α-Chimaerin exists in a functional complex with the Cdk5 kinase in brain

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Abstract Cyclin-dependent kinase 5 (Cdk5) in association with its neuronal activators p35 and p39 shows a complex involvement in the control of neurocytoskeletal dynamics. Here we show that α-chimaerin, a GTPase-activating protein specific for Rac and Cdc42, is a p35-binding protein. The interaction domains of p35 and α-chimaerin were delineated. In transfected HeLa cells, p35 and α-chimaerin displayed an overlapping distribution pattern and they could be co-immunoprecipitated from the cell lysate. As α-chimaerin has a regulatory function in actin repolymerization, these results suggested that the regulation of neurocytoskeleton dynamics by Cdk5 is mediated at least in part via α-chimaerin.

Key words: Cdk5; Cdk5 activator; Chimaerin; Protein–protein interaction

1. Introduction

As a unique member of the CDK family, cyclin-dependent kinase 5 (Cdk5) is highly enriched in neurons of mammalian central nervous tissues. Along with its neuron-specific activators, p35 and p39, Cdk5 plays key roles in a variety of neuronal functions [1]. In mammalian brain extracts, Cdk5 and its activators are present in macromolecular complexes with multiple protein components [2]. Over the last few years, a large number of Cdk5-p35-binding proteins have been identified by yeast two-hybrid screens or proteomic analyses, implicating the kinase in diverse cellular processes in neurons (see [3] for a review). In particular, many of the binding proteins are cytoskeletal proteins including protein subunits of neurofilaments (neurofilament heavy chain NF-H and middle chain NF-M), actin filaments and cytoskeleton-regulatory proteins such as tau and α-actinin 1 [4–6]. In addition, Rac1, which is a small GTPase protein implicated in the regulation of actin cytoskeletons, was shown to be a p35-binding protein [7]. These findings suggested that Cdk5 plays important roles in neurocytoskeleton dynamics. However, the molecular mechanism underlying Cdk5 actions is highly complex and far from clear.

Rac1 is a member of the Rho small GTPase family and implicated in signal pathways of neuronal growth cone activity [8]. The active form of Rac1 (GTP-bound form) is capable of activating a protein Ser/Thr kinase, PAK1, to induce remodeling of actin filaments, thus leading to the formation of lamellipodia and membrane ruffles. PAK1 is a substrate of Cdk5; phosphorylation of PAK1 by Cdk5 results in the inactivation of PAK1 [7]. All Rho family members possess intrinsic GTP hydrolysis activity that is stimulated by GTPase-activating proteins (GAPs), which convert the Rho members from the active form to the inactive form (GDP-bound form). In the present study, we have identified α-chimaerin, a GAP for Rac1 and Cdc42 [9,10], as a p35-binding protein in a yeast two-hybrid screen and demonstrated the interaction of α-chimaerin and p35-Cdk5 both in vitro and inside cultured cells. α-Chimaerin mediates actin cytoskeleton dynamics in a Rac1/Cdc42-dependent manner [11,12]. Results presented here suggest that α-chimaerin and Cdk5-p35 act cooperatively in Rac1 signaling pathways to modulate actin remodeling in neuronal growth cones.

2. Materials and methods

2.1. Yeast two-hybrid assay

The yeast two-hybrid system was employed as described in previous reports [4,13]. To search for p35-interacting proteins, p35 (SwissProt accession number Q28199) was engineered in the vector pAS2 (Clontech) as a bait and the prey is a human brain cDNA library constructed in pACT2 (Clontech). The bait and the prey were co-transformed into the yeast reporter strain CG-1945 for the interaction hunt. To test the interaction between α-chimaerin (SwissProt accession number P15882) and various p35 fragments, the p35-binding fragment of α-chimaerin in pACT2 and the p35 fragments in pAS2 were co-expressed in the yeast strain Y190 for reporter assays. Cdk5 (SwissProt accession number Q00535) and p39 (SwissProt accession number Q13319) were also subcloned into pAS2 for the interaction test.

2.2. Plasmid construction and recombinant protein preparation

The sequence of α_1-chimaerin (SwissProt accession number P15882) was amplified by a polymerase chain reaction from a human brain cDNA library and engineered into the vector pGEX-KT. Recombinant proteins in fusion with glutathione *S*-transferase (GST) or His_6 were prepared as described previously [13,14]. To remove GST from the fusion proteins, glutathione (GSH) beads coupled with the GST proteins were suspended in the cleavage buffer containing 25 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol (DTT), 0.15 M NaCl, and thrombin (Sigma). The cleavage was carried out at room temperature for 2 h. The beads were then removed by centrifugation. Supernatant was subjected to incubation with benzamidine-Sepharose (Sigma) at 4°C for 1 h to absorb thrombin. After the incubation, the beads were

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Abbreviations: Cdk, cyclin-dependent kinase; DTT, dithiothreitol; GSH, glutathione; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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pelleted by centrifugation to collect supernatant, which contained the GST-free recombinant proteins.

