Dynamin Is a Minibrain Kinase/Dual Specificity Yak1-related Kinase 1A Substrate*

The minibrain kinase (Mnbk)/dual specificity Yak 1-related kinase 1A (Dyrk1A) gene is implicated in the mental retardation associated with Down’s syndrome. It encodes a proline-directed serine/threonine kinase whose function has yet to be defined. We have used a solid-phase Mnbk/Dyrk1A kinase assay to aid in the search for the cellular Mnbk/Dyrk1A substrates. The assay revealed that rat brain contains two cytosolic proteins, one with a molecular mass of 100 kDa and one with a molecular mass of 140 kDa, that were prominently phosphorylated by Mnbk/Dyrk1A. The 100-kDa protein was purified and identified as dynamin 1. The conclusion was further supported by evidence that a recombinant glutathione S-transferase fusion protein containing dynamin isoform 1aa was phosphorylated by Mnbk/Dyrk1A. In addition to isoform 1aa, Mnbk/Dyrk1A also phosphorylated isoforms 1ab and 2aa but not human Mxα protein when analyzed by the solid-phase kinase assay. Upon Mnbk/Dyrk1A phosphorylation, the interaction of dynamin 1 with the Src homology 3 domain of amphiphysin 1 was reduced. However, when Mnbk/Dyrk1A phosphorylation was allowed to proceed more extensively, the phosphorylation enhanced rather than reduced the binding of dynamin 1 to amphiphysin 1. The result suggests that Mnbk/Dyrk1A can play a dual role in regulating the interaction of dynamin 1 with amphiphysin 1. Mnbk/Dyrk1A phosphorylation also reduced the interaction of dynamin with endophilin 1, whereas the same phosphorylation enhanced the binding of dynamin 1 to Grb2. Nevertheless, the dual function of Mnbk/Dyrk1A phosphorylation was not observed for the interaction of dynamin 1 with endophilin 1 or Grb2. The interactions of dynamin with amphiphysin and endophilin are essential for the formation of endocytic complexes; our results suggest that Mnbk/Dyrk1A may function as a regulator controlling the assembly of endocytic apparatus.

Minibrain kinase (Mnbk)† was originally identified in Drosophila as a mutation affecting neurogenesis (1). Mnbk mutant flies, which have low levels of kinase expression, possess fewer neuroblasts and a reduced brain volume as compared with the wild type (WT), especially in the optic lobes and central brain (1). The reduction in brain size leads to several distinct learning and behavioral defects. Because the Mnbk mutation does not appear to affect the development of Drosophila until late in the third instar, it was postulated that the Mnbk gene is required for the proliferation of neuroblasts during postembryonic neurogenesis (1).

Dual specificity Yak 1-related kinase (Dyrk) 1A was subsequently cloned (2–5) and identified as the mammalian homologue of the Drosophila Mnbk gene. The Mnbk/Dyrk1A gene is a member of a growing family of Dyrk-related genes (6) whose members include Yak1 (7), several Dyrks (8), ANPK (9), HIPK2 (10), Mirk (11), Myak (12), and Pom1p (13). Mammalian Mnbk/Dyrk1A gene contains either 763 or 754 amino acid residues as a result of alternative splicing (2). The kinase domain of Mnbk/Dyrk1A, consisting of ~320 residues, is located roughly in the center of the protein. In addition to the 11 subdomains characteristic of all protein kinases (14), the Mnbk/Dyrk1A kinase domain contains several signature motifs unique to the Dyrk family. These motifs include the sequence DFGSSC in subdomain VII, a substitution of a highly conserved arginine in subdomain VI-B by cysteine, and a YXX motif between subdomains VII and VIII (6). Outside the kinase domain, Mnbk/Dyrk1A contains a bipartite nuclear target sequence (15), a PEST (proline, glutamate, serine, and threonine) region, a 13-residue histidine repeat, and a serine/threonine repeat near the C-terminal end. Little is known about the functions of these structural features except for the bipartite nuclear targeting sequence. The targeting sequence has been shown to guide overexpressed Mnbk/Dyrk1A into the nucleus (8, 16).

Several lines of evidence suggest that the Mnbk/Dyrk1A gene plays a role in causing the mental retardation phenotype of Down’s syndrome. The human Mnbk/Dyrk1A gene maps to the q22.2 region of chromosome 21 (4, 17–20), a section of chromosome 21 known to associate with the mental retardation phenotype of Down’s syndrome (21). Mnbk/Dyrk1A is highly expressed in the cortex, spinal cord, and olfactory bulb in developing mouse embryos. The patterns of Mnbk/Dyrk1A expression appear to correlate well with the regions of brain

† The abbreviations used are: Mnbk, minibrain kinase; Amp(SH3), Src homology 3 domain of amphiphysin 1; DTT, dithiothreitol; Dyrk, dual specificity Yak-related kinase; PFLC, fast protein liquid chromatography; GST, glutathione S-transferase; MBP, myelin basic protein; SH, Src homology; WT, wild type; MES, 4-morpholineethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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affected the most by Down’s syndrome (3, 22). Significantly, when transgenic mice carry additional Mnbk/Dyrk1A genes, a situation mimicking Down’s syndrome, the animals exhibit various learning and memory defects (23, 24).

