Identification of Reelin-induced Sites of Tyrosyl Phosphorylation on Disabled 1*

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The study of mice with spontaneous and targeted mutations has uncovered a signaling pathway that controls neuronal positioning during mammalian brain development. Mice with disruptions in reelin, dab1, or both vildr and apoER2 are ataxic, and they exhibit severe lamination defects within several brain structures. Reelin is a secreted extracellular protein that binds to the very low density lipoprotein receptor and the apolipoprotein E receptor 2 on the surface of neurons. Disabled-1 (Dab1), an intracellular adapter protein containing a PTB (phosphotyrosine binding) domain, is tyrosyl-phosphorylated during embryogenesis, but it accumulates in a hypophosphorylated form in mice lacking Reelin or both very low density lipoprotein receptor and apolipoprotein E receptor 2. Dab1 is rapidly phosphorylated when neurons isolated from embryonic brains are stimulated with Reelin, and several tyrosines have been implicated in this response. Mice with phenylalanine substitutions of all five tyrosines (Tyr185, Tyr198, Tyr200, Tyr220, and Tyr232) exhibit a reeler phenotype, implying that tyrosine phosphorylation is critical for Dab1 function. Here we report that, although Src can phosphorylate all five tyrosines in vitro, Tyr198 and Tyr220 represent the major sites of Reelin-induced Dab1 phosphorylation in embryonic neurons.

The formation of the mammalian central nervous system involves a complex pattern of neuronal migration that results in the organization of several neuronal populations into precise layers (1, 2). Over the past five years, a great deal has been learned about the molecular mechanisms that control cell migration in the developing brain from the study of mutant mice. In particular, the identification of reelin, the gene mutated in the classic ataxic mutant mouse reeler, has uncovered a signaling pathway that is critical for cell positioning (3). Detailed histological studies of reeler mice revealed severe lamination defects in the cerebral cortex, cerebellum, hippocampus, and other laminated structures in the central nervous system (4). Remarkably, mutations in Disabled 1 (Dab1), an intracellular adapter protein (5–8), or both the very low density lipoprotein receptor and apolipoprotein E receptor 2 (9) result in identical defects in mice. The reelin gene encodes a large protein secreted by several populations of neurons in the developing brain, including Cajal-Retzius cells in the neocortex and granule cell precursors in the cerebellum (3, 10). Biochemical studies have demonstrated that Reelin serves as a ligand for lipoprotein receptors (11, 12). Taken together, these findings suggest a model in which Reelin binds to lipoprotein receptors present on target neurons that express Dab1, initiating an intracellular signaling cascade. Recently, reelin has been shown to be mutated in a rare form of autosomal lissencephaly in humans (13), and alterations in the Reelin pathway have also been proposed to be associated with other neurological disorders (14, 15).

Although the exact mechanism involved in Reelin signaling is unclear, it has been proposed that Dab1 acts as an intracellular adapter molecule downstream of Reelin. Dab1 was first identified as a Src-binding protein in a yeast two-hybrid screen (16). The amino terminus of Dab1 contains a PTB (phosphotyrosine binding) domain that is structurally similar to the PTB domains of Shc and Numb (17). Although PTB domains were originally identified as protein interaction domains that recognize tyrosyl-phosphorylated Asn-Pro-X-Tyr (NPXY) motifs present on target proteins, they have recently emerged as a more diverse family of protein modules that vary in their recognition sequences (17). The Dab1 PTB domain interacts with NPXY sequences within the cytoplasmic regions of several membrane-bound proteins, including lipoprotein receptors (18) and amyloid precursor protein family proteins (19, 20). The Dab1 PTB domain has a preference for the unphosphorylated NPXY motif, and phosphorylation of the tyrosine residue is inhibitory to the interaction of Dab1 PTB domain with amyloid precursor protein (20). The NPXY motif serves as an internalization signal for the low density lipoprotein receptor (21), and the interaction with Dab1 has been suggested to regulate the rate of receptor internalization (22).

