Cyclin D1-Cdk4 Induce Runx2 Ubiquitination and Degradation*

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Run Shen†, Xiumei Wang‡, Hicham Drissi§, Fang Liu†, Regis J. O’Keefe†, and Di Chen††

From the †Department of Orthopaedics, Center for Musculoskeletal Research, University of Rochester School of Medicine, Rochester, New York 14642 and the ‡Center for Advanced Biotechnology and Medicine, Rutgers, the State University of New Jersey, Piscataway, New Jersey 08854

Runx2 is a Runt domain transcription factor involved in the activation of genes encoding osteoblast and chondrocyte-specific proteins. Runx2 activity is regulated by transcriptional and post-transcriptional mechanisms. The functional significance of the post-translational modification of Runx2 has not been fully defined. We show that cyclin D1-Cdk4 induce Runx2 degradation in an ubiquitination-proteasome-dependent manner. Mutagenesis of Runx2 serine-472, a consensus Cdk site, to alanine increases the half-life of Runx2 and causes loss of sensitivity to cyclin D1-induced Runx2 degradation. The targeted Runx2 degradation by cyclin D1 identifies a novel mechanism through which Runx2 activity is regulated coordinately with the cell cycle machinery in bone cells.

Runx22 (runt-related gene 2) is a bone- and cartilage-specific transcription factor that belongs to the Runx family (1). The DNA-binding sites for Runx2 have been identified in the promoter regions of many osteoblast- and chondrocyte-specific genes (2–9). Runx2 binds to specific response elements in the promoters of Runx2 target genes and regulates the transcription of these genes. Runx2+/− mice die following birth and lack bone formation due to the absence of mature osteoblasts (10, 11). Heterozygous mutant mice have skeletal abnormalities, similar to those seen in a human mutation called cleidocranial dysplasia syndrome (12, 13) and have delayed development of intramembranous bones (10, 11).

Additional studies demonstrate delayed chondrocyte maturation in Runx2+/− mice (14, 15), suggesting that Runx2 also promotes chondrocyte differentiation. Dominant-negative Runx2 inhibits chondrocyte maturation (16, 17), whereas overexpression of Runx2 in chondrocytes restores chondrocyte maturation in Runx2+/− mice (18). Recent studies demonstrate that chondrocyte maturation was completely absent in Runx2 and Runx2 double knock-out mice (19), demonstrating that Runx2 and Runx3 are functionally redundant and play an essential role in chondrocyte maturation.

The mechanism through which Runx2 induces osteoblast and chondrocyte differentiation involves both the withdrawal from cell cycle and the activation of osteoblast and chondrocyte-specific genes. Runx2 is expressed in proliferating osteoblasts and chondrocytes and causes efficient withdrawal from the cell cycle prior to the differentiation process.

Recent data demonstrate a direct link between Runx2 and cell cycle regulation (20).

Cell cycle regulation is controlled by a combination of cyclins, Cdkks, and Cdk inhibitors, which together with the tumor suppressor retinoblastoma (Rb) are involved in the tight control of cell cycle machinery. Cdkks, in association with their regulatory partners, the cyclins, are key regulators of cell cycle progression. Cyclin D (D1, D2, D3)-Cdk4/Cdk6 and cyclin A/E-Cdk2 are involved in regulation of the G1/S transition (21, 22). Cyclin D-Cdk4/Cdk6 promotes the phosphorylation of Rb. The hypophosphorylated Rb proteins are known to inhibit the function of the E2F proteins, which promote transcription of factors essential for DNA synthesis (23). Thus, phosphorylation of Rb by the cyclin D-Cdk complexes relieves inhibition of Rb on the E2F function, promoting the entry of cells into S phase.

Recent observations implicate that in addition to their function as cell cycle regulators, the cyclin D proteins also serve as regulators for transcription factors and signaling proteins (24–26). In this report, we show that cyclin D1-Cdk4 induces Runx2 phosphorylation, ubiquitination, and proteasome degradation and thereby inhibits differentiation while stimulating proliferation.

MATERIALS AND METHODS

Cell Culture and Transfection—COS and C3H10T1/2 cells were cultured in Dulbecco’s modified Eagle’s medium and supplemented with 10% fetal calf serum at 37 °C under 5% CO2 condition. DNA plasmids were transiently transfected into COS or C3H10T1/2 cells in 6-cm culture dishes using the Lipofectamine 2000 reagents (Invitrogen). Empty vector was used to keep the total amount of DNA transfected constant. FLAG-EGFP plasmid was co-transfected as an internal control for transfection efficiency. Western blot and immunoprecipitation (IP) assays were performed 24 h after transfection.

