THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 49, Issue of December 7, pp. 46533–46543, 2001
Printed in U.S.A.

Proteasomal Degradation of Smad1 Induced by Bone Morphogenetic Proteins*

Received for publication, June 14, 2001
Published, JBC Papers in Press, September 24, 2001, DOI 10.1074/jbc.M105500200

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The bone morphogenetic proteins (BMPs) regulate early embryogenesis and morphogenesis of multiple organs, such as bone, kidney, limbs, and muscle. Smad1 is one of the key signal transducers of BMPs and is responsible for transducing receptor activation signals from the cytoplasm to the nucleus, where Smad1 serves as a transcriptional regulator of various BMP-responsive genes. Based upon the ability of Smad1 to bind multiple proteins involved in proteasome-mediated degradation pathway, we investigated whether Smad1 could be a substrate for proteasome. We found that Smad1 is targeted to proteasome for degradation in response to BMP type I receptor activation. The targeting of Smad1 to proteasome involves not only the receptor activation-induced Smad1 ubiquitination but also the targeting functions of the ornithine decarboxylase antizyme and the proteasome β subunit HsN3. Our studies provide the first evidence for BMP-induced proteasomal targeting and degradation of Smad1 and also reveal new players and novel mechanisms involved in this important aspect of Smad1 regulation and function.

The transforming growth factor β (TGF-β)[1] superfamily consists of a group of structurally related cytokines that regulate cell proliferation, differentiation, and apoptosis, as well as extracellular matrix deposition through transcriptional regulation of different target genes (1, 2). The bone morphogenetic proteins (BMPs) form a subfamily of the TGF-β superfamily and are key regulators of the morphogenesis and maintenance of multiple tissues and organs (1, 3–5). Each member of the TGF-β family ligands binds to a characteristic pair of type I and type II transmembrane serine/threonine kinase receptors, both of which are needed for signaling. The ligand first binds to the type II receptor, which then recruits and transphosphorylates a type I receptor-specific juxtamembrane motif, called the GS region, which is rich in serine and glycine (6). The type I receptor is normally bound to a cytoplasmic inhibitor FKBP12, which is released upon a type II receptor-induced phosphorylation event different from GS motif phosphorylation (7–10). The phosphorylation of the GS motif and the release of the inhibitor FKBP12 together contribute to the activation of the type I receptor kinase activity. The activated type I receptor subsequently recruits and phosphorylates the intracellular target molecules, among which are the Smad family signal transducers (11–13).

The Smad family proteins are a group of vertebrate proteins that exhibit high homology to the *Drosophila* Mad and *Caenorhabditis elegans* Smas, proteins first identified by genetic approaches to be signal transducers of TGF-β-like ligands in these invertebrate species (14, 15). Based upon their structural and functional properties, Smads are divided into three subclasses: 1) receptor-activated Smads (R-Smads), which are direct substrates of TGF-β family receptor kinases; 2) co-Smads, which participate in signaling by associating with R-Smads; and 3) anti-Smads, which act to inhibit the signaling functions of R-Smads. R-Smads interact transiently with specific ligand-activated type I receptors which directly phosphorylate the C-terminal SS(V/M)S motif of R-Smads. Smad2 and Smad3 are R-Smads specific for TGF-β and activin, whereas Smad1, -5, and -8 are R-Smads for BMPs (16). Smad6 and Smad7 are known as anti-Smads or inhibitory Smads (17), whereas Smad4 is the only known vertebrate co-Smad (18). After phosphorylation at the SS(V/M)S motif, the R-Smad associates with Smad4, and then together they translocate into the nucleus. In the nucleus, Smads are known to act as transcriptional regulators (11–13).

We have previously searched for protein interaction partners of Smad1 to better understand its signaling and regulation mechanisms in the signaling pathways of BMPs. We found that Smad1 binds to multiple proteins involved in proteasome-mediated degradation pathways such as HsN3, antizyme (Az), and ubiquitin.[2] HsN3 is one of the seven β subunits of the 20 S proteasome, the catalytic core of the 26 S proteasome (19–21). HsN3 was also shown to be involved in the targeting of p105 NF-κB subunit to proteasome for processing (22). Ubiquitin is well known for its role in covalently modifying proteasomal substrates for ubiquitin-dependent degradation (23–25). Az is a protein previously known to bind ornithine decarboxylase (ODC), the rate-limiting enzyme for polyamine synthesis. Interestingly, its physical interaction with ODC is necessary and sufficient for targeting ODC to the 26 S proteasome for ubiquitin-independent degradation (26, 27). Thus, ubiquitin and Az are two types of proteasome targeting proteins that mark pro-

* This work was supported by Virginia Mason Research Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations and trivial names used are: TGF-β, transforming growth factor β; BMP, bone morphogenetic protein; Az, antizyme; ODC, ornithine decarboxylase; PAGE, polyacrylamide gel electrophoresis; Ub, ubiquitin; HA, hemagglutinin; MG-132, *Z*-Leu-Leu-Leu-aldehyde; LLnL, *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal; LLM, *N*-acetyl-L-leucinyl-L-leucinal-L-methional.

