Cyclin-dependent kinase inhibitory proteins (CKIs) are negative regulators of cell cycle. Of all CKIs, only p57Kip2 plays an essential role(s) that other CKIs cannot compensate for in embryonic development. Recently, we found that p57Kip2 is degraded through the ubiquitin-proteasome pathway in osteoblastic cells stimulated to proliferation by transforming growth factor β1 (TGF-β1; Urano, T., Yashiroda, H., Muraoka, M., Tanaka, K., Hosoi, T., Inoue, S., Ouchi, Y., and Toyoshima, H. (1999) J. Biol. Chem. 274, 12197-12200). We report here that TGF-β1-induced p57Kip2 proteolysis is mediated through transcription by the Smad pathway. When the constitutively active form of the TGF-β type I receptor ALK5(TD) was ectopically expressed in osteoblastic cells, p57Kip2 that had been accumulated by serum starvation causing the cell-cycle arrest was rapidly degraded in a manner analogous to TGF-β1 stimulation. Moreover, Smad2 or Smad3 with Smad4 enhanced the proteolytic pathway of p57Kip2. The degradation of p57Kip2 evoked by TGF-β1 was blocked by forced expression of an inhibitory Smad called Smad7 or by the addition of actinomycin D or α-amanitin. These results indicate that accelerated degradation of p57Kip2 by TGF-β1/Smad signaling is mediated through a newly synthesized factor(s) that modifies p57Kip2 or the ubiquitin-proteasome pathway.

Cell division is determined by whether cells meet the requirements for entry into the S phase. Without these requirements, cells may progress from the G1 phase toward quiescence or terminal differentiation. In these regulatory processes, cyclin-dependent kinases (CDKs) positively drive the progression of the cell cycle, whereas CDK inhibitory proteins (CKIs) act as negative regulators by binding to cyclin-CDK complexes (reviewed in Refs. 1 and 2). CKIs have been classified into two families in mammals, i.e. the INK4 (inhibitor of CDKs) family and the Cip/Kip (CDK interacting protein/kinase inhibitory protein) family. The INK4 family (p16Ink4a, p15Ink4b, p18Ink4b, and p19Ink4b) inhibits only CDK4 and CDK6, whereas the Cip/Kip family (p21Cip1, p27Kip1, and p57Kip2) inhibits all CDKs functioning during the progression of the G1/S phase.

There are convincing lines of evidence addressing the importance of the Cip/Kip family proteins in the proliferation and/or differentiation of cells. Compared with the tremendous progress in the p21Cip1 and p27Kip1 studies, much less is known about the role of p57Kip2 in the cell cycle. Interestingly, only p57Kip2 is shown to be essential for mouse embryogenesis (3, 4), whereas the lack of p21Cip1 or p27Kip1 does not show gross developmental defects (2). Thus, it is of interest to explore the relationship between p57Kip2 expression and cell proliferation under physiological circumstances. However, p57Kip2 is hard to study due to its low expression level in many cells. We recently found that p57Kip2 accumulates in rat osteoblastic cells when arrested by serum starvation. Furthermore, TGF-β1 stimulates proliferation of osteoblastic cells induces rapid degradation of p57Kip2 through the proteasome-dependent pathway (5).

Current studies have advanced our understanding of the intracellular signaling pathway of TGF-β (reviewed in Refs. 6–8). TGF-β exerts its effect via two types of serine/threonine kinase receptors: TGF-β binds first to the type II receptor, which consequently activates the type I receptor by direct association. Signals from the activated type I receptor are known to be transmitted into the nucleus through various mediator molecules. Among these, the best characterized molecules are a family of proteins termed Smad (reviewed in Refs. 9–11). To date, eight different Smads have been identified in mammals, and these are classified into three subgroups, i.e. the receptor-regulated Smads (R-Smads), the common-partner Smads (Co-Smads), and the inhibitory Smads (I-Smads). R-Smads are phosphorylated by type I receptors, then complexed with Co-Smads, and ultimately translocate into the nucleus. The R-Smad/Co-Smad heterooligomers are capable of binding Smad; Co-Smad, common-partner Smad; α-MEM, α-minimal essential medium; FCS, fetal calf serum; HA, hemagglutinin; m.o.i., multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; E3, ubiquitin-protein ligase; ALK, activin receptor-like kinase; Kip, kinase inhibitory protein; Cip, CDK interacting protein.

