Identification of the p28 subunit of eukaryotic initiation factor 3(eIF3k) as a new interaction partner of cyclin D3

Xiaoyun Shen, Yanzhong Yang, Weicheng Liu, Maoyun Sun, Jianhai Jiang, Hongliang Zong, Jianxin Gu*

State key laboratory of Genetic Engineering and Gene Research Center, Shanghai Medical College of Fudan University, Shanghai 200032, PR China

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Abstract Cyclin D3 is found to play a crucial role not only in progression through the G1 phase as a regulatory subunit of cyclin-dependent kinase 4 (CDK 4) and CDK 6, but also in many other aspects such as cell cycle, cell differentiation, transcriptional regulation and apoptosis. In this work, we screened a human fetal liver cDNA library using human cyclin D3 as bait and identified human eukaryotic initiation factor 3 p28 protein (eIF3k) as a partner of cyclin D3. The association of cyclin D3 with eIF3k was further confirmed by in vitro binding assay, in vivo coimmunoprecipitation, and confocal microscopic analysis. We found that cyclin D3 specifically interacted with eIF3k through its C-terminal domain. Immunofluorescence experiments showed that eIF3k distributed both in nucleus and cytoplasm and colocalized with cyclin D3. In addition, the cellular translation activity in HeLa cells was upregulated by cyclin D3 overexpression and the mRNA levels are constant. These data provide a new clue to our understanding of the cellular function of cyclin D3.

Keywords: Cyclin D3; eIF3k; Interaction; Translation initiation

1. Introduction

As cells enter the cell cycle from quiescence, genes encoding D-type cyclins get activated at the beginning of the G1 phase [1]. The expression of cyclin D is largely dependent on extracellular signals and signaling cascades, so that D-type cyclins represent a fundamental link between mitogen, nutrient stimulation and the cell cycle machinery [2].

Among D-type cyclins, mouse cyclin D1 was first characterized and then cyclin D2 and cyclin D3 were discovered [3–5]. Compared with cyclin D1 and cyclin D2, little is known about the function of cyclin D3. Most of the studies of cyclin D3 focus on its association with cyclin-dependent kinase 4 (CDK 4) and CDK 6 to drive cells through a G1 restriction point. The studies of our group have shown that cyclin D3 is found not only to play a crucial role in progression through the G1 phase as a regulatory subunit of CDK 4 and CDK 6, but also to interact with CDK 11^{p58}, a G2/M CDK, in G2/M phase instead of G1/S phase as a partner of CDK 11^{p58}, and the elevated expression of cyclin D3 affects CDK 11^{p58} cellular distribution, resulting in enhanced kinase activity of CDK 11^{p58} [6]. The first observation regarding a new potential of D-type cyclins was reported when it was shown that cyclin D2 or cyclin D3 inhibit 32D myeloid cells to undergo neutrophil differentiation in response to granulocyte colony-stimulating factor [7], suggesting that cyclin D3 is able to modulate differentiation program. In addition, cyclin D3 is found to be involved in a complexity of networks of interactions and play new cdk-independent roles. Cyclin D3 can interact with different kinds of transcription factors. Like cyclin D1, cyclin D3 could form a specific complex with the estrogen receptor (ER) and activate ER-mediated transcription activity in a CDK 4 and ligand independent manner and show a less pronounced effect than cyclin D1 on ER [8,9]. Cyclin D3 is also a cofactor of retinoic acid receptors, modulating their activity in the presence of cellular retinoic acid-binding protein II [10]. Through direct binding, cyclin D3 could inhibit the growth-restraining capacity of the DMP1 [11]. These findings suggest that cyclin D3 may play an important role in the regulation of transcription. Surprisingly, cyclin D3 is found to activate Caspase 2 [12] and regulate apoptosis induced by T cell receptor activation in leukemic T cell lines [13], which suggests that a physical interaction between cyclin D3 and Caspase 2 connects the genetic networks that govern cell-cycle progression with those that govern cell death [12].

Altogether, cyclin D3 represents a central link between cell cycle progression, cell differentiation, transcriptional regulation and apoptosis. Since identification of cell-specific partners of cyclin D3 might help us to understand the genetic networks in which cyclin D3 might be involved more thoroughly and systematically, we performed a yeast two-hybrid screen of a human fetal liver cDNA library using human cyclin D3 as bait and subsequently identified human eukaryotic initiation factor 3 p28 protein (eIF3k) as a partner of cyclin D3.

Mammalian translation initiation factor 3 (eIF3) is the most complex and least understood of the protein synthesis initiation factors, containing at least 12 subunits with an apparent aggregate mass of approximately 700 kDa [14]. eIF3 binds to

* Corresponding author. Fax: +86-21-64164489.
E-mail address: jxgu@shmu.edu.cn (J. Gu).

Abbreviations: eIF3k, the p28 subunit of eukaryotic initiation factor 3; CDK, cyclin-dependent kinase; PVDF, polyvinylidene difluoride; GST, glutathione *S*-transferase; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription-polymerase chain reaction; ECL, enhanced chemiluminescence; HRP, horse radish peroxidase; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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the 40S ribosomal subunit, promotes the binding of methionyl-$tRNA_i$ and mRNA, and interacts with several other initiation factors to form the 43S initiation complex [15]. Twelve non-identical subunits have been identified in mammalian eIF3 and their cDNAs have been cloned and characterized: a (p170) [16], b (p116) [17], c (p110) [18], d (p66) [19], e (p48) [20], f (p47) [19], g (p44) [21], h (p40) [19], i (p36) [18], j (p35) [21], k (p28) [14] and l (p69) [22]. Since it was reported that eIF3k mRNA is highly expressed in the brain, testis and kidney tissues, especially in HeLa and Jurkat cell lines [14], we adopted HeLa cells to further our study on eIF3k in this work.

