Apolipoprotein E Receptors Are Required for Reelin-induced Proteasomal Degradation of the Neuronal Adaptor Protein Disabled-1*

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Received for publication, February 18, 2004 and in revised form, May 17, 2004 Published, JBC Papers in Press, June 2, 2004, DOI 10.1074/jbc.M401770200

The cytoplasmic adaptor protein Disabled-1 (Dab1) is necessary for the regulation of neuronal positioning in the developing brain by the secreted molecule Reelin. Binding of Reelin to the neuronal apolipoprotein E receptors apoER2 and very low density lipoprotein receptor induces tyrosine phosphorylation of Dab1 and the subsequent activation or relocalization of downstream targets like phosphatidylinositol 3 (PI3)-kinase and Nckβ. Disruption of Reelin signaling leads to the accumulation of Dab1 protein in the brains of genetically modified mice, suggesting that Reelin limits its own action in responsive neurons by down-regulating the levels of Dab1 expression. Here, we use cultured primary embryonic neurons as a model to demonstrate that Reelin treatment targets Dab1 for proteolytic degradation by the ubiquitin-proteasome pathway. We show that tyrosine phosphorylation of Dab1 but not PI3-kinase activation is required for its proteasomal targeting. Genetic deficiency in the Dab1 kinase Fyn prevents Dab1 degradation. The Reelin-induced Dab1 degradation also depends on apoER2 and very low density lipoprotein receptor in a dose-dependent manner. Moreover, pharmacological blockade of the proteasome prevents the formation of a proper cortical plate in an in vitro slice culture assay. Our results demonstrate that signal transduction through neuronal apoE receptors can activate the ubiquitin-proteasome machinery, which might have implications for the role of Reelin during neurodevelopment and in the regulation of synaptic transmission.

*This work was supported in part by National Institutes of Health Grants HL09848, HL63762, and NS43408, the Alzheimer's Association, the Wolfgang-Paul Award of the Humboldt Foundation, the Peet Foundation (to J. H.), and an Emmy Noether fellowship of the Deutsche Forschungsgemeinschaft (to P. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: Dab1, Disabled-1; apoER2, apolipoprotein E receptor 2; Cdk5, cyclin-dependent kinase 5; PI3-kinase, phosphatidylinositol 3-kinase; VLDLR, very low density lipoprotein receptor; VLDL, very low density lipoprotein; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; Z, carboxenoxyl.
express mutated Dab1 lacking Reelin-dependent tyrosine phosphorylation sites (4). Moreover, genetic deficiency of components of the Reelin-Dab1 signaling cascade results in a reduced striatochimerism or absence of Reelin-induced Dab1 tyrosine phosphorylation. These observations suggest that Reelin signaling might limit itself through a feedback mechanism, which regulates the protein levels of its target molecule Dab1 and depends on its tyrosine phosphorylation response to Reelin.

Here, we report that prolonged exposure of primary embryonic cultured neurons to Reelin reduces Dab1 protein levels. This effect of Reelin involves neither transcriptional nor translational regulatory mechanisms, but depends on the polyubiquitination and proteasomal targeting of Dab1. The proteasomal degradation of Dab1 requires Src family kinase but not P38-kine activation. Using genetic models of defective Reelin signaling, we demonstrate that Reelin-induced Dab1 degradation depends on apoER2 and VLDLR expression, and correlates with the number of functional Reelin receptor alleles. Pharmacological inhibition of the proteasome interferes with normal cortical plate development. Our results establish Dab1 ubiquitination and proteasomal degradation as an important mechanism of regulating the cellular response to Reelin and suggest that the ubiquitin-proteasome machinery participates in the regulation of neuronal positioning.