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Shigeki Nishimori,††, Yoshikazu Tanaka,‡‡, Tomoki Chiba,‡‡, Makiko Fuji,‡, Takeshi Imamura,‡, Kohei Miyazono, Tohru Ogasawara,*, Hiroshi Kawaguchi,††, Tetsuya Igarashi,§, Toshiro Fujita,§, Keiji Tanaka,§§, and Hideo Toyoshima,†† From the †Department of Molecular Oncology, and the ‡‡Department of Tumor Biochemistry, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan, the §Department of Internal Medicine, University of Tokyo Branch Hospital, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112-8688, Japan, the ¶¶Department of Orthopaedic Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan, the ‡Department of Biochemistry, The Cancer Institute of the Japanese Foundation for Cancer Research, 1-37-1 Kami-ikebukuro, Toshima-ku, Tokyo 170-8455, Japan, and the †Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Japan
to DNA directly or indirectly via other DNA-binding proteins and thus regulate positively or negatively the transcription of a multitude of target genes. In contrast, I-Smads inhibit the phosphorylation of R-Smads by activated type I receptors by interfering with their association, leading to prevention of the assembly of R-Smads with Co-Smads. In the TGF-β signaling pathway, Smad2 and Smad3 function as R-Smads, Smad4 as a Co-Smad, and Smad7 as an I-Smad (9–11).

In the present study, we examined whether or not the Smad pathway is responsible for the accelerated degradation of p57Kip2 in mouse osteoblastic cells treated with TGF-β1. To this end, we used adenovirus-based vectors able to obtain high efficiencies of transfection in primary cell culture for expression of various mediator proteins which are known to locate downstream of TGF-β1 signaling. We report here that protosomal degradation of p57Kip2 in osteoblastic cells derived from the calvariae of newborn mice treated by TGF-β1 is mediated by Smad proteins and that the TGF-β1-induced p57Kip2 proteolysis is regulated at the transcriptional level.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sources of materials were as follows: recombinant human TGF-β1 (R & D Systems Inc., Minneapolis, MN); MG132 (Z-Leu-Leu-Leu-aldehyde; Peptide Institute, Inc., Osaka, Japan); actinomycin D and aminomycin (a-amanitin; Sigma); α-mannosidase; Life Technologies, Inc., Grand Island, NY); fetal calf serum (JRH Biosciences, Lenexa, KS); antibiotics-antimycotic (Life Technologies, Inc., 100 units/ml of penicillin G, 100 units/ml of streptomycin, 0.1 μg/ml of amphotericin B); mouse monoclonal anti-HA antibody (Berkley Antibody Co., Richmond, CA); mouse monoclonal anti-FLAG M2 antibody (Sigma). Polyclonal antibodies against mouse p27Kip1 and p57Kip2 were raised in rabbits using synthetic peptides corresponding to the C-terminal amino acids of each protein (5).

**Isolation and Culture of Osteoblastic Cells**—Primary mouse osteoblastic cells were isolated from calvariae of 1-day-old mice, strain ddY, by sequential enzymatic digestion as described previously (12, 13). The cells prepared from calvariae by enzymatic digestion are composed of heterogeneous cell populations, but they express various osteoblastic phenotypes including the ability to form bone nodules in vitro, and are useful for examining sequential changes of phenotypes occurring during osteoblast differentiation, as reported before (13). The cells were cultured in α-MEM containing 10% fetal calf serum (FCS) and antibiotics-antimycotic. Cells at the second passage were frozen to stock for each experiment.

After thawing the cells, 5 × 10^5 cells in 6-well plastic plate or 2 × 10^4 cells in 12-well plate were cultured with α-MEM containing 10% FCS and antibiotics-antimycotic. Cells of the second passage were frozen to stock for each experiment.

**Constructions of Recombinant Adenoviruses**—To obtain a high transfection efficiency in primary osteoblastic cell cultures, we used a recombinant adenovirus system. Recombinant adenoviral vectors carrying hemagglutinin (HA)-tagged human TGF-β type I receptor (ALK-5), FLAG-tagged Smads, and β-galactosidase (LacZ) cDNAs were constructed as described previously (14). Infection of the recombinant adenovirus was performed at a multiplicity of infection (m.o.i.) of 50–100 plaque forming units/cell. More than 80% of the cells were infected as determined by staining of the cells for β-galactosidase. The expression of recombinant proteins with these adenoviruses was obtained at the peak about 3 days after infection, so we cultured osteoblastic cells for 3 or 4 days after viral infection prior to use.

