Cables Links Cdk5 and c-Abl and Facilitates Cdk5 Tyrosine Phosphorylation, Kinase Upregulation, and Neurite Outgrowth

Neuron, Vol. 26, 633–646, June, 2000, Copyright ©2000 by Cell Press

Cables Links Cdk5 and c-Abl and Facilitates Cdk5 Tyrosine Phosphorylation, Kinase Upregulation, and Neurite Outgrowth

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Summary

Cyclin-dependent kinase 5 (Cdk5) is a small serine/threonine kinase that plays a pivotal role during development of the CNS. Cables, a novel protein, interacts with Cdk5 in brain lysates. Cables also binds to and is a substrate of the c-Abl tyrosine kinase. Active c-Abl kinase leads to Cdk5 tyrosine phosphorylation, and this phosphorylation is enhanced by Cables. Phosphorylation of Cdk5 by c-Abl occurs on tyrosine 15 (Y15), which is stimulatory for p35/Cdk5 kinase activity. Expression of antisense Cables in primary cortical neurons inhibited neurite outgrowth. Furthermore, expression of active Abl resulted in lengthening of neurites. The data provide evidence for a Cables-mediated interplay between the Cdk5 and c-Abl signaling pathways in the developing nervous system.

Introduction

Cyclin-dependent kinases (Cdks) are a family of small serine/threonine kinases that require association with regulatory subunits known as cyclins for activation. In addition to binding to cyclins, posttranslational phosphorylation and dephosphorylation events regulate Cdk activity (reviewed by Morgan, 1995). Phosphorylation of the threonine residue in the T loop (T160 on Cdk2 or T161 on Cdc2) by Cdk-activating kinase (CAK) is an obligatory step in kinase activation, and a threonine to alanine mutation of this residue renders the Cdk inactive. On the other hand, phosphorylation of the threonine 14 and tyrosine 15 (Y15) residues by the Wee1 family of dual specificity kinases is inhibitory for the Cdks, and dephosphorylation of these residues by the Cdc25 family of phosphatases coincides with Cdk activation.

Initially identified as regulators of the cell division cycle, there is emerging evidence to suggest that some Cdks are involved in events unrelated to cell division. A function of Cdk5 has been demonstrated only in the development of the nervous system. Introduction of a dominant-negative Cdk5 mutant inhibited neurite outgrowth in cultured primary cortical neurons (Nikolic et al., 1996). A mouse strain lacking Cdk5 exhibited a late embryonic to perinatal lethality with obvious defects in the developing CNS (Ohshima et al., 1996). Indeed, Cdk5 protein is expressed at the highest levels in the nervous system, and its associated kinase activity has only been detected in brain lysates (Lew et al., 1992; Shetty et al., 1993; Tsai et al., 1993; reviewed by Lew and Wang, 1995). This temporal and spatial specificity of Cdk5 kinase activity is due to the restricted expression of its regulatory subunit p35 (Ishiguro et al., 1994; Lew et al., 1994; Tsai et al., 1994). p35 is a neuronal specific protein that is important in normal neuronal migration (Chae et al., 1997). The amino acid sequence of p35 does not resemble that of the cyclins, although it was suggested that the predicted ternary structure of p35 is similar to that of cyclin A (Tang et al., 1997). Activation of Cdk5 kinase activity can be achieved by mixing purified bacterially expressed p35 and Cdk5 proteins in vitro, indicating that posttranslational modification is not required for Cdk5 activation (Lew et al., 1994; Tsai et al., 1994). This is supported by the finding that CAK does not phosphorylate Cdk5 on the serine 159 residue (equivalent to T160 of Cdk2; Poon et al., 1997), and a serine to alanine mutation of Cdk5 on the 159 residue does not affect the extent of Cdk5 activation by p35 (Y. Ramos and L.-H. T., unpublished data). A kinase activity has been identified that phosphorylates Cdk5 on the threonine 14 residue and inhibits the p35/Cdk5 kinase (Matsuura and Wang, 1996), despite the fact that T14 phosphorylation of Cdk5 has not been demonstrated in vivo. Finally, tyrosine phosphorylation of Cdk5 was reported in lysates prepared from developing rat cerebellum (Lazaro et al., 1996), although the identity of the phosphorylated tyrosine residue and the consequences of tyrosine phosphorylation on Cdk5 kinase activity are not known.

First identified as the cellular homolog of the transforming gene of Abelson murine leukemia virus, *c-abl* encodes a nonreceptor tyrosine kinase related to the Src family. c-Abl is found in both the nuclear and cytoplasmic compartments, including the plasma membrane and actin cytoskeleton. Thus, c-Abl is likely to have multiple functions or integrate multiple signaling events. Mice with homozygous disruption of the *c-abl* gene display pleiotropic defects, including increased perinatal lethality, runtedness, lymphopenia, and abnormal head and eye development (Schwartzberg et al., 1991; Tybulewicz et al., 1991). Mice lacking both Abl and Arg (the Abl-related gene) nonreceptor tyrosine kinases show delayed closure of the neural tube, and the neuroepithelium buckles into the lumen of the neural tube (Koleske et al., 1998). In *Drosophila*, Abl is abundantly localized to the axons of the CNS (Gertler et al., 1993). While mutations in *Drosophila abl* have no detectable effect on the embryonic CNS, animals with heterozygous mutations or deletions of *disabled* (*dab*) in the *abl* mutant

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background are embryonic lethal and display defects in organization and fasciculation of axons (Gertler et al., 1989). A mammalian homolog of Dab, mDab1, was isolated by virtue of its interaction with Src (Howell et al., 1997a). mDab1 undergoes tyrosine phosphorylation in a developmental stage–specific manner, and it binds to phosphotyrosine-containing proteins. Strikingly, mouse strains with naturally occurring mutations in mDab1 (*scrambler*) or homozygous transgenic deletion of mDab1 display cortical lamination defects (Howell et al., 1997b; Sheldon et al., 1997; Ware et al., 1997), indicating a role of mDab1 in neuronal migration. These observations support a function of the c-Abl-mediated signal transduction pathway in the development of the nervous system.

We identified a Cdk5 binding protein that also interacts with c-Abl. This protein is named Cables because it is a Cdk5 and Abl enzyme substrate. Cables links Cdk5 and c-Abl, and enhances Y15 phosphorylation of Cdk5 by c-Abl tyrosine kinase. While Y15 phosphorylation on Cdc2 and Cdk2 is inhibitory, Y15 phosphorylation of Cdk5 by Cables and c-Abl increases the kinase activity of the p35/Cdk5 complex in developing neurons. Expression of antisense Cables in primary neurons causes axonal shortening, similar to expression of a dominant-negative Cdk5, while expression of active Abl results in axonal lengthening. The data suggest that Cables and Cdk5 tyrosine phosphorylation are involved in axon growth regulation.

Results

Cables Is a Novel Cdk5-Interacting Protein

To identify potential substrates of Cdk5, a yeast two-hybrid screen was performed using a kinase-inactive Cdk5 mutant ($Cdk5N^{144}$; van den Heuvel and Harlow, 1994) and a cDNA library made from embryonic day 14.5 (E14.5) whole mouse embryos (Vidal et al., 1996; Hu et al., 1997). A total of 120 clones were isolated that scored positively on at least two of three reporter genes (*ura3*, *his3*, and *lacZ*; see Experimental Procedures) and of which 80 encode complete or partial open reading frames of cyclins D1, D2, and D3. The D-type cyclins have been shown previously to associate with Cdk5 (Xiong et al., 1992). The other clones represent seven different cDNAs which encode proteins that associate with Cdk5 in yeast and in vitro binding assays. When tested for binding specificity to Cdk5, 6 of the clones displayed unique affinity for Cdk5. The seventh clone (Cables) bound to Cdk5 with high affinity but also bound with much reduced affinity to Cdk2 and Cdk3 (Figure 1A). A 4 kb cDNA of the seventh clone was isolated from a mouse neonatal brain cDNA library using the partial cDNA as a probe. This cDNA encodes a putative protein product of 568 residues (Figure 1B) with a predicted molecular size of 63 kDa. Cables displays little sequence homology to other known proteins in the databases. It does, however, show weak homology to cyclin A (Figure 1C) and weaker homology to cyclin C over an ~200 amino acid stretch in the C-terminal third of the protein that may be the Cdk-interacting region. Cables also contains six PXXP motifs (Cicchetti et al., 1992; Ren et al., 1993), defined as the minimal consensus for SH3 domain binding, and two tyrosine-based sorting motifs (YXXLE), which have been implicated in axonal growth cone sorting (Kamiguchi and Lemmon, 1998). It contains three serine proline/threonine proline minimal Cdk phosphorylation sites and at least one potential c-Abl phosphorylation site (YXXP; Songyang et al., 1995). A single 4 kb band was detected on Northern blots (Figure 1D), confirming that a full-length cDNA was identified. Among adult tissues, Cables mRNA is most highly expressed in the brain (Figure 1D). High levels of mRNA are also present in kidney, liver, and lung. During mouse embryonic development, Cables can be detected as early as E7, and its expression increases as development proceeds (Figure 1D).

