Minibrain kinase/dual-specificity tyrosine phosphorylation-regulated kinase (Mnb/Dyrk1A) is a proline-directed serine/threonine kinase encoded in the Down syndrome critical region of human chromosome 21. This kinase has been shown to phosphorylate dynamin 1 and synaptojanin 1. Here we report that amphiphysin I (Amph I) is also a Mnb/Dyrk1A substrate. This kinase phosphorylated native Amph I in rodent brains and recombinant human Amph I expressed in Escherichia coli. Serine 293 (Ser-293) was identified as the major site, whereas serine 295 and threonine 310 were found as minor kinase sites. In cultured cells, recombinant Amph I was phosphorylated at Ser-293 by endogenous kinase(s). Because mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) has been suggested to phosphorylate Amph I at Ser-293, our efforts addressed whether Ser-293 is phosphorylated in vivo by MAPK/ERK or by Mnb/Dyrk1A. Overnight serum-withdrawal inactivated MAPK/ERK; nonetheless, Ser-293 was phosphorylated in Chinese hamster ovary and SY5Y cells. Epigallocatechin-3-gallate, a potent Mnb/Dyrk1A inhibitor in vitro, apparently reduced the phosphorylation at Ser-293, whereas PD98059, a potent MAPK/ERK inhibitor, did not. High frequency stimulation of mouse hippocampal slices reduced the phosphorylation at Ser-293, albeit in the midst of MAPK/ERK activation. The endophilin binding in vitro was inhibited by phosphorylating Amph I with Mnb/Dyrk1A. However, phosphorylation at Ser-293 did not appear to alter cellular distribution patterns of the protein. Our results suggest that Mnb/Dyrk1A, not MAPK/ERK, is responsible for in vivo phosphorylation of Amph I at Ser-293 and that phosphorylation changes the recruitment of endophilin at the endocytic sites.

After exocytosis of neurotransmitters, rapid endocytosis for recycling synaptic vesicle constituents occurs via clathrin-mediated endocytosis. Many proteins associated with this endocytosis are phosphoproteins and undergo dephosphorylation-phosphorylation cycles during the exocytosis-endocytosis cycles of synaptic vesicles (1, 2). Dynamin, synaptojanin, and amphiphysin are phosphorylated-dephosphorylated synchronously during repeated stimulation of synaptic termini (3). Amphiphysin links endocytic proteins such as dynamin, synaptojanin, endophilin, clathrin, and AP2 complex at the clathrin-mediated endocytic sites. The C-terminal Src homology (SH3)2 domain of amphiphysin interacts with the proline-rich domain (PRD) of dynamin and synaptojanin (1, 4). Phosphorylation of the PRD inhibits the binding of these proteins to the SH3 domain of amphiphysin, whereas phosphorylation of amphiphysin results in reduction of binding to α-AP-2 (1). Cdc2/cdk5 is the first identified amphiphysin kinase. It phosphorylates amphiphysin I (Amph I) at five sites within the PRD located in the middle of the protein (5, 6). Cdc2/cdk5 also phosphorylates dynamin and synaptojanin and inhibits their binding to the SH3 domain (6, 7). As a second kinase, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) has been shown to phosphorylate Amph I at two sites, also within the PRD but distinct from those for cdc2/cdk5, and to negatively regulate the amphiphysin binding to AP2 (8).

Minibrain kinase/dual-specificity tyrosine phosphorylation-regulated kinase (Mnb/Dyrk1A) is a proline-directed serine/threonine kinase (9, 10) encoded by a gene located within the Down syndrome (DS) critical region of human chromosome 21 (11–13). Its expression is apparently elevated in individuals with DS (14, 15). Several endogenous substrates for this kinase have been identified, such as transcription factor FKHR (16), microtubule-associated protein Tau (17), and cAMP-response element-binding protein (18). We recently found that Mnb/Dyrk1A phosphorylated multiple proteins engaged in endocy-
tosis: dynamin 1 (19) and synaptojanin (20). The phosphorylated dynamin altered its affinity to amphiphysin SH3 domain depending upon the extent of phosphorylation (19). Mnb/Dyrk1A phosphorylates dynamin 1α primarily at Ser-857 in the PRD, and phosphorylation at this site reduces its binding affinity to amphiphysin. Interestingly, this reduction can be completely reversed by an additional phosphorylation at Ser-795 (21). The Mnb/Dyrk1A-directed phosphorylation also altered synaptojanin 1 binding to the SH3 domains of amphiphysin, intersectin, and endophilin. Here, we show that Amph I is also phosphorylatable by Mnb/Dyrk1A within the PRD in at least one major and two minor sites. The phosphorylation at the major site occurs in vivo, and the phosphorylated Amph I decreases endophilin binding. Our finding that three key proteins required for regulated endocytosis are substrates of Mnb/Dyrk1A strongly suggests that this kinase plays an important role in regulating endocytosis at synapses.

**EXPERIMENTAL PROCEDURES**

**Materials**—Nerve growth factor (NGF), LipofectAmine (PLUS and 2000), fetal bovine serum (FBS), horse serum, Dulbecco’s modified Eagle’s medium, and antibiotic mixtures (PLUS and 2000), fetal bovine serum (FBS), horse serum, Calbiochem, and rat brain Ca2+-transferase (GST)-endophilin 1 were described previously (19). Antibodies specific to Mnb/Dyrk1A and glutathione S-transferase (GST)-endophilin 1 were described previously (19). Antibodies specific to Mnb/Dyrk1A and the phosphorylated dynamin 1 at Ser-857 were generated in-house (21, 22). Anti-phospho-ERK antibody was from Cell Signaling Tech (Beverly, MA). Monoclonal anti-HA, monoclonal anti-poly-His, alkaline phosphatase-conjugated anti-mouse antibodies, and epigallocatechin-3-gallate (EGCG) were from Sigma. Alexa-labeled anti-mouse IgG was from Molecular Probes (Eugene, OR), and anti-endophilin and alkaline phosphatase-conjugated anti-goat IgG were obtained from Santa Cruz (Santa Cruz, CA). PD98059 was purchased from Calbiochem, and rat brain Ca2+/calmodulin-dependent kinase II and anti-Amph I antibody were from Signal Transduction, Inc. (San Diego, CA).

**Expression of Truncated and Full-length Amph I—cDNA coding for human full-length Amph I was generated by PCR from a human cDNA library with a set of primers (5’ primer, ATCGATGGCCGACATCAAGACGGCATC; 3’ primer, CTCCAGCCTAATCTAAGCGGTGAAGTTCTC) having Clal and Xhol sites, respectively. The PCR product was ligated into pGEX vector with modified restriction sites through the Clal and Xhol sites. Truncated Amph I was expressed either as N-terminal GST fusion or C-terminal poly-His fusion proteins.**

