Interaction between Dab1 and CrkII is promoted by Reelin signaling

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Summary

Reelin-induced Dab1 tyrosine phosphorylation has been implicated in the regulation of neuronal positioning during brain development. The downstream consequences of Dab1 tyrosine phosphorylation are not fully understood, however. Here we identify CrkII, CrkL and Dock1 in complexes bound to tyrosine-phosphorylated Dab1, through mass spectrometry. The CrkII-Dab1 interaction requires tyrosine phosphorylation of Dab1 at residues 220 or 232 and is promoted by Reelin treatment of embryonic forebrain neurons. Unlike other CrkII binding proteins, such as paxillin and p130Cas, expression of Dab1 interfered with CrkII-dependent cell migration of Nara Bladder Tumor II (NBT-II) cells, in a tyrosine phosphorylation-site dependent manner. Overexpression of CrkII GFP rescued the migration of these cells, suggesting that Dab1 makes Crk a limiting factor for migration. The Dock1-Dab1 association is indirect and requires CrkII. In organisms such as Drosophila melanogaster and Caenorhabditis elegans, signaling complexes, which contain Crk and Dock1 family members are conserved and act through Rac. We show that a rough-eye phenotype in Drosophila caused by exogenous expression of tyrosine-phosphorylated mouse Dab1RFP is partially rescued by a loss-of-function mutation in myoblast city, a Dock1-like gene in Drosophila. We propose a model that tyrosine-phosphorylated Dab1 engages the conserved Crk-Dock1-Rac signaling cassette, but when bound to Dab1 this signaling complex does not support migration.

Key words: Reelin, Dab1, Brain development, Crk, Dock1

Introduction

The cytoplasmic docking protein Dab1 mediates a signaling event that regulates the placement of neurons during brain development. In its absence, developmental anomalies are observed in the positioning of neurons in several brain regions including the neocortex, the hippocampus, the cerebellum, the olfactory bulb and the spinal cord (Goldowitz et al., 1997; Gonzalez et al., 1997; Howell et al., 1997; Phelps et al., 2002; Sheldon et al., 1997; Yip et al., 2000). Genetic and biochemical experiments place Dab1 downstream of two receptors of the LDL superfamily, VLDLR and ApoER2, with which it interacts through an N-terminal PTB/PI domain (D’Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1998; Trommsdorff et al., 1999). The extracellular domains of these receptors interact with Reelin, a secreted glycoprotein produced in discreet layers of the brain (D’Arcangelo et al., 1999; D’Arcangelo et al., 1999; Hiesberger et al., 1999; Ogawa et al., 1995). The Reelin-receptor interaction activates the Src family kinases, Src, Fyn and Yes (SFY), and stimulates Dab1 tyrosine phosphorylation, probably through a Reelin-induced multimerization of the receptors (Arnaud et al., 2003b; Bock and Herz, 2003; Strasser et al., 2004).

The Dab1 tyrosine phosphorylation sites have been shown to be essential for Dab1 activity during brain development (Howell et al., 2000). Mice that express only Dab1 molecules with tyrosine substitutions at five positions, the Dab1-5F homozygous mutant mice, have a phenotype reminiscent of the Dab1-null mice. Dab1-5F is expressed at comparable levels to the wild-type protein, but it is not detectably phosphorylated.

Tyrosine phosphorylation is known to potentiate downstream signal transduction by fostering interactions between proteins containing SH2 and other phosphotyrosine-dependent domains (Marengere and Pawson, 1994). The motifs around the three Dab1 phosphorylation sites used in brain development, Y198, Y220 and Y232, match consensus sequences for several SH2 domains (Songyang et al., 1993). Previously, Dab1 has been shown to interact with the SH2 containing proteins, P85 and Nckβ (Bock et al., 2003; Pramatarova et al., 2003). In addition, Dab1 interacts with Lis1, a protein with no SH2 domain, in a manner that depends on Dab1 phosphorylation at Y198 or Y220 (Assadi et al., 2003). These studies were not exhaustive and here we used an affinity purification approach to identify additional phosphotyrosine-dependent Dab1-binding proteins.

The related SH2-SH3-SH3 domain adaptor molecules Crk (CrkL and CrkII) and Crk-like (CrkL), and the Crk-binding protein, Dock1, were found to associate with Dab1 in a phosphorylation-dependent manner. Crk and Dock1 have been shown to regulate cell migration downstream of integrins in mammals (Cheresh et al., 1999; Gu et al., 2001; Klemke et al., 1998; Petit et al., 2000). The Crk family adaptor molecules are recruited to focal contact upon the tyrosine phosphorylation of their SH2 domain ligands, paxillin and p130Cas (Gu et al., 2001; Mielenz et al., 2001; Schaller et al., 1995). Integrin-induced tyrosine kinase activation has recently been implicated...
in adhesion turn over in the protrusive region of migrating cells (Webb et al., 2004). Cells that lack Fak, Src family kinases, which are activated by integrin signaling, or their substrates paxillin or p130Cas, have reduced rates of adhesion turn over and reduced rates of migration. Because Crk proteins are recruited to focal complexes downstream of integrin engagement and promote cell migration (Huang et al., 2003; Petit et al., 2000), we investigated a role for Dab1 in the regulation of this process. Using NB-11 cells that require Crk for migration on collagen (Petit et al., 2000), we show that expression of tyrosine-phosphorylated Dab1 impedes Crk-dependent cell migration.

