Regulation of Protein Tyrosine Kinase Signaling by Substrate Degradation during Brain Development

Lionel Arnaud, Bryan A. Ballif, and Jonathan A. Cooper*

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109

Received 30 June 2003/Returned for modification 4 August 2003/Accepted 5 September 2003

Disabled-1 (Dab1) is a cytoplasmic adaptor protein that regulates neuronal migrations during mammalian brain development. Dab1 function in vivo depends on tyrosine phosphorylation, which is stimulated by extracellular Reelin and requires Src family kinases. Reelin signaling also negatively regulates Dab1 protein levels in vivo, and reduced Dab1 levels may be part of the mechanism that regulates neuronal migration. We have made use of mouse embryo cortical neuron cultures in which Reelin induces Dab1 tyrosine phosphorylation and Src family kinase activation. We have found that Dab1 is normally stable, but in response to Reelin it becomes polyubiquitinated and degraded via the proteasome pathway. We have established that tyrosine phosphorylation of Dab1 is required for its degradation. Dab1 molecules lacking phosphotyrosine are not degraded in neurons in which the Dab1 degradation pathway is active. The requirements for Reelin-induced degradation of Dab1 in vitro correctly predict Dab1 protein levels in vivo in different mutant mice. We also provide evidence that Dab1 serine/threonine phosphorylation may be important for Dab1 tyrosine phosphorylation. Our data provide the first evidence for how Reelin down-regulates Dab1 protein expression in vivo. Dab1 degradation may be important for ensuring a transient Reelin response and may play a role in normal brain development.

Mammalian brain development involves coordinated migrations of different neuronal populations, resulting in highly organized laminar structures. Recent studies have led to the identification of a signaling pathway, known as the Reelin-signaling pathway, that plays a critical role during many of these migrations (43). Reelin is a large glycoprotein that is secreted by specific neurons and binds to the very-low-density lipoprotein receptor (VLDLR) and the ApoE receptor 2 (ApoER2) on other neurons, thereby regulating their migrations. Disruption of this pathway by mutations in the gene encoding Reelin (reln), or by mutation of both vldlr and apoER2, produces an indistinguishable “Reeler” phenotype characterized by ataxia, severe malformation of the cerebellum, inverted layering of the cortex, and neuronal ectopias in several other brain regions (9, 49).

Binding of Reelin to VLDLR and ApoER2 induces tyrosine phosphorylation of Dab1, a cytoplasmic adaptor protein that is expressed in Reelin-responsive neurons (5, 21, 25) and interacts with a conserved NPXY motif in the intracellular tails of both receptors (48, 49). Furthermore, the absence of Dab1 or the presence of a Dab1 point mutant that cannot be tyrosine phosphorylated produces a Reeler phenotype indistinguishable from that of Reelin-deficient mice (24, 45). Therefore, it has been suggested that Reelin-induced tyrosine phosphorylation of Dab1 relays Reelin cues inside migrating neurons by initiating a cascade of signaling events. Indeed, recent studies have shown that Reelin activates Src family kinases (SFKs) and Akt concomitantly with (and dependent on) Dab1 tyrosine phosphorylation (1, 3, 4, 6). In turn, SFKs are needed for efficient phosphorylation of Dab1 (1, 6). Further events remain largely unknown but may involve the glycogen synthase kinase 3β (GSK-3β) (4), a serine/threonine kinase implicated in the phosphorylation of the microtubule-associated protein Tau (27). Whether by regulating phosphorylation of Tau or by other pathways, Reelin-induced Dab1 tyrosine phosphorylation ultimately controls neuronal migrations.

While the Reelin-signaling pathway is becoming understood, little is known about how it is down-regulated. Most, perhaps all, signaling pathways have desensitization mechanisms to allow the system to reset its sensitivity to repeat stimulation (33). For receptor tyrosine kinases, the receptor itself is commonly down-regulated but for the insulin receptor the principal substrate, insulin receptor substrate 1 (IRS-1), is also down-regulated (34, 50). In the case of Reelin signaling, down-regulation may be important because recent data suggest that migrating neurons respond differently to Reelin signals at different times during the history of the cell (see the Discussion). This suggests that the Reelin response mechanism may be localized, spatially and temporally, within migrating neurons.

One mechanism for down-regulation of Reelin signaling may involve Reelin-dependent down-regulation of Dab1, because Dab1 protein levels are much higher in embryonic Reelin-deficient brain samples than in those of controls (25, 44, 45). Dab1 protein also accumulates when other components of the Reelin-signaling pathway (such as VLDLR, ApoER2, Fyn, or Src) are absent (1, 49). This down-regulation appears to be posttranscriptional, since dab1 mRNA expression is normal in Reelin-deficient mice (44). In this report, we show that in primary cultures of cortical neurons in vitro, Reelin stimulates tyrosine phosphorylation of a serine/threonine-phosphorylated subpopulation of Dab1 molecules which are then polyubiquitinated and degraded via the proteasome pathway. Reelin-induced degradation of Dab1 observed in vitro has biochemical

* Corresponding author. Mailing address: Fred Hutchinson Cancer Research Center, Maitland A2-025, 1100 Fairview Ave. N., Seattle, WA 98109-1024. Phone: (206) 667-4454. Fax: (206) 667-6522. E-mail: jcooper@fhcrc.org.

