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Identification of extracellular signal-regulated kinase 3 as a new interaction partner of cyclin D3

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Abstract

Cyclin D3, like cyclin D1 and D2 isoforms, is a crucial component of the core cell cycle machinery in mammalian cells. It also exhibits its unique properties in many other physiological processes. In the present study, using yeast two-hybrid screening, we identified ERK3, an atypical mitogen-activated protein kinase (MAPK), as a cyclin D3 binding partner. GST pull-down assays showed that cyclin D3 interacts directly and specifically with ERK3 in vitro. The binding of cyclin D3 and ERK3 was further confirmed in vivo by co-immunoprecipitation assay and confocal microscopic analysis. Moreover, carboxy-terminal extension of ERK3 was responsible for its association with intact cyclin D3. These findings further expand distinct roles of cyclin D3 and suggest the potential activity of ERK3 in cell proliferation.

Keywords: Cyclin D3; ERK3; Yeast two-hybrid; Protein interaction

Mammalian D-type cyclins, cyclin D1, D2, and D3, are rate limiting for the progression through G1 phase of cell cycle [1]. These three distinct D-type cyclins share 50–60% identity throughout the coding region and possess a similar function to drive cell cycle [2].

However, the features of individual D-type cyclins are not fully redundant. Compared with cyclin D1 and D2, cyclin D3 has its unique properties in several aspects. Cyclin D3 is shown to be expressed most ubiquitously [3], to be closely implicated in the differentiation of skeletal muscle cells [4–6] or non-muscle cell types [3,7,8], and to be involved in the regulation of apoptosis [9]. Additionally, unlike cyclin D1 or cyclin D2 knock-out mice, cyclin D3-deficient mice display defects in the development of immature T lymphocytes [10,11]. Altogether, these findings suggest that cyclin D3 plays a unique role in many physiological processes. Nevertheless, more detailed non-overlapping potential of this cyclin subtype remains far from being understood.

Extracellular signal-regulated kinase 3 (ERK3) was first characterized by virtue of its homology to MAP kinase ERK1 [12]. Unlike other ERKs, ERK3 is a highly unstable protein that is constitutively degraded by the ubiquitin–proteasome pathway in proliferating cells and also is ubiquitinated on its NH_2 terminus, not on its internal lysine residues [13,14]. Some recent studies suggest a possible role for ERK3 during embryo development and cellular differentiation [15–17]. Notably, nuclear accumulation of ERK3 potentially inhibits cell cycle progression, despite the fact that its subcellular location has been still controversial [13,18,19].

To identify novel cyclin D3 relevant partners that help to elucidate its unique properties, we performed a yeast two-hybrid screen using the full-length human cyclin D3 as bait. In this study, we demonstrate that ERK3, an atypical MAP kinase, interacts with cyclin D3 in vitro and in vivo.

Experimental procedures

Reagents and antibodies. The LexA two-hybrid system and human fetal liver LexA cDNA library were from Clontech. The T7-coupled rabbit reticulocyte lysate system was bought from Promega. Protein G–agarose

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and anti-GFP body were purchased from Roche Applied Science. The monoclonal antibody directed against GAPDH and human cyclin D3 was bought from Oncogene and Pharmingen, respectively. The goat anti-mouse secondary antibodies were from Santa Cruz Biotechnology.

Plasmid construction. The plasmid pLexA-cyclin D3 and cyclin D3 deletion mutants (ΔC1, aa1–88, ΔC2, aa1–153, and ΔN, aa154–292) were described previously [20]. The plasmids pcDNA3.0-GST-cyclin D1, cyclin D2, cyclin D3, and pcDNA3.0-GST have been constructed as previously described [21]. Using the pcDNA3.0-hERK3 plasmid (a gift from Sylvain Meloche, GenBank Accession No. NM_002748) as template, ERK3-ΔC (aa1–347) was amplified by PCR and then cloned into the *Eco*RI/*Xho*I sites of pB42AD. To generate the C-terminal GFP-tagged ERK3, ERK3 was amplified by PCR with no stop codon using pcDNA3.0-hERK3 as template and then ligated to pEGFP-N3. For generation of N-terminally GEP-tagged ERK3, EGFP sequence was obtained by PCR using pEGFP-N3 as template. The PCR products were first subcloned into the *Kpn*I/*Eco*RI sites of pcDNA3.0.The ERK3 coding sequence was then inserted into the *Eco*RI/*Not*I sites of the resulting vector. To construct pcDNA3.0-ERK3-ΔN, pB42AD-ERK3-ΔN (prey) derived from screening was digested with *Eco*RI/*Xho*I enzymes and then, the resulting ERK3-ΔN fragment (amino acid residues 348–675) was inserted into the pcDNA3.0 vector. All constructs were confirmed by DNA sequencing.

