

Identification of Nuclear Import Mechanisms for the Neuronal Cdk5 Activator^{*S}

Received for publication, November 28, 2005, and in revised form, September 5, 2006 Published, JBC Papers in Press, October 23, 2006, DOI 10.1074/jbc.M512663200

Xinrong Fu[‡], Yuk-Kwan Choi[‡], Dianbo Qu[§], Yan Yu[‡], Nam Sang Cheung[¶], and Robert Z. Qi^{†1}

From the [‡]Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China, the [§]Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673, and the [¶]Department of Biochemistry, National University of Singapore, 8 Medical Drive, Singapore 117597

The activation of Cdk5 by p35 plays a pivotal role in a multitude of nervous system activities ranging from neuronal differentiation to degeneration. A fraction of Cdk5 and p35 localizes in the nucleus where Cdk5-p35 exerts its functions via protein phosphorylation, and p35 displays a dynamic localization between the cytoplasm and the nucleus. Here, we examined the nuclear import properties of p35. In nuclear import assays, p35 was actively transported into the nuclei of digitonin-permeabilized HeLa cells and cortical neurons by cytoplasmic carrier-mediated mechanisms. Importin- β , importin-5, and importin-7 were identified to import p35 into the nuclei via a direct interaction with it. An N-terminal region of p35 was defined to interact with the above importins, serving as a nuclear localization signal. Finally, we show that the nuclear localization of p35 does not require the association of Cdk5. Furthermore, Cdk5 and importin- β /5/7 are mutually exclusive in binding to p35. These results suggest that p35 employs pathways distinct from that used by Cdk5 for transport to the nucleus.

As a distinct member of the cyclin-dependent kinase family, Cdk5 is not activated by any known cyclin (1, 2). Instead, p35 is found as a specific activator of Cdk5 in nervous system neurons, and just recently, p35 has also been detected in muscle cells (3–5). Despite little apparent sequence homology between p35 and cyclins, p35 assembles into a cyclin A-like structure with distinct features to specifically bind and activate Cdk5 (6). A body of evidence has accumulated to indicate the vital role of Cdk5-p35 in a multitude of neural functions, including cytoskeletal dynamics, cell adhesion, axonal guidance, cell signaling, and synaptic plasticity (1, 2). Neurotoxins induce the proteolytic transformation of p35 to an N-terminally truncated form, p25; the resulting complex Cdk5-p25 becomes neurotoxic (7, 8). Furthermore, the aberrant Cdk5 regulation by p25 is implicated in several neurodegenerative diseases, including Alzheimer disease (1, 2).

In cultured neurons, both Cdk5 and p35 are detected throughout the cell soma and neurites (9, 10). In addition, a number of studies have shown the presence of Cdk5 and p35 in the nucleus (9–12). Also, some of Cdk5-p35 associating proteins and substrates display nuclear localization and functions (2). For instance, Cdk5 and p35/p25 colocalize with the transcription factor MEF2 in the nucleus (11). Moreover, Cdk5-p35/p25 catalyzes the site-specific phosphorylation of MEF2, inhibiting MEF2 transcriptional activity (11). Another nuclear protein, SET, has been found to interact with Cdk5-p35, up-regulating its kinase activity (10). These observations suggest the nuclear functions of Cdk5-p35, which are linked to the phosphorylation of its substrates. Recently, it has been observed that neuregulin induces the elevation of p35 levels but not those of Cdk5 in the nuclei of myotubes, implicating a signal-mediated dynamic nucleocytoplasmic transport of p35 (13). The p35 uptake may regulate Cdk5 functions in the nucleus.

Protein transport between the cytoplasm and the nucleus occurs through the nuclear pore complex (NPC),² which allows the entry of small molecules by diffusion and the selective entry of large molecules via active transport. Importins are an expanding family of proteins that mediate active nuclear import through association with protein cargos (14, 15). Importin-cargo association is regulated by small GTPase Ran, which shifts between the GTP- and GDP-bound states (16). In the cytoplasm where Ran is primarily GDP-bound, importins are loaded with cargos and the complexes transit to the nucleus where Ran-GTP is accumulated. The binding of Ran-GTP to the importins triggers cargo release in the nucleus. Both importins and Ran recycle for efficient nuclear transport. In the classical import mechanism, importin- α (Imp α) binds to the nuclear localization signal (NLS) within the import cargos. Such binding couples the cargos to the transport carrier importin- β (Imp β), which moves the Imp α -cargo complexes into the nucleus (14, 15). In addition, a number of proteins interact directly with Imp β or other carrier importins, such as importin-5 (Imp5) and importin-7 (Imp7), for Imp α -independent transport (14, 15). Interestingly, various import properties have been observed for different cyclin family members. For example, cyclin E employs the Imp α /Imp β heterodimer pathway, while cyclin B is imported by Imp β without the involvement of

^{*} This work was supported by the Research Grants Council (HKUST6414/05M) and the University Grants Committee (AoE/B-15/01) of Hong Kong. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3.

¹ To whom correspondence should be addressed: Dept. of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China. Tel.: 852-2358-7273; Fax: 852-2358-1552; E-mail: qirz@ust.hk.

² The abbreviations used are: NPC, nuclear pore complex; aa, amino acid(s); Imp, importin; GFP, green fluorescent protein; GST, glutathione-S-transferase; NLS, nuclear localization sequence.

Imp α (17, 18). The different import pathways imply separate control mechanisms for each CDK/cyclin at the nucleocytoplasmic transport level.

In the present report, we investigated the nuclear import mechanisms of p35. In nuclear import assays using digitonin-permeabilized HeLa cells and cortical neurons, we found that p35 import requires soluble import factors and is in an energy-dependent and Ran-GTP/GDP-mediated manner. Subsequently, Imp β , Imp5, and Imp7 were isolated from rat brain to interact with p35. We show that the above importins can transport p35 into the nucleus via direct association with a basic region at the N terminus of p35. We also probed the role of Cdk5 in p35 nuclear import and show that p35 does not depend on the Cdk5 association for nuclear localization. In addition, Cdk5 does not form a ternary complex with p35 and Imp β , Imp5, or Imp7. Therefore, p35 and Cdk5 may undertake separate pathways for nuclear import.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Mutants and fragments of p35 were generated from a bovine p35 cDNA using PCR-based methods and were then cloned into pCI-neo, pEGFP-N3, pFlag-CMV2, and pGEX4T-1. We modified pCI-neo to express cloned genes with a double myc-tag at the C terminus. The p35 fragment aa 31–98 and its K61–63A mutant were also double tagged with GFP and GST by ligating a GST sequence amplified from pGEX in-frame to the C terminus of the p35 sequence in pEGFP-C3. Ran-GAP1 and Ran-BP1 were cloned by PCR from the cDNA preparations of a human cell line and were inserted into the vector pET14. Imp β /pET30a (obtained from Dr. Stephen A. Adam, Northwestern University Medical School) (19) was used to construct Imp β /pFlag-CMV2. We generated Imp β -(1–281) and Imp β -(1–483) by subcloning Imp β restriction fragments into pET30Xa/LIC. Imp5 was cloned from Imp5/pGEX4T (obtained from Dr. Günter Blobel, The Rockefeller University) (20) into pET30. Human CRM1 was subcloned from CRM1/pcDNA3.1 (obtained from Dr. Edward Seto, University of South Florida) (21) into pET14b.