**Protease Inhibitor**—Osteoblastic cells were treated with MG132 (Z-Leu-Leu-Leu-aldehyde) at a final concentration of 2.5 μM for 12 h prior to harvesting of cells to avoid cell toxicity.

**Immunoblot Analysis**—Cells were rinsed with ice-cold phosphate-buffered saline and lysed directly in SDS sample buffer. The lysates were sonicated briefly and clarified by centrifugation at 15,000 × g for 5 min at 4 °C. For immunoblot analysis, the samples were separated on 12.5% SDS-PAGE. Proteins were then electrotransferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA), immunoblotted with respective antibodies, and visualized using an enhanced chemiluminescence detection system (Renassiance; PerkinElmer Life Sciences, Boston, MA).

**Pulse-Chase analysis**—Serum-starved osteoblastic cells in 35-mm dishes were labeled for 16 h with 70 μCi/ml [35S]Met and [35S]Cys (Amersham Pharmacia Biotech, Piscataway, NJ) in α-MEM without methionine/cysteine (Sigma) containing 0.5% FCS, then chased with α-MEM containing 0.5% FCS with 0.4 mg/ml methionine for the time periods indicated in the presence or absence of TGF-β1 (1 ng/ml). Cells were lysed in Nonidet P-40 lysis buffer as described previously (5) and the soluble extracts were subjected to immunoprecipitation with polyclonal anti-p57Kip2 antibody. The resulting immunoprecipitates were analyzed by SDS-PAGE and autoradiography, and the p57Kip2 band was quantified with an image analyzer (Fujix BAS 2000).

**Northern Blot Analysis**—Total cellular RNAs from cultured cells were extracted using TRIZOL reagent (Life Technologies, Inc.). These RNAs were electrophoresed on 1.2% agarose formaldehyde gels and transferred onto nylon membrane filters (Nitran 0.45; Schleicher & Schuell, Keene, NH). Hybridization and washing the filters were carried out as described previously (15). The cDNA probe for a 377-base pair Smad1-HindIII fragment from mouse p57Kip2 was labeled with α-32P-dCTP using the multiprime labeling kit (Amersham Pharmacia Biotech).

**RESULTS**

**p57Kip2 Proteolysis Is Induced by TGF-β1 in Mouse Osteoblastic Cells**—Previously, we reported that p57Kip2 was degraded by TGF-β1 stimulation through the ubiquitin-proteasome pathway using rat osteoblastic cells (5). To further explore the mechanism of TGF-β1 signaling for p57Kip2 proteolysis, we used various adenovirus vectors carrying Smad cDNAs and osteoblastic cells isolated from the calvariae of newborn mice (14). At first, we tested whether or not TGF-β1 induces p57Kip2 degradation in the mouse osteoblastic cells as in rat cells. As shown in Fig. 1, p57Kip2 accumulated in serum-starved mouse osteoblastic cells. Treatment with TGF-β1 resulted in almost complete loss in the cellular level of p57Kip2 during 16–24 h. On the other hand, p27Kip1 remained unchanged irrespective of TGF-β1 stimulation, although its cellular level slightly increased due to serum deprivation.

Furthermore, addition of a proteasome inhibitor MG132 blocked the reduction of p57Kip2 caused by TGF-β1 without affecting the p57Kip2 level in the absence of TGF-β1 (Fig. 2), suggesting that TGF-β1 induces the instability of p57Kip2. To confirm directly this assumption, we conducted pulse-chase analysis. As shown in Fig. 3, endogenous [35S]p57Kip2 metabolically labeled with [35S]Met and [35S]Cys under serum-starved conditions was rapidly disappeared with an apparent half-life of 8–10 h when cells were cultured in the presence of TGF-β1, whereas [35S]p57Kip2 was fairly stable without treatment with TGF-β1, indicating that TGF-β1 certainly promotes the degradation of p57Kip2. MG132 treatment of these cells also appreciably increased the p27Kip1 level (Fig. 2), which is consistent with previous reports that the degradation of p27Kip1 is processed in a proteasome-dependent manner in a variety of cells (16).
Fig. 2. A proteasome inhibitor MG132 prevents the degradation of p57Kip2 in osteoblastic cells stimulated by TGF-β1. Serum-starved osteoblastic cells (see Fig. 1) were cultured for 24 h in the presence (+) or absence (−) of TGF-β1 (1 ng/ml). A proteasome inhibitor MG132 was added for 12 h at the final concentration of 2.5 μM prior to harvesting of the cells. The immunoblot analysis was carried out as for Fig. 1. All experiments were conducted in duplicate.

