p57\textsuperscript{Kip2} Is Degraded through the Proteasome in Osteoblasts Stimulated to Proliferation by Transforming Growth Factor $\beta1$\textsuperscript{*}

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Cyclin-dependent kinase inhibitory proteins are negative regulators of the cell cycle. Although all the cyclin-dependent kinase inhibitory proteins may be involved in cell cycle control during a differentiation process, only p57\textsuperscript{Kip2} is shown to be essential for embryonic development. However, the role of p57 in the control of the cell cycle is poorly understood. Using osteoblasts derived from the calvaria of rat fetus, we show that p57 is accumulated in cells starved by low serum. Cyclin-dependent kinase 2 activity was suppressed in these cells with a significant amount bound to p57. Treatment of the cells with transforming growth factor $\beta1$ dramatically reduced the amount of p57, resulting in an activation of cyclin-dependent kinase 2 activity and the stimulation of cell proliferation. The decrease in p57 was inhibited by treating the cells with proteasome inhibitors, Z-Leu-Leu-Leu-aldehyde or lactacystin, but not with Z-Leu-Leu-aldehyde, which is an inhibitor of calpain, indicating that p57 is degraded through the proteasome pathway. p57 was also shown to be ubiquitinated in vitro. Because transforming growth factor $\beta1$ not only stimulates the growth but also inhibits the differentiation of the cells in this system, our results may suggest a possible involvement of p57 in the control of osteoblastic cell proliferation and differentiation.

Proliferation of cells is controlled by positive and negative regulatory pathways of the cell cycle. The activity of cyclin-dependent kinases (CDKs) positively drives the progression of the cell cycle. As for negative regulation, a number of CDK inhibitory proteins (CKIs) are known (reviewed in Ref. 1). On the basis of homology and specificities of interactions, CKI can be classified into two distinct families. The INK family (p16, p15, p18, and p19) specifically inhibits cyclin D-CDK4/6 activity by binding to CDK4/6 (2–6), whereas the Cip/Kip family (p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1}, and p57\textsuperscript{Kip2}) inhibits multiple CKIs including both CDK4/6 and CDK2 by binding to cyclin-CDK complexes (7–15). The amounts of CKIs expressed in cells may be crucial in controlling cells regarding whether to start or to stop proliferating. p21 and p27 have been reported to be induced by various anti-proliferative stimuli (1). Conversely, the p27 level was shown to be decreased following mitogenic stimulation of quiescent cells in a number of systems (16–19). The ubiquitin-proteasome pathway is known to play an essential role in the control of p27 protein expression (17). CKIs may have important roles in the withdrawal of the cells from the cell cycle in the differentiation process. p21 and p27 are both shown to be induced during the differentiation process of certain cell types including muscle, neuronal, and hematopoietic cells and in some cases introduction of p21 or p27 into cognate precursor cells is shown to induce their differentiation (20–24). Also, the accumulation of CKIs has been shown in many terminally differentiated cells in mouse embryo (1). However, among the studies of mice deficient in CKIs, only p57 is shown to be essential for mouse embryogenesis, suggesting that p57 may have specific roles in mouse development that cannot be compensated by other CKIs (25, 26). Developmental defects shown in p57 knockout mice include a cleft palate and abnormal endochondral ossification, attributable to increased apoptosis or proliferation at the expense of cell differentiation.

In the present study, we sought to investigate the possible involvement of CKIs in the control of cell growth and differentiation of osteoblastic cells. Osteoblasts isolated from the calvaria of rat fetus are useful in an in vitro culture system for studying the mechanisms of osteoblast differentiation (27). By analyzing this system, we found that p57 is accumulated in cells starved by low serum. Growth stimulation of these cells by transforming growth factor $\beta1$ (TGF$\beta1$) resulted in a dramatic decrease in p57. Further analysis using a set of proteasome inhibitors demonstrated that p57 was degraded through the proteasomal degradation pathway. Because TGF$\beta1$ inhibits the differentiation of these cells, our findings suggest a possible involvement of p57 and its proteasomal degradation in the control of cell growth and differentiation of osteoblastic cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary rat osteoblastic cells were isolated from calvariae of 21-day-old Sprague-Dawley rats embryos as described previously (28). The cells were maintained in α-MEM containing 10% FCS and antibiotics. Cells at the second passage were used for all experiments.

