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Tyrosine phosphorylated Disabled 1 recruits Crk family adapter proteins

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Abstract

Disabled 1 (Dab1) functions as a critical adapter protein in the Reelin signaling pathway to direct proper positioning of neurons during brain development. Reelin stimulates phosphorylation of Dab1 on tyrosines 198 and 220, and phosphorylated Dab1 is likely to interact with downstream signaling proteins that contain Src homology 2 (SH2) domains. To search for such proteins, we used a Sepharose-conjugated peptide containing phosphotyrosine 220 (PTyr-220) of Dab1, as an affinity matrix to capture binding proteins from mouse brain extracts. Mass spectrometric analysis of bound proteins revealed that Crk family adapter proteins selectively associated with this phosphorylation site. We further show that Crk-I and Crk-II, but not CrkL, stimulate phosphorylation of Dab1 on tyrosine 220 in a Src-dependent manner. Our results suggest that Crk family adapter proteins may play an important role in the Reelin signaling pathway during brain development.
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The Reelin (RELN) signaling pathway plays a critical role in the proper positioning of neurons in the central nervous system during development [1]. Reelin is a secreted glycoprotein that is deficient in the classical ataxic mouse *reeler* [2]. In mice as well as in humans, Reelin deficiency is associated with dramatic disruptions in cell layering in laminated structures throughout the brain [3].

Reelin is a ligand for the very low density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2) on the surface of neurons [4,5]. The cytoplasmic regions of these receptors contain the Asn-Pro-X-Tyr (NPXY) sequence motif required for binding to the phosphotyrosine-binding (PTB) domain of Dab1, an intracellular adapter protein [6]. The most dramatic and well-characterized biochemical consequence of Reelin binding to the receptors is a rapid increase in tyrosine phosphorylation of Dab1 [7]. The biochemical data implicating Reelin, VLDLR, ApoER2, and Dab1 within a signaling pathway are consistent with the genetic data derived from mouse models. Mice lacking Dab1 [8,9] or both VLDLR and ApoER2 [10] exhibit behavioral and neuroanatomical phenotypes that are indistinguishable from *reeler*.

Dab1 was originally discovered as a Src-binding protein [11], and it contains several tyrosine residues that are readily phosphorylated by Src family kinases in vitro [12]. While Fyn phosphorylates Dab1 in vivo, Src can partially compensate for its role in Fyn-deficient mice [13,14]. A "knock-in" mouse mutant expressing Dab1 with phenylalanine substitutions in place of the five in vitro phosphorylated tyrosine residues exhibits a phenotype that is indistinguishable from *reeler* [15]. In mice lacking Reelin, or both VLDLR and ApoER2, Dab1 accumulates in a hypophosphorylated form [10,16], whereas treatment of neurons derived from the mutant embryos with Reelin rapidly induces tyrosine phosphorylation [7]. Therefore, tyrosine phosphorylation of Dab1 is critical for the function of the Reelin pathway during brain development. While relatively little is known about the signaling events that lie

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downstream of Dab1, phosphorylated tyrosine residues are likely to serve as docking sites for recruitment of downstream signaling proteins containing Src homology 2 (SH2) domains. Identification of these proteins is essential for elucidation of this important signaling pathway.

Previously, we showed that Reelin-induced phosphorylation of Dab1 in neurons occurs on tyrosines 198 (Tyr-198) and 220 (Tyr-220) [12]. Here we use a combination of peptide affinity capture and mass spectrometry to show that Crk family adapter proteins Crk-I, Crk-II, and CrkL bind to phosphorylated Dab1 via their SH2 domains. We also show that Crk-I and Crk-II, but not CrkL, specifically stimulate phosphorylation of Tyr-220, in the absence of Reelin or its receptors, in a Src-dependent manner. Therefore, our results raise the intriguing possibility that the Crk family adapter proteins play an important role in the intracellular signaling events triggered by Reelin stimulation during neuronal development.

Materials and methods

Peptide affinity capture and mass spectrometry. Snap-frozen postnatal day 8 (P8) mouse brains were purchased from Harlan (Indianapolis, IN). Extracts were prepared by homogenizing the brains in cell lysis buffer as described previously [17]. The extracts were clarified by centrifugation and then used for the peptide affinity captures. The following peptides corresponding to a Dab1 tyrosine phosphorylation sites were synthesized and conjugated to Sepharose by the Hartwell Center for Bioinformatics and Biotechnology (St. Jude Children's Research Hospital): Y198, DVEDPVYQYIVFEA; PY198, DVEDPVY(PO_4)QYIVFEA; Y220, CETEENIYQVPTSQK; PY220, CETEENIY(PO_4)QVPTSQK); Y232, SQKKEGVYDVPKSQPVS; and PY232, SQKKEGVY(PO_4)DVPKSQPVS. Peptide–Sepharose slurries were incubated with equal amounts of clarified brain extracts at 4 °C overnight. After extensive washing of bound proteins with Laemmli buffer they were separated by SDS–PAGE and visualized by SYPRO Ruby stain (Molecular Probes). Image capture was carried out using a Fujifilm LAS-1000 imaging system.

Protein bands of interest were excised and subjected to in-gel trypsin digestion. Tryptic peptides were desalted using ZipTips (Millipore), mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix, and then applied to a sample plate. Mass measurements were performed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry in a Voyager DE-RP instrument from Applied Biosystems. Measured peptide masses were matched with entries in the NCBInr protein database using the MSFit routine in Protein Prospector suite (http://Prospector.ucsf.edu).

DNA constructs, cell culture, and transfection. Dab1 expression plasmids and point mutants were described previously [12]. Additional point mutations at phosphorylation sites were carried out using QuickChange Plus site-directed mutagenesis kit (Stratagene). pCAGGS-CrkII, pEBG-CrkI, pEBG-CrkI-R38V, and pEBG-CrkI-W169A were provided by Dr. Sansana Sawasdikosol (NYU). pSG5-CrkL was obtained from Dr. John Groffen (USC, Children's Hospital, Los Angeles). pLNCX-Src-K295R was obtained from Dr. Joan Brugge (Harvard University). HEK293T cells were transfected using FuGene 6 (Roche Molecular Biochemicals, Indianapolis, IN), as described previously [12]. Forty hours after transfection, cells were harvested and lysed in the cell lysis buffer. In some instances, transfected cells were treated with the Src family kinase inhibitor PP2 or its inactive analog, PP3, for 1 h at 37 °C prior to cell lysis.