A specific antibody was raised against a $histidine_6$-tagged Cables (His_6-Cables). The affinity-purified antibody recognizes a protein doublet of about 68 kDa in cortical lysates on SDS–PAGE that comigrates with Cables synthesized in rabbit reticulocyte lysates in vitro and migrates slightly faster than Myc-tagged Cables transfected into COS7 cells (Figure 2A). Thus, the size of Cables estimated from mobility on gel electrophoresis is larger than the predicted size. Treatment of Cables immunoprecipitates with acid phosphatase increased the mobility of the lower band, while the upper band remained relatively unaltered, indicating that at least one component of the doublet is phosphorylated (Figure 2A). The nature of the doublet is currently under investigation. During cortical development, the appearance of Cables undergoes dynamic changes (Figure 2B). The lower band first appears at E15, peaks around birth, and gradually declines. In contrast, the abundance of the slower mobility species is first detectable by E17 and increases at the time of maturation of the cortex.

Association of Cables with Cdk5 In Vivo

Association of Cables with Cdk5 was verified by cotransfection in COS7 cells and in cortical lysates. Upon cotransfection, association between Cdk5 and Cables could be readily demonstrated (Figure 2C). However, anti-Cables immunoprecipitates from a triple transfection, including p35, Cdk5, and Cables, did not contain p35 (data not shown). Similarly, Cables was not present in anti-p35 immunoprecipitates from the triple transfection (Figure 2C), suggesting that p35 and Cables do not coexist stably in a complex with each other. However, the efficacy of the reagents may preclude our ability to detect the presence of p35 in the Cables compex or vice versa. Complex formation between endogenous Cables and Cdk5 could also be demonstrated in mouse cortical lysates, as Cdk5 was present in Cables immunoprecipitates, and Cables in Cdk5 immunoprecipitates (Figure 2D). p35 immunoprecipitates contain Cdk5 but not Cables (Figure 2D), and Cables immunoprecipitates contain Cdk5 without p35 (data not shown). These results demonstrate an in vivo association of Cdk5 and Cables and no evidence of p35 in the complex. Because of the limited but significant homology between Cables and cyclin A, we tested whether Cables could activate Cdk5. No Cdk5-associated histone H1 kinase activity was detected when Cdk5 and Cables were cotransfected into COS7 cells (data not shown).

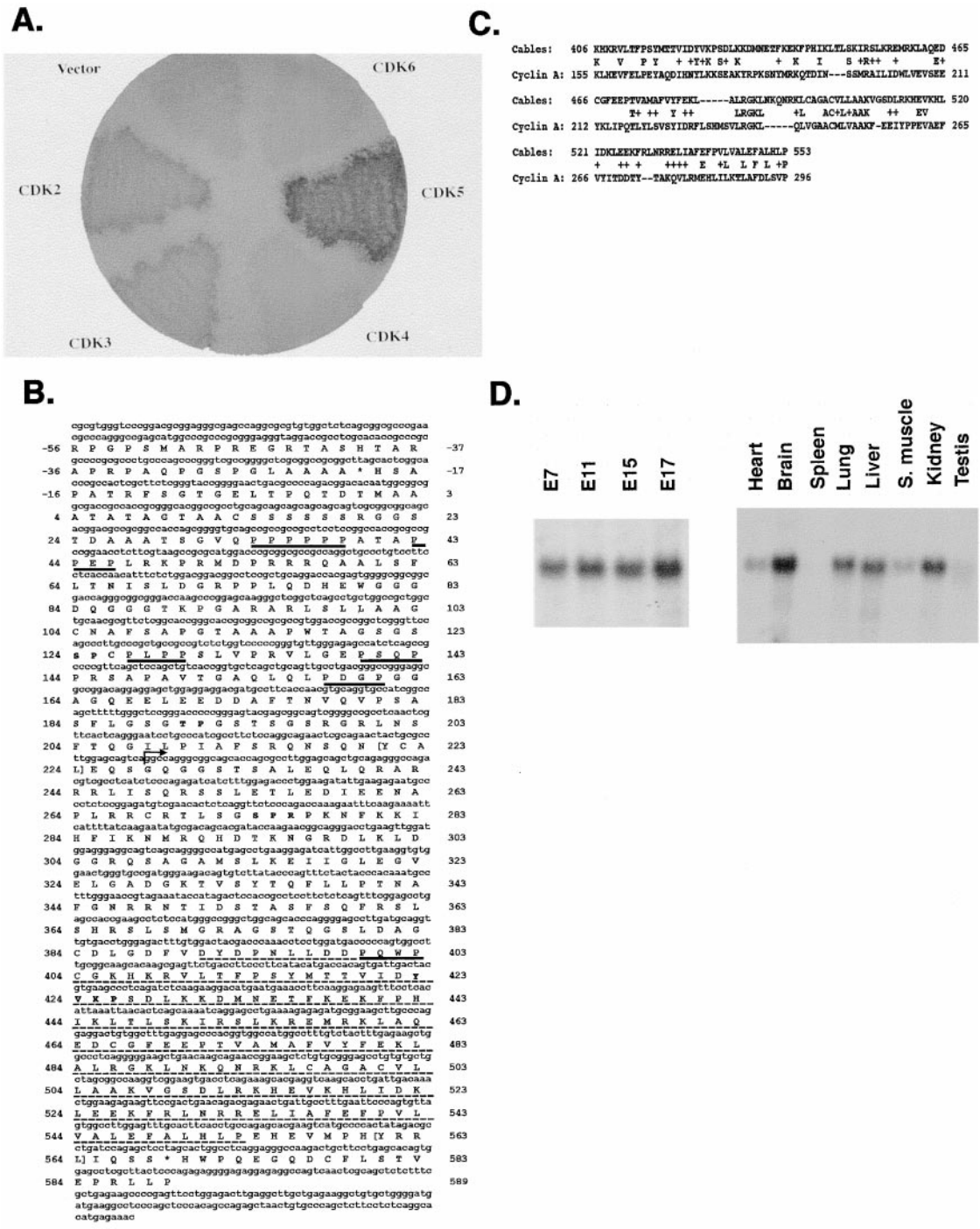


Figure 1. Cables Is a Novel Protein with Limited Homology to Cyclin A

(A) Cables interacts strongly with Cdk5 and weakly with Cdk2 and Cdk3. No interaction is seen with Cdk4 and Cdk6. Yeast strain MV101, containing Cables fused to the GAL4 transactivation domain, was transformed with different Cdk baits, and β-gal activity was detected by incubation of filters with X-gal.

(B) Nucleotide and amino acid sequence of Cables. The arrow indicates the position of the point of fusion of the cDNA recovered in the yeast two-hybrid screen. Potential serine/threonine and tyrosine phosphorylation sites are in bold type. PXXP domains are underlined. The axonal sorting motifs are bracketed. The region of homology to cyclin A is marked with a dashed underline.

(C) Region of homology between Cables and cyclin A using a gapped BLAST search (NCBI).

(D) Northern blots (Clontech) of multiple mouse tissues and whole embryonic mouse at different stages of development.

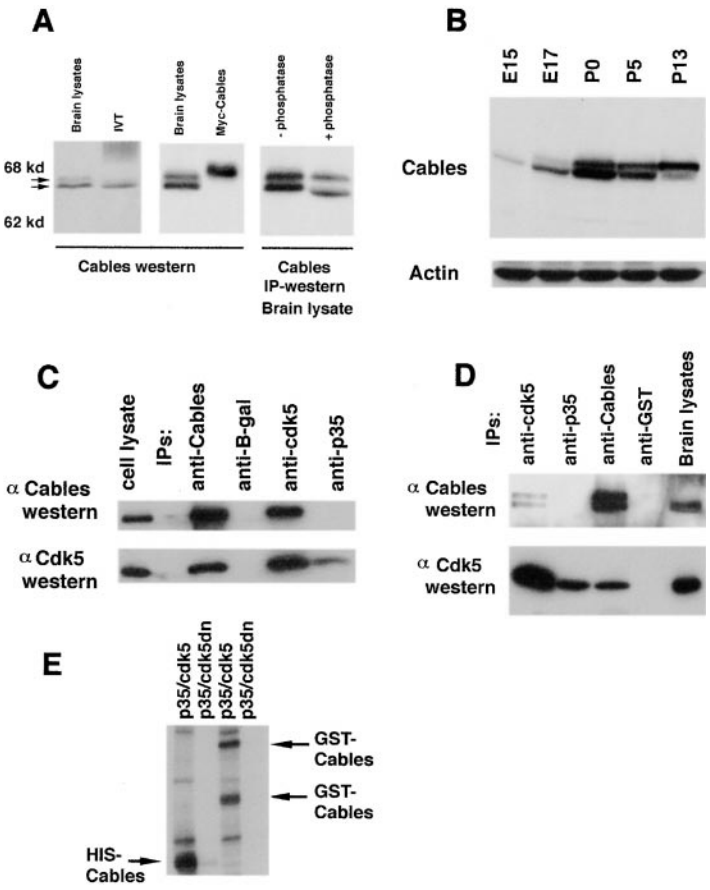


Figure 2. Cables Associates with Cdk5 in Brain Lysates

(A) Affinity-purified anti-Cables antisera recognize Cables in brain lysates as a doublet of 68 kDa that migrates with Cables synthesized in rabbit reticulocyte lysate (IVT), slightly faster than with Myc-tagged Cables transfected into COS7 cells. Cables immunoprecipitated from brain lysate was treated with or without phosphatase prior to an anti-Cables Western blot.

(B) A Western blot of brain lysates (100 μg) from different stages of mouse development was probed with anti-Cables antisera and reprobed with antibodies against actin as a loading control.

(C) COS7 cells were transfected with Cables, Cdk5, and p35. Immunoprecipitations with antibodies to Cables, Cdk5, p35, and β-gal were performed followed by anti-Cables and anti-Cdk5 Western blots.