Western Blot Analysis and IP Assays—Western blot and IP assays were performed as described previously (27).

PCR-based Site-directed Mutagenesis—FLAG-tagged mouse Runx2 cDNA (MASN isoform, NCBI accession number: NM_009820) was amplified by PCR, sequenced, and cloned into pcDNA3 and pCMV-Tag2 expression vectors (Stratagene, La Jolla, CA). Mutant Runx2 constructs (SA-Runx2 and SE-Runx2) were generated using Stratagene QuikChange site-directed mutagenesis kit and cloned into pcDNA3 and pCMV-Tag2 vector (Stratagene).

In Vivo Protein Decay Assay—Cells were seeded in 15-cm culture dishes, and equal amounts of FLAG-Runx2, FLAG-SA-Runx2, and FLAG-SE-Runx2 were transfected, respectively. 24 h after transfection, cells were trypsinized and split into five 10-cm dishes. 12 h after recovery, each sample was cultured in regular medium with 80 μg/ml cycloheximide (Calbiochem), and samples from time 0, 20, 60, 120, and 300 min were harvested. Western blot was performed to detect the decay of wild-type (WT) and mutant Runx2 proteins.
Cyclin D1 Targets the C-terminal Domain of Runx2—A putative SP motif was identified in the C terminus of the murine Runx2 protein at amino acids 472–475 (MASN isoform, amino acid 1–528). To determine whether the C-terminal region of Runx2 is important for the protein stability, a truncated Runx2 construct with a C-terminal 79-amino-acid deletion was generated (mRunx2, 1–449). We found that the mRunx2-(1–449) was more stable than WT-Runx2 in COS cells (Fig. 1c). When cyclin D1 was co-transfected with WT or mutant Runx2, cyclin D1 induced the degradation of WT but not mutant Runx2 containing a C-terminal 79-amino-acid deletion (Fig. 1d). These results suggest that the C-terminal region of Runx2 is important for Runx2 protein stability.

Serine-472 Is Required for Runx2 Degradation—To determine the importance of the serine-472 residue for Runx2 stability, site-directed mutagenesis was used to replace serine-472 with alanine (SA-Runx2) or glutamic acid (SE-Runx2). Prior work has established that replacement of serine with alanine abolishes phosphorylation, whereas replacement with glutamic acid mimics constitutive phosphorylation of cyclin-Cdk substrate proteins (26, 30). As expected, the Western blot analysis revealed that the SA-Runx2 was more stable than WT-Runx2 and that the SE-Runx2 was the most unstable in COS cells and mesenchymal pluripotent C3H10T1/2 cells (Fig. 2a). These results were further confirmed by a proteolytic decay assay. Runx2 protein levels were significantly reduced at 300 min after protein synthesis was stopped by the treatment of cells with a protein synthesis inhibitor, cycloheximide. The SA-Runx2 was stable during the entire 300-min period after cells were treated with cycloheximide. In contrast, the SE-Runx2 was significantly degraded at the 60–120-min period after cells were treated with cycloheximide (Fig. 2b). To determine whether the serine-472 residue of Runx2 plays a critical role in cyclin D1-induced Runx2 degradation, cyclin D1 expression plasmid was co-transfected with either WT- or SA-Runx2 plasmid into COS cells. Cyclin D1 induced WT-Runx2 degradation in a dose-dependent manner (Fig. 2c). In contrast, cyclin D1 had no effect on SA-Runx2 degradation (Fig. 2d). These results indicate that serine-472 is critical for Runx2 stability.

Cyclin D1 Induces Runx2 Phosphorylation—We then examined the role of cyclin D1 in Runx2 phosphorylation. Cyclin D1 plasmid was co-transfected with FLAG-Runx2 or FLAG-SA-Runx2 plasmids into COS cells, and IP was performed with the anti-FLAG antibody followed by the Western blot using the anti-phosphoserine antibody. Although WT-Runx2 was phosphorylated, minimal phosphorylation of SA-Runx2 was observed (Fig. 3a). To confirm this finding, GST–Runx2 and GST-SA-Runx2 fusion proteins were generated, and the in vitro phosphorylation assay was performed. The GST–Rb-(379–928) was used as a positive control, and GST–SmaD4 was used as a negative control (Matsura et al. (26)). The in vitro phosphorylation was induced by the incubation of recombinant proteins with purified cyclin D1-Cdk4 enzymes. Although strong phosphorylation was detected for WT-Runx2, a weak phosphorylation was detected for SA-Runx2. As reported previously, Rb protein was phosphorylated by cyclin D1-Cdk4 in this assay, but SmaD4 protein was not phosphorylated (Fig. 3b). The results demonstrate serine-472 as a target for cyclin D1-Cdk4-induced Runx2 phosphorylation.