Yeast two-hybrid screen and assays. Yeast two-hybrid screen and co-transformation assays were performed according to manufacturer's protocol. Briefly, the bait plasmid, pLexA-cyclin D3, was transformed into the yeast strain EGY48 to generate a stably pLexA/cyclin D3-transformed yeast clone; then this clone was transformed with human fetal liver cDNA library constructed in the pB42AD plasmid. Yeast transformation was performed by the lithium acetate procedure. The resulting transformants were screened on selective medium and the positive clones were isolated for further assays.

In vitro binding assays. The indicated proteins were in vitro produced using T7-coupled rabbit reticulocyte lysate system. As previously described [20], binding assays were performed in binding buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1% glycerol, 10 mM NaF, 0.1% Nonidet P-40, 1 mM $NaVO_4$, 10 μg/m aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF).

Cell culture and transfections. Human SMMC/7721 cells, a hepatocarcinoma cell line (Institute of Cell Biology, Academic Sinica), were maintained in RPMI-1640 medium and transfected with the indicated plasmids using LipofectAMINE reagents according to the manufacturer's recommendations.

Immunoprecipitations and immunoblotting. Cells were harvested in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 60 mM β-glycerophosphate, 1 mM sodium orthovanadate, 20 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF). Then, immunoprecipitations were carried out with indicated antibodies and protein G–agarose beads according to the manufacturer's protocol. The resulted immunoprecipitates were subjected to Western blot analysis according to standard procedures.

Immunofluorescence and confocal assay. Cells grown on coverslips were fixed, permeabilized, and blocked. Then, coverslips were incubated with anti-cyclin D3 antibody followed by rhodamine-conjugated secondary antibody. Coverslips were mounted on glass slides and viewed using a confocal microscope (Zeiss LSM 410). Image acquirement and confocal analysis were performed using Zeiss AIM software.

Results

Isolation of ERK3 as a binding partner of cyclin D3

The yeast two-hybrid screen of human fetal liver cDNA library was employed to identify proteins that interact with cyclin D3. Of approximately 3×10^6 transformants from the initial screen, 22 colonies positive for both two reporters (LEU2 and LacZ) were obtained. Among them, two independent clones encoding the ERK3 cDNA fragment were identified. The isolated ERK3 cDNA clone was truncated at the 5′ end and encoded C-terminal region (residues 348–675, ERK3-ΔN) of ERK3. Purified ERK3 AD fusion plasmid was reintroduced into yeast cells, along with the LexA vector fused to the indicated protein or alone, for co-transformation assays. Similar to the interaction of human p53 and large T-antigen, cyclin D3 interacted with ERK3 as evidenced by the growth of colonies on selective medium and activation of the lacZ reporter gene (Fig. 1A, area 4). No growth was observed in yeast cells transformed with LexA-lamin C or empty LexA vector and ERK3 AD fusion plasmid. Furthermore, yeast co-transformation assays between either full-length or N-terminal region (residues 1–347, ERK3-ΔC) of ERK3 and cyclin D3 were performed (Fig. 1B). The data showed that cyclin D3 also activated both two reporter gene expressions, when co-existing with full-length ERK3, but not with the N-terminus of ERK3. Together, these findings demonstrate that cyclin D3 interacts with the C-terminus of ERK3, but not with the N-terminus in yeast.