Dab1 accumulates in mice lacking Reelin or both very low density lipoprotein receptor and apolipoprotein E receptor 2 (6), suggesting that Dab1 is degraded as a consequence of Reelin signaling. In wild-type mice, Dab1 is phosphorylated on tyrosines during embryogenesis (16). In contrast, tyrosyl phosphorylation of Dab1 is greatly diminished in reeler mice, despite the elevated levels of the protein. However, stimulation of forebrain neurons from reeler embryos with exogenous Reelin can result in a rapid and dramatic induction of Dab1 tyrosyl phosphorylation (23). This has led to the hypothesis that tyrosyl phosphorylation of Dab1 is a critical step in the Reelin-signaling pathway. Indeed, mice expressing a mutant form of Dab1, in which a cluster of five tyrosine residues located immediately downstream of the PTB domain were replaced with phenylalanine, exhibit a reeler phenotype (24). Although this study supported a role for Dab1 phosphorylation in neuronal positioning, it did not identify the specific sites involved. Here,
we identify the sites of Reelin-induced tyrosyl phosphorylation on Dab1 in neurons. This is a critical first step in understanding the mechanisms that couple the molecular events triggered by Reelin to the cellular processes that mediate neuronal positioning in the developing brain.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids**—The GST-Dab1 fusion construct and Dab1-hemagglutinin expression plasmid have been described previously (19). GST fusions of the Dab1 PTB domain (GST-Dab11–179), middle region (GST-Dab1180–399), and the carboxy-terminal region (GST-Dab1400–555) were expressed in BL21 bacterial strains as described previously (19). Isopropyl-1-thio-
β-D-galactopyranoside-induced bacterial pellets were lysed by sonication in phosphate-buffered saline/Tween containing 5 mM EDTA, 2 mM phenylmethylsulfonlfuoride, 40 µg/ml aprotinin, and 40 µg/ml leupeptin. The lysates were incubated with glutathione-Sepharose (Amersham Pharmacia Biotech) at 4 °C. Both proteins were then used as substrates in Src kinase reactions in vitro.

**Kinase Reactions**—GST fusion proteins (~5 µg each) immobilized on glutathione-Sepharose were phosphorylated by ~15 units of purified active c-Src (Upstate Biotechnology) in 50 µl of kinase reaction buffer (25 mM Tris-HCl, pH 7.3, 25 mM MgCl2, 10 mM MnCl2, 0.5 mM EGTA, 0.05 mM sodium orthovanadate, 0.5 mM dithiothreitol, 100 µM ATP, and 25 µCi of [γ-32P]ATP). The kinase reactions were carried out at 30 °C for 30 min. The reactions were stopped by the addition of SDS sample loading buffer, and protein samples were boiled for 5 min. Phosphoproteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and visualized by autoradiography.

**Phosphopeptide Mapping**—Phosphopeptides were excised from nitrocellulose membranes and digested with trypsin essentially as described (25). Briefly, membrane pieces were first incubated in 0.5% polyvinylpyrrolidone (PVP-360, Sigma), 100 mM acetic acid for 30 min at 37 °C. After extensive washing with H2O, membranes were incubated for 2 h at 37 °C with 10 µg of 1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) in 50 mM NH4HCO3. This was followed by an overnight digestion with an additional 10 µg of trypsin. Samples were lyophilized and dissolved in alkaline PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 6 x urea, and bromophenol blue). The tryptic phosphopeptides were resolved by electrophoresis on an alkaline 40% acrylamide gel, as described (26). The samples were electrophoresed overnight at 7 mA until the tracking dye had migrated to 0.5 cm. The gel was dried, and phosphopeptides were detected by autoradiography.

**Phosphopeptide Antibodies and Immunoblotting**—Synthesis of peptides and generation of phosphopeptide antibodies were carried out by Alpha Diagnostic International (San Antonio, TX). Rabbits were immunized with KLH-coupled phosphopeptides PY185, CEQAVY(PO4)QT; PY198/200, CEDPVY(PO4)QY; PY220, CETQPVSKQ; and PY232, CKKEGVYPO4DVPKSP. Phosphopeptide PY185 and an unphosphorylated form of this peptide were conjugated to bovine serum albumin (BSA) by Imject maleimide-activated BSA (Pierce). Unphosphorylated GST-Dab1, Src-phosphorylated GST-Dab1, and GST-Dab1 mutant proteins were used in immunoblotting assays to characterize the antisera.

