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Dyrk1A Binds to Multiple Endocytic Proteins Required for Formation of Clathrin-Coated Vesicles[†]

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ABSTRACT: In spite of a nuclear targeting sequence, a substantial amount of dual-specificity tyrosine phosphorylation-regulated kinase (Dyrk1A) is located within the cytoplasm of neurons. Analysis of fractionated rat brains revealed that the majority of Dyrk1A was in the postnuclear precipitate. The kinase in this fraction was resistant to high salt and Triton X-100 extraction at pH 6.5. Hypothesizing that Dyrk1A binds tightly with cell constituents, we searched for Dyrk1A binding proteins in the Triton X-100-insoluble fraction extracted with urea and fractionated by column chromatography. An overlay assay using the recombinant kinase revealed that multiple proteins are capable of binding to Dyrk1A. Among them, we identified clathrin heavy chain and dynamin 1 as potential candidates. An overlay assay using purified and partially purified proteins showed the binding of Dyrk1A with both proteins. Under native conditions, Dyrk1A precipitated with newly formed clathrin cages and with dynamin via the GST-amphiphysin SH3 domain. We also identified another endocytic protein, endophilin 1, as an additional Dyrk1A binding protein. We then tested whether the clathrin-coated vesicle (CCV)-associated proteins could be phosphorylated by Dyrk1A. Multiple proteins apparently distinctive from the known substrates were phosphorylated in the brain CCV. Our findings suggest a role for Dyrk1A in controlling synaptic vesicle recycling processes.

Dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A)[1] is a proline-directed serine/threonine kinase (*1*, *2*) highly conserved among various organisms (*3*−*5*). The Dyrk1A gene homologue, minibrain, is required for the proliferation of neuroblasts during postembryonic neurogenesis in *Drosophila*. Deletion mutations of the gene cause a reduction in the level of kinase expression and in brain size (hence the name "minibrain"), but not the brain gross anatomy of adult flies (*6*). As in to its role in *Drosophila*, Dyrk1A is also involved in the early development of the central nervous system of vertebrates (*4*, *7*−*9*); it is expressed strongly in the central nervous system and heart, suggesting significant roles of Dyrk1A in the development of these organs (*8*). The Dyrk1A gene is located within the Down syndrome (DS) critical region of human chromosome 21 (*9*−*11*) and considered as one of the key genes underlying DS. The expression levels of Dyrk1A are elevated in individuals with DS (*12*, *13*), and transgenic mice overexpressing Dyrk1A exhibit symptoms similar to those of DS, including neurodevelopmental delay, cognitive deficits, and significant impairment in memory (*14*). Dyrk1A is expressed persistently throughout adulthood in selected brain regions, such as the entorhinal cortex, the olfactory bulb, the cerebellum, and the hippocampus (*10*, *15*, *16*), brain regions affected most severely in DS patients. The temporal and spatial distributions of Dyrk1A suggest that this gene plays an important role in controlling the functions of the adult brain. In addition, the level of Dyrk1A mRNA seems to be significantly elevated in the hippocampus in individuals with Alzheimer's disease (AD) (*17*). Overexpression of the kinase in immortalized hippocampal cells produced the neuropathological features of DS and AD (*18*). Thus, it is rational to believe that upregulation of Dyrk1A contributes to the abnormal neurobiological features seen in DS and AD patients.

Several substrates for this kinase have been identified such as microtubule-associated protein tau (*19*, *20*), transcription factors such as FKHR (*21*), cAMP response element-binding protein (*22*), NFAT (*23*, *24*), α-synuclein (*25*), and caspase 9 (*26*, *27*). We have found that Dyrk1A phosphorylates multiple proteins engaged in regulated endocytosis in neurons: dynamin 1a (*28*), synaptojanin 1 (*29*), and amphiphysin 1 (*30*). Dynamin 1 phosphorylation occurs primarily at Ser^{857} in the proline rich domain (PRD) (*31*). Phosphorylation at Ser^{857} directly contributes to a reduction in the level of binding of dynamin to the Src homology 3 (SH3) domains of amphiphysin 1 (*31*). Phosphorylation of synaptojanin 1 by Dyrk1A also reduced its level of binding to the SH3 domains of amphiphysin and intersectin (*29*). We expect that Dyrk1A phosphorylates synaptojanin 1 within its PRD, yet the exact phosphorylation site(s) remains to be mapped. Amphiphysin 1 is phosphorylated at Ser^{293} within a PRD located in the middle of

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[1]Abbreviations: 8D9, monoclonal anti-Dyrk1A antibody; AD, Alzheimer's disease; CCV, clathrin-coated vesicle; DS, Down syndrome; Dyrk1A, dual-specificity tyrosine phosphorylation-regulated kinase 1A; $Dyrk1A^{497}$, truncated Dyrk1A without the C-terminal PEST domain; GST, glutathione *S*-transferase; GT, glutathione; HA, hydroxylapatite; Hudy1, monoclonal anti-human dynamin 1 antibody; LC−MS/MS, liquid chromatography−tandem mass spectroscopy; MS, mass spectrometry; PRD, proline rich domain; PEST, proline-glutamic acid-serine-threonine rich sequence; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SH3, Src homology 3.

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the protein, and phosphorylated amphiphysin 1 displays a reduced level of binding to endophilin 1, but not to clathrin (*30*). Thus, our results suggest that Dyrk1A functions to control clathrin-mediated endocytosis in neurons.

Dyrk1A has a nuclear targeting sequence at its N-terminus, a PEST sequence at its C-terminus, and the kinase domain between these sequences (*2*). Because of the PEST sequence, this kinase is assumed to have a short half-life. In spite of the nuclear targeting sequence, a substantial amount of endogenous Dyrk1A is localized within the cytoplasm of the cells in the brains of humans, mice, and chickens (*4*, *15*, *16*, *32*). Subcellular localization of the kinase, however, has not been studied systematically. To search for mechanisms that regulate Dyrk1A function, we first aimed to identify Dyrk1A binding proteins in the postnuclear fraction from rat brain. We found that the majority of Dyrk1A was associated with postnuclear structures that are insoluble after extraction with high-salt/Triton X-100 and that at least three endocytic accessory proteins could function as the kinase binding proteins. In addition, multiple proteins associated with brain clathrin-coated vesicles (CCVs) were phosphorylated by Dyrk1A.