Copyright © 2003, American Society for Microbiology. All Rights Reserved.

0270-7306/03/$08.00
requirements that are consistent with the genetic requirements for Dab1 down-regulation in vivo. Furthermore, we show that only tyrosine-phosphorylated Dab1 molecules are targeted for degradation. Upstream components of the Reelin pathway do not appear to be down-regulated, making Dab1 degradation a primary mechanism for desensitization of the Reelin-signaling pathway.

MATERIALS AND METHODS

Antibodies and inhibitors. Anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology, anti-Fyn antibody (EYNN3) and anti-β-catenin (E-5) were from Santa Cruz Biotechnology, anti-Abi (NE9) was from BD Biosciences, anti-phospho-Src (Tyr418) antibody was from Biosource, anti-phospho-Akt(Ser473) was from Cell Signaling Technology, and anti-ubiquitinated proteins (FK2) were from Affinity Research. Affinity-purified anti-Dab1 polyclonal antibody (B3), a generous gift of B. W. Howell, has been previously described (1). Cycloheximide, MG132, and protein G beads (Protein G Sepharose 4 Fast Flow; Amersham Biosciences) were purchased from Sigma, and LY294002 was purchased from Lilly.

Mice. All mice used in this study were hybrid C57BL/6J*129S animals. The dab1 alleles dab1P16, dab1P77, and dab1P45 have been described elsewhere (19, 26). Mouse care, husbandry, and handling were performed in compliance with federal, state, and institutional regulations and policies.

Recombinant Reelin, neuron cultures, and Reelin stimulation. Stably transfected 293 cells secreting Reelin have been described previously (1). To obtain Reelin-containing and mock supernatants, respectively, stably Reelin-secreting and stably green fluorescent protein-expressing 293 cells were grown to subconfluence and then switched to Neurobasal medium (Gibco, Invitrogen Corp.) supplemented with 50 μ of penicillin (Gibco, Invitrogen Corp.)/ml and 50 μg of streptomycin (Gibco, Invitrogen Corp.)/ml for 2 days before supernatants were harvested. These were centrifuged at 4,000 × g for 15 min at 4°C, and aliquots were stored at −70°C.

Mouse embryonic day 16.5 (E16.5) embryo cortical neurons were isolated and grown in cultures essentially as previously described (19). After 5 days in vitro, neuron cultures were stimulated with Reelin-containing or mock supernatant at 37°C in 5% CO2 and then washed with ice-cold phosphate-buffered saline (PBS) and lysed in neuron RIPA buffer (10 mM phosphate buffer [pH 7.4], 150 mM NaCl, 1 mM Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 2 mM EDTA, 50 mM NaF, 1 mM Na2VO4, a cocktail of protease inhibitors, 10 μg of RNase A/ml, and 5 μg of DNase I/ml) for 20 min on ice. When required, neuron cultures were pretreated for 30 min with kinase inhibitors or vehicle (DMSO).

Western blot analysis and immunoprecipitation. Brains were dissected from E16.5 embryos and frozen at −70°C until lysis in neuron RIPA buffer. For Western blot analysis, equal amounts of proteins (20 μg for neuron culture lysates and 35 μg for brain lysates) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (better resolution of the electrophoretic mobility of these proteins). Primary cultures of cortical neurons isolated from mouse embryos at E16.5 were stimulated with Reelin-containing or mock supernatant, and levels of tyrosine-phosphorylated Dab1 and total Dab1 were analyzed over a period of 6 h. Dab1 tyrosine phosphorylation increased rapidly, was maximal by 5 min, remained high for 1 h, and decreased thereafter (Fig. 1A and B) (note that different SDS-PAGE conditions enabled resolution of Dab1 into two forms, which will be discussed in detail at the end of this section). By 4 h, Dab1 phosphotyrosine levels were lower than before stimulation (Fig. 1A, lane 11, and 1B). Interestingly, Dab1 protein levels declined progressively in Reelin-treated neuron cultures (Fig. 1A and B). Dab1 decrease over the first 4 h fit first-order kinetics (data not shown), with a half-life of 2 h 40 min. In contrast, Dab1 levels were not altered when neuron cultures were treated with mock supernatant (Fig. 1A, lanes 4 and 8, and 1C; see also Fig. 2A and 3A). The Reelin-dependent loss of Dab1 was highly specific for Dab1, since the levels of other proteins we tested remained stable upon Reelin stimulation. These proteins included members of the Reelin-signaling pathway like Fyn and ApoER2 (Fig. 1A and data not shown) and also β-catenin (Fig. 1A), Abl (Fig. 1C; see also Fig. 2A and 3B), Gab2, Dab2, and p130Cas (data not shown).