Immunoprecipitations and immunoblotting. Immunoprecipitations were carried out as described previously [12,17]. Whenever cells were transfected with GST-Crk-I, associated complexes were precipitated using glutathione–agarose beads in place of immunoprecipitation. Proteins in the immunoprecipitates or glutathione–agarose pull-downs were detected by Western blotting, as described previously [12]. The following antibodies were used: Crk, RasGap, and Nck (BD Transduction Labs); 4G10, CrkL, and Nck-β (Upstate Biotechnology); and Src (Santa Cruz). Anti-Dab1 (CT38; Rockland), which was described previously [17], was raised against a GST fusion of Dab1 carboxyl region. Therefore, these antisera contain a significant titer of anti-GST antibodies, and they were used to simultaneously detect Dab1 and GST-Crk-I in Western blots.

Gene expression analysis by in situ hybridization. Gene expression was evaluated by in situ hybridization using [α-^{33}P]UTP labeled riboprobes. This has been described previously [16]. Cryostat tissue sections (16 μm) were prepared from embryonic day 15.5 mouse embryos. Riboprobe templates for crk and crkL were generated using RT-PCR from neonatal mouse brain cDNA using the following primers: *crk* sense, 5′atggcgggcaacttcgactcggag3′, *crk* antisense, 5′tccaccactgctcttcaggc3′, *crkl* sense, 5′atgtcctccgccaggtttgattc3′, *crkl* antisense, 5′gttgagcatacgcatgagcagg3′. Autoradiography was allowed to proceed for 1–3 days at 4 °C. A nearby section in the tissue block used to analyze *crk* and *crkl* was stained with cresyl violet as a reference.

Results

Affinity capture of phospho-Dab1-binding proteins

To search for phospho-Dab1-binding proteins, mouse brain extracts were incubated with Sepharose-conjugates of non-phosphorylated or phosphorylated peptides corresponding to the PTyr-220 phosphorylation site. As shown in Fig. 1, several proteins bound to

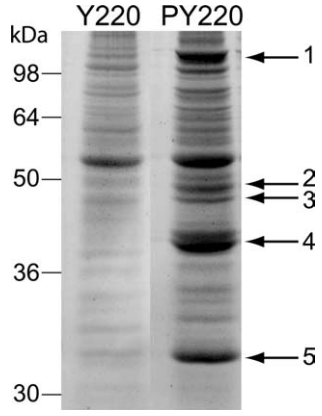

Fig. 1. Affinity capture of proteins from mouse brains that bind to Dab1 phosphorylation site PTyr-220. Sepharose-conjugated unphosphorylated (Y220) or phosphorylated (PY220) peptides corresponding to Dab1 phosphorylation site Tyr-220 were incubated with clarified extracts prepared from P8 mouse brains. After washing, bound proteins were eluted by Laemmli buffer, separated by SDS–PAGE, and detected by SYPRO Ruby stain using UV transillumination. The protein bands are labeled 1–5.

both the phosphorylated and the unphosphorylated peptides. However, at least five major proteins preferentially bound to the phosphorylated form of the peptide. The bands, labeled 1–5, correspond to proteins with observed molecular weights of approximately 110, 44, 40, 38, and 32 kDa. Band 4 was closely associated with a weaker band that migrated more slowly. Since it was difficult to separate the two, they were treated as a single gel band for the purpose of mass spectrometric analysis.

Identification of proteins by MALDI-TOF

To identify proteins that selectively bound to the phosphorylated form of the peptide, masses of tryptic peptides were determined by MALDI-TOF and matched to proteins in NCBInr protein database. The results are summarized in Table 1. A total of seven proteins were identified. Bands 1, 3, and 5 contained peptides that matched to RasGap, β-actin, and Crk-I, respectively. While band 2 appeared as a single protein on the gel (Fig. 1), MALTI-TOF analysis revealed the presence of peptides matching to Nck-1 and Nck-2. Further analysis confirmed the presence of two distinct populations of peptides that matched to non-overlapping sequences within Nck-1 or Nck-2. Indeed, the two proteins are nearly identical in their mass but share only 68% amino acid identity [18]. While the majority of peptides derived from the band 4 doublet matched to Crk-II, at least six peptides matched to unique sequences within the closely related protein CrkL.

The known molecular weights of the proteins identified correspond well to the observed size of the bands on the SDS–PAGE gel (Fig. 1). With the exception of β-actin, the identified proteins are known to contain SH2 domains. RasGap is an important regulator of Ras activity that is required for proper neuronal and vascular development [19]. Nck-1 and Nck-2 are closely related SH2/SH3 adapter proteins that play functionally redundant roles in the development of mesoderm-derived structures, including the notochord [20]. Recently, Pramatarova et al. [21] identified Nckβ (Nck-2) as a Dab1-binding protein using a modified yeast two-hybrid approach.

Crk family proteins selectively bind to PY220 and PY232

Crk family adapter proteins are known to participate in numerous signaling pathways that are relevant to cellular adhesion and motility [22]. Crk-I and Crk-II are alternatively spliced variants of a single transcript. Crk-I contains an amino terminal SH2 domain followed by a more carboxyl terminal SH3 domain, while Crk-II contains an additional linker region containing a key regulatory tyrosine (Tyr-221) followed by a second SH3 domain (Fig. 2A). CrkL also contains an amino terminal SH2 domain followed by two SH3 domains, and it shares a high degree of identity with Crk-II [22].

