Amphiphysin 1 is a phosphoprotein expressed at high levels in neurons, where it participates in synaptic vesicle endocytosis and neurite outgrowth. It is a substrate for cyclin-dependent kinase (cdk) 5, a member of the cyclin-dependent protein kinase family, which has been functionally linked to neuronal migration and neurite outgrowth via its action on the actin cytoskeleton. The yeast homologue of amphiphysin, Rvs167, functions in endocytosis and actin dynamics, is phosphorylated by the cdk5 homologue Pho85, and binds the Pho85 regulatory subunit Pcl2. We show here that amphiphysin 1 interacts with the cdk5-activating subunit p35 and that this interaction is mediated by the conserved NH2-terminal region of amphiphysin. Amphiphysin 1 colocalizes with p35 in the growth cones of neurons and at actin-rich peripheral lamellipodia in transfected fibroblasts. Amphiphysin is phosphorylated by cdk5 in a region including serines 272, 276, and 285. Amphiphysin 1 is also phosphorylated by the cdc2/cyclin B kinase complex in the same region and undergoes mitotic phosphorylation in dividing cells. These data indicate that phosphorylation by members of the cyclin-dependent kinase family is a conserved property of amphiphysin and suggest that this phosphorylation may play an important physiological role both in mitosis and in differentiated cells.

MATERIALS AND METHODS

Antibodies—Amphiphysin polyclonal antibodies CD5 and CD9 and monoclonal antibodies have been described previously (11, 13). p35 antibodies were obtained from Santa Cruz Biologicals (antibody C-19) or were generated as described previously (14). Anti-cdc2 antibody was obtained from Transduction Laboratories, and anti-cdk5 antibody was obtained from Upstate Biotechnology. Nonimmunocyte rabbit IgG was obtained from Sigma.

Glutathione S-Transferase (GST) Fusion Proteins—cDNAs encoding sequences of p35, p25, cdk5, or amphiphysin 1 were cloned into either pGEX2T, pGEX4T, or pGEX6P (Pharmacia) as described previously (14, 15). Fusion proteins were prepared according to the manufacturer’s instructions. Baculovirus-expressed human GST-cdc2/cyclin B1 with activating T14A and Y15F mutations as described previously (17) was a kind gift of Dr. Graham Warren (Yale University, New Haven, CT).

In Vitro Kinase Assay—p35 was [35S]methionine-radiolabeled using a coupled in vitro transcription/translation kit (Promega). 5 μl of the total reaction mixture was combined with 10 μg of recombinant GST protein fused to amino acids 1–161, 1–246, 1–306, 262–435, 411–581, and 545–695 of the human amphiphysin 1 sequence in 1 ml of buffer A (150 mM NaCl, 50 mM Tris- HCl, 5 mM EDTA, and 1% Triton X-100). These reactions were incubated for 1 h at room temperature, combined with 50 μl of 50% slurry of glutathione-Sepharose in buffer A, incubated for 30 min at room temperature, washed four times with 1 ml of buffer A, and then washed once with SDS-PAGE loading buffer and boiled for 1 min. The active protein bands were detected by fluorography.

In Vitro Kinase Assays—Kinase assays were performed as described previously (18), with the following modifications: 1 μl purified histone H1 (Roche) or amphiphysin fusion protein cleaved from the GST tag according to the manufacturer’s instructions was included in a total reaction volume of 50 μl containing either 10 μg of purified GST-cdk5 and 10 μg of purified GST-p25, anti-p35 immunoprecipitate from rat brain extract, or 1 μg of GST-tagged human cyclin B1/cdc2. [γ-32P]ATP was included at a final specific activity of 1–10 Ci/mmol in a total concentration of 200 μM. The reaction mixtures were incubated at room temperature for 30 min, and the reaction was stopped by adding sample buffer and boiling for 1 min. The proteins were separated by SDS-PAGE, the gels were dried to Whatman filter paper, and radioactive HPLC, high pressure liquid chromatography; CHO, Chinese hamster ovary.
protein bands were detected by autoradiography. Radioactive bands were quantitated on a STORM 860 PhosphorImager (Molecular Dynamics) or by scanning on a Gel Doc 2000 densitometer (Bio-Rad). For SDS-PAGE mobility assays, 1 μg amphiphysin 1 or mutant 2 was incubated with 200 μM ATP in the presence or absence of 1 μg of GST cyclin B1/cdc2 overnight at room temperature in a total volume of 50 μl. Proteins were then separated on 6% SDS-PAGE gels and probed by Western blot with monoclonal amphiphysin 1 antibody.