(D) Immunoprecipitations with antibodies to Cables, Cdk5, p35, and GST were performed from mouse brain lysate. Western blots show that Cables and Cdk5 coimmunoprecipitate.

(E) In vitro $^{32}P\gamma$-ATP kinase assays with transfected p35/Cdk5 (wild-type kinase) or p35/Cdk5dn (inactive kinase) were performed using bacterially produced His_6-Cables and GST-Cables as substrate. A parallel Western blot showed that the indicated bands reacted with anti-Cables antisera (arrows). GST-Cables prepared in *E. coli* contains several breakdown products.

Cables Is a Cdk5 Substrate

In light of the three potential Cdk phosphorylation sites present in Cables, we tested if Cables could be phosphorylated by the p35/Cdk5 kinase. Figure 2E shows that both GST-Cables and His_6-Cables fusion proteins could be readily phosphorylated by the p35/Cdk5 kinase in vitro. The extent of Cables phosphorylation by the p35/Cdk5 kinase is similar to that of histone H1, a well-known substrate of Cdk5, when identical amounts of protein were used in the kinase reactions (data not shown). Phosphatase treatment downshifted Cables on SDS–PAGE (Figure 2A), suggesting that Cables is a phosphoprotein in vivo.

Cables Is Expressed in Postmitotic Neurons

As it was shown previously that the p35/Cdk5 kinase is expressed in postmitotic neurons of the cerebral cortex, we asked if Cables was also present in postmitotic neurons. To examine protein expression, Cables immunohistochemistry was performed on coronal and sagittal sections of mouse brain of different developmental stages using affinity-purified anti-Cables antisera (Figure 3). Preabsorbed antiserum was used as a negative control to show specificity (Figure 3B). Strong staining is seen in neurons of the subplate, cortical plate, and marginal zone of E18 mouse embryos (Figure 3C). Cables staining is also present in the subventricular zone. No detectable staining was seen in neurons of the ventricular zone when E16 sections were examined (data not shown). In postnatal day 1 (P1) sections, staining is again seen in the subplate and marginal zone, as well as in the deep layers of the cortex (V/VI), but less staining is present in the more superficial cortical layers (Figure 3A). In P7 brain sections, strong staining is seen in all layers of the cerebral cortex with prominent staining in the cell body (Figure 3D). In situ hybridization was also performed on sections of various ages, which is consistent with the data obtained from immunohistochemistry. Collectively, these results indicate that Cables is present in postmitotic neurons. High levels of Cables were also seen in the CA1 and CA3 regions of the hippocampus, and less staining was seen in the dentate gyrus (Figure 3E). Hippocampal neurons prepared from E16 mice show that in immature neurons without a dominant process, Cables is present in the cell body and proximal region of the developing axonal shaft, as revealed by Deltavision deconvolution microscopy (Figure 3F). In more mature neurons with one dominant process, Cables is also present in the axonal growth cone but not in the distal part of the axon shaft or in dendritic growth cones (Figure 3G), suggesting a specific function of Cables in the axonal growth cones.

Association of Cables and c-Abl

In addition to the potential Cdk phosphorylation sites, Cables contains six minimal SH3 domain binding motifs (PXXP; Cicchetti et al., 1992; Ren et al., 1993), two of which contain additional proline residues (PPXP and

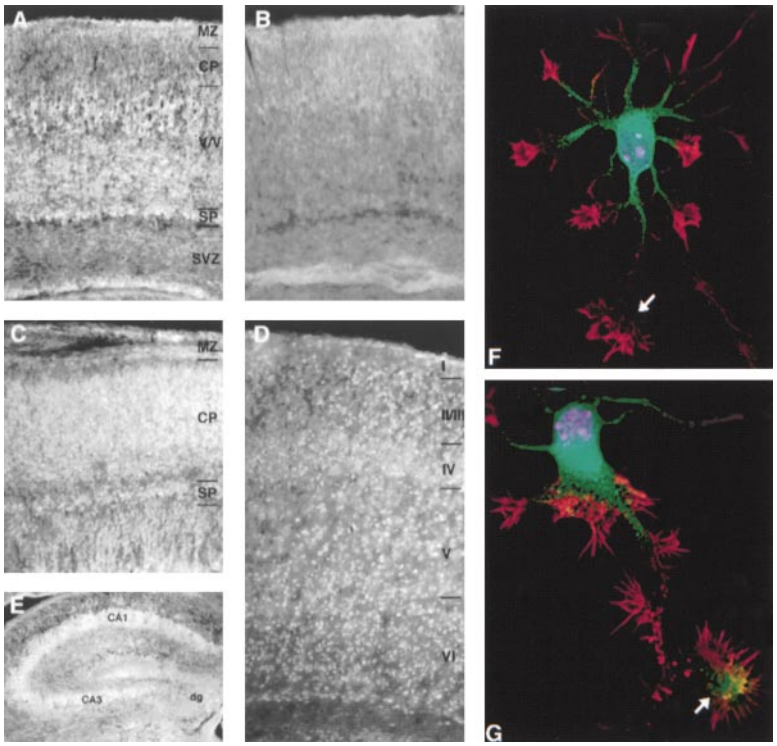


Figure 3. Cables Is Present in Cortical and Hippocampal Neurons

In coronal sections of E18 mouse brain, strong Cables immunostaining is seen in the neurons of the subplate (SP), cortical plate (CP), and marginal zone (MZ) (C). In P1 sections (A and B), staining is seen in the subplate, marginal zone, and early layers of the cortex (V/VI). No staining is seen with preabsorbed antisera (B). In P7 sections (D and E), strong staining is seen in all layers of the cerebral cortex and in the hippocampus (CA1 and CA3) with less staining of the dentate gyrus (dg). Magnification is 100× for (A) through (D), and 200× for (E). Embryonic hippocampal neurons were labeled with DAPI (blue), Texas red phalloidin (red), and antiserum to Cables followed by FITC-labeled secondary antibody (green). Texas red phalloidin labels both axonal and dendritic growth cones. At early stages, Cables is detected in the cell body and proximal region of the developing axon shaft but is not detected in the growth cone (arrow, [F]). As the axon elongates, Cables is detected in the axonal growth cone (arrow, [G]) but is not detected in the distal part of the axon shaft or in the dendritic growth cones. Magnification is 600×.

PXPP) with good potential for binding to the SH3 domain of the c-Abl tyrosine kinase (Cicchetti et al., 1992; Dai and Pendergast, 1995; Shi et al., 1995). To test if Cables binds to SH3 domain–containing proteins, Cables transcribed and translated in vitro was mixed with glutathione S-transferase (GST) fusion proteins containing various SH3 domains. Cables bound to GST-c-Src and GST-c-Abl SH3 domains but not GST-NCK or GST alone (Figure 4A). Compared with the 10% input in lane 1, nearly all of the available Cables bound to GST-SrcSH3 and GST-AblSH3, suggesting a high affinity interaction. We further verified these interactions in transfected cells. While a robust association was readily observed between Cables and c-Abl when coexpressed in COS7 cells, we failed to detect a similar association between Cables and c-Src, including the F527 mutant (Figures 4B and 4E). As the SH3 domain of c-Abl associated with Cables in vitro, an activated version of c-Abl with the SH3 domain deleted (ΔXB; Jackson and Baltimore, 1989) and a catalytically inactive mutant of c-Abl (K290M; Van Etten et al., 1994) were tested for binding to Cables in transfection assays. Surprisingly, the ΔXB mutant bound to Cables, albeit to a lesser extent than did wild-type c-Abl (Figure 4C). Conversely, the K290M mutant displayed a more stable interaction with Cables. The AblΔXB/K290M double mutant also associated with Cables (data not shown). These results suggest that both the SH3 domain and a region outside the SH2 and SH3 domains of c-Abl are involved in association with Cables. A dot blot of overlapping 15 amino acid c-Abl peptides was probed with Cables, and multiple binding regions were identified, many of which are in the Abl C terminus (data not shown). However, an Abl deletion mutant missing both the SH3 domain and C terminus maintained strong binding to Cables in vivo, suggesting that a region different from the SH3 and SH2 domains and the C terminus was also involved in binding (data not shown).

As mentioned, Cables contains six minimal SH3 domain binding motifs (PXXP); 5 of the 6 PXXP sites present in Cables are located in the N-terminal one-third of the protein. The yeast clone is missing these sites and showed little association with c-Abl (Figure 4F), suggesting that this region is important in c-Abl binding.

Cables Is a Substrate of c-Abl

In light of the association between Cables and c-Abl and the presence of a consensus tyrosine phosphorylation site for c-Abl in Cables, we determined if Cables could be phosphorylated by c-Abl. Strong tyrosine phosphorylation of Cables was evident upon coexpression of active Abl with Cables (Figure 4D) and, to a lesser extent, upon coexpression of Cables with active Src (SrcY527F). As no in vivo interaction of Cables and c-Src or the 527 mutant was detected (Figure 4E), the possible phosphorylation of Cables by c-Src remains to be determined. No tyrosine phosphorylation was seen when Cables was transfected alone. In cortical lysates of E15 mice, anti-phosphotyrosine antibody recognizes Cables, indicating that in vivo Cables exists as a tyrosine-phosphorylated protein (Figure 5E). Thus, Cables is likely to be an in vivo substrate of c-Abl.