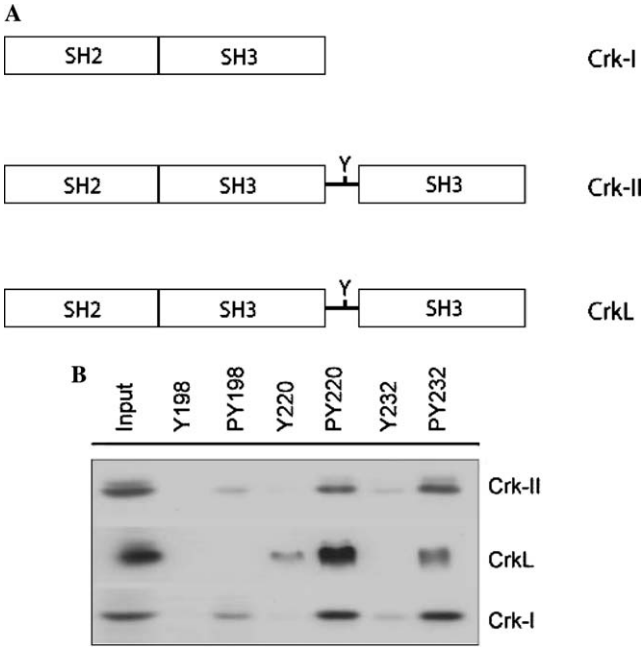

Fig. 2. Site-selective binding of Crk family adapter proteins. (A) Schematic representation of SH2 and SH3 domain arrangements within the Crk adapter family. (B) Sepharose-conjugated unphosphorylated (Y198, Y220, and Y232) or phosphorylated (PY198, PY220, and PY232) peptides corresponding to known phosphorylation sites on Dab1 were incubated with mouse brain extracts. Precipitated Crk proteins were detected by Western blotting using anti-Crk (Crk-I and Crk-II) and anti-CrkL antibodies. Approximately 5% of the brain extract was run on the side as positive control (Input).

Table 1
Identification of PY220-binding proteins by MALDI-TOF mass spectrometry

Band	Observed MW (kDa)	Protein	Matching peptides	Protein coverage (%)	Calculated MW (kDa)	SH2 domain
1	110	RasGap	28	35	94	+
2	44	Nck-1	15	38	43	+
		Nck-2	13	37	43	+
3	40	β-Actin	10	25	40	–
4	38	Crk-II	13	42	34	+
		CrkL	6	20	34	+
5	32	Crk-I	15	75	23	+

To confirm the results from mass spectrometric analyses, we used three sets of Sepharose-conjugated unphosphorylated and phosphorylated peptides, corresponding to the three known in vitro sites of phosphorylation on Dab1, to precipitate proteins from mouse embryonic brain extracts. As shown in Fig. 2B, immunoblots revealed that Crk-I, Crk-II, and CrkL bound selectively to PY220 and PY232 phosphopeptides with very little binding PY198 or to the non-phosphorylated peptides. Predictions based on phosphopeptide library screening [23] suggest that SH2 domains of the Crk adapter proteins exhibit preference for ligands containing a proline residue at the +3 position on the carboxyl side of the phosphotyrosine. Both phosphopeptides PY220 (**pY**QV**P**) and PY232 (**pY**DV**P**) contain the minimum sequences required for such binding.

Interaction of Dab1 with Crk proteins in transfected cells

The peptide pull-down experiments showed that the Dab1-derived phosphopeptides, PY220 and PY232, can precipitate Crk family proteins from brain extracts. To determine whether full-length Dab1 can interact with Crk-I in cells, we carried out transient transfection experiments using HEK293T cells. When a GST fusion of Crk-I was transfected into these cells together with Dab1, the two proteins co-precipitated in glutathione–agarose pull-downs (Fig. 3A). However, mutation of a critical arginine residue to valine (R38V) within the SH2 domain of Crk-I abrogated the interaction. In contrast, a mutation within the SH3 domain (W169A) did not have any effect on the Crk-I–Dab1 interaction. These results confirmed that the interaction between Dab1 and Crk proteins occurs in transfected cells via the SH2 domain.

Previously, we showed that Dab1 is phosphorylated by Src on tyrosines 198, 220, and 232 when exogenously expressed in HEK293T cells [12]. To further investigate the specific tyrosine residues on Dab1 involved in the interaction with Crk proteins, we transfected cells with a combination of Crk-II, constitutive active form of Src (Src^{527F}), and various forms of Dab1 containing phenylalanine substitutions at single or multiple phosphorylation sites. As shown in Fig. 3B, binding to Crk-II required at least one of tyrosines 220 and 232, and Dab1 did not bind to Crk-II when both residues were replaced with phenylalanine. In contrast, Tyr-198 was completely dispensable for the interaction of Dab1 with Crk-II. Similarly, the interaction between Dab1 and CrkL also required either Tyr-220 or Tyr-232 but not Tyr-198 (Fig. 3C). We repeatedly observed that the interaction of Dab1 with CrkL was noticeably weaker than that with Crk-II. Nevertheless, the SH2 domains of Crk and CrkL have a preference for PTyr-220 and PTyr-232. These findings are consistent with the results of our peptide capture experiments showing selective binding of Crk adapter proteins to PY220 and PY232.

