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Original Article

14-3-3 Binding to Cyclin Y contributes to cyclin Y/CDK14 association

Shan Li, Mei Jiang, Wenjuan Wang, and Jiangye Chen*

Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
*Correspondence address. Tel: +86-21-54921251; Fax: +86-21-54921011; E-mail: jychen@sibcb.ac.cn

Cyclin Y is a highly conserved cyclin among eumetazoans, yet its function and regulation are poorly understood. To search for Cyclin Y-interacting proteins, we screened a yeast two-hybrid library using human Cyclin Y (CCNY) as a bait and identified the following interactors: CDK14 and four members of the 14-3-3 family (ε, β, η, τ). The interaction between CCNY and 14-3-3 proteins was confirmed both *in vitro* and *in vivo*. The results showed that Ser-100 and Ser-326 residues in CCNY were crucial for 14-3-3 binding. Interestingly, binding of CCNY to 14-3-3 significantly enhanced the association between CCNY and CDK14. Our findings may add a new layer of regulation of CCNY binding to its kinase partner.

Keywords cyclin Y; 14-3-3 protein; 14-3-3 binding motif

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Introduction

Cyclin Y is a recently characterized member of the cyclin family. It is highly conserved among eumetazoan species [1], suggesting that Cyclin Y has important biological functions. In 2010, Liu *et al.* [2] found that Cyclin Y is essential for several developmental processes in *Drosophila*. Moreover, *cyclin Y* null mutant is homozygous lethal with most mutant animals arresting during pupal development. At the same time, another group reported that Cyclin Y is necessary for targeting presynaptic components to the axon in *Caenorhabditis elegans* [3]. An intriguing finding is that Cyclin Y is required *in vivo* for LRP6 phosphorylation, maternal Wnt signaling, and Wnt-dependent anteroposterior embryonic patterning in *Xenopus* [4].

The 14-3-3 proteins are a family of small acidic, dimeric proteins that appear to be present in all eukaryotic organisms. There are seven 14-3-3 isoforms in mammals: β, γ, ε, η, σ, τ, and ζ. All 14-3-3 proteins are highly conserved, with ~50% identity among amino acids both within and across species [5]. Although the 14-3-3 proteins have no detectable catalytic domain [6], they are involved in the regulation of almost all biological processes via regulation of hundreds of interacting proteins. The vast majority of cellular-binding partners interact with 14-3-3 proteins in a phosphorylation-dependent manner [7]. Two canonical 14-3-3 binding motifs have been defined as RSXpSXP and RXXXpSXP (where R is arginine, S is serine, X is any amino acid, pS is phosphoserine, and P is proline). Phosphothreonine (pT) could be replaced with pS [8,9]. The different regulatory role of 14-3-3 proteins can be grouped into three major modes of action [5,10,11]: (i) Directed conformational changes in the target protein. The highly α-helical nature of the 14-3-3 proteins is rigid, which upon binding to target protein leads to deformation of the target protein with little or no change in the structure of the 14-3-3 dimer [11]. By this way, 14-3-3 binding can alter the intrinsic catalytic activity of the target protein, either inhibiting or augmenting its function; (ii) Masking. 14-3-3 Proteins act as masks to physically occlude sequence-specific or structural features of their targets, thus to modify the target protein localization [12] or to protect the target from other modifications such as dephosphorylation [13]; (iii) Scaffolding. 14-3-3 Dimer can bridge two proteins together and serve as a phosphorylation-dependent scaffold protein [14].

Human Cyclin Y (CCNY) protein is composed of 341 amino acids, with the 143–249 amino acid residues forming a 'cyclin fold', a stack of five α-helices termed the cyclin box [1,15]. Conventional cyclins require two cyclin folds for their activity; it is unknown how CCNY binds to and activates its kinase partner with only a single cyclin fold [1,15]. We have previously reported that the interaction between CCNY and its kinase partner CDK14 requires the typical CDK-cyclin-binding domains, the 'PFTAIRE' motif in CDK14, and the cyclin-fold domain in CCNY. In addition, it also requires additional regions outside the cyclin box (85–136 aa and 244–341 aa) [16], implying the involvement of other regulatory mechanisms. Data in the present paper demonstrated that the binding of 14-3-3 proteins to CCNY enhanced the association between CCNY and CDK14, adding a new layer of regulation of CCNY binding to its kinase partner.