Phosphorylation of Cdk5Y15 by c-Abl

The observation that Cables interacts with a serine/threonine kinase and a tyrosine kinase raised the possibility of cross-regulation of the two kinases via Cables. We failed to detect a difference in the level of c-Abl tyrosine kinase activity in the presence of increasing amounts

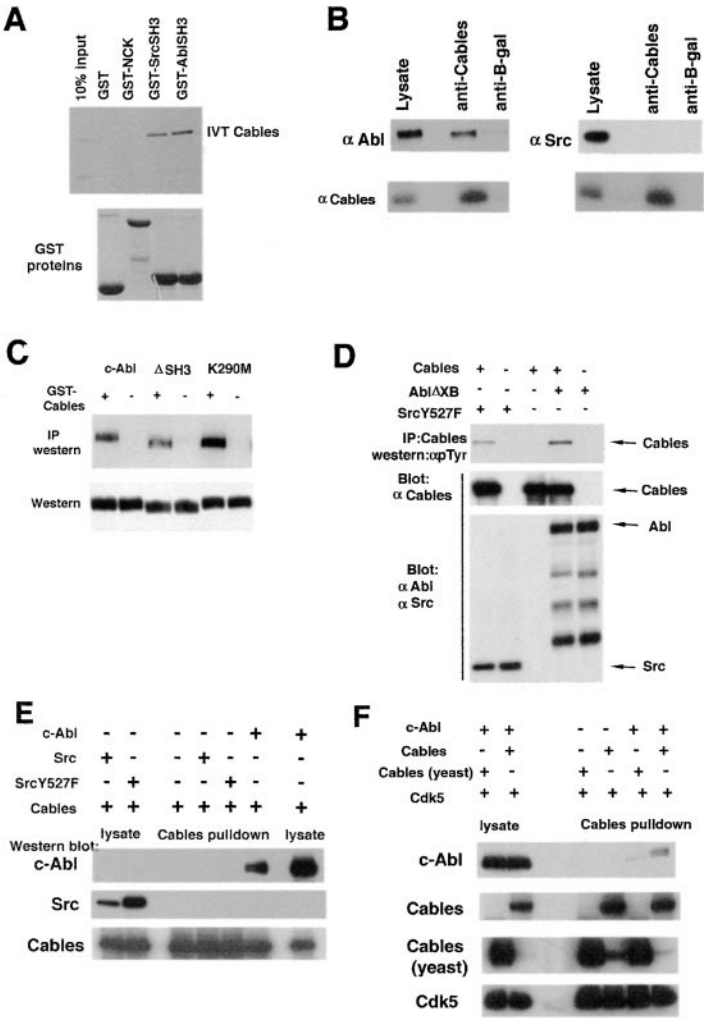


Figure 4. Cables Binds to and Is Tyrosine Phosphorylated by c-Abl

(A) In vitro translated (IVT) ^{35}S-labeled Cables was mixed with GSH beads bound to GST, GST-NCK, GST-SrcSH3, and GST-AblSH3. The bottom panel shows a Coomassie blue–stained gel of the proteins bound to the GSH agarose.

(B) COS7 cells were transfected with Cables and c-Abl or c-Src, and cell lysates were subjected to Cables or control (β-gal) immunoprecipitations followed by anti-Abl or anti-Src Western blots.

(C) Lysates of COS7 cells transfected with c-Abl, SH3-deleted Abl (ΔXB), or kinase-inactive Abl (K290M) with or without GST-Cables were subjected to a GSH pull-down and Abl Western blot. The bottom panel shows an Abl Western blot of the cell lysates.

(D) COS7 cells were transfected with Cables and active Src (SrcY527F) or active Abl (AblΔXB), and cell lysates were subjected to Cables immunoprecipitations followed by an anti-phosphotyrosine Western blot. The bottom panels show Cables, Src, and Abl in the cell lysate.

(E) COS7 cells were transfected with Cables and c-Abl, c-Src, or Src527 mutant, and cell lysates were subjected to Cables precipitations followed by anti-Abl or anti-Src Western blots.

(F) COS7 cells were transfected with Cdk5, c-Abl, and full-length or partial (yeast clone) Cables, and lysates were subjected to Cables precipitations followed by Abl, Cables, and Cdk5 Western blots.

of p35/Cdk5, as indicated by c-Abl autophosphorylation in an in vitro kinase assay (data not shown). Interestingly, Cdk5 became tyrosine phosphorylated when Cables or AblΔXB was overexpressed, and the level of tyrosine phosphorylation increased when both Cables and AblΔXB were coexpressed (Figure 5A). Phosphorylation of the Y15 residue is a known regulatory event for the cyclin-dependent kinases (Morgan, 1995). This residue is conserved in Cdk5. A Y15 to phenylalanine Cdk5 mutant (F15) was no longer phosphorylated by Cables and AblΔXB (Figure 5A), demonstrating that tyrosine phosphorylation of Cdk5 occurs on the highly conserved Y15 residue. The F15 Cdk5 mutant is able to interact with p35 and Cables to a wild-type extent (data not shown). To further verify the phosphorylation of Cdk5 by c-Abl, bacterially produced 6×His-Cdk5 fusion protein was used as an in vitro substrate for c-Abl. An anti-phosphotyrosine blot and ^{32}Pγ-ATP incorporation showed that Cdk5 is a direct substrate of the c-Abl kinase (data not shown). To determine if Cdk5 is tyrosine phosphorylated in vivo, an anti-phosphotyrosine Western blot was performed on Cdk5 immunoprecipitates from E15 mouse brain lysates. As demonstrated in Figure 5E, that Cdk5 is tyrosine phosphorylated in vivo. In addition, a phospho-Cdc2 (Tyr15) antibody that is specific for Y15-phosphorylated Cdc2 and Cdk2 reacted only with tyrosine-phosphorylated Cdk5, as evidenced by transfection of Cdk5 with or without active Abl (Figure 5F). The antibody reacted with Cdk5 from brain lysates, further suggesting that Cdk5Y15 is phosphorylated in vivo.

Y15 Phosphorylation of Cdk5 by c-Abl Enhances Cdk5 Kinase Activity

Y15 phosphorylation of Cdc2 and Cdk2 by the Wee1 family kinases is inhibitory and must be relieved by the Cdc25 family of phosphatases for kinase activation. Surprisingly, we found that Cdk5F15 is much less active (but does have some kinase activity) than is the wild-type kinase (Figures 5A and 5B), yet it binds to p35 as well as or better than wild-type Cdk5. This result suggests that Y15 phosphorylation is stimulatory for Cdk5 kinase activity. To test this hypothesis directly, kinase activity of p35/Cdk5 was compared in the absence or presence of Cables and c-Abl. Higher levels of p35/Cdk5 kinase activity were consistently observed in the

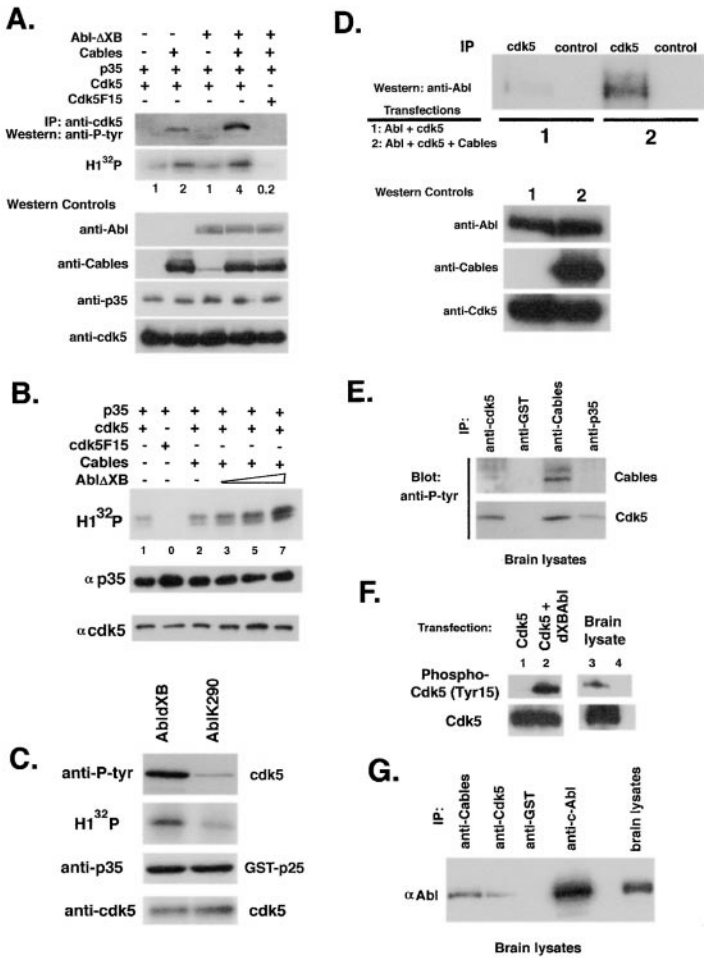


Figure 5. Cables Links Cdk5 and c-Abl, Facilitating Cdk5 Tyrosine Phosphorylation and Stimulation of Kinase Activity

(A) COS7 cells were transfected with p35, Cdk5, or Cdk5F15 and Cables, AblΔXB, or both, and cell lysates were subjected to Cdk5 immunoprecipitation followed by an anti-phosphotyrosine Western blot. Cdk5 activity was assessed by histone H1 kinase assay. In the lower panel, the lysates were reprobed with antibodies to c-Abl, Cables, p35, and Cdk5 to assess protein levels.

(B) COS7 cells were transfected with p35, Cdk5 or Cdk5F15, Cables, and increasing amounts of AblΔXB. Cell lysates were subjected to p35 immunoprecipitation followed by in vitro $^{32}P\gamma$-ATP kinase assays using histone H1 as a substrate. The lower panels show a parallel p35 immunoprecipitation followed by p35 and Cdk5 Western blots.

