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Identification of cyclin D3 as a new interaction partner of lamin A/C

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Abstract

Lamin A/C is a major component of the nuclear lamina. An intact nuclear lamina has been proposed to be necessary for muscle differentiation. Cyclin D3 is known to be upregulated in differentiated muscle cells and to form insoluble complexes with cell-cycle regulatory factors in these cells. We have examined the possibility of direct binding interactions between lamin A/C and cyclin D3 by in vitro binding assays and co-immunoprecipitation studies with muscle cells. Our results indicate that cyclin D3 binds specifically to amino acid residues 383–474 of lamin A/C and associates with lamin A/C in muscle cells. The identification of cyclin D3 as a novel binding partner of lamin A/C has important implications for a role for lamin A/C in muscle differentiation. © 2007 Elsevier Inc. All rights reserved.

Keywords: Nuclear lamina; Lamin A; Cyclin D3; Muscle differentiation

The major structural components of the nucleus are a group of proteins termed the lamins, which belong to the intermediate filament superfamily of proteins. Lamins are important for nuclear integrity and are involved in the organization of nuclear processes; increasing evidence suggests that lamins can also interact directly with proteins that function in diverse cellular pathways [1,2]. The A-type lamins (A and C) are 3' alternately spliced forms of the lamin A gene and have been detected primarily in differentiated cells. Mutations in the human lamin A/C gene have been linked to at least 10 highly degenerative, heritable disorders. As the majority of mutations cause Emery-Dreifuss muscular dystrophy (EDMD), there has been considerable interest in understanding a possible role for lamin A/C in muscle differentiation. Lamin A/C knock-out mice develop muscular dystrophy symptoms resembling EDMD [3]. Myoblasts from $Lmna^{-/-}$ mice show impaired differentiation [4] and expression of lamin A mutants results in inhibition of myoblast differentiation [2,5,6].

We have earlier shown that internal lamins are reorganized to a diffuse network during the differentiation of C2C12 myoblasts [7]. This process required cyclin D3 and retinoblastoma protein (pRb), which were sequestered on the insoluble nuclear matrix [8]. Cyclin D3 behaves as an atypical cyclin in muscle cells as it is activated severalfold when myoblasts differentiate [9], and a role for cyclin D3 in the induction and/or maintenance of terminal differentiation in both muscle and non-muscle cells has been proposed [10]. In this study, we have examined the possibility that cyclin D3 binds directly to lamin A/C by carrying out in vitro binding assays and co-immunoprecipitation experiments. Our data indicates that cyclin D3 binds specifically to amino acid residues 383–474 of lamin A/C and interacts with lamin A/C in muscle cells.

Materials and methods

Plasmid constructs. Restriction fragments of lamin A cDNA were subcloned into pGEX vectors as described earlier [11]. Restriction fragments of cyclin D3 (from pRC-CMV-cycD3-HA, provided by P. Hinds, Harvard Medical School, MA) and the cyclin D3 K112E mutant [8] were subcloned into pET vectors.

In vitro binding assays. Recombinant pGEX and pET clones were expressed in *Escherichia coli* BL21(DE3)-pLys(S). Cells expressing pGEX clones were lysed in 100 mM EDTA, 1% Triton X-100, 10 mM sodium

Abbreviations: Cdk, cyclin-dependent kinase; EDMD, Emery–Dreifuss muscular dystrophy; GST, glutathione-S-transferase; pRb, retinoblastoma protein.

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phosphate buffer, pH 7.2, 150 mM NaCl, 1 mM PMSF, 0.5 mM DTT, and protease inhibitors. The supernatants were incubated with glutathione agarose beads for 2–3 h at 4 °C, centrifuged and washed with lysis buffer. Subsequently, cultures of induced pET clones were lysed in 20 mM Hepes, pH 7.2, 0.4 mM EDTA, 1% Triton X-100, 10 mM NaF, 150 mM NaCl, 0.5 mM Na₃VO₄, 1 mM PMSF, 0.5 mM DTT, and protease inhibitors. The supernatants were incubated with equal quantities of beads bound to either GST or GST-fusion proteins for 3 h at 4 °C, centrifuged and washed with lysis buffer, separated by SDS–PAGE and analyzed by immunoblotting.