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Materials and Methods

Yeast two-hybrid assay

The yeast two-hybrid screening and yeast two-hybrid interaction assays were carried out as described previously [17,18]. A human adult brain LexA two-hybrid complementary DNA (cDNA) library (Clontech, Mountain View, USA) in which cDNA fragments inserted in pB42AD were used for two-hybrid screening. pGilda-CCNY (LexA−CCNY fusion) was used as a bait to screen the library. *Saccharomyces cerevisiae* strains used were EGY48 (*MATα*, *trp1*, *ura3*, *his3*, *leu2*, *lexA*$_{op(\times 6)}$*-leu2*) and CJY151 (*MATα*, *his3*, *trp1*, *LexA*$_{op(\times 6)}$*-leu2*, *LexA*$_{op(\times 8)}$*-lacZ*), which was generated by integrating the lacZ reporter and its upstream modulation sequence to the strain EGY48. The pGilda was used for the expression of LexA-tagged fusion proteins. The pB42AD was used for the expression of HA fusion proteins with the B42 activation domain under the control of *GAL1* promoter. The pSH17-4 was a positive control plasmid for LexA−AD fusion protein. The pRFHM1 was a negative control plasmid for expression of a LexA−human lamin C fusion protein. Filter assays for β-galactosidase activity were performed as described previously [19]. X-gal was used as a substrate. Colonies (2 mm diameter) on filters were frozen with liquid nitrogen for 1 min, then incubated with X-gal (334 μg/ml) at 30°C.

Plasmid construction

For over-expression in HEK-293T cells, human CCNY open reading frame (ORF) was cloned into a pMYCN3 or pEGFPN3 vector, where a C-terminal Myc-tag or GFP-tag was fused to CCNY [20]. Human CDK14 ORF was cloned into a pEGFPN3 vector. Site-directed mutagenesis of CCNY was generated by polymerization chain reaction amplification using a mutagenesis kit (Strategene, Wilmington, USA). Human 14-3-3ε, β, η, or τ ORF was cloned into a pcDNA3.1-N-Flag vector.

Cell culture

All cells in this study were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, USA.) and penicillin/streptomycin at 37°C and 5% CO_2. Cells were transfected by using Lipofectamine^{TM} 2000 (Invitrogen) according to manufacturer's instruction. An equal amount of empty vectors was used as control in all experiments.

Co-immunoprecipitation and immunoblotting

Transiently transfected HEK-293T cells were collected 24 h post-transfection, and then lysed with 1%-Trition lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, complete protease inhibitor cocktail (Roche, Basel, Switzerland), and PhosSTOP phosphatase inhibitor (Roche)]. The same amount of total proteins was incubated with anti-Myc beads for 3 h at 4°C. After washing, the bound proteins were eluted by boiling the beads and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Antibodies used in the present study were anti-14-3-3β (SC-25276; Santa Cruz, Santa Cruz, USA); anti-FLAG (A2220; Sigma, St Louis, USA); anti-C-MYC (C3956; Sigma); and anti-GFP (11814460001; Roche).

Subcellular localization

Transfected HEK-293T cells were plated at a low confluency close to 30% on coverslips in a 12-well plate. Twenty-four hours later, the cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min at room temperature, and then washed with PBS. To visualize nuclei, the cells were stained with 1 mg/ml 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min, and then washed with PBS. Coverslips were mounted and observed under a Zeiss confocal microscope.

Results

CCNY interacts with 14-3-3 proteins *in vitro* and *in vivo*

To identify CCNY-interacting proteins, a full-length human CCNY was used as a bait to screen a human brain Lex-A two-hybrid library. In addition to CDK14, which has been shown to be a kinase partner activated by CCNY, four 14-3-3 isoforms (ε, β, η, τ) were also identified. Their interactions were further validated by direct yeast two-hybrid analysis. The yeast CJY151 co-transformed with pGilda-CCNY and pB42AD-14-3-3 plasmids grew well on SC Gal His$^-$, Trp$^-$, Leu$^-$ medium and showed β-galactosidase activity in 4-h incubation at 30°C, confirming the CCNY-14-3-3 interaction *in vitro* (**Fig. 1A**). To further verify the physical interaction of these proteins *in vivo*, a co-IP assay was performed by using mouse brain extracts. The mouse brain extracts were immunoprecipitated by an anti-14-3-3β antibody, and the presence of CCNY was investigated by western blotting. CCNY was clearly detected in the 14-3-3β immunoprecipitant, but not in that of IgG control (**Fig. 1B**), confirming that CCNY interacted with 14-3-3 proteins *in vivo*.

