FEBS Letters 583 (2009) 2171–2178

FEBS Letters

journal homepage: www.FEBSLetters.org

Cyclin Y, a novel membrane-associated cyclin, interacts with PFTK1

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ARTICLE INFO

Article history:
Received 29 April 2009
Revised 1 June 2009
Accepted 4 June 2009
Available online 12 June 2009

Edited by Angel Nebreda

Keywords:
CCNY
PFTK1
Membrane localization
Interaction
Regulatory partner
Kinase activity

ABSTRACT

A novel cyclin, CCNY, was identified as a PFTK1 interacting protein in a yeast two-hybrid screen. The cyclin box in CCNY and the PFTAIRE motif in PFTK1 are both required for the interaction which was confirmed by in vivo and in vitro assays. Two transcripts (4 and 2 kb), of CCNY were detected by Northern blot analysis and CCNY was enriched at the plasma membrane due to an N-terminal myristoylation signal. We propose that binding of CCNY to PFTK1 enhances PFTK1 kinase activity and changes its intracellular location.

Structured summary:
MINT-7147585, MINT-7147598, MINT-7147614, MINT-7147628, MINT-7147647, MINT-7147665, MINT-7147680: *pftk1* (uniprotkb:O94921) *physically interacts* (MI:0915) with *CCNY* (uniprotkb:Q8ND76) by *two hybrid* (MI:0018)
MINT-7147725, MINT-7147743: *pftk1* (uniprotkb:O94921) *physically interacts* (MI:0914) with *CCNY* (uniprotkb:Q8ND76) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7147758: *pftk1* (uniprotkb:O35495) *physically interacts* (MI:0914) with *CCNY* (uniprotkb:Q8BGU5) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-7147695, MINT-7147713: *pftk1* (uniprotkb:O94921) and *CCNY* (uniprotkb:Q8ND76) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

The cyclins are a family of proteins, which are identified by a homologous region of about 100 amino acid residues, termed cyclin box which directs cyclin–CDK interaction [1]. Cyclins are regulatory subunits that associate with specific CDKs through the cyclin box to form functional protein kinase complexes. The CDKs are a family of Ser/Thr protein kinases that share a highly conserved PSTAIRE motif which is involved in cyclin binding and has been used to classify CDK-related kinases [2,3]. The activity of CDK is tightly regulated in part by its subcellular localization [4]. The specific subcellular compartment where CDKs are located to, may define a specific substrate to be phosporylated, and thus a specific function to be induced. Cyclins play a role in regulating the localization of CDKs. For instance, cyclin B1 can target CDK1 to the intracellular membranes, cytoplasm or nucleus during the cell cycle, whereas cyclin B2 retained CDK1 on intracellular membrane [5–7]. Different localization of the B-type cyclin–CDKs enables cyclin B1–CDK1 to cause chromosome condensation, microtubule reorganization, and disassembly of the nuclear lamina and of the Golgi apparatus, while restricts cyclin B2–CDK1 to disassembly of the Golgi apparatus [7].

PFTK1 has recently been characterized as a CDK that regulates cell cycle progression and cell proliferation. It can interact with cyclin D3 (CCND3), forming a ternary complex with the cell cycle inhibitor $p21^{Cip1}$ in mammalian cells. Its kinase activity is enhanced by CCND3 and inhibited by $p21^{Cip1}$ [3]. PFTK1 belongs to the subgroup of Cdc2-related kinase family, which includes mammalian PFTAIRE, PCTAIRE, PITSLRE, PISSLRE, etc. [3,8–14]. These kinases derive their names from the characteristic amino acid sequence in the cyclin binding motif PSTAIRE and display a peculiar pattern of expression with high levels in post-mitotic tissue [13]. This suggests that their function is not restricted to the regulation of proliferation, and also is involved in control of post-mitotic cellular process [10,13]. Relatively little is known, however, about the function of most of these kinases, as their cyclin partners remain unidentified. CDK5 was identified as PSSALRE and is also one member of the Cdc2-related kinase subfamily. Great advances have been made in understanding the function of CDK5 since the two activation regulators, p35 and p39, have been identified. Although p35 and p39 share little sequence identity with other cyclins in their amino acid sequences, they share similarity to cyclins in the ternary structure [15–19]. Like the CDK regulatory subunits (cyclins), p35 or p39, is required for the kinase

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.06.010

activity of CDK5 and confers the substrate specificity to CDK5 [16]. The subcellular distribution of CDK5 is also determined by p35 and p39 [17]. p35 and p39 are targeted to the cell membrane by N-myristoylation [16,20]. CDK5 alone does not show a specific distribution pattern, but is recruited to the cell membrane when associated with p35 or p39 [21]. Therefore, the transmembrane or membrane-associated proteins are likely to be physiological substrates of CDK5 [21]. Although CDKs are traditionally involved in regulating cell cycle progression, CDK5 functions in various events throughout the life span of neurons, from development to cell death. For instance, it plays critical roles in neuronal apoptosis, neuronal migration and axon guidance, synaptic neurotransmission, membrane transport, as well as regulation of the cytoskeleton [17,21,22].

We previously reported the isolation of human PFTAIRE1 (PFTK1) which is highly expressed in brain, pancreas, kidney, heart, testis and ovary [8]. To search its substrate and regulatory proteins, we screened a two-hybrid library and identified seven PFTK1 interacting factors including four 14-3-3 isoforms (β, ε, η, τ) [23], a septin family member KIAA0202 (SEPT8) [24], PLZF protein [25] and a novel cyclin-like protein, named cyclin Y (CCNY), which we study in this paper.