Here, we report that eIF3k is a new interaction partner of cyclin D3, which is the unique one among D-type cyclins that binds with eIF3k through its C-terminal domain. Its distribution is consistent with that of cyclin D3 by fluorescence microscopy. The whole cellular translation activity was apparently enhanced by cyclin D3 expression in a dose-dependent manner.

2. Materials and methods

2.1. Cell lines and reagents

HeLa cells were obtained from the Institute of Cell Biology, Academic Sinica. MATCHMAKER LexA two-hybrid system and human fetal liver MATCHMAKER LexA cDNA library were products of CLONTECH (Palo Alto, CA). Protein-G-agarose, glutathione Sepharose beads, and the mouse monoclonal anti-Myc antibody were purchased from Roche Company. The mouse monoclonal anti-cyclin D1, D2, and D3 antibodies were bought from PharMingen. The rabbit Myc-tag polyclonal antibody was purchased from Cell Signaling. The fluorescein goat anti-mouse IgG and mouse monoclonal anti-actin antibody were purchased from Oncogene. The goat anti-rabbit IgG-R was purchased from Santa Cruz Biotechnology. Leupeptin, aprotinin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., the goat anti-mouse-horseradish peroxidase (HRP) secondary antibody, [^{35}S]methionine, hybond polyvinylidene difluoride (PVDF) membrane, and the enhanced chemiluminescence (ECL) assay kit were purchased from Amersham Pharmacia Biotech. The TNT T7 coupled reticulocyte lysate system and the dual-luciferase reporter assay system were purchased from Promega. The LipofectAMINE^{TM} and Trizol reagents were bought from Invitrogen. Real-time reverse transcription-polymerase chain reaction (RT-PCR) kit was purchased from TaKaRa. The nuclear extract kit was purchased from Activemotif. Other reagents were commercially available in China.

2.2. Yeast two-hybrid assays

MATCHMAKER LexA two-hybrid system was used to perform yeast two-hybrid screening according to the manufacturer's instructions. The full-length cyclin D3 was cloned in-frame into LexA coding sequence to generate bait plasmid, pLexA-cyclin D3. A human fetal liver cDNA library in the pB42AD plasmid (CLONTECH) was screened for proteins that interact with cyclin D3 using EGY48 yeast strain. The transformed yeast was grown on plates lacking both histidine to select the bait plasmid and tryptophan to select the prey plasmid, respectively. The individual cDNA of interest was purified from the positive clones and re-transformed into yeast to confirm its specific interaction with cyclin D3.

2.3. Cell culture, transient transfection and cell fractionation

HeLa cells were cultured with DMEM supplemented with 10% FBS, 1% penicillin/streptomycin at 37 °C in 5% CO_2. Cells were plated at 70% confluence in 60 mm tissue culture dishes or six-well plates and the following day were transfected with relevant plasmids using LipofectAMINE^{TM} transfection reagent according to the manufacturer's recommendations. After 48 h, the cells were harvested for further analysis. The cytoplasmic and nuclear fractions from HeLa cells were collected using the nuclear extract kit under the manufacturer's instructions.

2.4. Western blot analysis

Western blot experiments were used to measure certain proteins. Briefly, the cells were lysed in lysis buffer (120 mM Tris (pH 7.4), 135 mM NaCl, 1 mM EDTA, 1% NP40, 0.1% SDS, 1 mM Na_3VO_4, 1 mM aprotinin, and 1 mM PMSF). An equivalent amount of protein from each sample was electrophoresed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to a PVDF membrane. After blocking with phosphate-buffered saline (PBS) containing 5% non-fat milk and 0.1% Tween 20 overnight, the membrane was incubated with primary antibody at 4 °C overnight. After washing with PBS containing 0.1% Tween 20 three times, each for 5 min, the membrane was then incubated with HRP-labeled secondary antibody for another 2 h at room temperature (RT). The membrane was then developed using the ECL detection systems.