(C) Baculovirus-produced and -purified GST-p25 and Cdk5 were mixed with Abl immunoprecipitates from cells transfected with active Abl (AblΔXB) or inactive Abl (AblK290M) in the presence of cold ATP and kinase buffer followed by a Western blot for anti-phosphotyrosine. Similarly, the baculovirus proteins were mixed with $^{32}P\gamma$-ATP and histone H1 in a kinase assay. When active Abl is mixed with histone H1, no kinase activity is seen. The lower panel shows that the levels of p25 and Cdk5 were the same in both reactions.

(D) COS7 cells were transfected with Cdk5 and c-Abl in the presence or absence of Cables, and cell lysates were subjected to Cdk5 or control immunoprecipitations followed by anti-Abl Western blot.

(E) Immunoprecipitations with antibodies to Cables, Cdk5, p35, and GST were performed from E15 mouse brain lysate and followed by anti-phosphotyrosine Western blot.

(F) Immunoprecitipations of Cdk5 (lanes 1–3) or control antibody (lane 4) from transfected cell lysates and E15 mouse brain lysates were probed with an antibody specific for Y15-phosphorylated Cdk5 and anti-Cdk5.

(G) Immunoprecipitations with antibodies to Cables, Cdk5, GST, and c-Abl were performed from E15 mouse brain lysates followed by anti-Abl Western blot.

presence of Cables and c-Abl or AblΔXB measured either by histone H1 phosphorylation (Figures 5A and 5B) or p35 autophosphorylation. The p35/Cdk5 kinase activity increased with increasing amounts of AblΔXB, up to 7-fold higher than did basal p35/Cdk5 kinase activity (Figure 5B), and correlated with Cdk5 tyrosine phosphorylation (Figure 5A). Further support for these observations was gained using purified components. p25/Cdk5 kinase was produced in insect cells after baculovirus infection, incubated with either AblΔXB or AblK290M immunoprecipitated from transfected COS7 cells, and tyrosine phosphorylation, as well as kinase activity of Cdk5, were evaluated. Like the Cdk5 produced in *E. coli*, Cdk5 synthesized in insect cell lysates was strongly phosphorylated by AblΔXB compared with AblK290M (Figure 5C). Histone H1 phosphorylation assays indicated that Cdk5 kinase activity correlated with the increase in tyrosine phosphorylation and was at least 3-fold higher in the presence of AblΔXB than in the presence of AblK290M (Figure 5C). No phosphorylation of histone H1 by AblΔXB was seen (data not shown). Together, these results strongly suggest that Y15 phosphorylation of Cdk5 by c-Abl upregulates its kinase activity. p35 immunoprecipitates from transfected cells or E15 brain lysates contain tyrosine-phosphorylated Cdk5 (Figure 5E), which is consistent with the notion that Y15-phosphorylated Cdk5 is more active when associated with p35 than is unphosphorylated Cdk5.

Cdk5, Cables, and c-Abl Trimolecular Complex Formation In Vivo

As tyrosine phosphorylation of Cdk5 by c-Abl appears to be enhanced by Cables, it is possible that Cables is necessary to recruit c-Abl to Cdk5. To examine this possibility, Cdk5 immunoprecipitations followed by c-Abl Western blots were performed from cells transfected with Cdk5 and c-Abl with or without Cables. Figure 5D shows that while only a marginal interaction was observed in the absence of Cables, a strong interaction between Cdk5 and c-Abl was observed when Cables was expressed. In addition, we do not have evidence for a direct interaction between Cdk5 and c-Abl in vitro.

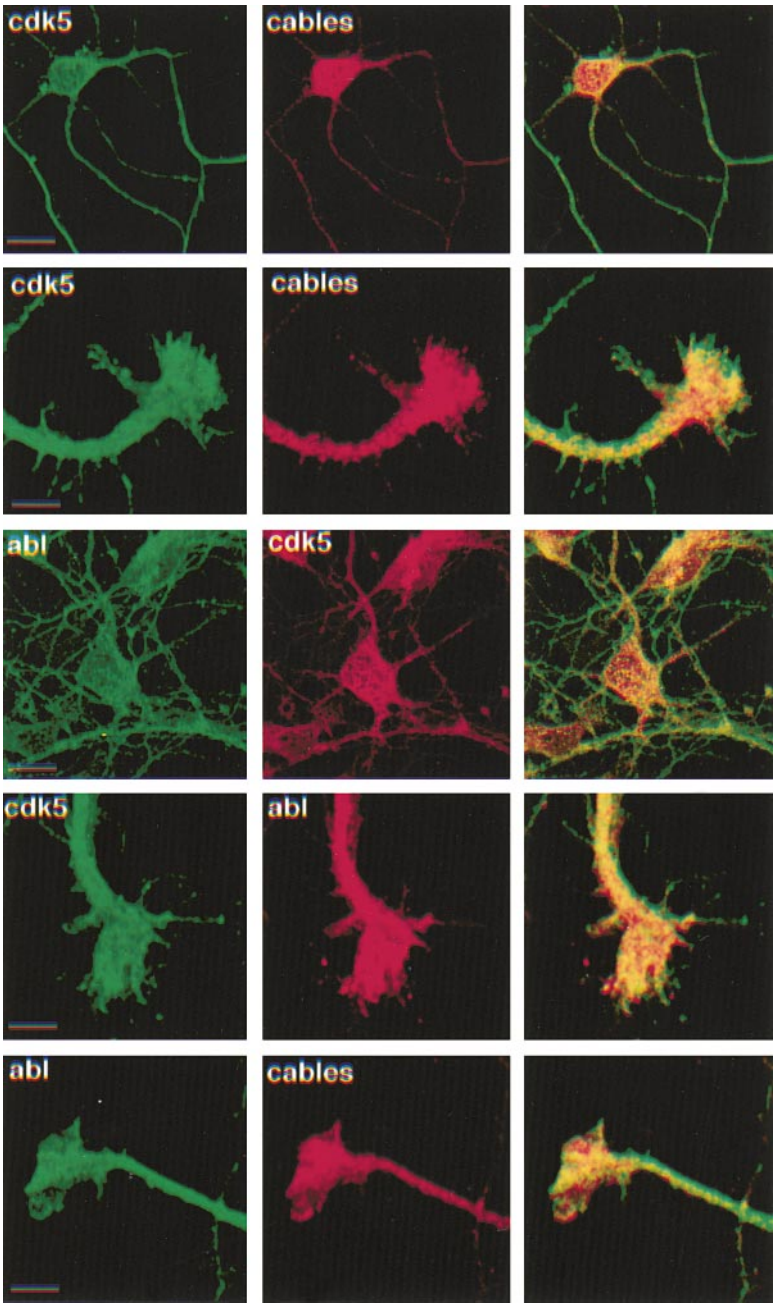


Figure 6. Cdk5, Cables, and c-Abl Colocalize in Primary Cortical Neurons

Immunostaining of Cdk5, c-Abl, and Cables was performed on cultured primary cortical neurons using confocal microscopy. As seen in Figure 3, intense Cables signal was present in the soma of the cultured neurons. In growth cones, Cdk5, c-Abl, and Cables colocalized in the central regions of the growth cones, while Cdk5 extended further to the periphery. Preabsorption of the Cables antibody with cognate antigen eliminated the staining signal, demonstrating the specificity of staining (data not shown). Scale bars, 10 μm.

Thus, it is suggestive that Cables facilitates the interaction between Cdk5 and c-Abl. To examine if a trimolecular complex was present in vivo, Cdk5 and Cables immunoprecipitations were performed using E17 cortical lysates followed by c-Abl Western blots. Figure 5G shows that c-Abl is present in both Cdk5 and Cables immunoprecipitates. This result corroborates data shown in Figure 5D and indicates the existence of trimolecular complexes containing Cdk5, c-Abl, and Cables in vivo. Therefore, Cables may act as an adaptor or cable between Cdk5 and c-Abl. To examine the extent of the subcellular colocalization of these proteins, immunostaining of Cdk5, c-Abl, and Cables was performed on cultured primary cortical neurons followed by confocal microscopy. This revealed that Cables predominates in the soma of cultured cortical neurons (Figure 6). Cables staining was evident in axonal growth cones in which Cdk5 is also enriched (Figure 6; Nikolic et al., 1996), and the two proteins colocalized. Cables seemed to be present more centrally in the growth cone, while Cdk5 extended to the peripheral lamelipodia. c-Abl appears to be highly enriched in neurites, in agreement with a previous report on c-Abl distribution in the nervous system (Koleske et al., 1998). In axonal growth cones, Cdk5, c-Abl, and Cables fully colocalize in central regions (Figure 6). Together, the immunostaining results suggest that association between Cables, c-Abl, and Cdk5 takes place in the neuronal peripheries, such as axonal growth cones in which dynamic actin cytoskeleton rearrangements occur.