Many of the signaling properties of the Crk and Dock1 proteins have been revealed from the study of their homologs in other species, such as Drosophila melanogaster and Caenorhabditis elegans. Crk and Dock1 have been shown to form an evolutionarily conserved signaling cassette that acts through Rac to regulate diverse cell responses such as migration, endocytosis and cell morphology (Albert et al., 2000; Brugnera et al., 2002; Grimsley et al., 2003; Gumienny et al., 2001; Wu et al., 2001). In Drosophila, dominant loss-of-function alleles of myoblast city (mhc), the Drosophila homolog of Dock1, rescues defects in external eye morphology of Drosophila caused by over expression of Rac (Nolan et al., 1998). However, heterozygous loss of mhc was unable to rescue the ommatidial defects caused by overexpression of Rho or CDC42. This suggests that Mhc is a principal regulator of RacGTP levels. We have previously reported that exogenous expression of Dab1RFP in the Drosophila visual system causes developmental aberrations in the external eye morphology (Pramatarova et al., 2003). Here we test if this rough eye phenotype is sensitive to mhc gene dose. We observe that a mutant mhc allele rescues the external eye morphology of Dab1RFP-expressing flies and suggest that Dab1 engages the conserved Crk-Dock1 signaling cassette to regulate Rac function.

Materials and Methods

Antibodies, vectors and virus production

The following antibodies were used in western blots and immunoprecipitation experiments: anti-Dab1 (Biodesign), anti-CrkII (Sigma), anti-Dock180 (C-19, and N-19; Santa Cruz) and anti-FAK pY397 (Biosource). The vectors expressing wild-type and tyrosine to phenylalanine substituted GST-Dab1 fusions encoding residues 1-257 of Dab1 have been described previously (Pramatarova et al., 2003). GST-fusion proteins were produced in bacteria and purified with Glutathione Sepharose beads using standard methods and tyrosine phosphorylated in vitro with Abl kinase (New England Biologicals) in kinase buffer containing 1 mM ATP (Pramatarova et al., 2003). The PECE-Dab1 (encoding wild-type or mutant Dab1) and pDsDab1RFP vectors (encoding Dab1RFP) used to express wild-type and mutant proteins have been described (Howell et al., 2000; Pramatarova et al., 2003). The expression vectors for CrkII (pCDNA Crk) and Dock1 (also known as Dock180; pCAGGS-Dock180) were gifts and have been described previously (Kiyokawa et al., 1998; Takino et al., 2003).

Lentiviral vector plVCMV-EGFP was constructed starting from the third generation self-inactivating vector DNA LV-lac (Pfeifer et al., 2001) by replacing the lacZ gene between Xbal and BamHI with the entire EGFP coding region (BD Biosciences Clontech). EGFP is expressed from the CMV immediate-early (IE) promoter. The expression vector encoding CrkII as an N-terminal in-frame fusion with GFP was constructed by insertion of Crk cDNA PCR product into LV-CMV-EGFP vector. The primers used for CrkII amplification were as follows: (forward) GCCGCCGCCGCCACCATGGGCGG- AACCTGAGCTCG and (reverse) CGGACACGTGCTGTGGAATCTG- CTATCCGGATT. The CrkII construct was sequenced and verified to the published sequence (GenBank accession number NM 016823).

The lentiviral vector packaging system has previously been described (Dull et al., 1998). HEK293T cells were cotransfected using Lipofectamine-2000 (Invitrogen) with vector DNA and the three support plasmids encoding the structural and regulatory proteins. Vector particles were harvested from cell supernatants by slow-speed centrifugation and filtration through 0.45 μm filters. They were concentrated 150× by ultracentrifugation.

Identification of Dab1-binding proteins

Embryonic brains were lysed (nine brains at stage embryonic day 16.5) in TX-IPB (0.1 M NaCl, 1% Triton X-100, 10 mM HEPES (pH 7.4), 2 mM EDTA, 50 mM NaF, 1 mM phenylarsine oxide, 0.1% 2-mercaptoethanol) by sonication on ice (5 seconds). Lysates were clarified by centrifugation at 20,000 g for 20 minutes. Sepharose beads containing either GST-Dab1-257, tyrosine phosphorylated fusion, or unphosphorylated fusion were incubated with the embryonic brain lysate at 4°C for 2 hours to permit binding of Dab1-interacting proteins (Pramatarova et al., 2003). Tyrosine phosphorylation of the fusion protein was done in vitro at 30°C using Abi kinase (100 U; New England Biologicals). The protein complexes were washed four times with TX-IPB. Complexes were eluted in TX-IPB containing 100 mM phenylphosphate, in order to preferentially elute proteins bound to Dab1 in a phosphotyrosine-dependent manner. Eluates were mixed with an equal volume of 2× sample buffer [4% sodium dodecyl sulfate, 40% glycerol, 0.2 M Tris-HCl (pH 6.8), 5.6 M 2-mercaptoethanol, 5 mM EDTA, 0.02% bromophenol blue] and boiled for 5 minutes prior to analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins were visualized with either Silver stain or colloidal Coomassie stain (Invitrogen). Bands were excised and prepared for mass spectrometry analysis. Tryptic peptides were formed by in-gel digestion and separated by high-pressure liquid chromatography which was coupled directly to a model LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) as previously described (Szajner et al., 2003). MS/MS spectra were obtained and searched against the non-redundant protein database using the BLAST program (NCBI).