MATERIALS AND METHODS

Materials. Rat brains (frozen or excised freshly and shipped on ice) were purchased from Pel-Freez (Rogers, AR). A monoclonal anti-Dyrk1A antibody (8D9) was produced "in house" as described in ref *16*. The specificity of this antibody is shown in Figure 1A. The monoclonal antibody against human dynamin 1 (Hudy 1) was obtained from Millipore (Billerica, MA). Glutathione (GT) resin and monoclonal anti-clathrin heavy chain antibody were from BD Transduction Laboratories (Franklin Lakes, NJ). Alkaline phosphatase-conjugated goat anti-mouse IgG (absorbed with rat serum proteins) and mass spectrometric (MS) grade trypsin were purchased from Sigma-Aldrich (St. Louis, MO). [γ-^{32}P]ATP was obtained from MP Biomedicals, Inc. (Costa Mesa, CA). CDP-Star reagent was from New England Biolab (Ipswich, MA).

Subcellular Fractionation of Dyrk1A from Rat Brain. Rat brain was homogenized with 3 volumes of 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.5 mM DTT, and 1 mM EGTA (homogenization buffer). The homogenate (H), filtered through four layers of gauze, was centrifuged at 1000*g* for 10 min. The precipitate was resuspended in 2 volumes of homogenizing buffer and centrifuged again. The supernatants were combined (S1), whereas the precipitate (P1, crude nuclear fraction) was resuspended in a DNase I digestion buffer [10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$]. S1 was centrifuged at 19000*g* for 40 min to collect the supernatant (S2) and precipitated (P2) fractions. S2 was further centrifuged at 150000*g* for 1 h, and both precipitated (P3) and supernatant (S3) fractions were collected. The P2 and P3 fractions were resuspended in homogenization buffer. Aliquots of the homogenate and the P1 suspension were incubated with DNase I (70−100 μg of total protein/μg of DNase I) at 37 °C for 30 min before use. An aliquot of the resuspended P2 fraction was extracted with 2% Triton X-100 (Tx-7.4) or 1 M NaCl (Na-7.4) at 4 °C for 30 min. For extraction at pH 6.5, an aliquot of P2 was resuspended in MES buffer [0.1 M MES (pH 6.5), 1 mM EGTA, 0.2 mM DTT, and 0.5 mM $MgCl_2$] containing 2% Triton X-100 (Tx-6.5). After centrifugation at 25000*g* for 30 min, the supernatant and precipitate fractions were collected. Protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) was added throughout the experiments.

The Triton X-100-insoluble fraction at pH 6.5 was mixed with 8 M urea at room temperature for 30 min. The urea extract, collected by centrifugation at 25000*g* for 30 min, was applied to a hydroxylapatite (HA) column (1.5 cm × 5 cm). The column-unbound fraction (ub) was collected, and the column was washed with 10 mM potassium phosphate (pH 7.5) containing 6 M urea and then eluted at 4 °C with a gradient of potassium phosphate from 10 to 500 mM in the presence of 6 M urea. Unbound and pooled fractions from column chromatography containing 8 or 6 M urea were concentrated in dialysis tubes against solid sucrose.

Preparation of CCVs and Other Proteins. Fresh rat brains or mouse livers were homogenized with a MES buffer, and the CCV preparations were obtained according to the method of Campbell et al. (*33*). The CCV-associated proteins were stripped by incubating liver CCVs with 0.8 M Tris-HCl (pH 7.4) according to the method of Manfredi et al. (*34*). Dynamin was purified from frozen rat brains as described previously (*31*). Recombinant full-length Dyrk1A and the GST-truncated kinase at the C-terminal PEST domain (at amino acid 497) (GST-Dyrk1A^{497}) was prepared as described in refs *28* and *35*, respectively. Dyrk1A^{497} with a poly-His tag (six-histidine repeat) at its N-terminus was generated by reconstruction of the pND vector using the same strategy as described in ref *35*. The GST-endophilin 1 (human, full-length) was prepared as described previously (*28*). Full-length endophilin 2 was prepared by PCR using a set of primers (5′-ccgcgtggatccatgtcggtggcggggctgaagaag-3′ and 5′-atcgatctcgagtcattatcactgcggcaggggcacaagc-3′ for the 5′-side and 3′-primer, respectively) using human brain QUICK-Clone cDNA (CLONTECH) as a template. The resultant PCR product was inserted into a pGEX vector at the BamHI and XhoI sites. The vector was confirmed by DNA sequencing, and the GST-endophilin 2 was expressed in *Escherichia coli*. Protein concentrations were determined by the Lowry method using BSA as a standard.

Dyrk1A Overlay Assay. Proteins of interest were separated via SDS−PAGE using a Tris-Tricine gel system (*36*) with gels consisting of either 7% acrylamide and 0.128% bisacrylamide or 8% acrylamide and 0.24% bisacrylamide and transferred to PVDF membranes. Mini-gels or regular size gels (14 cm × 15 cm, 1.1 cm lane width) were employed for the assay. After being blocked in 1% BSA and 2.5% milk in PBS-Tween (blocking buffer), the membranes were incubated with Dyrk1A at 4 °C overnight in blocking buffer. Positions of the membrane-bound Dyrk1A were revealed by 8D9.