2.3. Biochemical binding assay

GST-α_1-chimaerin was mixed and incubated with the recombinant proteins of Cdk5 and p35 in the binding buffer (25 mM Tris, pH 7.5, 1 mM DTT, 0.1 M NaCl, 0.1% Triton X-100 and 5 mg/ml bovine serum albumin) at 4°C for 1 h. GSH beads were then added for a further incubation at 4°C for 1 h to capture GST. The beads were then retrieved and washed extensively with the binding buffer. Proteins bound to the beads were dissolved in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer and subjected to immunoblotting using the antibodies of anti-p35 (C-19, Santa Cruz Biotech), anti-Cdk5 (C-8, Santa Cruz Biotech) and anti-His_6 (Amersham Pharmacia Biotech). Competitive binding assays were performed by incubating GST-α_1-chimaerin and His_6-p35(145–170) with increasing amounts of the His_6-tagged C48α1 fragment of protein C48 (SwissProt accession number Q9JLH5). After the pull-down of GST-α_1-chimaerin using GSH beads, bound His_6-p35(145–170) was determined by immunoblotting.

2.4. Protein phosphorylation

Phosphorylation of α_1-chimaerin was carried out at 30°C for 30 min. Reactions contained 20 mM MOPS, pH 7.5, 5 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (~5000 dpm/pmol), 1 mM DTT, 1 μg α_1-chimaerin and Cdk5-p25. The reaction was terminated by the addition of the SDS–PAGE sample loading buffer and boiling for 5 min. Proteins were then resolved on SDS–PAGE. Protein phosphorylation was visualized on autoradiograph.

2.5. Cell transfection, immunoprecipitation and immunostaining

HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were transfected using the calcium phosphate precipitation method at 40–60% confluence with 10 μg of plasmid DNA for a 100-mm plate. At 24 h post transfection, the cells were scraped from their plates and lysed in 1 ml of the ice-cold lysis buffer (50 mM HEPES, pH 7.2, 250 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM DTT, 10 μg/ml leupeptin, 1 μg/ml antipain, 2 mM phenylmethylsulfonyl fluoride and 100 μg/ml benzamidine) for 15 min at 4°C. Cell debris were then removed by centrifugation (15 min at 14 000×*g*).

Aliquots of the cell lysates (100 μg/μl) were diluted with phosphate-buffered saline (PBS) and incubated with the anti-p35 antibody for 2 h at 4°C, followed by 1 h incubation with 50 μl of 50% slurry of protein G-agarose beads. The beads were washed four times with PBS and bound proteins were released by the addition of the SDS–PAGE sample loading buffer and boiling. For immunostaining, transfected cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min, washed, and permeabilized with absolute methanol for 2 min. Primary antibodies (the anti-p35 antibody and the anti-HA monoclonal antibody 12CA5 at 1:50 and 1:60 dilutions respectively in PBS containing 3% bovine serum albumin) were added to the slide and stained for 1 h at room temperature. Likewise, secondary antibodies were added after removal of the primary antibodies by PBS washing. The cells were then visualized using confocal microscopy.

3. Results

3.1. Physical association of α-chimaerin and the neuronal Cdk5 kinase

As described in our earlier reports [4,13], the yeast two-hybrid system was employed to search for p35-binding proteins in a human brain cDNA library. One of the isolates was found to contain a sequence encoding the C-terminal 98 amino acid residues of α_1- and α_2-chimaerin. To further test the binding of α-chimaerin to p35, various truncated forms of p35 were examined for their ability to associate with the isolated α-chimaerin fragment. Two-hybrid assay results indicated that the interaction occurs within p25, which is the proteolytically truncated form of p35 existing in cells under neurotoxic conditions (Fig. 1) [15–17]. The assays of further truncated p35 fragments showed that the α-chimaerin-binding activity is retained in p35(145–291), but not in the shorter fragment p35(150–291) (Fig. 1). Mammalian nervous systems contain p39, which is a p35 homolog and displays a similar activity to activate Cdk5 specifically [18]. In our yeast two-hybrid assays, p39 is also capable of associating with α-chimaerin (data not shown). In addition, Cdk5 did not show any detectable interaction with the isolated α-chimaerin fragment in the two-hybrid assay (data not shown).