Mnbk/Dyrk1A was originally proposed to be a dual specificity protein kinase (2). However, the dual specificity appears to be limited only to Mnbk/Dyrk1A autophosphorylation because the kinase is unable to phosphorylate tyrosine residues on exogenous substrates (8). By using histone and synthetic peptides as the target for phosphorylation, Mnbk/Dyrk1A was subsequently determined to be a proline-directed serine/threonine kinase, and the preferred phosphorylation site was identified as RPP(S/T)P (25). Several potential Mnbk/Dyrk1A substrates were recently identified. These substrates include eukaryotic initiation factor 2Be, the microtubule-associated protein tau, transcription factor forkhead in rhabdomyosarcoma, and the cAMP-response element-binding protein (26–28). Despite this progress, the roles of the Mnbk/Dyrk1A gene in cellular processes are far from established. To unveil its functions, we have conducted a generalized search for the kinase’s substrates in rat brain. We show here that one of the major substrates in rat brain is dynamin 1 and that Mnbk/Dyrk1A phosphorylation modulates the interaction of dynamin with SH3 domain-containing proteins.

**MATERIALS AND METHODS**

**Clone Construction**—The full-length Mnbk/Dyrk1A gene was obtained by PCR from rat testis Quik-clone cDNA (CLONTECH) by using the following sequence-specific oligonucleotides, tctcatcgatgcatacagga- gggagccagggcaaccagaatttc and ctctetactgacagctagcagactgacctgatatgc, respectively, as the 5′ and 3′ primers for amplification. PCR produced a 2.3-kb amplicon, as expected for the full-length rat Mnbk/Dyrk1A gene. The PCR product was then digested with ClaI and XhoI (both restriction sites were introduced by the PCR primers) and cloned into restriction site-modified glutathione S-transferase (GST) vector pGEX-3X, as described previously (29). The coding region of the cloned Mnbk/Dyrk1A gene was sequenced. We found two mismatches between our clone and the published sequence (2); both of them are silent. The sequencing also disclosed that our clone encodes the 754-amino acid (residues 454–696) of the full-length human endophilin 1 (30, 31, 32). The primer pair ctctetactgacagctagcagactgacctgatatgc and ctctetactgacagtctagagctgacctgatatgc, respectively, were used for amplifying aphymiphin 1, whereas the primer pair ctctetactgacagctagcagactgacctgatatgc and ggtctctgagctgacttagatgagggcagagctgacctgatatgc, respectively, were used for generating endophilin 1. GST-dynamin 1aa (rat), GST-dynamin 2aa (rat), and GST-MxaA (human) clones were constructed from pCMV96-7 (33) and pCMV96-7 (33) and p78/2-Bb (34), respectively, by PCR and subcloning. Kinase-deficient GST-Mnbk/Dyrk1A harboring Y319F and Y321F double mutation (DF) was constructed by site-directed mutagenesis by using oligonucleotide ggtactgagctgacttagatgagggcagagctgacctgatatgc as the primer. The primer simultaneously converts both tyrosines to phenylalanines in a single step.

**Preparation of the GST Fusion Proteins**—GST fusion proteins were expressed in *Escherichia coli* as described previously (29). We found that GST-Mnbk/Dyrk1A purified by nickel-nitritrolactic acid resin (which binds to the 13-histidine repeat of Mnbk/Dyrk1A) tends to have a higher specific activity than that prepared by glutathione resin; GST-Mnbk/Dyrk1A purified by nickel-nitritrolactic acid resin as suggested by the manufacturer. All other GST fusion proteins were purified by using glutathione-Sepharose 4B as described previously (29). Protein concentration was determined by the Bradford method (35) with bovine serum albumin used as a standard.

**Kinase Assays**—The solid-phase kinase assay (denatured assay) was performed as follows. Proteins to be tested were separated by SDS-PAGE (minigel) and then transferred onto Immobilon-P membranes (Millipore). After the Mnbk/Dyrk1A membrane was blocked by shaking them in 20 ml of blocking buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl2, and 5 mM MnCl2) containing 5 μl cold ATP, 40 μCi of [γ-32P]ATP (specific activity, 7000 Ci/mmol), and 10 μg of GST-Mnbk/Dyrk1A at room temperature for 60 min. The membranes were then washed as described by Ferrell and Martin (36) to reduce the background. Phosphorylated peptides were detected by autoradiography. The solution kinase assay (native assay) was performed in a 30-μl reaction mixture containing kinase buffer, 1 μg of Sub-100 (dynamin 1), 5 μl ATP, and 2 μCi of [γ-32P]ATP (7000 Ci/mmol) if needed. The reaction was initiated by the addition of GST-Mnbk/Dyrk1A and allowed to proceed at 30°C. To determine the time course of phosphate incorporation, a 5-μl aliquot of each reaction mixture was withdrawn at the indicated times; precipitated together with 100 μg of bovine serum albumin in 2 ml of silicotungstic acid (4% in 3 N sulfuric acid), collected onto a glass fiber filter (Whatman GF/B), and quantified by a scintillation counter. To determine the kinetic parameters of phosphorylation, Sub-100 (0.5–5 μg) was phosphorylated with 0.05 μg of GST-Mnbk/Dyrk1A for 10 min at 30°C and quantified as described in the solution kinase assay. After correcting for the background (substrate and enzyme alone), the rate of phosphate incorporation was then used to fit the Michaelis-Menten equation to determine Vmax and Km for the phosphorylation reaction. The kcat was then calculated as Vmax/[Mnbk/Dyrk1A]. The data presented were the average of three independent assays.