If Dab1 protein is inherently unstable and is synthesized and degraded rapidly, then Reelin could induce a decrease in Dab1 protein level by inhibiting its synthesis. Alternatively, if Dab1 is normally stable then its decline after Reelin stimulation could only be due to increased degradation. To discriminate these possibilities, we assessed Dab1 stability in neuron cultures exposed to cycloheximide, an inhibitor of translation. As shown in Fig. 1C, Dab1 levels were not significantly altered by cycloheximide treatment, even after 6 h (lane 4). We estimate that Dab1 half-life in the absence of Reelin is longer than 12 h (data not shown). This implies that slow degradation is normally balanced by slow synthesis and that Reelin-induced loss of Dab1 is due to an increased degradation rate of an otherwise stable protein. Consistent with a slow rate of Dab1 protein synthesis, Dab1 protein levels were still recovering at 17 h after a 5-h Reelin treatment (Fig. 1D, lane 6).

Dab1 is polyubiquitinated and degraded via the proteasome pathway in response to Reelin. In animal cells, proteins are degraded principally by the proteasomal and the endosomal and lysosomal pathways. To investigate their respective roles in Reelin-induced degradation of Dab1, we made use of cell-permeable inhibitors: chloroquine, an inhibitor of lysosomal proteases, MG132, a reversible inhibitor of the proteasome, and epoxomicin, a highly specific and irreversible inhibitor of the proteasome. As shown in Fig. 2A, Reelin-induced degra-
Reelin stimulation induces degradation of Dab1 in primary cortical neurons. (A) Mouse cortical neuron cultures were left untreated (lanes 1 and 13) or treated with Reelin-containing (lanes 2 and 3, 5 to 7, and 9 to 12) or mock (lanes 4 and 8) supernatant for the indicated times. Total lysates were subjected to SDS–7.5% PAGE and Western blot analysis using an anti-phosphotyrosine antibody to detect tyrosine-phosphorylated Dab1 [pDab1(Tyr) WB], anti-Dab1 antibodies to detect total Dab1 (Dab1 WB), anti-β catenin antibody (β catenin WB), or anti-Fyn antibodies (Fyn WB). (B) Quantification of the data presented in panel A was used to determine relative levels of tyrosine-phosphorylated Dab1 [pDab1(Tyr)] and total Dab1 after Reelin stimulation for various times. In both cases, levels were normalized to those in untreated neuron cultures (set at 1.0). (C) Neuron cultures were treated with 20 µg of cycloheximide/ml (CHX; lanes 1 to 4) and either Reelin-containing (lane 5) or mock (lane 6) supernatant for the indicated times. Total lysates were subjected to SDS–8% PAGE and Western blot analysis using anti-Dab1 antibodies (Dab1 WB) or an anti-Abl antibody (Abl WB) as a loading control. (D) Neuron cultures were treated for 5 h with mock (lanes 1, 3, and 5) or Reelin-containing (lanes 2, 4, and 6) supernatant, washed, and returned to normal growth medium for the indicated times. Total lysates were subjected to SDS-PAGE and Western blot analysis using anti-Dab1 antibodies to detect total Dab1 (Dab1 WB) or an anti-Abl antibody (Abl WB) as a loading control. (E) Dab1 can be resolved into two forms, Dab1 p80L and p80U. Lysates were prepared from neuron cultures treated for 15 min with Reelin-containing or mock supernatant as indicated. A portion was analyzed directly by Western blotting of serial diluted samples: 50 (lanes 1 and 2), 25 (lane 3), 12.5 (lane 4), and 6.25 (lane 5) µg. A second portion was immunoprecipitated (IP) with an anti-phosphotyrosine antibody (4G10 IP), and the immunoprecipitates were analyzed in parallel with the total lysates by SDS–9% PAGE (see Materials and Methods for details) and Western blotting with an anti-phosphotyrosine antibody to detect tyrosine-phosphorylated Dab1 [pDab1(Tyr)] or anti-Dab1 antibodies (Dab1 WB). The respective positions of Dab1 p80 of slower electrophoretic mobility (upper band [p80U]) and Dab1 p50 of faster mobility (lower band [p50L]) are indicated. Note that only Dab1 p80U is tyrosine phosphorylated and that Dab1 p80U represents approximately two-thirds of total Dab1 after 15 min of Reelin stimulation. The quantity of Reelin-induced tyrosine-phosphorylated Dab1 in the 4G10 IP is equivalent to that present in 25 µg of total lysate, while the quantity of Dab1 p80U in the 4G10 IP is equivalent to two-thirds of that present in 6.25 µg of total lysate; therefore, after 15 min of Reelin stimulation, tyrosine-phosphorylated Dab1 represents ~15% (6.25/25 × 2/3 = 1/6 × 15%) of total Dab1 equivalent to ~25% (6.25/25 = 1/4 [25%]) of Dab1 p80U. (F) Brain lysates prepared from E16.5 embryos were subjected to immunoprecipitation with anti-Dab1 antibodies (Dab1 IP). The immunoprecipitate was treated with λ protein phosphatase (λ-PPase) (lane 1) or left untreated (lane 2) before analysis using SDS–9% PAGE and Western blotting with anti-Dab1 antibodies (Dab1 WB) was performed. (G) Neuron cultures were treated for 1 h with 0.4 µM (lanes 2 and 5), 2 µM (lanes 3 and 6), or 10 µM (lanes 4 and 7) calyculin A (lanes 2 to 4), okadaic acid (lanes 5 to 7), or DMSO (lane 1). Total lysates were subjected to SDS–9% PAGE and Western blot analysis using anti-Dab1 antibodies (Dab1 WB).
determination of Dab1 was inhibited in the presence of the proteasome inhibitors, MG132, and epoxomicin (lanes 4 and 5) but not in the presence of vehicle (DMSO; lane 3) or chloroquine (lane 6). The inhibition by MG132 or epoxomicin of Reelin-induced degradation of Dab1 also resulted in sustained tyrosine-phosphorylation of Dab1 (Fig. 3A, lanes 1 and 4), indicating that these inhibitors did not prevent earlier Reelin-signaling pathway events, such as Reelin-induced tyrosine phosphorylation of Dab1. Taken together, these results indicated that Reelin stimulation targeted Dab1 to degradation via a proteasome pathway and that upstream signaling to Dab1 continues unabated when the proteasome is inhibited.