Co-immunoprecipitation and binding assays

DNA vectors encoding Dab1 (3 μg, PECE III-Dab1) SrcY527F (1 μg, pLXSHD-Src Y527F) and CrkII (3 μg, pCDNA-CrkII) were transfected into HEK293T cells (5×10⁶) by incubating the DNA mixture with 21 μl of Fugene (Roche) for 20 minutes at 21°C in 800 μl of serum-free media before addition to cultured cells. PCDNA 3.1 vector (3 μg) was added for transfections in which either Dab1 or CrkII was omitted. Cells were lysed 48 hours after transfection with TX-IPB buffer (1 ml) containing 10% glycerol. Lysates were clarified by centrifugation and anti-Dab1 (2 μl) or anti-CrkII antibodies (10 μl) were incubated for 1 hour on ice followed by adsorption onto protein A Sepharose beads by rotating samples at 4°C for 1 hour. Complexes were washed three times with TX-IPB buffer and resolved by SDS-PAGE. Co-immunoprecipitation was assayed by immunoblotting anti-CrkII precipitated samples with anti-Dab1 antibodies, and Dab1 samples with anti-CrkII antibodies. The percentage of immunoprecipitated protein was determined by comparing the intensity of Dab1 or CrkII in the co-immunoprecipitation with the amount of the same protein in total cell lysate and adjusting for the fractions of total sample loaded. The co-immunoprecipitation from cultured primary neurons was done essentially the same as above from lysates of neurons (5×10⁶ in a 60 mm dish) untreated, treated for 15
minutes with control conditioned media (CCM), or with Reelin conditioned media (RCM). Collection of and concentration of CCM and RCM and neuronal cell culture was done essentially as previously described (Pramatarova et al., 2003). Western blotting to assay for Dab1 in anti-CrkII immunoprecipitates was done with the anti-Dab1 H monoclonal antibody (gift from Andre Goffinet) followed by a biotin-SP conjugated anti-mouse antibody (Jackson ImmunoResearch) and finally streptavidin-horseradish peroxidase (Life Technologies). The intense signal from the immunoprecipitating antibody precluded detection of Crk in the anti-Dab1 precipitates.

NBT-II mobility assay and immunocytochemistry

The NBT-II cell migration assay used in this report was modified from what has been previously been reported (Petit et al., 2000). NBT-II cells were transfected with Fugene (Roche)-DNA complexes at a ratio of 6:1 using 3 µg of either RFP, GFP, Dab1RFP or Dab1RFP-5F. After 24 hours in culture, cells were trypsinized and plated on collagen-treated microscope chambers (Biorad). Prior to use in experiments, collagen-treated surfaces were blocked with 2% BSA for 1 hour to prevent binding of serum proteins to the glass surfaces. Cells were allowed to attach for 2 hours at 37°C. Cells were then visualized every 6 minutes by fluorescence microscopy, while being maintained at 37°C in a heated microscope chamber and perfused with fresh media. We used a DeltaVision microscope system (Applied Precision), which enabled us to collect images from approximately 20 visual fields in each experiment. To ensure the GFP-expressing and Dab1RFP- or Dab1RFP-5F-expressing cells were exposed to the same environmental parameters, we plated both GFP- and RFP-expressing cells in the same dishes. The control and experimental samples were visualized in the same experiment by alternating between green and red fluorescent microscopy. In experiments in which CrkIGFP was expressed in NBT-II cells, virus was added to NBT-II cultures at a concentration of 5 x 10⁵ infectious particles per ml, 48 hours prior to visualization. Movies were produced with the DeltaVision software and the cell traces were generated from projections of the movies.

To prepare samples for immunocytochemistry, transfected cells were plated on coverslips treated with collagen 24 hours before processing. The coverslades were washed in phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde in PHEM buffer (60 mM PIPES (pH 7.0), 25 mM HEPES (pH 7.0), 10 mM EGTA (pH 8.0) 2 mM MgCl₂, 0.12 M sucrose) for 30 minutes at 37°C, rinsed in PBS and then permeabilized in 0.1% Triton-X100 for 20 minutes at 4°C. Filamentous actin was visualized by incubating the coverslips in fluorescein isothiocyanate (FITC)-conjugated phalloidin (Molecular Probes) for 0.5 hours. Coverslades were then washed in PBS buffer and mounted on microscope slides for analysis.

Drosophila stocks and culture

Fly culture and genetic manipulation were done according to standard protocols with lines that have been previously described (Nolan et al., 1998; Pramatarova et al., 2003). The UAS-Dab1RFP and UAS-RFP lines were maintained as homozygous animals and bred with a homozygous GMR-GAL line to generate the flies with the genotypes UAS-Dab1RFP/+;GMR-GAL4/+ and UAS-RFP/GMR-GAL4. To generate the flies with the genotype UAS-Dab1RFP; GMR; mbc2.35, we crossed UAS-Dab1/UAS-Dab1; mbc2.35/Tb flies with GMR/GMR flies and selected progeny that did not display the tubby phenotype. The mbc2.35 line was kindly provided by J. Settleman.

Results

In order to identify Dab1-binding proteins potentially involved in Reelin signaling, we employed affinity chromatography purification to isolate proteins that bind to Dab1 in a phosphotyrosine-dependent manner. We chose embryonic mouse brain at 16 days of gestation as a source tissue because this stage marks the end of neurogenesis, but neurons are still actively migrating and responding to Reelin (Howell et al., 1999a). Proteins from embryonic mouse brain lysate that bound tyrosine-phosphorylated GST-Dab1, which was immobilized on Sepharose beads, were preferentially eluted using a solution containing phenyl phosphate, a phosphotyrosine analog. Several proteins were observed to bind GST-Dab1 independent of the phosphorylation status (Fig. 1A). Proteins that were eluted only from the tyrosine-phosphorylated GST-Dab1 were potential phosphotyrosine-dependent Dab1-binding proteins. At least two proteins fit this criterion (Fig. 1A, arrows). These bands were excised and the peptide sequence was obtained from them by mass spectrometry.