2. Materials and methods

2.1. Plasmid construction

A 2.1 kb insert containing full length ORF of CCNY obtained from the yeast two-hybrid screening and a cDNA containing full length ORF of PFTK1 were used for further subcloning. The CCNY cDNA and its deletion fragments were amplified by PCR and subcloned into pGilda or pB42AD for BD fusion or AD fusion construction. PFTK1 mutants (K164R, H224N, D256A, F176Δ, S119A/S282A) were generated by PCR amplification using Mutagenesis kit (Strategene). PFTK1 and its mutants were subcloned into pGilda or pB42AD for BD fusion or AD fusion construction for yeast two-hybrid assay. Wild type and point mutation variants including G2A and N3A of CCNY were amplified by PCR and subcloned into different expression vectors including pEGFP-N3 (Clontech), pEGFP-C2, pcDNA3-2HA and pMycN3 [26] for ectopic expression. The PFTK1 and PFTK1 F176Δ mutant fragments were inserted into pEGFP-C2 for expression in 293T cells.

2.2. Yeast two-hybrid assay

The yeast two-hybrid screen and yeast two-hybrid interaction assay were carried out as described [23].

2.3. Northern blot analysis

Human multiple tissue Northern blots (Clontech) were used for analyzing the expression pattern of CCNY in human tissues. Total RNA was made from different cell lines, loaded and analyzed by the probes in parallel. A 1026 bp cDNA fragment containing whole coding region of the CCNY (CCNY probe) and a 162 bp cDNA corresponding to nucleotide 1–162 of the CCNY ORF (CCNY-N probe) were used as probes for Northern hybridization analysis. Human β-actin cDNA was used as loading control. Northern blot analysis was performed according to standard laboratory manual.

2.4. Cell fractionation and Western blot analysis

Cell fractionation was performed using the Mem-PER Eukaryotic Membrane Protein Extraction Kit (Pierce) and the NE-PER Nuclear and Cytoplasmic Fractionation Kit (Pierce) according to the manufacturer's instructions. The purity of these fractions was determined using a mouse monoclonal antibody to GAPDH as a cytoplasmic marker (1/5000, Kangchen), rabbit polyclonal to Histone H3 (1/5000; Abcam) as a nuclear marker and goat mAb to TFR (1/300; Santa Cruz) as a membrane marker. Western blot analysis was performed according to standard laboratory manual.

2.5. Co-immunoprecipitation and kinase assays

Transfected 293T cells were lysed with lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 0.5% sodium deoxycholate) and complete EDTA-free protease inhibitor cocktail (Roche). Immunoprecipitations were performed using protein G (Roche) according to the manufacturer's instructions. For kinase assays, the activity of PFTK1 was determined as described [27] with some modifications. Briefly, the immunoprecipitates were washed three times with the same lysis buffer and once with the wash buffer (50 mM HEPES pH 7.5, 1 mM DTT). The 25 μl reaction mixture, containing 50 mM HEPES pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 20 μM ATP, 10 μCi γ-[^{32}P]ATP and 0.4 μg retinoblastoma protein (Rb^{C137}) (Abcam), was added to the immunoprecipitate, and the enzyme reaction was continued for 30 min at 30 °C. Reactions were stopped by the addition of SDS–PAGE loading buffer, and the samples were separated on SDS–PAGE and visualized by autoradiography.

2.6. Immunofluorescence microscopy

Transfected 293T cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed with phosphate-buffed saline (PBS) and permeabilized with 0.1% Triton X-100 in blocking solution (5% BSA in PBS) for 1 h. For immunostaining, the cells were incubated with either anti-Myc (Santa Cruz, 1:150 dilution), or anti-HA (Cell Signaling Technology, 1:150 dilution) in blocking solution for 3 h followed by incubation with secondary antibodies coupled to FITC or TRITC (Sigma, 1:400 dilution) for 1 h. To visualize nuclei, cells were incubated in 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in PBS. Coverslips were mounted and observed under Zeiss confocal microscope.

3. Results and discussion

3.1. Identification of CCNY as a PFTK1 interacting protein by yeast two-hybrid screening

Human PFTK1 was first reported as a Cdc2-related kinase highly expressed in human brain [8] and identified as a CDK involved in cell cycle regulation [3]. When we used PFTK1 as a bait to screen a human brain LexA two-hybrid library [23], a novel cyclin, named cyclin Y (CCNY) was isolated. The assignment of CCNY as a cyclin is primarily based on its structural similarity to other known cyclins. Interactions between the full length PFTK1 and CCNY were confirmed by direct yeast two-hybrid analysis (Fig. 1A).

From analysis of the primary sequence, CCNY contains a cyclin box which is an essential characteristic of cyclin family in its central region. Comparison of the cyclin box sequences in CCNY, cyclin A (CCNA) and cyclin D (CCND) showed a high similarity between these proteins (Fig. 1B). To determine whether the cyclin box is crucial for interaction with PFTK1, seven CCNY deletion mutants were constructed for the yeast two-hybrid assay. As shown in Fig. 1C, the CCNY Δ(154–195) mutant abolished the β-galactosidase activity, indicating that the central cyclin box in CCNY is crucial for the interaction with PFTK1. Besides the CCNY Δ(154–195) mutant, the interaction was also lost in the CCNY (137–341) and CCNY (1–243) mutants. The CCNY (84–341) mutant also showed a lower

