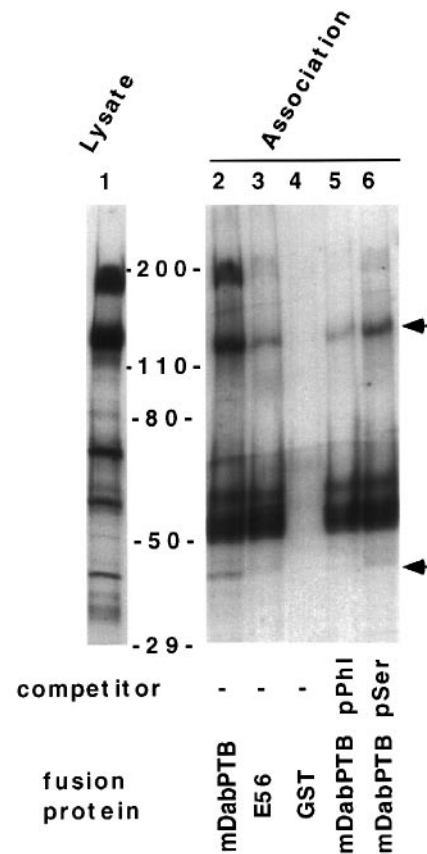


Fig. 7. The mDab1 PTB associates with tyrosine-phosphorylated proteins from embryonic mouse heads. Proteins from an E13 mouse head were incubated with a GST fusion protein containing the mDab1 PTB (lane 2), a mutant mDab1 PTB in which Arg56 was changed to Glu (mDab1 56E; lane 3) or GST alone (lane 4). Phenylphosphate (pPnl, lane 5) or phosphoserine (pSer, lane 6) were included in the binding buffer to investigate binding specificity. Bound proteins were eluted, resolved and immunoblotted with anti-phosphotyrosine antibodies; 20% of the lysate was run in parallel (lane 1). Phosphoproteins which are competed by addition of phenylphosphate to the binding buffer and do not bind to the E56 mutant are indicated by the arrowheads.

association with tyrosine-phosphorylated proteins. Genetic analysis will be required to determine whether the *mdab1* and *mdab2* genes have distinct or overlapping functions.

During neuronal differentiation of P19 EC cells, expression of mDab1 p60, p80 and p120 is induced, and the proteins are first tyrosine phosphorylated prior to neurite extension (Figure 3A). Tyrosine phosphorylation of p80 correlates with axonogenesis becoming maximal at day 5 (Figure 3A, data not shown). The expression pattern and phosphorylation of mDab1 are also regulated during mouse embryonic development. At E10.5, mDab1 expression was detected only in developing nerves (Figure 4), but at E13 additional expression was observed in developing bone, possibly in precursors to osteoclasts. mDab1 p120 is expressed maximally at E9 and E10 and then declines, while mDab1 persists in adults (Figure 3B). Tyrosine phosphorylation of both of these forms of mDab1 was observed in embryos but not in adults. mDab1 is, therefore, a substrate of a kinase which is active during neural development.

Although mDab1 is tyrosine phosphorylated *in vitro* by Src, in Src-transformed cells, and at times during neural

development where Src activity increases, mDab1 is not exclusively a Src substrate. We observed no decrease in mDab1 phosphotyrosine levels in *src*⁻, *fyn*⁻ or *abl*⁻ mutant embryos (data not shown). Therefore, mDab1 is phosphorylated by other tyrosine kinases, potentially including receptor PTKs.

The tyrosine phosphorylation of mDab1 provides one of at least two ways in which mDab1 can bind to other proteins. Analysis in yeast and *in vitro* shows that tyrosine-phosphorylated mDab1 binds strongly to the SH2 domains of Src and Fyn, and more weakly to Abl (Figure 5 and Table I). mDab–Src complexes are formed when mDab1 is overexpressed in fibroblasts transformed by activated mutant Src. Moreover, mDab1 p60 and p80 are associated with Src after induction of P19 cell differentiation along neuronal lineages (Figure 6). The tyrosine phosphorylation and, therefore, the potential for subsequent SH2 domain interaction is developmentally regulated. Ligands for the Src SH2 domain may activate Src by competition for an intramolecular repressive interaction (Brown and Cooper, 1996). mDab1 p80 may act in this way, and may induce Src activation when mDab1 is phosphorylated by upstream kinases that do not activate Src directly. Differential splicing of mDab1 creates proteins containing distinct potential tyrosine phosphorylation sites. mDab1 p80 and p45 contain two sequences of Val·Tyr·Gln·Xaa·Ile (Tyr185 and Tyr198) which may represent Src or Fyn binding sites and two sequences of Ile/Val·Tyr·Gln/Asp·Val·Pro (Tyr220 and Tyr232) that may represent binding sites for Abl and/or Crk (Songyang *et al.*, 1993). Mutation of one of the potential Src binding sites (Tyr198) significantly reduced Src association (Figure 5A, bottom). Other mDab1 proteins may contain sites for binding to other non-receptor tyrosine kinases.