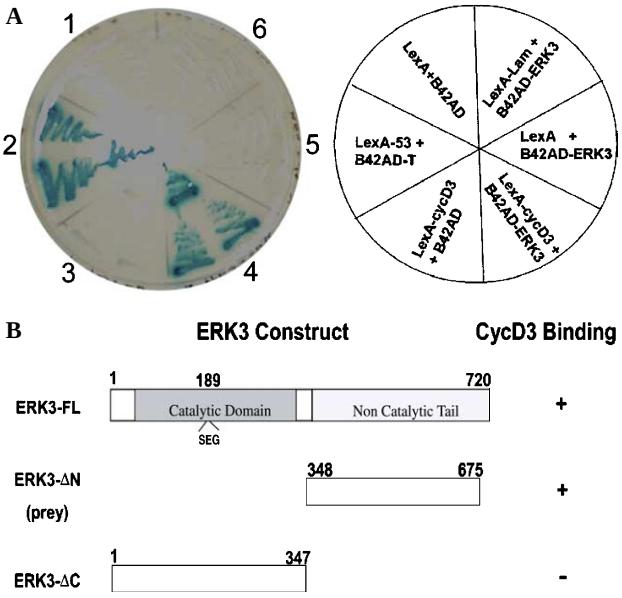

Fig. 1. Interaction of cyclin D3 with ERK3 in yeast two-hybrid system. (A) Cyclin D3 associates with C terminus of ERK3 (ERK3-ΔN) in yeast. Yeast plasmids, plexA and pB42AD that contained the indicated cDNA, were co-transformed into yeast EGY48 and streaked onto selective media (media lacking histidine, tryptophan, leucine, and uracil) in the presence of X-β-gal. The colonies can grow and turn blue ($LEU2^+$/$LacZ^+$), indicating interaction in yeast cells. The interacting proteins, p53 and SV40 large T-antigen, fused with the BD and the AD vectors, respectively, were used as a positive control. Co-transformation with empty vector plexA or plexA-lamin C and BD plasmid was used as a negative control. (B) Schematic representation of ERK3 and interaction in yeast between different domains of ERK3 and cyclin D3. The association between full-length or deletion constructs of ERK3 and cyclin D3 was tested as described in (A). The right column summarizes whether constructs did (+) or did not (−) interact.

Cyclin D3 binds to ERK3 in vitro

To verify protein interactions revealed with the yeast two-hybrid assay, binding assays were performed with in vitro translated, ^{35}S-labeled proteins. ^{35}S-labeled C-terminus (ERK3-ΔN) of ERK3, obtained from two-hybrid screening, was incubated with the GST-fused cyclin D3 or GST alone. As shown in Fig. 2A, C-terminus of ERK3 could bind significantly to GST-fused cyclin D3, but not to GST.

D-type cyclins share high sequence homology and overlapping functions. Thus, we also examined whether ERK3 interacted with additional cyclin D proteins. Likewise, the labeled full-length ERK3 was incubated with GST-fused cyclin D1, D2, D3 or GST alone for binding analysis. Similar to ERK3 C-terminus, full-length ERK3 bound strongly to GST-cyclin D3 (Fig. 2B). Very weak binding with GST-fused cyclin D1, D2, similar in intensity to that seen with GST alone, was observed, most likely representing non-specific association. The specificity of cyclin D3 association with ERK3 was also confirmed by yeast co-transformation assays (data not shown). Altogether, the results indicate that cyclin D3 directly and specifically bound to ERK3.

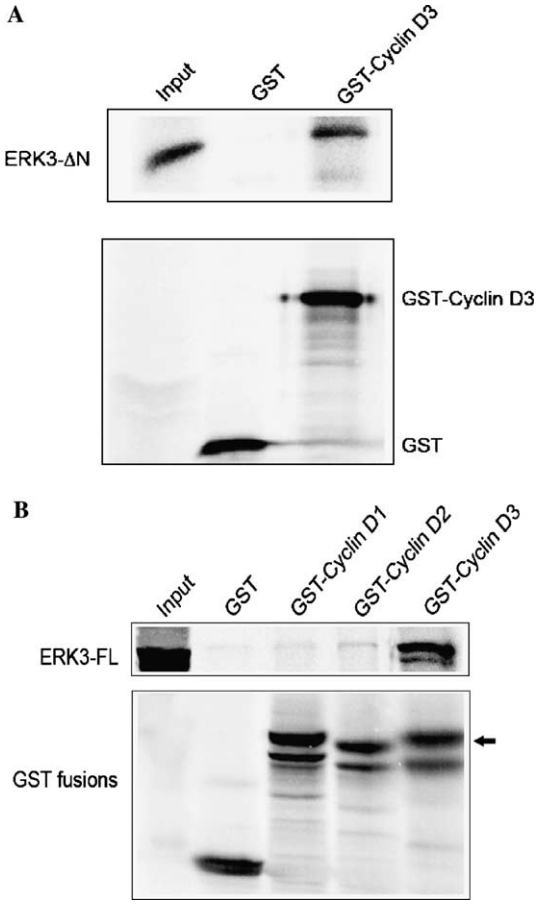

Fig. 2. Binding of cyclin D3 and ERK3 in vitro. (A) In vitro interaction of cyclin D3 with ERK3-ΔN. ^{35}S-labeled ERK3-ΔN was in vitro translated and incubated with a similarly labeled GST or GST-cyclin D3 fusion protein in the presence of glutathione–Sepharose beads. Bound proteins were analyzed by SDS–PAGE followed by autoradiography. Input represents 20% of the starting material. (B) Special interaction of cyclin D3 and full-length ERK3 (ERK3-FL). Where indicated, a similar assay was carried out with ^{35}S-labeled cyclins D1–D3 and ERK3-FL. Lane 1 (upper panel) corresponds to the input (20%). ^{35}S-labeled cyclin D1, D2, and D3 are indicated by the arrow (lower panel).