Both N- and C-terminal regions of CCNY are required for 14-3-3 binding

CCNY contains a single cyclin box domain (143−243 aa), which is required for binding to CDK14, and long N- and C-terminal regions outside the cyclin box domain. To investigate the regions of CCNY that are necessary for interaction with 14-3-3 proteins, we constructed various deletion mutants of pGilda-CCNY for interaction analysis between CCNY and 14-3-3 proteins using a yeast two-hybrid system (**Fig. 2A**). The yeast strains harboring CCNY or CCNY mutants with 14-3-3β were incubated on leucine containing

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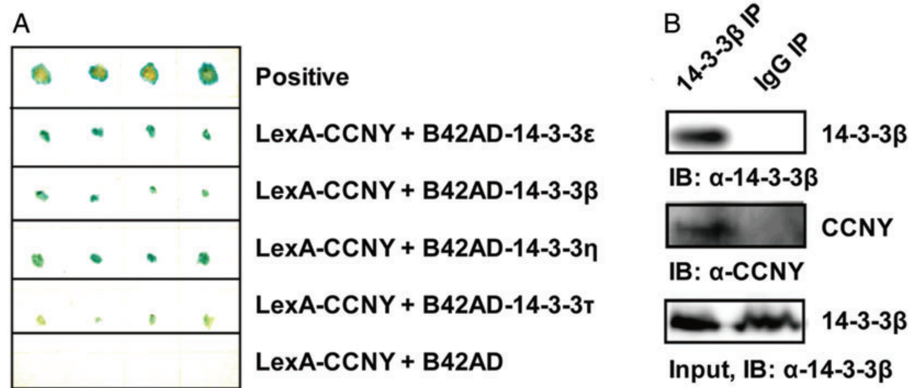

Figure 1. CCNY interacts with 14-3-3 proteins (A) Interaction between CCNY and four 14-3-3 isoforms (ε, β, η, τ) in a yeast two-hybrid system. The pGilda-CCNY and pB42AD-14-3-3 plasmids were co-transformed into CJY151. pSH17-4 containing LexA-AD fusion protein was used as a positive control. The interactions were analyzed using a β-galactosidase activity assay on SC Gal His^-, Trp^-, Leu^- media. (B) Interaction of CCNY with 14-3-3β *in vivo*. Lysates from mouse brain were prepared and immunoprecipitated by mouse monoclonal anti-14-3-3β or IgG as a control. The immunoprecipitates were blotted with an anti-14-3-3β antibody or anti-CCNY antibody. The mouse brain lysates were probed with an anti-14-3-3β antibody as a loading control.

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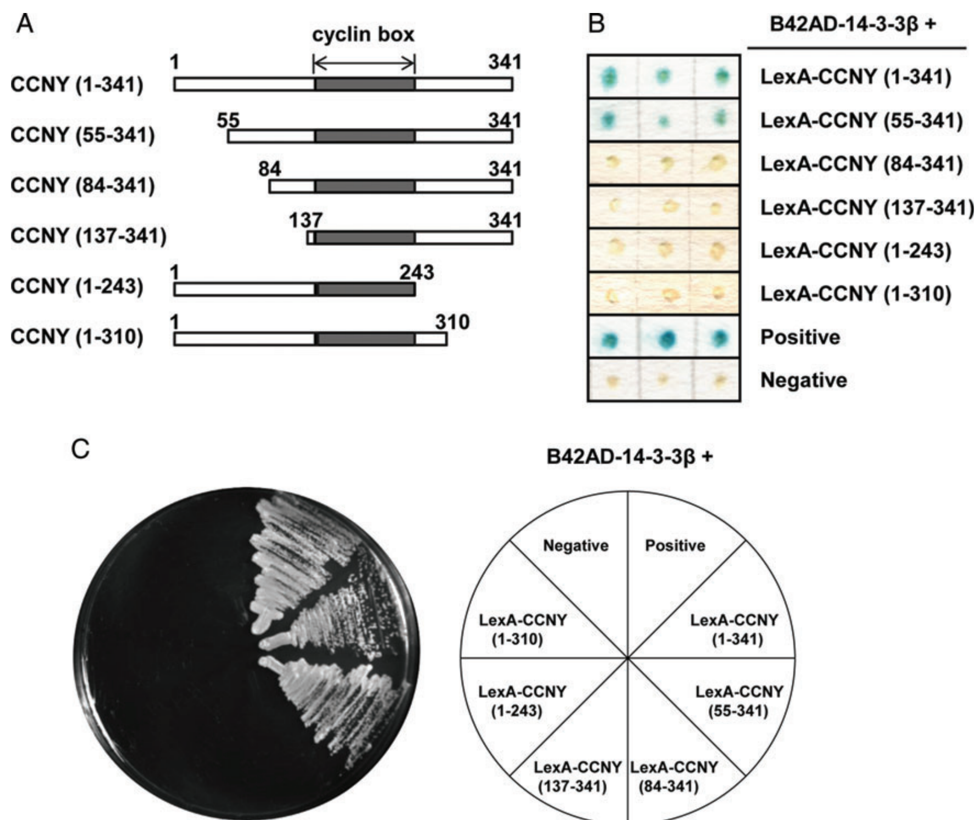