Identification of Dyrk1A Binding Proteins. Proteins were separated via 7% SDS−PAGE using regular size gels in duplicate sample sets. Each sample set was arranged to be sandwiched between two marker protein lanes. The gels with one set of lanes were stained with Coomassie Blue and stored in a destaining solution, whereas the proteins from the other set of lanes were transferred to PVDF membranes. Each sample lane of the membrane was cut into three strips. The strips next to the marker protein lane were stained with Coomassie Blue. The middle strips were overlaid with Dyrk1A, and the third strips were kept in the blocking buffer as a control. The latter two strips were subjected to immunoblotting using 8D9. All three strips for each sample lane were reassembled, and the protein bands stained positive for Dyrk1A in the overlaid lane but negative in the control lane were identified in the membrane strip stained with Coomassie Blue. The putative Dyrk1A binding proteins were then identified in the

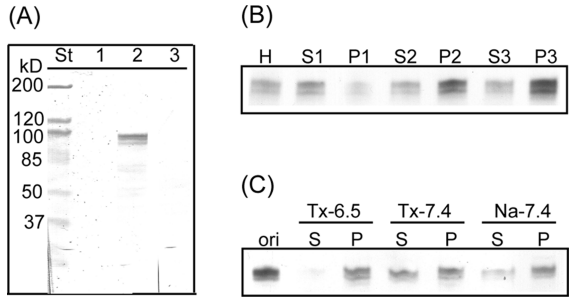

FIGURE 1: Patterns of extraction of Dyrk1A from rat brain. (A) Specificity of monoclonal anti-Dyrk1A (8D9) antibody. Rat brain homogenate (50 μg/lane) was separated via 7% SDS−PAGE and transferred to a PVDF membrane. Each lane was cut out, blocked, and incubated without primary antibody (lane 1), with 8D9 (lane 2), and with 8D9 premixed with an excess amount of Dyrk1A (lane 3). For lane 3, the full-length kinase was boiled briefly in the presence of 1% SDS and diluted ($^1/_{10}$) in a blocking buffer before being mixed with 8D9. (B) Fractionation of Dyrk1A from rat brain into homogenate (H) and subcellular fractions S1, P1, S2, P2, S3, and P3 was conducted as described in Materials and Methods. Sixty micrograms of proteins per lane was separated via 7% SDS−PAGE and subjected to immunoblotting with 8D9. (C) Effects of Triton X-100, NaCl, and pH on extraction of Dyrk1A from postnuclear precipitate 2. P2 (ori) was extracted with 2% Triton X-100 at pH 6.5 (Tx-6.5), at pH 7.4 (Tx-7.4), or with 1 M NaCl (Na-7.4). The supernatant (S) was removed, and the precipitate (P) was reconstituted with the starting volume of the homogenization buffer. Equal volumes of samples were used per lane for immunoblots with 8D9.

corresponding positions of the polyacrylamide gels stained with Coomassie Blue. The bands of interest were cut out, avoiding both edges of the each band, and processed by in-gel digestion for MS analyses.

LC−MS/MS Analysis. The in-gel digestion procedure was based on the method of Shevchenko et al. (*37*) with modifications. Briefly, the excised gels stained with Coomassie Blue were destained twice with 300 μL of 40% acetonitrile [in 0.1 M NH_4HCO_3 (pH 8.0)] for 30 min at 37 °C in 1.5 mL centrifuge tubes with a screw cap. The proteins were subsequently modified by incubating the gel slices first with 10 mM DTT and 0.1 M NH_4HCO_3 for 30 min at 60 °C and then with 100 mM iodoacetamide and 0.1 M NH_4HCO_3 in the dark for 45 min at room temperature. The gel slices were washed twice with 300 μL of digestion buffer (9% acetonitrile and 40 mM NH_4HCO_3) and dried in a speed-vac concentrator. A small volume (5−10 μL) of trypsin (0.02 μg/μL of digestion buffer) was applied directly onto each dried gel slice. Additional digestion buffer was subsequently added to cover the slice, and the gels were incubated at 37 °C overnight. The tryptic peptides, recovered in the supernatant after a brief centrifugation, were purified on a C18 OMIX tip (Varian, Inc., Lake Forest, CA). The purified peptides were analyzed by an LC−MS/MS system (Waters Corp.) equipped with a nanoflow electrospayer by scanning from m/z 200 to 1990 at 1.1 s intervals (Supporting Information).

RESULTS

Although substantial amounts of Dyrk1A are detected in cytoplasm by immunostaining of brain sections, subcellular localization of the kinase has not been systematically addressed. Therefore, we first estimated the amount of Dyrk1A associated with various subcellular fractions from rat brains. The homogenate (H) was first centrifuged at low speed to produce a nuclear precipitate (P1) and postnuclear supernatant (S1). S1 was then centrifuged at high speed to produce a postnuclear precipitate (P2) and supernatant (S2) fractions. S2 was further ultracentrifuged to produce a microsomal precipitate (P3) and cytosol (S3) fractions. As seen in Figure 1B, a substantial amount of kinase was recovered in S1, whereas the kinase level in the crude nuclear fraction, P1, was clearly low. Dyrk1A seemed to be concentrated in the postnuclear precipitates of P2 and P3, but much less in the cytosol fraction. On the basis of the total proteins recovered in each fraction and the relative reactivity of the anti-Dyrk1A antibody, we estimated that approximately 70 and 7% of the kinase in brain homogenate were recovered in the P2 and P3 fractions, respectively (Table 1). Recombinant Dyrk1A is a soluble protein; therefore, these results suggest that the most of the endogenous kinase was associated with cell constituents recovered in the postnuclear precipitates. Similar results were obtained from the cultured CHO cells (in confluent); the nuclear fraction had low but detectable amounts of kinase, whereas the majority of the kinase was again associated with the postnuclear precipitates (data not shown).

Table 1: Recovery of Total Proteins and Relative Amounts of Dyrk1A in Various Fractions from Rat Brain

		Dyrk1A estimated		
fraction	total protein (mg)	units[a]/mg	units[a] total	recovery (%)
homogenate	226	102[b]	23052	100
1000*g* supernatant (S1)	176	104	18251	79
1000*g* precipitate (P1)	31	39	1215	5
19000*g* supernatant (S2)	58	66	3811	17
19000*g* precipitate (P2)	83	193	16019	69
150000*g* supernatant (S3)	45	60	2700	12
150000*g* precipitate (P3)	6	265	1588	7

[a]The unit represents an arbitrary unit from the scanning. [b]The immunoblot shown in Figure 1B was scanned using an Epson V700 scanner, and the staining intensities of each band were quantified using Image-J.