**RESULTS**

**Dab1 Is Phosphorylated by Src in the Middle Region**—Previous studies demonstrated that Dab1 binds to members of the Src family of kinases and that Dab1 can be phosphorylated by Src in transfected cells (16, 24). To determine if Src phosphorylates Dab1 directly, we used purified active Src (Src527F) to phosphorylate GST fusion proteins containing Dab1. Indeed, as shown in Fig. 1A, Src efficiently phosphorylated GST-Dab1, whereas GST alone was not phosphorylated. As a first step toward identifying...
A mixture of peptide mapping and mutagenesis approaches was used to identify the specific sites of phosphorylation within Dab1. GST fusion proteins containing single phenylalanine substitutions at tyrosines 185 (185F), 198 (198F), 200 (200F), 220 (220F), and 232 (232F) and a GST-Dab1 fusion protein containing phenylalanine substitutions in place of tyrosines 198 (198F), 200 (200F), 220 (220F), 232 (232F), or both 198 and 200 (198F/200F) were phosphorylated using Src. The phosphorylated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and located by autoradiography. The proteins were trypsinized off the membrane, and the tryptic fragments were resolved by 2D-gel electrophoresis. The phosphopeptides were visualized by autoradiography. A, a schematic showing the identity of the tryptic phosphopeptides, including the sites of phosphorylation within each peptide.

The in vitro sites of phosphorylation, we used GST fusion proteins containing three distinct Dab1 regions as substrates. As illustrated in Fig. 1B, these domains comprised the PTB domain (GST-Dab11–179), the middle region (GST-Dab1180–399), and the carboxyl terminus (GST-Dab1400–555) of Dab1. Interestingly, only the middle region of Dab1 was phosphorylated by Src (Fig. 1A). This region contains six tyrosines, five of which were previously suggested as sites of Dab1 phosphorylation in transient transfection assays.

Mapping of the in vitro Phosphorylation Sites—A combination of peptide mapping and mutagenesis approaches was used to identify the specific sites of phosphorylation by Src in vitro. Phosphopeptides generated by tryptic digestion of in vitro phosphorylated Dab1 were readily separated by one-dimensional 40% polyacrylamide gel electrophoresis. As shown in Fig. 2A, complete digestion of phosphorylated wild-type GST-Dab1 consistently yielded four phosphopeptides. These phosphopeptides were designated numbers 1–4 starting from the most slowly migrating peptide. Occasionally, an additional phosphopeptide (indicated by an asterisk, Fig. 2A) was also observed to migrate at a slightly faster migration rate than phosphopeptide 3. It is possible that this band represented a variant form of phosphopeptide 3 with a migration rate that would depend on which of the other tyrosine residues was phosphorylated within the single tryptic fragment. The exact identity of this phosphopeptide was difficult to establish, because this band was absent in digestes of all mutant forms of GST-Dab1 fusion proteins. Consistent with the possibility that phosphopeptide 3 resulted from phosphorylation of Tyr198 and phosphorylation of either Tyr185 or Tyr200, both phosphopeptides 3 and 4 were absent from tryptic digestes of the GST-Dab1 fusion protein containing phenylalanine substitutions at both residues 198 and 200. However, since phosphopeptide 3 persisted in the absence of Tyr200, it is likely that Tyr185 was also phosphorylated by Src in vitro. Therefore, the most straightforward interpretation of our results is that phosphopeptide 3 resulted from phosphorylation of Tyr198 and phosphorylation of either Tyr185 or Tyr200. Although it was difficult to establish the relative contributions of Tyr185(P) and Tyr200(P) toward the composition of phosphopeptide 3, our results suggest that Tyr198 and Tyr200 were major in vitro phosphorylation sites, and Tyr185, Tyr200, and Tyr232 were minor Src phosphorylation sites.