The second mechanism for protein interaction with mDab1 is provided by the putative PTB domain. The importance of the mDab1 PTB is suggested by its conservation across the Dab family (Figure 1B), but it is sufficiently divergent in sequence from the Shc family of PTBs to question whether it adopts a similar structure (Figure 1C). PTBs are difficult to recognize by primary sequence alone. The IRS-1 PTB is highly divergent in primary sequence, yet is fully functional and has a similar structure to the ShcA PTB (Zhou *et al.*, 1996). PTBs can bind to phosphotyrosyl peptides and to polyphosphoinositides (Zhou *et al.*, 1995). Sequence conservation in the mDab1 PTB is too low to predict whether it binds phosphoinositides, but it appears to bind to phosphotyrosine.

The mDab1 PTB formed complexes with unidentified tyrosine-phosphorylated proteins of 40, 50–65, 120 and 200 kDa from the heads of embryonic mice. Complex formation with the 40 and 120 kDa proteins was competed more effectively by phenylphosphate than by phosphoserine, and was reduced by mutation of a basic residue in the mDab1 PTB, which was predicted, on the basis of alignment with the ShcA PTB, to contact the bound phosphotyrosine. Since the mDab1 PTB domain is the most highly conserved part of the protein, the identification of the ligands may be central to understanding mDab1 function.

In *Drosophila*, *dab* acts as a genetic enhancer of *dAbl*. However, it is not clear whether Dab and dAbl physically interact, nor whether Dab is regulated by tyrosine phos-

phorylation by dAbl. The requirement for Dab is unveiled under conditions where dAbl tyrosine kinase activity is absent. Thus, Dab must be functional under conditions where it is not tyrosine phosphorylated by dAbl. dSrc may phosphorylate Dab under these conditions. Overexpression of kinase-defective dSrc during embryogenesis interferes with longitudinal connections in the CNS (Kussick *et al.*, 1993), reminiscent of *dAbl dab* double mutants (Gertler, 1989). The detection of mammalian mDab1, its ability to be phosphorylated on tyrosine and then bind SH2-containing PTKs, such as Src and Abl, and the binding of its PTB to tyrosine-phosphorylated proteins in embryonic extracts suggest that mDab1 might be regulated by PTKs during mammalian neurogenesis.

Materials and methods

Yeast two-hybrid screen

A modified yeast two-hybrid system as described elsewhere (Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995) was employed to identify Src-interacting proteins. The *Saccharomyces cerevisiae* strain L40 [*Matα His3delta200 trp1-900 leu2-3, 112 ade2 LYS2::(lexAop)_g-HIS3 URA3::(lexAop)_g-lacZ GAL4*] was transformed with the BTM116-Src(wt) plasmid and a mixed E9.5 and E10.5 library in the vector pVP16 (Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995). Transformants were grown on minimal media lacking tryptophan, leucine and histidine for 2 days, and colonies were picked and analyzed for β-galactosidase expression by a filter lift assay. Yeast that expressed levels of β-galactosidase detectable within 3 h were grown in media containing tryptophan and characterized for the loss of the pBTM116-Src(wt) plasmid. Individual library isolates were placed into groups based on β-galactosidase production in the progeny of the crosses with the L40 strain containing the library isolate and the AMR70 strain [*Matα his3 lys2 trp1 leu2 URA3::(lexAop)_g-lacZ Gal4*] expressing different LexA fusion proteins. These LexA fusion proteins included LexA–lamin (Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995), LexA–Src(wt), LexA–Src(FF), LexA–Src(ΔSH3), LexA–Src(SH2') and LexA–Src(295R) (Table I). Total DNA was purified from selected library isolates, transformed into the XL-1 Blue bacterial strain (Stratagene) and purified. DNA was sequenced with a primer that hybridizes to the pVP16 vector, 5'-GCAAGATCTTAGGGATCG-ATTGG-3', and M13 universal primer. Sequence comparisons were done with the Genetics Computer Group (GCG) programs and compared against the databases GenBank, SwissProt and PirProtein using the FASTA program (Pearson and Lipman, 1988).

cDNA cloning and sequence analysis

The mDab555 and 271 full-length cDNAs were obtained using standard techniques (Sambrook *et al.*, 1989) from a pCDNAI (Invitrogen) library of E15–17 mouse brain cDNAs (gift from V.Dixit) and mDab217 was from a λ YES (Stratagene) library made with embryonic stem cell cDNAs (from Z.Chen), using the mDab(B3) cDNA as a probe. cDNAs were subcloned into pBS (Stratagene). Nested deletion mutants were generated with sequential exonuclease III and S1 nuclease treatments at 37°C (Sambrook *et al.*, 1989). Automated DNA sequencing was performed with plasmid templates on a BioSequencer (Applied Biosystems) and overlapping sequences were obtained for both strands.