EXPERIMENTAL PROCEDURES

Reagents—The pharmacological inhibitors PP1 (4-amino-1-tetra-butylylcyanopyrazole[3,4-d]pyridine), PP2 (4-amino-5-(4-chroropheryl)-7-t-tuty1pyrazole3,4-dpyridimine), ALLM (calpain inhibitor II, N-acetyl-Leu-Leu-methionin), CA-074Me (N-[t-3 transpropyl(3-carboxoxyl-oxirane-2-carboxyl)-t-isoleucyl-t-proline methyl ester), Z-Leu-Leu-CHO (carboxbenzyo-leucyl-leucinal), rocevostin (2-(R)-1-ethyl-2-hydroxyethylamino)-6-benzylamo-9-isopropyluripine), and olomoucine (2-9-hydroxyethylaminobenz-hemazin-9-methylpyridine), were from Biomol Research Laboratories (Plymouth, PA). We obtained MG-132 (carboxbenzyo-leucyl-leucinal), ALLM (calpain inhibitor I, acetyl-leucyl-leucyl-norleucinal), MDL-28170 (carboxbenzyo-valinylphenyalanin), cycloheximide, epoxomicin, and chloroacetoxycystatin C from Biochembio (Merck Biosciences, Darmstadt, Germany). LY294002 (2-[4-morpholinyl]-8-phenyl-4-propylcarbonyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline methyl ester), were purchased from Cell Signaling Technology (Beverly, MA). Wortmannin and chloroquine were from Sigma. 1000 stock solutions of the pharmacological inhibitors were prepared in Me2SO (Sigma), except for chloroquine (dissolved in water). Poly-n-lysine (P-7280), N-ethylmaleimide, and the phosphatase inhibitor mixtures (Complete, EDTA-free) were from Roche Diagnostics. Protein A-agarose (P-3476) was from Sigma, and protein G-Sepharose 4 Fast Flow was obtained from Amersham Biosciences. Neurobasal medium and B-27 serum-free supplement were purchased from Invitrogen. The production of par-}

...tially purified concentrated Reelin- or control-conditioned medium has been described elsewhere (3, 6).

Immunocytochemistry—Embryonic neurons were treated with Reelin or control medium for 15 min, fixed, and incubated with the monoclonal antibody FK2 (Affiniti Research Products), which recognizes ubiquitinated proteins but not free ubiquitin (20). 4,6-Diamidino-2-phenylindole was used as a nuclear counterstain. Bound primary antibody was detected with donkey antibody labeled goat anti-mouse antibody (Molecular Probes, Eugene, OR). Digital images were acquired with a Zeiss Axiovert 100M fluorescence microscope at ×63 magnification.

Plasmin and Transient Transfections—The plasmid pcDNA3.1 zeol|(*) mDab55 encoding murine full-length Dab1 (9) and the plasmid pEP1a-FA-UBQ (Dr. Zhijian Chen, Dallas), which encodes amino acids 165–411 of human ubiquitin protein tagged by an epitope consisting of the influenza hemagglutinin (HA) protein (YPYDVPDYD) under the control of the EF1a promoter (21), have been described elsewhere. HEK-293 cells (CRL-1573, ATCC) grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum were transfected with Fu-}

...gine 6 transfection reagent (Roche Diagnostics) and harvested 24 to 36 h after transfection.

Immunoblotting, Immunoprecipitation, and Antibodies—Cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 2 mM EGTA, 5 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) SDS, 0.25% (w/v) sodium deoxycholate with protease and phosphatase inhibitor mixtures). The lysates were cleared by centrifugation, mixed with concentrated gel-loading buffer and separated by SDS-gel electrophoresis under reducing and denaturing conditions. After protein transfer to Hybond-C extra nitrocellulose membranes (Amersham Biosciences), the membranes were blocked in phosphate-buffered saline, pH 7.4, with 0.05% Tween 20 and 4% nonfat dry milk, and incubated with primary antibodies diluted in blocking buffer, followed by three washing steps with phosphate-buffered saline/Tween and incubation with secondary antibodies coupled to horseradish peroxidase (Amersham Biosciences). Signals were detected by enhanced chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce) on a Kodak X-OMAT Blue film. Scanned images of exposed films were processed with Adobe Photoshop and Illustrator Software (San Jose, CA). Bands were quantified using ImageJ (Scion Image Beta 4.0.2i, Scion Corp.). Immunoprecipitation of Dab1 from cellular lysates (immunoprecipitation buffer: 20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 1 mM CaCl2, 1 mM MgCl2, 1% (v/v) Triton X-100, protease and phosphatase inhibitor mixtures) was carried out as described (8). For the detection of ubiquitinated Dab1, N-ethylmaleimide (10 mM) was added freshly to the immunoprecipitation buffer (22). The rabbit polyclonal antibodies raised against the carboxyl-terminal 13 amino acid residues of mouse Dab1 and of mouse LDL receptor and the monoclonal anti-Reelin antibody G10, which was kindly provided by Dr. André Goffinet (Brussels), have been described elsewhere (18, 23, 24). The monoclonal antibody HA.11 against hemagglutinin (anti-HA) was from Roche Diagnostics (Beverly, MA) and the polyclonal antibody against serine 473-phospho-
results