**Phosphorylation of Amphiphysin I by Mnb/Dyrk1A**

Phosphorylation of Amphiphysin I by Mnb/Dyrk1A was expressed in *Escherichia coli* (strain BL21) as an N-terminal GST fusion or C-terminal poly-His fusion proteins. With either tag, the proteins were recovered poorly from affinity resins. Therefore, we used conventional purification methods. The *E. coli* cell pellets from a 250-ml culture were suspended in 25 ml of sonication buffer (20 mM sodium phosphate at pH 8.0, 1 mM dithiothreitol, and 1 mM Triton X-100) and sonicated for 10 min on ice followed by stirring for 30 min. After centrifugation at 27,000 × g for 20 min, the supernatant fractions were made to 33% saturation of ammonium sulfate and centrifuged for 10 min. The aqueous fractions were removed carefully, and the fluffy top layer and materials adhering to the tube walls were recovered. These fractions were suspended in 25 ml of buffer A (10 mM sodium phosphate at pH 8.0, 0.2 mM EDTA, 0.2 mM EGTA, and 1 mM dithiothreitol), dialyzed overnight against the same buffer, and ultracentrifuged at 150,000 × g for 1 h. The resultant supernatants were then dialyzed against 10 ml sodium acetate (pH 4.5) for several hours, and aggregates were recovered by centrifugation at 27,000 × g for 10 min. The pellets were then suspended in a small volume of buffer A, dialyzed for at least 36–48 h against the same buffer, and centrifuged at 150,000 × g for 1 h. The supernatant fractions were used as Amph I preparations. All purification steps were carried out at 4 °C, and protease inhibitors (0.1 mM aprotinin, and pepstatin A) were included throughout the steps. GST–PRD or PRD–poly-His was purified from *E. coli* by extracting the cell pellets with a buffer containing 50 mM Tris–HCl (pH 8.0), 10 mM imidazole (pH 7.4), 5 mM 2-mercaptoethanol, 1% Triton X-100, and 0.15 M NaCl followed by purification using either glutathione (GT) or nickel nitritotriacetic acid resins. Protein concentrations were measured by the Lowry method using BSA as a standard. Phosphorylation of recombinant amphiphysin and its fragments in solution and in solid phase were performed as described previously (19).

**Identification of Phosphorylation Sites**—Purified PRD-poly-His was phosphorylated extensively by Mnb/Dyrk1A with [32P]ATP or cold ATP. After precipitation and washing 3 times with 5% trichloroacetic acid, the acid-insoluble fraction was rinsed 3 times with ether to remove trichloroacetic acid and was subjected to tryptic digestion in 0.1 M ammonium bicarbonate at 37 °C for 16 h. The digests were dried, resuspended twice in a small volume of water, re-dried, and extracted with 0.1% trifluoroacetic acid for application to HPLC as described previously (24). All peaks from HPLC were collected, and the amount of radioactivity associated with each peak was measured after drying and reconstruction with 0.5 ml of 0.5% trifluoroacetic acid. Aliquots of the radioactive fractions were subjected to phosphoamino acid analysis and to manual Edman degradation (24). To identify the phosphopeptides, mass analyses of the peptide peaks were performed on a matrix-assisted laser desorption/
Phosphorylation of Amphiphysin I by Mnb/Dyrk1A

ionization time-of-flight mass spectrometer (Voyager Elite, Applied Biosystems; Foster City, CA). The matrix solution contains α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 0.1% trifluoroacetic acid and 50% acetonitrile. Nonionic detergent, n-octyl β-D-glucopyranoside, was included in the matrix solution to optimize the detection of large or less soluble peptides as described in Cohen and Chait (25).

**Monoclonal Antibodies**—Monoclonal antibody (mAb) specific to the phosphorylated Ser-293 (4A2AF) was generated as follows. A synthetic peptide corresponding to residues 288–298 of Amph I with an additional cysteine at the C terminus (288SAPRPRPSQTRC) (2 mg) was phosphorylated extensively by Mnb/Dyrk1A in a final volume of 0.5 ml with a total of 90 μg of Mnb/Dyrk1A. After reduction of the Cys residue by triethylphosphine (21), the peptide was conjugated to Super Carrier (Pierce), as instructed by the manufacturer, and used for immunization. mAbs were first screened against the peptide phosphorylated by Mnb/Dyrk1A and HPLC-purified. 4A2AF was selected by further screening against both phosphorylated and unphosphorylated GST–PRD as well as GST full-length Amph I. mAb against Amph I (7C6) was raised against the PRD-poly-His. The clone was selected on the basis of its reactivity to recombinant PRD and full-length Amph I as well as native protein expressed in rat brains. Phosphorylation of amphiphysin by Mnb/Dyrk1A did not alter the reactivity of the protein to 7C6.

**Transfection of Mammalian Cells**—Cells (5–10 × 10⁵ cells/35-mm dish) grown overnight in a growth medium were incubated with the premixed LipofectAmine (either PLUS or 2000) with vector DNA (1–6 μg) complexes, as suggested by the manufacturer. The transfected cells were grown for an additional 24–48 h before use. For immunostaining, cells grown on coverslips were fixed by cold methanol (−20°C) for 15 min, blocked with 5% FBS and 5% goat serum in phosphate-buffered saline for 1 h, and incubated with primary antibodies at room temperature for 1 h followed by Alexa Fluor-488- or -568-conjugated secondary antibodies for 1 h. Images were captured by a Nikon PCM2000 dual laser scanning confocal microscope.

**High Frequency Stimulation of Brain Slices**—Mouse hippocampal slices were prepared from 3–5-month-old CD-1 mice (26). The slices were first maintained at low frequency stimulation (0.03 Hz) to establish stable potential and then stimulated at 100 Hz pulse for 1 s three times for every 10 s as described in El-Sherif et al. (26). The control and stimulated slices were frozen immediately on dry ice and stored at −70°C until use for immunoblotting.

**Immunoblotting**—Brain slices or harvested cells were homogenized or sonicated in 50–100 μl of lysis buffer (0.15 M NaCl, 1% Nonidet P-40, 1% deoxycholate, 10 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 10 mM NaF, 10 mM levamisole, 10 mM sodium molybdate, 5 mM sodium vanadate, and various protease inhibitors). After microcentrifugation for 10 min, the supernatant fractions were recovered. The protein concentrations for each sample were measured by the Bradford method using BSA as a standard. SDS-PAGE was performed using either the Tris-glycine (27) or Tris-Tricine (28) buffer system. The proteins transferred to Immobilon-P membranes (Millipore; Bedford, MA) were blocked with 5% BSA or nonfat milk and incubated with primary antibodies. The membranes were then processed for color development or chemiluminescence detection (PhotoStop-Star reagent, New England Biolabs; Beverly, MA) after incubation with alkaline phosphatase-conjugated secondary antibodies.

**Cells**—CHO and NIH3T3 wells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS and penicillin G sodium, whereas SY5Y cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FBS and 5% horse serum. PC-12 cells were grown over rat collagen-coated culture dishes in F-12K medium containing 12.5% horse serum and 2.5% FBS.

**RESULTS**

**Phosphorylation of Full-length Human Amph I by Mnb/Dyrk1A**—Amph I contains a sequence, 290RPRSP294, that matches the consensus Mnb/Dyrk1A phosphorylation site (RPXS/TP) (10) as well as the dynamin 1xa phosphorylation site (RPESP) (21). Therefore, we speculated that human Amph I could be phosphorylated by Mnb/Dyrk1A. We first tested whether native Amph I in rat brain is phosphorylatable by the kinase in vitro. Phosphorylation of native Amph I by an unknown kinase(s) in synaptosomes (3) or by cdc2/cdk5 (5) slows electrophoretic mobility of the protein in SDS-PAGE. Therefore, we tested the mobility changes of Amph I after incubating rat brain extract with Mg²⁺-ATP and with exogenous Mnb/Dyrk1A (Fig. 1). The addition of phosphatase inhibitors alone (lane 2) or the kinase without ATP (not shown) did not change the mobility of endogenous Amph I, whereas the addition of ATP and phosphatase inhibitors reduced the mobility (lane 3). The addition of both ATP and Mnb/Dyrk1A with (lane 4) or without (lane 5) phosphatase inhibitors caused a further shift of the mobility, whereas the addition of Ca²⁺-calmodulin kinase II caused no change (lane 6). These results suggest that Amph I is phosphorylatable by Mnb/Dyrk1A.