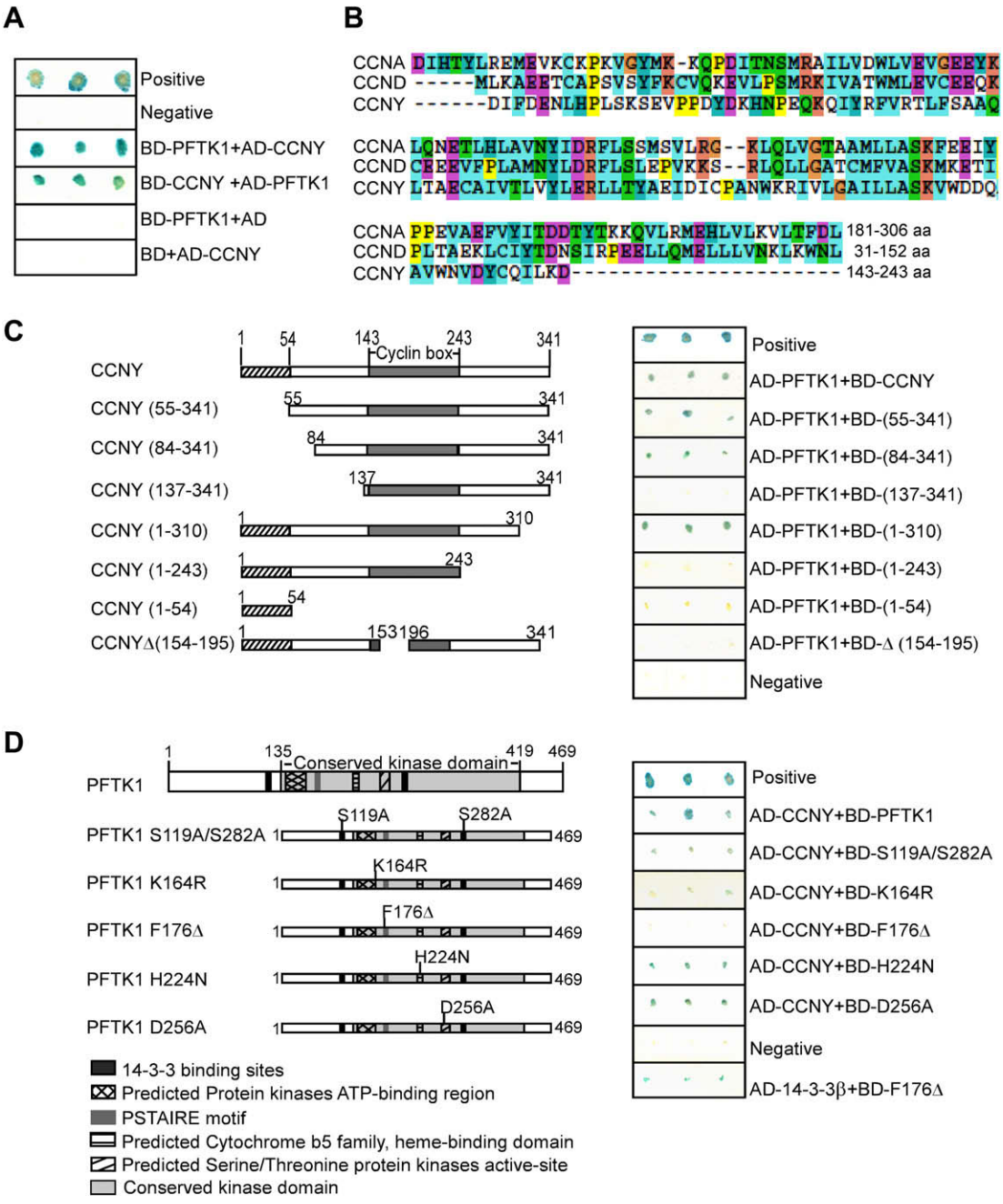

Fig. 1. Interaction between CCNY and PFTK1 in a yeast two-hybrid system. (A) Identification of CCNY as a PFTK1 interacting protein. (B) Alignment of cyclin box sequences in human CCNY, cyclin A and cyclin D. Sequence alignments were performed using ClustalX program. Members of a similar residue group are indicated in same color. (C) Mapping the binding regions on CCNY for PFTK1. (left) Schematic representation of CCNY deletion. (right) Two-hybrid assay. (D) Mapping the CCNY binding sites on PFTK1. (left) Schematic representation of PFTK1 mutants. (right) Two-hybrid assay. The motifs and domains are predicted by ScanProsite (http://www.expasy.ch/tools/scanprosite/). The results are representative of 2–4 independent experiments.

β-galactosidase activity in two-hybrid assay. Taken together, we conclude that both the cyclin box domain and regions flanking the cyclin box are required for the interaction with PFTK1 (Fig. 1C).

PFTK1 contains many predicted domains and motifs conserved in protein kinases, including ATP binding region, Ser/Thr kinase signature, etc. [23]. To further map the CCNY-interacting region of PFTK1, we constructed a series of PFTK1 mutants on these predicted domains and motifs. Besides the PFTK1 F176Δ mutant, all the mutants showed β-galactosidase activity (Fig. 1D). The K164R substitution occasionally showed no β-galactosidase activity in repeated experiments, suggesting that the putative ATP binding site K164 may contribute to the binding of CCNY. The PSTAIRE motif in many CDKs is the primary site of intermolecular recognition in cyclin binding [2]. The motif is also a specific binding site in CCNY binding since the deletion of the phenylalanine in the PFTAIRE motif (PFTK1 F176Δ) prevents the binding of CCNY but has no effect on the binding of 14-3-3 proteins (Fig. 1D). Taken together, our data demonstrate an interaction between PFTK1 and CCNY. Both the cyclin box in CCNY and the PFTAIRE motif in PFTK1 are required for the interaction, consisting with the features for specific binding between cyclins and CDKs.