**Purification of Sub-100**—Sub-100 was purified from rat brain aqueous extracts as follows. All procedures were performed at 4°C or 0–4°C unless stated otherwise. Adult rat brains (unstripped) were obtained from PelFreez Biological (Rogers, AK) and homogenized in homogenization buffer (3.5 ml brain containing 25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 2 mM EDTA, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride by using a glass Dounce homogenizer. This buffer released at least 50% of total cellular Sub-100. Brain lysate was obtained by centrifugation at 30,000 × g for 20 min, followed by a second centrifugation at 100,000 × g for 60 min. The recovered supernatant was dialyzed overnight against Tris-buffer A (25 mM Tris-HCl, pH 7.4, and 25 mM NaCl) plus 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride. After removing precipitates by centrifugation (12,000 × g for 20 min), the brain lysate was loaded onto a Mono Q (HR 10/10) column (Amersham Biosciences) attached to a fast protein liquid chromatography. The column was then eluted with a 0–0.4 M NaCl gradient (0.02 × NaCl gradient/min in Tris-Buffer A and 1 mM DTT; flow rate, 3 ml/min) at room temperature, and 20 one-minute fractions were collected. Each fraction was analyzed for the presence of Sub-100 by the solid-phase kinase assay. Sub-100 was eluted from Mono Q column with 0.16–0.22 M NaCl. Sub-100 fractions were pooled and mixed with an equal volume of Tris-Buffer A (25 mM Tris-HCl, pH 7.0, 0.1 M NaCl, and 1 mM DTT). This mixture was then applied to a Ni2+–nitrilotriacetic acid resin as described previously (29). The precipitates were collected by centrifugation (12,000 × g for 10 min), dissolved in 3 ml of Tris-buffer A, and dialyzed overnight against 1000 ml of buffer containing 25 mM MES (pH 6.5), 2.5 mM NaCl, and 1 mM DTT. Sub-100 precipitated during dialysis. The precipitates were then collected by centrifugation and extracted three times with 100 μl of Tris-buffer A. This extraction recovered about 70% of the precipitated Sub-100. The extraction of 100 μl of Tris-buffer A was then added to Sub-100 solution to stabilize the protein. The proteins were stored at −70°C until use. Purification of Sub-140 has been achieved recently and will be described elsewhere. Brain SDS extract was prepared by homogenizing rat brains in homogenization buffer containing 1% SDS followed by centrifugation at 30,000 × g for 20 min.

**Protein Sequencing**—Sub-100 to be sequenced was further purified on an 8% Tricine SDS-PAGE gel and transferred onto an Immobilon-CD membrane (Millipore). The area of membrane containing Sub-100 was cut into small strips and placed into a 1.5-mL screw-cap microcentrifuge tube. Enough cyanoagen bromide solution (20 mg/ml in 70% formic acid) to cover membrane strips was added to the tube, followed by incubation at room temperature under nitrogen and in the dark for 16 h. Digested peptides were recovered in formic acid, dried under vacuum, and then resolved on a 12% Tricine SDS-PAGE gel. Separated peptides were then transferred to an Immobilon-P membrane, stained with Coomassie Blue, and sequenced as membrane-bound material by using an Applied Biosystems automatic sequencer.