Recombinant Protein Preparation—Protein expression was induced in *Escherichia coli* BL21(DE3) with 0.2 mM isopropyl β -D-thiogalactopyranoside at 18 °C for 16 h. Recombinant proteins were purified using GSH-Sepharose (GE Healthcare) for GST fusion proteins and Ni²⁺-nitrilotriacetic acid beads (Qiagen) for His₆-tagged (His₆) proteins (22). After purification, proteins were dialyzed in a buffer solution of 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 110 mM potassium acetate, 5 mM magnesium acetate, 0.5 mM EGTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamide. Imp7 and NTF2 were prepared from *Xenopus* Imp7/pQE9 (obtained from Dr. Dirk Görlich, Zentrum für Molekulare Biologie der Universität Heidelberg, Germany) and NTF2/pET23b (obtained from Dr. Larry Gerace, The Scripps Research Institute), respectively, following published protocols (23–25).

Isolation of p35-binding Proteins and Protein Binding Assays—Proteins interacting with p35 were isolated from rat brain using GST-p35 fragments as reported previously (10, 22). The binding of p35 to importins was tested by incubating GST-p35 proteins with His₆-tagged importins at 4 °C for 1 h in 0.5 ml of the

binding buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.2% Triton X-100) plus 0.5 mg/ml bovine serum albumin. After the GST pull down, the bound proteins were analyzed on immunoblots. The antibodies used were anti-Imp β (mAb3E9, Affinity BioReagents), anti-Imp5 (obtained from Dr. Günter Blobel), anti-Imp7 (Santa Cruz Biotechnology), and anti-His₆ (H-15, Santa Cruz Biotechnology).

Nuclear Import Assays—HeLa cells were grown on coverslips in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen). Cultures of primary cortical neurons were prepared from cortices of mouse embryos as detailed in a previous report (10). Nuclear import assays were performed as described (24, 26). Briefly, cells were permeabilized with 60 μ g/ml of digitonin (Sigma) in the import buffer (20 mM Hepes-KOH, pH 7.5, 110 mM potassium acetate, 2 mM magnesium acetate, 250 mM sucrose, and 0.5 mM EGTA). Import assays were conducted at 30 °C for 30 min in the import buffer containing import substrates (3 μ M), an energy-regenerating system (1 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, and 50 μ g/ml creatine kinase), and 30% rabbit reticulocyte lysate (Promega). Alternatively, a mixture of 3 μ M Ran-GDP, 0.3 μ M Ran-GAP1, 0.3 μ M Ran-BP1, 0.3 μ M NTF2, and 2 μ M of Imp β , Imp5, or Imp7 was used instead of rabbit reticulocyte lysate. Where indicated, nuclear import was also tested under the following conditions: 50 units/ml apyrase (Sigma) was applied instead of the energy-regenerating system, the nuclei were preincubated with 0.1 mg/ml wheat germ agglutinin (Sigma) in the import buffer for 15 min at room temperature, and 3 μ M Ran-Q69L-GTP or Ran-GDP was present in the reactions. Following the reactions, the cells were washed with the cold import buffer and were fixed for immunofluorescence staining. The anti-GST antibody was from Sigma. Secondary antibodies were Alexa Fluor 488- or Alexa Fluor 594-conjugated goat antibodies (Invitrogen). Nuclear DNA was labeled by 1 μ M Hoechst 33258 (Sigma). Images were acquired on an epifluorescence microscope (Nikon model TE2000).

Transfection and Immunoprecipitation—Transient transfection of COS-7 and HEK293T was carried out using Lipofectamine Plus Reagent (Invitrogen). Cell lysates were prepared in 50 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, and the protease inhibitor mixture (Roche Applied Science). Anti-FLAG immunoprecipitation was performed using anti-FLAG M2-agarose (Sigma). The immunoprecipitates were analyzed on immunoblots with antibodies where indicated. The antibodies recognizing GFP, Cdk5 (anti-Cdk5 DC17), and p35 (anti-p35 C-19) were from Santa Cruz Biotechnology.

Confocal Laser Scanning Microscopy—The nucleocytoplasmic localization of GFP in fusion with p35 or p35 mutants was examined under a confocal microscope (Olympus Fluoview BX61). To determine the ratio of nuclear/cytoplasmic p35 fluorescence ($F_{n/c}$), the quantification of fluorescence intensity in the nucleus (F_n), the cytoplasm (F_c), and un-transfected background areas (F_b) was performed using the MetaMorph software (Universal Imaging). In each cell, at least three different

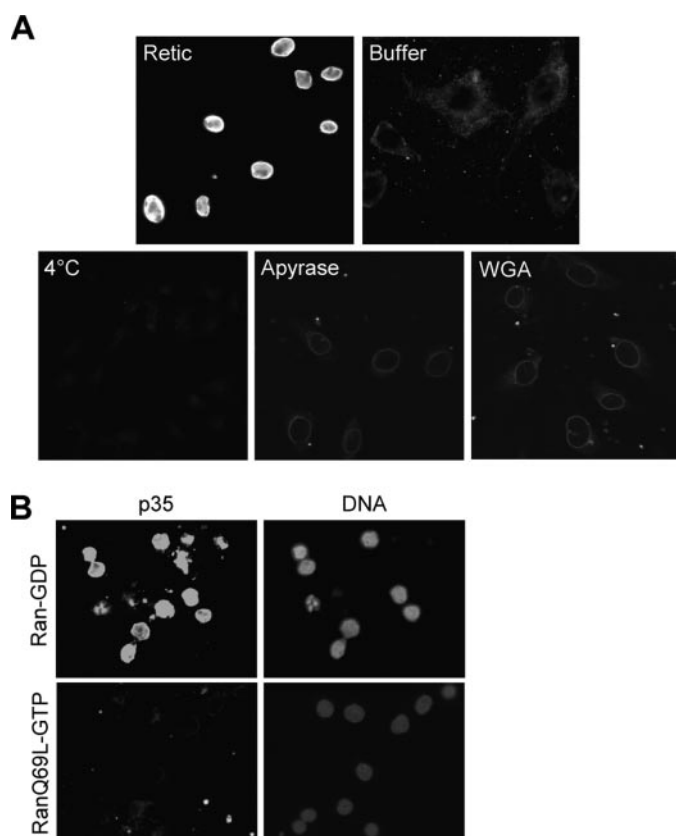


FIGURE 1. p35 is actively imported into the nucleus via the NPC. *A*, import assays were performed on digitonin-permeabilized HeLa cells at 30 °C with 3 μ M GST-p35, rabbit reticulocyte lysate (*Retic*) and an energy-regenerating system. The import was also assayed under the following conditions: rabbit reticulocyte lysate was replaced with the import buffer (*Buffer*), the import reaction was conducted at 4 °C, apyrase was applied instead of the energy-regenerating system, and wheat germ agglutinin (*WGA*) was added to the import reaction. *B*, Ran-Q69L-GTP interferes with p35 nuclear import. The nuclear import of GST-p35 was tested on digitonin-treated cortical neurons with rabbit reticulocyte lysate and an energy-regenerating system in the presence of either Ran-GDP or Ran-Q69L-GTP (3 μ M). The cells were stained for GST-p35 (anti-GST). DNA was labeled with Hoechst 33258.

fields in the nucleus and in the cytoplasm excluding areas of protein aggregates were measured to obtain average nuclear and cytoplasmic pixel intensities, respectively. $F_{n/c}$ was derived from the formula $F_{n/c} = (F_n - F_b)/(F_c - F_b)$ (27). Statistical analysis was performed using Student's *t* test (unpaired, two-tailed).