2.5. Plasmid construction

The full-length cyclin D3 (GenBank Accession No. NM_001760) isolated from the original fetal cDNA was cloned into the *Eco*RI/*Xho*I site of pLexA in-frame with the DNA binding domain of LexA. The eukaryotic expression vectors pcDNA3.0-GST-CDK 4, pcDNA3.0-cyclin D1, D2, and D3, pcDNA3.0-GST have previously been described [6]. The full-length eIF3k from the original fetal cDNA was cloned into pLexA vector by *Eco*RI and *Xho*I. The deficient mutants of cyclin D3 were designated as N1 (1–88 aa), N (1–153 aa), and C (154–292 aa), and performed by PCR using pLexA-cyclin D3 as template. N1 was generated from primer 1 (5′-gCGAATTCATGGAG-CTGCTGTGTTGC-3′) and primer 2 (5′-TTTCTCGAGCTACAGG-TAGCGATCCAGGTAG-3′), N from primer 1 and primer 3 (5′-ATGCTCGAGCTAAGCAGCCAGGTCCCACTTG-3′), and C from primer 4 (5′-GGGAATTCATGGCTGTGATTGCACATGAT-TTC-3′) and primer 5 (5′-GATCTCGAGCTACAGGTGTATGGC-TG-3′). The *Eco*RI site underlined in the forward primers and the *Xho*I site underlined in the reverse primers were used to clone these fragments into pB42AD plasmid. The full-length fragments of cyclin D2, D3 were cut from pcDNA3.0-cyclin D2, D3 by *Eco*RI and *Xho*I, and cloned into pB42AD vector. The full-length eIF3k without the stop codon was cloned into pcDNA3.1/Myc-His(−) C vector with primer 6 (5′-ACACTCGAGATGGCGATGTTTGAGCAG-3′, *Xho*I site underlined) and primer 7 (5′-TGGAATTCTCTGGGAGGAGGCCATGAT-3′, *Eco*RI site underlined). To make cyclin D3 siRNA plasmid, we designed two complementary oligonucleotides targeted to the cyclin D3 gene *CCND3*: CD3iF (5′-GATCCCCGATGCTGGCTTACTGGATG-TTCAAGAGACATCCAGTAAGCCAGCATCTTTTTGGAAA-3′) and CD3iR (5′-AGCTTTTCCAAAAAGATGCTGGCTTACTGGA-TGTCTCTTGAACATCCAGTAAGCCAGCATCGGG-3′). The two complementary oligonucleotides were then synthesized, annealed, and ligated into pSUPERIOR vector by *Bgl*II/*Hin*dIII site. All the plasmids produced by PCR were confirmed by sequencing.

2.6. In vitro binding analysis

Cyclins D1, D2, D3, glutathione *S*-transferase (GST), GST-CDK 4 and GST-eIF3k were [^{35}S] methioninelabeled in vitro with the TNT coupled reticulocyte lysate system (Promega) at 30 °C for 90 min according to the user manual. Twenty percent of these samples were removed for direct analysis in an SDS–polyacrylamide gel, and the remainders were incubated with glutathione-Sepharose beads in protein binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, 10 mM NaF, 1% Nonidet P-40, 1 mM NaVO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) at 4 °C for 2 h with constant mixing. The beads were then washed three times with the same buffer and the bound proteins were subjected to 12% SDS–PAGE analysis. The gel was then dried and autoradiographed.

2.7. In vivo interaction assay

Forty eight hours posttransfection, cells were washed three times with ice cold PBS and solubilized with lysis buffer (50 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 0.1 mM Sodium orthovanadate, 0.1 mM NaF, 0.1 mM benzamide, 10 μg/ml aprotinin, 10 g/ml leupeptin, and 1 mM PMSF). Detergent insoluble materials were removed by centrifugation at 12 000 rpm for 15 min at 4 °C. Ten percent of these samples were removed for direct analysis in an SDS–polyacrylamide gel, and the remainders were incubated with the mouse monoclonal anti-Myc antibody at 4 °C for 2 h. Pre-equilibrated protein-G agarose beads (Roche) were then added, and collected by cen-

trifugation after 2 h of incubation, and then gently washed three times with the lysis buffer. The bound proteins were eluted by boiling in SDS sample buffer and resolved on a 12% SDS–PAGE gel. The proteins were transferred onto a PVDF membrane and performed as Western blot analysis.

2.8. Immunofluorescence

HeLa cells were plated onto coverslips and were transfected with relevant plasmids. Forty eight hours after transfection, cells were washed with cold PBS and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature (RT). Cells were rinsed three times with PBS and permeabilized with 100% methanol at −20°C for 10 min. Cells were washed with PBS again and blocked in PBS containing 5% bovine serum albumin (BSA) and then BSA was removed. The coverslips were then incubated with mouse monoclonal cyclin D3 (1:100 dilution) antibody and rabbit polyclonal anti-Myc antibody (1:200 dilution) for 2 h at RT. Coverslips were washed three times with PBS for 5 min each and then incubated with fluorescein goat anti-mouse IgG (1:100 dilution) and goat anti-rabbit IgG-R (1:100 dilution) in dark for 1 h at RT. Following incubation, coverslips were washed three times with PBS and contained with Hoechst for 5 min and washed once again in dark. The coverslips were then sealed and examined immediately in a Leica confocal microscope. Digitized images of the fluorescent-antibody-stained cells were acquired with the software provided by Leica.

2.9. In vivo reporter synthesis assay

HeLa cells were co-transfected with 400 ng/well pRL control vector (Promega) and pcDNA3.0-cyclin D1, D2, D3 or pSUPERIOR-cyclinD3 with the dose from 100 to 600 ng in six-well plates in triplicate. The total amount of plasmid DNA was adjusted to 1.0 μg/well with empty pcDNA3.0. After 48 h, cells were lysed in passive buffer and Renilla luciferase assays were performed with the dual-luciferase reporter assay system (Promega) and measured using the ultra sensitive tube luminometer (Berthold). Each experiments condition was measured in triplicate.