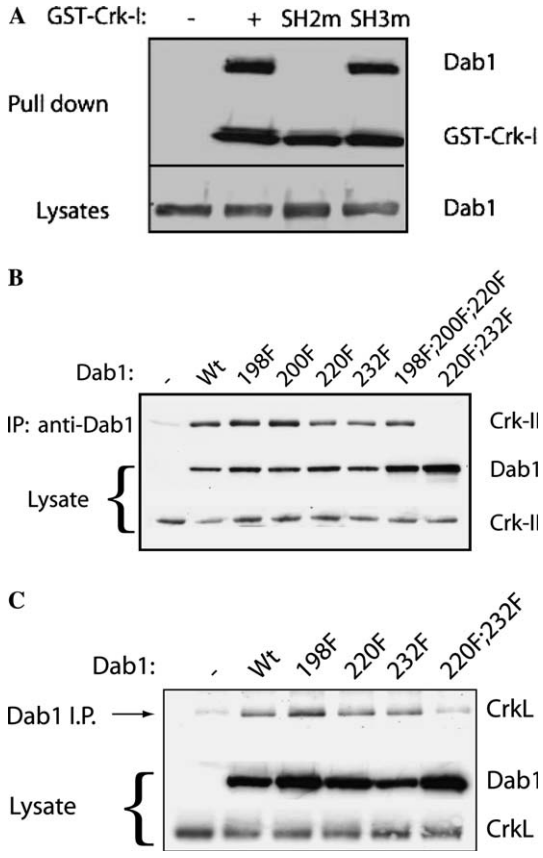

Fig. 3. Interaction of Dab1 with Crk adapters in transfected HEK293T cells. (A) Lysates of cells transfected with Dab1 alone (−) or Dab1 co-transfected with wild-type GST-Crk-1 (+), GST-Crk-I with a mutant SH2 domain (SH2m), or GST-Crk-I with a mutant SH3 domain (SH3m) were incubated with glutathione–agarose, and precipitated proteins were detected by immunoblotting with anti-Dab1 antibodies. (B and C) Cells were transfected with Crk-II or CrkL alone (−) or with either wild-type Dab1 (Wt) or various Dab1 mutants harboring single or multiple phenylalanine substitutions in place of tyrosine sites of phosphorylation. Constitutively active Src was also co-expressed in all transfectants. Dab1 immunoprecipitates from the lysates were then analyzed for the presence of Crk-II or CrkL. Lysates were also run separately for determination of Dab1, Crk-II, and CrkL levels.

Crk-I and Crk-II stimulate tyrosine phosphorylation of Dab1

Since the retroviral oncogene *v-crk* is known to induce tyrosine phosphorylation of cellular proteins [24], we asked whether co-expression of GST-Crk-I and Dab1 can induce tyrosine phosphorylation of Dab1. We expressed Dab1 along with either GST-Crk-I, Crk-II or CrkL in HEK293T cells and determined the phosphotyrosine content of Dab1 in the absence of exogenous Src. As shown in Fig. 4A, co-transfection of Dab1 with GST-Crk-I or Crk-II resulted in a dramatic induction of tyrosine phosphorylation, whereas CrkL did not have any effect. Interestingly, mutation within the SH2 domain in Crk-I abrogated this effect, whereas an SH3 domain mutant was able to induce Dab1 phosphorylation to the

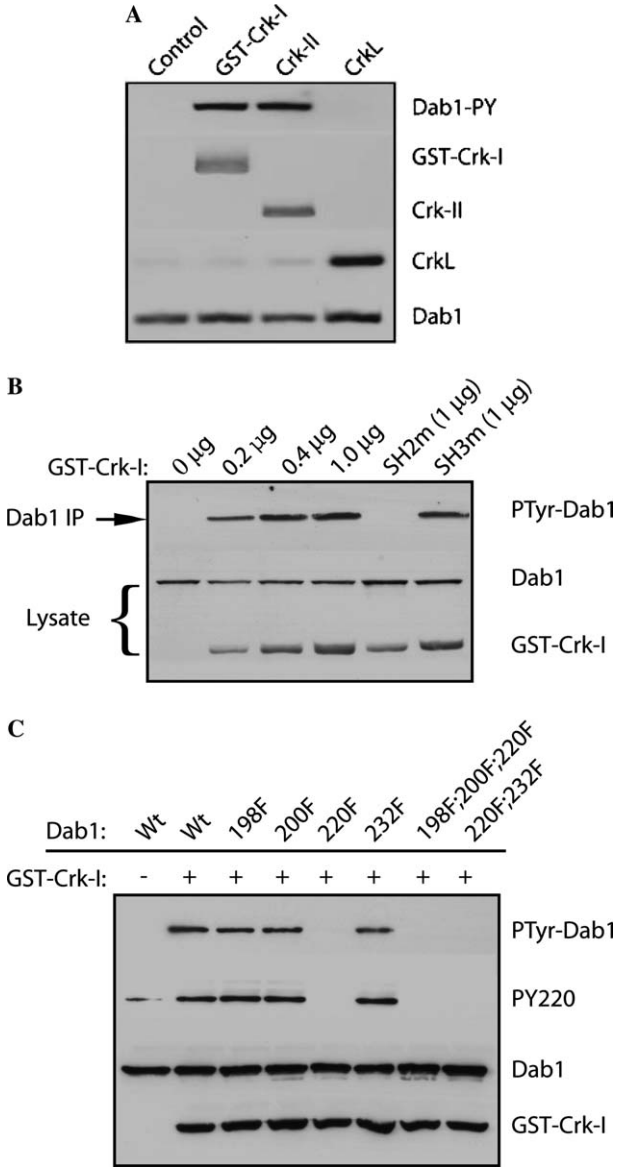

Fig. 4. Crk-I and Crk-II stimulate Dab1 tyrosine phosphorylation. (A) Lysates of HEK293T cells transfected with Dab1 alone or in combination with either GST-Crk-I, Crk-II, or CrkL were immunoblotted with anti-phosphotyrosine, anti-Crk, anti-CrkL or anti-Dab1 antibodies. (B) Cells were transfected with Dab1 alone or in combination with increasing amounts of GST-Crk-I. Alternatively, Dab1 was transfected together with either an SH2 or SH3 mutant of GST-Crk-I. Lysates were immunoblotted with anti-phosphotyrosine, anti-Dab1, and anti-Crk antibodies. (C) Wild-type Dab1 or Dab1 with single or multiple phenylalanine substitutions at the phosphorylation sites was co-transfected with GST-Crk-I. Lysates were immunoblotted with anti-phosphotyrosine, anti-PY220, or anti-Dab1 antibodies.

same extent as wild-type Crk-I (Fig. 4B). Therefore, the SH2 domain of Crk-I is required for the induction of Dab1 tyrosine phosphorylation.