We then tested whether Mnb/Dyrk1A could phosphorylate recombinant human Amph I purified from E. coli. As shown in Fig. 2A, phosphorylation levels of the protein increased in parallel to the increased amounts of Mnb/Dyrk1A and incubation time. Assuming the amphiphysin preparation to be 100% pure, in Fig. 2A a maximum of 0.5 mol of phosphate was incorporated per mol of protein. Based on the apparent purity of the GST-Amph I seen in Fig. 2B, this stoichiometry was likely underestimated. Autoradiography of ³²P-labeled Amph I showed that...
Phosphorylation of Amphiphysin I by Mnb/Dyrk1A

A, time course with various concentrations of kinase. GST-Amph I (5 μg) was incubated with 0.1 μg (□), 0.5 μg (△), or 1.0 μg (○) of Mnb/Dyrk1A in the presence of [γ-32P]ATP as described under “Experimental Procedures.” B, Coomassie Blue-stained Amph I preparation used for the phosphorylation assay. Proteins were separated by SDS-6% PAGE (28). Lane 1, marker proteins; lane 2, GST-Amph I; lane 3, Amph I-poly-His. C, phosphoamino acid analysis of the phosphorylated Amph I by Mnb/Dyrk1A. Aliquots of the reaction mixtures from A were used for the analysis after precipitation with 10% trichloroacetic acid (24). After high voltage electrophoresis, the TLC plate was exposed to an x-ray film for detection of 32P-labeled amino acids. Pi, free phosphate.

the majority of the radioactivity was associated with Amph I (data not shown). Phosphoamino acid analysis of 32P-labeled Amph I preparations revealed that both Ser and Thr residues were phosphorylated with serine as the major residue (Fig. 2C). Regardless of its name “dual specificity kinase,” Mnb/Dyrk1A does not phosphorylate Amph I at Tyr. This result is consistent with the earlier findings that Mnb/Dyrk1A is a Ser/Thr kinase (9, 10, 16, 17, 21).

Localization of the Mnb/Dyrk1A Phosphorylation Sites—We then proceeded to identify the Mnb/Dyrk1A phosphorylation site(s) on Amph I. For this purpose we first expressed several deletion mutants of Amph I in E. coli that have GST and poly-His tags at the N- and C-termini, respectively (see Fig. 3A for summary) and used them as substrates for Mnb/Dyrk1A. The E. coli cell homogenates containing these constructs were subjected to a solid-phase kinase assay (19) as well as immunoblotting using anti-poly His antibody. As seen in Fig. 3B, Mnb/Dyrk1A phosphorylated full-length, and all three of the C terminus deletion mutants, Amp-ΔSH3, -ΔC254, and -ΔC355, which contain the PRD. In contrast, the kinase did not phosphorylate the N terminus deletions Amp-ΔN321 and -Δ454 or the Amph I lacking the PRD (Amp-ΔPRD). This finding suggests that the kinase sites are located within the PRD.

Phosphorylation of PRD by Mnb/Dyrk1A—To confirm that the PRD is the domain for the phosphorylation, we next used the GST- and poly-His-tagged amphiphysin PRD for further analysis. Under the conditions used, phosphorylation occurred at a maximum stoichiometry of 1 mol/mol of PRD for both constructs. Subsequent phosphoamino acid analysis revealed that both Ser and Thr residues were phosphorylated in the PRD, with serine being the major residue (Fig. 4B). The pattern of phosphorylated amino acids was similar to that of the full-length Amph I. This result confirms that the Mnb/Dyrk1A sites are located within the PRD.

Subsequently, we determined the specific Mnb/Dyrk1A phosphorylation sites in the PRD. To proceed, we first emphasized on the human Amph I PRD region 287APARPRSPSQ296, a segment containing the consensus Mnb/Dyrk1A site. GST fusion protein containing the sequence 287APARPRSPSQ296 was generated and subjected to phosphorylation by Mnb/Dyrk1A. The fusion protein was phosphorylated. To determine whether the Ser-293 is indeed the kinase site, we mutated the Ser-293 to either Asp or Ala (S293D and S293A). In vitro, Mnb/Dyrk1A phosphorylated the S293D and S293A mutants at significantly reduced levels from that of the wild type (WT) (Fig. 4A). This result indicates that Ser-293 is indeed phosphorylatable by the kinase and likely the major kinase site. Phosphorylation of PRD-WT, S293D, and S293A was, again, kinase concentration-dependent (Fig. 4A).

Fig. 4B summarizes the phosphoserine and phosphothreonine levels detected in the PRDs (WT, S293D, and S293A) upon incubation with different amounts of kinase shown in Fig. 4A. Although the mutation at Ser-293 did not completely eliminate
Phosphorylation of Amphiphysin I by Mnb/Dyrk1A

Phosphorylation, the amount of phosphoserine was significantly reduced for both mutants over the entire range of kinase concentration (Fig. 4B). On the other hand, the relative amounts of phosphothreonine were unchanged among WT, S293D, and S293A.

Identification of Other Mnb/Dyrk1A Sites in PRD—To simplify, we used PRD-S293A-poly-His as the starting material. After phosphorylation, the $^{32}$P-labeled protein was digested by trypsin and subjected to HPLC (Fig. 5A) for isolation of the phosphopeptides. Aliquots of each peptide were subjected to both phosphoamino acid analysis and manual Edman degradation. A majority of the radioactivity was eluted between 7.5 and 9 min (fraction 8/9) (Fig. 5A) (Table 1). This fraction contained phosphoserine and phosphothreonine. $^{32}$P]Phosphate was released from the peptide(s) in fraction 8/9 at the second and third cycles of Edman degradation (Fig. 5B, panel a). The phosphopeptide(s) in this fraction was identified by mass spectrometry by analyzing the corresponding tryptic fragments similarly prepared with cold ATP. We found that fraction 8/9 contains two phosphopeptides, i.e. $^{309}$VTPTK and $^{293}$APSQTR, which include 1 mol each of phosphate. Some radioactivity was also associated with fraction 18a eluted at $\sim$18 min. This fraction contained phosphoserine only, and the phosphate was released at the fifth cycle of Edman degradation. Trypsin does not cut K/R-P peptide bond. Judging from all possible tryptic cleavage sites, no peptide could be generated from the PRD having a Ser residue at the fifth position from its N terminus. Therefore, the peptide(s) in fraction 18a was not derived from the PRD. Several fractions contained low levels of $^{32}$P]phosphate. All these fractions contained both phosphoserine and phosphothreonine. $^{32}$P]Phosphate was associated with the second and third amino acids for all fractions. Based upon the tryptic cleavage sites within the PRD, we assumed those minor fractions (18b-23/24) to be the peptides of $^{309}$VTPTK and $^{293}$APSQTR that were incompletely digested at their C termini. The recovery rates of the $^{32}$P]phosphate as well as the types and positions of phosphoamino acids for each peptide peak are summarized in Table 1. The combined data suggest that Mnb/Dyrk1A is capable of phosphorylating the PRD-S293A at Ser-295 and Thr-310.