Sequence information was obtained for several peptides from each band (Table 1). These were compared against the non-redundant protein databases using blast search. Peptides derived from band I correspond to mouse Dock1 (also known as Dock180). The peptides derived from band II are encoded by two genes, including the Crk gene, which produces two alternatively spliced transcripts, CrkI and CrkII, and the CrkL gene. The CrkI and CrkII transcripts encode common SH2 domain and SH3 domains. CrkL, the larger transcript, encodes an additional C-terminal SH3 domain and this protein has a molecular weight of 38 kDa, which matches the size of band

![Fig. 1. Isolation and identification of phosphotyrosine-dependent Dab1-binding proteins from embryonic mouse brain. (A) Two protein bands are observed to bind specifically to phosphorylated GST-Dab1 by silver staining samples resolved by SDS- PAGE (arrows). Embryonic brain lysates were incubated with immobilized tyrosine-phosphorylated GST-Dab1 (pY, lane 1) or unphosphorylated Dab1 (Y, lane 2) and bound proteins were eluted with 100 mM phenyl phosphate solution. The band at approximately 180 kDa was more distinct in the preparative gels used for protein identification. (B) Dock1, associated with tyrosine-phosphorylated GST-Dab1 (lane 1), but not unphosphorylated GST-Dab1 (lane2), was detected by western blotting samples eluted with sample buffer. (C) Crk was observed to interact with tyrosine-phosphorylated GST-Dab1 (lane 1) but not unphosphorylated GST-Dab1 (lane 2) by western blotting samples eluted with sample buffer.](Image 376x202 to 501x417)
2. The CrkL is homologous to CrkII and shares a high degree of sequence similarity through the SH2 and two SH3 domains. We decided to focus on the CrkII and Dock1 proteins, because CrkL is likely to act analogously to CrkII. The proteins collected from the affinity purification were compared by western blotting with anti-Crk and anti-Dock1 antibodies (Fig. 1B,C). In this experiment, bound proteins were eluted with sample buffer instead of phenyl phosphate, in order to elute all bound material. The affinity chromatography using the resin with the tyrosine-phosphorylated Dab1 enriched for both Dock1 and CrkII compared with the resin containing the unphosphorylated Dab1. The Crk polypeptides are known to interact directly with tyrosine-phosphorylated proteins through their SH2 domains (Songyang et al., 1993). Dock1, in contrast, is not known to directly bind to tyrosine phosphorylated peptide sequences. However, Dock1 is a known CrkII and CrkL-binding protein, and it is possible that its interaction with Dab1 was mediated by CrkII or CrkL (Hasegawa et al., 1996; Li et al., 2003).

To determine whether Dock1 interacts with Dab1 directly or indirectly, we assayed whether Dock1 required CrkII to associate with tyrosine-phosphorylated GST-Dab1. We overexpressed Dock1 and CrkII alone or together in HEK293T cells and incubated lysates from these cells with tyrosine-phosphorylated GST-Dab1 Sepharose beads (Fig. 2A). CrkII bound to phosphorylated GST-Dab1, in both the presence and absence of overexpressed Dock1 (Fig. 2A, lanes 4 and 6, lower panel). However, Dock1 was only seen to associate with the phosphorylated GST-Dab1 when CrkII was co-expressed (Fig. 2A, lane 3, lower panel). This suggests that Dock1 binds to Dab1 indirectly through CrkII or another adaptor protein.

To determine whether the CrkII-Dab1 interaction is of high enough affinity to support binding in vivo, we assayed for co-immunoprecipitation of these proteins from HEK293T cells transfected with cDNAs encoding both proteins. To ensure high levels of Dab1 tyrosine phosphorylation, the activated Src527F mutant was included in the transfections. To compare the relative level of Dab1 or CrkII expression, lysates were analyzed by western blotting with antibodies to the respective proteins, 48 hours after transfection (Fig. 2B). CrkII associated with tyrosine-phosphorylated Dab1 in co-expressing cells. Dab1 was detected in anti-CrkII immunoprecipitates from cells expressing both Dab1 and CrkII, by western blot (Fig. 2B, lane 3, lower panel). Lysate that did not contain CrkII did not support Dab1 precipitation (Fig. 2B, lane 1, lower panel), showing that Dab1 does not precipitate non-specifically, under the assay conditions. Similarly, CrkII was detected in anti-Dab1 immunoprecipitates, only from lysates that expressed both proteins (Fig. 2B, lane 10, lower panel) and not from cells expressing only CrkII (Fig. 2B, lane 9, lower panel). In this experiment, in which moderate levels of Dab1 and high levels of CrkII were expressed, approximately 50% of the total Dab1 and 1% of the total CrkII were precipitated through association with the other protein (data not shown).

To determine which tyrosine-phosphorylated residues supported the interaction between CrkII and Dab1, we co-expressed CrkII and tyrosine substituted Dab1 mutants, which represent the sites shown to be phosphorylated in brain using phosphospecific antibodies (Keshvara et al., 2001) (B.W.H., unpublished). Individual substitution of Y198, Y220 or Y232 with phenylalanine did not abolish the Crk-II-Dab1 interaction (Fig. 2B, lanes 4-6 and 11-13, lower panels). The Crk SH2 domain is known to bind to proteins that have a proline, three residues C-terminal to the phosphorylated tyrosine (Songyang et al., 1993). This matches the sequence downstream of Y220 and Y232, which are YQVP and YDVP, respectively. Mutation of either of these reduced the efficiency of co-immunoprecipitation of Dab1 and CrkII. It therefore seemed likely that the Crk SH2 domain could bind to either the Y220 or Y232 phospho-epitopes. To test this, the Dab1 Y220F-Y232F double mutant was used. This mutant failed to support co-immunoprecipitation, suggesting both sites are ligands for the Crk SH2 domain (Fig. 2B; lane 7 and 14, lower panels). The Dab1-5F mutant, which has previously been shown not to be tyrosine phosphorylated in Src transformed cells (Howell et al., 2000), also failed to support co-immunoprecipitation (data not shown). Similar results were obtained for CrkII binding to tyrosine-phosphorylated GST-Dab1. The single-point mutants bound to CrkII, but the double Y220F-Y232F mutant did not (data not shown).