2.10. Quantitative real-time RT-PCR

Total RNAs were isolated with Trizol reagent from HeLa cells transfected with pRL and pcDNA3.0-cyclin D1, D2, D3 or pSUPERIOR-cyclinD3 with different doses, 100, 300 and 600 ng, in triplicate. Real time RT-PCR was performed using TaKaRa real time RT-PCR kit with SYBR Green I (Roche) and amplified in BioRad iCycler according to the manufacturer's instructions. PCR was carried out at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Melting curves were performed to determine the specificity of the amplicon. Each sample was completed in triplicate and a template-free negative control (the total RNA extracted from untransfected HeLa cells) was included. Cycle threshold (Ct) during the exponential phase of amplification was determined by real time monitoring of fluorescent emission. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control gene. Primers for GAPDH are 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′ and 5′-GGTATCGTGGA-AGGACTCATGAC-3′. Primers for Renilla luciferase are 5′-ATGATCCAGAACAAAGG-3′ and 5′-GAACTCGCTCAACGAACG-3′. Renilla Luciferase mRNA levels were demonstrated as the absolute number of copies normalized against GAPDH mRNA.

3. Results

3.1. Screening eIF3k as a cyclin D3-associated protein

To identify proteins that interact with cyclin D3, a screening of human fetal liver cDNA library was performed with cyclin D3 as bait using yeast two-hybrid system. Database searching revealed that one of the clones encode the full-length human eIF3k. To further confirm the interaction in yeast system, the two yeast vectors were exchanged by moving cyclin D3 from the pLexA to pB42AD vector and eIF3k from the activation domain (pB42AD) to the DNA-BD vector (pLexA) and the newly constructed plasmids were co-transformed into EGY48 yeast cells. This reversed two-hybrid assay also confirmed their interaction in yeast (Fig. 1A, 1–4). Characterization of the other clones is in progress in our laboratory.

3.2. Mapping of the cyclin D3 region that interacted with eIF3k

To determine which domain of cyclin D3 was important for this interaction, we searched the putative pattern of cyclin D3 in the PROSITE database. Three deficient mutants were generated corresponding to different domains of cyclin D3 (designed as N1, N and C) as shown in Fig. 1B. Yeast cells were co-transformed individually with these deficient mutant constructs N1, N, C, the full-length constructs of cyclins D1, D2, D3 and pLexA-eIF3k. As shown in Fig. 1A, 4–12, the C-terminal part of cyclin D3 (154–292 aa) did interact with eIF3k, while the N-terminal part of cyclin D3 lost this ability. This suggested that the amino acids 154–292 within the C-terminal region of cyclin D3 were responsible for eIF3k binding. Interestingly, no interaction was observed with the cyclin D1 and cyclin D2 co-transformants, which implied that among D-type cylcins, cyclin D3 might uniquely interact with eIF3k. This prompts us to investigate their interaction pattern in following experiments.

3.3. In vitro interactions between eIF3k and D-type cyclins

To investigate whether cyclin D3 was the unique one in the D-type cyclins that could interact with eIF3k, GST pull-down experiments were performed. GST-eIF3k, GST-CDK 4, GST, cyclins D1, D2, and D3 were synthesized and isotopically labeled in vitro, respectively. The labeled proteins were incubated in different combinations. As shown in Fig. 2, though all different proteins were generated as indicated (lanes 1–5), only cyclin D3 could bind with GST-eIF3k (lane 8) but not the other two cyclin Ds (lanes 6,7), suggesting that this interaction was specific in vitro, which was consistent with the result mentioned in yeast.

3.4. Binding of cyclin D3 with eIF3k in vivo

Because we have been unsuccessful in obtaining antibodies specific for eIF3k at present, an expression vector containing Myc-tagged full-length eIF3k was constructed for experiments. HeLa cells were transiently transfected with pcDNA3.1-Myc-eIF3k or pcDNA3.1-Myc empty vector, respectively. As shown in Fig. 3A, the endogenous cyclin D1, D2 or D3 was detected individually by anti-cyclin D1, D2 or D3 monoclonal antibody and the expression of Myc-eIF3k was confirmed by an anti-Myc monoclonal antibody. The remainder cell lysates were immunoprecipitated with anti-Myc monoclonal antibody, followed by immunoblotting using anti-cyclin D1, D2 or D3 mouse monoclonal antibody, respectively. As shown in Fig. 3B, cyclin D3 was co-immunoprecipitated with Myc-eIF3k, whereas cyclin D1 and D2 could not be detected in immunoprecipitation. This result further verified that cyclin D3 did associate with eIF3k in vivo and this interaction was specific.

3.5. The cellular distribution analysis of eIF3k

From Fig. 4A, we found that endogenous cyclin D3 localized both in nucleus and cytoplasm in untransfected HeLa cells and no fluorescence was seen when detected by the Myc antibody. Immunofluorescence microscopy using an anti-Myc antibody revealed that eIF3k also localized both in nucleus and cytoplasm in HeLa cells transfected with Myc-eIF3k, and merging of the two images showed a yellow