**Protein-Protein Binding Assays**—Binding of dynamin to SH3 domain-containing proteins was performed as follows. Proteins to be tested were separated by SDS-PAGE (minigel) and then transferred onto Immobilon-P membranes (Millipore). After the Mnbk/Dyrk1A membrane was blocked by shaking them in 20 ml of blocking buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl2, and 5 mM MnCl2) containing 5 μl cold ATP, 40 μCi of [γ-32P]ATP (specific activity, 7000 Ci/mmol), and 10 μg of GST-Mnbk/Dyrk1A at room temperature for 30 min at 30°C, but no radioactive ATP was included in the reaction. The phosphorylated dynamin was then diluted with binding buffer (25 mM Tris, pH 7.4, 50 mM NaCl, 5 mM EDTA, and 1 mM DTT) to 200 μl and mixed with a 50-μl suspension of glutathione resin precoated with GST or GST fusion protein for 16 h at 4°C. The resin was collected by centrifugation and washed five times by tumbling in...
the washing buffer (25 mM Tris, pH 7.4, 150 mM NaCl, and 1% Triton X-100) for 5 min at room temperature. Following the washings, the resin-bound proteins were eluted with 60 µl of buffer containing 100 mM Tris, pH 8.0, and 20 mM glutathione and subjected to Western blotting analysis. The blot was probed with mouse monoclonal anti-dynamin antibody HDy-1 (Upstate Biotechnology, Lake Placid, NY) (1:5000 dilution) and visualized by using sheep anti-mouse alkaline phosphatase-conjugated secondary antibody (Sigma) (1:5000 dilution) and CDP-Star chemiluminescence reagent (Applied Biosystems/Tropix, Foster City, CA). The quantitation of dynamin was performed by scanning x-ray films and analyzing the scanned images with the National Institutes of Health Image program. Precoating of glutathione resin with GST or GST fusion protein was prepared by incubation with tumbling of 200 µl (bed volume) of washed (twice with washing buffer) glutathione resin with 1.6 nmol of proteins at 4 °C for 4 h. The resin was then washed twice and resuspended to 25% slurry in washing buffer. Approximately 80–90% GST or GST fusion proteins bound to the resin, which is equivalent to at least 80 pmol GST or GST fusion proteins/50 µl resin suspension.

Brain cytosol was prepared by following the protocols described by Slepnev et al. (38). Phosphorylation was performed by incubating the cytosol at 30 °C for 30 min in the presence of 2 mM ATP, 2 mM MgCl₂, 0.2–10 µM either WT or DF GST-Mnbk/Dyrk1A (if needed), and a mixture of phosphatase inhibitors (2 µM cyclosporine, 0.2 µM okadaic acid, and 1 mM sodium orthovanadate). After stopping the reaction by the addition of 5 mM EDTA, the treated cytosol was then subjected to binding assay by mixing 0.5 µl of cytosol with 50 µl of glutathione resin precoated with GST proteins for 16 h at 4 °C. Bound dynamin was eluted from the resin and determined as described.

RESULTS

Development of the Mnbk/Dyrk1A Solid-phase Kinase Assay—A rapid and reliable assay is essential for the purification of kinase substrates. Therefore, a solid-phase kinase assay was developed for Mnbk/Dyrk1A by adapting the combination of two previously described protocols (36, 39). Briefly, proteins to be tested are separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, blocked by bovine serum albumin, and then subjected to Mnbk/Dyrk1A phosphorylation in the presence of [γ-³²P]ATP. Under the conditions specified, the method detected 10 ng of immobilized myelin basic protein (MBP) (Fig. 1).

Detection of Mnbk/Dyrk1A Substrates in Rat Brain—Rat brain aqueous extract was prepared and subjected to the solid-phase kinase assay. As shown in Fig. 2A, Mnbk/Dyrk1A prominently phosphorylated two proteins in adult rat brain: a 100-kDa protein (Sub-100) and a 140-kDa protein (Sub-140). In addition, several minor phosphorylated proteins were also detected in the assay. Phosphorylation of these two proteins did not appear to be due to auto phosphorylation because no labeled phosphate incorporation was detected in the assay. Phosphorylation of these two proteins did not appear to be due to autophosphorylation because no labeled phosphate incorporation was detected in the assay. Phosphorylation of these two proteins did not appear to be due to autophosphorylation because no labeled phosphate incorporation was detected in the assay. Phosphorylation of these two proteins did not appear to be due to autophosphorylation because no labeled phosphate incorporation was detected in the assay.

Sub-100 was purified from adult rat brains under native conditions (Fig. 3A). Like the denatured Sub-100 used in the solid-phase assay, purified Sub-100 was efficiently phosphorylated by Mnbk/Dyrk1A under native conditions. This result suggests that the Mnbk/Dyrk1A phosphorylation site on Sub-100 is naturally exposed. The extent of phosphate incorporation was highly dependent on the GST-Mnbk/Dyrk1A concentration (Fig. 3B). The molar ratio of phosphate incorporation would only reach a certain level if Sub-100 was incubated with a low amount of kinase. On the other hand, the ratio could easily exceed 1 if a high level of kinase was used for the assay. By using native Sub-100 as a substrate and a constant kinase level of 0.05 µg/assay, the apparent K₉ and k₉ values of the reaction were determined to be 1.17 µM and 0.15 s⁻¹, respectively.