3.2. Two transcripts of CCNY are expressed in tissues and cell lines

CCNY is a 341 amino acid protein. The cDNA sequence encoding for CCNY was submitted to GenBank with the accession number

AY504868 (this sequence was replaced by GI: 71658801, Q8ND76). By BLAST searching the up-to-date human genome database (NCBI), the *CCNY* gene has been mapped to human chromosome 10p11.21, flanked by *CREM* gene and *GJD4* gene.

To determine tissue distribution of the *CCNY* transcripts, Northern blot hybridization was performed on poly(A) RNA isolated from 16 types of adult human tissues, with a CCNY probe. Two transcripts of 4 and 2 kb were detected (Fig. 2A). The 4 kb transcript was relatively ubiquitously expressed, being detected at low levels in all the tissues examined. The 2 kb transcript was more restricted in its expression, being detected at very high level in testis, medium levels in heart and skeletal muscle, and low levels in the other tissues, except in lung and thymus where the 2 kb transcript was hardly detected. A weak hybridization band about 4.5 kb was detected in testis, ovary and brain which may reflect more alternatively spliced *CCNY* transcripts existing in these tissues.

To examine the transcriptional levels of *CCNY* in cell lines, we extracted total RNA from 10 types of cell lines for Northern blot analysis, using the CCNY probe for hybridization. As expected, two transcripts of 4 and 2 kb were observed (Fig. 2B). The 4 kb transcript was detected at low levels in all cell lines examined, except in HepG2, where its expression level is higher than the others. The 2 kb transcript was expressed at low level in the cell lines, but abundant in HepG2. Besides the 4 and 2 kb transcripts, we also observed a weak hybridization band about 3.8 kb in A431, HepG2, B16 and NIH3T3, suggesting that the *CCNY* may have different spliced forms in these cell lines.

From the database, two of *CCNY* isoforms representing alternatively spliced CCNY transcripts are released. The one we isolated encodes a 341 aa protein, another one encoding for a protein of 287 residues which was reported by Li et al. named as cyclin X [28]. The two proteins differ in their N-terminal sequences. The CCNY has a 54 amino acid extension at its N-terminus and is identical to the cyclin X through the rest of coding region. To determine whether *cyclin Y* (*CCNY*) and *cyclin X* representing the 4 and 2 kb transcripts, respectively, we performed Northern blot analysis by using CCNY-N probe, which is specific for *CCNY* (Fig. 2C). Surprisingly, both the 4 and 2 kb transcripts could be hybridized with the CCNY-N probe, and showed a similar hybridization pattern given by the CCNY probe. Combining our data and the information from database suggests that the *CCNY* could be mainly transcribed into two different transcripts, the *cyclin X* should be a shorter splicing form of *CCNY* and other alternatively spliced *CCNY* transcripts could also exist in different tissues or cell lines.

3.3. Ectopic expressed CCNY enriches at plasma membrane

To examine the cellular distribution of CCNY, CCNY was fused with a C-terminal GFP tag (CCNY-GFP), expressed in 293T cells and observed under a fluorescence microscope. The results showed that CCNY-GFP was localized to the plasma membrane (Fig. 3B). A similar distribution was observed in ECV304, NIH3T3 and SHSY-5Y cells (data not show). Fig. 3A shows an N-terminal myristoylation signal motif exists in CCNY which is highly conserved in *Mus musculus*, *Rattus norvegicus*, *Xenopus laevis*, *Danio rerio*, *Drosophila pseudoobscura* and *Caenorhabditis elegans* CCNY orthologues. To determine whether this myristoylation signal is essential for targeting CCNY to the membrane, the conserved glycine at position 2 was mutated to alanine. The G2A mutant protein showed a diffuse cytoplasmic distribution, with stronger nuclear accumulation (Fig. 3B) contrasting to wild type CCNY which is associated with the plasma membrane. As a control, the N3A mutant in which the third asparagine residue was substituted with alanine was retained at the plasma membrane just like the wild type CCNY. In