By using the same strategy, we repeated the experiment with PRD-WT to confirm the Mnb/Dyrk1A sites found in S293A. Again, most of the radioactivity was recovered in fraction 8/9 (Table 1). This fraction contained both phosphoserine and phosphothreonine.
phosphothreonine, although phosphoserine was much more abundant. The peptides recovered in fraction 8/9 from WT were again identified to be $^{309}$VTPTK and $^{293}$SPSQTR by mass spectrometry and released $[^{32}P]$phosphate at the first and second positions but not at the third position by manual Edman degradation (Fig. 5B, panel b, Table 1). However, although the total recovery from HPLC was low, fractions 18b, 19/20a, and 20b/21a contained the peptides phosphorylated at the second and third amino acids (Table 1). These fractions were, again, assumed to contain the peptides of $^{309}$VTPTK and $^{293}$SPSQTR and the third amino acids (Table 1). These fractions were, again, assumed to contain the peptides phosphorylated at the second and third amino acids (Table 1). These fractions were, again, assumed to contain the peptides phosphorylated at the second and third amino acids (Table 1).

The specificity of this antibody was tested by using rat brain extract phosphorylated in vitro with and without additional Mnb/Dyrk1A (Fig. 8A). 4A2AF did not react to a detectable that the Thr-312 may be phosphorylated when Thr-310 is replaced with Ala. However, both single- and double-mutated PRD at the Thr residues were phosphorylated to the same level. Mutations at all four positions (S293A/S295A/T310A/T312A), although showing the lowest levels, did not completely eliminate phosphorylation by Mnb/Dyrk1A. Mutating the Mnb/Dyrk1A sites to Asp gave slightly different results. The Asp mutation at either Ser-293 (S293D) or at both Ser-293 and Ser-295 (S293D/S295D) produced the same effect on phosphorylating PRD; both exhibited the lowest phosphorylation levels (Fig. 6B). The reduction levels in the phosphorylation were greater than those of S293A and S293A/S295A. Single mutation at Thr-310 (T310D) and double mutation at Thr-310 and Thr-312 (T310D/T312D) gave very similar phosphorylation efficiencies; both mutants were phosphorylated to lesser extents (Fig. 6B) than were those of the equivalent Ala mutants (Fig. 6A). Mutations at four sites to Asp (S293D/S295D/T310D/T312D) did not have the additive reduction effect of T310D/T312D on S293D/S295D. Instead, they had a higher phosphorylation level than S293D/S295D.

To further confirm the Mnb/Dyrk1A sites in the parent protein, effects of mutations on the phosphorylation efficiencies were also studied using full-length Amph I. GST-Amph I, WT and mutant, were purified simultaneously to minimize conditional variations during purification steps. Fig. 6A, panels a and b, shows representative phosphorylation patterns of Amph I preparations, and Fig. 7B shows autoradiography of the phosphorylated proteins from Fig. 7A. The S293A and S293A/S295A mutants had almost the same phosphorylation efficiencies, which are at or less than half the WT level (Fig. 7A, panel a). The Ala mutation at all four sites, nonetheless, did not noticeably lower the phosphorylation levels from those of S293A and S293A/S295A. Overall phosphorylation patterns of the full-length Amph I with Ala mutations were similar to those of GST-PRD. Likewise, we analyzed GST-Amph I Asp mutants (Fig. 7A, panel b). The S293D and S293D/S295D mutants were phosphorylated at similar levels and exhibited the lowest phosphorylation levels by the kinase. Thus, regardless of whether full-length or the PRD, the Ser-293 mutants with and without Ser-295 mutation had the same phosphorylation efficiencies. Interestingly, full-length Amph I T310D/T312D mutant had a higher phosphorylation level than WT, indicating that replacing the amino acids at Thr-310/Thr-312 with Asp created other possible phosphorylation sites. It should be noted that the GST-Amph I-Asp mutant at four positions degraded rapidly during purification and could not be used in the experiment.

**TABLE 1**

| Fraction | % recovery | Phosphoamino acids | Positions of $[^{32}P]$-labeled amino acids |
|----------|------------|--------------------|-------------------------------------------|
| Peptides from PRD-S293A mutant | 8/9 | 47.2 | Ser, Thr | 2, 3 |
| 18a | 15.3 | Ser | 5 |
| 18b | 16 | ND | ND |
| 19 | 2.5 | ND | ND |
| 20a | 4.1 | Ser, Thr | 2, 3 |
| 20b | 6.5 | Ser, Thr | 2, 3 |
| 21a | 6.8 | Ser, Thr | 2, 3 |
| 21b | 2.3 | ND | ND |
| 23/24 | 8.4 | Ser, Thr | 2, 3 |

*ND, not determined.*

**Peptides from PRD-WT**

| Fraction | % recovery | Phosphoamino acids | Positions of $[^{32}P]$-labeled amino acids |
|----------|------------|--------------------|-------------------------------------------|
| 8/9 | 56.0 | Ser, Thr | 1, 2 |
| 18a | 16 | Ser | 5 |
| 18b | 4.3 | ND | 1, 2, 3, 5 |
| 19/20a | 8.3 | ND | 2, 3 |
| 20b/21a | 10.2 | Ser, Thr | 2, 3 |

**Phosphorylation of Amphiphysin I by Mnb/Dyrk1A**

**FIGURE 6.** Phosphorylation of variously mutated PRDs by Mnb/Dyrk1A. The GST-PRD mutants (10 µg each) were phosphorylated with either 1 µg (A) or 0.25 µg (B) of Mnb/Dyrk1A for various periods as in Fig. 2. A, Ala mutants; B, Asp mutants; C, WT; D, S293A or S293D; E, S293A/S295A or S293D/S295D; F, T310A or T310D; G, T310A/T312A or T310D/T312D; H, S293A/S295A/T310A/T312A or S293D/S295D/T310D/T312D.
Phosphorylation of Amphilphysin I by Mnb/Dyrk1A

level with native Amphilphysin I in the rat brain Triton extracts (lane 1). Incubation of the brain extract with Mg2+-ATP produced a single band that reacted with 4A2AF (lane 2). The addition of Mnb/Dyrk1A and Mg2+-ATP to the brain extract further increased the levels of the 4A2AF-reactive band (lane 3). Anti-PRD antibody (7C6) (Fig. 8B) and a commercially obtained anti-amphiphysin antibody (Fig. 8C), both, appear to recognize the same band that reacted with 4A2AF. The reactivity of 7C6 or anti-amphiphysin antibody did not appear to be affected by the phosphorylation.