prolonged treatment of cultured neurons with Reelin down-regulates Dab1 protein levels—Previous studies on Reelin signaling have focused on the mechanisms leading to the tyrosine phosphorylation of the neuronal intracellular adaptor molecule Dab1, its interacting partners, and downstream signaling cascades. Far less is known about the termination of the Reelin-induced signal. In line with earlier observations reporting down-regulation of Dab1 in neurons in response to long-term treatment with Reelin (16), we found a robust decrease in Dab1 protein levels after stimulating cultured forebrain neurons with Reelin for 8 h (Fig. 1A). A range of different membrane and cytosolic proteins was not affected by Reelin incubation (Fig. 1A), indicating that the effect on Dab1 levels was specific. Activation of the serine-threonine kinase AKT, a downstream target of Reelin, which requires tyrosine phosphorylation of Dab1 (6), was not observed after prolonged treatment, which is in line with the disappearance of tyrosine-phosphorylated Dab1 (Fig. 1A). We found that the protein levels of Dab1 were reduced to about one-half of the initial amount after 2.5 h and were almost undetectable by immunoblotting after 6.5 h of treatment (Fig. 1B), whereas the levels of the serine-threonine kinase Cdk5 did not change.

Reelin Induced Decrease of Dab1 Protein Levels in Cultured Embryonic Neurons—Northern blot analysis of neurons treated for 8 h revealed no decrease of Dab1 mRNA levels in response to Reelin (Fig. 1C). These results were confirmed by real time PCR. Two different primer pairs were designed as described under “Experimental Procedures,” which amplify either all three major Dab1 transcripts (bridging exons 2 and 3, named Dab1 in Fig. 1C), or full-length Dab1 (named Dab555) only (13, 26). As shown in Fig. 1C, the expression of Dab1 mRNA was not altered in response to Reelin treatment for 4, 8, or 16 h. Cycloheximide, a protein synthesis inhibitor, did not abolish the difference in Dab1 levels of control- and Reelin-treated neurons (Fig. 1D). Altogether, these data suggest that the Reelin effect on Dab1 protein levels is neither transcriptionally regulated nor because of suppression of translation.

Reelin Targets Dab1 for Proteasomal Degradation—Next, we used different pharmacological inhibitors to investigate if the Reelin-induced destruction of Dab1 is mediated by proteolytic degradation of Dab1. ALLN and ALLM, two peptide aldehyde inhibitors, blocked the Dab1 degradation (Fig. 2A, lanes 4 and 6). Both compounds, also known as calpain inhibitors I and II, inhibit calpains as well as the proteasome (28), a large enzyme complex that degrades ubiquitin-tagged cellular proteins (29). ALLM also blocks cathepsins, lysosomal cystein proteases (30). To distinguish between the different inhibitory activities, more specific inhibitors were used. Chloroquine, an inhibitor of lysosomal proteases (31), and CA-074Me, a selective and irreversible cathepsin-B inhibitor, had no effect on Dab1 degradation at concentrations of 100 and 50 μM, respectively (Fig. 2A, lanes 8 and 10).