Characterization of Phosphopeptide-specific Dab1 Antibodies—A combination of metabolic radiolabeling of cells with orthophosphate and tryptic phosphopeptide mapping has been the traditional approach to identify in vivo sites of protein phosphorylation. Due to inefficient radiolabeling of Dab1 in vivo and the presence of multiple tyrosines (Tyr185, Tyr198, and Tyr200) within a single tryptic fragment, it was difficult to employ this technique to identify the in vivo sites of Dab1 phosphorylation. Therefore, we chose to prepare phosphopeptide-specific antibodies to confirm the in vitro phosphorylation sites and to investigate in vivo phosphorylation of Dab1. Peptides containing single phosphoryrosines Tyr185(P) (PY185), Tyr200(P) (PY200), and Tyr232(P) (PY232) were used to immunize rabbits. Given the close proximity of Tyr185 and Tyr200, we used a synthetic peptide containing both tyrosines in the phosphorylated form (PY185/200) for immunization. The efficacy of each antibody was determined by Western blot assays in

with double substitutions at residues 198 and 200 (198F/200F). The mutant GST-Dab1 fusion proteins were phosphorylated by Src in vitro, tryptic phosphopeptide maps were generated, and the results are shown in Fig. 2. Phenylalanine substitution at residues 220 and 232 resulted in the loss of phosphopeptides 2 and 4, respectively. Therefore, Src phosphorylated Tyr220 and Tyr232 of Dab1 in vitro. However, given the low intensity of the band corresponding to peptide 4, Tyr232 was considered to be a minor site of in vitro phosphorylation. The remaining three potential sites of phosphorylation, Tyr185, Tyr198, and Tyr200, are located within a single tryptic peptide fragment, which complicates analysis. Single substitution of either Tyr185 or Tyr200 did not result in the loss of any of the four major phosphopeptides, indicating that these two tyrosines were not major phosphorylation sites. In contrast, substitution of Tyr198 with phenylalanine resulted in a dramatic loss of phosphopeptide 1 and phosphopeptide 3 and the appearance of a novel minor peptide (indicated by an arrow, Fig. 2A). Therefore, phosphopeptide 1 probably represented a tryptic fragment that was almost exclusively phosphorylated on Tyr198, whereas phosphopeptide 3 likely represented a doubly phosphorylated form of the same tryptic fragment containing Tyr198(P) and either Tyr185(P) or Tyr200(P). The novel minor phosphopeptide in tryptic digest of GST-Dab1198F probably appeared as a result of a single phosphate present on the same peptide at either Tyr185 or Tyr200. Interestingly, tryptic digest of wild-type GST-Dab1 contained an additional phosphopeptide (indicated by an asterisk, Fig. 2A) with a slightly faster migration rate than phosphopeptide 3. It is possible that this band represented a variant form of phosphopeptide 3 with a migration rate that would depend on which of the other tyrosine residues was phosphorylated within the single tryptic fragment. The exact identity of this phosphopeptide was difficult to establish, because this band was absent in digestes of all mutant forms of GST-Dab1 fusion proteins. Consistent with the possibility that phosphopeptide 3 resulted from phosphorylation of Tyr198 and phosphorylation of either Tyr185 or Tyr200, both phosphopeptides 3 and 4 were absent from tryptic digestes of the GST-Dab1 fusion protein containing phenylalanine substitutions at both residues 198 and 200. However, since phosphopeptide 3 persisted in the absence of Tyr200, it is likely that Tyr185 was also phosphorylated by Src in vitro. Therefore, the most straightforward interpretation of our results is that phosphopeptide 3 resulted from phosphorylation of Tyr198 and phosphorylation of either Tyr185 or Tyr200. Although it was difficult to establish the relative contributions of Tyr185(P) and Tyr200(P) toward the composition of phosphopeptide 3, our results suggest that Tyr198 and Tyr200 were major in vitro phosphorylation sites, and Tyr185, Tyr200, and Tyr232 were minor Src phosphorylation sites.