Fig. 1. Interaction of p35 and its fragments with α-chimaerin in the yeast two-hybrid assay. p35 fragments in pAS2 and the C-terminal 98 amino acids of α-chimaerin in pACT2 were co-transformed into the yeast Y190 for the reporter assays.

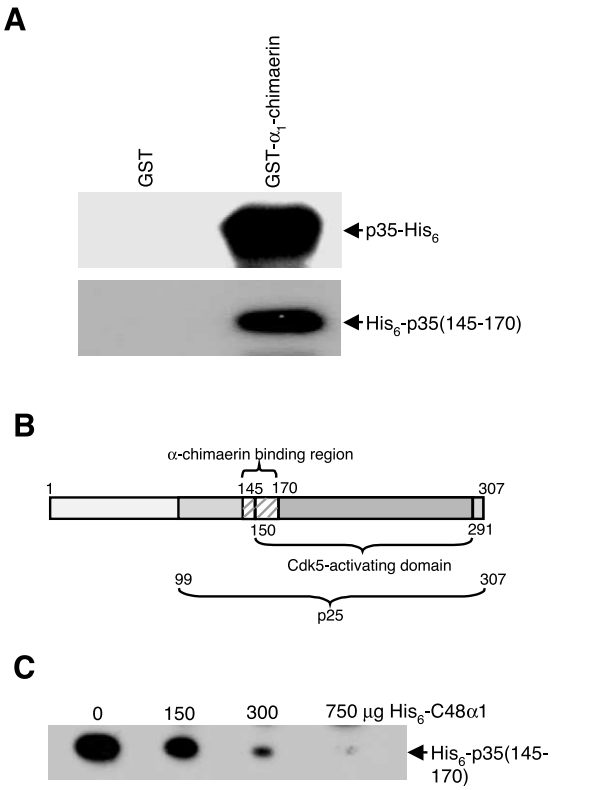

Fig. 2. Biochemical binding assay of α-chimaerin and Cdk5-p35. A: Binding of GST-α_1-chimaerin and His_6-tagged p35 proteins. GST or GST-α_1-chimaerin (30 μg) was incubated with 15 μg of the indicated His_6-tagged p35 proteins at 4°C for 1 h. The GST proteins were then retrieved using GSH beads. Co-precipitated p35 proteins were detected by Western blots using the anti-His_6 antibody. B: Schematic representation of the functional regions of p35. C: α-Chimaerin competes with C48 in binding to p35. GST-α_1-chimaerin (30 μg) was incubated with His_6-p35(145–170) (15 μg) in the presence of increasing amounts of the C48 fragment C48α1. After the pull-down of GST-α_1-chimaerin with GSH beads, the amounts of His_6-p35(145–170) bound to the beads were determined by an anti-His_6 immunoblot.

Direct binding of α-chimaerin to p35 was validated in a biochemical binding assay using recombinant proteins of p35 and α_1-chimaerin. Incubation of p35-His_6 with GST-α_1-chimaerin followed by affinity precipitation of GST-α_1-chimaerin resulted in the co-precipitation of p35 as revealed on

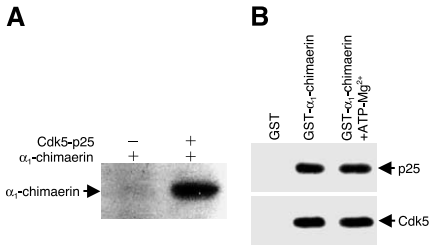

Fig. 3. The Cdk5 kinase is enzymatically active in the complex of α-chimaerin. A: Phosphorylation of α_1-chimaerin by Cdk5. 1 μg of α_1-chimaerin was subjected to phosphorylation reactions in the presence or absence of Cdk5-p25. After incubation at 30°C for 30 min, reactions were terminated by boiling in the SDS–PAGE sample loading buffer. Proteins were then resolved by SDS–PAGE to visualize protein phosphorylation by autoradiography. B: The α_1-chimaerin phosphorylation does not affect its association with the Cdk5 kinase. 10 μg of GST-α_1-chimaerin was incubated with the Cdk5-p25 complex in the presence or absence of ATP-Mg^{2+} at 30°C for 30 min. GST was used in the control in place of GST-α_1-chimaerin. After the GST proteins were recovered with GSH beads, bound proteins were immunoblotted using the anti-Cdk5 and anti-p35 antibodies.