**Tryptic Digestion and Identification of Phosphorylated Peptides—**50 μg of recombinant amphiphysin 1 was 32P-radioabeled in vitro using p35 immunoprecipitates from rat brain as described above. The reaction products were separated by SDS-PAGE and stained with Coomassie Blue, and the amphiphysin band was excised. In conjunction with the Keck Foundation Biotechnology Resource Laboratory at Yale University, this sample was tryspin-digested, and peptides were eluted from the gel and separated by HPLC as described previously (19, 20). Radioactive fractions were detected by Cerenkov counting, and constituent peptides were determined by matrix assisted-laser desorption ionization mass spectrometry as described previously (21) and Edman sequencing on a Procise cLC instrument (PerkinElmer Life Sciences) as per the manufacturer’s protocols.

**Mutagenesis—**Constructs containing the mutations T260L, S262A, S265A (Mut 1), S272A, S276A, S265A (Mut 2), T260L, S262A, S265A, S272A, S276A, and S265A (Mut 3) in the human amphiphysin 1 sequence were generated by polymerase chain reaction, digested with restriction enzyme DpnI, and transformed into competent bacteria as described previously (22).

**Mitotic Synchronization of CHO Cells—**CHO cells stably transfected with amphiphysin 1 were synchronized with nocodazole as described previously (23). Mitotic cells were harvested and homogenized or aliquoted to progress to G1 phase before harvesting. Proteins were analyzed by SDS-PAGE and Western blotting.

**Cell Culture and Transfections—**Culture of primary rat cortical neurons was performed as described previously (24, 25). An amphiphysin stable cell line was generated by transfecting Chinese hamster ovary cells maintained in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum with human amphiphysin cDNA in pRC/RSV vector (InviGen). Transfected cells were selected by neomycin resistance, and serial dilutions were performed to obtain single clones. Cos7 cells were maintained in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum. Transient transfections were performed using p35 or amphiphysin 1 in vector pcDNA3 and LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer’s instructions.

**Miscellaneous—**Immunoprecipitation, immunostaining, and SDS-PAGE were performed as described previously (15). Triton X-100 extracts of whole rat brain were prepared as described previously (15).

**RESULTS**

**Amphiphysin and p35 Interact in Brain and in Transfected Cells—**Recently, amphiphysin has been identified as a substrate and interactor of the cdk5 kinase complex (10). To further investigate this interaction, we performed immunoprecipitation experiments from rat brain extracts. Anti-amphiphysin antibodies purified the amphiphysin 1–2 heterodimer and co-purified p35, the activating subunit of the cdk5 kinase complex. Likewise, anti-p35 antibodies coprecipitated amphiphysin (Fig. 1). cdk5 was not detected in anti-amphiphysin immunoprecipitates, indicating that amphiphysin does not interact directly with the kinase and that a ternary complex of amphiphysin, p35, and cdk5 is not present at significant levels (data not shown).

We probed the interaction of amphiphysin with p35 by reconstituting it in a transfected cell system. To this end, a CHO cell line stably transfected with amphiphysin 1 was transiently transfected with either p35 or empty control vector. As shown in Fig. 2, immunoprecipitation with antibodies directed against either amphiphysin or p35 precipitated a complex containing both proteins. cdk5 was endogenously expressed in this cell line and could be immunoprecipitated by anti-p35 antibodies (Fig. 2B, bottom panel). However, in agreement with our observation in brain extracts, amphiphysin immunoprecipitates did not contain cdk5.

**Amphiphysin 1 and p35 Colocalize in Vivo—**Based on the results of coprecipitation experiments, we investigated whether p35 and amphiphysin colocalize in cells. Both p35/cdk5 and amphiphysin have been implicated in neurite outgrowth and neuronal migration (14, 26–29). As shown by Fig. 3, A and B, endogenous amphiphysin 1 and p35 colocalize in the growth cones of rat cortical neurons in culture, consistent with a possible role for their interaction in growth cone navigation.