Because both of the CrkII binding sites on Dab1 are tyrosine phosphorylated in response to Reelin signaling (Ballif et al., 2004; Keshvara et al., 2001), we investigated whether the Crk-II-Dab1 complex is formed by Reelin stimulation of neurons. As shown previously, stimulation of primary forebrain neurons in culture with RCM induced Dab1 tyrosine phosphorylation as compared with stimulation with CCM or no treatment (Fig. 3, lanes 1-3). Dab1 protein levels were relatively unaffected by the treatment. It has recently been shown that Dab1 protein levels decline after treatment with

Table 1. Peptide sequence obtained for phosphotyrosine-dependent Dab1-binding proteins by mass spectrometry

<table>
<thead>
<tr>
<th>Molecular mass</th>
<th>Peptide sequences</th>
<th>Identity</th>
</tr>
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<tbody>
<tr>
<td>Band I</td>
<td>LLPGDINQIR, NDIVTVQGDFDK, LTQNVDDLLLK, DEDGNIQPSTSIISLFR</td>
<td>DOCK1(Dock180)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGI:104686</td>
</tr>
<tr>
<td></td>
<td>170-190 kDa</td>
<td></td>
</tr>
<tr>
<td>Band II</td>
<td>QEAVALQQR, HGVFLVR, DSSTPGDVLYSLYSENKR, VSHEYIINSSGFRPPVPPPAQPGVSSPR, ALDFNGNEEIDLFFK, IGDQFDSLPALLEFYK</td>
<td>CrkL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGI:88508</td>
</tr>
<tr>
<td></td>
<td>38-42 kDa</td>
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Protein bands were digested by trypsin and the peptide sequence of the peptide mixture was determined by mass spectrometry. Blast searches were used to identify proteins that matched the various peptides. Accession numbers for mouse genome informatics (MGI) and Unigene cluster databases are provided.
Reelin promotes a Crk-Dab1 interaction

RCM with a half life of 120 minutes, and therefore the slight decrease in Dab1 protein levels in the RCM-treated sample is expected (Arnaud et al., 2003a). Dab1 co-immunoprecipitation was augmented in the Reelin-treated sample, whereas levels of immunoprecipitated CrkII were relatively constant between treatments (Fig. 3, lanes 4-6). Because these cultures were prepared from wild-type animals, the low levels of Reelin in the unstimulated cultures may be partially responsible for the low level of Dab1 detected in complex with CrkII from the untreated and CCM-treated samples. This demonstrates that the exposure of embryonic neurons to Reelin promotes the formation of Dab1-CrkII complexes.

The interaction of Crk with the tyrosine kinase substrates, paxillin and p130Cas, has previously been shown to regulate cell migration (Cary et al., 1998; Klemke et al., 1998; Petit et al., 2000; Takino et al., 2003). We therefore employed the NBT-II cell line to determine whether Dab1-Crk complexes have a role in regulating cellular mobility. NBT-II cells have previously been shown to migrate on collagen in a Crk-dependent manner (Petit et al., 2000). Expression of dominant-negative versions of Crk or phosphorylation-site mutants of paxillin reduces the migration of these cells on collagen (Petit et al., 2000). Because expressing Dab1 with an activated kinase, such as Src, could complicate the results by promoting cell migration (Cary et al., 1996), we needed another means to activate Dab1 tyrosine phosphorylation. Conveniently, when Dab1 is expressed as an RFP fusion in Rat-2 cells or in the Drosophila eye, it becomes tyrosine phosphorylated in the absence of overexpressed kinases (Pramatarova et al., 2003). Dab1 dimerization, induced by AP20187 treatment of cells expressing Dab1FKBP fusions, has recently been shown to induce its tyrosine phosphorylation, suggesting that Dab1

**Fig. 2.** CrkII mediates the Dock1 interaction with Dab1 and requires Dab1 Y220 or Y232 for binding. (A) Dock1 (lanes 1-3, upper panel) and CrkII (lanes 4-6, upper panel) were detected by western blotting lysates from HEK293T cells transfected with Dock1 (lanes 2,3), and/or CrkII (lanes 1,3). Dock1 only associated with the immobilized tyrosine-phosphorylated GST-Dab1 in the presence of overexpressed CrkII (lane 3, lower panel) and not in its absence (lane 2, lower panel). In contrast, Crk II associated with GST-Dab1 in both the absence (lane 4, lower panel) and presence (lane 6, lower panel) of Dock1. (B) Expression of Dab1 wild-type (lanes 1,3, upper panel), the indicated Dab1 tyrosine to phenylalanine mutants (lanes 4-7, upper panel) and CrkII (lanes 9-14, upper panel) was detected in total cell lysates of transfected HEK293T cells, which were cotransfected with the activated SrcS27F mutant. Co-immunoprecipitation of Dab1 and CrkII was detected by immunoprecipitation with anti-CrkII (lanes 1-7, lower panel) or anti-Dab1 (lanes 8-14, lower panel) and western blotting with anti-CrkII antibodies (lanes 8-14, lower panel). Wild-type Dab1 (lanes 3,10, lower panel) and single-site substituted Dab1 (lanes 4-6 and 11-13, lower panel), but not the Dab1 Y220F-Y232F double mutant (lanes 7,14, lower panel) supported co-immunoprecipitation with CrkII. Dab1 molecules that are phosphorylated at Y232 have reduced electrophoretic mobility as compared with unphosphorylated Dab1 or Dab1 with phenylalanine substitutions at this position (compare lanes 1 and 3-5 with 6 and 7, lower panel) (see also Howell et al., 2000). This figure shows representative results obtained in three independent experiments.