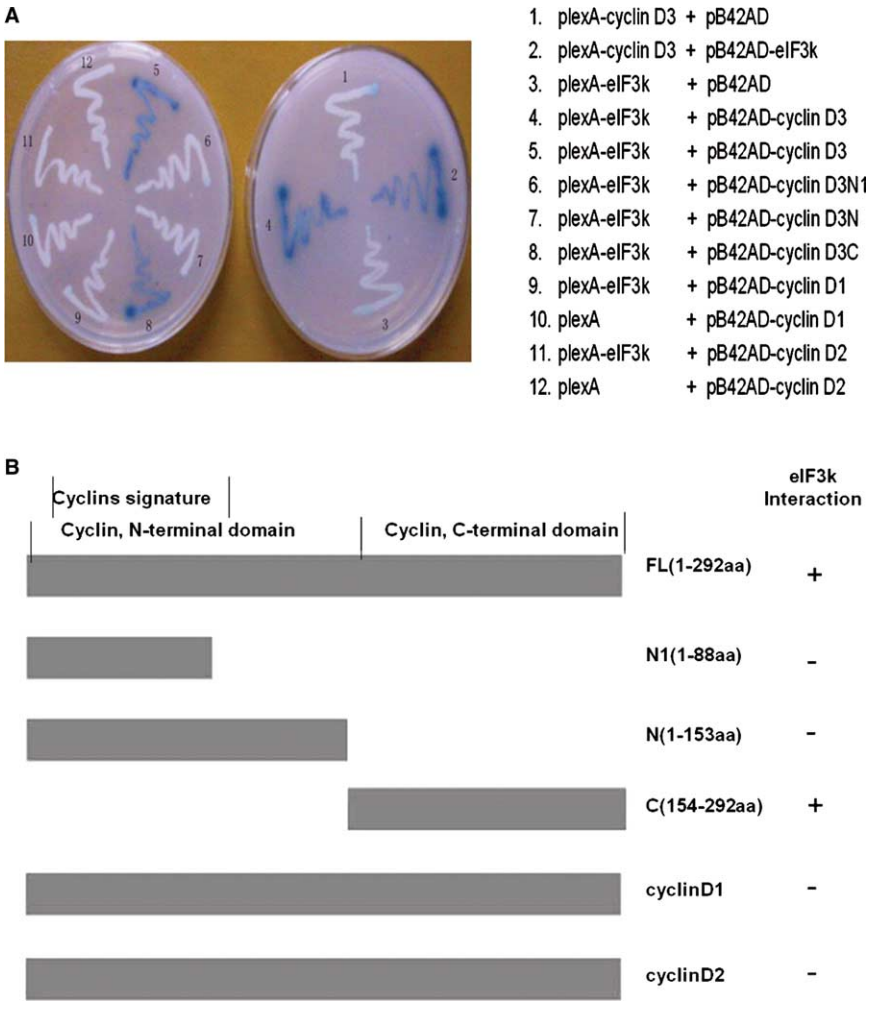

Fig. 1. The yeast two-hybrid assays of D-type cyclins and deficient mutants of cyclin D3 with eIF3k. (A) Individual EGY48 cells, containing relevant plasmids (indicated as 1–12). were streaked with toothpicks onto the SD induction plates (His$^-$,Ura$^-$, Trp$^-$, Leu$^-$ containing X-gal). The positive colonies can grow on the induction plates and turn blue. (B) Mapping of cyclin D3 region required for binding to eIF3k. The top diagram illustrates full-length eIF3k and the domains and residue numbers of deletion mutants of cyclin D3 are indicated. Columns on the right summarize whether constructs did (+) or did not (−) interact.

color (Fig. 4B), indicating that eIF3k co-localizes with cyclin D3, especially in cytoplasm. To confirm this observation, we performed cell fractionation and Western blotting analysis. Again, we observed both a cytoplasmic and nuclear pool of eIF3k and cyclin D3 (Fig. 4C). This result was consistent with our findings in their cellular distribution in HeLa cells and further confirmed their interaction mentioned above.

3.6. *Upregulation of the cellular translation activity in HeLa cells upon cyclin D3 overexpression*

Since eIF3k could interact with cyclin D3, the question arises whether cyclin D3 could play a role in regulating cellular translation activity? HeLa cells were transiently co-transfected with Renilla luciferase reporter plasmid and pcDNA3.0-cycln D1, D2, or D3 with a dose response assay in triplicate. The Renilla luciferase activity was then measured and the mRNA levels were determined after 48-h transfection. We found that Renilla luciferase synthesis was significantly elevated in the HeLa cells transfected with cyclin D3 in a dose dependent manner (Fig. 5A). In contrast, Renilla luciferase activity was not affected in the HeLa cells transfected with pcDNA3.0-cyclin D1 or D2 (Fig. 5A). To further confirm the upregulation of the cellular translation activity in HeLa cells upon cyclin D3 overexpression, the cyclin D3 siRNA expression vector was addressed to suppress the endogenous cyclin D3. HeLa cells were co-transfected with pSUPERIOR-cyclinD3 under the same condition. As shown in Fig. 5A, Renilla luciferase activity of cyclin D3 siRNA was decreased comparing to that of cyclins D1, D2 and D3. The expression of cyclin D1, D2, or D3 was confirmed by immunoblotting in transfected HeLa cells (Fig. 5B). Real-time RT-PCR analysis excludes the transcriptional effect of cyclins D1, D2 (data not shown), D3 (Fig. 5C) and cyclin D3 siRNA (Fig. 5C). Taken together, cyclin D3 upregulated the cellular protein synthesis in vivo at the translation level, which might be achieved through its interaction with eIF3k. This needs to be further investigated.