Most proteasome substrates are marked with polyubiquitin chains (20, 22), although ubiquitin-independent proteasomal degradation has also been described previously (2, 28). To determine whether Dab1 is polyubiquitinated, we first tested for the presence of high-molecular-mass species of Dab1 upon Reelin stimulation. Neuron cultures were treated with Reelin-containing mock supernatant in the presence of MG132 to inhibit proteasomal degradation, lysed in the presence of MG132 and ubiquitin-aldehyde to prevent deubiquitination, and immunoprecipitated with anti-Dab1 antibodies. The immunoprecipitates were then analyzed by Western blotting with anti-Dab1 antibodies, revealing the presence of high-molecular-mass Dab1 species (>180 kDa) in neuron cultures treated with Reelin (Fig. 2B, lane 2). High-molecular-mass Dab1 species were also detected in mock-treated neuron cultures (Fig. 2B, lane 1), albeit at much lower levels, probably reflecting basal processing of Dab1 due to low-level stimulation of the Reelin pathway by endogenously produced Reelin (32). To test for polyubiquitinated Dab1, neuron culture lysates were immunoprecipitated with the monoclonal antibody FK2 that recognizes ubiquitinated proteins (13) and immunoprecipitates were analyzed by Western blotting with anti-Dab1 antibodies (Dab1 WB). The respective positions of unmodified Dab1 (Dab1) and high-molecular-mass Dab1 species (arrow) are indicated. (C) Neuron cultures were treated as described for panel B except that MG132 was added after 0 min (lanes 1 and 2) or 15 min (lanes 3 and 4) of mock (lanes 1 and 3) or Reelin (lanes 2 and 4) treatment. Ubiquitinated proteins were immunoprecipitated from neuron lysates with the FK2 antibody (FK2 IP) and analyzed by SDS–7% PAGE and Western blotting with anti-Dab1 antibodies (Dab1 WB). The position of polyubiquitinated Dab1 (Ubn-Dab1) is indicated.

Reelin-induced degradation of Dab1 correlates with tyrosine phosphorylation of Dab1 and activation of SFKs. We and others have previously shown that SFKs are activated by Reelin and that treatment of neuron cultures with SFK inhibitors prevents Reelin-induced tyrosine phosphorylation of Dab1 (1, 6). To test whether SFK activity was important for Reelin-induced degradation of Dab1, Dab1 levels were measured in neuron cultures treated with the SFK inhibitor PP2. As expected, PP2 treatment completely abolished SFK activation (monitored using anti-phospho-Src[Tyr418] antibodies) (Fig. 3A), as well as Dab1 tyrosine phosphorylation, after 15 min of exposure to Reelin (Fig. 3A, lane 3). PP2 also prevented Dab1 degradation in neuron cultures treated with Reelin for 5 h (Fig. 3A, lane 9). In contrast, treatment with PP3, a structural analog of PP2 that is inactive on SFKs, did not prevent Reelin-induced tyrosine phosphorylation or degradation of Dab1 (Fig. 3A, lanes 4 and 10). These results suggest that Dab1 tyrosine phosphorylation and/or SFK activation is required for activation of the Reelin-induced Dab1 degradation pathway.