Fig. 3. Pulse-chase analysis of endogenous p57Kip2 in osteoblastic cells. Cells were cultured in serum-starved media containing [35S]Met and [35S]Cys for 16 h, then chased in the presence or absence of TGF-β1 (1 ng/ml) as a function of time. The immunoprecipitated [35S]p57Kip2 was subjected to SDS-PAGE and autoradiography (upper panel). Relative intensities of p57Kip2 bands determined with an image analyzer are shown (lower panel). The data show typical results of three independent experiments which were essentially the same.

To test whether TGF-β1 affects the synthesis of p57Kip2, we measured the mRNA level of p57Kip2 after TGF-β1 stimulation by Northern blot analysis. As shown in Fig. 4, the amount of p57Kip2 mRNA slightly decreased after 24 h and thus TGF-β1 may decrease or increase the p57Kip2 synthesis, affecting partly the level of p57Kip2 in osteoblastic cells. In addition, the possibility that TGF-β1 also regulates p57Kip2 at the translational level cannot be completely ruled out. Nevertheless, considering almost complete loss of cellular p57Kip2 level upon TGF-β1 stimulation, it is clear that TGF-β1 predominantly regulates p57Kip2 proteolysis by the proteasome pathway.

Degradation of p57Kip2 Is Induced by Constitutively Active Form of TGF-β Type I Receptor—It is conceivable that TGF-β1 binds first to the type II receptor, which subsequently activates the type I receptor (6–8). Of seven different type I receptors of the TGF-β superfamily (originally termed activin receptor-like kinase (ALK)-1 to ALK-7), ALK-5 is the type I receptor for TGF-β1 (17, 18). Therefore, we next examined whether a constitutively active form of ALK-5 operates the down-regulation of p57Kip2 in osteoblastic cells. Replacement of Thr at position 204 in ALK-5 by acidic amino acids such as Asp (termed ALK-5(TD)) leads to constitutive activation of the type I receptor without ligands or type II receptor (19). Thus, we expressed HA-tagged ALK-5(TD) using an adenoviral vector in serum-starved osteoblastic cells. As shown in Fig. 5 (upper panel), p57Kip2 was decreased, depending on the increment of the ALK-5(TD) protein (lower panel), and disappeared after 72 h viral infection. However, no obvious change in the p57Kip2 level was found in the osteoblastic cells infected with control vector that carried a β-galactosidase (LacZ) cDNA or nothing. The level of p27Kip1 remained unchanged, irrespective of the expression of the ALK-5(TD) protein (Fig. 5, middle panel). Thus, it was concluded that p57Kip2 is surely degraded through the downstream signaling pathway of the TGF-β type I receptor.

The Smad Pathway Accelerates the Degradation of p57Kip2 by TGF-β1—To date, it becomes clear that the Smad family proteins are involved in the signaling pathway evoked by the TGF-β type I receptor (9–11). Therefore, we examined whether Smad-mediated transcription accelerates the instability of p57Kip2. The activated TGF-β type I receptor, ALK-5 phosphorolytes R-Smads, such as Smad2 and Smad3, whereas an I-Smad, such as a Smad7, interferes with the phosphorylation of Smad2 and Smad3 by preventing their interaction with ALK-5. If the Smad pathway is involved in the down-regulation of p57Kip2, I-Smad would suppress the reduction of p57Kip2 induced by TGF-β1. When mouse osteoblastic cells were infected with the adenoviruses carrying the Smad7 cDNA prior to TGF-β1 treatment, the signal-dependent breakdown of p57Kip2 was considerably inhibited (Fig. 6A). In addition, the similar expression of Smad7 also prevented the decrease in p57Kip2 induced by ALK-5(TD) (Fig. 6B). However, the p27Kip1 level showed no significant change in these conditions.