immunoblots (Fig. 2A, upper panel). In the control assay, p35 was not detected in the precipitate of GST, indicating the specific binding of p35 and α_1-chimaerin. A short peptide spanning the residues 145–170 of p35 was previously identified as the region for the competitive binding of at least three proteins, C42, C48 and C53 [14]. Given the yeast two-hybrid result that α-chimaerin binds to a region located within 145–291 amino acids of p35, the 145–170-amino acid fragment was tested in the pull-down of GST-α_1-chimaerin. Results indicated that this short region of p35 is capable in binding to α_1-chimaerin (Fig. 2A, lower panel). Fig. 2B is a schematic graph of the binding fragments of p35. Further, when the α1 fragment of protein C48 was applied at increasing amounts in the binding assays, it competed off GST-α_1-chimaerin from the p35(145–170) association (Fig. 2C). Taken together, α-chimaerin binds p35 in the same region as C42, C48 and C53 and competes with these proteins in the p35 association.

3.2. *The complex of α-chimaerin, Cdk5 and p35 is enzymatically functional*

In brain, p35 or p25 exists with and activates Cdk5. It is therefore important to examine whether α-chimaerin can bind to the p25- or p35-Cdk5 complex. Using GST-α_1-chimaerin, we performed the pull-down assays and found that both p35 and Cdk5 could be co-precipitated by α_1-chimaerin. Moreover, there are two potential Cdk5 phosphorylation sites in the α_1-chimaerin sequence [19,20], and in an in vitro phosphorylation reaction α_1-chimaerin was readily phosphorylated by Cdk5-p25 (Fig. 3A), suggesting that α_1-chimaerin is a potential physiological substrate of Cdk5. To investigate whether the α_1-chimaerin phosphorylation affects its association with Cdk5-p25, the pull-down assay was performed under the phosphorylation condition. As shown in Fig. 3B, GST-α_1-chimaerin precipitated the same amount of Cdk5-p25 under both the non-phosphorylating and phosphorylating conditions, indicating that the α_1-chimaerin association is not mediated by the α_1-chimaerin phosphorylation.

Next, we examined the potential effects of α-chimaerin on the Cdk5 activation. Cdk5 can be highly activated by reconstitution with p35 or its truncated form p25 [15,21]. In our tests, the Cdk5 kinase was reconstituted from recombinant Cdk5 and p25 proteins in the presence or absence of α_1-chimaerin. Kinase assays showed that α_1-chimaerin had no marked effect on the Cdk5 activation by p25 even when α_1-chimaerin was present in excess (data not shown). On the other hand, α-chimaerin is a GAP to enhance the intrinsic GTPase activity of Rac1 or Cdc42. In order to determine whether the Cdk5-p35 association affects its GAP activity, a GTPase assay was performed according to the published protocols to measure the GAP activity of α_1-chimaerin towards Rac1 [22,23]. From the assays, α_1-chimaerin that was preincubated with or without an excessive amount of Cdk5-p25 did not show any detectable difference in its GAP activity (data not shown), indicating that the association of Cdk5-p25 does

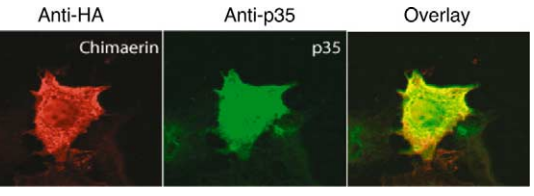

Fig. 4. Co-localization of α_2-chimaerin and p35 in transfected cells. HeLa cells were co-transfected with plasmids expressing p35 and HA-α_2-chimaerin. Double immunostaining was performed on the transfected cells with the anti-HA (red) and anti-p35 (green) antibodies. The merged image shows the region of co-localization in yellow.