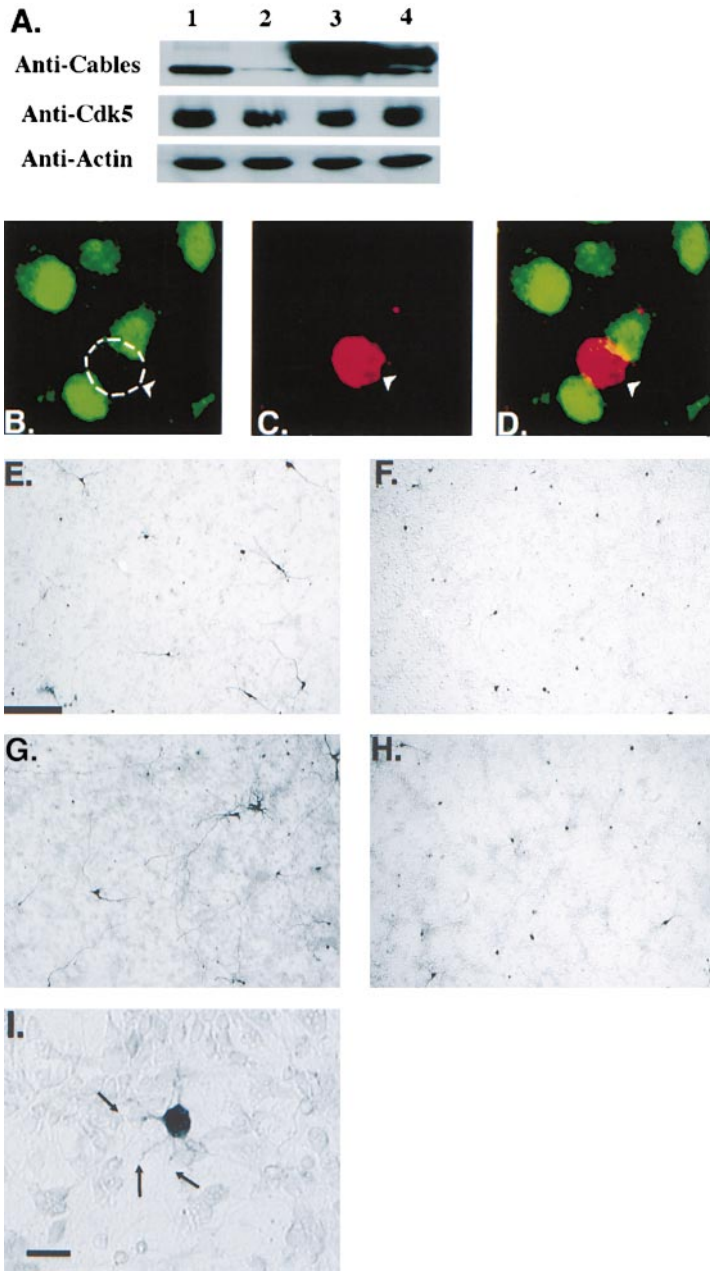


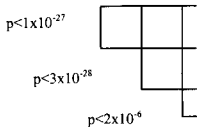
Figure 7. Expression of Antisense Cables in Primary Cortical Neurons Inhibits Neurite Outgrowth

Western blot of HeLa cells transfected with empty vector (lane 1), antisense Cables (lane 2), sense (Myc-tagged) Cables (lane 3), or sense + antisense Cables (lane 4) and probed with antibodies to Cables, Cdk5, and β-actin (A). Immunostaining is shown of endogenous Cables (green) and β-gal (red) in cultured cortical neurons transfected with β-gal and antisense Cables. Antisense Cables–expressing neuron is devoid of Cables compared with nontransfected neurons (B–D). Cultured cortical neurons were transfected with the following CMV expression constructs: β-gal alone (E), β-gal in combination with Cables antisense (F), β-gal in combination with active Abl (AblΔSH3) (G), and β-gal in combination with Cdk5dn (H). High-magnification Nomarski image of antisense Cables–transfected neuron is shown in (I). Scale bar, 200 μM (E–H) and 20 μM (I).

Cables Is Essential for Neurite Outgrowth

Cortical neurons obtained from E17–E18 rat embryos mature morphologically during the course of culturing and can be made to express ectopic protein by transfection using a calcium phosphate method (Nikolic et al., 1996; Dudek et al., 1997). This approach has been used previously to assess the effect of Cdk5 and p35 on neurite outgrowth (Nikolic et al., 1996). It was shown that dominant-negative Cdk5 (Cdk5dn, Cdk5N[144]) markedly inhibited neurite outgrowth. To evaluate the role of Cables in the axonal growth cones, we analyzed neurite length after transfection of sense and antisense Cables constructs in primary cortical neurons. The antisense Cables construct was demonstrated to reduce endogenous levels of Cables protein, but not Cdk5 or actin, when overexpressed in HeLa cells (Figure 7A). In addition, the antisense Cables construct decreased Cables protein levels in cortical neurons, as evidenced by immunostaining (Figures 7B–7D). Expression of the antisense Cables construct in cortical neurons caused extensive neurite shortening comparable to that of Cdk5N[144] (Figures 7E, 7F, 7H, and 7I; Table 1). This phenotype was reversed by coexpression of sense and antisense Cables. Rare neurons transfected with antisense Cables had long axons, possibly from expression of only the marker protein LacZ. Expression of sense Cables produced a minor inhibitory effect that might be due to a dominant-negative effect of expressing nonphysiological levels of Cables. In addition, to examine if neurite outgrowth was affected by the Cables-associated c-Abl

Table 1. Neurite Length



Identity	# of cells	<50μM (S.E.*)	>50μM	>100μM	>200μM	>300μM
βgal	218	5.4% (3.4%)	94.6% (3.4%)	78.6% (2.8%)	35.4% (1.1%)	13.8% (3.8%)
Sense Cables	261	16.0% (1.0%)	84.0% (1.0%)	53.8% (4.9%)	21.1% (4.1%)	8.5% (1.2%)
Anti-Sense Cables	341	49.7% (4.0%)	50.2% (4.1%)	24.4% (2.4%)	7.8% (0.8%)	4.9% (3.7%)
Sense + Anti-Sense	195	18.9% (10.4%)	80.9% (10.1%)	60.7% (13.8%)	30.9% (7.0%)	12.8% (3.1%)
DNK5	213	52.1% (3.0%)	48.0% (2.9%)	17.9% (4.5%)	5.9% (2.8%)	1.7% (1.7%)
AblΔSH3	190	10.2% (3.2%)	89.9% (3.1%)	71.1% (0.1%)	47.9% (8.9%)	37.0% (13.0%)
AblK290M	189	12.8% (2.2%)	87.2% (2.2%)	54.6% (4.6%)	16.6% (1.4%)	8.6% (2.5%)

Asterisk denotes standard error. Significant ANOVA p values (compared with β-gal) are indicated.

kinase, active Abl was expressed in the primary neurons. An increase in neurite length was found with expression of active Abl but not inactive Abl (Figures 7E and 7G; Table 1).

To quantitate the differences between neurons with β-galactosidase (β-gal), Cdk5dn, Cables sense and antisense constructs, and Abl constructs, the neurite lengths of transfected cells were examined using a cooled charge-coupled device (CCD) camera and Metamorph image analysis software (Universal Imaging). For each transfection, 200–300 positive cells were examined, as described in the Experimental Procedures. All measurements were performed in a "blind" manner, and each transfection was analyzed by at least three observers. The longest neurite emanating from each cell was measured, and only if it was longer than the diameter of the cell body. Analysis of the obtained data revealed that 50% and 24% of the cells transfected with antisense Cables elaborated neurites longer than 50 and 100 μm, respectively, in contrast to 95% and 79% of mock transfected neurons (Table 1). Similarly, less neurons transfected with the antisense construct showed neurites longer than 200 μm compared with the other transfections (Table 1). This effect was reversed when coexpressed with sense Cables. In contrast, 37% of cells transfected with active Abl elaborated neurites longer than 300 μm compared with 14% of mock transfected cells or cells expressing inactive Abl. Statistical analysis using ANOVA (Table 1) showed that compared with β-gal, transfection of Cdk5dn ($p < 3.2 \times 10^{-28}$) and antisense Cables ($p < 1.0 \times 10^{-27}$) resulted in significantly shorter neurites, which was reversed when sense and antisense Cables were coexpressed ($p = 0.069$).

Discussion

Cdk5 plays an important role in neuronal migration and neurite outgrowth. Mice lacking Cdk5 exhibit late embryonic to perinatal lethality and show many CNS abnormalities, including lamination defects of the cerebral cortex, abnormal hippocampal formation, and cerebellar defoliation (Ohshima et al., 1996). To date, only a few Cdk5-interacting molecules are known, including the Cdk5 activators p35 and p39 (Lew et al., 1994; Tsai et al., 1994; Tang et al., 1995). In this paper, we describe a novel protein, Cables, that binds directly to Cdk5 and associates with Cdk5 in brain lysates. Cables is expressed at the highest levels in the brain, where it is present in postmitotic neurons of the cerebral cortex. Furthermore, Cables and Cdk5 colocalize at the tips of growing axons. Based on the expression pattern, colocalization, and strong association in brain lysates, it is likely that the association of Cdk5 and Cables is part of the signaling pathway that operates during brain development.

Cables Contains Sequence Motifs for Cdk and SH3 Binding

Cables is a 568 amino acid protein that exhibits little homology to other proteins in the databases. The C-terminal portion of Cables binds to Cdk5, as indicated by the partial clone isolated from the two-hybrid yeast screen. A 200 amino acid stretch in the C terminus of Cables is 25% identical and 45% similar to cyclin A in a region that coincides with the cyclin box, which is a key element of the cyclin–Cdk interface. Cables contains the conserved alanine residues that are characteristic of the cyclin fold and also the lysine and glutamic acid residues that contact the backbone of Cdk2 in the cyclin molecules (Jeffrey et al., 1995). This region interacts with the PSTAIRE helix of the Cdks. Cdk5 contains the PSSALRE sequences in place of PSTAIRE, and this region is necessary for p35 binding to Cdk5 (Y. Ramos and L.-H. T., unpublished data). Thus, it appears that both Cables and p35 bind to regions of Cdk5 that include the PSSALRE sequence. Therefore, stable binding of both Cables and p35 to Cdk5 at the same time may be difficult, which fits with our experimental data.