RESULTS

p35 Is Actively Imported into the Nucleus—We examined p35 nuclear import properties in nuclear import assays. When the assays were conducted at 30 °C on digitonin-permeabilized HeLa cells with rabbit reticulocyte lysate and an energy regenerating system, GST-p35 was avidly transported into the nuclei (Fig. 1*A*). Moreover, the p35 import failed to occur under the following conditions (Fig. 1*A*). First, rabbit reticulocyte lysate, which was the source of cytosolic import factors, was omitted in the assay. Second, the assay was conducted at 4 °C, which inhibits active protein import but not passive diffusion. Third, apyrase was used instead of the energy system. Fourth, wheat germ agglutinin was used to block the NPC (28). These results demonstrate that p35 is actively imported into the nucleus by

cytosolic factors via the NPC. The same import results were obtained using the protein p35-His₆ (data not shown), indicating that the tag moiety of the recombinant proteins did not affect p35 import behavior. As p35 is a neuronal protein, we also conducted the import assays on digitonin-permeabilized cortical neurons. The results were in complete agreement with those of HeLa cells (supplemental Fig. S1), suggesting that both cell types are suitable for p35 nuclear import assays.

Ran-GTP mediates nuclear import by dissociating cargos from carrier importins (14, 15). The Ran-GTP effect on p35 import was probed by using the GTPase-deficient mutant Ran-Q69L. p35 import assays were performed with rabbit reticulocyte lysate as the source of all import protein factors. In contrast to the nuclear uptake of p35 in the assay with Ran-GDP, p35 import was inhibited in the presence of Ran-Q69L-GTP (Fig. 1*B*), revealing that p35 nuclear import is mediated by the Ran-GTP/GDP system. Therefore, we conclude that p35 is imported into the nucleus by import factors in a Ran-mediated manner.

p35 Binds to Imp β , Imp5, and Imp7—To seek for nuclear import receptors of p35, we carried out a biochemical isolation of p35-interacting proteins from rat brain. As described previously, two recombinant proteins, GST-p16 comprising aa 1–149 and GST-p25 comprising aa 99–307 of p35, were used in the isolation (10, 22). Tandem mass spectrometric data revealed the specific pull down of Imp β , Imp5, and Imp7 by GST-p16 (data not shown). To confirm the isolation results, we probed the pull downs on immunoblots. As seen in Fig. 2*A*, Imp β , Imp5, and Imp7 coprecipitated with GST-p16 but not with GST or GST-p25, indicating the specific association of the above importins with the N-terminal fragment of p35.

Next, we conducted biochemical binding assays using purified recombinant proteins. As detected on the immunoblots, Imp β , Imp5, and Imp7 were pulled down by GST-p35 but not by GST (Fig. 2*B*), showing direct interaction between p35 and the importins. We also examined the effect of the Ran-GTPase on p35 interaction with Imp β /5/7. Imp β , Imp5, and Imp7 failed to coprecipitate with p35 in the presence of GTP-loaded Ran-Q69L, in contrast to the coprecipitation of the importins with p35 when Ran-GDP was used (Fig. 2*C*). Therefore, GTP-bound Ran abrogates p35 association with Imp β /5/7. In a previous report, Imp β and Imp7 have been found to cooperate in binding to histone H1 for histone H1 import (29). In our assays, we did not observe any cooperative effect between Imp β and either Imp7 or Imp5 in the p35 association (data not shown).

Imp β contains a protein cargo binding site at the N-terminal half (aa 1–462) (17, 24). We tested p35 binding to two N-terminal fragments of Imp β , aa 1–281 and aa 1–483. The binding assays were performed with the p35 fragment p10, which contains the Imp β -binding domain (see Fig. 7*A*). Like the full-length Imp β , Imp β -(1–483) exhibited a strong p10 binding activity (Fig. 3). In contrast, Imp β -(1–281) did not bind to p10 in the assay (Fig. 3). These results show that like other cargo proteins such as cyclin B1 and rpL23a, p35 binds to an N-terminal region of Imp β , which overlaps with the Ran-GTP-binding region (17, 24, 30).

Imp β /5/7 Can Import p35 into the Nucleus in Vitro—The import activities of Imp β /5/7 toward p35 were tested on HeLa