Similar observations were made in Cos7 cells cotransfected with amphiphysin 1 and p35 cDNAs. In these cells, a large fraction of both proteins had a diffuse cytosolic distribution. In addition, pools of amphiphysin 1 and p35 were colocalized in ruffles and lamellipodia at the cell periphery (Fig. 3, C and D). These structures were also positive for filamentous actin, as demonstrated by phalloidin immunostaining. These findings are consistent with previous observations that both amphiphysin and the p35/cdk5 complex are present at actin-rich lamellipodial structures in neuronal growth cones (14, 28).

**p35 Binds the NH2-terminal Region of Amphiphysin 1—**To map the domain of amphiphysin 1 that binds to p35, we radiolabeled p35 by coupled in vitro transcription/translation and performed in vitro binding experiments with glutathione S-transferase-tagged fragments of amphiphysin 1. As shown in Fig. 4, the first 306 amino acids of amphiphysin 1 were sufficient to retain in vitro translated p35 on glutathione-Sepharose beads. These data confirm the interaction of amphiphysin 1 with p35 and demonstrate that the binding site for p35 resides in the NH2-terminal domain of the molecule.
Amphiphysin 1 is Phosphorylated by cdk5 between Amino Acids 254 and 320—

We next investigated cdk5 phosphorylation of amphiphysin 1. To this end, we prepared immunoprecipitates of the p35/cdk5 complex from rat brain using anti-p35 antibodies. These immunoprecipitates were then used in an in vitro kinase reaction with recombinant amphiphysin 1 or histone H1 as substrates. As shown in Fig. 5, lanes 1–6, p35 immunoprecipitates, but not control IgG immunoprecipitates, incorporated radiolabeled phosphate into both histone H1 and amphiphysin 1. In vitro kinase reactions performed with bacterially expressed recombinant p35/cdk5 instead of immunoprecipitates gave similar results, indicating that amphiphysin 1 is phosphorylated by cdk5 and not by a contaminating kinase from brain extracts (data not shown).

To determine what region of amphiphysin 1 is phosphorylated by cdk5, recombinant peptides corresponding to overlapping regions of the protein were tested in an in vitro kinase reaction. This strategy demonstrated the presence of a major phosphorylation site or sites selectively in amphiphysin 1 fragments comprising amino acids 1–306 and 262–435 (Fig. 5). Strikingly, the region of overlap between these two peptide fragments, but not other regions of the two fragments, contains...
Members of the cyclin-dependent kinase family of kinases require similar sequence motifs in their respective substrates (30). Two other proteins implicated in clathrin-mediated endocytosis and actin function, epsin and eps15, are substrates for cyclin-dependent kinases in mitotic cells (23, 31). Whereas amphiphysin 1 is expressed at high levels in other cells, including dividing cells (11, 12). In addition, it is overexpressed in some breast tumors and breast cancer cell lines and was identified as a paraneoplastic syndrome autoantigen in patients with breast cancer (2, 11, 32). These considerations prompted us to investigate whether amphiphysin undergoes mitotic phosphorylation.

Based on substrate sequence preference similarities among cyclin-dependent kinases, we tested whether amphiphysin 1 is phosphorylated in vitro by the mitotic cyclin-dependent kinase cdc2/cyclin B1. To this end, we used a purified recombinant cdc2/cyclin B1 complex in which cdc2 harbors an activating mutation (17). As shown in Fig. 7A, full-length amphiphysin 1 was phosphorylated by this complex. Using amphiphysin fragments, we mapped the major cdc2/cyclin B1 phosphorylation site(s) to the region between amino acids 262 and 306, i.e. the same region that is phosphorylated by cdk5 (Fig. 7A). Both cdc2/cyclin B1 and cdk5 showed reduced phosphorylation of the amphiphysin mutant (Mut 2) harboring serine to alanine substitutions at positions 272, 276, and 285, indicating that amphiphysin is phosphorylated at these residues by both of these kinases (Fig. 7B).