Cell culture, extraction and DNA transfections. C2C12 mouse skeletal myoblasts were maintained in DMEM containing 20% FBS and induced to differentiate by transfer to DMEM containing 2% horse serum for 48 h. Cells plated on coverslips were extracted with 0.4% Triton X-100, followed by nucleases and 2 M NaCl as described [12], fixed with formaldehyde and immunostained. Transient transfection of myoblasts with pRC-CMV-cycD3-HA was carried out with Lipofectamine (Invitrogen).

Immunochemical techniques. Immunofluorescence assays were carried out as described [8]. Samples were viewed on a Zeiss LSM510 META confocal microscope (Carl Zeiss, Oberkochen, Germany). Immunoblot analysis was carried out as described [8]. For immunoprecipitation assays, the procedure described by Capanni et al. [13] was used with small modifications. Briefly, cells were lysed with cold buffer containing 50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 5 mM ATP, 1 mM PMSF, 50 mM NaF, 0.1 mM Na₃VO₄, and protease inhibitors. The precleared samples were immunoprecipitated with primary antibody followed by protein A-sepharose beads and the bound proteins were analyzed by immunoblotting. The primary antibodies used were polyclonal antibodies to lamin A/C or HA epitope tag from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), mouse monoclonal antibodies to cyclin D3 from Cell Signaling Technology (Beverly, MA) and to the T7 fusion peptide from Novagen (Madison, WI).

Results

In vitro interactions of lamin A and cyclin D3

We initially determined whether cyclin D3 could bind directly to lamin A in a solid-phase binding assay. When bacterial lysates of pET-expressed cyclin D3 were incubated with full-length GST-lamin A bound to glutathione agarose beads, cyclin D3 was detected in the bound fraction (Fig. 1A and B). This interaction was specific as binding was not observed with GST alone. To delineate the binding site on lamin A, GST was fused with deletion fragments of lamin A encompassing different domains [1,2,14]. In binding assays with these fragments, the smallest fragment of lamin A which demonstrated specific binding to cyclin D3 was narrowed down to amino acid residues 383–474 (Fig. 1A and B). This region is common to both lamin A and C as lamin C encompasses residues 1–566 of lamin A followed by six unique residues.

Next, various deletion and mutant constructs of cyclin D3 were expressed in the pET vector and binding assays were carried out with GST-lamin 356–665 (which was convenient to use experimentally as it was more soluble than full-length lamin A). Both wild-type cyclin D3 and the kinase inactive K112E mutant associated specifically with GST-lamin 356–665, whereas binding to lysates expressing the empty pET vector was negligible (Fig. 2). Also, both N-terminal and C-terminal deletion fragments of cyclin D3 displayed binding to GST-lamin 356–665.

Association of cyclin D3 with lamin A/C in mammalian cells

Although endogenous cyclin D3 is known to form insoluble complexes with cell cycle regulatory factors in differentiated muscle cells [15], its pattern of localization in nuclear matrices prepared from differentiated cells has not been reported. We have analyzed the expression and location of endogenous cyclin D3 in C2C12 myotubes that have been extracted with detergent and treated with nucleases and salt to remove soluble nuclear components. Cyclin D3 was detected in a punctate stained pattern in insoluble nuclear matrix preparations, at levels comparable to those observed in unextracted differentiated cells (Fig. 3). Lamin A/C was detected in a typical pattern at the nuclear rim. Extracted samples were observed to be depleted of DNA as a negative control. Myoblasts expressed very low levels of cyclin D3, as expected.

To determine whether cyclin D3 interacted with lamin A/C in vivo, lysates of myotubes were immunoprecipitated



Fig. 1. Binding assays with lamin A fragments. (A) Coomassie-blue stained gel of purified GST-lamin fragments. (B) Immunoblot of bound cyclin D3 probed with anti-T7 antibody. (C) Summary of data. Molecular mass markers are: phosphorylase b, 94 kDa; albumin 67 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 30 kDa.



Fig. 2. Binding assays with cyclin D3 fragments. (A) Input cyclin D3 fragments and (B) cyclin D3 fragments bound to GST-lamin 356–665, detected by immunoblotting with anti-T7 antibody. (C) Summary of data. Asterisk, non-specific protein.