As mentioned before, Smad7 competes with Smad2 and Smad3 for its binding to activated ALK-5; thus the inhibitory effect of Smad7 suggests the involvement of Smad2 and/or Smad3 in the destabilization of p57Kip2. Therefore, we examined the effects of Smad2 or Smad3 with Smad4 (Co-Smad), a partner molecule collaborating with R-Smad in the presence or absence of Smad7 on the instability of p57Kip2. In this experiment, a low concentration of TGF-β1 incapable of promoting p57Kip2 proteolysis (Fig. 7, left lane) was added for 24 h after viral infection, which might be required for phosphorylation of expressed Smad2 and Smad3. When Smad2 or Smad3 was coexpressed with Smad4, p57Kip2 disappeared in the osteoblastic cells infected with adenoviruses carrying these Smads (Fig. 7). The forced expression of Smad7 prevented the decrease in the p57Kip2 level induced by coexpressions of Smad2 or Smad3 with Smad4. No significant change in the p57Kip2 level was observed in the control expressing LacZ. These results strongly indicate that the degradation of p57Kip2 by TGF-β1 is mediated through the Smad2/4 or Smad3/4 pathway. In contrast, the p27Kip1 level remained unchanged, irrespective of coexpression in any given combinations of Smads (Fig. 7).

To further test whether Smad-mediated transcription is regulated for p57Kip2 degradation, we tested the effect of several transcription inhibitors. When osteoblastic cells were treated with actinomycin D for 24 h in the absence of TGF-β1, the p57Kip2 level was not affected, however, the TGF-β1-induced degradation of p57Kip2 was blocked (Fig. 8A), implying that a protein(s) newly synthesized by TGF-β1 stimulation is involved in the proteolytic elimination of p57Kip2. The similar suppressive effect for the TGF-β1-induced decrease of p57Kip2 was observed by treatment with another transcription inhibitor, α-amanitin (Fig. 8B). Considering the fact that the p57Kip2 level remained unchanged for 24 h during exposure to actinomycin D or α-amanitin (Fig. 8), p57Kip2 is found to be appa-
ently stable without TGF-β1 stimulation (see “Discussion”). This also indicates that the disappearance of p57Kip2 by TGF-β1 treatment is not attributed to the down-regulation of the p57Kip2 production. Taken together, these results suggest that Smad-mediated transcription is involved in the induction of p57Kip2 proteolysis, which may play an essential role in the proteolysis of p57Kip2 by the Smad pathway.
absence of actinomycin D (abbreviated Act. D) and α-amanitin (α-amanin) at the same m.o.i. were used as a control. The immunoblot analysis was carried out as for Fig. 1, except that the expression of FLAG-tagged Smad2, Smad3, Smad4, and Smad7 was measured by reblotting the same membrane with anti-FLAG M2 antibody (bottom panel).

FIG. 7. The effect of forced expressions of various Smad proteins on the stability of p57Kip2 and p27Kip1. Serum-starved osteoblastic cells (see Fig. 6) were infected for 48 h with Smad2/4 or Smad3/4 with or without Smad7 as indicated (m.o.i. of 100 for each). A low concentration of TGF-β1 (0.03 ng/ml) was added for 24 h after viral infection which had no effect of the level of p57Kip2. Adenoviruses carrying β-galactosidase (LacZ) at the same m.o.i. were used as a control. The immunoblot analysis was carried out as for Fig. 1, except that the expression of FLAG-tagged Smad2, Smad3, Smad4, and Smad7 was measured by reblotting the same membrane with anti-FLAG M2 antibody (bottom panel).

FIG. 8. The effect of actinomycin D (A) and α-amanitin (B) on the level of p57Kip2 and p27Kip1 in the presence or absence of TGF-β1. Serum-starved osteoblastic cells (see Fig. 1) were cultured for 24 h with (+) or without (−) TGF-β1 (1 ng/ml) in the presence (+) or absence (−) of actinomycin D (abbreviated Act. D) (0.1 μg/ml) or α-amanitin (5 μg/ml). The immunoblot analysis was carried out as for Fig. 1. All experiments were conducted in duplicate.

growth control of osteoblastic cells in vivo.

On the other hand, actinomycin D or α-amanitin caused disappearance of p27Kip1, irrespective of TGF-β1 stimulation (Fig. 8). These results indicate that p27Kip1, unlike p57Kip2, are rapidly turned over.