Characterization of Phosphopeptide-specific Dab1 Antibodies—A combination of metabolic radiolabeling of cells with orthophosphate and tryptic phosphopeptide mapping has been the traditional approach to identify in vivo sites of protein phosphorylation. Due to inefficient radiolabeling of Dab1 in neurons and the presence of multiple tyrosines (Tyr185, Tyr198, and Tyr200) within a single tryptic fragment, it was difficult to employ this technique to identify the in vivo sites of Dab1 phosphorylation. Therefore, we chose to prepare phosphopeptide-specific antibodies to confirm the in vitro phosphorylation sites and to investigate in vivo phosphorylation of Dab1. Peptides containing single phosphoryrosines Tyr185(P) (PY185), Tyr200(P) (PY200), and Tyr232(P) (PY232) were used to immunize rabbits. Given the close proximity of Tyr185 and Tyr200, we used a synthetic peptide containing both tyrosines in the phosphorylated form (PY185/200) for immunization. The efficacy of each antibody was determined by Western blot assays in
which the antisera were tested against unphosphorylated GST-Dab1 and GST-Dab1 phosphorylated with Src in vitro. As shown in Fig. 3A, the antisera raised against PY198/200, PY220, and PY232 recognized only phosphorylated GST-Dab1, with no significant cross-reactivity with unphosphorylated GST-Dab1. Western blotting using anti-Dab1 antibodies confirmed that equivalent levels of unphosphorylated and phosphorylated Dab1 were present. Antisera from three different rabbits immunized with the PY185 peptide failed to show any specific reactivity toward phosphorylated GST-Dab1. This lack of reactivity was not due to a low titer, because the antisera strongly recognized bovine serum albumin-conjugated PY185 phosphopeptide in a Western blot assay without any cross-reactivity toward an unphosphorylated form of the peptide. Therefore, since the antisera recognized a synthetic phosphopeptide but did not recognize Src-phosphorylated GST-
Dab1, it can reasonably be concluded that Tyr\textsuperscript{185} was not phosphorylated by Src \textit{in vitro}. This finding was consistent with our phosphopeptide mapping results, which indicated that Tyr\textsuperscript{185} was a minor site of \textit{in vitro} phosphorylation.

Although the data in Fig. 3A show that the phosphopeptide-specific antibodies specifically recognized phosphorylated Dab1, the utility of these antibodies requires that they also exhibit sequence specificity. To determine sequence specificity, the antibodies were tested in Western blots against unphosphorylated and Src-phosphorylated wild-type GST-Dab1 or GST-Dab1 fusion proteins containing single phenylalanine substitutions at Tyr\textsuperscript{198}, Tyr\textsuperscript{200}, Tyr\textsuperscript{220}, and Tyr\textsuperscript{232} or double mutations of Tyr\textsuperscript{198} and Tyr\textsuperscript{200}. As shown in Fig. 3B, the antibodies raised against PY198/200, PY220, and PY232 exhibited strong sequence specificity. The anti-PY220 and anti-PY232 antibodies did not recognize phosphorylated GST-Dab1 containing single phenylalanine substitutions in place of the corresponding tyrosines. The specificity of anti-PY198/200 was determined by testing it on GST-Dab1 fusion proteins that contained either single phenylalanine substitutions in place of Tyr\textsuperscript{198} and Tyr\textsuperscript{200} or double substitution of both Tyr\textsuperscript{198} and Tyr\textsuperscript{200}. The antibodies did not recognize Src-phosphorylated GST-Dab1 when Tyr\textsuperscript{198} alone or both Tyr\textsuperscript{198} and Tyr\textsuperscript{200} were substituted with phenylalanine. In contrast, the antisera contained partial reactivity toward Dab1 containing a single mutation of Tyr\textsuperscript{200}, indicating that Tyr\textsuperscript{198}(P) is the major epitope for this antibody but both Tyr\textsuperscript{198}(P) and Tyr\textsuperscript{200}(P) are required for optimal recognition of phosphorylated Dab1.