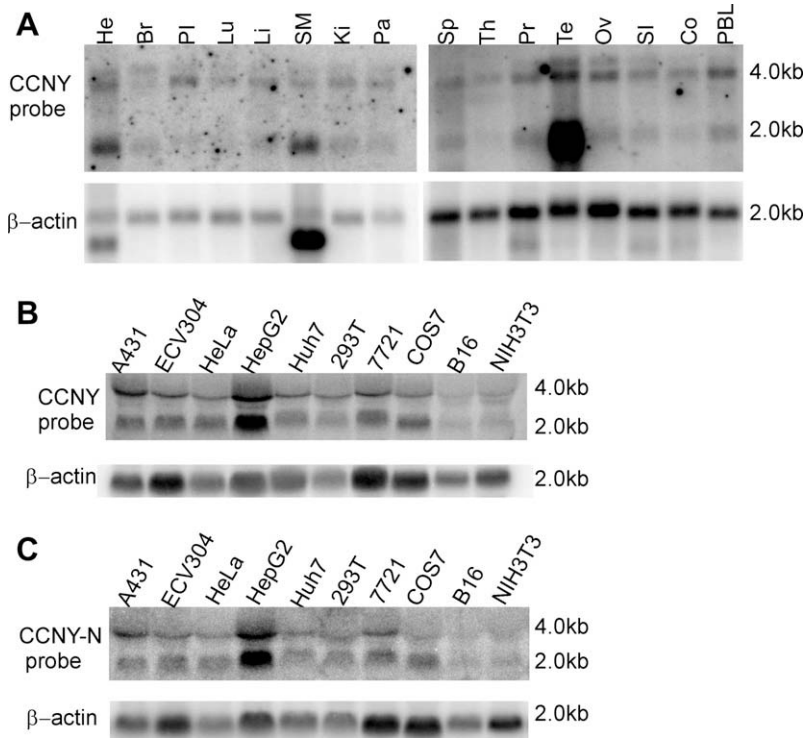

Fig. 2. Expression pattern of CCNY mRNA in human tissues and cell lines. (A) Northern blot analysis of poly(A) + RNAs from various human tissues. Northern blots were hybridized with the CCNY probe (upper). The membranes were stripped and reprobed with a human β-actin probe (lower). He: heart; Br: brain; Pl: placenta; Lu: lung; Li: liver; SM: skeleton muscle; Ki: kidney; Pa: pancreas; Sp: spleen; Th: thymus; Pr: prostate; Te: testis; Ov: ovary; SI: small intestine; Co: colon; PBL: peripheral blood leukocyte. The image for CCNY was obtained after 7 days exposure and for β-actin was 24 h exposure. (B) Northern blot analysis of total RNAs from various cell lines. Northern blots were hybridized with the CCNY probe or the CCNY-N probe. The β-actin probe was used as a loading control.

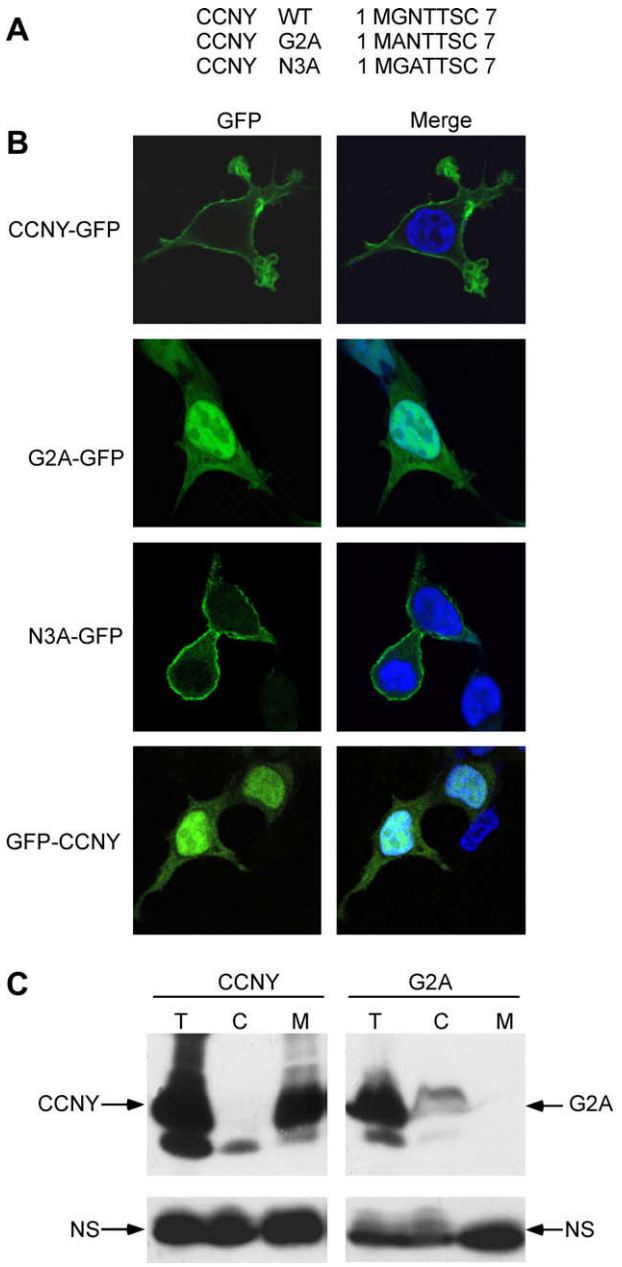

Fig. 3. Cellular localization of CCNY and CCNY mutants. (A) N-terminal sequences of wild-type CCNY, G2A mutant and N3A mutant. CCNY G2A represents a substitution of second glycine residue with alanine, Gly → Ala. CCNY N3A represents a substitution of third asparagine residue with alanine, Asn → Ala. (B) The second Gly is required for membrane localization of CCNY. The pEGFPN3-CCNY (CCNY-GFP), pEGFPN3-CCNY-G2A (G2A-GFP), pEGFPN3-CCNY-N3A (N3A-GFP) and pEGFPC2-CCNY (GFP-CCNY) constructs were separately transfected into 293T cells for observation of the cellular localization. (C) Subcellular distribution of CCNY and G2A in fractionated 293T cell lysates after transfection. Total protein (T), membrane (M) and cytosol/nuclear (C) fractions were analyzed by Western blot analysis with anti-GFP antibody. NS: non-specific bands.

addition, the N-terminal GFP tagged CCNY (GFP-CCNY) also showed nuclear localization which was in good agreement with the fact that in this case the N-terminal myristoylation site was inactivated by the fusion tag (Fig. 3B).