Inhibition of SFKs abolishes not only Reelin-induced tyrosine phosphorylation of Dab1 but also downstream activation of Akt dependent on the presence of phosphatidylinositol 3-kinase (PI 3-kinase) (3) (Fig. 3A, lane 3). To determine
whether PI 3-kinase was required for the degradation of Dab1, we made use of the PI 3-kinase inhibitor LY294002. Treatment of neuron cultures with LY294002 did not prevent Reelin-induced degradation of Dab1 (Fig. 3A, lanes 11 and 12). The presence of LY294002 effectively inhibited PI 3-kinase, because Reelin-induced activation of Akt [monitored using anti-phospho-Akt(Ser473) antibodies] (Fig. 3A) was completely abolished. Therefore, PI 3-kinase and downstream Akt activation are not required for Reelin-induced degradation of Dab1.

If tyrosine phosphorylation of Dab1 is required for its degradation, Dab1SF, a mutant form of Dab1 which cannot be tyrosine phosphorylated (26), should be protected from degradation upon Reelin stimulation. To test this, neuron cultures were prepared from littermate embryos derived from dab1SF/+ parents and subsequently genotyped. The cultures corresponding to dab1SF/ (lanes 1 and 2 and lanes 5 and 6) or dab1SF/ + SF (lanes 3 and 4 and lanes 7 and 8) embryos were treated with Reelin-containing or mock supernatant as indicated for 20 min (lanes 1 to 4) or 5 h (lanes 5 to 8). Total lysates were subjected to SDS-PAGE and Western blot analysis using anti-Dab1 antibodies to detect total Dab1 (Dab1 WB), an anti-phosphotyrosine antibody to detect tyrosine-phosphorylated Dab1 [pDab1(Tyr) WB], anti-phospho-Src(Tyr418) antibodies [pSrc(Y418) WB], or anti-phospho-Akt(Ser473) antibodies [pAkt(S473) WB]. (B) Neuron cultures were prepared from littermate embryos derived from dab1SF/ + SF (left graph n = 18) or dab1WTWT (right graph n = 18) parents, and Dab1 protein levels were determined by Western blot analysis using anti-Dab1 antibodies (examples are shown above each graph) and the anti-βIII tubulin antibody TUJ1 for normalization of the loading (data not shown) (standard deviation, 1.8%; n = 36). The relative levels of Dab1 proteins were normalized to the average Dab1 level in wild-type embryos (set to 1.0) (standard deviation, 4.1%; n = 11) and were plotted against the genotypes of embryos as indicated. Error bars represent standard deviations.
induced decline (Fig. 3B, lanes 5 and 6). Furthermore, in contrast to the results seen with wild-type control cultures (Fig. 3C; compare lanes 2 and 6), the presence of the proteasome inhibitor MG132 had no effect on Dab1 levels in dab1^{5F/5F} neuron cultures treated for 5 h with Reelin (Fig. 3C; compare lanes 4 and 8). Together, these results indicate that Dab1^{5F} is completely protected from Reelin-induced degradation via the proteasome.

If Dab1 tyrosine phosphorylation is required for Dab1 down-regulation in vivo, then Dab1 levels should be much higher in dab1^{5F/5F} than in wild-type embryos. However, embryonic dab1^{5F/5F} brains have only modestly increased Dab1 levels (26). To resolve this discrepancy, we reassessed the level of Dab1^{5F} protein in vivo. Brain lysates were prepared from E16.5 embryos derived from intercrosses between dab1^{5F/+} mice. Since the dab1^{5F} allele is a cDNA knockin, samples were also prepared from embryos derived from intercrosses of dab1^{WT/WT} mice in which dab1^{WT} is the wild-type cDNA knockin allele (26). Dab1 levels were 1.5-fold higher in dab1^{5F/5F} embryo brains than in their dab1^{+/+} littermates (Fig. 3D, left panel). Surprisingly, Dab1 levels were decreased two-fold in dab1^{WT/5F} embryo brains compared to those seen with their dab1^{+/+} littermates (Fig. 3D, right panel), probably due to altered expression of knockin alleles. Thus, Dab1 levels were approximately threefold higher in dab1^{5F/5F} embryos than in control dab1^{WT/WT} embryos, a difference similar to that measured in relnrl/rl or fn^{−/−} src^{+/+} embryos compared to wild-type controls (1). Thus, the Reelin-induced degradation of Dab1 that we observed in vitro correlates well with the in vivo regulation of Dab1 protein levels by Reelin signaling, SFK mutation, and the dab1^{5F} allele.

**Reelin-induced tyrosine phosphorylation of Dab1 is necessary for its degradation.** The preceding experiments show that Reelin-stimulated, SFK-dependent, Dab1 tyrosine phosphorylation correlates with Dab1 degradation in vivo and in vitro. However, genetic and biochemical manipulations that alter Dab1 tyrosine phosphorylation also cause parallel changes in SFK activity, since Dab1 tyrosine phosphorylation and SFK activation are interdependent (1, 3, 6). Therefore, our results did not distinguish whether Dab1 tyrosine phosphorylation per se or activation of SFKs or a downstream pathway is required for Dab1 degradation.