Calpains are proteases that participate in cellular functions like cytoskeletal rearrangement, signal transduction, and apo-
ptosis (32) and have been implicated in neurodegenerative diseases. Interestingly, the neuronal proteins p35 and p39 can be cleaved by calpains, thereby changing the activity and subcellular localization of Cdk5 (33). We used two specific inhibitors, MLD-28170 and Z-Leu-Leu-CHO, to examine a possible involvement of calpains in Reelin-triggered proteolysis of Dab1. Only the last compound partially inhibited Dab1 degradation by the proteasome. A peak of Dab1 protein, which was immunoprecipitated from the cell lysates and separated by SDS-PAGE, was detected with an antibody against the HA tag (top panel). B, rat forebrain neurons were pretreated with 10 μM MG-132 after incubation with control or Reelin for the indicated periods, the neurons were lysed in immunoprecipitation (IP) buffer containing 12 mM N-ethylmaleimide, which was added to inhibit ubiquitin-cleaving isopeptidases (22), and immunoprecipitated with a Dab1-specific antibody (lanes 1–4) or a sera control (lanes 5–7). After SDS-PAGE and transfer to membrane filters, ubiquitination was detected by immunoblotting with the monoclonal antibody P4D1. A peak of Dab1 ubiquitination appears after ~15 min Reelin treatment (lane 3). Lysate and immunoprecipitation supernatant (5% of total) were loaded in lanes 6 and 7 (Dab1 immunoblot, bottom panel). The asterisk (*) indicates the position of the IgG heavy chain (lanes 1–5, immunoprecipitates). C, treatment with Reelin (bottom panel) induces protein ubiquitination in the process tips (white arrowheads) of cultured primary embryonic neurons. Ubiquitinated proteins were detected by immunofluorescence using the FK2 monoclonal antibody (red), nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue).
Reelin-induced Proteasomal Degradation of Dab1

A. Proteasome inhibition in Reelin-treated neurons blocks degradation of tyrosine-phosphorylated Dab1. Protein levels of total Dab1 (second panel), tyrosine-phosphorylated Dab1 (top panel) and serine 473-phosphorylated AKT (third panel from top), which correlates with PI3-kinase activation, in cellular lysates from control- or Reelin-treated neurons were measured by immunoblotting. Proteasome inhibition with 10 μM MG-132 (lanes 3 and 4) not only blocked the Reelin-induced degradation of total Dab1 (lane 2), but also prevented the reduction of tyrosine-phosphorylated Dab1 (lane 4), which correlates with sustained PI3-kinase activation (lane 4, third panel from top). Cdk5 served as a loading control. B. Inhibition of Src kinase activity (measured by immunoblotting for cellular phosphotyrosine levels, second panel from top) with PP1 (lane 3) or PP2 (lane 4) during the treatment of cultured neurons with Reelin (lanes 2–4; top panel) blocks the degradation of Dab1 protein (third panel from top). Src family kinase inhibition alone has no effect on the Dab1 levels (lane 5). C, Down-regulation of Dab1 levels by overnight (ON) treatment with Reelin (lanes 3 and 4). The remaining Dab1 protein is still tyrosine-phosphorylated (middle panel, lane 4, asterisk) in response to subsequent short term Reelin treatment. Lanes 1 and 2, no overnight pretreatment; lanes 5 and 6, overnight control treatment.

Role of Src Family Kinase Activity in Reelin-dependent Proteasomal Degradation of Dab1—Using an antibody specific for protein-conjugated ubiquitin (FK2), we noticed a rise in immunofluorescence at the tip of processes (Fig. 3C, white arrowheads) of Reelin-treated cultured embryonic neurons (Fig. 3C, bottom panel). Here, the ubiquitinated proteins are present in a pattern that is indistinguishable from that of Reelin-induced phosphoprotein products (6). Because the Reelin signal specifically targets Dab1 for proteasomal degradation (Fig. 1A), we asked whether the Reelin-induced tyrosine phosphorylation of Dab1 might be critical for its proteasomal destruction. As shown in Fig. 4A, the coinoculation with a proteasome inhibitor leads to an accumulation of tyrosine-phosphorylated Dab1 (top panel, lane 4), which corresponds to the blocked degradation of total Dab1 protein (middle panel).