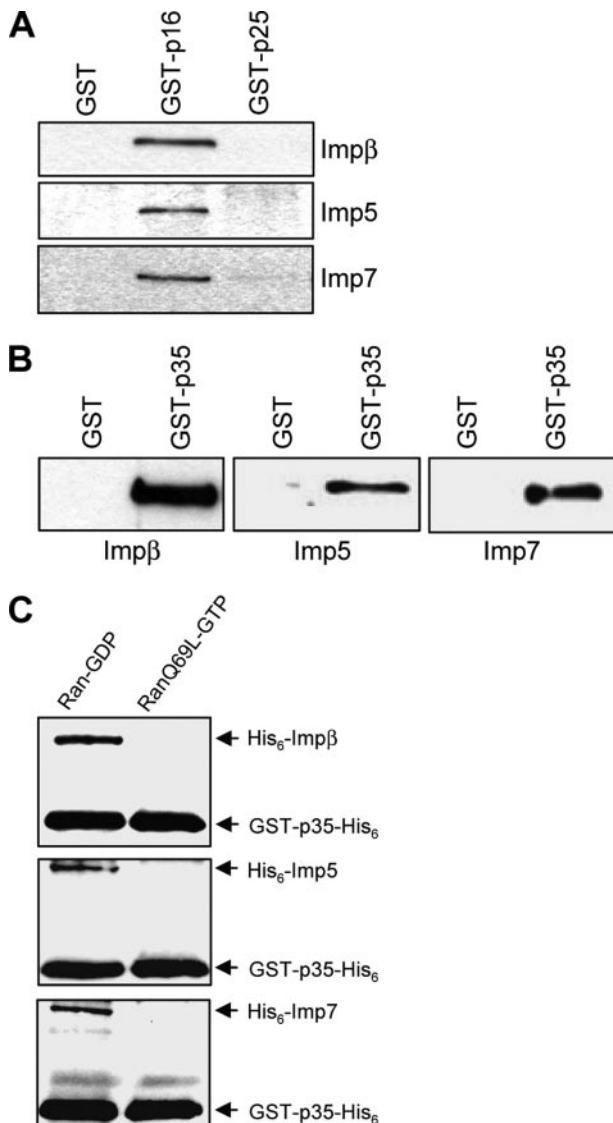


FIGURE 2. p35 binds to Impβ, Imp5, and Imp7. A, pull down of Impβ, Imp5 and Imp7 from a rat brain lysate. 20 μg of GST, GST-p16, or GST-p25, pre-coupled to GSH-beads was incubated with 1 mg of brain lysate protein at 4 °C for 1 h. Proteins associated with the beads were analyzed on immunoblots for Impβ, Imp5, and Imp7. B, protein binding assays using recombinant proteins. 3 μg of GST or GST-p35, pre-coupled to GSH beads, was subjected to binding with 6 μg of His₆-Impβ, His₆-Imp5, or His₆-Imp7. The bound proteins were detected by anti-His₆ immunoblotting. C, Ran-Q69L-GTP ablates the interaction between p35 and Impβ/5/7. GST-p35-His₆ (5 μg) was incubated with His₆-Impβ, His₆-Imp5, or His₆-Imp7 (5 μg) in the presence of either Ran-GDP or Ran-Q69L-GTP (5 μM). After the pull down of GST-p35-His₆, the bound proteins were analyzed by anti-His₆ immunoblotting.

nuclei with import factors supplied from purified recombinant proteins. When the assay was performed with Impβ, strong and exclusive nuclear staining of p35 was observed, whereas the p35 import completely failed in the absence of any importin (Fig. 4). The nuclear uptake of p35 was also detected in the assays of Imp5 or Imp7 (Fig. 4). Likewise, when the Impβ-triggered import was tested on digitonin-permeabilized cortical neurons, p35 was readily imported into the nuclei (supplemental Fig. S2). Therefore, each of Impβ, Imp5, and Imp7 can stimulate the nuclear import of p35.

p35 and Cdk5 Are Imported by Separate Pathways—Given that p35 is a primary regulator of Cdk5, we explored the poten-

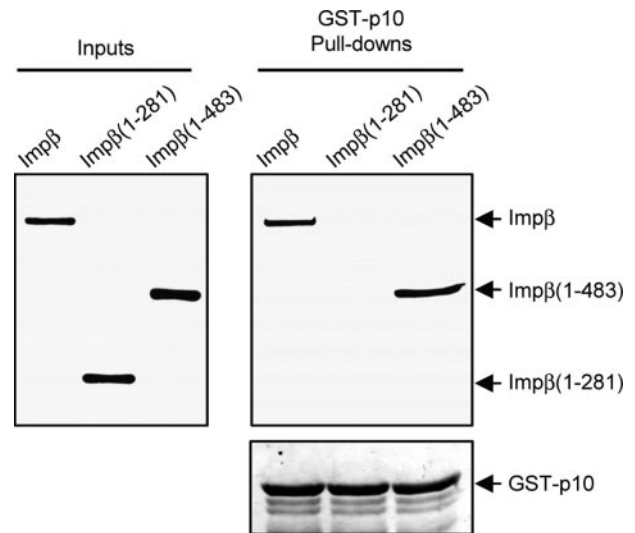


FIGURE 3. p35 binds to an N-terminal region of Impβ. GST pull downs were performed using 5 μg of GST-p10 and 10 μg of His₆-Impβ or the Impβ fragments. The bound fractions and the input Impβ proteins (10%) were analyzed on anti-His₆ immunoblots. GST-p10 retrieved by the beads was visualized by staining the membrane with Ponceau S.

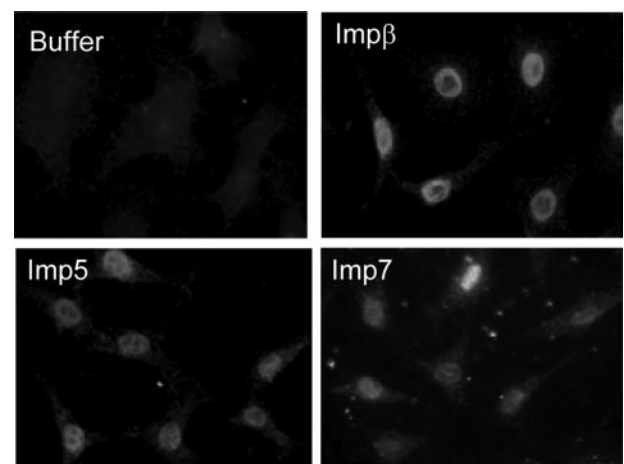


FIGURE 4. Nuclear import of p35 by Impβ, Imp5, and Imp7. Nuclear import assays performed on digitonin-treated HeLa cells. The assay mixtures contained GST-p35, an energy-regenerating system, and purified recombinant import factors. The importins were omitted in the control (Buffer). The nuclear uptake of GST-p35 was visualized by anti-GST immunofluorescence.

tial effects of Cdk5 on p35 association with Impβ, Imp5, and Imp7. In a binding test, Cdk5 did not exhibit any significant binding activity to Impβ/5/7 (Fig. 5A). Interestingly, after pre-incubation of GST-p35 with excess Cdk5, GST-p35 failed to pull down Impβ/5/7 (Fig. 5A), revealing that Cdk5 blocked p35 binding to the importins. In this and the experiments below, the amount of the GST proteins retrieved by GSH beads remained unchanged under various conditions (data not shown). To assess the effect of Impβ/5/7 on the interaction between p35 and Cdk5, p35 was incubated with an increasing amount of Impβ, Imp5, or Imp7 and was then subjected to a Cdk5 binding test. As shown in Fig. 5B, the binding of p35 with Cdk5 was inhibited by the importins in a dose-dependent manner. Collectively, these results indicate that Cdk5 and Impβ/5/7 are mutually exclusive in binding to p35.

To determine whether Cdk5 association is required for p35 nuclear localization, we generated two p35 mutants,