In Vivo Phosphorylation at Ser-293 Mediated by Mnb/Dyrk1A—A recent study by Shang et al. (8) suggested that MAPK/ERK phosphorylates the Ser-285 and Ser-293 residues of Amphilphysin I. They concluded this based upon the observations that 1) NGF stimulation caused GFP-Amphilphysin I phosphorylation in PC12 cells, which was inhibited by MEK inhibitor PD98059, and 2) mutations at Ser-285 and Ser-293 significantly reduced the NGF-stimulated phosphorylation. To test whether phosphorylation at Ser-293 is mediated by MAPK/ERK, Mnb/Dyrk1A, or both, we expressed GFP-Amphilphysin I in multiple mammalian cell lines and tested the effects of inhibitors for Mnb/Dyrk1A and MAPK/ERK on the phosphorylation at Ser-293. We first used CHO cells because of their high transfection efficiency and relatively high expression levels of endogenous Mnb/Dyrk1A. Transiently transfected CHO cells were serum-starved overnight. Then the cells were incubated with EGCG (100 μM) or PD98059 (50 μM/ml) or without inhibitor. One set of cells were further stimulated with FBS with or without inhibitors. Fig. 9A shows immunoblots of the cell lysates detected with 4A2AF, 7C6, and anti-phospho-ERK antibodies. Although 4A2AF gave a single band for the rat brain extract in Fig. 8, when cultured cells were analyzed, this antibody reacted strongly with an unidentified protein(s) migrating close to but slightly slower than GFP-Amphilphysin I (Figs. 9 and 10, lanes 1). By expressing GFP-Amphilphysin I in CHO cells, 4A2AF gave a second reactive band, which was identified as GFP-Amphilphysin I by 7C6 (Fig. 9A, panels a and b).

FIGURE 7. Phosphorylation of full-length Amphilphysin I-WT and mutants by Mnb/Dyrk1A. A, time course. GST full-length Amphilphysin I harboring various mutations at the kinase sites was incubated with 0.25 μg of Mnb/Dyrk1A and γ[32P]ATP as in Fig. 2. WT; □, S293A or S293D; ○, S293A/S295A or S293D/S295D; ▲, S2923A/S295A/T310A/T312A; △, T310D/T312D. B, Coomassie staining (CB) and autoradiography ([32P] of the phosphorylated Amphilphysin I from A. Lane 1, WT; lane 2, S293A; lane 3, S293A/S295A; lane 4, S293A/S295A/T310A/T312A; lane 5, S293D; lane 6, S293D/S295D; lane 7, T310D/T312D.

FIGURE 8. Specificity of mAb 4A2AF to the phosphorylated Amphilphysin I. Rat brain extract (30 μl) was incubated at 30 °C for 30 min in the presence of a mixture of protease inhibitors with/without ATP and with/without recombinant Mnb/Dyrk1A as described in Fig. 1. Aliquots of the reaction mixtures were subjected to SDS-6% PAGE (28) followed by immunoblotting. A–C, blots incubated with 4A2AF, 7C6, and commercially obtained anti-Amphilphysin I antibody, respectively. Lanes 1, no ATP; lanes 2, + ATP; lanes 3, + ATP and exogenous Mnb/Dyrk1A. *, nonspecific bands generated by the secondary antibody.

Under this condition, GFP-Amphilphysin I remained phosphorylated at Ser-293 (panel a, lane 2). By stimulating the serum-starved cells with FBS, MAPK/ERK became phosphorylated (panel c, lane 5), indicating that the kinase was activated (29, 30). However, this activation did not change the phosphorylation level at Ser-293. In addition, despite PD98059 inhibited the serum-mediated activation of MAPK/ERK, it failed to reduce the phosphorylation at Ser-293 (panels a and c, lanes 7). In contrast, incubation of the serum-starved CHO cells with EGCG, a potent Mnb/Dyrk1A inhibitor in vitro in a purified system (31), mildly reduced the phosphorylation at Ser-293 (lane 3). However, this inhibitory effect was largely diminished by the addition of serum (lane 6). The reactivity of 4A2AF with the unidentified protein(s), on the other hand, was inhibited strongly by EGCG even after stimulation with FBS. Interestingly, EGCG enhanced phosphorylation of MAPK/ERK both in the absence and presence of FBS (panel c, lanes 3 and 6), suggesting that EGCG functioned to activate MAPK/ERK in CHO cells.

A similar study was carried out using a neuronal cell line, SY5Y (Fig. 9B). As seen in CHO cells, phosphorylated
MAPK/ERK was at undetectable levels after overnight serum starvation, whereas GFP-Amph I was still phosphorylated at Ser-293 (panels a–c, lane 2). Stimulation of the starved cells with sera clearly activated MAPK/ERK; however, it failed to enhance the phosphorylation at Ser-293 (lane 5). EGCG somewhat reduced the phosphorylation if cells were incubated in the serum-free medium (lane 3). On the other hand, PD98059 significantly reduced the serum-mediated MAPK/ERK activation in the cells without changing the levels of Ser-293 phosphorylation (lane 7).

We also employed PC-12 cells to test whether phosphorylation at Ser-293 can be altered by NGF stimulation. GFP-Amph I was expressed in PC-12 cells at a moderate level (Fig. 10A, panel b). By incubating the cells overnight without sera, MAPK/ERK was completely inactivated. Under these conditions, 4A2AF recognized GFP-Amph I weakly just below the strongly stained nonspecific bands (panels a and c, lanes 1 and 2). Such poor phosphorylation of Amph I at Ser-293 became apparent when immunoblots are compared between the extracts of PC-12 and SY5Y cells. EGCG treatment reduced the staining of the nonspecific bands in PC-12 cells.

Although the phosphorylation levels were low, EGCG lowered Ser-293 phosphorylation as well (lane 3). Incubation of the serum-starved cells with NGF clearly activated MAPK/ERK but failed to enhance Ser-293 phosphorylation at an appreciable level (lane 5). Again, as seen in CHO and SY5Y cells, treatment of PC-12 cells with PD98059 was unsuccessful in reducing the phosphorylation at Ser-293 both without (lane 4) and with (not shown) NGF stimulation. These results strongly suggest that MAPK/ERK is not involved in phosphorylating Amph I at Ser-293, even in PC-12 cells.

We then carried out a co-expression study of GFP-Amph I and Mnb/Dyrk1A to show that overexpression of the kinase enhances Amph I phosphorylation at Ser-293. Among the cell lines tested, NIH3T3 cells expressed endogenous Mnb/Dyrk1A at the lowest levels. The recombinant Amph I expressed in the cells showed a slight phosphorylation at Ser-293 (Fig. 10B, panel a, lanes 1 and 2). By overexpressing Mnb/Dyrk1A, the phosphorylation levels of GFP-Amph I at Ser-293 were clearly enhanced (panels a and c, lanes 3 and 4). Thus, our results from Figs. 9 and 10 strongly suggest that in vivo phosphorylation at

**FIGURE 9.** Phosphorylation of GFP-Amph I in CHO (A) and SY5Y (B) cells. GFP-Amph I was transiently expressed in the cells by transfecting with pEGFP-Amph I (4 μg of DNA) mixed with Lipofectamine™ 2000 and by culturing for 48 h. The cells were then serum-starved overnight. One group of cells was treated with EGCG (100 μM) or PD98059 (50 μg/ml) for 1 h in serum-free medium (lanes 1–4). The other group of cells was similarly treated with EGCG or PD98059 and then incubated in a growth medium with corresponding inhibitors for additional 15 min, as indicated in the figure. After rinsing twice with ice-cold phosphate-buffered saline, the cells were lysed in 100 μl of lysis buffer, sonicated briefly, and microcentrifuged. The resultant supernatants were subjected to immunoblotting as described in Fig. 8. The same membrane was stripped and reused with another antibodies. For SY5Y cells, 50 μg EGCG was used throughout the experiment. Panel a, 4A2AF; panel b, 7C6; panel c, anti-P-ERK antibody. The proteins applied per lane were 23 and 20 μg for A and B, respectively.