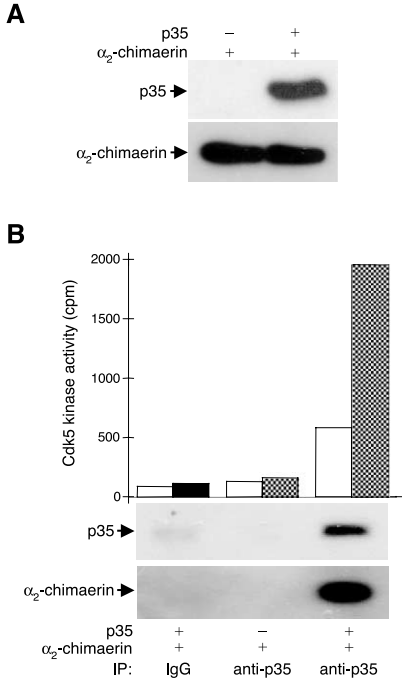

Fig. 5. In vivo association of α-chimaerin and Cdk5-p35. HeLa cells were singly transfected with the construct expressing HA-α_2-chimaerin or doubly transfected with the HA-α_2-chimaerin and p35 constructs. A: The expression of HA-α_2-chimaerin and p35 was examined in the cell lysates by Western blotting using anti-HA and anti-p35 antibodies, respectively. B: Immunoprecipitation was performed using the anti-p35 antibody or non-specific IgG from the cell lysates containing 500 μg proteins. The immunoprecipitates were probed on Western blots with the anti-HA and anti-p35 antibodies. The Cdk5 kinase activity in the immunoprecipitates was measured with (solid bar) and without (empty bar) adding the exogenous GST-Cdk5 protein (0.6 μg).

not affect the GAP activity of α-chimaerin. Thus, it is tempting to conclude from these results that Cdk5, p35 and α-chimaerin form a ternary protein complex with both the kinase and GAP activities.

3.3. α-Chimaerin associates with the neuronal Cdk5 kinase in vivo

To examine the cellular localizations of α-chimaerin and p35, immunofluorescent staining was performed. HeLa cells were doubly transfected with the constructs expressing p35 and α_2-chimaerin. Using the antibodies, the α_2-chimaerin protein was found to localize predominantly in cytoplasm and plasma membranes, displaying a very similar pattern to that of p35 (Fig. 4). Co-localization of p35 and α_2-chimaerin was observed mainly in the plasma membrane region (Fig. 4). In order to determine their association in the cells, the cell lysates that contained similar amounts of the expressed proteins were selected for the immunoprecipitation experiment (Fig. 5A). Fig. 5B shows that the HA-α_2-chimaerin protein was co-precipitated with p35 from the co-transfected lysate, whereas the negative controls, which are the p35 immunoprecipitation from the singly transfected HA-α_2-chimaerin lysate and the non-specific IgG immunoprecipitation from the p35 and α_2-chimaerin co-transfected lysate, did not precipitate any α_2-chimaerin protein. Moreover, when the immunoprecipitates were assayed for Cdk5 activity with or without added GST-Cdk5 protein, the α_2-chimaerin-p35 co-immunoprecipitate exhibited endogenous Cdk5 kinase activity, which can be further activated by the addition of the exogenous Cdk5 protein (Fig. 5B), corroborating the results of in vitro reconstitution assays that p35 and Cdk5 can form an enzymatically active complex with α-chimaerin.

4. Discussion

Here, using the yeast two-hybrid system, α-chimaerin was identified to interact with the p35 activator of Cdk5. In vitro studies demonstrated the association of α-chimaerin with Cdk5-p35 to form a ternary protein complex. The α-chimaerin interaction was located in p35 to a short peptide spanning the residues 145–170. Given the facts that this region interacts with the C42, C48 and C53 proteins in a competitive manner and C48 exhibited competition with α_1-chimaerin in the binding assay [14], C42, C48 and C53 could mediate the association of p35 and α-chimaerin in vivo.

α-Chimaerin exists as two alternatively spliced variants, α_1- and α_2-chimaerins, sharing the structure of a cysteine-rich domain followed by the GAP domain [20]. The difference between the two variants lies at the N-terminus with a SH2 domain in α_2- but not in α_1-chimaerin. The isolated p35-binding sequence comprises the C-terminal 98 amino acids of both α_1- and α_2-chimaerins, which overlaps the GAP domain. The two forms of α-chimaerin have slightly different expression patterns in brain and the expression of α_2-chimaerin was found to resemble that of p35 [11]. In the transfected cells, α_2-chimaerin displayed an overlapping localization pattern with p35 and the two proteins as well as Cdk5 could be co-immunoprecipitated from the cell lysate. These results suggested that α-chimaerin, Cdk5 and p35 exist in a complex in neurons. Moreover, both the kinase activity of Cdk5 and the GAP activity of α-chimaerin are retained in the protein complex. Meanwhile, the protein complex may contain additional protein molecules such as Rac1 and PAK1 [7,11]. Although the functional significance of the protein complex is unclear yet, the possibility that various activities in the complex are coordinated in the regulation of actin dynamics may be considered. For example, α-chimaerin may facilitate locating the Cdk5 kinase to its targets in the signaling pathways. On the other hand, α-chimaerin, by facilitating the conversion of Rac1 from the GTP- to GDP-bound form [9,10], and Cdk5, by phosphorylating and inhibiting PAK1 [7], bring about the down-regulation of the Rac1 signaling. Thus, the association of α-chimaerin and Cdk5-p35 may be required for their coordinated involvements in remodeling of actin filaments.

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