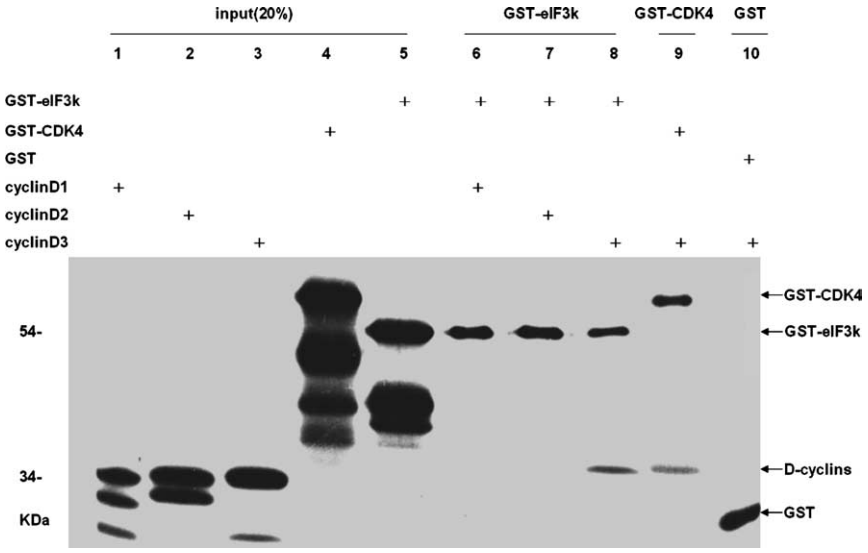

Fig. 2. Binding of cyclin D3 and eIF3k in vitro. Constructs were engineered as described by Section 2. In vitro translated [^{35}S] labeled GST-eIF3k was incubated with [^{35}S] labeled D-type cyclins in the presence of glutathione-Sepharose beads with in vitro translated [^{35}S] labeled GST-CDK 4 incubated with cyclin D3 as a positive control, and [^{35}S] labeled GST incubated with [^{35}S] labeled cyclin D3 as a negative control. After incubation, the beads were washed three times with the binding buffer and analyzed by autoradiography after SDS–PAGE. Lanes from left to right are 20% cyclin D1 input (lane 1), 20% cyclin D2 input (lane 2), 20% cyclin D3 input (lane 3), 20% GST-CDK 4 input (lane 4), 20% GST-eIF3k (lane 5) input, GST-eIF3k + cyclin D1 (lane 6), GST-eIF3k + cyclin D2 (lane 7), GST-eIF3k + cyclin D3 (lane 8), GST-CDK 4 + cyclin D3 (lane 9), and GST + cyclin D3 (lane 10).

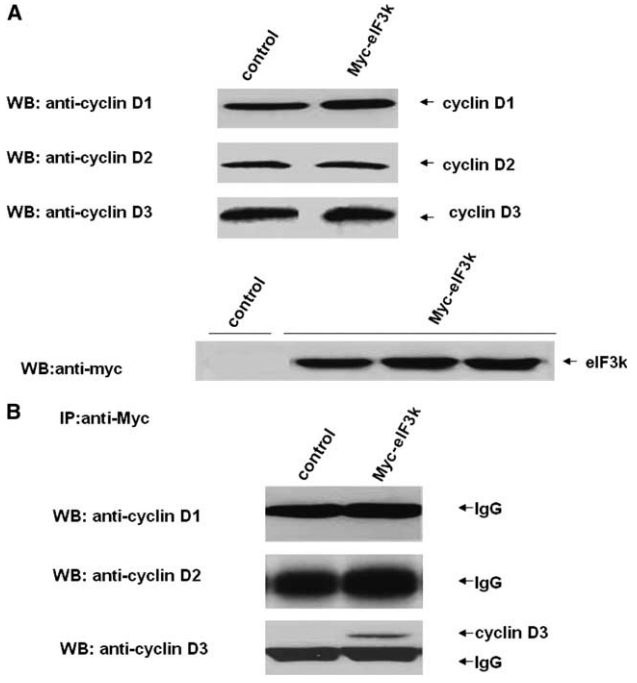

Fig. 3. Association of cyclin D3 with eIF3k in vivo. (A) The expression of cyclin D1, D2, D3 (10% input) and Myc-eIF3k (10% input) in pcDNA3.1-Myc (control) and pcDNA3.1-Myc-eIF3k transfected HeLa cells by Western blotting. Myc-eIF3k expression was detected using a monoclonal antibody against the Myc-tag. The D-type cyclins were detected using a monoclonal antibody against the cyclin D1, D2 or D3, respectively. (B) The interaction between D-cyclins and eIF3k in pcDNA3.1-Myc(control) and pcDNA3.1-Myc-eIF3k transfected HeLa cells. Cell lysates were immunoprecipitated (IP) with an anti-Myc monoclonal antibody. The immunoprecipitates were immunoblotted (WB) with a monoclonal antibody against cyclin D1, D2 or D3, respectively.