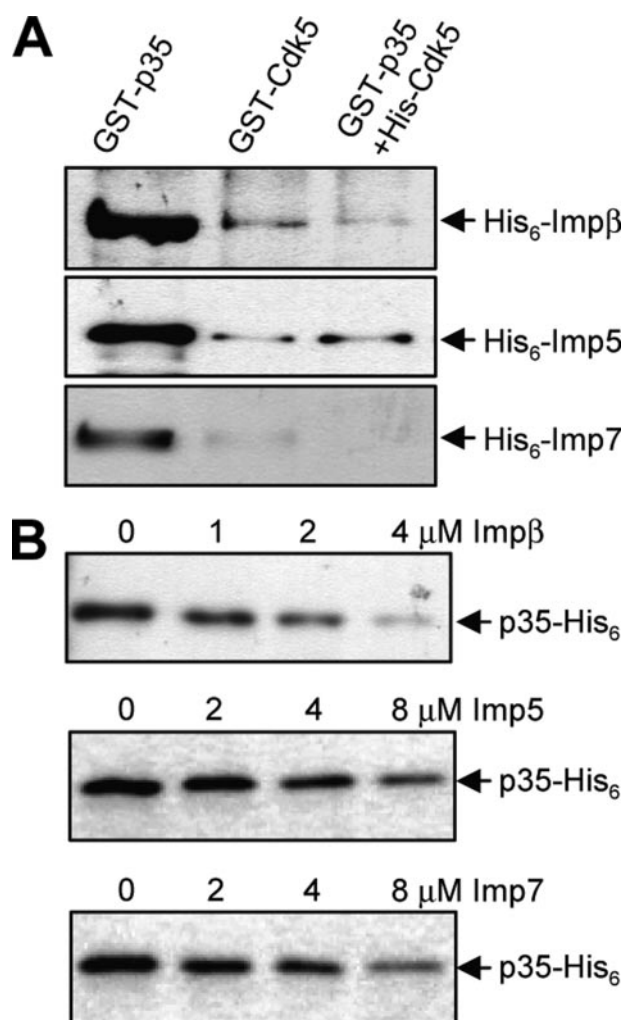


FIGURE 5. Cdk5 and Impβ/5/7 are mutually exclusive in binding to p35. A, Cdk5 blocks p35 association with Impβ, Imp5, and Imp7. 5 μg of GST-p35, GST-Cdk5, or the complex of GST-p35 and His₆-Cdk5, precoupled to GSH-beads, was tested for binding to His₆-Impβ, His₆-Imp5, or His₆-Imp7 (5 μg). Proteins bound to the beads were subjected to anti-His₆ immunoblotting. B, Impβ, Imp5, or Imp7 blocks p35 binding to Cdk5. p35-His₆ (5 μg) was preincubated with various amounts of His₆-Impβ, His₆-Imp5, or His₆-Imp7. GST-Cdk5 (5 μg) was then added for further incubation. After the incubation, GST-Cdk5 was retrieved using GSH beads to detect bound p35-His₆ by anti-His₆ immunoblotting.

p35(L151,152N) and p35(D288A,L289A), which are devoid of the Cdk5 binding activity (31). Indeed, these p35 mutants were not able to coimmunoprecipitate Cdk5 from the transfected cells in contrast to the wild type (Fig. 6A). To examine the subcellular localization, p35 and the non-Cdk5-binding mutants were ectopically expressed with GFP. The expression of GFP in fusion with the p35 proteins was verified on an immunoblot of the cell lysates (Fig. 6B). GFP fluorescence showed that these mutations did not alter the nucleocytoplasmic distribution of p35 (Fig. 6B). Thus, p35 does not require Cdk5 association for its nuclear localization.

An N-terminal Region of p35 Can Serve as an NLS—As shown above, Impβ, Imp5, and Imp7 were isolated from the brain extract with aa 1–149 of p35 but not with p25. We further mapped the Impβ/5/7-binding site at the N terminus of p35. In binding assays, Impβ, Imp5, and Imp7 bound to aa 1–99 and aa 31–98 but not to the further truncated fragments aa 1–52 and

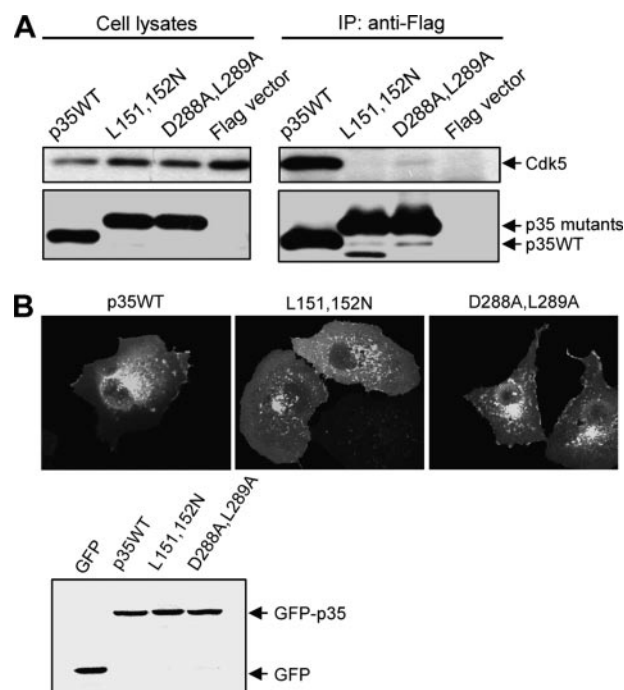


FIGURE 6. Cdk5 is dispensable in p35 nuclear localization. A, p35(L151,152N) and p35(D288A,L289A) are deficient of the Cdk5 binding activity. Anti-FLAG immunoprecipitation was performed with HEK293T lysates transiently expressing FLAG-tagged p35 proteins or the vector. Protein expressions and anti-FLAG immunoprecipitates were analyzed by anti-Cdk5 and anti-FLAG immunoblots. B, fluorescence of p35-GFP and p35 mutants in COS-7 cells. Protein expressions are shown by an anti-GFP immunoblot of the cell lysates. p35WT, wild type p35; L151,152N, p35(L151,152N), and D288A,L289A, p35(D288A,L289A).

aa 31–90 (Fig. 7A). Thus, aa 31–98 is responsible for the binding of the above importins. In addition, we found that p10 did not bind to the nuclear export receptor CRM1 in a binding assay (Fig. 7B). As a control, CRM1 was readily detected in the pull down of cyclin D1 (Fig. 7B), a known cargo protein of CRM1 (32). This result provides additional support to the specificity of the interaction between the N terminus of p35 and the importins.

The sequence of aa 31–98 is rich in basic amino acids, reminiscent of the BIB domain (β-like import receptor binding domain) of the ribosomal protein rPL23a (24). Particularly, five Lys residues were found in a short stretch, aa 61–67 (Fig. 7C). To determine whether these Lys residues are required for the importin associations, we substituted Ala for the three consecutive lysines ⁶¹KKK⁶³, generating the K61–63A mutant of p35. This mutation did not affect p35 association with Cdk5 (Fig. 7D). However, it abrogated the Impβ binding activity (Fig. 7E), demonstrating the requirement of these basic residues in the Impβ association. In addition, the K61–63A mutation reduced the Imp5 binding activity but did not significantly affect the Imp7 binding activity of p35 (data not shown). We went on to assess the import activity of the K61–63A mutant in nuclear import assays. When the assays were performed with rabbit reticulocyte lysate, the mutant was detected at only minimal levels in the nuclei compared with the avid nuclear uptake of parent p35 (Fig. 7F). Moreover, in the assay using recombinant Impβ the mutant failed to be imported into the nuclei (sup-