\textbf{Phosphorylation of Dab1 in Transfected 293T Cells—}Dab1 is efficiently phosphorylated by Src in transfected cells. Mapping studies using mass spectrometry to identify tryptic phosphopeptides showed that Tyr\textsuperscript{198} and Tyr\textsuperscript{232} were phosphorylated by Src (24). However, tyrosyl phosphorylation of Dab1 remained undiminished when these two residues were mutated to phenylalanine, suggesting that Dab1 was phosphorylated on other tyrosine residues as well. Phenylalanine substitution of all five tyrosines (Tyr\textsuperscript{185}, Tyr\textsuperscript{198}, Tyr\textsuperscript{200}, Tyr\textsuperscript{220}, and Tyr\textsuperscript{232}) present immediately downstream of the PTB domain abrogates Src phosphorylation in transfected cells (24). To identify specific sites of tyrosyl phosphorylation, we transfected 293T cells with Src and with either wild-type Dab1 or Dab1 carrying single phenylalanine substitutions at Tyr\textsuperscript{198}, Tyr\textsuperscript{200}, Tyr\textsuperscript{220}, Tyr\textsuperscript{232} or a double substitution of Tyr\textsuperscript{198} and Tyr\textsuperscript{200}. Western blots using the phosphopeptide-specific antibodies were then carried out to determine the phosphorylation status of each of the mapped tyrosine residues. As shown in Fig. 4A, Tyr\textsuperscript{220} and Tyr\textsuperscript{232} were clearly phosphorylated by Src. The antibody raised against the doubly phosphorylated PY198/200 peptide did not recognize Dab1 if Tyr\textsuperscript{198} alone or both Tyr\textsuperscript{198} and Tyr\textsuperscript{200} were mutated to phenylalanine. In contrast, mutation of Tyr\textsuperscript{200} did not have any effect on the ability of this antibody to recognize phosphorylated Dab1, implying that Tyr\textsuperscript{200} was not phosphorylated. To determine if Tyr\textsuperscript{185} was phosphorylated by Src, Dab1 was expressed either with Src or without Src in 293T cells, and anti-Dab1 immunoprecipitates were immunoblotted with anti-PY185. As shown in Fig. 4B, Tyr\textsuperscript{185} was not phosphorylated, even though Dab1 was tyrosyl-phosphorylated in the presence of Src. Therefore, these results show that Dab1 is phosphorylated by Src on Tyr\textsuperscript{198}, Tyr\textsuperscript{220}, and Tyr\textsuperscript{232} in 293T cells.

\textbf{Identification of Sites of Reelin-induced Phosphorylation—}Dab1 is tyrosyl-phosphorylated in a Reelin-dependent manner, and Reelin-induced phosphorylation of Dab1 in embryonic neurons is an important biochemical assay for Reelin function. As shown in Fig. 5A, tyrosyl phosphorylation of Dab1 increased dramatically when neurons from \textit{reeler} embryos were treated with Reelin. We used the phosphopeptide antibodies to identify the sites of Reelin-induced tyrosyl phosphorylation on Dab1 to detect Dab1 in cultured neurons. Neurons isolated from embryonic brains of \textit{reeler} mice were either left untreated or treated with Reelin. Anti-PY185, anti-PY198/200, anti-PY220, and anti-PY232 were used in Western blots to detect phosphorylated Dab1 in the anti-Dab1 immunoprecipitates. As shown in Fig. 5, only the anti-PY198/200 and anti-PY220 recognized Dab1 in Reelin-treated neurons, indicating that Tyr\textsuperscript{220} and either Tyr\textsuperscript{198} alone or both Tyr\textsuperscript{198} and Tyr\textsuperscript{200} were phosphorylated in neurons stimulated with Reelin. In three independent experiments, anti-PY185 and anti-PY232 did not react against Dab1 from Reelin-treated neurons, suggesting that these two tyrosines were not phosphorylated to any significant extent in response to Reelin treatment. Tyrosyl phosphorylation of Dab1 was very faint in the absence of Reelin, and only the anti-PY220 antisera reacted against Dab1 from unstimulated neurons. Therefore, although it is possible that Dab1 is basally phosphorylated on other tyrosines, our results suggest that Tyr\textsuperscript{220} is the major site of phosphorylation in absence of Reelin.