**Antibodies and Immunological Methods**—Rabbit antiserum against mouse p27, p57, and CDK2 were raised against synthetic peptides corresponding to the C terminus of each protein. Anti-mouse p21 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All antibodies cross-react efficiently with the respective rat homologs. Cells were plated in 6-well plastic dishes with α-MEM containing 10% FCS and were cultured in α-MEM containing 0.5% FCS for 72 h prior to

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1 The abbreviations used are: CDK, cyclin-dependent kinase; CKI, CDK inhibitory protein; TGF$\beta$, transforming growth factor $\beta$; MG132, Z-Leu-Leu-Leu-aldehyde; Z-LL-H, Z-Leu-Leu-aldehyde; α-MEM, α-minimum essential medium; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.
the experiment. The cells were then treated with TGFβ1 (1 ng/ml) for the times indicated. Cells were rinsed twice with ice-cold phosphate-buffered saline and lysed in 180 μl of Nonidet P-40 lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM NaF, 5 mM EDTA, 5 mM EGTA, 2 mM sodium vanadate, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, and 0.1% Nonidet P-40. The lysates were cleared by centrifugation at 15,000 × g for 5 min at 4 °C. For immunoblot analysis, the samples were separated on 9 or 12.5% SDS-PAGE. Western blotting was performed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

**CDK2 Binding to CKIs**—To investigate the amount of CDK2 bound to each of the CKIs, total cell lysates were immunoprecipitated with rabbit polyclonal antibodies against p27 and p57 or monoclonal antibody against p21. Briefly, 2 μl of antisera or 2 μg of a monoclonal antibody was added to 200 μg of total cell lysates and incubated for 2 h at 4 °C. The immunocomplexes were brought down with protein G-Sepharose beads (Amersham Pharmacia Biotech), and the pellets were washed four times with ice-cold Nonidet P-40 lysis buffer. The samples were separated on 12.5% SDS-PAGE and analyzed by immunoblotting for CDK2, as described above.

**Histone H1 Kinase Assays**—Immunoprecipitations with anti-CDK2 antibodies were performed as described above. Immunoprecipitates were then washed twice with kinase buffer (80 mM sodium β-glycerophosphate, 5 mM EGTA, 7.4, 20 mM Mg(OAc)2, 15 mM MgCl2, 5 mM dithiothreitol) and were mixed with 10 μl of kinase buffer containing 50 μM ATP, 1.25 μM of [γ-32P]ATP, and 1 μg of histone H1. The samples were incubated at 25 °C for 30 min, and the reactions were terminated by addition of 30 μl of Laemmli sample buffer. The samples were separated on 12.5% SDS-PAGE; the gel was dried and visualized by autoradiography.

**Proteasomal Degradation of p57 by TGFβ1**—Our data demonstrated that the dramatic reduction of p57 expression by the TGFβ1 treatment may have important roles in the control of osteoblastic cell growth stimulation. Although little is known about the control of p57 expression, the related CKI, p27, has been shown to be controlled by the ubiquitin-proteasome degradation pathway (17). Therefore, we tested whether the proteasomal degradation pathway is involved in the TGFβ1-induced decrease in p57. Addition of MG132, a proteasome and calpain inhibitor (32–34), to the culture medium completely blocked the decrease in p57 protein by TGFβ1 treatment (Fig. 3A). The levels of p57 but not to p21. As expected, after growth stimulation by TGFβ1, a decreased amount of CDK2 bound to p27, and none to p57, nor p21 were seen in these cells. Also, the activity of CDK2 in these cells, as determined by phosphorylation of histone H1 with anti-CDK2 immunoprecipitates, was induced by TGFβ1 treatment (Fig. 2B). These results suggest that TGFβ1 stimulates the cell proliferation of osteoblastic cells by reducing the amounts of p57 and p27 and releasing CDK2 bound to the CKIs, which in turn results in the induction of CDK2 activities.

**RESULTS**

**Down-regulation of p57 by TGFβ1**—TGFβ1 has been shown to induce proliferation of osteoblastic cells isolated from the calvaria of rat embryos (30, 31). We examined the expressions of p21, p27, and p57 in this system to see if these CKIs are involved in the regulation. All three CKIs were expressed in osteoblastic cells serum-starved in α-MEM containing 0.5% FCS. Treatment of these cells with TGFβ1 for 24 h resulted in a 5-fold increase in [3H]thymidine incorporation (data not shown), which is consistent with previous reports (30, 31). The amount of p57 was dramatically decreased within the first 12 h, whereas some decrease was also seen in p21 and p27 expressions during the growth induction by TGFβ1 treatment (Fig. 1). These results suggested that the decrease in the expressions of CKIs may have important roles in the growth stimulation of rat osteoblastic cells by TGFβ1.

**Binding of CKIs with CDK2**—The Cip/Kip family proteins (p21, p27, and p57) control cell cycle progression by binding and inhibiting cyclin-CDK complexes, including cyclin E/A-CDK2 complexes (7–15). Therefore, we examined possible changes in the amount of CDK2 bound to each CKI before and after TGFβ1 stimulation (Fig. 2A). Before the TGFβ1 treatment, a significant amount of CDK2 was shown to be bound to p27 and p57 but not to p21. As expected, after growth stimulation by TGFβ1, a decreased amount of CDK2 bound to p27, and none to p57, nor p21 were seen in these cells. Also, the activity of CDK2 in these cells, as determined by phosphorylation of histone H1 with anti-CDK2 immunoprecipitates, was induced by TGFβ1 treatment (Fig. 2B). These results suggest that TGFβ1 stimulates the cell proliferation of osteoblastic cells by reducing the amounts of p57 and p27 and releasing CDK2 bound to the CKIs, which in turn results in the induction of CDK2 activities.