Cyclin D3 interacts with ERK3 in vivo

As previously reported, ERK3 is an unstable protein. Addition of large N-terminal tag stabilizes, and thus dramatically augments the expression of ERK3 [14]. According to this, N-terminally or C-terminally GFP-tagged ERK3 was constructed and transfected into cells, followed by immunoblotting. In agreement with what was previous-

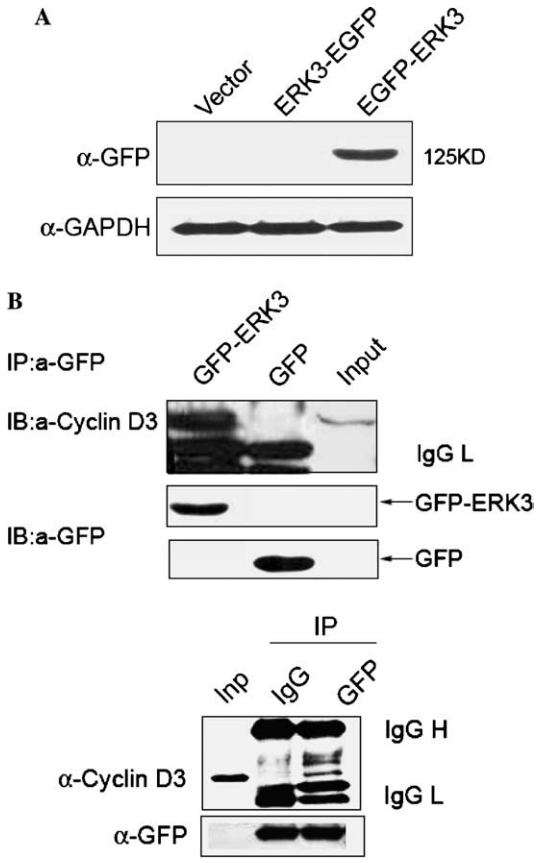

Fig. 3. Association of cyclin D3 and ERK3 in vivo. (A) Expression of ectopic ERK3 in SMMC/7721 cells. N-terminally or C-terminally GFP-tagged ERK3 (designated as EGFP-ERK3 or ERK3-EGFP) and GFP vector were transiently transfected into 7721 cells. After 48 h, total cell extracts were analyzed by immunoblotting using antibodies against GFP (upper panel) or GAPDH (lower panel) as a loading control. (B) Co-immunoprecipitation assay of cyclin D3 and ERK3. 7721 cells were transfected with EGFP-ERK3 fusion vector or GFP vector (control) and cell lysates were immunoprecipitated with anti-GFP antibody. Immunoprecipitates were analyzed by Western blotting using either anti-cyclin D3 or anti-GFP antibody (upper panels). Similarly, lysates of 7721 cells transfected with EGFP-ERK3 fusion vector were immunoprecipitated using anti-GFP antibody, followed by immunoblotting of the indicated antibodies. Herein, control immunoprecipitations were performed using mouse normal IgG (lower panels). Input represents 10% whole cell lysates. IgG L and IgG H denote IgG light chain and heavy chain, respectively.

ly observed [14], N-terminally GFP-tagged ERK3 (EGFP-ERK3) was expressed at high levels while fusion of GFP to C-terminus was insufficient to stabilize ERK3 (Fig. 3A).

Next, the interaction of cyclin D3 with ERK3 was further investigated in co-immunoprecipitation experiments using 7721 cells overexpressing EGFP-ERK3 or GFP. The whole cell lysates were immunoprecipitated with GFP antibody followed by immunoblotting using cyclin D3 antibody. It was shown that cyclin D3 was co-precipitated from cells transfected with EGFP-ERK3, but not from the cells transfected with GFP vector (Fig. 3B). Alternatively, EGFP-ERK3 transfected cells were immunoprecipitated with GFP antibody or control IgG, respectively, and the precipitates were examined for the presence of cyclin D3. As expected, cyclin D3 was clearly detected when precipitated with anti-GFP antibody, but not when precipitated with control antibody (Fig. 3B). Thus, these data confirm that ERK3 and cyclin D3 interact in vivo.