**Fig. 3.** CrkII forms a Reelin-promoted complex with Dab1 in embryonic forebrain neurons. Total cell lysates (TCL) were collected from cultured embryonic forebrain neurons which were unstimulated (0; lanes 1,4), stimulated with control conditioned media (CCM; lanes 2,5), or stimulated with Reelin conditioned media (RCM; lanes 3,6) for 15 minutes. Western blotting these samples with anti-phosphotyrosine antibody (pY; 4G10) shows the induction of the tyrosine phosphorylation of an 80 kDa protein, which corresponds to Dab1 (lanes 1-3, upper panel), whereas the Dab1 protein levels are relatively constant between samples (lanes 1-3, lower panel). Equal levels of CrkII are detected in the anti-CrkII immunoprecipitates from these cell lysates (lanes 4-6, upper panel); however, increased co-immunoprecipitation of Dab1 was detected from lysates of Reelin-treated samples (compare lanes 4-6, lower panel). Approximately 100% of CrkII was immunoprecipitated under the assay conditions, and 5% of total Dab1 was detected in the co-immunoprecipitation (not shown).
multimerization is sufficient to activate its phosphorylation (Strasser et al., 2004). In line with our previous findings, Dab1RFP was tyrosine phosphorylated in NBT-II cells, but the Dab1RFP-5F mutant was not (data not shown). Therefore, Dab1RFP expression can be used to monitor the effects of tyrosine-phosphorylated Dab1 on Crk-dependent migration.

Because Dab1RFP is a fluorescent molecule, it was straightforward to detect and monitor the movement of the cells expressing this fusion by fluorescence microscopy. We first examined the mobility of RFP-expressing cells and observed that they migrated on collagen-coated dishes, indicating that the visualization conditions did not impact NBT-II cell movement (data not shown). To ensure that assay conditions were permissive for migration, we plated both Dab1RFP- and GFP-expressing cells in the same temperature-controlled dishes. We monitored the movement of both of these cell populations over an 8-hour period (Fig. 4). Whereas the GFP-expressing cells were observed to migrate randomly around the microscope chambers, the Dab1RFP-expressing cells moved very little (Fig. 4A, compare green and red trajectories). To determine whether this influence on cell mobility was dependent upon the Dab1 tyrosine-phosphorylation sites, we monitored the movement of the tyrosine-substituted Dab1 mutant, Dab1RFP-5F. Cells expressing Dab1RFP-5F migrated as well as cells expressing GFP alone (Fig. 4B). The Dab1RFP were typically more rounded than the Dab1RFP-5F- and GFP-expressing cells (data not shown). To distinguish whether the loss of migration in the Dab1RFP-expressing cells is specific to lost Crk function or if it is unrelated to a Dab1-Crk interaction, we overexpressed CrkIII GFP in the Dab1RFP-expressing cells. NBT-II cells were infected with a lentivirus expressing Dab1RFP and CrkIIGFP co-expressing cells was less rounded and flatter than cells expressing Dab1RFP alone in the majority of cells observed. CrkII GFP expression restored the migration of the Dab1RFP-expressing NBT-II cells (Fig. 4C). The morphology of the majority of Dab1RFP and CrkIIGFP co-expressing cells was less rounded and flatter than cells expressing Dab1RFP alone (data not shown). This suggests that in this assay Dab1RFP hinders cell migration by interfering with a Crk-dependent process. It is formally possible, however, that signaling upstream of Crk is attenuated by Dab1RFP and this is rescued by increasing the dose of Crk.

Regulation of actin dynamics is known to be instrumental in the control of cellular migration. Hence, we examined the actin cytoskeletons of NBT-II cells expressing RFP, Dab1RFP or Dab1RFP-5F. Visualizing the cells with FITC-labeled phalloidin showed differences in the cell morphology, cell polarity and in the arrangement of actin filaments (Fig. 5, green). The RFP- and Dab1RFP-5F-expressing cells were polarized and had characteristics of migrating cells such as lamellipodia and stress fibers (Fig. 5A,C). The Dab1RFP-expressing cells were not polarized, but instead the majority had a circular appearance (Fig. 5B). We did not detect lamellipodia-like structures in these cells. In contrast, filopodia were the prominent actin feature in Dab1RFP-expressing NBT-II cells.

To determine whether the subcellular localization of Crk might be altered in the presence of tyrosine-phosphorylated Dab1, we examined its distribution in cells expressing either Dab1RFP or the unphosphorylated mutant, Dab1RFP-5F. Endogenous CrkII was found to co-localize with Dab1RFP at a subset of sites at the cell periphery of NBT-II cells, whereas it was not observed to co-localize with Dab1RFP-5F (compare Fig. 6A and Fig. 6B).

We also examined the possibility that expression of Dab1RFP might hinder integrin signaling. Fak tyrosine phosphorylation at Y397 is an early event downstream of integrin activation. If integrin signaling was in someway disrupted, we reasoned that Fak phosphorylation at Y397 would be reduced. We therefore examined the lower plane of cells transfected with Dab1RFP and Dab1RFP-5F with an antibody specific to the autophosphorylation site. Dab1RFP, Dab1RFP-5F and untransfected NBT-II cells had similar levels of tyrosine-phosphorylated Fak at the surface in contact with the extracellular matrix (Fig. 6C,D and data not shown).