We further examined the intracellular distribution of CCNY by subcellular fractionation. Wild type CCNY (CCNY-GFP) was more abundant in the membrane fraction. In contrast, the CCNY G2A mutant (G2A-GFP) was enriched in the cytosolic/nuclear fraction (Fig. 3C). The results of the fractionation experiment agree well with these localization patterns shown in Fig. 3B. These results indicate that CCNY is also targeted to the membrane by N-myristoylation, in a similar manner to p35 and p39 [20].

3.4. CCNY recruits PFTK1 to the plasma membrane

CCNY is a PFTK1 interacting protein (Figs. 1 and 5). However, the two interacting proteins seem to be located within different subcellular compartments. Ectopic expressed PFTK1 mainly localized in cytoplasm while ectopic expressed CCNY localized in the plasma membrane (Figs. 3B and 4C). Overexpression of proteins may cause them mislocalization, we decided to investigate whether endogenous CCNY and PFTK1 are located in different subcellular compartments. We were unable to detect the endogenous CCNY and PFTK1 proteins by immunofluorescence in any cell types studied presumably because of limited sensitivity of the antibodies available. Therefore, we used subcellular fractionation of mouse testis that had high CCNY and PFTK1 mRNA levels to examine their localization. The results showed that both CCNY and PFTK1 were predominantly localized in the membrane and cytosol fractions in mouse testis, with a small amount detected in the nuclear fraction (Fig. 4B).

CCNY alone could bind to the membrane (Fig. 4C), while PFTK1 alone does not show a specific distribution in the membrane (Fig. 4C). When PFTK1 co-expressed with CCNY-Myc, the two proteins are co-localized in the membrane (Fig. 4D), indicating that the CCNY determines the membrane localization of PFTK1.

We noticed that N-terminal HA tagged CCNY (HA-CCNY) showed predominantly nuclear staining with some cytoplasmic staining (Fig. 4C). There was reduced nuclear expression of HA-CCNY compared with GFP-CCNY. Thus the remarkable nuclear localization of GFP-CCNY might be an artifact caused by GFP tag (Fig. 3B). When we co-expressed GFP-PFTK1 with HA-CCNY in 293T cells, HA-CCNY could not direct GFP-PFTK1 to the nucleus, in contrast, the two proteins were co-localized in cytoplasm (Fig. 4D). The results suggested that the CCNY might direct PFTK1 to the plasma membrane, once released from the membrane, CCNY could still co-localize with PFTK1 in the cytoplasm.

3.5. Binding of CCNY to PFTK1 enhances the PFTK1 kinase activity

Interaction between the PFTK1 and CCNY was proved in the yeast two-hybrid assay, and provided the first evidence that CCNY is a regulatory partner of the PFTK1. To examine the endogenous association between CCNY and PFTK1, we used a rabbit polyclonal anti-PFTK1 antibody to immunoprecipitate PFTK1 from mouse brain. We could readily detect CCNY in the PFTK1 immunoprecipitates but could not detect any endogenous CCNY in the control rabbit IgG precipitates, indicating that the two proteins indeed interact each other in vivo (Fig. 5A). The result was consistent with the yeast two-hybrid data. We further analyzed the interaction between PFTK1 and the membrane-associated form or cytoplasmic form of CCNY by co-immunoprecipitation analysis. The co-IP experiments showed that both CCNY-Myc (Fig. 5B) and HA-CCNY (Fig. 5C) could specifically bind to PFTK1 but not to PFTK1 F176Δ mutant, demonstrating that the CCNY interacts with PFTK1 independently of its cytoplasmic or membrane-associated form.

We have showed that CCNY could recruit PFTK1 to the plasma membrane and regulate the subcellular distribution of PFTK1. The cellular localization of proteins is closely related to their functions. Therefore, we tried to find the different substrates of PFTK1–CCNY complex corresponding to their subcellular localizations. Firstly, we found that the membrane-associated CCNY was phosphorylated. The exogenously expressed CCNY-Myc showed a hybridization band with anti-Phospho-Serine antibody (Fig. 5D), but not with anti-Phospho-Threonine or anti-Phospho-Tyrosine