Cell lines and viruses

P19 EC cells (gift from J.C.Bell and N.Abraham) were grown and differentiated as described elsewhere (Rudnicki and McBurney, 1987). Rat-1 and 293T cells were obtained from R.Eisenman and were grown in DME with 10% fetal bovine serum.

Virus was produced by co-transfecting retroviral DNAs into 293T cells (Afar *et al.*, 1994), with the retroviral vectors indicated. Virus was collected 60 h post-infection, mixed with polybrene (4 μg/ml) and filtered through 0.45 μm filters prior to addition to target cells. Cells were selected in hygromycin (50 μg/ml; Calbiochem) or l-histidinol (2 mM; Sigma) starting 24 h after addition of virus.

Antibodies

Rabbit polyclonal antibodies against mDab1 were prepared by immunizing New Zealand White female rabbits with a GST–mDab1 fusion corresponding to residues 107–243 (B3) or with peptide N (CELQVAA-

AVKTSAKKDSRKK) and peptide C (CGEPPSGGDNISPODGS) that correspond to the mDab555 sequence beginning at residues 6 and 542 respectively. All sera were affinity purified with the corresponding antigen immobilized on cyanogen bromide-activated Sepharose (Sigma) or SulfoLink (Pierce), and are designated anti-mDab(B3), anti-mDab(N) or anti-mDab(C). The Src polyclonal 3060 was raised to a peptide corresponding to residues 519–533 of c-Src (Cooper *et al.*, 1986). The anti-phosphotyrosine monoclonal 4G10 was a gift from D.Morrison (Druker *et al.*, 1989).

Plasmid constructions

The Src cDNA, and mutants, were shuttled into the pBTM116 vector (Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995) by digesting the PCR product of the oligonucleotides 5'-CTCGGATCTCATGGGAGCA-GCAAGAGCA-3' and 5'-CTCATGCATCCTATAGGTTCTCTCCAGG-3' directed to the N- and C-terminus of Src respectively, with the restriction enzymes *Bam*HI and *Nsi*I and ligating this product into the *Bam*HI and *Pst*I cloning sites in the vector. An in-frame fusion with *lexA* DNA binding protein and the Src cDNA is produced. The construction of the Src mutants has been described elsewhere (Cooper and MacAuley, 1988; MacAuley and Cooper, 1988; Seidel-Dugan *et al.*, 1992) except for the SH2 domain mutant. The mutation in the Src SH2 was generated by PCR and changed the critical Arg175 in the phosphate binding pocket to Lys and the adjacent Glu175 to Ser, introducing a unique *Sal*I site in the process. This mutation, referred to as Src(SH2') (Table I), was confirmed by restriction and sequence analysis. This mutation was predicted to reduce binding to tyrosine-phosphorylated peptides since Arg175 makes contacts with the phosphate of bound peptides (Waksman *et al.*, 1992) and the equivalent substitutions in the Abl SH2 domain abolished binding (Mayer *et al.*, 1992).

Two GST fusion constructs were made with the mDab555 cDNA. Both were cloned between the *Bam*HI and *Eco*RI sites in the pGex-2T (Pharmacia) polylinker. The first corresponds to the region cloned in the yeast two-hybrid screen comprised of residues 107–243 that was PCR amplified with the oligonucleotide pairs 5'-CGCGGATCCCATCACC-ATGCTGTTCATGAA-3' and 5'-CGCGAATTCGACGGGAGAAAAG-GCATCAC-3'. The second fusion protein contains the region corresponding to the mDab1 PTB, residues 29–197 that was PCR amplified with the oligonucleotides 5'-CGCGGATCCGCCACTTTG-ATAAAGAGGT-3' and 5'-CCGGAATTCACGGGATCTTCCACATC-3'. The GST–Src(SH2), GST–Fyn(SH2), GST–Abl(SH2) (gift from L.Shive, S.Baragonia, J.Y.Wang) and GST–CSK(SH2) constructs have been described elsewhere (Okada *et al.*, 1993; Duyster *et al.*, 1995).

For expression in mammalian cells, the mDab555 cDNA was ligated into the *Bam*HI site of the pLXSH retroviral vector (Miller *et al.*, 1993). The entire open reading frame was PCR amplified with the oligonucleotides 5'-CGCGGATCCAGGATGTCAACTGAGACA-3' and 5'-CGCGGATCCTTCACTGGGCGACTGTGAGT-3'. The Tyr198 and 200 to Phe mutations were generated simultaneously by oligonucleotide-directed mutagenesis as described previously (Kunkel *et al.*, 1987), with the oligonucleotide 5'-CACAAATGAACTGGAAGACGGGATCTTCCAC-3'. Mutants were identified by screening colonies for the introduction of a unique *Bbs*I site into the mDab555 cDNA and were confirmed by sequence analysis.