Identification of Sub-100 kDa as Dynamin 1—Because Sub-100 could not be sequenced directly, it was first cleaved with cyanogen bromide. Cyanogen bromide cleaves Sub-100 into eight visible fragments with apparent molecular masses ranging from 4 to 16 kDa (4.5, 5, 6, 8, 10, 11, 13, and 16 kDa) when analyzed on a 12.5% SDS-Tricine gel (polypeptides smaller than 4 kDa were not resolved by this gel). Edman degradation showed that the largest fragment, the 16-kDa polypeptide, contained the sequence (M)(T/E)DLIPLVNRL. BLAST search identified this fragment as unique to rat dynamin 1. This 16-kDa fragment corresponds to the 135-residue peptide (calculated mass, 14,528 Da) resulting from cyanogen bromide cleavage at methionine-4 and methionine-140 of dynamin 1. Then the 6-kDa peptide was sequenced and found to contain the sequence MGDGXXDAR. This sequence again matches dynamin, but it is common to all dynamin isoforms. Dynamin is encoded by three distinct genes, each expressed as several closely related spliced variants (40). We inferred that Sub-100 is primarily, if not exclusively, dynamin 1 based on the following reasoning. First, Sub-100 matches dynamin 1 in size and contains a dynamin 1-specific peptide. Second, dynamin 1 is the dominating isoform in brain, the source for Sub-100. Third, Sub-100 precipitates at pH 6.5 (Materials and Methods).
which is consistent with the isoelectric point of dynamin 1 but not with that of dynamin 2 and dynamin 3. Fourth, Sub-100 lacks the larger cyanogen bromide-cleavable peptide (calculated mass, 16,825 Da) predicted from the sequence of dynamin 2, which indicates that Sub-100 does not contain a significant amount of dynamin 2. For convenience, Sub-100 will be referred to as dynamin 1.

**Phosphorylation of E. coli-expressed Dynamin Isoforms by Mnbk/Dyrk1A**—To confirm that dynamin 1 is an Mnbk/Dyrk1A substrate, a GST fusion clone containing the dynamin 1aa isoform was constructed from plasmid pCMV96-7 (33). Upon induction, the clone produced a 125-kDa protein (Fig. 4A, 1), which can be recognized by anti-human dynamin monoclonal antibody Hudy-1 (37) (data not shown). This protein was then analyzed by the solid-phase kinase assay. As shown in Fig. 4B, 1, GST-dynamin 1aa was phosphorylated by Mnbk/Dyrk1A similarly to the purified rat brain dynamin 1. This observation substantiates the conclusion that dynamin 1aa is a Mnbk/Dyrk1A substrate. In addition to isoform 1aa, Mnbk/Dyrk1A also phosphorylated dynamin-1ab (data not shown) and dynamin 2aa (Fig. 4A and B, 2) under the same condition. Dynamin 1ab is identical to the 1aa isoform except for 20 or so residues at the C terminus, whereas dynamin 2 shares about 78% overall homology with dynamin 1. In contrast, human MxA protein, a distant dynamin homologue that is inducible by type 1 interferon and some viruses (34), was not a Mnbk/Dyrk1A substrate (Fig. 4, A and B, 3).

**Effects of Mnbk/Dyrk1A Phosphorylation on the Interaction of Purified Dynamin 1 with Amphiphysin**—The binding of dynamin to amphiphysin is required for incorporation of dynamin into endocytic complexes (41). The interaction is inhibited when dynamin is phosphorylated (38). However, the kinase (or kinases) responsible for the dynamin phosphorylation has not been identified. Therefore, it was of interest to test whether Mnbk/Dyrk1A could assume the role of the unknown kinase. To perform the experiment, purified rat brain dynamin 1 was phosphorylated by Mnbk/Dyrk1A in solution and then allowed to bind to the immobilized GST fusion protein containing the SH3 domain of human amphiphysin 1, GST-Amp(SH3). After recovering the immobilized GST-Amp(SH3), the presence of dynamin in the complex was determined with the anti-dynamin antibody Hudy-1. As expected, GST-Amp(SH3) bound

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Purification and phosphorylation of native Sub-100 by GST-Mnbk/Dyrk1A. A, Coomassie Blue staining of purified Sub-100. B, time course of phosphate incorporation. Purified native Sub-100 (1 μg) was phosphorylated by GST-Mnbk/Dyrk1A (0.05–0.8 μg) in the solution kinase assay in a total volume of 30 μl. At the indicated times, a 5-μl aliquot of labeled Sub-100 was withdrawn and precipitated with silicotungstic acid and quantified as described under “Materials and Methods.” Parallel experiments, performed with Sub-100 alone and with kinase alone, were used for background correction. The numbers represent the average of three independent experiments. The amounts of GST-Mnbk/Dyrk1A used for the assays were 0.05 (●), 0.1 (■), 0.4 (▲), and 0.8 μg (▲).

![Figure 4](http://www.jbc.org/)

**FIG. 4.** The production and phosphorylation of dynamin isoforms and the MxA protein. GST fusion proteins were expressed in *E. coli* strain BL21(DE3) as described under “Materials and Methods.” Cells from 200-μl aliquots were collected by centrifugation, boiled in 0.5 volume of SDS-PAGE loading buffer, and electrophoresed on duplicate 8% Tricine-SDS gels. One gel was stained with Coomassie Blue (A), and the other was blotted and subjected to solid-phase Mnbk/Dyrk1A phosphorylation (B). IPTG –, controls; IPTG +, isopropyl-β-D-thiogalactopyranoside-induced samples. Asterisks indicate the induced GST fusion proteins. 1, GST-dynamin 1aa; 2, GST-dynamin 2aa; 3, GST-MxA.
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Dynamin 1 as opposed to the GST control (Fig. 5A). Interestingly, the ability to bind GST-Amp(SH3) was reduced when dynamin was phosphorylated by GST-Mnbk/Dyrk1A because DF, a mutant protein possessing a fraction of catalytic activity of the WT (2), had little inhibitory effect on the binding (Fig. 5A). The reduction in binding required the dynamin phosphorylating activity of GST-Mnbk/Dyrk1A because DF, a mutant protein possessing a fraction of catalytic activity of GST-Mnbk/Dyrk1A-phosphorylated and nonphosphorylated dynamin 1 (Fig. 7), it further ruled out the possibility that the reduction observed (Fig. 5A) was caused by the inability of Hudy-1 to recognize the Mnbk/Dyrk1A-phosphorylated dynamin 1.