We then tested whether Dyrk1A was loosely associated with the cellular constituents, by incubating the P2 fraction with a detergent or high salt (Figure 1C). At pH 7.4, the kinase became soluble to some extent after treatment with Triton X-100 (Tx-7.4) and 1.0 M NaCl (Na-7.4). However, at pH 6.5, treatment with neither Triton X-100 (Tx-6.5) nor NaCl (not shown) released the kinase in the supernatant at a significant level. Dyrk1A or its binding components apparently bind to cell constituents in the P2 fraction more tightly at pH 6.5 than at pH 7.4. Therefore, we searched for Dyrk1A binding proteins in the Triton X-100-insoluble fraction at pH 6.5 assuming that the binding protein(s) remains insoluble at this pH. The insoluble fraction (Tx-6.5) was extracted with urea at pH 7.4 and fractionated using a HA column in the presence of urea. The column-unbound and -bound fractions were processed in duplicate; one set was subjected to immunoblotting directly with 8D9 (Figure 2A, panel I), and the other set was incubated first with recombinant full-length Dyrk1A (overlay) and then blotted with 8D9 (panel II). Fractions 15−25 contained endogenous Dyrk1A (~90 kDa), as revealed in the membranes without an overlay (arrow). The broad and less intense band at approximately 60 kDa (arrowhead) appeared to be caused by nonspecific binding of antibody (8D9 and/or goat anti-mouse IgG) due to the high protein levels in this band. For the Dyrk1A-overlaid membranes, 8D9 clearly stained multiple new bands (asterisks); the column-unbound fraction (lane ub) contained at least four Dyrk1A-interacting bands (ranking from 70 to 120 kDa), while the column-bound fractions, eluted in

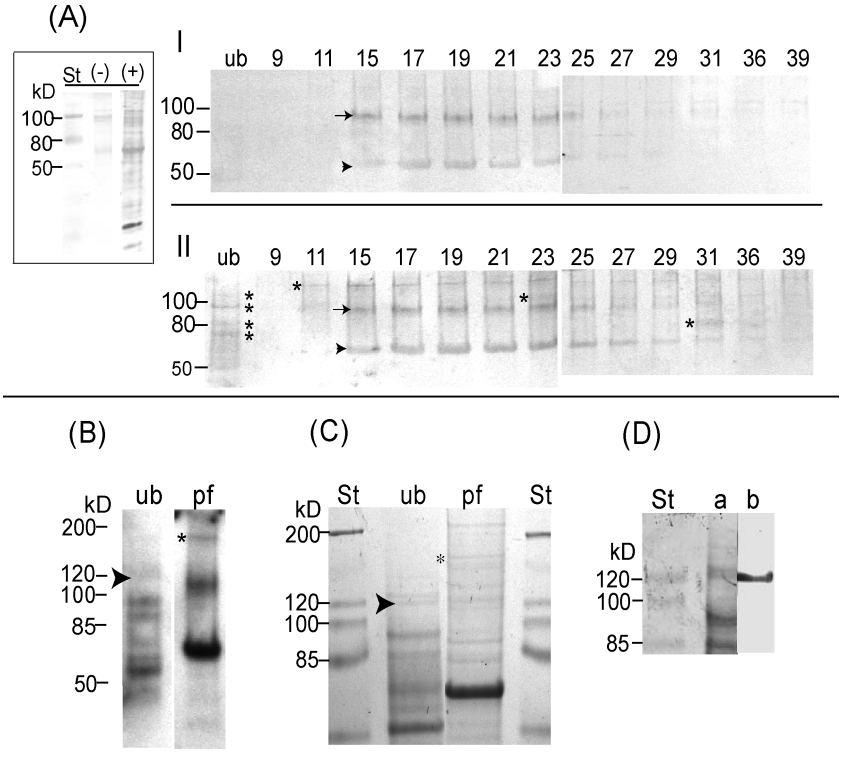

FIGURE 2: Separation of the Dyrk1A binding proteins by HA column chromatography. (A) Dyrk1A overlay assay of the fractions from a HA column (I and II) and the total urea extract (inset). The urea extract from the Tx-6.5 precipitate fraction was applied to a HA column as described in Materials and Methods. Equal volumes of selected fractions from the column were subjected to 8% SDS−PAGE in duplicate sets and then transferred to PVDF membranes. After blocking, one set of the membranes was kept in blocking buffer as a control [panel I and (−)], and the other set was overlaid with 4 μg/mL full-length Dyrk1A [panel II and (+)]. The bound kinase was revealed by 8D9: (arrow) endogenous rat brain Dyrk1A, (arrowhead) abundant protein around 60 kDa, and (asterisk) specific bands seen only after Dyrk1A overlay. The low-molecular mass proteins, < 50 kDa and seen in the total urea extract (inset) after Dyrk1A overlay, were not detected via column chromatography. (B) Overlay assay of the concentrated unbound (ub) and pooled fractions (pf) 15−25. Aliquots of the concentrated fractions were mixed with an equal volume of 2× SDS sample buffer, separated via 8% SDS−PAGE, and subjected to the Dyrk1A overlay assay. (C) Coomassie Blue staining of unbound and pooled fractions. Aliquots of the samples from panel B were separated via 7% SDS−PAGE using regular size gels. After the Dyrk1A overlay, the 8D9 positive bands from ub (arrowhead) and pf (asterisk) lanes were cut out and processed for LC−MS/MS analysis. (D) Migration position of dynamin 1 under our SDS−PAGE conditions. An aliquot of the ub fraction from panel B was subjected to SDS−PAGE as described for panel C and transferred to a PVDF membrane. The sample lane was cut in half; one strip with protein standards was stained with Coomassie Blue (a), while the other was immunoblotted by using anti-dynamin 1 antibody (Hudy1) (b).

fractions 9−36, had at least three Dyrk1A-interacting bands with apparent molecular masses of 70, 100, and 150 kDa.