We next tested phosphorylation of amphiphysin in mitotic cells. CHO cells stably transfected with amphiphysin 1 were mitotically synchronized using nocodazole, and the electrophoretic mobility of amphiphysin 1 was analyzed by Western blotting in the mitotic and G1 stages. As shown by Fig. 7C, amphiphysin 1 undergoes a mobility shift in mitotic cells. This slower mobility, which has been observed for several other mitotic phosphoproteins, could be reversed by alkaline phosphatase treatment of the mitotic extract, as expected for a shift due to phosphorylation (Fig. 7C, lane 2). Interestingly, cdc2/cyclin B1 could induce an electrophoretic shift in recombinant amphiphysin 1 in in vitro kinase reactions (Fig. 7D). This shift was reduced in the Mut 2 amphiphysin construct harboring serine to alanine mutations at residues 272, 276, and 285. Note, however, that even the Mut 2 variant of amphiphysin runs slightly slower than wild type amphiphysin in SDS-PAGE after the in vitro kinase reaction with cdc2/cyclin B1 complex. This observation suggests that under these in vitro conditions, cdc2 can phosphorylate additional sites.

**DISCUSSION**

In this study we report evidence for a functional link between amphiphysin 1 and the cdk5 kinase complex. We have demonstrated the in vitro occurrence of an interaction between amphiphysin and the cdk5 regulatory subunit p35 by immunoprecipitation and immunofluorescence. This interaction is analogous to the Pcl2-Rvs167 interaction in yeast. We have also shown that p35 binds to the evolutionary conserved NH2-terminal region of amphiphysin 1, in agreement with the reported binding of Pcl2 to the NH2-terminal moiety of Rvs167 (3). Thus, our findings point to a further similarity between the mammalian p35/cdk5/amphiphysin 1 and the yeast Pcl2/Pho85/Rvs167 protein networks. We also mapped the main cdk5 phosphorylation site to a small fragment containing three serine residues in the central region of amphiphysin 1, just upstream of the binding sites for clathrin and the clathrin adaptor AP-2, although phosphorylation of this site did not appear to affect AP-2 or clathrin binding. Because Rvs167, like several other members of the amphiphysin family, does not contain recognizable clathrin or AP-2 binding sites (33–35), it is not unexpected that cdk5 phosphorylation may not represent a conserved mechanism to regulate these interactions.

Studies of p35/cdk5 have revealed a key role of this complex in the regulation of actin function at the leading edge of neu-
ronal processes with important functional implications for neuronal migration and growth cone navigation (14, 26, 36). Likewise, independent studies have suggested a role of amphiphysin and its binding partners in growth cone dynamics (28). Amphiphysin has also been implicated in endocytosis, a process for which a key role of actin is emerging (37, 38). Although a function of cdk5 phosphorylation in endocytosis has not been reported, in yeast, Pho85, Plc2, and Rvs167 mutations have strikingly similar effects on both actin function and endocytosis (3). We suggest therefore that amphiphysin, p35, and cdk5 may be interrelated in their physiological functions in vivo. Our demonstration that amphiphysin 1 and p35 colocalize with each other and with actin in lamellipodia of transfected cells supports this possibility. We note that an interaction between amphiphysin 2 and the cAbl kinase has been reported (39). cAbl has recently been shown to phosphorylate and activate cdk5 and to bind cdk5 through the bridging protein cables (40). In addition, cAbl is known to have regulatory actions on the actin cytoskeleton and participate in neuronal development (41, 42).

Finally, we have shown that amphiphysin 1 undergoes mitotic phosphorylation. The same region that is a target for cdk5 phosphorylation can also be phosphorylated by the mitotic
cdk2/cyclin B1 complex. Thus, phosphorylation of amphiphysin 1 in this region may have a similar conserved function in both neurons and dividing cells. One of the critical events that correlates with mitosis is the dramatic rearrangement of the peripheral cytoplasm that results in the partial dissociation of cells from the substratum. Cyclin-dependent kinase phosphorylation of amphiphysin may help to produce local changes in the actin cytoskeleton that are crucial for the dynamic properties of dividing cells, neuronal growth cones, the leading edge of migrating cells, and the function of the mature synapse.

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