To determine if Crk-I induces phosphorylation of specific tyrosines, we carried out experiments in which GST-Crk-I was co-transfected with various point mutants of Dab1. As shown in Fig. 4C, Crk-I-induced Dab1 tyrosine phosphorylation was not detected when tyrosine 220 was mutated to phenylalanine either alone or together with other point mutations. In contrast, mutation of other tyrosine residues in Dab1 did not alter the extent of phosphorylation induced by Crk-I. Also, immunoblotting analysis using site-specific anti-PTyr220 antibodies confirmed that tyrosine 220 was phosphorylated in response to co-expression of Crk-I (Fig. 4C). In contrast, we were unable to detect Crk-I-induced Dab1 tyrosine phosphorylation using antibodies specific for PTyr-198 and PTyr232 (data not shown). Therefore, Crk-I specifically stimulates phosphorylation of tyrosine 220 on Dab1.

Crk-I-induced Dab1 tyrosine phosphorylation is mediated by Src

Since the Crk proteins do not have a catalytic kinase domain of their own, and Dab1 is phosphorylated by the Src family kinases in vitro and in vivo, we asked whether Crk-I-induced increase in Dab1 tyrosine phosphorylation is mediated by Src family kinases. HEK293T cells transiently expressing both Dab1 and GST-Crk-I were treated with the Src inhibitor PP2 or PP3, an inactive structural analog of PP2. As shown in Fig. 5A, the Crk-I-induced increase in Dab1 tyrosine phosphorylation was significantly reduced in the presence of PP2 although it

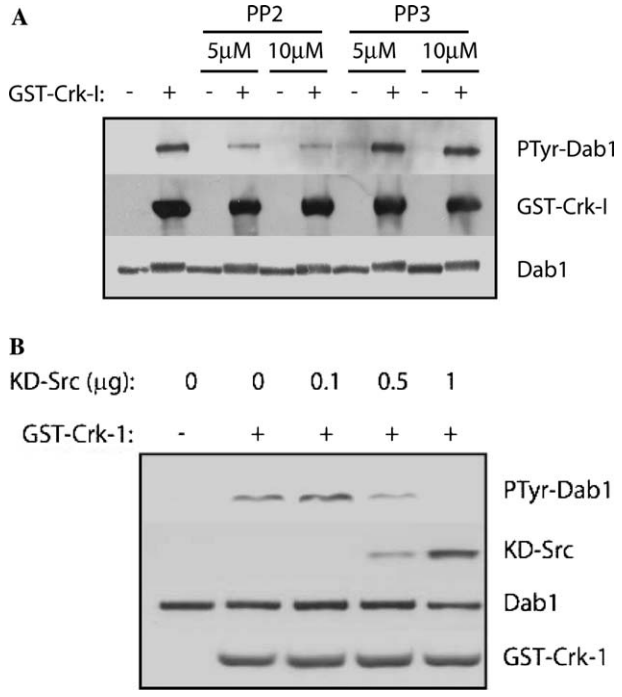

Fig. 5. Crk-induced Dab1 tyrosine phosphorylation is Src-dependent. (A) Cells were transfected with Dab1 only (−) or together with GST-Crk-I (+) and then treated with 5 and 10 μM PP2 or PP3. Lysates from the treated cells were immunoblotted with anti-phosphotyrosine, anti-Crk-I or anti-Dab1 antibodies. (B) Cells were transfected with Dab1, GST-Crk-I, and increasing amounts of kinase-inactive (KD) Src.

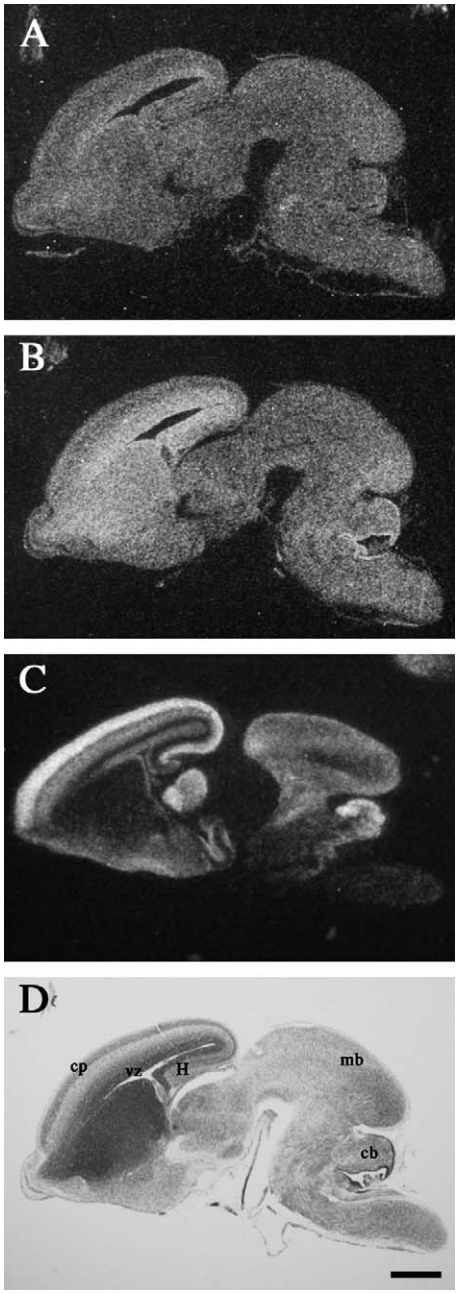

Fig. 6. it Expression patterns of crk, *crkl*, and *dab1*. *Crk*, *crkl,* and *dab1* are expressed in overlapping cell populations in the developing brain. The temporal and spatial expression of *crk*, *crkl*, and *dab1* mRNA was analyzed by in situ hybridization in embryonic day 15.5 mouse brain. (A) *crk*, (B) *crkl*, (C) *dab1*, (D) cresyl violet reference section. The scale bar in (D) represents approximately 800 μm. *Abbreviations:* cp, cortical plate; vz, ventricular zone; H, hippocampus; mb, midbrain; cb, cerebellum.

was not affected by PP3. Since PP2 is known to inhibit several other tyrosine kinases, we further probed the specific role of Src in Crk-I-induced Dab1 tyrosine phosphorylation by co-expressing a dominant negative form of Src (KD-Src). As shown in Fig. 5B, co-expression of increasing amounts of kinase dead Src resulted in a dose-dependent decrease in tyrosine phosphorylation of Dab1. While we cannot rule out the possibility that the dominant negative form of Src also inhibited Fyn or other Src family kinases, Crk-I-induced tyrosine phosphorylation of Dab1 is most likely mediated by Src in HEK293T cells.