The Crk-Dock interaction is conserved in Drosophila, C. elegans and mammals, and the genes that encode these proteins have been shown to interact genetically with the Rac homologs in these species (Galletta et al., 1999; Nolan et al., 1998; Reddien and Horvitz, 2000; Wu et al., 2001). Dock1 interacts with nucleotide-free Rac and promotes GTP loading (Brugnera et al., 2002; Grimsley et al., 2003). In Drosophila the Dock1 homolog, myoblast city (mbc), has been implicated in myoblast fusion, dorsal closure and border cell migration. An mbc allele has also been identified in a screen to identify mutations that rescued a rough eye phenotype caused by Rac overexpression in Drosophila (Nolan et al., 1998). Therefore, the Drosophila compound eye is an applicable model to test whether tyrosine-phosphorylated Dab1 regulates signaling that affects Rac function.

We have shown that exogenous expression of mouse Dab1RFP in the developing compound eye of Drosophila produces anomalies in the external eye morphology
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(Pramatarova et al., 2003). To determine whether hyperactivation of the Drosophila Crk-Mbc signaling could be a cause of the rough eye phenotype, we introduced a loss-of-function mbc mutation into the Dab1RFP-expressing line. The eyes of flies that expressed Dab1RFP, and which were heterozygous for the mbc mutation, were less affected than eyes from flies that expressed Dab1RFP alone (Fig. 7Bi,Ci). Expression of RFP alone is very similar to the normal fly eye and it is shown for comparison (Pramatarova et al., 2003). In high-power images, it was apparent that in flies expressing Dab1RFP, the rows of the ommatidial facets were straighter when mbc was heterozygous (compare Fig. 7Bii and 7Cii). This suggests that expressing tyrosine-phosphorylated mouse Dab1, in the developing Drosophila visual system, is capable of activating a pathway that is sensitive to the mbc gene dose.

Discussion

Reelin signaling regulates Dab1 tyrosine phosphorylation in the developing nervous system, and genetic studies have demonstrated that Dab1 tyrosine phosphorylation sites are required for normal brain development (Howell et al., 1999a; Howell et al., 2000). However, the cell biological consequences of Reelin presentation and the molecular events downstream of Dab1 tyrosine phosphorylation are only partially understood.

Fig. 5. The expression of Dab1RFP alters the morphology and profile of filamentous actin in NBT-II cells plated on collagen. (A) The filamentous actin distribution detected by FITC-phalloidin (green) was polarized and features of migrating cells such as lamellipodia, filopodia and stress fibers were apparent in RFP-expressing (red) NBT-II cells. This was comparable to untransfected cells (not shown). (B) Dab1RFP expression (red) resulted in a morphological change in the cell and loss of lamellipodia and stress fibers, but filopodia were apparent. (C) Expression of Dab1RFP-5F (red) did not lead to the loss of lamellipodia or stress fibers (Bar, 10 μm). Nuclei were detected by Dapi staining (blue). Arrowhead, lamellipodia; arrow, stress fibers. The cells shown are representative of the majority of cells from each transfection.

Fig. 6. Expression of Dab1RFP in NBT-II cells alters CrkII distribution but does not alter phosphorylation of Fak at Y397. (A) Endogenous CrkII (green) was observed to co-localize (yellow areas) with Dab1RFP (red) at the cell membrane of transfected NBT-II cells. (B) In cells expressing Dab1RFP-5F (red) the CrkII signal was not observed to overlap. (C) In cells expressing Dab1RFP, Fak phosphorylated at Y397 (green) was observed in the plane closest to the coverglass. (D) Dab1RFP-5F-transfected cells also expressed phosphorylated Fak.

Fig. 7. Reduced gene dosage of the Drosophila Dock1 homolog, mbc, partially rescues the rough eye phenotype caused by expression of Dab1RFP. (A) The compound eyes of Drosophila with the genotype UAS-RFP/GMR-GAL4 appeared normal as visualized by scanning electron microscopy. (B) Expression of tyrosine-phosphorylated Dab1RFP in Drosophila with the genotype UAS-Dab1RFP/+;GMR-GAL4/+ led to the development of a rough eye morphology characterized by fusions of ommatidia and loss of linearity of the rows of the ommatidial facets (Pramatarova et al., 2003). (C) Heterozygous mutation of mbc partially rescued the developmental phenotype caused by Dab1RFP expression. Drosophila with the genotype UAS-Dab1RFP/+; GMR-GAL4/+; mbc+/+ had fewer ommatidial fusions, and the rows of ommatidia are more linear. Magnification: Ai-Ci, 200× (Bar, 100 μm); Aii-Cii, 800×.
Here we identify CrkII and CrkL as phosphotyrosine-dependent Dab1-binding proteins and demonstrate that Dock1 interacts with the CrkII-Dab1 complex in vitro. Reelin stimulation of primary neuronal cultures augments the association of endogenous levels of CrkII and Dab1, suggesting that this complex is physiologically relevant to the Reelin response. Crk signaling complexes regulate a variety of cellular processes, including cell migration downstream of the integrin-mediated activation of tyrosine kinases (Feller, 2001). We found that Dab1 acted to suppress Crk-dependent cell migration using an NBT-II cell culture assay. This activity was dependent upon the Dab1 tyrosine phosphorylation sites. We suggest Dab1 acts on the conserved Crk-Dock1-Rac pathway because a rough eye phenotype caused by Dab1 overexpression is rescued by reduced gene dose of the Drosophila mbc, the Dock1 homolog in this organism.