Figure 2. Mapping regions of CCNY that interact with 14-3-3 proteins (A) Schematic representation of CCNY deletion. (B) Interactions of CCNY deletion mutants with 14-3-3β in a yeast two-hybrid system. The pGilda-CCNY deletion mutants were co-transformed with pB42AD-14-3-3β into CJY151. The protein–protein interactions were analyzed using a β-galactosidase activity assay on SC Gal His^-, Trp^- media. (C) Interactions between CCNY deletion mutants and 14-3-3β were analyzed using a growth ability assay. The yeast strains shown in (B) were streaked onto SC Gal His^-, Trp^- Leu^- plates and incubated at 30°C for 3 days. Plasmid pSH17-4 containing a LexA–AD fusion was used as a positive control and pRFHM1 containing a LexA–human lamin C fusion was used as a negative control.

media for β-galactosidase activity assay. Results showed that CCNY lacking 1–54 aa retained the ability to interact with 14-3-3β, but CCNY lacking 1–83 aa was unable to interact with 14-3-3β, suggesting that the 55–83 amino acid residues were required for 14-3-3 binding. Furthermore, CCNY lacking 311–341 aa in the C-terminus did not interact with 14-3-3β either, indicating that this region was also required for 14-3-3 binding (**Fig. 2B**). We further examined the growth abilities of the yeast strains on leucine-depleting media. Interaction between CCNY and 14-3-3β resulted in

growth of the yeast strains on Leu^{-} media. The growth ability assay confirmed the results of β-galactosidase activity assay (**Fig. 2C**). These data indicated that both N- and C-terminal regions outside the cyclin box were required for the interaction between CCNY and 14-3-3 proteins.

The Ser-100 and Ser-326 are crucial for the interaction between CCNY and 14-3-3 proteins

The 14-3-3 proteins mostly bind to their ligands through pS/pT motifs with a few exceptions [21]. We performed a co-IP assay to examine whether the interaction between CCNY and 14-3-3 proteins is phosphorylation-dependent. As shown in **Fig. 3A**, when the cell lysates were pre-treated with lambda protein phosphatase, Flag-14-3-3β was hardly pulled down by CCNY-Myc (**Fig. 3A**), indicating that dephosphorylation would abolish 14-3-3 binding to CCNY. We then searched the N- and C-terminal regions of CCNY for 14-3-3 binding sites, and found four putative 14-3-3 binding sites at Thr-67, Ser-83, Ser-100, and Ser-326 (**Fig. 3B**) based on the two canonical 14-3-3 binding phosphopeptide motifs: RSXpSXP and RXXXpSXP.

To determine whether these predicted residues can provide docking sites for 14-3-3 proteins, we mutated the Thr and/or Ser residues to non-phosphorylatable Ala. Co-IP assay and western blotting revealed that T67A and S83A mutations did not affect the association of CCNY with 14-3-3β. However, the mutation S100A or S326A greatly reduced the association of CCNY with 14-3-3β. Double mutation of Ser-100 and Ser-326 completely abolished 14-3-3 binding with CCNY (**Fig. 3C**), suggesting that the phosphorylation of CCNY at Ser-100 and Ser-326 was critical for 14-3-3 binding. Phosphorylation at Ser326 has been detected in both nuclear and cytoplasmic fractions [22,23]. Our unpublished data also showed that Ser-100 and Ser-326 of CCNY were phosphorylated. Sequence alignment showed that the residues Ser-100 and Ser-326 were highly conserved among eumetazoans, from hydra to human (**Fig. 3D**), suggesting that the interaction between CCNY and 14-3-3 proteins was evolutionary conserved.