To address whether Dab1 tyrosine phosphorylation or a downstream pathway regulates Dab1 degradation, we determined whether Dab1^{5F} is degraded when it is coexpressed with tyrosine-phosphorylated Dab1. Rather than using wild-type Dab1, which cannot be resolved from Dab1^{5F} by SDS-PAGE, we made use of Dab1^{p45}, another mutant form of Dab1 which lacks the carboxy-terminal half and runs at ~45 kDa (19). Dab1^{p45} is tyrosine phosphorylated with the same kinetics as full-length Dab1 upon Reelin stimulation (19) and mediates downstream Reelin signaling, including Akt activation (3).

Neuron cultures were prepared from littermate embryos derived from a cross between a dab1^{5F/+} female and a dab1^{p45/p45} male and treated with Reelin-containing or mock supernatant for various times. Upon Reelin stimulation, Dab1^{p45} was degraded with the same kinetics as full-length Dab1 (Fig. 4A, lanes 8, 10, 12, and 16, and 4C; note that like full-length Dab1, Dab1^{p45} was resolved into two bands, p45U and p45L, which will be discussed in detail below). This result shows that Reelin-induced degradation of Dab1 does not require the carboxy-terminal half of Dab1 and is in agreement with previous data showing that Dab1^{p45} levels are negatively regulated by Reelin in vivo (19). In dab1^{p45/p45} neuron cultures, there was no inhibition of Dab1^{p45} degradation due to the presence in the same neurons of Dab1^{5F} (Fig. 4A, lane 14, and 4C), indicating that the Dab1 degradation pathway was fully active in these neurons. In contrast, Dab1^{5F} was not degraded upon Reelin stimulation of dab1^{p45/p45} neuron cultures (Fig. 4A, lane 14, and 4C). Together, these results demonstrate that Reelin-induced tyrosine phosphorylation of Dab1 is required for targeting Dab1 to degradation and that activation of downstream pathways is not sufficient.

**Additional levels of Dab1 regulation.** We observed that Dab1 could be resolved by SDS-PAGE as a doublet of ~80 kDa (Fig. 1C and 1D). By adjusting the electrophoreses conditions, it was possible to reproducibly separate two forms of Dab1, p80U (upper) and p80L (lower) (Fig. 1E and G, 2A, and 3). In contrast, under the same electrophoreses conditions, tyrosine-phosphorylated Dab1 ran as a single band comigrating with Dab1 p80U, as demonstrated by immunoprecipitation with an anti-phosphotyrosine antibody and Western blot analysis with anti-Dab1 antibodies (Fig. 1E; compare lanes 5 and 7). This suggests that only Dab1 p80U is tyrosine phosphorylated. However, three lines of evidence suggest that tyrosine phosphorylation does not cause the mobility shift: both forms existed before Reelin stimulation (Fig. 1E, lane 1); both forms were observed with Dab1^{5F} that cannot be tyrosine phosphorylated (Fig. 3B and C); and tyrosine phosphorylation of Dab1 induced by coexpression with SFKs in 293 cells did not cause a mobility shift (1). Nevertheless, Dab1 p80U and p80L could be converted into a single lower band by phosphatase treatment in vitro (Fig. 1F, lane 1) or conversely into a single higher band by treatment of neuron cultures with the serine/threonine phosphatase inhibitors calyculin A and okadaic acid (Fig. 1G, lanes 4 and 7). Therefore, serine/threonine phosphorylation (likely serine phosphorylation) (23) appears to account for the mobility shift (1). Nevertheless, Dab1 p80U was tyrosine phosphorylated upon Reelin stimulation.

Dab1^{p45} was also resolved by SDS-PAGE into upper and lower bands (p45U and p45L, respectively) (Fig. 4A). As for Dab1 p80U, only Dab1 p45U was tyrosine phosphorylated upon Reelin stimulation (Fig. 4B, compare lanes 2 and 4) and Reelin-induced tyrosine phosphorylation was not responsible for the existence of Dab1 p45U since it was present before stimulation (Fig. 4A, mock-treated sample lanes). This indicates that the serine/threonine phosphorylation causing the mobility shift occurred in the amino-terminal half of Dab1 that is common between Dab1^{p45} and full-length Dab1.

By comparing the relative quantities of Dab1 p80U and tyrosine-phosphorylated Dab1, we estimate that only ~25% of Dab1 p80U (~15% of total Dab1) becomes tyrosine phosphorylated after 15 min of Reelin stimulation (Fig. 1E; see figure legend for details) when Reelin-induced tyrosine phosphorylation of Dab1 is maximal (Fig. 1B). This suggests that tyrosine phosphorylation of Dab1 might be a limited process. Furthermore, even though neuron cultures prepared from relnrl/rl embryos contain ~4-fold more Dab1 than wild-type embryos (1), Reelin stimulation of relnrl/rl and wild-type neurons raises the
amount of tyrosine-phosphorylated Dab1 to the same level (data not shown). Also, tyrosine phosphorylation of Dab1 was constant over a range of Reelin concentrations, indicating that the dose of Reelin was saturating (reference 5 and data not shown). Altogether, these results indicate that the number of Dab1 molecules that are tyrosine phosphorylated is limited by something other than Dab1 quantity and Reelin concentration. Thus, Reelin-induced tyrosine phosphorylation of Dab1 is a limited process, affecting a restricted subpopulation of Dab1 p80U molecules at any given time.