Intriguingly, if dynamin was phosphorylated with higher concentrations of Mnbk/Dyrk1A (Fig. 5B), specifically with an amount of Mnbk/Dyrk1A that was able to promote >1 mol phosphate incorporation/mol of dynamin (Fig. 3B), the phosphorylation resulted in enhancement rather than reduction of the binding of dynamin to GST-Amp(SH3). This observation suggests that Mnbk/Dyrk1A phosphorylation plays a dual role in regulating the amphiphysin binding property of dynamin.

Effects of Mnbk/Dyrk1A Phosphorylation on the Interaction of Dynamin with Amphiphysin in Crude Brain Extract—We further examined the effects of Mnbk/Dyrk1A phosphorylation on dynamin-ampiphysin binding in crude brain extracts. A low-salt aqueous extract (cytosol) containing many proteins involved in endocytosis was prepared from adult rat brains by following the protocols of Slepnev et al. (38). After incubation with ATP and phosphatase inhibitors, cytosol was mixed with resin-immobilized GST-Amp(SH3), and the amount of dynamin co-precipitated with the resin was analyzed. As opposed to the Triton X-100 extract (38), the addition of ATP and phosphatase inhibitors did not cause an appreciable reduction in the binding of dynamin to GST-Amp(SH3) (data not shown). This result suggests that the cytosol fraction lacked the necessary kinase to modulate the binding of dynamin to GST-Amp(SH3). Therefore, the cytosol was used for testing the effects of GST-Mnbk/Dyrk1A supplementation on the binding of dynamin to GST-Amp(SH3). Similarly to purified dynamin 1 (Fig. 5), the addition of the wild-type GST-Mnbk/Dyrk1A, ATP, and phosphatase inhibitors inhibited the binding of dynamin to GST-Amp(SH3) (Fig. 8). Again, GST-DF had little effect on the binding, indicating that the kinase activity is essential for the inhibition of dynamin-ampiphysin binding (Fig. 8). This result shows that Mnbk/Dyrk1A phosphorylation can inhibit dynamin-ampiphysin binding in a defined as well as in a more complex system, such as crude brain extract. When cytosol was treated with a higher concentration of Mnbk/Dyrk1A, more dynamin was found to bind GST-Amp(SH3) than the untreated control (data not shown). Thus, the dual effect of Mnbk/Dyrk1A phosphorylation on dynamin and GST-Amp(SH3) binding was also observed in crude extract.

Effects of Mnbk/Dyrk1A Phosphorylation on the Interaction of Dynamin with Endophilin 1 and Grb2 in Crude Brain Extract—We then analyzed whether Mnbk/Dyrk1A phosphorylation affected the binding of dynamin to other SH3 domain-containing proteins. Endophilin 1 (SH3p4) (42) and Grb2 (43) were chosen for the study because of their roles in mediating the cellular function of dynamin. To perform the assay, GST fusion of full-length human endophilin 1 and Grb2 was prepared, immobilized onto resin, and used as the affinity matrix for binding dynamin. Both endophilin 1 and Grb2 bound dynamin under the same assay conditions used for analyzing