Crk and CrkL expression analysis

Mice defective in Dab1 display distinct anatomical defects most noticeable in the cerebral cortex and the cerebellum, and Dab1 is expressed in these structures throughout development [16]. Since we were unable to demonstrate a convincing co-immunoprecipitation of Dab1 with Crk family adapters from extracts prepared from these structures (data not shown), we sought to analyze the expression patterns of *crk*, *crkl*, and *dab1* by in situ hybridization in embryonic (Fig. 6) and postnatal mouse brain (not shown). Throughout development, *crk* and *crkl* display very similar expression patterns. *Crk* is uniformly expressed at a low level throughout the embryonic brain (Fig. 6A). *crkl* shows a similar pattern as *crk*, however, the signal intensity for *crkl* mRNA appears higher in proliferative compartments like the ventricular zone of the cerebral cortex and in the neuroepithelium in the developing cerebellum (Fig. 6B). *crk* and *crkl* mRNA overlap with the distribution of *dab1* mRNA. *dab1* is expressed in many of the same anatomical locations as *crk* and *crkl*, however its mRNA distribution is more specific (Fig. 6C) [16]. These analyses demonstrate that *crk*, *crkl*, and *dab1* mRNAs are temporally and spatially expressed in comparable cell populations during development of the central nervous system.

Discussion

The Reelin signaling pathway governs cellular events required for proper positioning of neurons during brain development. Reelin-induced tyrosine phosphorylation of Dab1 is a critical early event within this pathway. While our understanding of the molecular events that lie downstream of Dab1 phosphorylation is still limited, the specific tyrosine residues of Dab1 that are phosphorylated in response to Reelin are believed to serve as docking sites for proteins containing SH2 domains. Identification of such proteins is critical for understanding how this important pathway functions. We used an approach involving a combination of peptide affinity capture and mass spectrometry to identify Dab1-binding proteins. Several proteins, including RasGap as well as Crk and Nck family adapter proteins, were found to be specifically associated with phosphopeptide PY220. We characterized the interaction of Dab1 with Crk family adapters in a greater detail, and here we

show that Crk-I, Crk-II, and CrkL bind to Dab1 at PTyr-220, a major Reelin-induced site of phosphorylation. We also show that Crk-I and Crk-II, but not CrkL, stimulate phosphorylation of tyrosine 220 in Src-dependent manner. While we were unable to convincingly demonstrate the existence of Dab1-Crk/CrkL complex in vivo by co-immunoprecipitations from brain extracts (data not shown), in situ hybridization studies showed overlapping expressions of transcripts for these proteins. Therefore, these results serve as the first critical step towards elucidation of molecular components that may function downstream of Dab1 phosphorylation.

Dab1 contains a cluster of five tyrosine residues (Tyr-185, -198, -200, -220, and -232) that are known to be phosphorylated in vitro by Src [12]. Previously, we showed that Tyr-198, Tyr-220, and Tyr-232 are also phosphorylated by Src when co-expressed in heterologous systems, such as in HEK293T cells [12]. Of these three residues, only Tyr-198 and Tyr-220 are detectably phosphorylated in primary neurons in response to Reelin stimulation. In our studies, we did not detect phosphorylation of Tyr-232 using our phosphorylation site-specific antibodies, however, Brian Howell and colleagues (NINDS) have been able to detect in vivo phosphorylation of this site using different antibodies (personal communication). All three tyrosines (Tyr-198, -220, and -232) are present within amino acid sequences related to motifs that bind to SH2 domains [23], but there are also important differences. Based on the phosphopeptide selectivity studies reported by Songyang et al. [23], PTyr-198 would be predicted to bind to the SH2 domains of Src, SHP-2, and PLC-γ, whereas PTyr-220 and PTyr-232 are likely to be selective for SH2 domains of the Nck and Crk adapter family proteins. Therefore, it is not surprising that Crk-I, Crk-II, and CrkL bind to Dab1 at PTyr-220 and PTyr-232 but not PTyr-198. While we did not further characterize the interaction of Dab1 with Nck proteins, Pramatarova et al. recently showed that Nckβ has a preference for PTyr-220 and PTyr-232 sites while exhibiting minimal binding to PTyr-198. Therefore, our approach involving the use of phosphopeptide affinity capture and mass spectrometry to identify phosphorylation site-binding proteins is a valid one, and it is consistent with theoretical predictions for phosphopeptide-SH2 recognition selectivity.

It is intriguing that Crk-I and Crk-II, but not CrkL, induced tyrosine phosphorylation of Dab1 in HEK293T cell in the absence of Reelin or the lipoprotein receptors. It is not clear why CrkL did not induce Dab1 phosphorylation, even though the two proteins share roughly 60% identity [25]. Since this phosphorylation was Src-dependent, it is possible that Crk-I induced Src activation, which then resulted in increased Dab1 phosphorylation. Indeed, the viral oncogene product of Crk (v-Crk), encoded by avian sarcoma virus CT10, is known to increase tyrosine phosphorylation of numerous proteins during cellular transformation of chicken embryo fibroblasts [24]. Src activity is known to mediate the effects of v-Crk, although the mechanism by which v-Crk activates Src is unclear [26,27]. However, we were unable to detect any Crk-dependent activation of endogenous Src, nor were able to clearly demonstrate the existence of a tripartite complex involving Dab1, Crk-I, and Src (data not shown). An alternate explanation is that the increase in phosphorylation was simply a result of protection from phosphatase activity by Crk binding. Crk has been shown to increase phosphorylation of epidermal growth factor receptor (EGFR) in this manner [28].