DISCUSSION

Previously, we reported that TGF-β1 causes rapid degradation of p57Kip2 in osteoblastic cells isolated from the calvariae of rat fetuses that had been serum-starved to induce the cellular p57Kip2 level (5). The process was inhibited by various proteasome inhibitors, such as lactacystin and MG132, and a polyubiquitylation of p57Kip2 was detected in an in vitro assay using TGF-β1-treated cell extracts, strongly indicating that the ubiquitin-proteasome pathway operates in the degradation of p57Kip2. In the present work, first, we observed the proteasomal degradation of p57Kip2 in mouse osteoblastic cells induced to proliferate by TGF-β1 (Figs. 1 and 2). Thus, the TGF-β1-dependent selective degradation of p57Kip2 in mouse osteoblastic cells was essentially the same as rat cells, except that the degradation of p57Kip2 in the latter cells was considerably faster compared with that in former cells for unknown reasons.

Second, we found that this effect of TGF-β1 is mediated by the TGF-β type I receptor, because forced expression of the constitutively active form of the TGF-β type I receptor ALK-5(TD) resulted in the accelerated degradation of p57Kip2 analogous to TGF-β1 stimulation (Fig. 5), and because one member of the I-Smads, Smad7, blocked its degradation induced by TGF-β1 or ALK-5(TD) (Fig. 6). Third, we observed a stimulatory effect on the down-regulation of p57Kip2 when Smad2 or Smad3 was expressed simultaneously with Smad4 under the low concentration of TGF-β1 (Fig. 7). We also observed that Smad7 greatly suppressed the down-regulation of p57Kip2 induced by coexpressions of Smad2 or Smad3 with Smad4. These findings strongly indicate that the Smad pathway mediates the degradation of p57Kip2 in osteoblastic cells stimulated to proliferate by TGF-β1. However, the possibility that the TGF-β type I receptor also activates Smad-independent pathways, i.e. extracellular signal-regulated kinase/mitogen-activated protein (MAP) kinase, c-Jun N-terminal kinase/serum-activated protein kinase, and p38 MAP kinase pathways (20–24) to cause p57Kip2 degradation cannot be completely excluded, because we have not yet examined the effect of the compounds capable of inhibiting these pathways. Nonetheless we favor the idea that the Smad pathway plays a central role in the destabilization of p57Kip2, because coexpression of R-Smad2/3 and Co-Smad4 mimicked the effect of TGF-β1 treatment causing loss of p57Kip2 and because loss of p57Kip2 induced by these R- and Co-Smads, like TGF-β1 stimulation, was suppressed by I-Smad, Smad7. In the present experimental conditions, we have added a low concentration of TGF-β1 (0.03 ng/ml) that was insufficient to induce p57Kip2 proteolysis for activation of Smad2 and Smad3. Thus, Smad7 blocks competitively for the binding of Smad2 or Smad3 to the activated TGF-β type I receptor, although the possibility that Smad7 directly suppresses the binding of Smad2 or Smad3 to Smad4 cannot be completely ruled out. Whatever Smad7 acts, it is clear that the Smad pathway is involved in p57Kip2 proteolysis induced by TGF-β1.

Finally, we found that the TGF-β1-induced degradation of p57Kip2 was abrogated by actinomycin D (Fig. 8A) or α-amanitin (Fig. 8B), which had no effect on the p57Kip2 level per se. The effect of these transcriptional inhibitors is quite interesting in considering the mechanistic insights of how TGF-β1 accelerates the degradation of p57Kip2. It is well known that the TGF-β1/Smad signaling system acts as transcriptional factors capable of activating a diverse spectrum of target genes (9–11). According to this scenario, a newly synthesized protein(s) may affect the stability of p57Kip2. For this, two possible mechanisms can be considered. One possibility is that the presumptive new protein may activate the p57Kip2 protein, which directs p57Kip2 to the ubiquitin-proteasome machinery. To date, various signals are known to act as a degradation signal for a multitude of cellular proteins (reviewed in Ref. 25). Indeed there is accumulating evidence addressing the importance of phosphorylation of most target molecules involved in the cycle progression or signal transduction, such as p27Kip1, cyclin E, IκB, and β-catenin, as a prerequisite for their ubiquilation and subsequent proteasomal degradation (reviewed in Refs. 26–28). Therefore, it is likely that p57Kip2 is also phosphorylated prior to its ubiquilation, although phosphorylation of p57Kip2 has not yet been reported so far. Moreover, to our
knowledge, there is no available information that p57Kip2 is modified post-translationally in other ways, such as acetylation or oxidation. Further study is required to clarify whether p57Kip2 is actually modified in response to TGF-β stimulation.