Cables also contains six PXXP sites, which is the minimal requirement for SH3 domain binding (Cicchetti et al., 1992; Ren et al., 1993). Based on the PXXP sites, we tested and found that Cables binds strongly to the SH3 domains of c-Src and c-Abl, but not other SH3-containing proteins, like Nck, in vitro. Furthermore, we found that Cables bound to full-length c-Abl, but not c-Src or the 527 mutant, upon cotransfection. The c-Abl binding was not dependent on the SH3 domain of c-Abl, as an SH3 deletion mutant bound almost as strongly as did wild-type c-Abl. A dot blot of overlapping 15 amino acid c-Abl peptides showed that Cables bound to multiple overlapping peptides of c-Abl, many in the C terminus. A deletion mutant of c-Abl lacking both the SH3 domain and C terminus bound Cables, suggesting that Cables can interact with c-Abl at many sites. The multiple binding sites of Cables to c-Abl in regions with no Src homology would support our finding that Cables does not stably bind c-Src in vivo. Other c-Abl interactors, such as the recently described Abi-2 protein, interact with at least two domains in c-Abl, the SH3 and C-terminal domains (Dai and Pendergast, 1995).

Cables exists as a doublet of ~68 kDa on Western blots of brain lysates using two different affinity-purified antisera. The reason for the doublet is not clear at this time, but both forms associate with Cdk5. Although strong binding of Cables and Cdk5 can be demonstrated using either Cdk5 or Cables antibodies, no association of Cables and p35 has been detected in transfected cells or brain lysates. This suggests that Cdk5 stably exists in at least two separate complexes, one with p35 and the other with Cables. It is possible that Cdk5 translocates from Cables to p35 while remaining Y15 phosphorylated, such that there is a transient but unstable interaction among Cdk5, p35, and Cables. The presence of such a complex would also allow the p35/Cdk5 kinase to phosphorylate Cables. Alternatively, heterologous p35/Cdk5 complexes may phosphorylate Cables.

Cables Is a Substrate of Cdk5 and c-Abl

Cables exists in vivo as a phosphoprotein; treatment with phosphatase shifts at least the faster migrating species but does not condense the doublet. In addition to Cdk5 binding, Cables may be an in vivo substrate of Cdk5, since it contains three potential Cdk5 phosphorylation sites (serine proline/threonine proline), one of which is followed by basic residues and is a preferred Cdk5 phosphorylation site (Beaudette et al., 1993; Songyang et al., 1996). Cables is readily phosphorylated by the p35/Cdk5 kinase in in vitro kinase assays. Cotransfection of Cables with active Abl resulted in Cables tyrosine phosphorylation. Thus, Cables interacts with and can be phosphorylated by c-Abl, suggesting that it is an in vivo substrate. Phosphorylation of Cables by Cdk5 and c-Abl may cause rapid dissociation of Cables from this complex and allow free p35/Cdk5 to phosphorylate substrates involved in neurite outgrowth and neuronal migration.

Cables Enhances Y15 Phosphorylation of Cdk5 by c-Abl Tyrosine Kinase

Cables associates with both the Cdk5 serine/threonine kinase and c-Abl nonreceptor tyrosine kinase. We found that Cdk5 became tyrosine phosphorylated in the presence of c-Abl, and this was enhanced by inclusion of Cables. In transfection experiments, expression of either c-Abl or Cables resulted in Cdk5 tyrosine phosphorylation. Endogenous c-Abl and Cables are detected in the cell lysates, which may explain why c-Abl alone phosphorylates Cdk5. When transfected together, there was an increased level of Cdk5 tyrosine phosphorylation. It is not clear why transfection of Cables itself leads to Cdk5 tyrosine phosphorylation. Endogenous c-Abl is thought to be inhibited by cellular inhibitors that bind to the SH3 domain of c-Abl (Pendergast et al., 1991; Wen and Van Etten, 1997). It is possible that Cables acts not only as an adaptor molecule but also as a c-Abl activator. Cables binds to the SH3 domain of c-Abl, and perhaps this binding displaces inhibitor molecules, leading to Cdk5 tyrosine phosphorylation. Mutants of Cables that no longer bind to the SH3 domain of c-Abl will be useful for examining this hypothesis.

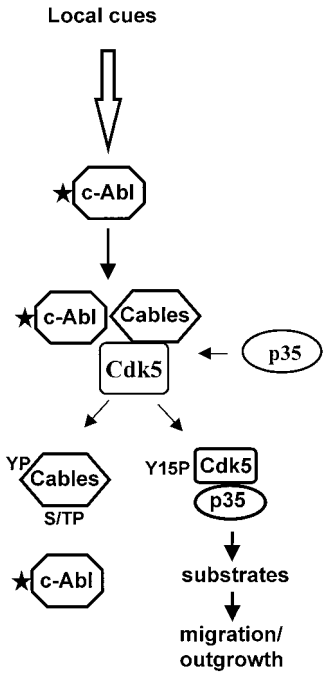


Figure 8. Model Illustrating Possible Cdk5/Cables/c-Abl Pathway Involved in Neuronal Development

c-Abl is activated in response to local stimuli and phosphorylates the Y15 residue of Cdk5, which is enhanced by Cables. p35 binds to Y15-phosphorylated Cdk5, and the Cdk5/Cables/c-Abl complex dissociates. c-Abl and p35/Cdk5 may both phosphorylate Cables.

Y15 Phosphorylation Augments p35/Cdk5 Kinase Activity

Evaluation of Cdk5 tyrosine phosphorylation, using an F15 mutant of Cdk5, showed that tyrosine phosphorylation occurred on Y15, an important Cdk regulatory site. Despite the sequence homology to Cdk2 and Cdc2, it was previously shown that Cdk5 is not activated by CAK and is neither phosphorylated nor inhibited by the Wee1 kinase (Poon et al., 1997). There is a report of in vivo Cdk5 tyrosine phosphorylation in rat cerebellum, but the site of phosphorylation and the kinase responsible were not known (Lazaro et al., 1996). Our data implicate the nonreceptor tyrosine kinases, especially c-Abl, as Cdk5 tyrosine kinases. The Y15 to F15 mutant of Cdk5 had dramatically less kinase activity, despite binding to p35 and Cables as well as or better than wild-type Cdk5. The data suggest that Cdk5 Y15 phosphorylation is a stimulatory posttranslational modification that upregulates Cdk5 kinase activity. Evidence for an inhibitory kinase activity distinct from Wee1 and Myt1 (Matsuura and Wang, 1996) was found to affect threonine 14 of Cdk5. Cdk5Y15 phosphorylation may also affect substrate specificity and/or localization of the kinase. Furthermore, an effect on Cdk5 activity imposed by tyrosine phosphorylation of p35 cannot be dismissed.

Cables, c-Abl, and Cdk5 Colocalize at the Neuronal Periphery

In the developing cerebral wall, Cables is expressed in postmitotic neurons but is absent in neuronal precursors. Cables is discontinuously expressed in the growth

cones of developing axons, where it colocalizes with Cdk5 and c-Abl. Similar expression is not seen in the growing tips of dendrites. We examined Cdk5 kinase activity in *abl*$^{-/-}$ mice but could not detect a difference (data not shown). If Cdk5 activity is only transiently upregulated in the axonal growth cones in developing neurons, a Cdk5 in vitro kinase assay may not be sensitive enough to detect a difference. Alternatively, c-Abl-related genes, such as *arg* (Kruh et al., 1990), may be redundant in phosphorylation of Cdk5. This is supported by the finding that the *c-abl* knockout mice lack a neuronal phenotype, while the *c-abl/arg* double knockout mice die at E9.5 with neural tube defects (Koleske et al., 1998). The early lethality of the double mutant makes it difficult to assess the profile of Cdk5 phosphorylation in these animals.

A Role for Cables in Axon Growth

In light of the preferential distribution of Cables in the growing tips of axons rather than dendrites, a possible role of Cables in axon growth was evaluated by overexpression of sense and antisense Cables constructs in primary cortical neurons. Transfection using the calcium phosphate method has been shown to be efficient in ectopic gene expression in postmitotic neurons (Nikolic et al., 1996; Dudek et al., 1997) and for assessing neurite outgrowth following overexpression of various molecules (Nikolic et al., 1996). Expression of the antisense Cables construct caused neurite shortening similar to that seen for dominant-negative Cdk5. This effect could be rescued using sense Cables, suggesting that the phenotype was specific. In contrast, expression of active Abl produced remarkably longer neurites than did inactive Abl or the control β-gal transfection, consistent with genetic data implicating Abl in axonal development and organization (Gertler et al., 1989, 1993).

When taken together, our data suggest that Cables serves as an adaptor molecule, facilitating Cdk5 tyrosine phosphorylation and regulation by c-Abl (Figure 8). Phosphorylation of key substrates involved in actin and microtubule dynamics by active Cdk5 is likely to contribute to its role in neuronal migration and neurite outgrowth. Furthermore, Cdk5 was shown to downregulate N-cadherin-mediated cell adhesion (Kwon et al., 2000). Data presented in this communication suggest that Cables mediates an interaction between c-Abl and Cdk5, and may positively affect brain development and neurite outgrowth by enhancing Cdk5 tyrosine phosphorylation and upregulation of kinase activity. Cables may also mediate an interaction between Cdk5 and mDab1 by binding to both Cdk5 and c-Abl.