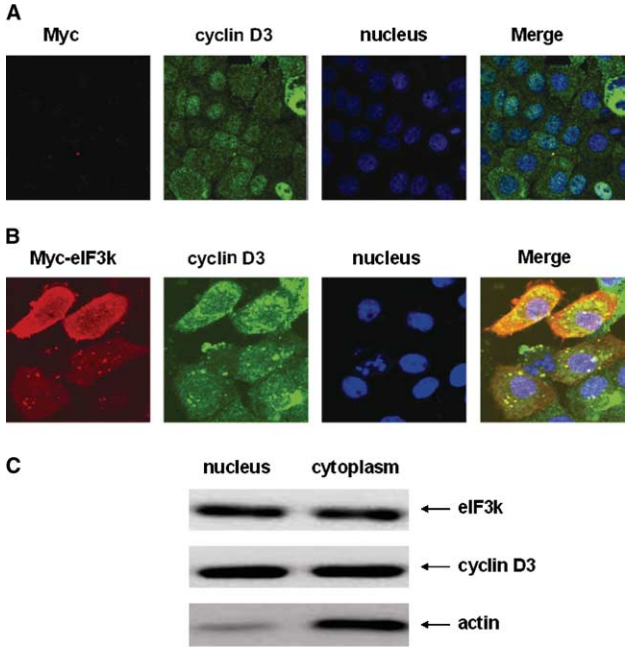

Fig. 4. The co-localization of cyclin D3 and eIF3k in HeLa cells. HeLa cells were contained with Hoechst (blue) to indicate the nuclear position. The localization of cyclin D3 (green) was detected with the mouse monoclonal anti-cyclin D3 antibody and eIF3k (red) was with the rabbit Myc-tag polyclonal antibody. (A) The localization of cyclin D3 in untransfected HeLa cells. (B) The co-localization of cyclin D3 and eIF3k in HeLa cells transfected with pcDNA3.1-Myc-eIF3k. (C) The localization of cyclin D3 and eIF3k by cell fractionation and Western blotting. Twenty micrograms of fractionated HeLa cells lysates were used for each lane, with mouse monoclonal anti-cyclin D3 antibody, mouse monoclonal anti-Myc antibody and mouse monoclonal anti-actin antibody(as a marker control) for immunoblotting.

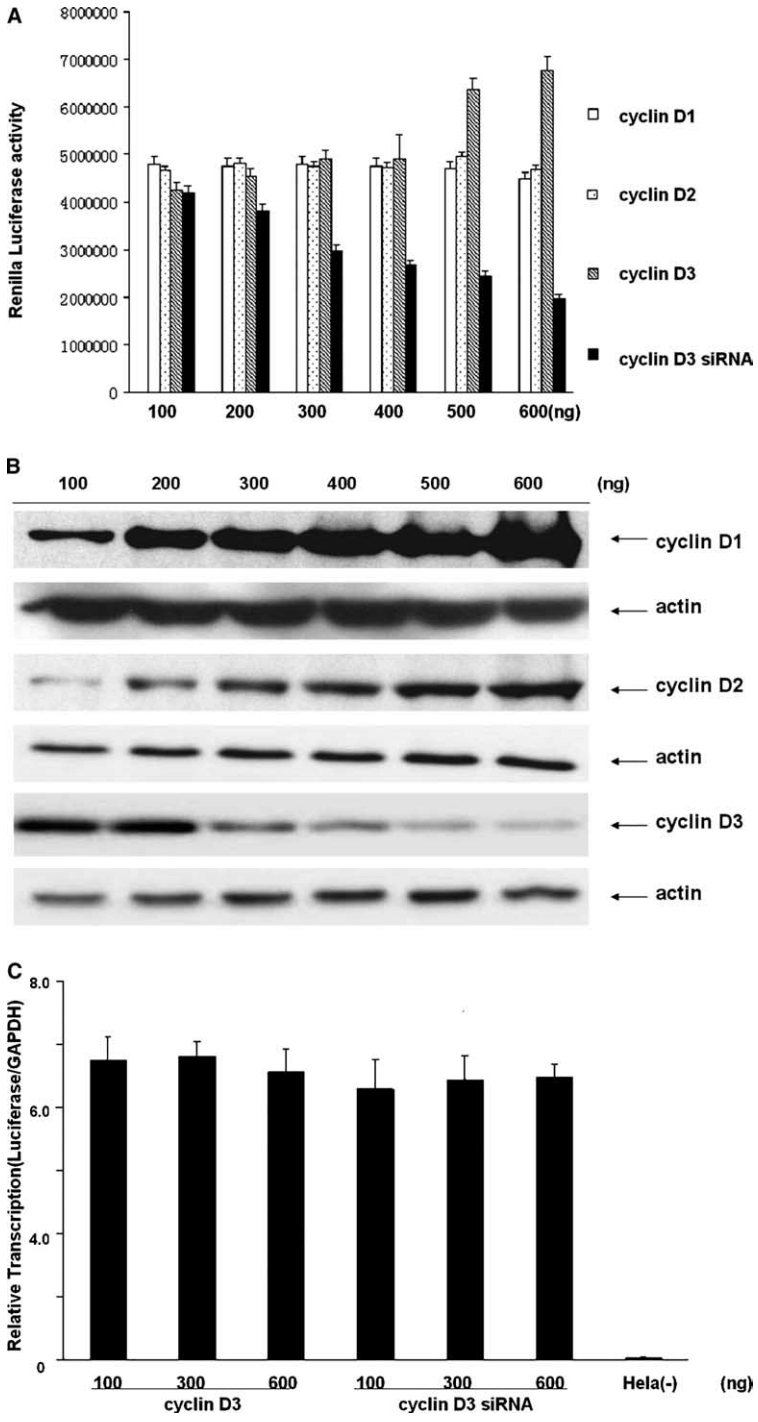

Fig. 5. Upregulation of the cellular translation activity in HeLa cells upon cyclin D3 overexpression. (A) The Renilla luciferase activity of cyclins D1, D2, D3 and D3 siRNA. HeLa cells were co-transfected with 400 ng Renilla luciferase reporter plasmid and pcDNA3.0-cyclin D1, D2, D3 or D3 siRNA plasmid from 100 to 600 ng in triplicate. The total amount of plasmid DNA was adjusted to 1.0 μg/well with empty pcDNA3.0. Forty eight hours after transfection, cells were lysed and Renilla luciferase activity was measured. (B) The expression of cyclin D1, D2 or D3 in HeLa cells transfected individually with pcDNA3.0-cyclin D1, D2 or D3 siRNA plasmid from 100 to 600 ng. Twenty micrograms of HeLa cells lysates was used for each lane. Actin was served as a quantitative control. (C) Quantitative real time RT-PCR analysis of the Renilla luciferase in HeLa cells. HeLa cells were transfected with cyclin D1 (data not shown), D2 (data not shown), D3 or D3 siRNA with the doses 100, 300, and 600 ng in triplicate. Real time RT-PCR was performed using TaKaRa real time RT-PCR kit with SYBR Green I (Roche) and amplified in BioRad iCycler according to the manufacturer's instructions. Renilla luciferase mRNA levels were normalized against GAPDH. Untransfected HeLa cells (HeLa(−)) served as a negative control.