The other possibility is that the degradation machinery itself may be activated by Smad-mediated transcription. In the ubiquitination pathway, ubiquitin-protein ligase, E3, plays an important role in the selection of proteins for degradation, because it specifically binds to the protein substrate (25–28). Therefore, it is tempting to speculate that the newly induced protein by TGF-β might belong to E3, which selectively targets p57Kip2 for ubiquilation. Consistent with this notion, recently it was reported that an F-box protein acting as a substrate recognition module of a large multisubunit ubiquitin-ligase called SCF (skp1-cdc53 or a cullins-F-box protein complex) is extremely unstable and regulated at transcriptional level (29–31). In other cases, for example, the anaphase-promoting complex or cyclosome E3-ligase complex is known to be controlled by phosphorylation during the M-phase traverse of the cell cycle (32). Therefore, TGF-β may affect the activity of a specific E3 capable of ubiquilating p57Kip2. To determine the mechanism underlying this hypothesis, it is essential to search for the E3-ligase responsible for ubiquilation of p57Kip2, which is in progress.

It is noteworthy that the level of p57Kip2 is very low in proliferating osteoblastic cells, but serum deprivation caused its dramatic accumulation (Ref. 5 and this study). The exposure of TGF-β1 to such nongrowing cells induces down-regulation of p57Kip2, strongly indicating the importance of p57Kip2 proteolysis in regulating proliferation and possible differentiation of osteoblastic cells. This notion is also supported by the findings that MG132 prevented the TGF-β1-induced loss of p57Kip2 (Fig. 2) and TGF-β1 had a little effect on the level of the p57Kip2 mRNA (Fig. 4), indicating that TGF-β1 affects primarily the degradative process of p57Kip2. Moreover, actinomycin D or α-amanitin showed no appreciable alteration of the level of p57Kip2 without TGF-β1 stimulation, suggesting that p57Kip2 is fairly stable under nondividing conditions (Fig. 8). This assumption was ascertained by pulse-chase analysis of metabolically labeled [35S]p57Kip2. p57Kip2 did not disappear appreciably in the absence of TGF-β1, while it was rapidly degraded with an apparent half-life of 8–10 h under the presence of TGF-β1 in osteoblastic cells (Fig. 3).

On the other hand, the level of p27Kip1 was unaffected in TGF-β1 stimulation and was reduced by treatment with actinomycin D or α-amanitin irrespective of treatment with TGF-β1, indicating that turnover of p27Kip1 is rapid in osteoblastic cells (Fig. 8). Our results also indicate that regulatory mechanisms for these two CKIs, p27Kip1 and p57Kip2, considerably differ in osteoblastic cells. However, serum starvation also caused accumulation of p27Kip1 to a lesser extent (Fig. 1), indicating that it may somehow be involved in the growth control of osteoblastic cells.

TGF-β provides a variety of signals for numerous cells and interestingly often exerts apparently contradictory effects, depending on the type of cells (6–8). For instance, it mainly suppresses the growth in many epithelial cells, but promotes it in certain cells, such as osteoblastic cells. The mechanism of these diverse effects remains unknown, but it could be due to how the regulatory factors that positively (e.g. CDKs) and negatively (e.g. CKIs) drive the cell-cycle progression are induced by the TGF-β signaling. However, the details are still unknown at present.

Notably, TGF-β, which is locally produced by osteoblasts and accumulated abundantly in bone matrix, is thought to have important roles in bone remodeling (33). It is worth noting that, among the gene knockout studies of the Cip/Kip family proteins, only p57Kip2-deficient mice showed developmental abnormalities (3, 4). 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. Therefore, it is tempting to speculate that p57Kip2 may affect the regulation of cell growth and differentiation of cells of a certain cell lineage, including osteoblasts and chondrocytes. In considering these in vivo roles of p57Kip2, the new regulatory system of p57Kip2 by TGF-β signaling linked to proteolysis sheds new light on the mechanisms underlying the development and/or differentiation of osteoblasts and perhaps certain other cells in vivo.

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