Co-localization of cyclin D3 and ERK3 in mammalian cells

To further characterize the interaction of cyclin D3 and ERK3 in vivo, EGFP-ERK3 or GFP vector was transfected into SMMC/7721 cells. The subcellular distribution of cyclin D3 and ERK3 was determined by immunofluorescence with cyclin D3 antibody or by direct observation of GFP fluorescence. As described previously [18], ERK3 was evenly distributed in the cytoplasmic and nuclear compartments in many types of cells. Cyclin D3 also exhibited a diffuse distribution in the cells. Merging of the two images showed a yellow color in the cells transfected with EGFP-ERK3, but not in the cells transfected with GFP (Fig. 4). The data showed the co-localization of cyclin D3 and ERK3, confirming in vivo interaction of both two proteins.

Mapping of the cyclin D3 region required for the interaction with ERK3

We have showed that the C-terminal truncated form of ERK3 is able to associate with cyclin D3. Then, we investigated the cyclin D3 region involved in the interaction with ERK3. Similar to cyclin D1 [22], the functional domains of cyclin D3 are schematically shown (Fig. 5). Accordingly, we generated three truncated constructs of cyclin D3: ΔC1 (amino acids 1–88), ΔC2 (amino acids 1–153), and ΔN (amino acids 154–292). These constructs were co-transformed with either ERK3 AD fusion plasmid or empty AD vector into yeast cells and transformants were analyzed for growth of colonies and activation of β-galactosidase. No interaction was measured between individual deletion constructs of cyclin D3 and ERK3, suggesting that the full length of cyclin D3 might be necessary for its binding to ERK3.

Discussion

In the present study, we isolated ERK3 as a binding partner of cyclin D3. The interaction between cyclin D3 and ERK3 was confirmed both in vitro and in vivo. We also demonstrated that cyclin D3 interacted specifically with ERK3. Furthermore, we identified C-terminal

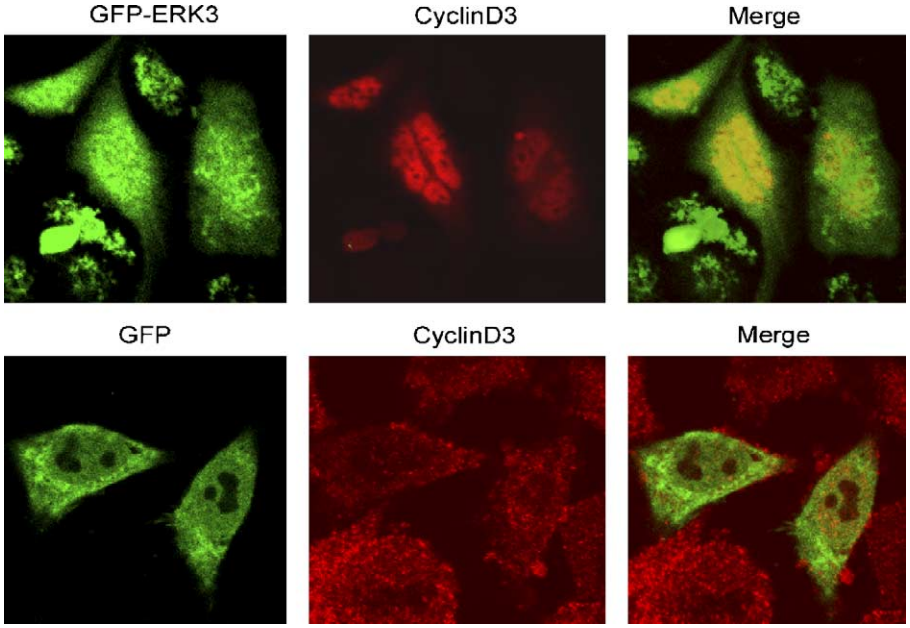

Fig. 4. Confocal microscopic analysis of cyclin D3 association with ERK3. SMMC/7721 cells cultured on coverslips were transfected with expression vectors encoding either EGFP-ERK3 or GFP (control). After 48 h, cells were processed for immunofluorescence. The images were captured with a confocal microscope and software provided by Carl Zeiss. EGFP-EKR3 or GFP was directly visualized (green channels, on left panels), and cyclin D3 was visualized by staining with an anti-cyclin D3 antibody (red channels, on middle panels). The merged image of green and red channels is shown (right panels). These images are representative cells over the entire field. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