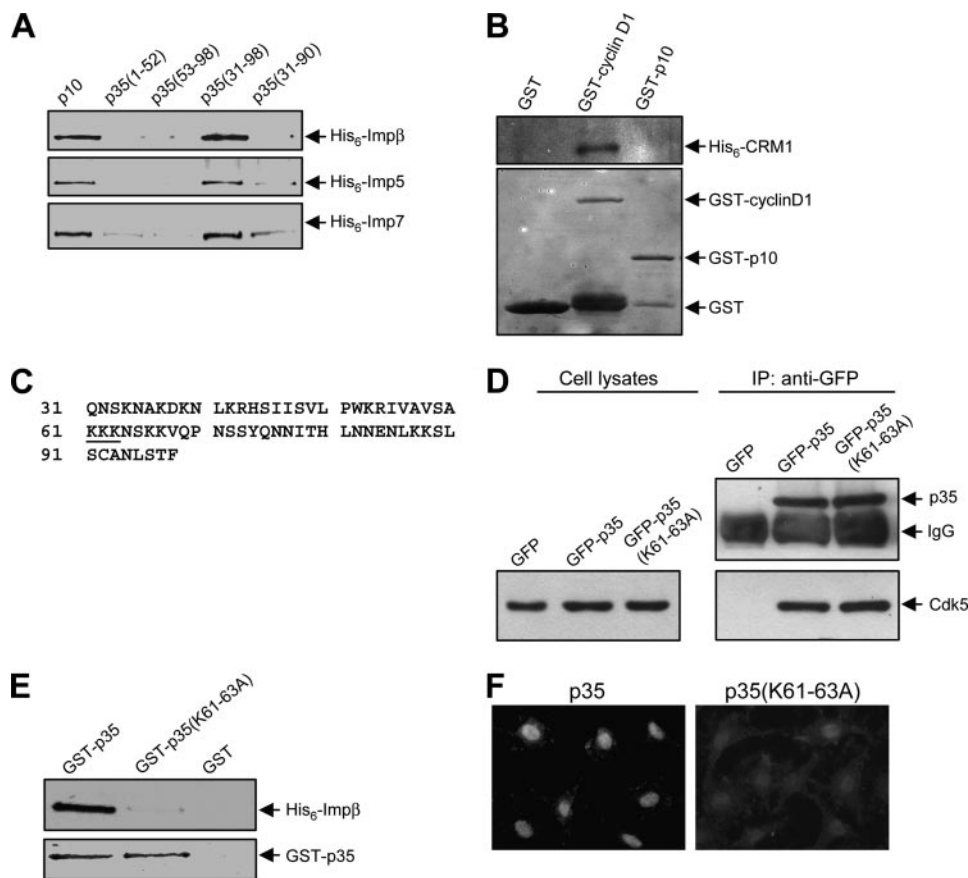


FIGURE 7. Mapping the Imp β /5/7-binding site of p35. A, Imp β /5/7 interacts with an N-terminal fragment of p35. 5 μ g of GST-tagged p35 fragments was subjected to a binding test with 10 μ g of His $_6$ -tagged Imp β , Imp5, or Imp7. After retrieval of the GST proteins, proteins retained on the beads were examined by anti-His $_6$ immunoblotting. B, p10 does not bind to the transport receptor CRM1. 1 μ g of GST, GST-cyclinD1, GST-p10 was tested in a binding assay with 1 μ g of His $_6$ -CRM1 in the presence of 1 μ g GTP-charged His $_6$ -Ran-Q69L. After retrieval of the GST proteins, proteins retained on the beads were analyzed for CRM1 (anti-His $_6$ immunoblotting) and the membrane was stained with Ponceau S to visualize the retrieved GST proteins. C, the sequence of aa 31–98 is shown with 61 KKK 63 underlined. D, mutation of 61 KKK 63 to Ala does not alter the Cdk5 binding activity of p35. COS-7 cells were transfected with a GFP construct of p35 wild type (p35WT) or p35(K61–63A) or with the vector. Anti-GFP immunoprecipitates were probed for p35 and Cdk5. Cdk5 expression is shown by an anti-Cdk5 immunoblot of the cell lysates. E, the K61–63A mutation abolishes p35-Imp β interaction. The binding assay was performed with 1 μ g of the GST proteins and 6 μ g of His $_6$ -Imp β . GST pull downs were analyzed on anti-His $_6$ and anti-p35 immunoblots. F, the K61–63A mutation impairs the nuclear import activity of p35. Import assays were performed on HeLa nuclei with rabbit reticulocyte lysate and an energy system. The import of GST-p35 or GST-p35(K61–63A) was detected by anti-GST immunofluorescence.

plemental Fig. S3). Thus, the mutation interferes with the import activity of p35.

To demonstrate the NLS function of aa 31–98, we transiently expressed this fragment or its K61–63A mutant in fusion with both GFP and GST, a double tag that does not traffic between the cytoplasm and the nucleus (33). In fusion with aa 31–98, GFP-GST exhibited exclusive nuclear localization, whereas the K61–63A counterpart was found primarily in the cytoplasm (Fig. 8A). Therefore, aa 31–98 is a functional NLS and the function requires lysines at positions 61–63. Next, we assessed the effect of the K61–63A mutation on the nucleocytoplasmic distribution of p35. Wild type and mutant p35 were transiently expressed to examine their subcellular localizations (Fig. 8B). The K61–63A mutant did not exhibit a dramatic difference from the wild type in their nucleocytoplasmic localizations (Fig. 8B). To define the distributions precisely, p35 fluorescence

ratios between the nucleus and the cytoplasm were determined. As shown in Fig. 8C, the mutation reduced the ratio of nuclear/cytoplasmic p35 fluorescence by 20%, indicating the involvement of this region in the nuclear localization of p35.

DISCUSSION

In association with p35, Cdk5 exerts its functions via the phosphorylation of its substrates in various subcellular compartments including the nucleus. As p35 is primarily a neuronal protein, we established a procedure to test p35 nuclear import using digitonin-permeabilized cortical neurons, in addition to the widely used assay based on HeLa nuclei. From the assays of both cell types, we conclude that p35 import is well controlled and is mediated by cytoplasmic carriers. In addition, p35 import is sensitive to Ran-GTP. Thus, like other cytoplasmic carrier-dependent imports, p35 nuclear import is guided by the asymmetric distribution of Ran-GDP/Ran-GTP in the cytoplasm and the nucleus.

An N-terminal region of p35, aa 31–98, can be recognized by Imp β /5/7, acting as an NLS. This p35 region is rich in basic residues, which is a common feature of the Imp β /5/7-binding site in their substrates (24, 34). Further truncation of aa 31–98 abrogated the importin binding activities, suggesting that the efficient recruitment of p35 to Imp β /5/7 requires a folded large structure instead of a short motif.

The binding of p35 to Imp β /5/7 can be blocked by Ran-GTP, consistent with the Ran-GDP/GTP control of the p35 import. Also, p10 does not interact with CRM1, a nuclear export receptor that recognizes leucine-rich motifs (14, 15). Therefore, the N terminus of p35 serves as a specific binding site of the importins in p35 nuclear import.

The association of p35 with Imp β /5/7 suggests that multiple import receptors may account for p35 nuclear import. Moreover, the alteration of the three consecutive lysines (61 KKK 63) to Ala interfered with the NLS function of aa 31–98 and reduced the nuclear distribution of p35 to a certain extent, indicating that aa 31–98 is a functional NLS. In addition, the appearance of p35(K61–63A) in the nucleus points to the existence of alternative mechanisms for p35 nuclear import. This idea is supported by the observation that p25, which lacks the N-terminal 98 aa, localizes in the nuclei at high levels (8, 11).