Binding to 14-3-3 does not alter membrane localization of CCNY

We next investigated the regulatory role of 14-3-3 binding in CCNY. In some cases, 14-3-3 proteins are implicated in regulating the subcellular localization of their target proteins by acting as a mask [6]. Ectopically expressed CCNY is enriched at the plasma membrane due to myristoylation at N-terminal Gly-2 [16]. We investigated whether 14-3-3

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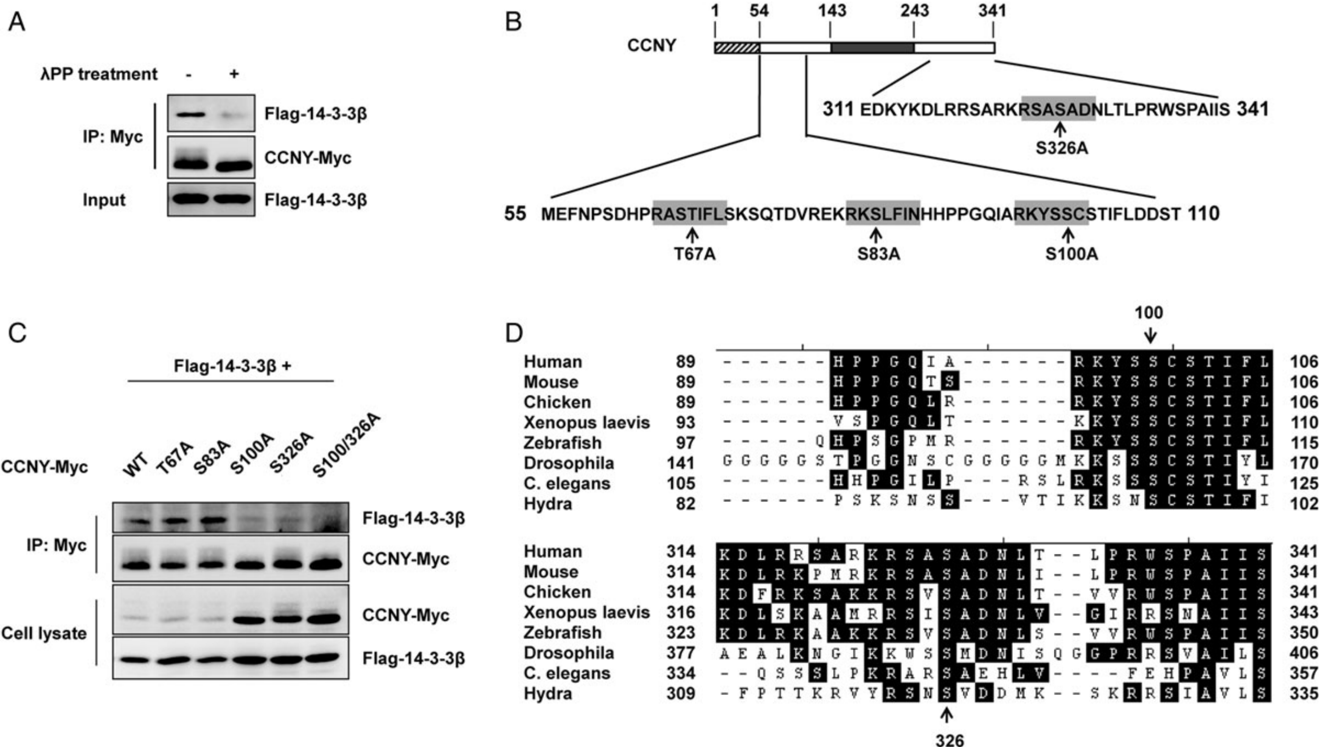

Figure 3. Identification of 14-3-3 binding sites on CCNY (A) 14-3-3β associated with phosphorylated CCNY. CCNY-Myc and Flag-14-3-3β were co-transfected in HEK-293T cells. Cell lysates were pre-treated with or without lambda protein phosphatase for co-IP assays. (B) Prediction of 14-3-3 binding sites on CCNY. (C) Co-IP assays of the interactions between CCNY mutants and 14-3-3β. (D) Sequence alignment of the two 14-3-3 binding sites across different eumetazoan species. DNA STAR Lasergene MegAlign V 7.1.0 was used for sequence alignments, and identical amino acids are highlighted in black.

binding will regulate the membrane localization of CCNY. The following plasmids were transfected: wild-type CCNY-GFP, S100A, S326A, or S100/326A mutants into HEK-293T cells, and their subcellular localizations were visualized by fluorescence confocal microscopy. All these mutants showed clear membrane-localization, similar to the wild-type CCNY (**Fig. 4**), suggesting that 14-3-3 binding was not required for membrane localization of the CCNY.