Experimental Procedures

Yeast Two-Hybrid Screen

A genetic screen using the yeast interaction trap was performed as described (Vidal et al., 1996). The bait plasmid (pPC97, Leu2) Cdk5dn contained full-length Cdk5 with a point mutation at the aspartic acid 144 position (D144 to N144) fused to a yeast transcription factor GAL4 DNA binding domain. It was transformed into yeast strain MV101, which contained three reporter genes, *ura3*, *his3*, and *lacZ*. This strain in turn was transformed with an embryonic mouse E14 library fused to the GAL4 activation domain in the reporter plasmid (pPC86, Trp1). Two million primary library transformants were amplified and screened on Trp-Leu-His containing 10 mM 3-aminotriazole. Transformants (120) were selected as meeting the following criteria: (1) they grew on Ura-His + Trp-Leu plates or (2) they grew on Ura + His-Trp-Leu plates and turned blue when incubated with X-gal. Plasmids from these interacting clones were isolated by transformation of *E. coli* strain DH5α.

Cell Culture and Transfection

COS7 cells were propagated in Dulbecco's modified Eagle's medium with 4.5 g/l of glucose, 10% calf serum, and penicillin/streptomycin. Neuronal cortical cell cultures were prepared as described (Nikolic et al., 1996). Similarly, cultures of embryonic hippocampal neurons were prepared from E16 mouse hippocampus as previously described for E18 rat (Goslin and Banker, 1989). Transient transfections in COS7 cells were performed using the calcium phosphate method with 10–20 μg of total DNA (2.5 of μg CMV-Cdk5, 5 μg of CMV-p35, 5 μg of CMV-Cables, 5 μg of CMV-Abl, and 5 μg of CMV-Src). Transfections of primary cortical cultures were carried out on 3-day-old cortical cultures as described previously (Nikolic et al., 1996).

DNA Constructs

Full-length Cables was obtained from a mouse neonatal brain cDNA library (Clontech) and sequenced. For production of tagged mammalian constructs and bacterial fusion proteins, the Cables cDNA was subcloned in-frame into the eukaryotic GST expression vector PEBG (gift of B. Mayer, Children's Hospital, Boston, MA) at the BamHI site, PCDNA3.1/Myc-His B (Invitrogen) at the BamHI site, PCDNA3.1 (Invitrogen) at the BamHI site, pGEX-4T-2 (Pharmacia) at the BamHI site, and PET15b (Novagen) at the BamHI site. For production of the antisense Cables construct, Cables was cloned in reverse orientation into PCDNA3.1 (Invitrogen) at the BamHI site.

The mammalian Abl expression vector pcDNA-c4 Abl and its various internal deletion mutations (c4ΔSH3 = AblΔXB, c4ΔSH3ΔBcl) and point mutants (c4K290M) have been described previously (Jackson and Baltimore, 1989; Van Etten et al., 1994). The Src and SrcY527F constructs were gifts of D. Morgan (University of California, San Francisco). GST-SrcSH3, GST-AblSH3, and GST-NCK constructs and protein were a gift of B. Mayer, Children's Hospital.

To make Y15F Cdk5, human Cdk5 cDNA was cloned into the BamHI site of pAlter (Promega) and was mutagenized using the Promega Altered Sites II kit according to the manufacturer's instructions. The following oligonucleotide was used for mutagenesis: 5′-GAAGGCACCTTCGGAACTGTGTTC-3′. The mutant was confirmed by sequencing. The insert was cloned into the BamHI site of pcDNA3 (Invitrogen).

Protein Analysis

Transfected cell or brain lysate was produced in E1A lysis buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA [pH 8.0], 0.1% Nonidet P-40, 5 mM DTT, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml Na_3VO_4) and RIPA lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, 5 mM DTT, 10 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml Na_3VO_4). Proteins were analyzed by direct Western blotting (50 μg/lane) or blotting after immunoprecipitation (300–1000 μg/immunoprecipitation). COS7 cell extracts were immunoprecipitated with glutathione-Sepharose (GSH) beads, anti-Cdk5 (pAb C-8, Santa Cruz), anti-p35 (pAb C-19, Santa Cruz), and anti-Cables (pAb 64, see above). Immunoprecipitates were collected by binding to protein A–Sepharose or protein G–Sepharose. Western blots were probed with anti-Cdk5 (mAb DC17), anti-p35 (pAb C-19), anti-Cables (pAb 64), anti-Abl (mAb 8E9, Pharmigen), anti-phosphotyrosine (mAb 4G10, Upstate Biotechnology), anti-Src (mAb 327, gift of J. Brugge, Harvard Medical School, Boston, MA), anti-phospho-Cdc2 (Tyr15; pAb, New England Biolabs).

Production and Purification of GST-Tagged or His-Tagged Proteins

GST and His fusion proteins were expressed in *E. coli* and purified with GSH beads or nickel-charged beads according to the manufacturer's instructions (Novagen).

Immunohistochemistry

Primary neuronal cultures were fixed for 10 min in 4% paraformaldehyde and 4% sucrose in 1× phosphate-buffered saline (PBS) prewarmed to 37°C for 10 min. Fixed cells were washed three times in PBS containing 0.1% Triton X-100 and 10 mM glycine, once in PBS containing 0.1% Triton X-100, and incubated with 10% goat serum, 3% bovine serum albumin (BSA), and 2% Tween-20 in PBS for 1 hr at room temperature to block nonspecific binding of antibodies. Embryonic brains were fixed in 4% paraformaldehyde in PBS for 10–12 hr. After fixation, brains were equilibrated in a 20% sucrose solution and frozen in OCT compound (VWR, Boston, MA). Sections were thawed at room temperature, equalized in PBS, and blocked with 10% goat serum and 2% Triton X-100 in PBS for 1 hr at room temperature. The indicated primary antibodies diluted in 3% BSA and 0.2% Tween-20 in PBS were incubated with coverslips/slides for 16 hr at 4°C. Subsequently, the coverslips/slides were washed in PBS and exposed to FITC or Texas red–conjugated secondary antibodies for 1 hr at room temperature. After washing extensively in PBS, they were mounted in nonfade (Molecular Probes).

The following antibodies were used for immunohistochemistry at the indicated dilutions. Primary antibodies were Cdk5 (mAb DC39, 1:5), c-Abl (mAb K-12, Santa Cruz, 1:10), β-gal (mAb, Promega, 1:300; pAb, 1:500), and Cables (pAb 64, raised against whole protein, affinity purified, and concentrated against protein A, 1:25; specificity was evaluated by [1] using preabsorbed antisera and [2] staining transfected versus untransfected Swiss-3T3 cells, which contain undetectable protein by Western blot). Secondary antibodies were fluorescein- (FITC-) conjugated, affinity-purified goat anti-mouse IgG, 1:150 (Cappel); fluorescein- (FITC-) conjugated, affinity-purified goat anti-rabbit IgG, 1:150 (Sigma); Texas red–conjugated, affinity-purified goat anti-rabbit IgG, 1:150 (Cappel); Texas red–conjugated, affinity-purified $F(ab')_2$ sheep anti-mouse IgG, 1:150 (Cappel); and biotinylated, affinity-purified goat anti-rabbit IgG, 1:100 (Vector Laboratories).

Neurite Measurement

Cells were analyzed using a Zeiss LSM 410 confocal scanning microscope and a Deltavision deconvolution microscope, and sections, using a Nikon TE300 immunofluorescent microscope. Neurite length was analyzed using a Nikon TE300 microscope, a CCD camera, and Metamorph image analysis software (Universal Imaging) as described previously (Nikolic et al., 1996). Transfected neurons and their neurites were distinguished by immunodetection of β-gal as a marker protein. The axons and dendrites were measured for each neuron; however, only one longest neurite per cell was used for comparative purposes with other neurons. Lengths were determined as the distance between the edge of the cell body and the tip of the growth cone.

In Vitro Kinase Assay

Kinase assays were performed by washing immunoprecipitates three times with lysis buffer and once with kinase buffer (50 mM HEPES [pH 7.0], 10 mM $MgCl_2$, and 1 mM DTT). p35 levels, which have been shown to be limiting (Patrick et al., 1998), were equalized by Western blotting prior to immunoprecipitation. Subsequently, the beads were incubated with kinase buffer containing 2 μg of substrate and 5 μCi of $^{32}P\gamma$-ATP in a final volume of 50 μl at room temperature for 30 min. Substrates added included histone H1, GST-Cables, and His_6-Cables; for autophosphorylation of p35, no substrate was added.

Acknowledgments

The authors would like to thank Yolande Ramos and Shih-Te Wen for DNA constructs and technical support; Ming Sum Lee, Vivien Tannoch, Young Kwon, Deanna Smith, and Elena Porro for help in preparation of this manuscript; and members of the Tsai laboratory for helpful discussions. L. Lanier is supported by the Anna Fuller Fund, and F. Gertler is supported by Merck and Company. This work was supported in part by National Institutes of Health grant RO1-NS37007 (to L.-H. T.). L.-H. T. is an assistant investigator of the Howard Hughes Medical Institute, a Rita Allen Foundation scholar, and a recipient of an Esther A. and Joseph Klingenstein Foundation Fund.

Received December 8, 1998; revised April 4, 2000.

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GenBank Accession Number

The GenBank accession number for the mouse *Cables* sequence reported in this paper is AF133208.