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**p57 Is Ubiquitinated in Vitro**—Ubiquitination is known to have important roles in the control of the proteasomal degradation pathway. Therefore, we tested whether p57 can also be modified by ubiquitination. p57 was prepared by in vitro translation in rabbit reticulocyte lysate and was incubated with an addition of osteoblast cell extract, with or without bacterially produced GST-ubiquitin (Fig. 4). After 3 h of incubation, in addition to slower migrating bands seen in both reactions that may correspond to the ubiquitinated p57, several distinct bands were seen in the sample incubated with GST-ubiquitin (Fig. 4, last lane). Of these, a 92-kDa band nicely corresponds to p57 covalently modified by one GST-ubiquitin, as GST-ubiquitin is approximately 35 kDa. The fact that these bands were only seen in the sample incubated with GST-ubiquitin indicated that the bands indeed represent GST-ubiquitinated p57s, which confirms that p57 can be ubiquitinated.
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Fig. 2. TGFβ1 increases CDK2 activity. A, the level of CDK2 associated with p27, p57, and p21 as indicated in the top row, in cells without (−) or with (+) TGFβ1 treatment were examined by immunoprecipitation (IP) with respective antibodies. B, anti-CDK2 immunoprecipitates from TGFβ1 untreated (−) or treated (+) cells were assayed for the kinase activity using histone H1 (H1) as a substrate.

Fig. 3. p57 is degraded through the proteasome pathway. A, serum-starved osteoblastic cells were treated without (−) or with (+) TGFβ1 and without (−) or with (+) a proteasome inhibitor MG132 as indicated. Extracts were prepared at the times shown in the top row. B, cells stimulated without (−) or with (+) TGFβ1 were treated for 8 h without (−) or with (+) a series of proteasome and protease inhibitors as indicated.

Fig. 4. p57 is ubiquitinated in vitro. p57, transcribed and translated in vitro, was submitted to an ubiquitination assay as described under “Experimental Procedures.” Each reaction was incubated without (−) or with (+) GST-ubiquitin (GST-Ub) for the time indicated. p57 denotes the original position of unmodified p57. The asterisks indicate the additional bands appearing in the right-hand lane that correspond to p57 covalently bound with GST-ubiquitin. Positions of molecular size markers are indicated on the left side.

DISCUSSION

TGFβ is thought to have important roles in bone remodeling. TGFβ is locally produced by osteoblasts and is accumulated abundantly in bone matrix tissue (31, 36, 37), and several studies have shown that TGFβ may regulate not only cell growth but also differentiation procedure (36–38). Consistent with the previous report (38), induction of cellular alkaline phosphatase activity, a bone phenotype marker that precedes the onset of mineralization, was inhibited during TGFβ1-induced stimulation of primary rat osteoblast proliferation in our experiments (data not shown). CKIs are shown to play important roles in the induction of cell differentiation (20–24); therefore, our results strongly suggest that p57 may also play an important role in regulating the differentiation procedure of osteoblasts. It is worth noting that among the gene knockout studies of the Cip/Kip family proteins, only p57-deficient mice showed developmental abnormalities (25, 26). Abnormalities shown in these mice include short limbs, a defect attributable to abnormal endochondral ossification, which may be caused by delayed cell cycle exit during chondrocyte differentiation (25, 26). Similar ossification defects have been observed in double-knockout mice of the retinoblastoma-related proteins, p107 and p130 (39). Chondrocytes and osteoblasts are thought to be derived from stem cells of the same origin, and interestingly, primary osteoblastic cells used in this experiment express p107 and p130 but not retinoblastoma, and p107 became phosphorylated when the cells were stimulated by TGFβ1. Therefore, it is tempting to speculate that p57, together with p107, can be involved in the regulation of cell growth and differentiation of cells of certain lineage, including osteoblasts and chondrocytes.

Our results also indicated that the expression level of p57 can be mainly regulated by the ubiquitin-proteasome protein degradation pathway, as is the case with its related CKI, p27. The increasing importance of ubiquitin-mediated proteolysis of the regulators of cell proliferation such as cyclin D, cyclin E, and p27 has been extensively examined in a number of recent studies (reviewed in Refs. 40–42), and our findings have added another CKI in this category. It will be important to study how signals from TGFβ1 can be delivered to the regulation of this machinery.

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