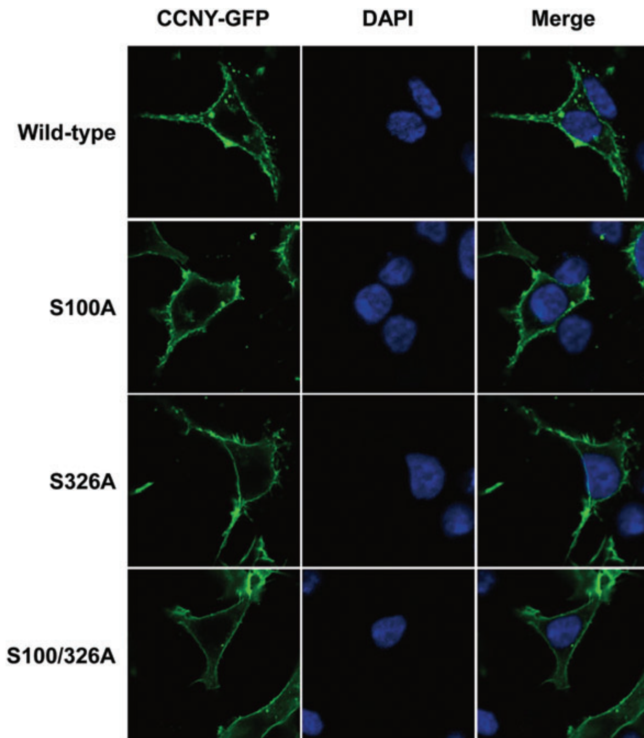

Figure 4. Subcellular localization of CCNY mutants HEK-293T cells transfected with the indicated vectors were plated on coverslips in 12-well plates. 24 h later, cells were fixed and then stained with DAPI.

Binding to 14-3-3 proteins enhances the association of a CCNY/CDK14 kinase complex

Another important regulatory activity of 14-3-3 proteins is to alter the binding ability of the target proteins. We decided to examine whether 14-3-3 binding affects the interaction between CCNY and its kinase partner CDK14. In a co-IP assay, co-transfection of the 14-3-3 isoforms significantly increased the amount of CDK14-GFP pulled down by CCNY-Myc (**Fig. 5A**), with the 14-3-3τ isoform showing less effect on this association. Thus, 14-3-3 binding could enhance the association of a CCNY/CDK14 complex.

The 14-3-3 proteins can play two possible roles in mediating CCNY/CDK14 association. One is that 14-3-3 proteins could act as a scaffold to bridge the interaction between CCNY and CDK14, as 14-3-3 proteins have been proven to bind to CDK14 in our previous report [17]. Another one is that 14-3-3 binding might induce conformational changes in CCNY or CDK14, which could lead to a higher binding affinity. To address which one is true in this context, we performed co-IP assays. As shown in **Fig. 5B**, co-expression of 14-3-3β could increase the interaction between CCNY and wild-type CDK14 as well as between CCNY and 14-3-3 binding-deficient mutant $CDK14^{S119A}$ (**Fig. 5B**). However, the interaction between 14-3-3 binding-deficient mutant $CCNY^{S100/326A}$ and wild-type CDK14 could not be enhanced by ectopically expressed 14-3-3β (**Fig. 5B**). If 14-3-3 proteins act as a scaffold, both 14-3-3 binding-deficient mutants $CDK14^{S119A}$ and $CCNY^{S100/326A}$ should weaken the role of 14-3-3, whereas only $CCNY^{S100/326A}$ but not $CDK14^{S119A}$ abolishes the function of 14-3-3 proteins in mediating CCNY/CDK14 association. Thus, binding with 14-3-3 probably induces conformational changes in CCNY, leading to a higher affinity binding with CDK14.