![Figure 5. Interaction of Mnbk/Dyrk1A-phosphorylated dynamin with the SH3 domain of amphiphysin 1. A, amphiphysin binding property of phosphorylated dynamin. Purified dynamin 1 (1 μg) was phosphorylated by 0.1 μg of Mnbk/Dyrk1A and then bound to glutathione resin pre-coated with GST or GST-Amp(SH3) as described under "Materials and Methods." After binding, glutathione resin was collected, and co-precipitated dynamin was detected by anti-dynamin antibody. Dynamin incubated with ATP but without the addition of kinase was used as the phosphorylation control. GST, experiment performed with GST-coated resin; Amp(SH3), experiment performed with GST-Amp(SH3)-coated resin. ATP, control phosphorylation with ATP alone; DF, phosphorylated by GST-Mnbk/Dyrk1A double mutant plus ATP; WT, phosphorylated by wild-type GST-Mnbk/Dyrk1A plus ATP. Bars, which have been normalized to the ATP control (ATP control = 1), represent the mean ± S.D. of three independent experiments. A paired t test was performed for the treatment pairs ATP-WT, DF-WT, and ATP-DF. ATP and DF were found to be statistically different from WT (*, p < 0.01), whereas no difference was found between ATP and DF. B, amphiphysin binding of dynamin phosphorylated by different concentrations of Mnbk/Dyrk1A. Purified dynamin 1 (1 μg) was phosphorylated with either 0, 0.1, 0.4, or 0.8 μg of WT Mnbk/Dyrk1A and then subjected to the amphiphysin binding assay as described above. Dynamin 1 treated with 0.1 or 0.8 μg of WT Mnbk/Dyrk1A was found to be significantly different from the untreated control in binding amphiphysin (*, p < 0.01, paired t test).
Dynamin is a large GTPase known to play an essential role in clathrin-mediated endocytosis and synaptic vesicle recycling (40, 41, 44). It has been proposed that dynamin, which assemble around the necks of invaginated clathrin-coated pits, is responsible for constraining and pinching coated vesicles from the plasma membrane through a concerted conformational change (45–48). However, evidence also exists that dynamin may function as a regulator in receptor-mediated endocytosis rather than as a mechanochemical enzyme directly involved in generating vesicles (49). Dynamins consist of four recognizable structural domains: a highly conserved GTPase domain in the amino terminus, an effector domain, and the least conserved proline-rich domain at the carboxyl terminus (40). This phenomenon may be attributed to the fact that purified GST-Mnbk/Dyrk1A is rather unstable in vitro. Dynamin can be phosphorylated to >1 mol phosphate/mol protein if sufficient kinase is present. The observation implies that dynamin may contain multiple Mnbk/Dyrk1A phosphorylation sites. Using a constant level of 0.05 μg of Mnbk/Dyrk1A, the $K_{m}$ and $k_{cat}$ values of the phosphorylation reaction were determined to be 1.17 μM and 0.15 s⁻¹, respectively. These values are similar to those of Mnbk/Dyrk1A phosphorylation of MBP (25).

**DISCUSSION**

A solid-phase kinase assay for analyzing substrates of Mnbk/Dyrk1A was developed and used to probe rat brain extract (Fig. 1). Two cytosolic substrates of Mnbk/Dyrk1A, one of 100 kDa and the other of 140 kDa, were revealed by the assay (Fig. 2). The 100-kDa protein was purified and determined to be dynamin 1. It was further confirmed that dynamin 1 is a Mnbk/Dyrk1A substrate by showing that recombinant GST-dynamin 1 proteins (both the dynamin 1aa and 1ab isoforms) were efficiently phosphorylated by Mnbk/Dyrk1A (Fig. 4). In addition to dynamin 1, Mnbk/Dyrk1A was also shown to phosphorylate recombinant GST-dynamin 2aa but not the human Mxα protein in the solid-phase assay (Fig. 4). Mnbk/Dyrk1A is capable of phosphorylating native dynamin (Fig. 3A), indicating that phosphorylation sites are naturally accessible to the kinase. The extent of phosphorylation was highly dependent on the amounts of input kinase (Fig. 3B). For example, a high phosphorylation ratio could not be achieved even with prolonged incubation if one started with a low level of kinase (Fig. 3B). This phenomenon may be attributed to the fact that purified GST-Mnbk/Dyrk1A is rather unstable in vitro. Dynamin can be phosphorylated to >1 mol phosphate/mol protein if sufficient kinase is present. The observation implies that dynamin may contain multiple Mnbk/Dyrk1A phosphorylation sites. Using a constant level of 0.05 μg of Mnbk/Dyrk1A, the $K_{m}$ and $k_{cat}$ values of the phosphorylation reaction were determined to be 1.17 μM and 0.15 s⁻¹, respectively. These values are similar to those of Mnbk/Dyrk1A phosphorylation of MBP (25).
amphiphysin and dynamin 1 are highly enriched in nerve terminals, and they are the major binding partners to each other (50). By virtue of its ability to interact with various components of endocytic complexes, such as clathrin, adaptins, synaptojanin, and others, amphiphysin appears to function as an adapter protein for incorporating dynamin into the endocytic apparatus (41, 51). In neuronal cells, dynamin undergoes an activity-dependent phosphorylation-dephosphorylation cycle: it is phosphorylated when the cell is in the resting state and is rapidly dephosphorylated when the cell is depolarized (52, 53). It has been shown that phosphorylation of dynamin inhibits its interaction with amphiphysin and the subsequent incorporation into the endocytic apparatus (38). The kinase (or kinases) responsible for the phosphorylation has not been identified. Our results demonstrate that Mnbk/Dyrk1A can fulfill the role of the unidentified kinase in vitro.