Nuclear Import of the Neuronal Cdk5 Activator

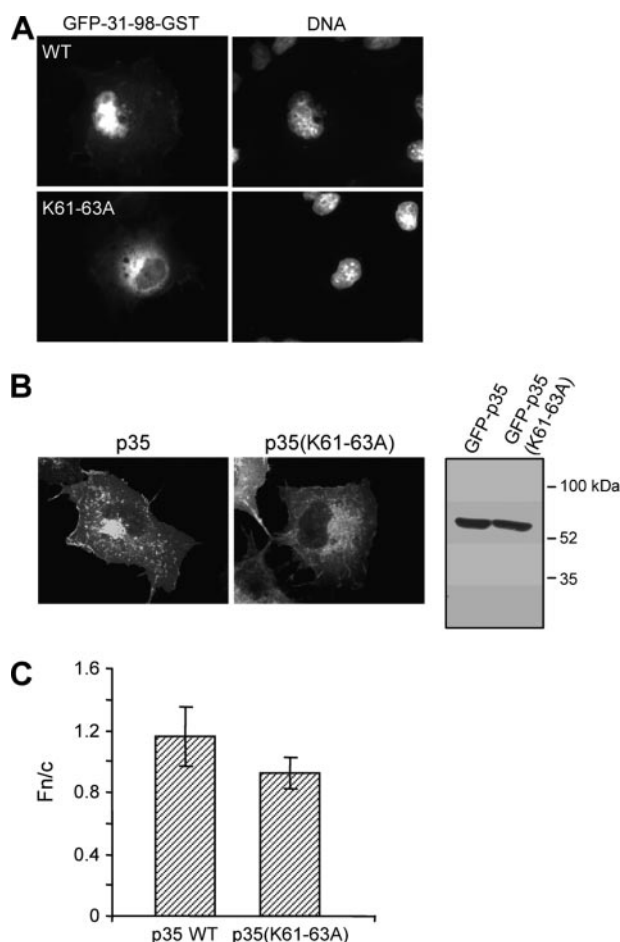


FIGURE 8. The N-terminal region of p35 can function as an NLS. *A*, the GFP-GST fusion protein of the fragment aa 31–98, but not the K61–63A mutant, localizes to the nucleus. COS-7 cells transiently expressing GFP-GST in fusion with aa 31–98 (WT) or aa 31–98 (K61–63A) were subjected to epifluorescence microscopy. DNA was stained by Hoechst 33258. *B* and *C*, COS-7 cells transiently transfected with a GFP fusion construct of the wild type (WT) or the K61–63A mutant of full-length p35 were analyzed by confocal scanning microscopy. Protein expressions were verified by anti-GFP immunoblotting (*B*). Representative images are shown in *B*. Fluorescence ratios between the nucleus and the cytoplasm ($F_{n/c}$) were determined (*C*) from 50 cells each of p35WT and p35(K61–63A). $F_{n/c}$ p35WT = 1.16 ± 0.19 ; p35(K61–63A) = 0.92 ± 0.10 . The results are statistically significant ($p < 0.0002$, Student's *t* test).

Obviously, it undergoes nuclear import by mechanisms distinct from those conferred by aa 31–98. The existence of multiple import pathways may ensure the efficient delivery of p35 to the nucleus for its actions. It also remains possible that some of the mechanisms are engaged as the major import pathways for p35 under certain import-triggering conditions.

In addition to being nuclear transport receptors, Imp β , Imp5, and Imp7 function as molecular chaperones for basic proteins, preventing the aggregation of exposed basic regions with cytoplasmic polyanions (35). p35 is a sticky basic protein, and the N terminus is rich in basic residues (isoelectric point of p10: ~ 10.6). We have observed that p35 forms cytoplasmic aggregates when highly expressed in mammalian cell cultures.³ Therefore, we reason that the association of Imp β /5/7 protects

basic patches exposed on the surface of p35 from undesired interactions and keeps the protein soluble in the cytoplasm.

From our studies and those of others, it has been shown that a proportion of p35 locates in the nucleus (Fig. 8, *B* and *C*, and Refs. 9–11). The following may contribute to the tight control of p35 nuclear localization. First, p35 associates with the plasma membrane and a number of cytoplasmic proteins and structures (1, 2), restraining it from transport to the nucleus. For instance, p35 localizes to the Golgi apparatus and the microtubule and actin cytoskeletons (1, 36). Moreover, p35 contains a myristoylation signal which targets the protein to the plasma membrane (8). Indeed, the disruption of the myristoylation signal enriches p35 in the nuclei (8). Therefore, there appears to be a tight control of p35 association with nuclear import receptors and the subsequent nuclear import. However, the precise control mechanism remains to be determined. Second, p35 nucleocytoplasmic distribution could be the equilibrium of the nuclear import and export. We have probed whether p35 is exported from the nucleus by the classical CRM1-dependent mechanism using leptomycin B, an inhibitor of CRM1 (37). However, the leptomycin B treatment did not alter the nucleocytoplasmic distribution of p35 (our unpublished data). Therefore, the p35 nuclear export is insensitive to CRM1 inhibition. Given that several nuclear export receptors have been identified recently (14, 38), it remains likely that p35 undertakes pathways other than CRM1 for nuclear export. Therefore, the nucleocytoplasmic distribution of p35 may represent the net effect of controlled p35 nuclear import and export.

As p35 is an activator of Cdk5, we asked whether p35 is transported in complexes with Cdk5 to the nucleus. We have shown that Cdk5 association is dispensable for p35 localization to the nucleus and that the p35 complexes of Imp β /5/7 do not contain Cdk5. The kinase activity of Cdk5 is highly stimulated by the association with p35 (39). When activated, Cdk5 catalyzes p35 phosphorylation, labeling p35 for degradation via the proteasome pathway (40). A conceivable benefit of separate import pathways for p35 and Cdk5 is to avoid Cdk5 activation and thus p35 phosphorylation and degradation during the transport. The independent regulation of Cdk5 and p35 nuclear imports is supported by a recent observation showing that the neuregulin treatment enhances the nuclear levels of p35 but not those of Cdk5 (13). The dynamic transport of p35 into the nucleus in response to the stimulation suggests that its regulated import may set a control mechanism of Cdk5 activity in the nucleus.

The present study identifies mechanisms governing p35 nuclear import, which has a critical impact on Cdk5 functions in the nucleus. Cdk5 has been implicated in a variety of neuronal activities, including learning and memory-related synaptic plasticity (1). In addition, Cdk5-p35 is up-regulated in regenerating axons and the Cdk5 kinase activity is required for axonal regeneration after nerve crush (41). Interestingly, it has been found that Imp α and Imp β mediate the retrograde transport of signaling molecules from distal neuronal processes to the cell body in synaptic plasticity and during axonal regeneration (42, 43). Therefore, our findings raise the intriguing possibility that p35 is transported by Imp β /5/7 from neuronal processes to the nucleus to mediate many of the neuronal activities, such as

³ X. Fu, Y.-K. Choi, and R. Z. Qi, unpublished data.

synaptic plasticity and nerve regeneration. Future studies will be of great interest to elucidate specific functions of Cdk5 and p35 in these neuronal events.