4. Discussion

In this study, we identified a p28 subunit of eIF3k as a new interaction partner of cyclin D3. The interaction between cyclin D3 and eIF3k is specific through the C-terminal domain within cyclin D3, as demonstrated by the inability of the other two D-type cyclins to associate with eIF3k. Cyclin D3 colocalizes with eIF3k both in nucleus and cytoplasm. And we also found that cyclin D3 could enhance whole cellular translation activity in a dose-dependent way.

The three D-type cyclins exhibit homology. The human and mouse cyclin D3 genes were originally identified by cross-hybridizing cyclin D1 probes with a mouse phage genomic library or a human cDNA library, respectively [23]. However, there is more evidence that the three D-type cyclins are not equivalent in many ways, such as the tissue-specific expression patterns [24], different affinities to CDKs [25], different inductions by various signals in a cell lineage-specific manner [24,26], different phenotypes of the knockout mice [27] and the transgenic mice [28].Our present study identified that eIF3k interacted exclusively with cyclin D3 rather than with cyclin D1 or cyclin D2. The peptide sequence alignment of the three D-type cyclins revealed that they had high similarity. (Fig. 6) One of the most important differences among cyclins D1, D2 and D3 was at the C-terminal end of the molecule. The eIF3k-binding site within the cyclin D3 domain was located in residues 154–284 (Fig. 1), which corresponded to the low homologous domain of the D-type cyclins. Therefore, this might account for the specific interaction between cyclin D3 and eIF3k.

Considerable attention has been paid to the role of D-type cyclins in controlling the G1 phase progression by regulating CDK 4 and 6 activation and Rb function. It was reported that the overexpression of cyclin D1 or D3 using the same experimental system caused a similar alteration of the cell cycle profile in asynchronous cells. Likewise, constitutive overexpression of cyclin D2 or D3 in 32Dcl3 myeloid cells was shown to cause contraction of G1 without concomitant increase in the population doubling time [29]. The phenotypic similarity of the effects of overexpression of various D cyclins does not, however, lead to the conclusion that all operate through identical mechanisms. In our study, it was found that eIF3k did interact with cyclin D3, and overexpression of cyclin D3 could increase the cellular translation activity, suggesting that cyclin D3 might be an important translation regulator, and the translation regulating function of cyclin D3 to accelerate protein synthesis did not result from the shortened G1 by cyclin D3 overexpressed, because the same phenomenon was not observed by cyclin D1 or D2 (Fig. 5).

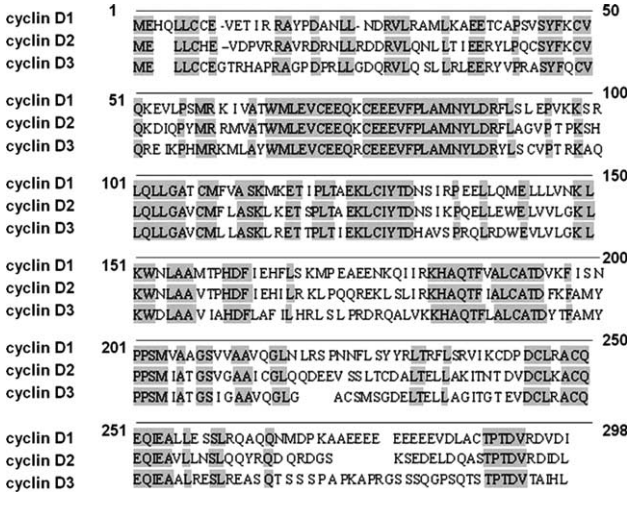

Fig. 6. Sequence alignment of D-type cyclins. The boxes in gray indicate all three identical residues among the sequences of cyclin D1, D2 or D3.

Sequence homology with eIF3k is found in the genomes of *Caenorhabditis elegans*, *Arabidopsis thaliana* and *Drosophila melanogaster*, and a homologous protein has been reported to be present in wheat eIF3 [30]. No sequence similar to eIF3k was found in *S. cerevisiae* and *S. pombe* [30], indicating that eIF3k may play a specialized and important regulatory role in the higher eukaryotic eIF3 complex. Considerable data support the fact that eIF3k is a true subunit of mammalian eIF3. eIF3k, for instance, can interact directly with eIF3c, eIF3g and eIF3j in vitro and form a stable, immunoprecipitatable complex with the eIF3 core [14]. In this study, we also show by confocal microscopy that there are both a cytoplasmic and a nuclear pool of eIF3k. This phenomenon is uncommon. Only several other proteins, such as eIF4E [31,32], eIF4G [33], eIF5A [34] and eIF3f [35] can also be found in the nucleus as well as in the cytoplasm. The data suggest that eIF3k may also have a nuclear function, which is under investigation in our laboratory.

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