Mnbk/Dyrk1A appears to play a dual role in mediating dynamin and amphiphysin binding (Fig. 5B). When dynamin was phosphorylated by a low concentration of Mnbk/Dyrk1A, the dynamin-amphiphysin binding was inhibited by the phosphorylation. However, if dynamin was phosphorylated with a larger amount of Mnbk/Dyrk1A, the phosphorylation resulted in the enhancement of dynamin binding to amphiphysin. The amount of Mnbk/Dyrk1A needed for the changeover appeared to be between 0.1 and 0.4 μg Mnbk/Dyrk1A/μg dynamin 1 (Fig. 5B). This level of kinase is roughly equivalent to the concentration required for promoting >1 mol phosphate incorporation/mol dynamin (Fig. 3B). The level of phosphate incorporation and the ability to bind amphiphysin must be related; therefore, we speculate that dynamin may contain two Mnbk/Dyrk1A phosphorylation sites, which are phosphorylated by Mnbk/Dyrk1A with different rates (or recognized by Mnbk/Dyrk1A with different affinities). Phosphorylation at the fast site can be achieved with low kinase concentrations and accounts for the reduction in amphiphysin binding, whereas phosphorylation at the slow site can only be obtained with high kinase concentrations and can reverse the effects of the fast site phosphorylation. This hypothesis explains the dual effect of Mnbk/Dyrk1A phosphorylation. Apparently, the slow site phosphorylation does not influence the binding of dynamin to endophilin 1 and Grb2 because these bindings are not affected by the level of dynamin phosphorylation. Furthermore, the determined \( K_a \) and \( k_{cat} \) values probably represent the phosphorylation of the fast site because the experiments were performed with a low concentration of kinase. Because the level of residual phosphorylation in purified rat dynamin 1 was not determined, alternatively, the property of dynamin 1 phosphorylated with varying amounts of Mnbk/Dyrk1A may reflect distinct sensitivities of different phosphorylation sites to dephosphorylation during dynamin 1 purification.

Mnbk/Dyrk1A phosphorylation also reduced the interaction of dynamin with endophilin 1 (Fig. 8). Like amphiphysin, endophilin 1 is highly enriched in nerve terminals (42, 54). Studies suggest that this protein may be involved in multiple steps of synaptic vesicle recycling, ranging from clathrin-coated vesicle invagination to fission and possibly to the uncoating of vesicles (55, 56). To accomplish these functions, endophilin 1 appears to require the lysophosphatidic acid acyltransferase activity of dynamin 1 as well as the direct participation of dynamin (57). The finding that Mnbk/Dyrk1A phosphorylation inhibits dynamin and endophilin 1 binding further suggests a potential role for the kinase in the endocytic pathways.

Mnbk/Dyrk1A phosphorylation enhanced the interaction of dynamin with Grb2. The role of the Grb2 in tyrosine kinase signal transduction is well established (58). Grb2 consists solely of SH2 and SH3 domains and serves as an adaptor linking different signal transduction pathways, such as receptor tyrosine kinases and the mitogen-activated protein kinase cascade. The ability of Grb2 to bind dynamin implies an involvement of dynamin in the tyrosine kinase signaling pathway. Studies have shown that the dynamin-Grb2 interaction recruits dynamin to the insulin signaling complex and subsequently promotes tyrosine phosphorylation of dynamin (59, 60). Furthermore, dynamin may also participate in the signaling pathways of G-protein-coupled receptors through interaction with Grb2. G-protein-coupled receptor, such as β2-adrenergic receptor, is known to associate with Grb2 as a result of tyrosine phosphorylation on G-protein-coupled receptor (61). Conceivably, the dynamin-Grb2 interaction could bring dynamin to phosphorylated G-protein-coupled receptor and promote receptor internalization, which may subsequently lead to termination of receptor signaling (61) or stimulation of the downstream kinase cascade for some receptors (62). These connections indicate that Mnbk/Dyrk1A may regulate the signal transduction pathway of receptors through dynamin phosphorylation.

Mnbk/Dyrk1A has been shown to be a proline-directed kinase (25). This conclusion is consistent with our preliminary data showing that all Mnbk/Dyrk1A phosphorylation sites appear to be localized in the proline-rich domain of dynamin 1. 2 This may also explain why MxA, a protein lacking proline-rich domain, was not a Mnbk/Dyrk1A substrate (Fig. 4). With the use of histone and synthetic combinatorial peptides, it was determined that Mnbk/Dyrk1A preferentially phosphorylates a site with the sequence RX(S/T)P (25). Dynamin 1xa isoforms contain the sequence RXRPEESP, localized near the C-terminal end (33, 53). Interestingly, the sequence is located about 14 residues away from the overlapping amphiphysin and endophilin 1 binding sites of dynamin 1 (63, 64). Nevertheless, it should be pointed out that although both dynamin 1ab and dynamin 2aa isoforms are efficiently phosphorylated by Mnbk/Dyrk1A in the solid-phase assay, the sequence RX(S/T)P is not present in either isoform. Clearly, Mnbk/Dyrk1A allows certain degrees of variation for its phosphorylation site. Work to map the Mnbk/Dyrk1A phosphorylation sites on dynamin is currently under way.

On the basis of its ability to phosphorylate dynamin and modulate its interaction with amphiphysin and endophilin, we suggest that Mnbk/Dyrk1A is involved in regulating synaptic vesicle recycling. Disrupting the function of dynamin has been shown to affect the synaptic activity required for memory retrieval in Drosophila (65, 66). Our findings shed light on animal model studies reporting that either over- or underexpression of the Mnbk/Dyrk1A gene caused behavioral, learning, and cognitive defects (1, 23, 24).

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