**FIGURE 10.** Phosphorylation of recombinant Amph I in PC-12 and NIH3T3 cells. A, phosphorylation in PC-12 cells. The cells were transfected with pEGFP-Amph I and incubated in the serum-free medium as in Fig. 9. One set of cells was incubated with EGCG (50 μM) or PD98059 (50 μg/ml) for 1 h. The other set of cells were treated similarly and then stimulated with NGF (50 ng/ml) with or without respective inhibitor for 15 min, as indicated in the figure. The cell lysates (18 μg/lane) were subjected to SDS-PAGE followed by immunoblotting with 4A2AF (panel a), 7C6 (panel b), and anti-phospho-ERK (panel c) as in Fig. 9. B, effect of co-transfection with Mnb/Dyrk1A on phosphorylating GFP-Amph I. NIH3T3 cells were transfected with pEGFP-Amph I (0.6 μg) alone or together with pCMV-Mnb/Dyrk1A (1.8 μg) by using LipofectaminePlus. Cells were grown for 48 h and serum-starved overnight. Cell lysates (8 μg of protein) were applied per each lane for immunoblotting with 4A2AF (panel a), 7C6 (panel b), or anti-Mnb/Dyrk1A (panel c). Lanes 1 and 2, pEGFP-Amph I alone; lanes 3 and 4, pEGFP-Amph I and pCMV-Mnb/Dyrk1A.
Ser-293 is mediated by Mnb/Dyrk1A, not by MAPK/ERK. It is interesting to note that in contrast to CHO cells, EGCG did not enhance the MAPK/ERK phosphorylation in the serum-starved SY5Y cells and that in PC-12 cells EGCG effectively prevented MAPK/ERK from the NGF-mediated activation (not shown).

**Dephosphorylation of Amph I at Ser-293 by High Frequency Electrophysiological Stimulation of Hippocampal Slices**—Many proteins involved in regulated endocytosis such as dynamin, amphiphysin, and synaptojanin are known to display an activity-dependent phosphorylation-dephosphorylation cycle (3, 6, 32, 33). By employing a specific antibody against the phosphorylated Ser-293, we studied whether high frequency stimulation can alter the phosphorylation level of Amph I in brains. When mouse hippocampus slices were stimulated at 100 Hz pulse for 1 s three times every 10 s, a protocol routinely used for stimulating long term potentiation, the phosphorylation levels of Amph I at Ser-293 were significantly reduced (Fig. 11a). The slices were then processed for immunoblotting against antibodies 4A2AF (panel a), 7C6 (panel b), or P-ERK (panel c). The same blotting membrane was stripped and used repeatedly for different antibody.

**Effect of Phosphorylation by Mnb/Dyrk1A on Amph I-Endophilin Binding**—We then tested whether phosphorylation of Amph I at the PRD by Mnb/Dyrk1A alters its interaction with known binding proteins. Endophilin is known to bind to amphiphysin at its PRD (35). We first performed a pull-down assay after incubating GST-PRD with rat brain extract. As shown in Fig. 12A, lane 3, rat brain endophilin bound to the GST-PRD and was recovered from GT resins. Endophilin-GST-PRD binding was specific, as it was detected only when both GST-PRD and brain extract were included. Prior phosphorylation of GST-PRD by Mnb/Dyrk1A significantly reduced the endophilin binding to GST-PRD/GT-resins (Fig. 12A, lane 4). Neither clathrin nor AP complex bound to the GST-PRD used in this study (not shown). To further test the effect of Mnb/Dyrk1A-mediated phosphorylation on endophilin binding, full-length Amph I without GST fusion was phosphorylated and incubated with GST-endophilin. The unphosphorylated Amph I was recovered from the GT resins, and the recovery was specific to the presence of GST-endophilin (Fig. 12B, lanes 5 and 7). Upon phosphorylation with Mnb/Dyrk1A, the binding of Amph I to GST-endophilin was drastically reduced (lane 6), similar to that of GST-PRD.

**Immunocytochemical Studies of Recombinant Human Amph I**—We examined whether phosphorylation at Ser-293 alters the cellular distribution of Amph I. For this study, an HA-tagged Amph I instead of a GFP moiety on amphiphysin assembly (36). First, transfected CHO cells were immunostained with 7C6 and 4A2AF antibodies (Fig. 13, a–f). The staining with 7C6 revealed the distribution of Amph I in both diffused and dotted patterns (a–c). In the cells expressing a minor level of Amph I, the dotted pattern was more prevalent (c) than those expressing higher levels of the protein (a). mAb 4A2AF staining revealed that the phosphorylated Amph I at Ser-293 also distributed in diffuse (d), dotted (f), or both (e) patterns. Because anti-Amph I anti-
body specific to the unphosphorylated form is not available, we expressed the GFP-tagged Amph I WT for co-staining with 4A2AF. Distribution of GFP-Amph I (g) and phosphorylated Amph I at Ser-293 (i) overlapped at most parts of the cell (j). Some structures, however, showed stronger staining either in green or red. These results suggest that both phosphorylated and unphosphorylated Amph I localize similarly within cells. To confirm this conclusion, we then expressed the HA-Amph I mutants, S293A/S295A/T310A/T312A (j and k) or S293D/S295D/T310D/T312D (l and m). Both mutants showed dotted structures distributed uniformly throughout the cells, indistinguishable from that of the WT. The other mutants (S239A, S293D, S239A/S295A and S239D/S295D) also exhibited similar distribution patterns (data not shown). These results suggest that phosphorylation by Mnb/Dyrk1A at Ser-293 does not alter cellular distribution of Amph I.

**DISCUSSION**

To study the physiological significance of amphiphysin phosphorylation by Mnb/Dyrk1A, we first identified the kinase sites by employing conventional approaches. Ser-293 was shown to be the major site that was phosphorylated in vivo in cultured cells and brain slices. Phosphorylation was inhibited by incubating cultured cells with EGCG but not with PD98059. The phosphorylation level at Ser-293 was also reduced by high frequency electrophysiological stimulation of mouse hippocampal slices, conditions under which MAPK/ERK was activated. Amph I phosphorylated by Mnb/Dyrk1A decreased endophilin binding in vitro. From these results we conclude that Amph I at Ser-293 is phosphorylated by Mnb/Dyrk1A and that the phosphorylation has physiological significance in controlling the interaction of amphiphysin with endocytic accessory proteins.

We found that most if not all of Mnb/Dyrk1A sites are located within the PRD positioning in the middle of Amph I. By using the PRD WT and S293A mutant, we identified one major (Ser-293) and two minor (Ser-295 and Thr-310) kinase sites. Such major and minor sites might represent high and low affinity sites for Mnb/Dyrk1A, respectively. Although the phosphorylation level was drastically reduced, elimination of all possible kinase sites did not abolish phosphorylation by Mnb/Dyrk1A either in the PRD or full-length Amph I. Given the indistinguishable phosphorylation efficiencies between S293A and S293A/S295A as well as between S293D and S293D/S295D, we speculate that the persistent low phosphorylation may be due to exposure of new minor site(s) as a result of mutation-triggered conformational changes within the PRD (see the discussion below) rather than a unidentified minor kinase site(s). By using specific antibody, Ser-293 was shown to be phosphorylatable in native Amph I expressed in rat and mouse brains (Figs. 8 and 11), and the phosphorylation level was increased after incubation of rat brain extracts with exogenous Mnb/Dyrk1A (Fig. 8). In cultured cell lines recombinant human Amph I was phosphorylated at Ser-293 by endogenous kinase(s) (Figs. 9 and 10). In mouse hippocampal slices Amph I was phosphorylated at Ser-293 in the resting stage, and importantly, the phosphorylation level was readily reduced upon electrophysiological stimulation (Fig. 11), which was used routinely to promote long term potentiation. This decrease was in accord with that of dynamin 1xa phosphorylation at Ser-857. Changes in the phosphorylation levels of amphiphysin, dynamin, and synaptotagmin have been known to follow depolarization and polarization of isolated synaptosomes (3). Therefore, we conclude that phosphorylation of Amph I at Ser-293 is a physiological event.

