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

Brian W. Howell, Frank B. Gertler and Jonathan A. Cooper

Fred Hutchinson Cancer Research Center Seattle, 1124 Columbia Street, Seattle, WA 98104, USA

1Corresponding author

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Here, we identify a mouse homolog of the Drosophila Disabled (Dab) protein, mDab1, and show it is an adap- tor 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 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 sig- naling 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 neuronal 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 Dabl 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. mDabl 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 in brains of age-matched mice. During 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.

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**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 5×10^4 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. 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 codons 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., 1995; 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 shows 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 which is capped with a charged α-helix (Zhou et al., 1995). A specific phosphopeptide can bind antiparallel to the β5 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).
The residues amino-terminal to the phosphorylated tyrosine in the phosphopeptide bound to the ShcA PTB include the Asn-Pro-X-pTyr (NPXpY) consensus sequence (Batzer et al., 1995; Kavanaugh et al., 1995; Songyang et al., 1995; van der Geer et al., 1995). These residues contact the β-sheet and α3.

Alignment of the mDab1 and ShcA PTBs (Figure 1C) was facilitated by the solution structure of the ShcA PTB (Zhou et al., 1995) and secondary structure predictions for mDab1 and the dNumb PTB (Zhou et al., 1995), which represents a phylogenetic intermediate between the Shc and Dab family PTBs. mDab1 has residues that correspond to critical amino acids in the ShcA PTB that contact the phosphate moiety, including Arg67, Ser151 and Lys169. However, the region between ShcA β1 and β2 is quite divergent in mDab1, and there is no apparent homolog of Arg175 of ShcA. The PTB of IRS-1 is also divergent in this region (Eck et al., 1996; Zhou et al., 1996). Some of the residues that contact the peptide ligand amino-terminal to the phosphotyrosine are conserved. In particular, Phe198 in α3 of ShcA is conserved in Dab family members and dNumb. This residue contacts the side chain of Asn−3 of the ligand. These sequence similarities suggest that the amino-terminal region of mDab1 may adopt a similar fold to the ShcA PTB and may function to bind to phosphorylated proteins or peptides.

**Characterization of mdab1 gene position and expression**

The chromosomal location of the mdab1 gene was mapped using Southern blotting to follow polymorphic restriction fragments, in the progeny of the backcross (C57BL/6J×Mus spretus)F1×C57BL/6J, and the reciprocal backcross. mdab1 was localized to mouse chromosome 4, at offset 70.6. The mdab1 map position is depicted at the Jackson Labs Web site (http://www.jax.org/resources/documents/cmdatas/maps/4MapPage.html). This portion of the mouse chromosome is syntenic with the human chromosome 1p32–31 region.

Northern blot analysis of adult mouse tissues showed that mDab1 expression is largely restricted to brain (Figure 2). Expression was also high in E10.5 (10.5 days postcoitus) embryos. Three transcripts of 5.5, 4.0 and 1.8 kb were detected. The 1.8 kb mRNA probably encodes the mDab1 217 isoform. The 5.5 and 4.0 transcripts are larger than the largest cDNA we uncovered, suggesting that mDab1 has an extensive 5′-untranslated region or that additional spliced forms exist.

**Expression and tyrosine phosphorylation of mDab1 in cultured cells**

In a survey of cultured cells, we find that mDab1 expression is limited to differentiated P19 EC cell cultures and various hematopoietic cell lines. mDab1 expression was not detected in the neuroblastoma- or neural crest-derived
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).

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 Mr of 75 kDa. When expressed in fibroblasts, mDab555 migrates at 80 kDa (see below), possibly due to phosphorylation, and colocalizes 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
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 1). 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 over-expressed 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-mDab 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
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. 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(ΔSH3), Src SH3 deletion mutant; Src(SH2'), SH2 domain mutant of Src which does not bind to phosphotyrosine; Src(295R), kinase-inactive version of Src.

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

<table>
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<tr>
<th>Vector</th>
<th>VP16</th>
<th>VP16-B3</th>
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<tr>
<td>LexA–lamin</td>
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<tr>
<td>LexA–Srt(wt)</td>
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<td>LexA–Src(FF)</td>
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<td>LexA–Src(ΔSH3)</td>
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<tr>
<td>LexA–Src(SH2')</td>
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<td>–</td>
</tr>
<tr>
<td>LexA–Src(295R)</td>
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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(ΔSH3), Src SH3 deletion mutant; Src(SH2'), SH2 domain mutant of Src which does not bind to phosphotyrosine; Src(295R), kinase-inactive version of Src.
mDab1, a Src binding protein

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-32P]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
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 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 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 (pPnI, lane 5) or phosphoserine (pSer, lane 6) were included in the binding buffer to investigate binding specificity. Bound proteins were eluted, resolved and immobilblotted 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.
development where Src activity increases, mDab1 is not exclusively a Src substrate. We observed no decrease in mDab1 phosphotyrosine levels in src-; fnr- 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 1). 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 phosphophorysine. 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 phosphorylation 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 His3Δ200 trp1-901 leu2-3, 112 ade2 lys2::lexAop-HIS3 ura3::lexAop-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). Transforms 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-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(ASH3), LexA–Src(ASH2) 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'–GGACAATCTTATGGATCGATTGC–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 mDab1 cDNA as a probe. cDNA clones were subcloned into pBS (Stratagene). Nested deletion mutants were generated with sequential exonuclease III and XI 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 DMEM 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 0.005% 2-mercaptoethanol (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 (CELVQVA-
AVKTSADKSRKK) and peptide C (CGEPPSAGGDIIPQDGS) that correspond to the mDb555 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-mDb(B3), anti-mDb(N) or anti-mDb(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 (Vojet et al., 1993; Hollenberg et al., 1995) by digesting the PCR product of the oligonucleotides 5'-CTCGGATCCAGGATGTCAACTGAGACA-3' and 5'-CTTTTGATTCATCATATAGTTTCTTCACG-3' directed to the N- and C-terminus of Src respectively, with the restriction enzymes BamHI and NsiI and ligating this product into the BamHI and PsI 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 Arg173 in the phosphoryte binding pocket to Lys and the adjacent Glu175 to Ser, introducing a unique Sall site in the process. This mutation, referred to as Src(SH2)1 (Table I), was confirmed by restriction and sequence analysis. This mutation was predicted to reduce binding to tyrosine-phosphorylated peptides since Arg173 makes contacts with the phosphate of bound peptides (Wakman 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 mDb555 cDNA. Both were cloned between the BamHI and EcoRI sites in the pGex-2T (Pharmacia) polyclinker. The first corresponds to the region cloned in the yeast two-hybrid screen comprised of residues 107–243 that was amplified with the oligonucleotide pairs 5'-CCGGGATCCATGATCTCATTCCACGGGATGCATCAC-3' and 5'-CCGGGATCCGCCACTTTGACAAGGAAATAGGCTGAATG-3'. The second fusion protein contains the region corresponding to the mDb1 PTB, residues 29–197 that was PCR amplified with the oligonucleotides 5'-CCGGGATCCGCCACTTTGATAAGAGGTT-3' and 5'-CCGGGATCCGGATGCTCATCAGGTCAGCA-3'. TheGST-Src(SH2), GST-Fyn(SH2), GST-Abl(SH2) (gift from A.Vojtek) was prepared ... constructs have incubated individually were identical to results obtained when used alone.

**Purification of GST fusion proteins**

The construction of the retroviral vector pLXSH-Src(527F) has been...the DDBJ/EMBL/GenBank nucleotide sequence database with the following accession numbers: Y08379, Y08380, Y08381 and Y08382.

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**References**