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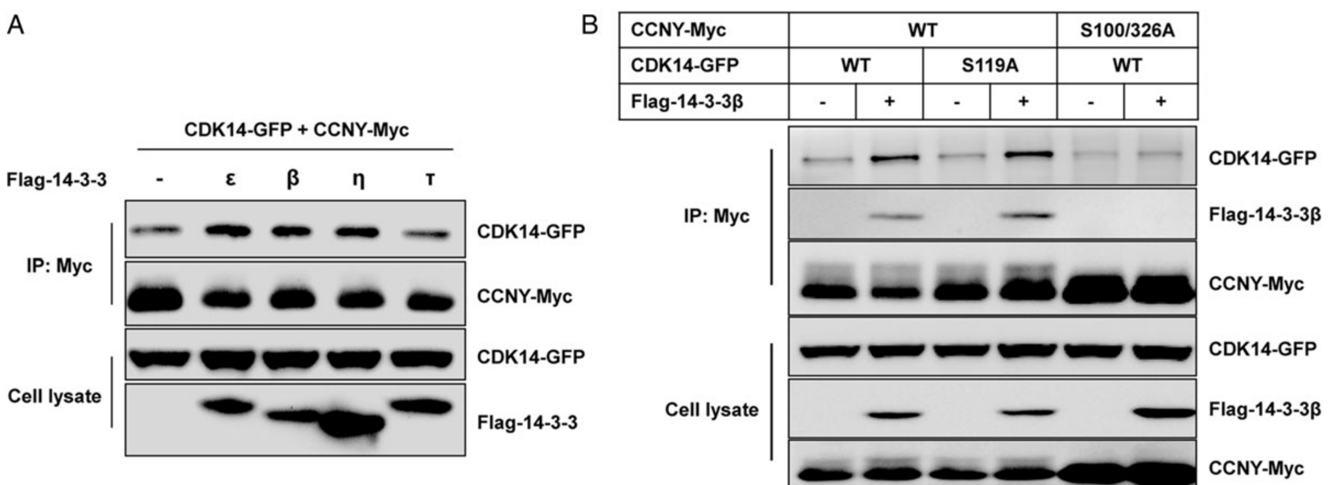

Figure 5. 14-3-3 Proteins enhance CCNY binding to CDK14 (A) CCNY-Myc and CDK14-GFP were co-transfected with Flag-14-3-3ε, β, η or τ. 24 h post-transfection, the interactions between CCNY and CDK14 were analyzed by co-IP assays. (B) HEK-293T cells were transfected as indicated, and the interactions between CCNY and CDK14 were analyzed by co-IP assays.

Discussion

In the current study, four 14-3-3 isoforms (ε, β, η, τ) were identified as novel CCNY-interacting partners in a yeast two-hybrid screening. CCNY contains two functional 14-3-3 binding sites, Ser-100 and Ser-326, in which mutation of these two sites to the nonphosphorylatable Ala residues completely abrogated 14-3-3 binding to CCNY. We also found that ectopically expressed 14-3-3 proteins could enhance the association of a CCNY/CDK14 complex. 14-3-3 proteins can bind to CCNY as well as CDK14. It seems that only the association with CCNY contributes to promote CCNY/CDK14 interaction. Based on these observations, we proposed that 14-3-3 probably acts as a scaffold protein to mediate the association of CCNY and CDK14, and that binding with 14-3-3 may also induce conformational changes in CCNY, leading to a higher affinity binding with CDK14.

Cyclins are generally very different from each other in primary structure, but they all contain a similar tertiary structure of compact domain of five α-helices, termed cyclin box [24]. Conventional cyclins usually have two cyclin folds: the N-terminal fold is conserved and necessary for CDK binding and activation; and the C-terminal fold is required for the proper folding of the cyclin molecule [1]. The regulatory role of 14-3-3 proteins in CCNY presented here may provide a new mechanism by which CCNY binds to and activates its kinase partners with only a single cyclin fold. The CCNY protein harbors two docking sites for 14-3-3 proteins: ser-100 which is located to the N-terminal of the cyclin box, and Ser-326 which is located to the C-terminal. 14-3-3 Binding to CCNY probably exposes the cyclin box, thus allowing its access to the 'PFTAIRE' motif of CDK14. Furthermore, the two 14-3-3 binding sites on CCNY exist in all metazoans, indicating that the interaction between CCNY and 14-3-3 proteins is highly conserved in evolution. The 14-3-3 proteins are likely to play important regulatory roles in the full function of CCNY.

Funding

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