These data strongly suggest that the post-translational modification of Dab1 by tyrosine phosphorylation in response to Reelin is a requirement for its subsequent ubiquitination and targeting for proteasomal destruction, and that the tyrosine-phosphorylated Dab1 is preferentially degraded. Inhibition of Src family kinase activity, which is induced by Reelin treatment of neurons (2, 3), by coinoculation with the pharmacological inhibitors PP1 (Fig. 4B, lane 3) and PP2 (lane 4) indeed blocked the Reelin-triggered decrease of Dab1 levels (Fig. 4B, third panel from top). The remaining Dab1 protein left after overnight Reelin treatment can still be tyrosine-phosphorylated in response to a short stimulation with Reelin (Fig. 4C, lane 4, asterisk).

Dab1 Degradation Is Independent of PI3K Activity—Proteasome inhibition not only prevents tyrosine-phosphorylated Dab1 from degradation but also maintains the activation of its downstream target PI3-kinase, measured by the degree of phosphorylation of AKT at serine 473 (Fig. 4A, lane 4, third panel from top), which is otherwise not increased after several hours of Reelin treatment (lane 2; see also Fig. 1A). In a similar setting in HepG2 cells, activation of PI3-kinase by insulin has been reported to be important for the ubiquitination and proteasomal degradation of the forkhead transcription factor Foxo1 (34). This lead us to investigate whether PI3-kinase activity might be required for the effect of Reelin on Dab1 degradation. Coincubation with the PI3-kinase inhibitor LY294002 did not block the Reelin-induced Dab1 decrease, however (Fig. 5A, lane 6), indicating that Src family kinase but not PI3-kinase activity is necessary for the Reelin-triggered targeting of Dab1 for proteasomal destruction. The same result was obtained with wortmannin, another inhibitor of PI3-kinase (not shown).

The Dab1 Protein Sequence Contains Three Putative PEST Domains—Regulation of the ubiquitin-proteasome machinery can be achieved at the level of substrate ubiquitination or at the level of proteasome activity. Target proteins often contain defined motifs that are recognized specifically by the ubiquitin-conjugating system. A computer-based motif search revealed the presence of three putative PEST domains in the Dab1 protein sequence, two of which are located in the carboxyl-terminal part of the molecule (Fig. 5C). PEST sequences are defined as hydrophilic stretches of at least 12 amino acid residues in length that contain proline (P), glutamate (E) or aspartate (D), and serine (S) or threonine (T) residues, flanked by regions enriched in positively charged residues (35). Phosphorylation has been described to unmask latent PEST signals. Remarkably, the tyrosyl residue 220, which is phosphorylated in response to Reelin in neurons (36), lies within the first putative Dab1 PEST region. This might turn a latent PEST signal into an active recognition signal for the ubiquitin-proteasome machinery and offers one potential explanation for the Src family kinase dependence of Reelin-triggered Dab1 degradation.

In addition to tyrosine phosphorylation, Dab1 has been reported to be phosphorylated at the carboxyl-terminal serine residues 491 and 515 by Cdk5 in vitro (14). The physiological consequence of this phosphorylation is not known. As those residues are located within (Ser515) or immediately adjacent (Ser491) to the two carboxyl-terminal PEST regions (marked by □ in Fig. 5C), we wondered whether Cdk5 phosphorylation of Dab1 might influence the targeting of Dab1 for proteasomal destruction by Reelin. Coincubation of Reelin-treated neurons with the Cdk5 inhibitors roscovitine (Fig. 5B, lanes 1 and 2) or olomoucine (not shown) had no effect on Dab1 degradation, however.