The unbound and pooled fractions (from 15 to 25) from the column were concentrated and used to identify Dyrk1A binding proteins. In the unbound fraction, the kinase overlay assay revealed a faintly stained band with a molecular mass of ~120 kDa (arrowhead) and multiple bands with masses ranging from 70 to 90 kDa, which were stained more intensely than the 120 kDa band (Figure 2B, lane ub). In contrast, the pooled fraction contained one sharp band of ~150 kDa (asterisk) and diffuse bands with molecular masses of 110−120 kDa (lane pf). In this study, we focused on the proteins at 120 kDa in the unbound fraction and at 150 kDa in the pooled fraction because they were clearly distinguishable from the other protein bands in the Coomassie Blue-stained gel (Figure 2C). LC−MS/MS analysis of the 120 kDa band gave five peptides corresponding to dynamin 1, whereas the 150 kDa band produced 18 peptides of clathrin heavy chain (see Table 2 of the Supporting Information for a summary). Although the apparent size (120 kDa) was larger than that of dynamin 1 (100 kDa), parallel staining with Coomassie Blue and Western blotting using monoclonal anti-human dynamin 1 antibody (Hudy 1) for the strips cut out from a single lane confirmed that the 120 kDa band indeed contained dynamin 1 (Figure 2D). The presence of urea in the sample as well as the running conditions for SDS−PAGE (with a Tris-Tricine buffer system instead of Tris-Glycine) likely altered the migration pattern of dynamin. By conducting a similar assay, we confirmed the 150 kDa band in the pooled fraction as the clathrin heavy chain (not shown).

To further substantiate the findings, we tested whether Dyrk1A interacted with purified dynamin 1 and clathrin heavy chain (Figure 3). First, the dynamin purified from rat brain was incubated with immobilized Dyrk1A on a PVDF membrane, and the membrane-bound dynamin 1 was detected with Hudy 1 (Figure 3A). Recombinant full-length Dyrk1A usually gives multiple bands after being stained with 8D9; they are likely degradation products of the kinase. The dynamin overlay assay also revealed multiple bands detected by Hudy 1, where dynamin 1 apparently bound better to a degraded Dyrk1A of 85 kDa. Increasing the amount of immobilized kinase increased Hudy 1 reactivity, whereas without the dynamin overlay, the Hudy 1 reactivity was negative, confirming the interaction. Similar results were obtained by overlaying Dyrk1A on immobilized dynamin (Figure 3B), where Dyrk1A bound to the dynamin in a dynamin concentration-dependent manner. Likewise, clathrin heavy chain−Dyrk1A binding was confirmed by an overlay assay no matter whether clathrin or the kinase was immobilized (Figure 3C).

We then analyzed the dynamin−Dyrk1A interaction under native conditions by two approaches. In the first approach, using the GST-Dyrk1A (full-length) and GT-resins failed to pull down dynamin (not shown), which was consistent with

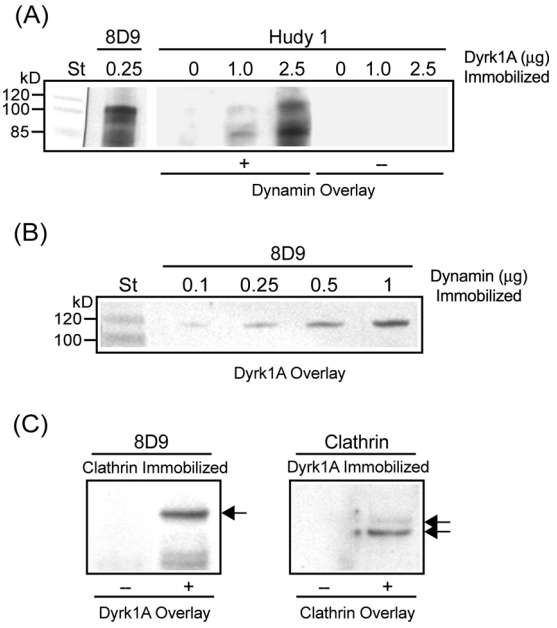

FIGURE 3: Overlay assays of purified dynamin, clathrin, and Dyrk1A. (A) Dynamin overlay assay. Various amounts of full-length Dyrk1A were separated via 7% SDS−PAGE and transferred to PVDF membranes in duplicate. The membranes were blocked and incubated without (−) or with (+) purified rat brain dynamin (1 μg/mL) in blocking buffer. The bound dynamin 1 was revealed by Hudy 1. The immunoblot of the recombinant Dyrk1A (full-length, 0.25 μg/lane) (8D9), used for the binding assay, is shown next to the protein standards (St). (B) Dyrk1A overlay assay. Purified rat brain dynamin in the indicated amounts was transferred to a PVDF membrane as in panel A for the overlay assay with Dyrk1A (4 μg/mL), and the membrane-bound kinase was revealed with 8D9. (C) Clathrin heavy chain−Dyrk1A overlay assay. A crude clathrin preparation extracted from mouse liver CCVs was used for the overlay assay as described for both panels A and B. Bound Dyrk1A and clathrin heavy chain were revealed by using corresponding antibodies and are denoted with arrows.