Previous studies have identified two other kinases, cdk5/cdc2 and MAPK/ERK, that are capable of phosphorylating Amph I. Cdk5/cdc2 phosphorylates Amph I at five sites, Ser-261, Ser-272, Ser-276, Ser-285, and Thr-310, within the PRD (5, 6). In this study the residue Thr-310 was also identified as the minor Mnb/Dyrk1A site. MAPK/ERK has been reported recently as an Amph I kinase (8). It phosphorylates GST-Amph I in vitro. In vivo, NGF induces phosphorylation of GFP-Amph I in PC-12 cells and in embryonic primary neurons. The phosphorylation is inhibited by PD98059 in both cell types. Based upon a consensus sequence for the kinase, they deduced that Ser-285 and Ser-293 are the MAPK/ERK sites, and indeed, the Amph I mutated at either site to Ala exhibits a reduction in phosphorylation level both in vitro and in vivo (8). To address which kinase is responsible for phosphorylating Ser-293 in vivo, we expressed recombinant human Amph I in various cell lines and treated the cells with two inhibitors, PD98059 and EGCG, after overnight serum
starvation. PD98059 is a specific inhibitor for MEK, the upstream activator of MAPK/ERK. EGCG is a potent inhibitor for Mnb/Dyrk1A (IC$_{50}$ of 0.33 μM, from _in vitro_ kinase assay) (31). In all cell types overnight serum withdrawal nearly completely inactivated MAPK/ERK. In contrast, in CHO cells, EGCG reduced the phosphorylation at Ser-293 despite significantly activating MAPK/ERK (Fig. 9A). EGCG also inhibited Amph I phosphorylation in SY5Y and PC-12 cells (Figs. 9 and 10). Furthermore, high frequency electrophysiological stimulation activated MAPK/ERK by more than 2-fold but reduced phosphorylation at Ser-293 by about 50% in mouse hippocampal slices (Fig. 11). Phosphorylation at Ser-293 could be significantly enhanced by co-expressing recombinant Mnb/Dyrk1A (Fig. 10B). Thus, our observations are consistent with the conclusion that Mnb/Dyrk1A is the primary kinase for phosphorylating Ser-293 in _in vivo_. However, the possibility that other EGCG-sensitive kinase(s) may also be involved in _in vivo_ cannot be completely ruled out. Despite potent inhibition of Mnb/Dyrk1A _in vitro_, EGCG had moderate inhibitory effects on Amph I phosphorylation _in vivo_. This might be due to the fact that Mnb/Dyrk1A is not the only cellular target of EGCG (37) and that EGCG is unstable and oxidized quickly in cell culture conditions.

Endophilin is required for efficient fission of synaptic vesicles (38). Endophilin binds to amphiphysin at the PRD (35); however, no studies have been conducted to determine whether phosphorylation at the PRD affects endophilin binding. Although our PRD construct is shorter than those described in other studies (1, 39), endogenous endophilin I in rat brain bound to our PRD. We found that Mnb/Dyrk1A-mediated phosphorylation of the PRD and full-length Amph I resulted in inhibiting the endophilin binding (Fig. 12). Phosphorylation and dephosphorylation of the endocytic proteins are required for regulated synaptic vesicle endocytosis (2, 40). Phosphorylation by either cdc2/cdk5 or MAPK/ERK reduces Amph I binding to AP-2 (6, 8). NGF-stimulated phosphorylation resulted in reduction of Amph I binding to AP-2 in PC-12 cells. In addition, the S285A or S293A mutants rendered the AP-2-Amph I binding more resistant to NGF stimulation (8). Future studies are required to address whether and how the Mnb/Dyrk1A-mediated phosphorylation of Amph I modulates the binding of AP-2, clathrin, and dynamin and whether and how coordinated phosphorylation at the PRD by multiple kinases regulates overall Amph I functions in a timely manner.

We subsequently determined whether phosphorylation by
Mnb/Dyrk1A alters cellular distribution of Amph I. The recombinant Amph I phosphorylated at Ser-293 was distributed diffusely throughout the cells and associated with vesicles/organelles, not different from distribution of total Amph I (Fig. 13). At this moment it is not possible to estimate the ratio of phosphorylated to unphosphorylated Amph I at Ser-293 in vivo. To address this problem, we also included various Amph I mutants at the phosphorylation sites in the subcellular distribution studies. Both Ala and Asp mutants at the Mnb/Dyrk1A sites displayed distribution patterns similar to those of the WT and of the phosphorylated Ser-293. These results strongly suggest that phosphorylation at Ser-293 does not function to redistribute Amph I by dissociating from vesicles or organelles. Of course, the possibility remains that these mutants do not fully mimic the properties of phosphorylated and unphosphorylated Amph I and that mutation of amino acids within the PRD alters the conformation of Amph I (see below).

GST-Amph I-WT did not bind well to GT affinity resins. In addition, recombinant Amph I with or without the GST moiety showed a tendency to aggregate. Thus, prolonged dialysis at pH 8.0 after precipitation at pH 4.5 was necessary to recover Amph I in the supernatant fraction after ultracentrifugation. Amphiphysin isoform IIb has a bent structure, where the SH3 domain interacts intramolecularly with the PIP2 binding region of exon 10 (41). Amph I is also proposed to have a bent structure formed by intramolecular binding of the SH3 domain to the PRD (39). Hence, one of the explanations for our observations could be that GST-Amph I-WT comprises either monomers with a folded structure between the SH3 domain and the PRD or polymers formed by intermolecular binding of the two domains. The core sequence for the SH3 domain binding, PXXP, where X denotes any amino acid (42), exists within the major Mnb/Dyrk1A site, RPRSPSQ (Ser-293 is shown in boldface). We found that the GST-Amph I mutants, S293A/S295A, T310A and T310A/T312A, bound well to the GT resins, whereas the S293A mutant behaved similar to the WT (data not shown). It is unlikely that the replacement of serine at 293 and 295 with Ala affects the affinity between the PRD and SH3 domain even though the SH3 domain binds to the PRD at this peptide region. In addition, Thr-310 and Thr-312 are not located within the core sequence for the SH3 binding. For these reasons, we believe that the mutation drastically changes the conformation of the PRD, which results in the exposure of the GT binding site in the GST moiety and in creation of artificial phosphorylation sites for Mnb/Dyrk1A.

In summary, we have found that Mnb/Dyrk1A phosphorylates Amph I, a scaffolding protein required for the onset of clathrin-mediated endocytosis, in addition to two other key endocytic proteins, dynamin I and synaptojanin. The phosphorylated Amph I reduces its binding to endophilin, whereas the phosphorylated dynamin 1xa and synaptojanin 1 alter their binding to the SH3 domains of Amph I and endophilin. Together, our findings suggest that Mnb/Dyrk1A plays an important role in controlling regulated endocytosis. This mechanism may underlie the altered neuronal plasticity associated with Down syndrome.