\textbf{DISCUSSION}

Dab1 plays a critical role in controlling neuronal positioning in the developing brain. It functions as an intracellular adapter protein in the Reelin-signaling pathway, although the mechanism by which it mediates downstream signaling is unknown. The most important clue to Dab1 function is that it accumulates in a hypophosphorylated form in \textit{reeler} mice, and binding of Reelin to lipoprotein receptors triggers tyrosyl phosphorylation of Dab1 in neurons (11, 12). The phosphorylation sites
were suspected to be contained within a cluster of five tyrosines located immediately downstream of the Dab1 PTB domain. Indeed, Howell et al. (24) show that a mutant mouse expressing Dab1 with phenylalanine substitutions in place of these five tyrosines exhibited the same phenotype as reeler mice. This provided genetic evidence to support the critical role of Dab1 tyrosyl phosphorylation in the Reelin-signaling pathway. The biochemical consequences of Dab1 tyrosyl phosphorylation are not known, but it is possible that the phosphorylated tyrosines serve to recruit signaling molecules that bind to phosphorylated Dab1 via their SH2 domains. Alternatively, tyrosyl phosphorylation could affect the stability of Dab1 or its interaction with lipoprotein receptors. Therefore, identification of the Reelin-induced phosphorylation sites on Dab1 is critical to identifying downstream signaling components of this pathway.

Dab1 was originally identified in a yeast two-hybrid screen as a Src-binding protein, and it was subsequently shown to be phosphorylated in an Src-dependent manner in cell lines (16). Given the likely role of Src in Dab1 phosphorylation, we used recombinant active Src to map the in vitro sites of Dab1 tyrosine phosphorylation. Using GST fusions of three distinct Dab1 domains as substrates for Src in vitro, we determined that Src phosphorylates several tyrosines that are clustered in the middle region of Dab1 immediately downstream of the PTB domain. Using a combination of site-directed mutagenesis and tryptic phosphopeptide mapping, we showed that the major sites of Src-catalyzed in vitro phosphorylation are Tyr^{198} and Tyr^{220}, and the minor sites of phosphorylation are Tyr^{185}, Tyr^{200}, and Tyr^{232}. Using antibodies raised against phosphopeptides corresponding to these sites of phosphorylation, we confirmed that Tyr^{198}, Tyr^{200}, Tyr^{220}, and Tyr^{232} are in vitro Src phosphorylation sites. In transfected 293T cells, Src phosphorylated Dab1 on Tyr^{198}, Tyr^{200}, and Tyr^{220}. Recently, a mass spectrometric approach was used to identify Tyr^{185} and Tyr^{232} as sites of Src-catalyzed phosphorylation on Dab1 in transiently transfected 293T cells (24). However, since Dab1 mutants with single phenylalanine substitutions of these two residues did not significantly reduce Dab1 tyrosyl phosphorylation, it was concluded that additional sites must be phosphorylated that are not detected by mass spectrometry. Our finding that Tyr^{220} is also phosphorylated is consistent with this conclusion. Since the antibodies raised against a synthetic peptide corresponding to the Tyr^{185}(P) phosphorylation site failed to react with phosphorylated Dab1, Tyr^{185} is unlikely to be a major phosphorylation site.

To identify the sites phosphorylated in response to Reelin, we treated reeler embryonic neurons with Reelin and used phosphopeptide antibodies raised against these putative sites in Western blots. The results of these experiments clearly indicate that Tyr^{198} and Tyr^{220} are phosphorylated in response to Reelin treatment. We were unable to determine whether Dab1 is phosphorylated on tyrosine 198 alone or both tyrosines 198 and 200, because the antibody against the doubly phosphorylated PY198/200 phosphopeptide retains reactivity in the absence of Tyr^{200}(P). However, Tyr^{200} was not a major Src phosphorylation site in vitro or in transfected cells. Therefore, it is unlikely that this tyrosine is a major site of Reelin-induced phosphorylation. Antibodies against PY185 and PY232 did not recognize Dab1 in either Reelin-treated or untreated cells, suggesting that these tyrosines are not phosphorylated to any significant extent in response to Reelin stimulation. Despite the elevated levels of Dab1, tyrosyl phosphorylation of Dab1 is significantly diminished in reeler mice. This makes it very difficult to identify tyrosines that are phosphorylated in the absence of Reelin. However, we were able to detect a very low level of phosphorylation of Tyr^{220} in untreated neurons. Therefore, our results suggest that a population of Dab1 may be constitutively phosphorylated on this tyrosine in the absence of Reelin.