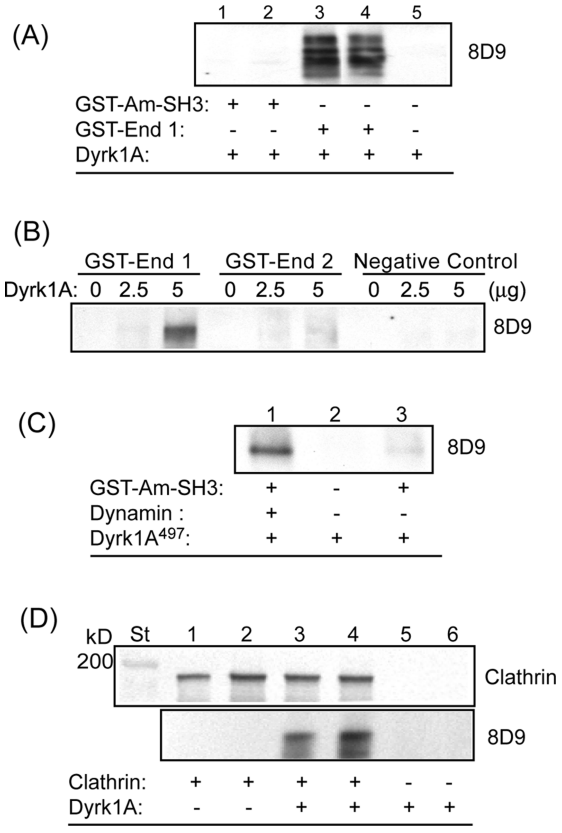

FIGURE 4: Binding of Dyrk1A under native conditions. (A) Affinity precipitation of Dyrk1A by the GST-endophilin 1. The GST-amphiphysin SH3 domain (GST-Am-SH3) or GST-endophilin 1 (GST-End 1) (7 μg each) was incubated with Dyrk1A (4 μg) and GT-resin (15 μL as a suspension) in a binding buffer [20 mM HEPES (pH 7.4), 1% Triton X-100, 0.12 M NaCl, 0 1 mM EGTA, 0.1 mM EDTA, and 0.1 mM DTT] in a final volume of 50 μL. After incubation at 4 °C overnight, the resins were washed three times with binding buffer and the resin-bound Dyrk1A was eluted with 20 mM GT and analyzed by immunoblotting using 8D9. (B) Isoform-specific binding of endophilin to Dyrk1A. Various concentrations of Dyrk1A were incubated without (negative control) or with a fixed amount (10 μg) of GST-endophilin 1 (GST-End 1) and GST-endophilin 2 (GST-End 2), and the GT-resin-bound kinase was analyzed as described above. (C) Affinity precipitation of the dynamin-bound Dyrk1A. GT-resin was mixed with poly-His-tagged Dyrk1A^{497} (4 μg) in the presence or absence of GST-Am-SH3 (16 μg) and purified dynamin (1 μg) as described for panel A. The resin-bound Dyrk1A^{497} was detected with 8D9. (D) Coprecipitation of Dyrk1A with clathrin cages. A stock solution of full-length Dyrk1A was precleared by centrifugation at 150000*g* for 2 h prior to use. Aliquots of the stripped proteins from liver CCVs were mixed with and without Dyrk1A (1 μg) in a final volume of 25 μL. The mixtures were then dialyzed using Slide-A-Lyzer mini dialysis units (Pierce) overnight against a MES buffer, and the clathrin cages formed during the dialysis were collected by ultracentrifugation as described by Manfredi et al. (*34*). The resultant precipitates were subjected to immunoblotting using anti-clathrin heavy chain antibody and 8D9. Duplicate samples are shown.

earlier work (*28*). We then took advantage of the ability of dynamin PRD to bind the SH3 domain; we tested whether Dyrk1A could be recovered with dynamin in GT-resin/GST-SH3 domain precipitates. Two sources of the SH3 domain were used as anchors for the precipitation assay: GST-full-length endophilin (GST-End 1) and GST-amphiphysin SH3 domain (GST-Am-SH3). By testing whether Dyrk1A itself binds these proteins, we unexpectedly found that Dyrk1A bound tightly to GST-endophilin (Figure 4A, lanes 3 and 4). The kinase did not bind to GT-resin (lane 5) and very little to GST-Am-SH3 (lanes 1 and 2), suggesting that the Dyrk1A binding is specific to the endophilin 1 moiety. In addition, we found that the Dyrk1A binding was specific to the endophilin 1 isoform because GST-End 1 pulled down Dyrk1A in a concentration-dependent manner, but not GST-End 2 (Figure 4B). Therefore, dynamin−Dyrk1A binding under native conditions was tested by using GST-Am-SH3 for the pull-down assay. The full-length kinase preparations always contained multiple protein bands; some of them are assumed to be variously degraded kinase (just as seen in Figures 3A and 4A), whereas the truncated kinase without the C-terminal PEST domain (Dyrk1A^{497}) can be purified at high purity. Therefore, we used the poly-His-tagged Dyrk1A^{497} for the pull-down assay (Figure 4C). Dyrk1A^{497} was pulled down with GST-Am-SH3 when dynamin was present (lane 1). Under the same assay conditions, Dyrk1A^{497} did not bind to the GT-resin (lane 2) and bound very weakly to GST-Am-SH3 without dynamin (lane 3), suggesting that dynamin and Dyrk1A^{497} bind under native conditions.

Similarly, clathrin−Dyrk1A binding under native conditions was tested by cosedimenting Dyrk1A with clathrin cages. Since liver expresses very little Dyrk1A, dynamin 1, and endophilin 1, we used the stripped proteins from mouse liver CCVs for the study. Clathrin cages, formed by dialyzing the stripped proteins against MES buffer at pH 6.5 (*34*), were collected by ultracentrifugation (Figure 4D). As expected, there was no detectable level of Dyrk1A in the liver clathrin cages (lanes 1 and 2).

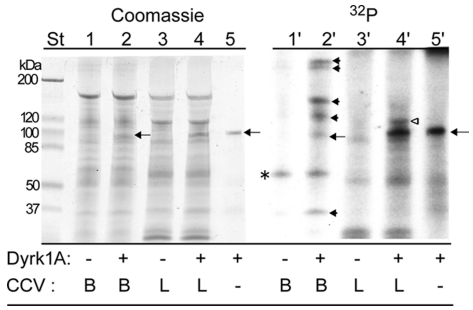

FIGURE 5: Phosphorylation by Dyrk1A of the CCV preparations from brain and liver. CCV preparations (20 μg) were incubated with 0.1 mM [γ-^{32}P]ATP, 25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.1 M NaCl, 0.1 mM DTT, and a protease cocktail with (+) or without (−) the GST-Dyrk1A^{497} (3.7 μg) at 30 °C for 30 min in a final volume of 30 μL. After the reaction had been stopped with 10 mM EDTA, a mixture of phosphatase inhibitors (2.5 mM Na_2VO_4, 5 mM Na_2MoO_4, 10 mM NaF, 2 μM cyclosporine, 0.6 μM okadaic acid, and 20 nM cypermethrin) was added to each tube, and the samples were subjected to 7% SDS−PAGE followed by autoradiography. Total proteins applied per each lane were 4.2 and 12.5 μg for brain (B) and liver (L) CCVs, respectively: (arrows) recombinant GST-Dyrk1A, (asterisk) endogenously phosphorylated protein, (filled arrowheads) phosphorylated proteins specific to brain, and (empty arrowheads) phosphorylated protein specific to liver.

Dyrk1A itself is soluble and did not sediment after dialysis (lanes 5 and 6). Dyrk1A was recovered in the sediments when the CCV extract was included during dialysis (lanes 3 and 4), indicating a direct interaction of Dyrk1A with the clathrin cages. The presence of Dyrk1A did not alter the recovery levels of clathrin heavy chains after the dialysis (lanes 1−4). This indicates that Dyrk1A did not affect the assembly of the clathrin cages.