Acknowledgments—We thank Dr. S. A. Adam, Dr. G. Blobel, Dr. D. Görlich, Dr. L. Gerace, and Dr. E. Seto for providing plasmid constructs and reagents. We also thank Dr. Y. P. Ng for his assistance on confocal microscopy, Dr. J. H. Wang for helpful discussions, and Dr. K. A. W. Lee for critical reading of the manuscript.

REFERENCES

- Dhavan, R., and Tsai, L. H. (2001) *Nat. Rev. Mol. Cell. Biol.* **2**, 749–759
- Lim, A. C., Qu, D., and Qi, R. Z. (2003) *Neurosignals* **12**, 230–238
- Fu, A. K., Fu, W. Y., Cheung, J., Tsim, K. W., Ip, F. C., Wang, J. H., and Ip, N. Y. (2001) *Nat. Neurosci.* **4**, 374–381
- Lew, J., Huang, Q. Q., Qi, Z., Winkfein, R. J., Aebbersold, R., Hunt, T., and Wang, J. H. (1994) *Nature* **371**, 423–426
- Tsai, L. H., Delalle, I., Caviness, V. S., Jr., Chae, T., and Harlow, E. (1994) *Nature* **371**, 419–423
- Tarricone, C., Dhavan, R., Peng, J., Areces, L. B., Tsai, L. H., and Musacchio, A. (2001) *Mol. Cell* **8**, 657–669
- Lee, M. S., Kwon, Y. T., Li, M., Peng, J., Friedlander, R. M., and Tsai, L. H. (2000) *Nature* **405**, 360–364
- Patrick, G. N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L. H. (1999) *Nature* **402**, 615–622
- Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F., and Tsai, L. H. (1996) *Genes Dev.* **10**, 816–825
- Qu, D., Li, Q., Lim, H. Y., Cheung, N. S., Li, R., Wang, J. H., and Qi, R. Z. (2002) *J. Biol. Chem.* **277**, 7324–7332
- Gong, X., Tang, X., Wiedmann, M., Wang, X., Peng, J., Zheng, D., Blair, L. A., Marshall, J., and Mao, Z. (2003) *Neuron* **38**, 33–46
- Ino, H., and Chiba, T. (1996) *Brain Res.* **732**, 179–185
- Fu, A. K., Fu, W. Y., Ng, A. K., Chien, W. W., Ng, Y. P., Wang, J. H., and Ip, N. Y. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 6728–6733
- Fried, H., and Kutay, U. (2003) *Cell Mol. Life Sci.* **60**, 1659–1688
- Gorlich, D., and Kutay, U. (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 607–660
- Gorlich, D., Pante, N., Kutay, U., Aebi, U., and Bischoff, F. R. (1996) *EMBO J.* **15**, 5584–5594
- Moore, J. D., Yang, J., Truant, R., and Kornbluth, S. (1999) *J. Cell Biol.* **144**, 213–224
- Takizawa, C. G., Weis, K., and Morgan, D. O. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 7938–7943
- Chi, N. C., Adam, E. J., Visser, G. D., and Adam, S. A. (1996) *J. Cell Biol.* **135**, 559–569
- Yaseen, N. R., and Blobel, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4451–4456
- Yang, W. M., Tsai, S. C., Wen, Y. D., Fejer, G., and Seto, E. (2002) *J. Biol. Chem.* **277**, 9447–9454
- Lim, A. C., Hou, Z., Goh, C. P., and Qi, R. Z. (2004) *J. Biol. Chem.* **279**, 46668–46673
- Gorlich, D., Dabrowski, M., Bischoff, F. R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997) *J. Cell Biol.* **138**, 65–80
- Jakel, S., and Gorlich, D. (1998) *EMBO J.* **17**, 4491–4502
- Paschal, B. M., and Gerace, L. (1995) *J. Cell Biol.* **129**, 925–937
- Adam, S. A., Marr, R. S., and Gerace, L. (1990) *J. Cell Biol.* **111**, 807–816
- Lam, M. H., Briggs, L. J., Hu, W., Martin, T. J., Gillespie, M. T., and Jans, D. A. (1999) *J. Biol. Chem.* **274**, 7391–7398
- Finlay, D. R., Newmeyer, D. D., Price, T. M., and Forbes, D. J. (1987) *J. Cell Biol.* **104**, 189–200
- Jakel, S., Albig, W., Kutay, U., Bischoff, F. R., Schwamborn, K., Doenecke, D., and Gorlich, D. (1999) *EMBO J.* **18**, 2411–2423
- Kutay, U., Izaurralde, E., Bischoff, F. R., Mattaj, I. W., and Gorlich, D. (1997) *EMBO J.* **16**, 1153–1163
- Tang, D., Chun, A. C., Zhang, M., and Wang, J. H. (1997) *J. Biol. Chem.* **272**, 12318–12327
- Alt, J. R., Cleveland, J. L., Hannink, M., and Diehl, J. A. (2000) *Genes Dev.* **14**, 3102–3114
- Rosorius, O., Heger, P., Stelz, G., Hirschmann, N., Hauber, J., and Stauber, R. H. (1999) *BioTechniques* **27**, 350–355
- Freedman, N. D., and Yamamoto, K. R. (2004) *Mol. Biol. Cell* **15**, 2276–2286
- Jakel, S., Mingot, J. M., Schwarzmaier, P., Hartmann, E., and Gorlich, D. (2002) *EMBO J.* **21**, 377–386
- Paglioni, G., Peris, L., Diez-Guerra, J., Quiroga, S., and Caceres, A. (2001) *EMBO Rep.* **2**, 1139–1144
- Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S., and Beppu, T. (1994) *J. Biol. Chem.* **269**, 6320–6324
- Mingot, J. M., Bohnsack, M. T., Jakle, U., and Gorlich, D. (2004) *EMBO J.* **23**, 3227–3236
- Qi, Z., Huang, Q. Q., Lee, K. Y., Lew, J., and Wang, J. H. (1995) *J. Biol. Chem.* **270**, 10847–10854
- Patrick, G. N., Zhou, P., Kwon, Y. T., Howley, P. M., and Tsai, L. H. (1998) *J. Biol. Chem.* **273**, 24057–24064
- Namgung, U., Choi, B. H., Park, S., Lee, J. U., Seo, H. S., Suh, B. C., and Kim, K. T. (2004) *Mol. Cell. Neurosci.* **25**, 422–432
- Thompson, K. R., Otis, K. O., Chen, D. Y., Zhao, Y., O'Dell, T. J., and Martin, K. C. (2004) *Neuron* **44**, 997–1009
- Hanz, S., Perlson, E., Willis, D., Zheng, J. Q., Massarwa, R., Huerta, J. J., Koltzenburg, M., Kohler, M., van Minnen, J., Twiss, J. L., and Fainzilber, M. (2003) *Neuron* **40**, 1095–1104