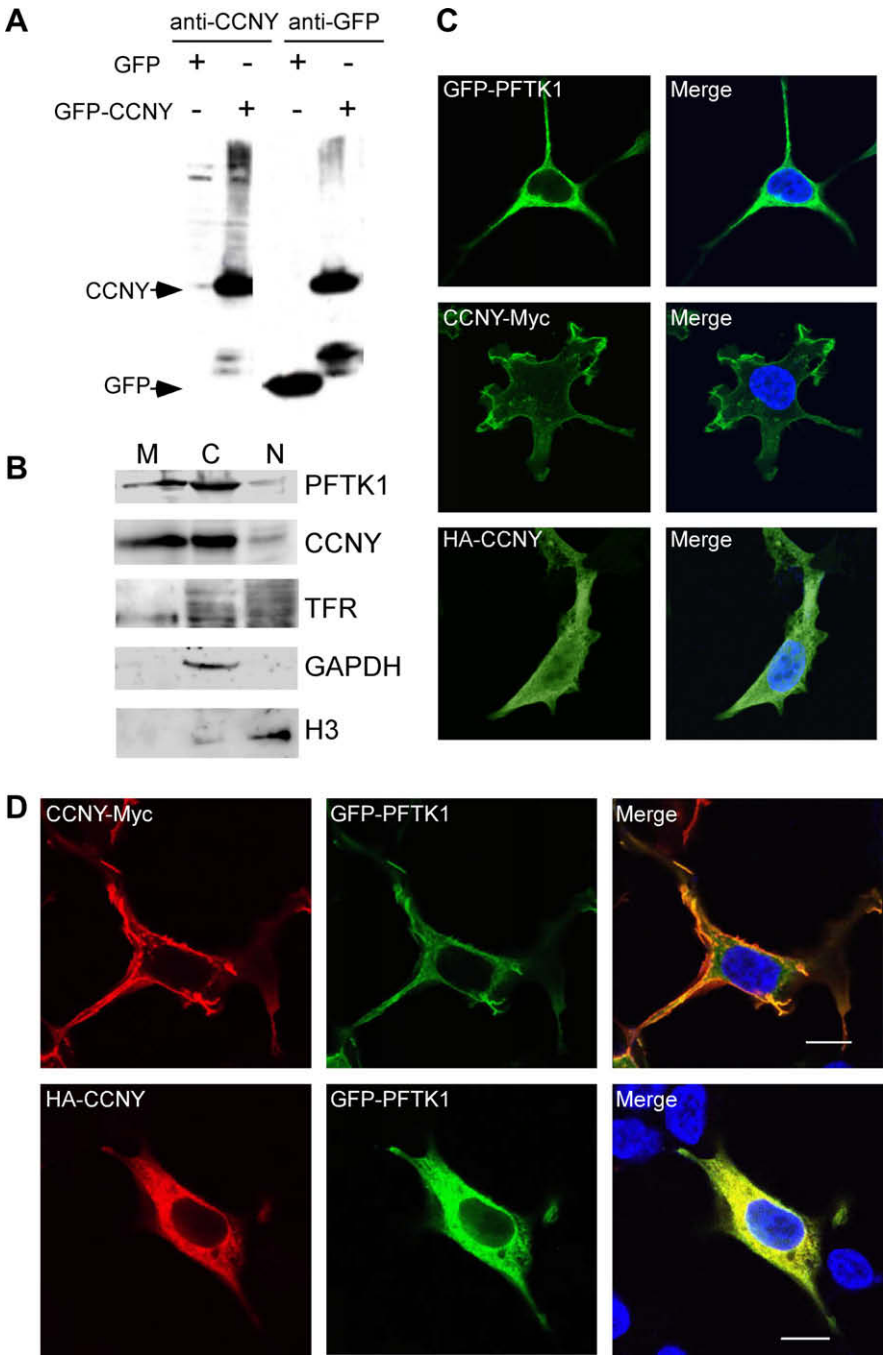

Fig. 4. Intracellular localization of CCNY–PFTK1 complex. (A) Characterization of a polyclonal rabbit antibody against full length CCNY. The 293T cells were transfected with the plasmid of pEGFPC2-CCNY or pEGFPC2. Cell lysates were subjected to 8% SDS–PAGE and blotted with rabbit polyclonal anti-CCNY antibody or with anti-GFP antibody (Santa Cruz) as a control. The anti-GFP antibody (Santa Cruz) recognized both the proteins, whereas the anti-CCNY antibody specifically recognized CCNY but not GFP. (B) Subcellular localization of endogenous CCNY and PFTK1 in fractionated testis. The mouse testis extracts were fractionated into membrane (M), cytosolic (C) and nuclear (N) fractions. The fractions were analyzed by Western blot. (C) Subcellular localization of exogenous CCNY and PFTK1 in 293T cells. 293T cells were transiently transfected with pMycN3-CCNY (CCNY-Myc), pcDNA3-3HA-CCNY (HA-CCNY) and pEGFPC2-PFTK1 (GFP-PFTK1), respectively for indirect immunofluorescence analysis. (D) Co-localization of exogenous PFTK1 and CCNY in 293T cells. Bars represent 10 μm.

antibody (data not shown), suggesting that the membrane-associated CCNY was phosphorylated at serine residue(s). The serine-phosphorylation was only observed in membrane-associated CCNY, but not in cytoplasmic CCNY (HA-CCNY) nor nuclear CCNY (CCNY-G2A-Myc) (data not shown), suggesting that the CCNY could be phosphorylated in membrane. PFTK1 might be required for the phosphorylation of CCNY, since the phosphorylation was enhanced by co-expression with PFTK1 (Fig. 5D). To demonstrate the phosphorylation of CCNY is catalyzed by PFTK1, the CCNY was co-expressed with PFTK1 F176Δ mutant (CCNY binding site) or PFTK1 K164R mutant (Predicted kinase ATP binding site). The expression of PFTK1 F176Δ or K164R did not increase CCNY phosphorylation level (data not shown). The results suggest that PFTK1 is involved in regulation of CCNY phosphorylation in membrane, although we cannot rule out the possibility that CCNY is also phosphorylated by other protein kinases. To confirm the