The construction of the retroviral vector pLXSHD–Src(527F) has been described (Howell and Cooper, 1994).

Analysis of mdab mRNA levels

The Northern blot (gift from A.Vojtek) was prepared as previously described (Vojtek and Cooper, 1993) and was incubated under standard hybridization conditions (Sambrook *et al.*, 1989), with the heat-denatured *mdab1* (B3) cDNA that was radioactively labeled with [α -³²P]dCTP using the random prime DNA labeling kit (USB) according to the manufacturer.

Purification of GST fusion proteins

All GST fusion proteins were affinity purified out of lysates of TG-1 *Escherichia coli* by adsorption onto glutathione–agarose resin (Pharmacia), followed by four washes with lysis buffer [phosphate buffered saline (PBS), 1% Triton X-100, 20 μ g/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and either used directly as affinity matrices or eluted with 5 mM reduced glutathione as previously described (Okada *et al.*, 1993). All GST fusion protein concentrations were determined by comparison with known amounts of protein on Coomassie blue-stained SDS–polyacrylamide gels.

Immunoprecipitation and Western blot detection

Cells (1×10^6) were lysed on ice in 1 ml of TX-IPB (0.1 M NaCl, 1% Triton X-100, 10 mM HEPES pH 7.4, 2 mM EDTA, 0.1% 2-mercaptoethanol, 20 μ g/ml aprotinin, 50 mM NaF, 0.2 mM Na₂VO₄, 2 mM PMSF, 1 mM phenylarsine oxide), incubated for 10 min on ice and clarified by centrifuging at 20 000 g for 30 min. Lysates were cleared with Sepharose CL-4B (Sigma) and adjusted to 3 mg of protein/ml unless otherwise stated. Antibodies, that were pre-bound and chemically cross-linked to protein A–Sepharose beads by treatment with dimethyl pimelimidate (Schneider *et al.*, 1982), were then mixed with the lysates for 90 min at 4°C, followed by four washes with TX-IPB buffer. When immunoprecipitates were employed for kinase assays, a further two washes were done with PAN buffer (100 mM NaCl, 10 mM PIPES pH 7.0, 20 μ g/ml of aprotinin) prior to incubation in UKB (10 mM PIPES pH 7.0, 10 mM MnCl₂, 0.50 μ M [γ -³²P]ATP [3000 Ci/mmol]) for 15 min at 30°C. All reactions were stopped and proteins were eluted by addition of twice-concentrated gel loading buffer (4% SDS, 40% glycerol, 0.2 M Tris–HCl pH 6.8, 5.6 M 2-mercaptoethanol, 5 mM EDTA, 0.02% bromophenol blue) and samples were boiled for 10 min prior to analysis by SDS–polyacrylamide gel electrophoresis. Samples analyzed by re-immunoprecipitation were lyophilized to remove the 2-mercaptoethanol, and then diluted 1:50 (from the original volume) into TX-IPB and mixed with antibodies overnight at 4°C.

Binding assays and analysis were done essentially as described for immunoprecipitations with 5 μ g of GST fusion protein immobilized on glutathione–agarose beads. Phenylphosphate and phosphoserine were used at 50 mM for competition experiments.

Immunoblotting was performed as described previously (Howell and Cooper, 1994) and immunoreactive proteins were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham).

Immunohistochemistry of whole mount embryos

Embryos were fixed and permeabilized as described (Hogan *et al.*, 1994). Subsequent incubations were for 12 h each at 22°C in 2% instant skim milk in PBS. Immunodetection of mDab1 was accomplished with anti-mDab(B3) antibody and sequential additions of goat anti-rabbit antibody, followed by fluorescein isothiocyanate-conjugated donkey anti-goat antibody. Axons were detected using the monoclonal antibody 2H3 (Placzek *et al.*, 1990) (gift from T.Jessell) and subsequent incubations with sheep anti-mouse and Texas red-conjugated donkey anti-sheep antibodies (Jackson ImmunoResearch Laboratories). Fluorescence patterns observed when anti-mDab(B3) and anti-2H3 antibodies were incubated individually were identical to results obtained when used together. Indirect fluorescence was detected with a Delta Vision microscope (Applied Precision Inc.). All secondary–tertiary antibody alone and mDab1 pre-immune controls were negative.

Accession numbers

The sequences of the cDNAs mentioned in this manuscript have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database with the following accession numbers: Y08379, Y08380, Y08381 and Y08382.

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