There are multiple ways in which Crk could mediate the effects of Reelin on cellular dynamics. Recently, Dab1 phosphorylation was shown to be required for Reelin-induced activation of the phosphatidylinositol-3-kinase (PI3K)–Akt pathway [29–31]. While it is not clear if the activation of this pathway involves a direct interaction between Dab1 and the p85 regulatory subunit of PI3K, it is possible that Crk serves as an intermediate between Dab1 and PI3K. Indeed, v-Crk is known to activate PI3K during cellular transformation [32]. Recently, tyrosine phosphorylated Dab1 was shown to be polyubiquitinated and degraded through the proteasome pathway [33]. It will be interesting if Crk proteins play a role in Dab1 degradation. Alternatively, Crk adapter proteins may also mediate downstream signaling effects of Reelin by directly activating small GTPase proteins that regulate cytoskeletal dynamics during cellular adhesion and movement. Indeed, Crk-II has been shown to activate Rac1 during cellular adhesion and migration [34,35]. Interestingly, Crk plays an important role in the neuronal differentiation of PC12 cells [36] as well as in the axonal growth of neurons [37]. Recently, Reelin signaling was reported to play an important role in the dendrite development of hippocampal neurons [38]. It will be interesting to see if Crk has a role within the Reelin pathway in this aspect of neurodevelopment.

Definitive experiments supporting the role of Crk proteins in the Reelin pathway require appropriate mouse models. Mice with targeted disruption of the *crkl* gene die before birth [39], but analysis of the mutant embryos reveals defects in the neural crest development resembling the human neurocristopathic disease, DiGeorge syndrome. While Crk 'knockout' mice have not been reported, one study reported identification of a mouse strain generated by gene trap insertional mutagenesis that expressed a truncated Crk [40]. Since the mice do not exhibit any overt phenotype, it is thought that the truncated Crk, which closely resembles Crk-I, is sufficient for most functions. Therefore, transgenic mice with conditional and tissue-specific expression of the Crk adapter proteins will be required to evaluate their role in neurodevelopment.