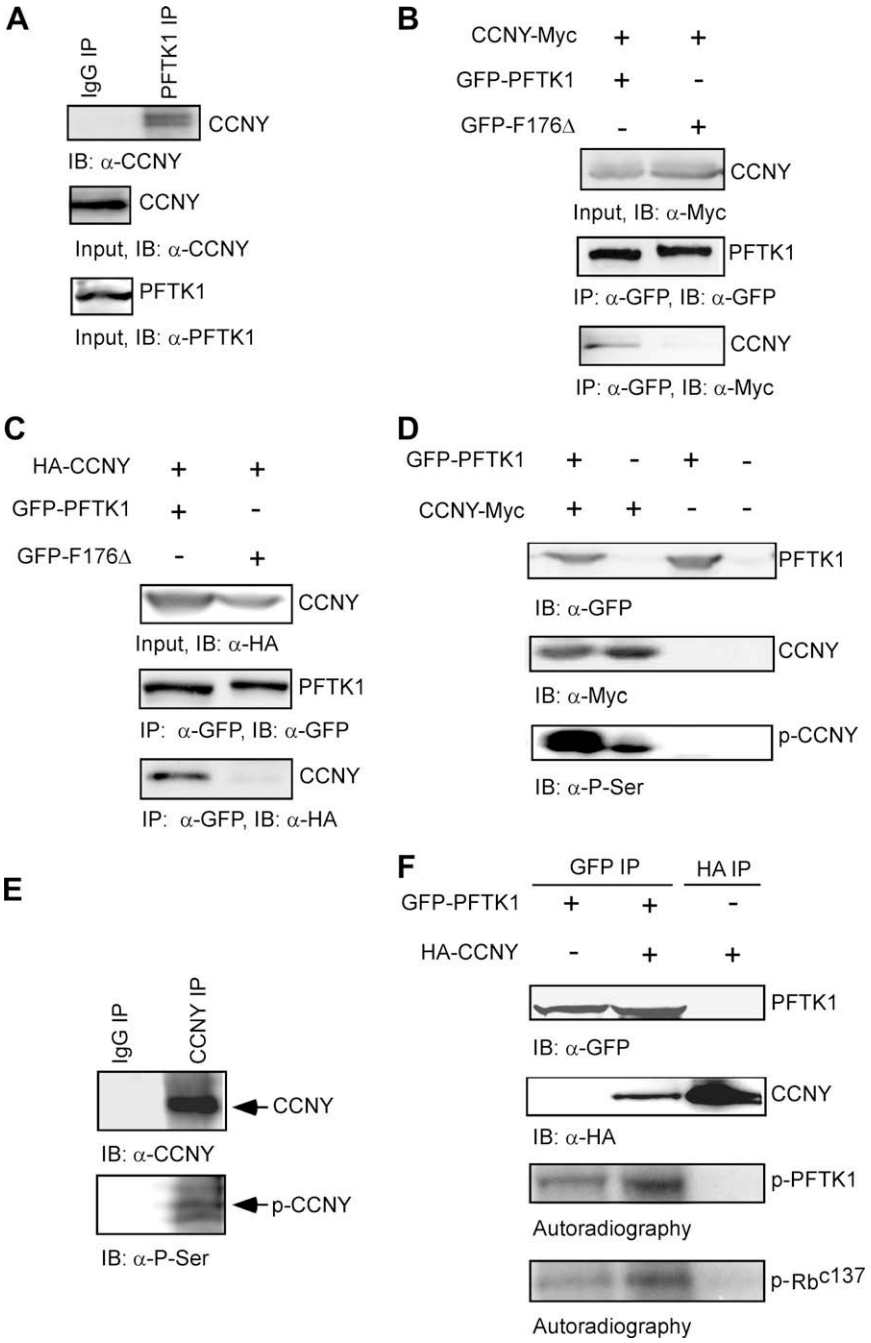

Fig. 5. CCNY interacts with PFTK1 in vivo and enhances its kinase activity. (A) Endogenous interaction between CCNY and PFTK1 in mouse brain. Lysates from mouse brain were immunoprecipitated using rabbit polyclonal anti-PFTK1 or rabbit IgG as a control. The immunoprecipitates were blotted with anti-CCNY. (B) Co-immunoprecipitation of CCNY-Myc and GFP-PFTK in 293T cells. (C) Co-immunoprecipitation of HA-CCNY and GFP-PFTK1 in 293T cells. (D) Enhanced phosphorylation of CCNY through its association with PFTK1. The 293T cells transfected with indicated constructs were harvested. Equal amounts of cell lysates were subsequently blotted with anti-GFP, anti-Myc or anti-phosphoserine antibody. Untransfected 293T cells were used as a control. (E) CCNY is phosphorylated at serine residue(s) in vivo. Lysates from the mouse brain were prepared and immunoprecipitated by anti-CCNY antibody or IgG as a control. The immunoprecipitates were blotted with anti-CCNY antibody or anti-phosphoserine (anti-P-Ser). (F) Kinase assay of PFTK1 from cytoplasm fraction. The 293T cells expressed with GFP-PFTK1 and HA-CCNY both or separately were harvested. One half of the immunoprecipitants was used for blotting with anti-GFP or anti-HA (upper). The other half was used for kinase assay (lower).

phosphorylation of CCNY in vivo, immunoprecipitates from mouse brain by anti-CCNY antibody were blotted with anti-CCNY antibody or anti-phosphoserine. Hybridization band was observed with both anti-CCNY and anti-phosphoserine antibodies (Fig. 5E), consistent with the results from exogenously expressed CCNY-Myc. Next, we examined whether the PFTK1 kinase activity is regulated by CCNY in the cytoplasm. Consistent with previous reports [3], both the autophosphorylation of PFTK1 and phosphorylation of Rb^{C137} could be detected. These phosphorylations were both slightly enhanced in the presence of HA-CCNY (Fig. 5F). Although histone H1 and myelin basic protein have also been examined as substrates, neither of them was found to be phosphorylated by the CCNY–PFTK1 complex (data not shown). Taken together, we suggest that the CCNY is a regulatory partner of the PFTK1, as binding of CCNY to the PFTK1 activates PFTK1, changes its cellular distribution and may also alter its substrate specificity.

Acknowledgements

We thank Dr. Lan Bao for kindly providing pMycN3. This work was supported by the Chinese National Natural Science Foundation (30830003) and Chinese 863 grant 2006AA02Z178.

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