Proteasome Inhibition Interferes with Formation of a Normal Cortical Plate—The observation that Reelin induces the proteasome-mediated degradation of Dab1 in neurons led us to investigate the functional effect of proteasome inhibition on neuronal positioning in the developing neocortex. To this end, we made use of a recently established embryonic slice culture system, which allows to recapitulate cortical plate development in vitro (19, 37). As shown in Fig. 6, treatment of E13.5 cortical slices (top panel) with 20 μM epoxomicin induces malformation of the cortical plate after 2 days in culture (bottom panel), whereas vehicle alone does not interfere with the formation of a dense cortical plate framed by a marginal zone and a cell-poor intermediate zone. Immunoblotting of homogenized slices with the FK2 monoclonal antibody demonstrated increased protein ubiquitination in the epoxomicin-treated slices (Fig. 6, inset),
Targeting of Dab1 for Proteasomal Destruction Depends on ApoE Receptors and Fyn—It is well established that steady-state Dab1 protein levels are up-regulated in brain tissue and cultured neurons derived from mice that are deficient for key components of the Reelin signaling pathway (2, 3, 17, 18), namely the Reelin receptors apoER2 and VLDLR, the Dab1 kinase Fyn, and Reelin itself. As would be expected, exogenous Reelin added to cultured neurons from reeler mice induces down-regulation of cellular Dab1 levels (Fig. 7A, middle panel, lane 2) to a comparable or even greater extent than in wild type neurons (lane 4). The Reelin-induced Dab1 degradation depends on Src family kinase activation (Fig. 4B), which requires Reelin binding to its receptors, apoER2 or VLDLR (3). We therefore investigated whether the decrease in Dab1 requires the expression of apoER2 or VLDLR (Fig. 7B). Genetic deficiency in both receptors (lanes 1–4 and 13–16) completely blocked the decrease of Dab1 levels after Reelin stimulation (lanes 3 and 4, 15 and 16). Remarkably, the down-regulation in neurons expressing one functional receptor allele of both Apoer2 and Vldlr (lanes 5–8) was almost as pronounced as in wild type neurons, whereas the effect was clearly mitigated in neurons with only one functional Reelin receptor allele left (lanes 11 and 12). This gene-dosage effect of Dab1 degradation in response to Reelin is mirrored by the gradual increase in steady-state Dab1 protein levels of cultured neurons (Fig. 7C) or brains (18, 38), which roughly correlates with the number of functional Reelin receptors. Our data suggest that other Reelin-binding receptors, like cadherin-related neuronal receptors or integrins (39, 40), are not required for the Reelin-triggered targeting of Dab1 for proteasomal degradation.

Finally, we sought to clarify the role of the Src family kinase Fyn, which phosphorylates Dab1 in vivo (2, 3), in the Reelin-dependent Dab1 down-regulation. We stimulated neurons from Fyn-deficient mouse embryos with Reelin and compared the Dab1 protein levels with those from wild type control littermates (Fig. 7D). Fyn deficiency almost completely prevented the Reelin-induced destruction of Dab1 (lane 4), which is observed in wild type neurons (lane 2). This result is consistent with the observed increase of steady-state Dab1 levels in Fyn-deficient animals.

DISCUSSION

A critical step in the transmission of the Reelin signal to postmitotic migrating neurons is the tyrosine phosphorylation...
Reelin-induced Proteasomal Degradation of Dab1

Tyrosine phosphorylation of Dab1 and its ubiquitination and proteasomal degradation are both Reelin-dependent, and occur preferentially at the tips of neuronal processes. We hypothesized that either the tyrosine phosphorylation of Dab1 itself or an activated downstream target might be required for the activation of the ubiquitin-proteasome machinery. Pharmacological inhibition of Src family kinases, which blocks tyrosine phosphorylation of Dab1 by Reelin, also prevented its Reelin-induced degradation (Fig. 4B). Because tyrosine-phosphorylated Dab1 activates Src kinases in a feed-forward fashion, two interpretations of this result are possible: 1) the activation of Src kinases or downstream targets is required for the activation of the ubiquitin-proteasome system, for example, by phosphorylation of the ubiquitin ligase; or 2) tyrosine phosphorylation of Dab1 itself provides a recognition signal for the ubiquitination machinery, similar to the prolactin-dependent tyrosine phosphorylation, ubiquitination, and degradation of Janus kinase-2 (42).