Dyrk1A could bind multiple endocytic accessory proteins, and various proteins required for CCV formation are known to be phosphoproteins. We therefore tested whether the isolated CCV could be phosphorylated by Dyrk1A by using the CCV preparations purified from brains (B) and livers (L) (Figure 5). By incubation with [γ-^{32}P]ATP, the CCVs prepared from rat brains produced primarily one radioactive protein band (asterisk, lane 1′). This is consistent with an earlier report (*38*) and suggests that the labeled protein is the μ-subunit of the AP2 complex. Incubation with both Dyrk1A and [γ-^{32}P]ATP resulted in the phosphorylation of multiple protein bands in the brain CCV with estimated molecular masses of approximately 400, 350, 145, 120, and 34 kDa (filled arrowheads) in addition to the autophosphorylated GST-Dyrk1A^{497} (arrow, lanes 2′ and 5′). The ^{32}P-labeled 145 kDa band did not coincide with a major band of clathrin heavy chain and migrated slightly faster than that (lanes 2 and 2′). In contrast, the liver CCV lacked these phosphorylated proteins. Instead, a band of 100−110 kDa was phosphorylated by Dyrk1A (empty arrowhead, lane 4′). Also the phosphorylation level of the 50 kDa band seemed to be enhanced by the kinase (lanes 3′ and 4′). The autophosphorylated Dyrk1A was seen as two different sizes: monomers (arrow) and aggregates at the top of the gel (lane 5′). The autophosphorylation became less significant when the samples were incubated with the brain CCVs. We also tested whether endophilin 1 could be a Dyrk1A substrate. We found that the GST-endophilin 1 was not phosphorylated by the kinase at a noticeable level (data not shown). Thus, our results indicate that neither clathrin heavy chain nor endophilin 1 is a substrate for the kinase.

Dynamin 1, amphiphysin 1, and synaptojanin 1 were previously identified as Dyrk1A substrates (*28*−*30*). To test whether these proteins contributed to the phosphorylation in the brain CCV, we analyzed the levels of these proteins in the CCV preparations. Western blotting revealed that amphiphysin 1 and synaptojanin 1 were below the detection levels. Dynamin 1 was found in the brain CCV; however, its levels varied with the preparations (not shown). Nevertheless, the size of dynamin 1 did not match any of the phosphorylated protein bands in Figure 5 (lane 2′). Therefore, we concluded that Dyrk1A phosphorylated a new set of proteins associated with the brain CCV.

DISCUSSION

The majority (70%) of Dyrk1A in the rat brain was found in postnuclear precipitates. The kinase was apparently associated with the postnuclear precipitates. Extraction of the precipitates with a buffer at pH 7.4, including high salt (1 M NaCl) or detergent (Triton X-100), released the kinase to some extent, but not with a buffer at pH 6.5. Two proteins capable of binding to Dyrk1A were identified from the Triton-insoluble fraction: clathrin heavy chain and dynamin 1. One more Dyrk1A binding protein, endophilin 1, was found during the study. All three proteins are required for the regulated endocytosis in neurons. Dyrk1A phosphorylated five distinct protein bands associated with brain CCVs. Some of the known substrates for Dyrk1A are also brain-specific endocytic accessory proteins. Therefore, binding to the multiple endocytic proteins may play a role in anchoring Dyrk1A near its substrates, thus assisting the kinase in controlling endocytic processes.

Localization of Kinase. Martí et al. (*15*) have shown that the majority of Dyrk1A expressed in adult mouse brain is recovered in postnuclear supernatant, which agrees with our current data. They subfractionated the postnuclear supernatant by a discontinuous sucrose gradient centrifugation and found that Dyrk1A segregated into two peaks. However, neither peak matched well with the positions of calretinin (soluble protein), SNAP-25 (plasma membrane protein), or p38 (synaptic vesicle protein) (*15*). Their results imply that Dyrk1A is associated with multiple cellular constituents. Our estimation of the kinase distribution in various subcellular fractionations indicates that 87% of the Dyrk1A recovered in the low-speed (1000*g*) postnuclear supernatant was precipitated by higher-speed centrifugation (19000*g*). Only a small fraction of Dyrk1A from the S1 fraction was found in the soluble fraction (cytosol). Regardless of its nuclear targeting sequence, the total amount of Dyrk1A recovered in the crude nuclear precipitate was only 5% (Figure 1 and Table 1). On the basis of the soluble nature of the recombinant Dyrk1A, we hypothesized that cells employ a mechanism(s) to retain the kinase in the insoluble extranuclear pools, which prevents the kinase from freely translocating to nuclei. It may also function to modulate the kinase action (or activity) and/or retain the kinase nearby its cytoplasmic substrates. Therefore, we sought to identify Dyrk1A binding proteins in the P2 fraction after Triton X-100 extraction at pH 6.5, assuming that the binding proteins are either insoluble or form insoluble aggregates with other cellular components at slightly acidic pH.

Dyrk1A Binding Proteins. So far, five cytoplasmic proteins have been reported to bind to Dyrk1A: phytanoyl-CoA α-hydroxylase-associated protein 1 (PAHX-AP1, also named PHYHIP) (*39*), α-synuclein (*25*), 14-3-3 (*40*, *41*), Sprouty2 (*42*), and caspase 9 (*26*, *27*). PAHX-AP1 is shown to interact with phytanoyl-CoA α-hydroxylase (PAHX or PHYH), a Refsum's disease gene product. In contrast to the endogenous kinase, most

recombinant Dyrk1A expressed in cultured cells is known to be located in the nucleus. However, when Dyrk1A and PAHX-AP1 were cotransfected into PC12 cells, Dyrk1A redistributed from the nucleus to the cytoplasm and colocalized with PAHX-AP1 (*39*). α-Synuclein is the major component of Lewy bodies and is observed in the brains of DS patients who have AD (*43*). 14-3-3 is a family of proteins with molecular masses of 28−31 kDa (*44*). The binding of 14-3-3β to its targets depends on phosphorylated Ser or Thr residues in the target proteins, and 14-3-3β bound to Dyrk1A autophosphorylated at Ser520 located within the PEST domain (*41*). In contrast, the 14-3-3ε bound to the kinase irrespective of autophosphorylation (*40*). In both cases, binding to 14-3-3 enhanced Dyrk1A activity almost 2-fold. Sprouty2 (35 kDa) has inhibitory effects on receptor tyrosine kinase, closely associated with FGF signaling (*45*, *46*). Dyrk1A forms complexes with Sprouty2 and phosphorylates it, which in turn impairs the inhibitory activity of Sprouty2. Expression of Sprouty2 and Dyrk1A overlaps in several structures in mouse brain, and both proteins copurified with synaptic plasma membranes (*42*). Similarly, recent studies have shown that Dyrk1A binds to caspase 9 and phosphorylates it at Thr125, a key site in inactivating the protease (*26*, *27*). Thus, Dyrk1A regulates apoptosis by inhibiting caspase 9 activation.