Cyclin D1-induced Runx2 Degradation Is Ubiquitin-Proteasome-dependent—To determine the role of cyclin D1 in Runx2 ubiquitination, FLAG-Runx2 plasmid was co-transfected with cyclin D1 in the presence or absence of proteasome inhibitor 1 (PS1). The IP was performed using the anti-FLAG antibody, and Runx2 ubiquitination was detected by Western blot using an anti-HA antibody. Cyclin D1 significantly induced Runx2 ubiquitination. In the presence of PS1,
cyclin D1-induced Runx2 ubiquitination was increased (Fig. 3c). To determine the role of serine-472 in Runx2 ubiquitination, the expression plasmids of WT-, SA-, and SE-Runx2 were co-transfected with HA-ubiquitin into COS cells. The ubiquitinated protein ladder of WT-Runx2 was detected. When the SE-Runx2 was immunoprecipitated, a stronger Runx2 ubiquitination band was observed. In contrast, no ubiquitination was detected when SA-Runx2 was immunoprecipitated (Fig. 3d). To determine whether the serine-472 site is critical for Runx2 degradation, COS cells were transfected with WT- and SA-Runx2 and then treated with PS1. The results showed that treatment with PS1 increased the protein level of WT-Runx2 but not SA-Runx2 (Fig. 3f). These results further demonstrate that cyclin D1 induces Runx2 ubiquitination and that proteasome-dependent degradation and serine-472 is critical for cyclin D1-induced Runx2 degradation.

**The Biological Significance of Cyclin D1-induced Runx2 Degradation**—To determine whether the cyclin D1-induced Runx2 degradation alters Runx2 transcriptional activity, Runx2 expression plasmid was co-transfected with different amounts of cyclin D1 plasmid and the Runx2 reporter, 6xOSE2-Luc, into C3H10T1/2 cells. Although Runx2 significantly increased reporter activity (6-fold), co-transfec-
tion with cyclin D1 dose-dependently inhibited Runx2-induced reporter activity (Fig. 4a). To determine the effect of serine-472 on Runx2 transcriptional activity, WT-, SA-, and SE-Runx2 expression plasmids were co-transfected with 6xOSE2-Luc reporter into C3H10T1/2 cells. The SA-Runx2 had higher activity, and SE-Runx2 had lower activity compared with WT-Runx2 (Fig. 4b). The results demonstrate that cyclin D1 induces Runx2 degradation, leading to changes in Runx2 transcriptional activity. Runx2 plays a key role in osteoblast differentiation and regulates the expression of osteoblast marker genes such as ALP. To further determine the biological significance of serine-472 of Runx2, WT-, SA-, and SE-Runx2 expression plasmids were co-transfected into C3H10T1/2 cells, and changes in ALP mRNA expression and ALP activity were examined. We found that transfection of WT-Runx2 significantly stimulated ALP mRNA expression (2.83-fold increase) and activity (3.56-fold increase) in C3H10T1/2 cells. SA-Runx2 had much higher stimulatory effects on ALP mRNA expression (8.1-fold increase) and activity (11.63-fold increase). In contrast, the effects of SE-Runx2 on ALP mRNA expression and activity were 66 and 72% lower, respectively, when compared with WT-Runx2 transfection group (Fig. 4c). These results demonstrate that Ser-472 of Runx2 plays an important role in Runx2-induced osteoblast differentiation, p27 expression, and cell cycle regulation.

**DISCUSSION**

In the present studies, we demonstrate for the first time that cell cycle proteins regulate the key transcription factor Runx2 by inducing its phosphorylation and degradation through the ubiquitin-proteasome pathway. The serine-472 residue plays a critical role in cyclin D1-induced Runx2 phosphorylation and subsequent ubiquitination and degradation. Furthermore, cyclin D1-Cdk4 controls Runx2 transcriptional activity and physiological function in osteogenic progenitor C3H10T1/2 cells.

Although regulation by cell cycle proteins has not been characterized for Runx2 in bone and cartilage, regulation of differentiation-associated transcription factors by cell cycle proteins has been well established in other cell types, such as myoblasts. MyoD is a key transcription factor.
controlling the myoblast differentiation. Its phosphorylation and degradation is controlled and regulated by cyclin B-Cdk1/Cdk2. Cyclin B induces phosphorylation of serine-200 of MyoD protein and triggers MyoD ubiquitination and rapid degradation (32, 33).