Reelin treatment promotes the formation of the CrkII-Dab1 complex. If the Dab1-CrkII complex has the same influence on migrating neurons as it did in NBT-II cells, we would predict that in the absence of Reelin signaling, neurons would migrate too far. Many Reelin-responsive neurons fail to meet this criterion, such as Purkinje cells, and some cortical neurons, which do not migrate far enough when Reelin or downstream components of the pathway are missing. However, the preanglionic autonomic spiral cord neurons (AMNs) appear to migrate beyond their target zones in the absence of Reelin (Phelps et al., 2002; Yip et al., 2000). The migratory pathway of these neurons is complex. They first migrate out from the ventricular zone, then dorsally in the spinal cord. In the absence of Reelin, most of the AMNs then migrate medially inward along radial glia, whereas in the presence of Reelin most of these neurons remain in the lateral spinal cord (Yip et al., 2003). Thus, the late-phase medial migration of the AMNs observed in the Reeler mutants could conceivably be prevented by Reelin-induced Dab1-CrkII complexes. It is also possible that complexes between Dab1 and Crk may have other roles in different neuronal classes. Crk signaling complexes have varied roles in the regulation of biological processes, such as alterations in cell morphology (Escalante et al., 2000; Feller, 2001; Lamorte et al., 2002). It has recently been demonstrated that Reelin regulates the branching of dendrites of hippocampal neurons and the morphology of radial glia in a Dab1-dependent manner (Hartfuss et al., 2003; Niu et al., 2004). Further work outside the scope of this manuscript is required to determine the full range of cell biological consequences of Reelin-induced Dab1-CrkII complexes.

Genetic studies in C. elegans and Drosophila have been instrumental in elucidating conserved members of the Crk signaling complex that promote Rac activation (Albert et al., 2000; Galletta et al., 1999; Nolan et al., 1998; Reddien and Horvitz, 2000; Tosello-Trampont et al., 2001; Wu et al., 2001). It has been shown that members of the Dock1 and ELMO family interact, and act as an unconventional exchange factor for Rac (RacGEF) when recruited into Crk-containing signaling complexes (Brugnera et al., 2002). Here we use a Drosophila model to show that a rough eye phenotype generated by expression of tyrosine-phosphorylated Dab1 is sensitive to the gene dose of the Drosophila Dock1 homolog, mbc. The mbc mutation has previously been shown to rescue a rough eye phenotype caused by Rac overexpression. Because the rough eye phenotype was not observed in flies that express the tyrosine phosphorylation site mutant Dab1, Dab1RFP-5F, the rescue data suggests that Dab1 tyrosine phosphorylation sites signal to activate Rac in this model. Previously, we have identified Nckβ as a Dab1-binding partner. Like Crk, Nckβ also binds to Dock1. It is possible therefore that Dab1RFP recruits either the Drosophila Crk or Nckβ homologs to the membrane, leading to the inappropriate activation of Rac and producing a rough eye. Further work is required to determine in what context this pathway may be activated by Reelin signaling in mammalian neurons.

It has recently been demonstrated that the subcellular targeting of the Crk-Dock1-ELMO complex is critical for its biological outcome. ELMO mutants, which have deletions in the N-terminal region and which are unable to localize to lamellipodia, fail to promote cell migration, although they retain the ability to bind Dock and cooperatively act as a RacGEF (Grimsley et al., 2003). In the NBT-II cell assay, Dab1 binding to Crk may work in a similar manner to cause the redistribution of Crk complexes away from focal contacts. We observe colocalization of CrkII and Dab1 at the cell membrane in NBT-II cells expressing Dab1RFP, but not in cell expressing Dab1RFP-5F, suggesting that Dab1 may be acting to redistribute CrkII. Importantly, it appears that Dab1 acts differently to other Crk-binding proteins such as paxillin and p130Cas, which promote cell migration in similar assays (Cary et al., 1998; Huang et al., 2003; Klemke et al., 1998; Petit et al., 2000).

Several signaling pathways are now thought to be activated downstream of Reelin signaling. Reelin activates PI3K, leading to activation of AKT, suppression of Gsk3β and decreased Tau phosphorylation (Ballif et al., 2003; Befert et al., 2002). PI3K activation is at least in part accomplished by the recruitment of the p85 subunit of PI3K to tyrosine-phosphorylated Dab1 (Bock et al., 2003). CrkII provides another possible link between Dab1 and PI3K signaling (Feller, 2001). While this manuscript was in preparation another independent report has shown that Rap1 is activated by Reelin signaling through C3G, another Crk and CrkL binding protein (Ballif et al., 2004). However, they were unable to demonstrate that Reelin increases RacGTP levels, using a biochemical assay. Work presented here suggests that Dock1 and Rac signaling may be downstream components of the Reelin signaling cascade. The proposed regulation of Rac by Reelin may be spatially or temporally regulated, in a manner that makes it difficult to detect biochemically. In some ways, the complexity of Reelin stimulation may be viewed to be analogous to signaling through receptor tyrosine kinases. Instead of activating a kinase that recruits signaling molecules to autophosphorylation sites, Reelin activates cytoplasmic kinases through the ligation of receptors that lack kinase activity. Dab1, which is anchored to the membrane through association with the Reelin receptors and phospholipids, acts to bind SH2 domain-containing molecules such as P85, Nckβ and Crk and activate downstream signaling pathways (Howell et al., 1999b; Trommsdorff et al., 1999). Further study will be required to determine the sum of downstream pathways that are activated by Reelin signaling and elucidate how the interplay of these pathways determines neuronal positioning.

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