References

1. Slenev, V. I., Ochoa, G. C., Butler, M. H., Grabs, D., and De Camilli, P. (1998) Science 281, 821–824
2. Cousin, M. A., and Robinson, P. J. (2001) Trends Neurosci. 24, 659–665
3. Bauerfeind, R., Takei, K., and De Camilli, P. (1997) J. Biol. Chem. 272, 30984–30992
4. Cesta, G., Castagnoli, L., Dente, L., Minenková, O., Petrelli, A., Migone, N., Hoffmüller, U., Schneider-Mergener, J., and Cesareni, G. (1999) J. Biol. Chem. 274, 32001–32007
5. Floyd, S. R., Porro, E. B., Slenev, V. I., Ochoa, G. C., Tsai, L.-H., and De Camilli, P. (2001) J. Biol. Chem. 276, 8104–8110
6. Tomizawa, K., Sunada, S., Lu, Y.-F., Oda, Y., Kinuta, M., Ohshima, T., Saito, T., Wei, F.-Y., Matsushita, M., Li, S.-T., Tsutsui, K., Hisanaga, S., Mikoshiba, K., Takei, K., and Matsui, H. (2003) J. Cell Biol. 163, 813–824
7. Lee, S. Y., Wenk, M. R., Kim, Y., Nairn, A. C., and De Camilli, P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 546–551
8. Shang, W. H., Adachi, Y., Nakamura, A., Copeland, T., Kim, S. P., and Kamata, T. (2004) J. Biol. Chem. 279, 40890–40896
9. Kentrup, H., Becker, W., Heukelbach, J., Wilmes, A., Schürmann, A., Huppertz, C., Kainulainen, H., and Joost, H. G. (1996) J. Biol. Chem. 271, 3488–3495
10. Himpel, S., Teige, W., Frank, R., Leder, S., Joost, H.-G., and Becker, W. (2000) J. Biol. Chem. 275, 2431–2438
11. Guimerà, J., Casas, C., Pucharcó,s, C., Solans, A., Domènech, A., Planas, A. M., Ashley, J., Lovett, M., Estivill, X., and Pritchard, M. A. (1996) Hum. Mol. Genet. 5, 1305–1310
12. Shindoh, N., Kudo, J., Maeda, H., Yamaki, A., Minoshima, S., Shimizu, Y., and Shimizu, N. (1996) Biochem. Biophys. Res. Commun. 225, 92–99
13. Song, W. J., Sternberg, L. R., Kasten-Sportès, C., Van Keuren, M. L., Chung, S. H., Slack, A., Miller, D. E., Glover, T. W., Chiang, P. W., Lou, L., and Kurnit, D. M. (2006) Genomics 38, 331–339
14. Guimerà, J., Casas, C., Estivill, X., and Pritchard, M. (1999) Genomics 57, 407–418
15. Tassone, F., Lucas, R., Slavoy, D., Kavans, V., Cnric, L., and Gardiner, K. (1999) J. Neural Transm. Suppl. 57, 179–195
16. Woods, Y. L., Rena, G., Morrice, N., Barthel, A., Becker, W., Guo, S., Unterman, T. G., and Cohen, P. (2001) Biochem. J. 355, 597–607
17. Woods, Y. L., Cohen, P., Becker, W., Jakes, R., Goedert, M., Wang, X., and Proud, C. G. (2001) Biochem. J. 355, 609–615
18. Yang, E. J., Ahn, Y. S., and Chung, K. C. (2001) J. Biol. Chem. 276, 39819–39824
19. Chen-Hwang, M. C., Chen, H. R., Elzenga, M., and Hwang, W. Y. (2002) J. Biol. Chem. 277, 17597–17604
20. Berisha, F., Cheng-Hwang, M., Murakami, N., Wang, R., and Hwang, Y. (2002 Mol. Biol. Cell 13, 94
21. Huang, Y., Chen-Hwang, M. C., Dolios, G., Murakami, N., Padovan, J., Wang, R., and Hwang, Y. W. (2004) Biochemistry 43, 10173–10185
22. Wegiel, J., Kuchna, I., Nowicki, K., Frackowiak, J., Dowjat, K., Silverman, W. P., Reisberg, B., DeLeon, M., Wisniewski, T., Adayev, T., Chen-Hwang, M. C., and Hwang, Y. W. (2004) Brain Res. 1010, 69–80
23. Sung, Y. J., Carter, M., Zhong, J. M., and Hwang, Y. W. (1995) Biochemistry 34, 3470–3477
24. Murakami, N., Chauhan, V. P. S., and Elzinga, M. (1998) Biochemistry 37, 1989–2003
25. Cohen, S. L., and Chait, B. T. (1996) Anal. Chem. 68, 31–37
26. El-Sherif, Y., Tesoriero, J., Hogan, M. V., and Wieraszko, A. (2003) J. Neurosci. Res. 72, 454–460
27. Lammlé, U. K. (1970) Nature 227, 680–685
28. Chagger, H., and Jagow, G. (1987) Anal. Biochem. 166, 367–379
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29. Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991) EMBO J. 10, 885–892
30. Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. J. (1997) Cell 90, 859–869
31. Bain, I., McLauchlan, H., Eliotte, M., and Cohen, P. (2003) Biochem. J. 371, 199–204
32. Cousin, M. A., Tan, T. C., and Robinson, P. J. (2001) J. Neurochem. 76, 105–116
33. Tan, T. C., Valova, V. A., Malladi, C. S., Graham, M. E., Berven, L. A., Jupp, O. J., Hansra, G., McClure, S. J., Sarcevic, B., Boadle, R. A., Larsen, M. R., Cousin, M. A., and Robinson, P. J. (2003) Nat. Cell Biol. 5, 701–710
34. English, J. D., and Sweatt, J. D. (1997) J. Biol. Chem. 272, 19103–19106
35. Micheva, K. D., Ramjaun, A. R., Kay, B. K., and McPherson, P. S. (1997) FEBS Lett. 414, 308–312
36. Peter, B. J., Kent, H. M., Mills, I. G., Vallis, Y., Butler, P. J. G., Evans, P. R., and McMahon, H. T. (2004) Science 303, 495–499
37. Ermakova, S., Choi, B. Y., Choi, H. S., Kang, B. S., Bode, A. M., and Dong, Z. (2005) J. Biol. Chem. 280, 16882–16890
38. Ringstad, N., Gad, H., Löw, P., Di Paolo, G., Brodin, L., Shupliakov, O., and De Camilli, P. (1999) Neuron 24, 143–154
39. Farsad, K., Slepnev, V., Ochoa, G., Daniell, L., Hauke, V., De Camilli, P. (2003) Neuropharmacol 45, 787–796
40. Marks, B., and McMahon, H. T. (1998) Curr. Biol. 8, 740–749
41. Kojima, C., Hashimoto, A., Yabuta, I., Hirose, M., Hashimoto, S., Kanaho, Y., Sumimoto, H., Ikegami, T., and Sabe, H. (2004) EMBO J. 23, 4413–4422
42. Kay, B. K., Williamson, M. P., and Sudol, M. (2000) FASEB J. 14, 231–241