To distinguish these two possibilities we examined first if activation of PI3-kinase, a downstream target of Reelin and Dab1, might be involved in the proteasomal targeting of Dab1. Activation of PI3-kinase has been shown to be required for the growth factor-dependent ubiquitination and degradation of other proteins, e.g., the cytosolic adaptor protein IRS-1 (43, 44) or forkhead transcription factors (34, 45). Reelin-induced Dab1 degradation did not depend on PI3-kinase activation, however (Fig. 5A).

Proteasome inhibition in Reelin-treated cultured neurons dramatically increased the half-life of tyrosine-phosphorylated Dab1 (Fig. 4A), whereas total Dab1 protein levels did not differ between control- and Reelin-treated cells. Moreover, short term incubation of neurons with Reelin followed by long term incubation with control medium did not trigger Dab1 degradation (not shown). These data suggest that it is the tyrosine phos-
Reelin-induced Proteasomal Degradation of Dab1

...phorylation itself and not the activation of downstream targets that preferentially targets Dab1 for degradation and thereby limits the cellular response to the Reelin signal. Incubation with sodium orthovanadate does not increase the stoichiometry of Dab1 tyrosine phosphorylation in cultured neurons (16), indicating that dephosphorylation by tyrosine phosphatases does not significantly contribute to the regulation of the Reelin signal in target neurons.

Altogether, these results support the hypothesis that the Reelin-dependent phosphorylation of tyrosyl residues 198 and 220 in neurons directly triggers the interaction of Dab1 with the ubiquitin-proteasome machinery. This view is corroborated by an elegant experiment reported by Arnaud and colleagues (41). Neurons simultaneously expressing two mutant forms of Dab1, a full-length protein lacking tyrosine phosphorylation sites (4) and a carboxyl-terminal truncated hypomorphic form, p45 (13), were treated with Reelin. Although p45 is tyrosine-phosphorylated in response to Reelin and supports Src family kinase activation (7), no Reelin-induced degradation of the phosphorylation site-deficient full-length Dab1 was observed in these neurons. We observed that genetic inactivation of the main Dab1 kinase Fyn almost completely blocked the Reelin-mediated degradation of Dab1 in neurons (Fig. 7D), although other Src family kinases can partly compensate for the absence of Fyn in mediating the phosphorylation of Dab1 (2, 3). It is possible that Fyn has additional specific functions that are required for the proteasomal targeting of Dab1, or that spacial restrictions because of the localization of Src family kinases into different membrane compartments play a role (8, 46).

How could tyrosine phosphorylation of Dab1 trigger its ubiquitination and degradation by the proteasome? A computer-based motif search revealed the presence of three putative PEST domains within the Dab1 protein sequence (Fig. 5C). These domains were first described in proteins undergoing targeted proteolytic destruction and can serve as constitutive or conditional targeting motifs for the ubiquitin-proteasome destruction pathway (35, 47). Tyrosine 220 lies within the amino-terminal PEST sequence of Dab1. Phosphorylation of this residue could provide a docking site for ubiquitinating enzymes, or change the overall conformation of the molecule and facilitate ubiquitination. Two more potential PEST sequences are found in the extreme carboxyl-terminal part of Dab1.

Interestingly, although carboxyl-terminal truncated Dab1 protein levels are regulated by Reelin in vivo and in cultured embryonic neurons (13, 41), p45 is expressed at higher levels than full-length Dab1 in hemizygous mice (13), indicating that the carboxyl-terminal region is involved in regulating protein expression levels of Dab1. The in vivo Cdk5 phosphorylation site of Dab1 at serine 491 (14) lies in one of the predicted COOH-terminal PEST domains. Phosphorylation by Cdk5 at this site might regulate overall Dab1 expression levels and represent a means of biochemical cross-talk between the Reelin and Cdk5 signaling pathways. A similar regulation of substrate turnover by Cdk5 phosphorylation has been documented for the regulatory protein p35 (48). Pharmacological inhibition of Cdk5 activity did not significantly influence Dab1 protein levels in control- or Reelin-treated cultured neurons, however (Fig. 5B), and Dab1 protein levels are unaltered in brains of mice lacking Cdk5 activity (49, 50).