Fig. 3. Association of cyclin D3 with the nuclear matrix. Immunostaining of cyclin D3 and lamin A/C in unextracted and extracted samples of (A) C2C12 myotubes and (B) myoblasts. Bar, 10 μ m.



Fig. 4. Co-immunoprecipitation of cyclin D3 and lamin A/C. Lysates from (A) C2C12 myotubes or (B) cyclin D3-transfected myoblasts were immunoprecipitated with antibodies to lamin A/C, HA tag or control IgG and analyzed by immunoblotting with the indicated antibodies. Asterisk, IgG.

with anti-lamin A/C antibody and analyzed by Western blotting for the presence of cyclin D3. Endogenous cyclin D3 was detectable in the lamin A/C immunoprecipitate,

as shown in Fig. 4. These proteins were not immunoprecipitated with purified normal IgG used as a control, thus confirming the specificity of the interaction between cyclin D3 and lamin A/C. In further experiments, lysates of myoblasts ectopically expressing HA-tagged cyclin D3 were immunoprecipitated with antibodies to HA or lamin A/C in separate reactions and analyzed by Western blotting. Both cyclin D3 and lamins A and C were detectable in the HA immunoprecipitate. Conversely, lamins A and C as well as cyclin D3 were detectable in the lamin A/C immunoprecipitate. These data indicate that cyclin D3 and lamin A/C interact in vivo.

Discussion

Our present findings demonstrate that cyclin D3 binds to amino acid residues 383–474 of lamin A/C in in vitro binding assays. Furthermore, this interaction is physiologically relevant as endogenous lamin A/C and cyclin D3 are co-immunoprecipitated from mammalian cell lysates.

Lamin A/C has been shown to bind to gene regulatory factors such as pRb, SREBP1, MOK2, and c-Fos and modulate their activities [16-19]. The binding region for both pRb and MOK2 spans coil 2 of the rod domain, whereas the binding site for c-Fos has been narrowed down to the Ig-fold domain (436-544). The smallest fragment, lamin 383-474, that we have determined to bind to cyclin D3 is in the globular tail domain in a region common to lamin A and C, and excludes the rod domain as well as most of the Ig-fold. This segment includes the NLS for lamin A/C at position 417–422 as well as a putative chromatin-binding domain between residues 396-430 [20]. Interestingly, the segment from 386–402, which is rich in arginine residues, has been proposed to be involved in associations of lamin dimers [21]. Furthermore, phosphorylation of the critical Ser 392 residue during mitosis causes dissociation of linear lamin polymers [22]. The arginine residues at 386 and 453 positions are critical for physiological functions as the R386K and R453W mutations cause EDMD [23]. Expression of these mutants has deleterious effects on muscle differentiation in cultured cells [2,5]; moreover, R386K assembles into large aggregates, leading to abnormal nuclear morphology. Thus, the 383-474 segment is an important region for protein-protein interactions.

Cyclin D3 associates with a number of cell cycle regulatory proteins such as cdks, cdk inhibitors, and pRb [15,24]. Binding of cyclin D3 has also been demonstrated for the small retinoic acid-binding protein, CRABPII [25], the p28 subunit of eukaryotic initiation factor 3 (eIF3k) [26], and p58^{PITSLRE}, a G2/M specific kinase [27]. Our binding data indicates that segment 76–292 of cyclin D3, which lacks the pRb-binding site at residues 3–7 [24] is able to bind to lamin A/C. Thus, the region of cyclin D3 that binds to lamin A/C does not overlap with the pRb-binding domain of cyclin D3.

In earlier studies, pRb has been shown to bind to residues 247–355 of lamin A/C [16]. Our finding that cyclin D3 binds to residues 383–474 of lamin A/C indicates that pRb and cyclin D3 bind to non-overlapping, contiguous

regions of lamin A/C. Thus our binding data and co-immunoprecipitation analysis are consistent with the formation of a multimeric complex of cyclin D3, pRb and lamin A/ C in muscle cells, which has important implications for a role for lamin A/C in muscle differentiation.

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