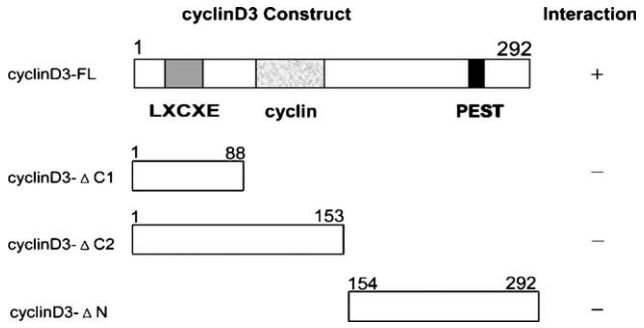

Fig. 5. Mapping of cyclin D3 regions responsible for the association with ERK3. Schematic representation of the full length of cyclin D3 is shown. Several functional domains in cyclin D3 described previously, including a LXCXE motif, a cyclin box, and a putative C-terminal PEST sequence, are indicated. The corresponding truncated mutants of cyclin D3 were constructed in the pLexA vector. Yeast co-transformation assays were performed with full-length or truncated mutants of cyclin D3 subcloned into pLexA vector and pB42AD-ERK3 plasmid, respectively. The interactions were scored positive (+) or negative (−) based on cell growth and β-galactosidase assay.

extension of ERK3 as interaction region and also showed that the entire structure of cyclin D3 was required for its interaction with ERK3.

As mentioned above, cyclin D3 has additional properties not found in the related isoforms cyclin D1 and cyclin D2. Besides, there have been some reports of specific interaction between cyclin D3 and its binding partners, also suggesting distinct roles of cyclin D3. Recently, it has been shown that both RAR and CRABPII are associated with cyclin D3, but not with cyclin D1 or cyclin D2 [23]. Our previous findings have demonstrated that only cyclin D3 functions as a regulatory partner of $p58^{PITSLRE}$ kinase in G2/M phase [21]. Our more recent studies have also indicated that specific binding of cyclin D3 to regulatory factors, such as VDR, ATF5, and eIF3k is involved in modulation of cellular transcription or translation activity [20,24,25]. Here, we show that cyclin D3 was the only D-type cyclin that could interact physically with ERK3, further extending previous observations that cyclin D3 possessed its distinct features.

Compared with the classical MAP kinases ERK1/2, ERK3 displays several unique features structurally. Most notably, the highly conserved TEY motif in activation loop of ERK1/2 is substituted by SEG in that of ERK3 and ERK3 has a long C-terminal extension, which is absent in other MAP kinases [12,26,27]. Recently, two independent groups have reported that C-terminal extension of ERK3 is necessary for ERK3 association with MK5 as well as for MK5 translocation and activation [15,17]. Another recent study has revealed that ERK3 C-terminus is responsible for directing its subcellular location. [19]. Similarly, the results presented here indicate that the carboxy-terminal region (amino acids 348–675) of ERK3 is capable of binding to cyclin D3, whereas the amino-terminal region is not. Collectively, carboxy-terminal extension, which is unique to ERK3, may result in its special functions.

ERK1/2 is mainly regulated by their phosphorylation and subcellular location after extracellular stimulation and thereby controls various physiological processes, including cell division cycle [28,29]. Remarkably, ERK3 is also found to be related with cell cycle progression, although its regulation, determined at least in part by protein stability, differs from ERK1/2 regulation. Ectopic expression of ERK3 markedly inhibits S phase entry in NIH3T3 cells, suggesting that ERK3 functions as a negative regulator of cell cycle [13]. Nuclear accumulation of ERK3 apparently causes cell cycle arrest, whereas the accumulation of ERK3 in cytoplasm decreases the ability [18]. Bind et al. have also reported that enrichment of ERK3 in nuclear is cell cycle dependent [19]. We demonstrate here that ERK3 was able to associate with cyclin D3, a specific cyclin of G1 phase, providing a clue for the possible activity of ERK3 in cell cycle progression. These observations strongly suggest the potential roles of ERK3 in cell proliferation. Briefly, we have showed in the study that cyclin D3 interacted specifically with an atypical MAP kinase ERK3. The functional consequences of the interaction need to be further investigated to elucidate the involvement of ERK3 in cell cycle regulation.

Acknowledgments

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