Experiments with cultured neurons from Reelin receptor-deficient mice indicate that the expression of apoER2 or VLDLR is required for down-regulation of Dab1 protein by Reelin, and that this effect is gene dose-dependent (Fig. 7B). These results explain the correlation between Dab1 protein levels and the number of functional alleles of Apoer2 or Vldlr in brains (18) or neurons (Fig. 7C) from mutant mice. Additionally, up-regulation of Dab1 in Vldlr<sup>+/−</sup>, Apoer2<sup>−/−</sup> brains was more pronounced than in Apoer2<sup>+/−</sup>, Vldlr<sup>−/−</sup> brains (Fig. 7C, lanes 2 and 3). This is consistent with a more severely reduced level of Reelin-induced Dab1 phosphorylation in neurons lacking apoER2 than in neurons lacking VLDLR (38), and provides additional support for the view that tyrosine-phosphorylated Dab1 is preferentially degraded in response to Reelin. The gene-dose effect of apoER2 and VLDLR on Dab1 degradation suggests a stoichiometric requirement of these receptors for Dab1 phosphorylation and subsequent proteasomal degradation after Reelin binding. It has been shown that dimerization of Dab1 is necessary and sufficient for its tyrosine phosphorylation (51). These data are consistent with a model where the di- or oligomerization of Dab1 molecules, which are associated stoichiometrically with the cytoplasmic tails of apoER2 and VLDLR, is induced by secreted oligomeric Reelin binding to and clustering its receptors (51). Conformational changes might then favor Dab1 tyrosine phosphorylation and its recognition by the ubiquitin-proteasome machinery. In this regard, it is interesting to note that di- or oligomerization is a prerequisite for the proteasomal degradation of activating transcription factor 2 (52) and p53 (53).

As the substrate specificity of the ubiquitination reaction is largely mediated by E3 ligases, which display a high degree of diversity, the characterization of the Dab1 ubiquitin ligase will be a focus of future work. Of the many neuronal ubiquitin ligases c-Cbl is a potential candidate because it is phosphorylated by Fyn, but targeted deletion in mice does not result in neurodevelopmental defects reminiscent of the reeler phenotype (54). Another candidate is the ubiquitin ligase Siah-1A, which has recently been identified in a yeast two-hybrid screen for Dab1-interacting partners (55).

Impairment of the ubiquitin-proteasome machinery has been implicated in the pathogenesis of neurodegenerative diseases, including Alzheimer’s (56), but reports about a role of ubiquitination in neuronal migration disorders are sporadic (57, 58). Hence, the identification of the ubiquitin-proteasome system as a downstream target of the Reelin signaling cascade that regulates the availability of Dab1 in responsive neurons and the direct demonstration of its role in cortical plate formation are an important step toward a better understanding of its role in neurodevelopment.

An increasing number of studies document the importance of the ubiquitin-proteasome pathway for synapse formation, transmission, and plasticity (reviewed in Ref. 59). It is therefore interesting to note that Reelin and Dab1 are important for synaptic connectivity in the retina (60), and a role for Reelin and its apoE receptors in hippocampal dendrite maturation (61) and in the enhancement of synaptic transmission in the adult hippocampus has been demonstrated recently (62). ApoER2-deficient mice had more pronounced defects in long term potentiation than mice lacking VLDLR (62), which correlates with the higher efficiency of apoER2 in mediating Reelin-induced Dab1 phosphorylation and degradation. It is tempting to speculate that activation of the ubiquitin-proteasome system by the Reelin signaling cascade in the adult brain might be relevant for its role in synaptic connectivity.

**Acknowledgments**—We are indebted to Huichuan Reyna, Jill Fairless, and Wen-Ling Niu for excellent technical assistance. We thank Megan Davenport and Richard Gibson for invaluable help with animal care and Jeff Cormier for real time PCR. We thank Navadar Sever, Russell DeBoise-Boyd, Eckart Förster, and Yiu-Kee Ho for helpful discussions and reagents, and Michael Frotscher and André Goffinet for critical reading of the manuscript.