**DISCUSSION**

We have found that following Reelin stimulation, tyrosine phosphorylation targets Dab1 for degradation via the ubiquitin-proteasome pathway. Dab1 is normally quite stable, with a
half-life around 12 h, but after Reelin stimulation up to 60% of Dab1 molecules are degraded with a half-life of 2 h 40 min (Fig. 1A). This increased degradation is sufficient to explain the pronounced accumulation of Dab1 protein in embryonic brains lacking Reelin or VLDLR and ApoER2 or one or more SFKs (1, 44, 49). Evidence that Dab1 tyrosine phosphorylation is required for degradation comes from studies with tyrosine kinase inhibitors (Fig. 3A) and from comparing the mutant Dab1\(^{5F}\), which cannot be tyrosine phosphorylated in vitro or in vivo, with Dab1\(^{WT}\), a wild-type control expressed from the same the chromosomal context (26). We find that Dab1\(^{5F}\) is expressed at significantly higher levels than Dab1\(^{WT}\) in vivo (Fig. 3D) and correspondingly resists Reelin-induced degradation in vitro (Fig. 3B and C). In addition, Dab1\(^{p45}\), a truncated form of Dab1 which lacks the carboxy-terminal half of the protein, is degraded in response to Reelin in vitro and is down-regulated by Reelin in vivo (Fig. 4) (19), showing that the amino-terminal region, including the PTB domain and tyrosine phosphorylation sites, is sufficient for Reelin-regulated degradation. Thus, the results of all relevant in vivo studies are consistent with the measurements made in vitro.

To test whether Dab1 degradation is signaled by Dab1 tyrosine phosphorylation per se, rather than by activation of downstream pathways, we made use of neurons from embryos transheterozygous for two dab1 alleles. These neurons contain Dab1\(^{5F}\), which cannot be tyrosine phosphorylated (26), and Dab1\(^{p45}\), which can be tyrosine phosphorylated and activates downstream pathways (3, 19). This experiment is formally equivalent to a transfection experiment with a tagged mutant, but in this case we know the alleles are expressed approximately equally in all cells and at a physiological level. We found that Dab1 lacking phosphotyrosine was not degraded even though the Dab1 degradation pathway was activated in these neurons. This supports a model in which tyrosine phosphorylation is the signal for degradation of Dab1.

Our results imply that tyrosine-phosphorylated Dab1 is recognized directly or indirectly by the ubiquitination machinery. This contrasts with the results seen with IRS-1, an important mediator of insulin signaling, which is degraded after insulin stimulation. While insulin-stimulated IRS-1 degradation is triggered by IRS-1 tyrosine phosphorylation, a multiple phenylalanine mutant of IRS-1 that cannot be tyrosine phosphorylated is also degraded (50). It appears that tyrosine phosphorylation of endogenous IRS-1 stimulates PI 3-kinase activity that then promotes degradation of both endogenous and phenylalanine mutant IRS-1; therefore, tyrosine phosphorylation per se is not required (50). IRS-1 degradation may thus be triggered by PI 3-kinase-dependent serine/threonine phosphorylation (by, e.g., Akt).

There are abundant examples in which serine/threonine phosphorylation primes a protein for ubiquitination (16, 29, 40). Targeting by tyrosine phosphorylation has also been reported. Activated forms of the SFKs Src and Blk are ubiquitinated and degraded more rapidly than their normal counterparts, although it isn’t clear whether this lability is due to altered sites of tyrosine phosphorylation or to an altered conformation (15, 17, 42). The Abl tyrosine kinase and its substrates Abi1 and Abi2 are degraded more rapidly when Abl is active (8, 10). In addition, many receptor tyrosine kinases are routed for endocytosis and proteasomal degradation contingent on their tyrosine phosphorylation and recognition by the E3 ligase Cbl (47). Cbl contains a phosphotyrosine recognition domain comprising a EF-hand and SH2-like structure through which it binds phosphotyrosine residues followed by a hydrophobic residue at pY + 4 (36, 37), such as are found at phosphorylation sites Tyr 185 and 198 of Dab1. Cbl is abundant in cortical neurons (data not shown) and is thus a candidate to mediate Dab1 degradation. However, we have been unable to detect either a complex between Cbl and Dab1 or Cbl tyrosine phosphorylation in Reelin-stimulated neuron cultures (data not shown). Cbl tyrosine phosphorylation is thought to be a prerequisite for its activation as an E3 ligase (35). Therefore it is unlikely that Cbl directs Dab1 degradation, although related proteins such as Cbl-b (30) or Hakai (14) may be involved. It is also possible that tyrosine-phosphorylated Dab1 is targeted indirectly by virtue of association with activated SFKs. Nearby Dab1 molecules lacking phosphotyrosine would not be associated with activated SFKs and would not be targeted. Whatever the mechanism of Dab1 degradation, it may be specific to neurons, since tyrosine-phosphorylated and unphosphorylated Dab1 proteins are equally stable when expressed in 293 cells (1).