In this study, we identified three new Dyrk1A binding proteins, clathrin heavy chain, endophilin 1, and dynamin 1. Dynamin 1 (xa isoform) is a Dyrk1A substrate (*28*), but clathrin heavy chain and endophilin are not. The proteins identified from the LC−MS/MS analysis of the Coomassie Blue-stained gels were confirmed as kinase binding proteins by using purified or partially purified proteins (Figure 3). In overlay assays, one of the binding partners was denatured and partially renatured on PVDF membranes. Thus, it was possible that the observed binding on the PVDF membrane might be an artifact. However, in our study, clathrin heavy chain as well as dynamin 1 did bind to Dyrk1A no matter which interacting partner was immobilized on PVDF membranes. Furthermore, the fact that Dyrk1A coprecipitated with newly formed clathrin cages (Figure 4D) suggests that it indeed binds to clathrin heavy chain under native conditions. Similarly, Dyrk1A−dynamin 1 binding was also observed under native conditions (Figure 4C). These observations validate the overlay assay as an appropriate method for looking for binding partners. Although the GST-Dyrk1A failed to pull down dynamin, GST-Am-SH3 was used successfully to cosediment the dynamin 1−Dyrk1A complex. Because Dyrk1A bound weakly to GST-Am-SH3 (Figure 4) but bound well to dynamin PRD (T. Adayev et al., unpublished observation), we concluded that the binding partner of Dyrk1A was dynamin, not GST-Am-SH3. This suggests that the conformational change of dynamin occurred by binding to anchor proteins on a solid surface under native conditions, which would expose the Dyrk1A-interacting site. As shown in Figure 2, the overlay assay disclosed additional Dyrk1A binding proteins with molecular masses between 70 and 110 kDa in the P2 fraction. On the basis of their molecular size, they are likely to be a different set of kinase-binding proteins.

Kinase, Its Binding Proteins, and Endocytosis. Although Dyrk1A coprecipitated with clathrin cages, the amounts of Dyrk1A associated with the isolated CCV from rat brains were not considerably high and varied from preparation to preparation (data not shown). Even though our CCV preparation was not pure, phosphorylation of the CCV-associated proteins by endogenous kinase(s) was limited to only a single protein band. This is consistent with reports by others; the single labeled band is likely the μ2 subunit of AP2 complex phosphorylated by the copurified cyclin G-associated kinase/adapter-associated kinase (*38*, *47*). The finding that Dyrk1A did not accumulate or concentrate in the isolated CCV suggests that binding of Dyrk1A to clathrin heavy chain (and also to dynamin 1 and endophilin 1) is modulated by other factors. A certain level of bound Dyrk1A could be dissociated from clathrin heavy chain during the isolation process of CCVs if the binding constant was low. Alternatively, binding of Dyrk1A to clathrin heavy chain may occur temporally in living cells, and the kinase may be subsequently replaced with other clathrin binding proteins during the formation of coated pits (*48*). Further studies are required to determine the binding site for Dyrk1A on clathrin heavy chain, binding affinity, and competition with other clathrin binding proteins. In addition, it is necessary to study how the kinase binding to the endocytic proteins is regulated and how the binding affects the kinase activity.

Phosphorylation of Brain-Specific Endocytic Proteins by Dyrk1A. Dyrk1A phosphorylated five distinct protein bands in brain CCVs with respective molecular masses of roughly 400, 350, 145, 120, and 35 kDa. These proteins are expressed in a brain-specific manner. Many endocytic proteins expressed in the brain are phosphoproteins (*49*). We have found that dynamin 1 (*28*), amphiphysin 1 (*30*), and synaptojanin 1 (*29*) are substrates for Dyrk1A. These key proteins regulate the clathrin-mediated endocytosis (*49*, *50*), and their phosphorylation statuses change synchronously with the stimulation of synaptic termini (*51*). More importantly, Dyrk1A is also expressed in the brains of adult animals (*8*, *13*, *15*). The apparent molecular masses of 400 and 350 kDa of the phosphorylated protein bands clearly differ from those of any known Dyrk1A substrates. In addition, amphiphysin 1 and synaptojanin 1 were not present in the CCV preparation at detectable levels. Dynamin 1 did not match the ^{32}P-labeled band of 120 kDa by immunoblotting. Therefore, these results suggest that the phosphorylated proteins of 145 and 120 kDa in the brain CCV were not dynamin, amphiphysin, or synaptojanin.

Dyrk1A is strongly expressed in the central nervous system, and its expression is persistent throughout adulthood in selected brain regions (*8*, *10*, *15*, *16*). Multiple substrates for Dyrk1A have been identified. Many of them are specifically expressed in the brain and are required for the regulated endocytosis or associated with the isolated CCV. Therefore, Dyrk1A may play a role in regulating clathrin-mediated endocytosis. In addition, clathrin heavy chain, dynamin 1, and endophilin 1 may serve to anchor Dyrk1A near its substrates. Levels of expression of both Dyrk1A (*12*, *13*) and the calcineurin inhibitor RCAN1 (DSCR1/MCIP1) (*52*) are elevated in the brains of DS individuals. Such elevation could result in aberrant phosphorylation of substrates, which may in turn lead to changes in synaptic vesicle formation and thereby neuroplasticity.

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SUPPORTING INFORMATION AVAILABLE

Table 2 and the LC−MS/MS methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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