An inverse relationship between proliferation and differentiation has been reported in many developmental processes, such as myogenesis and neural development. Previous findings suggest that this inverse relationship is regulated at different levels. For example, cyclin-Cdk complexes phosphorylate MyoD and induce its degradation (10). Forced expression of MyoD induces p21 expression in myoblasts (11). In addition, hypophosphorylated Rb acts as a co-repressor for E2F-induced gene expression of cell cycle proteins, and it also serves as a co-activator for Runx2-induced osteoblast differentiation (34). In the present studies, we report that cyclin D1-Cdk4 directly phosphorylate Runx2 and induce Runx2 ubiquitination and proteasome degradation, suggesting that coordinated relationship between proliferation and dif-

FIGURE 3. Cyclin D1 Induces Runx2 phosphorylation, ubiquitination (Ub), and proteasome degradation. a, equal amounts of FLAG-Runx2 and SA-Runx2 plasmids were transfected into COS cells and then treated with PS1 (10 μM, 4-hour incubation). Cell lysates were extracted 24 h after transfection. Phosphorylation of Runx2 was detected by Western blot (WB) with the anti-phospho-serine antibody after IP with the anti-FLAG antibody. Mutation of serine-472 into alanine dramatically decreases the phosphorylation of Runx2 protein. P-Runx2, phosphorylated Runx2; F-FLAG, b, recombinant GST-Rb-(379–928), GST-Runx2, GST-SA-Runx2, and GST-Smad4 were mixed with equal amounts of cyclin D1-Cdk4 enzyme and incubated for 40 min at 30 °C, and then the reactions were stopped by sample buffer and subjected to SDS-PAGE gel analysis. The gels were either stained for Coomassie Blue for protein loading or stained for phosphorylated protein using the Pro-Q Diamond phosphoprotein staining kit. P-Protein, phosphorylated protein. c, FLAG-Runx2 and HA-ubiquitin plasmids were co-transfected with different amounts of cyclin D1 expression plasmid (0.2 and 0.6 μg/dish, 6-cm culture dish) into COS cells in the presence or absence of PS1 (10 μM, 4-h incubation). Cyclin D1 induced Runx2 ubiquitination in a dose-dependent manner. In the presence of PS1, cyclin D1-induced Runx2 ubiquitination is further enhanced. d, FLAG-tagged WT-, SA-, or SE-Runx2 was co-transfected with HA-ubiquitin plasmid into COS cells in the presence of PS1 (10 μM, 4-hour incubation). Runx2 ubiquitination was detected in the WT- and SE-Runx2 groups but not in the SA-Runx2 group. e, FLAG-Runx2 expression plasmid was co-transfected with different amounts of cyclin D1 expression plasmid (0.2, 0.6, and 1.8 μg/dish) into COS cells. Cells were treated with PS1 (10 μM) for 4 h after transfection. Transfection of cyclin D1 induced a dose-dependent degradation of Runx2, and treatment with PS1 completely reversed cyclin D1-induced Runx2 degradation. f, WT- and SA-Runx2 expression plasmids were transfected into COS cells. Cells were treated with PS1 (10 μM) for 4 h after transfection. The addition of PS1 increased the protein level of WT-Runx2 but not SA-Runx2.
Differentiation also exists during skeletal development (Fig. 4e). The existence of these mutual inhibitions provides the handles for regulation of cellular functions by different signaling pathways.

Previously, we have shown that the ubiquitin-protein isopeptide ligase (E3) Smurf1 induces Runx2 degradation (35) and that Smad6 enhances Smurf1-induced Runx2 degradation (27). In the present studies, we have analyzed the effect of Smurf1 on the degradation of WT- and SA-Runx2 and found that Smurf1 induced WT as well as SA mutant Runx2 in a similar efficiency, suggesting that Smurf1 is not involved in the cyclin D1-induced, phosphorylation-dependent degradation of Runx2. Further investigation is required to identify the novel E3 ligase that is involved in the cyclin D1-Cdk4-induced Runx2 ubiquitination and degradation.

Proper development of bone tissues requires precise spatial and temporal control of cell proliferation and differentiation. Although Runx2 is expressed during early skeletal development (embryonic days 11.5–14.5), the formation of hypertrophic chondrocytes and mature osteoblasts does not occur in most bones until embryonic days 14.5–15.5 (15). These observations suggest that Runx2 function must be suppressed during early bone development to allow chondrocyte and osteoblast pools to proliferate and expand. One possible mechanism for the suppression of Runx2 function during early bone development is
through cyclin D1-Cdk4-induced degradation. More detailed in vivo studies need to be conducted to further investigate this hypothesis.

In summary, our findings demonstrate that the cyclin D1-Cdk4 complex phosphorylates Runx2 and induces Runx2 degradation in an ubiquitin-proteasome-dependent manner. This study reveals a tightly regulated mechanism between bone cell growth and differentiation.

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