The finding that Reelin triggers phosphorylation of Tyr^{198} and Tyr^{220} has important implications. Although the immediate biochemical consequences of Reelin-induced Dab1 tyrosyl phosphorylation are unknown, tyrosyl-phosphorylated Dab1 is likely to be involved in protein-protein interactions. Dab1 has been shown to interact with SH2 domains of various signaling proteins in vitro (16, 24), but the in vivo interaction of Dab1 with any of these proteins has not been demonstrated. Our results suggest that physiologically relevant interactions of Dab1 with SH2-containing proteins would be mediated by Tyr^{198}(P) and Tyr^{220}(P). The amino acid sequence surrounding Tyr^{198} is very similar to the juxtamembrane region of platelet-derived growth factor β-receptor that binds to the SH2 domains of Src family kinases (27). It is conceivable that phosphorylation of this tyrosine would serve to recruit Src family kinases. These kinases may then phosphorylate other proteins that may be associated with Dab1. Alternatively, such kinases may also play a role in amplifying the Reelin signal by increasing Dab1 phosphorylation in a positive feedback loop. The amino acid sequence downstream of Tyr^{220} represents a minimal consensus sequence, YXXP, for interaction with SH2 domains of various signaling proteins, such as Abl, phospholipase Cγ, Crk, and Nck (28). Thus, Reelin-induced phosphorylation of Tyr^{198} and Tyr^{220} could result in formation of a Dab1-associated signaling complex that would mediate the downstream effects of Reelin.
The kinases responsible for in vivo tyrosyl phosphorylation of Dab1 are not known. It is interesting that the Src-catalyzed in vitro phosphorylation sites are also phosphorylated in neurons stimulated with Reelin. The amino acid sequences upstream of the two major sites of phosphorylation, Tyr\(^{195}\) and Tyr\(^{220}\), share a marked sequence similarity. A hydrophobic residue at the –1 position and acidic residues at positions –3, –4, –6, and –8 relative to the tyrosines are characteristic of phosphorylation sites on substrates of the Src family of kinases (29). Indeed, we have been able to phosphorylate Dab1 in vitro using various tyrosine kinases, including Src and Fyn, and to a lesser extent, Abl. Several Src family tyrosine kinases are widely expressed in the brain during embryogenesis (30). Although Fyn has been suggested to play a role in synaptic plasticity (31), no overt brain phenotype has been associated with src or fyn mutations. Furthermore, tyrosyl phosphorylation of Dab1 is undiminished in mice lacking several Src family kinases (16). However, functional redundancy within this family of kinases has made it very difficult to elucidate the roles of individual kinases by genetic approaches, and it is possible that Dab1 is phosphorylated by more than one Src family kinase. A hydrophobic residue at the 1 position and acidic residues at positions 3, 4, and 6 may also help identify other signaling pathways that cross-talk with the Reelin-Dab1 pathway.

Identification of the sites of Reelin-induced Dab1 tyrosyl phosphorylation is an important step toward understanding the biochemical function of Dab1 in the Reelin-signaling pathway. Synthetic peptides containing Tyr\(^{195}\) and Tyr\(^{220}\) can be used in biochemical assays to identify and purify tyrosine kinases involved in Dab1 phosphorylation. Furthermore, the availability of antibodies specific for these phosphorylation sites makes it possible to identify other upstream signaling pathways that may cross-talk with the Reelin pathway. Tyrosine phosphorylation of Dab1 is an obligatory step in Reelin-Dab1 signaling, and Reelin-induced phosphorylation served as an important assay for identifying lipoprotein receptors as key components of the Reelin-signaling pathway. The availability of phosphorylation site-specific antibodies may not only allow us to identify additional components of this pathway, but they may also help identify other signaling pathways that cross-talk with the Reelin-Dab1 pathway.

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