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References

[1] D.S. Rice, T. Curran, Role of the Reelin signaling pathway in central nervous system development, Annu. Rev. Neurosci. 24 (2001) 1005–1039.
[2] G. D'Arcangelo, G.G. Miao, S.C. Chen, H.D. Soares, J.I. Morgan, T. Curran, A protein related to extracellular matrix proteins deleted in the mouse mutant reeler, Nature 374 (1995) 719–723.
[3] S.E. Hong, Y.Y. Shugart, D.T. Huang, S.A. Shahwan, P.E. Grant, J.O. Hourihane, N.D. Martin, C.A. Walsh, Autosomal recessive lissencephaly with cerebellar hypoplasia is associated with human RELN mutations, Nat. Genet. 26 (2000) 93–96.
[4] G. D'Arcangelo, R. Homayouni, L. Keshvara, D.S. Rice, M. Sheldon, T. Curran, Reelin is a ligand for lipoprotein receptors, Neuron 24 (1999) 471–479.
[5] T. Hiesberger, M. Trommsdorff, B.W. Howell, A. Goffinet, M.C. Mumby, J.A. Cooper, J. Herz, Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation, Neuron 24 (1999) 481–489.
[6] M. Trommsdorff, J.P. Borg, B. Margolis, J. Herz, Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein, J. Biol. Chem. 273 (1998) 33556–33560.
[7] B.W. Howell, T.M. Herrick, J.A. Cooper, Reelin-induced tryosine phosphorylation of disabled 1 during neuronal positioning, Genes Dev. 13 (1999) 643–648.
[8] B.W. Howell, R. Hawkes, P. Soriano, J.A. Cooper, Neuronal position in the developing brain is regulated by mouse disabled-1, Nature 389 (1997) 733–737.
[9] M. Sheldon, D.S. Rice, G. D'Arcangelo, H. Yoneshima, K. Nakajima, K. Mikoshiba, B.W. Howell, J.A. Cooper, D. Goldowitz, T. Curran, Scrambler and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice, Nature 389 (1997) 730–733.
[10] M. Trommsdorff, M. Gotthardt, T. Hiesberger, J. Shelton, W. Stockinger, J. Nimpf, R.E. Hammer, J.A. Richardson, J. Herz, Reeler/disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2, Cell 97 (1999) 689–701.
[11] B.W. Howell, F.B. Gertler, J.A. Cooper, Mouse disabled (mDab1): a Src binding protein implicated in neuronal development, EMBO J. 16 (1997) 121–132.
[12] L. Keshvara, D. Benhayon, S. Magdaleno, T. Curran, Identification of Reelin-induced sites of tyrosyl phosphorylation on disabled 1, J. Biol. Chem. 276 (2001) 16008–16014.
[13] L. Arnaud, B.A. Ballif, E. Forster, J.A. Cooper, Fyn tyrosine kinase is a critical regulator of disabled-1 during brain development, Curr. Biol. 13 (2003) 9–17.
[14] H.H. Bock, J. Herz, Reelin activates SRC family tyrosine kinases in neurons, Curr. Biol. 13 (2003) 18–26.
[15] B.W. Howell, T.M. Herrick, J.D. Hildebrand, Y. Zhang, J.A. Cooper, Dab1 tyrosine phosphorylation sites relay positional signals during mouse brain development, Curr. Biol. 10 (2000) 877–885.
[16] D.S. Rice, M. Sheldon, G. D'Arcangelo, K. Nakajima, D. Goldowitz, T. Curran, Disabled-1 acts downstream of Reelin in a signaling pathway that controls laminar organization in the mammalian brain, Development 125 (1998) 3719–3729.
[17] L. Keshvara, S. Magdaleno, D. Benhayon, T. Curran, Cyclin-dependent kinase 5 phosphorylates disabled 1 independently of Reelin signaling, J. Neurosci. 22 (2002) 4869–4877.
[18] L. Buday, L. Wunderlich, P. Tamas, The Nck family of adapter proteins: regulators of actin cytoskeleton, Cell Signal. 14 (2002) 723–731.
[19] M. Henkemeyer, D.J. Rossi, D.P. Holmyard, M.C. Puri, G. Mbamalu, K. Harpal, T.S. Shih, T. Jacks, T. Pawson, Vascular system defects and neuronal apoptosis in mice lacking ras GTPase-activating protein, Nature 377 (1995) 695–701.
[20] F. Bladt, E. Aippersbach, S. Gelkop, G.A. Strasser, P. Nash, A. Tafuri, F.B. Gertler, T. Pawson, The murine Nck SH2/SH3 adaptors are important for the development of mesoderm-derived embryonic structures and for regulating the cellular actin network, Mol. Cell. Biol. 23 (2003) 4586–4597.
[21] A. Pramatarova, P.G. Ochalski, K. Chen, A. Gropman, S. Myers, K.T. Min, B.W. Howell, Nck beta interacts with tyrosine-phosphorylated disabled 1 and redistributes in Reelin-stimulated neurons, Mol. Cell. Biol. 23 (2003) 7210–7221.
[22] S.M. Feller, Crk family adaptors-signalling complex formation and biological roles, Oncogene 20 (2001) 6348–6371.
[23] Z. Songyang, S.E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W.G. Haser, F. King, T. Roberts, S. Ratnofsky, R.J. Lechleider, et al., SH2 domains recognize specific phosphopeptide sequences, Cell 72 (1993) 767–778.
[24] B.J. Mayer, M. Hamaguchi, H. Hanafusa, A novel viral oncogene with structural similarity to phospholipase C, Nature 332 (1988) 272–275.
[25] J. ten Hoeve, C. Morris, N. Heisterkamp, J. Groffen, Isolation and chromosomal localization of CRKL, a human crk-like gene, Oncogene 8 (1993) 2469–2474.
[26] H. Sabe, S.E. Shoelson, H. Hanafusa, Possible v-Crk-induced transformation through activation of Src kinases, J. Biol. Chem. 270 (1995) 31219–31224.
[27] H. Sabe, M. Okada, H. Nakagawa, H. Hanafusa, Activation of c-Src in cells bearing v-Crk and its suppression by Csk, Mol. Cell. Biol. 12 (1992) 4706–4713.
[28] R.B. Birge, J.E. Fajardo, B.J. Mayer, H. Hanafusa, Tyrosine-phosphorylated epidermal growth factor receptor and cellular p130 provide high affinity binding substrates to analyze Crk-phosphotyrosine-dependent interactions in vitro, J. Biol. Chem. 267 (1992) 10588–10595.
[29] H.H. Bock, Y. Jossin, P. Liu, E. Forster, P. May, A.M. Goffinet, J. Herz, Phosphatidylinositol 3-kinase interacts with the adaptor protein Dab1 in response to Reelin signaling and is required for normal cortical lamination, J. Biol. Chem. 278 (2003) 38772–38779.
[30] B.A. Ballif, L. Arnaud, J.A. Cooper, Tyrosine phosphorylation of Disabled-1 is essential for Reelin-stimulated activation of Akt and Src family kinases, Brain Res. Mol. Brain Res. 117 (2003) 152–159.
[31] U. Beffert, G. Morfini, H.H. Bock, H. Reyna, S.T. Brady, J. Herz, Reelin-mediated signaling locally regulates protein kinase B/Akt and glycogen synthase kinase 3beta, J. Biol. Chem. 277 (2002) 49958–49964.
[32] T. Akagi, T. Shishido, K. Murata, H. Hanafusa, v-Crk activates the phosphoinositide 3-kinase/AKT pathway in transformation, Proc. Natl. Acad. Sci. USA 97 (2000) 7290–7295.

[33] L. Arnaud, B.A. Ballif, J.A. Cooper, Regulation of protein tyrosine kinase signaling by substrate degradation during brain development, Mol. Cell. Biol. 23 (2003) 9293–9302.
[34] P.W. Reddien, H.R. Horvitz, CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in *Caenorhabditis elegans*, Nat. Cell Biol. 2 (2000) 131–136.
[35] Y.A. Abassi, K. Vuori, Tyrosine 221 in Crk regulates adhesion-dependent membrane localization of Crk and Rac and activation of Rac signaling, EMBO J. 21 (2002) 4571–4582.
[36] S. Tanaka, S. Hattori, T. Kurata, K. Nagashima, Y. Fukui, S. Nakamura, M. Matsuda, Both the SH2 and SH3 domains of human CRK protein are required for neuronal differentiation of PC12 cells, Mol. Cell. Biol. 13 (1993) 4409–4415.
[37] D.E. Weinstein, K. Dobrenis, R.B. Birge, Targeted expression of an oncogenic adaptor protein v-Crk potentiates axonal growth in dorsal root ganglia and motor neurons in vivo, Brain Res. Dev. Brain Res. 116 (1999) 29–39.
[38] S. Niu, A. Renfro, C.C. Quattrocchi, M. Sheldon, G. D'Arcan-Arcangelo, Reelin promotes hippocampal dendrite development through the VLDLR/ApoER2-Dab1 pathway, Neuron 41 (2004) 71–84.
[39] D.L. Guris, J. Fantes, D. Tara, B.J. Druker, A. Imamoto, Mice lacking the homologue of the human 22q11.2 gene CRKL phenocopy neurocristopathies of DiGeorge syndrome, Nat. Genet. 27 (2001) 293–298.
[40] T. Imaizumi, K. Araki, K. Miura, M. Araki, M. Suzuki, H. Terasaki, K. Yamamura, Mutant mice lacking Crk-II caused by the gene trap insertional mutagenesis: Crk-II is not essential for embryonic development, Biochem. Biophys. Res. Commun. 266 (1999) 569–574.