Our results suggest that Dab1 tyrosine phosphorylation is subjected to several novel control mechanisms (Fig. 5). First, Reelin stimulates rapid tyrosine phosphorylation of a subpopu-
lation (approximately 15%) of Dab1 molecules in neuron cultures (Fig. 1E). The quantity of tyrosine-phosphorylated Dab1 does not increase when Dab1 levels are higher (data not shown), when degradation is blocked with proteasome inhibitors (Fig. 2A), or when phosphotyrosine dephosphorylation is inhibited with orthovanadate (data not shown). This implies that another component is limiting for Dab1 tyrosine phosphorylation. In principle, this component could be the Reelin receptors (VLDLR and ApoER2), the SFKs, or some unknown molecule or cellular compartment. This limiting factor is represented as a box in our model (Fig. 5). Second, Dab1 is primed for tyrosine phosphorylation by one or more prior serine/threonine phosphorylations (presumably on serine) that are Reelin independent (Fig. 1D). These phosphorylations cause an electrophoretic mobility shift and are in the amino-terminal half of the molecule, since Dab1 is similarly modified (Fig. 4A and B). This makes it unlikely that the Cdk5 phosphorylation sites identified in the Dab1 carboxy terminus are involved (31). Third, because Dab1 and SFKs are in a positive-feedback loop (1, 6), tyrosine phosphorylation of Dab1 seems to continue as long as Dab1 is available. As tyrosine-phosphorylated Dab1 molecules are removed by degradation, more serine/threonine phosphorylated Dab1 molecules are recruited into the limiting compartment (Fig. 4A and 4D). When proteasomal degradation is inhibited, Dab1 tyrosine phosphorylation continues unchecked for at least 5 h (Fig. 2A). This implies that components in the signaling pathway up-stream of Dab1 remain active until the reaction is terminated by proteasomal degradation. Unlike the EGF receptor, which is desensitized by downstream kinase cascades after EGF stimulation (7, 46), the Reelin response machinery remains active as long as Dab1 substrate is available.

Our results provide a potential explanation for the long-standing observation of increased Dab1 levels in the cerebral cortex of Reelin-deficient mice (44). While dab1 mRNA levels are approximately equal in wild-type and rehr-deficient mice, the levels of Dab1 protein are increased throughout the depth of the Reelin-deficient cortical plate. This implies that cells at all levels of the wild-type cortical plate have down-regulated Dab1 protein, and our results suggest that the mechanism is tyrosine phosphorylation-dependent polyubiquitination and proteasome-dependent degradation. The slow synthesis rate of Dab1 may be important for maintaining low Dab1 levels over extended periods of time after neurons first encounter Reelin. This might explain why cells deep in the cortical plate have down-regulated Dab1. These cells are no longer in contact with Reelin-producing cells and would have first encountered Reelin in the marginal zone several days before those at the top of the cortical plate. Since Dab1 synthesis is slow, new Dab1 protein may not be made in significant quantities after the initial down-regulation.

Dab1 degradation may be important for proper location of neurons in the developing brain. Reelin is expressed by specialized cells in the outermost region of the cortex, the marginal zone (43). Cortical neurons migrate from deeper layers towards the marginal zone but do not enter it unless rehr or other genes in the Reelin pathway are mutated. Thus, it was once thought that Reelin-Dab1 signaling commenced when a migrating neuron reached the Reelin in the marginal zone. However, it is now evident that some cortical neurons have an ascending process that extends into the marginal zone (38) and ascend by somal translocation (39). Moreover, the migrating neurons are daughters of cells called radial glia (41), which have a process that extends into the marginal zone. Radial glia can themselves respond to Reelin (11, 12, 18). Thus, it now seems that Reelin acts on radial glia as well as their daughters, postmitotic neurons. In this case, the Dab1 tyrosine phosphorylation induced by Reelin may need to be transient to allow the signaling pathway to be reactivated upon subsequent exposure to Reelin. We hypothesize that Dab1 degradation may be important for limiting the duration and perhaps spatial spread of the Reelin signal within cells, thereby fine-tuning the Reelin response.

ACKNOWLEDGMENTS

We are deeply grateful to Priscilla Kronstad for her excellent technical assistance with mice. We thank B. H. Howell for the B3 antibody. We thank M. Le Gall, A. Orian, and A. Mukherjee for helpful discussions.

This work was supported by grant R37-CA41072 from the National Cancer Institute.

REFERENCES