[2] Y. Lin, J. Martin, C. Gruendler, J. Farley, X. Meng, B.-Y. Li, R. Lechleider, C. Huff, R. Kim, W. Grasser, V. Paralkar, and T. Wang, submitted for publication.

teins for both ubiquitin-dependent and ubiquitin-independent degradation by the 26 S proteasome. Currently, it is not clear how proteasome recognizes ubiquitinated proteins or Az-bound ODC. The ability of Smad1 to bind to ubiquitin and Az as well as HsN3, which is a proteasome component, suggests an interesting link between Smad1 and the proteasome targeting events involving ubiquitin, Az and HsN3. Studies were carried out to test whether the physical interaction between Smad1 and proteins involved in proteasomal degradation pathways (HsN3, Az, and Ub) may lead to: 1) proteasomal degradation of Smad1 or 2) proteasomal degradation of Smad1 interacting proteins. Concomitant with our studies, recent studies by others in the field have demonstrated several important roles of proteasomal degradation in regulating the protein levels of Smads and Smad-interacting proteins (28–35). In the signaling pathways of BMPs, it has been shown that Smad1 interacts with an ubiquitin E3 ligase, Smurf1, which regulates proteasomal degradation of Smad1 independent of BMP type I receptor activation (30). Here we provide the first evidence that proteasomal degradation of Smad1 is also induced upon the activation by the BMP type I receptor. Furthermore, our data reveal novel roles of two Smad1 interactors, Az and HsN3, in proteasomal targeting and degradation of Smad1, in addition to Smad1 ubiquitination.

EXPERIMENTAL PROCEDURES

Mammalian Cell Lines—P19 cells (mouse teratocarcinoma cells) were cultured in α-minimal essential medium containing 10% bovine calf serum and 50 units/ml penicillin-streptomycin at 37 °C in presence of 5% CO_2. 293 cells (human kidney cells transformed with adenovirus 5 DNA) were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin-streptomycin, and 2 mM glutamine at 37 °C in presence of 5% CO_2.

Constructs, Antibodies, Proteasome Inhibitors, BMPs, and Polyamine Treatment—For the 293 overexpression system, Smad1, HsN3, and antizyme were cloned into a modified pCMV6 vector that had the Flag epitope placed upstream of the multiple cloning site. The expression constructs for the wild-type BMP type I (ALK3) receptor, type II (bRII) receptor, and activated type I receptor (ALK3Q233D) were gifts from Dr. J. Massagué. The expression construct of R1 (rat homologue of ALK2) was made by polymerase chain reaction, followed by subcloning the polymerase chain reaction fragment of full-length R1 into pCMV6. R1K235R and R1Q207D were made by site-directed mutagenesis. FKBP12 has been described previously (8). HA-ubiquitin was a kind gift from Drs. M. Treies and D. Dohmann. Anti-Smad1 (367), a rabbit polyclonal antibody, was obtained from A. Roberts at NCI; anti-Smad1-P, a rabbit polyclonal antibody, was purchased from Upstate Biotechnology.

Lactacystin was purchased from E. J. Corey's laboratory at Harvard University (Cambridge, MA); *Z*-Leu-Leu-Leu-aldehyde (MG-132) was purchased from Affinity Research; *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL) and *N*-acetyl-L-leucinyl-L-leucinal-L-methional (LLM) were purchased from Sigma. MG-132, LLnL, and LLM were made in Me_2SO as a stock solution of 50 mM and then added directly into cell culture medium, to be diluted to a final concentration of 50 μM 8 h before cells were harvested for analyses. Lactacystin was made in H_2O as a stock solution of 10 mM and added directly into cell culture medium, to be diluted to a final concentration of 25 μM.

BMP7 was obtained from Creative Biomolecules Inc. (Hopkinton, MA); BMP2 and BMP4 were from the Genetics Institute Inc. For BMP treatment, the growth medium was changed to serum-free medium and then the stock solutions of BMP2, -4, or -7 were added directly into the cell culture medium to reach a final concentration of 400 ng/ml (for BMP2/4) and 250 ng/ml for BMP7.

Putrescine and aminoguanidine were purchased from Sigma. Putrescine and aminoguanidine were made in H_2O as a stock solution of 1 M and then added directly into cell culture medium, to be diluted to a final putrescine concentration of 25 mM and an aminoguanidine concentration of 2.5 mM 8 h before cells were harvested for analyses.

Transient Transfections and Immunoprecipitation/Western Blot—Equal amounts of plasmids were used to transfect 293 cells transiently with the calcium-phosphate precipitation method (36). Twenty-four hours after transfection, cells were lysed in lysis buffer (50 mM Hepes, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.5) containing protease and phosphatase inhibitors (Sigma). The protein concentration was determined using the Bio-Rad protein assay. Standard SDS-PAGE and Western blot assays were carried out to determine the expression levels of each transfected construct.

For immunoprecipitation assays, cell lysates were rotated at 4 °C for 5 h or overnight in the presence of an antibody as indicated and 40 μl of 50% protein G-Sepharose. Afterward the beads were washed once with lysis buffer, then three times with modified lysis buffer (0.1% Triton X-100 instead of 1%). After spinning down and removing the supernatant, the Sepharose beads were mixed with 50 μl of 2× sample buffer containing 10% 2-mercaptoethanol and boiled for 5 min. The total elute was used for SDS-PAGE and Western blot.

Pulse-Chase Analysis—To assure that each group of cells have the same transfection efficiency, 293 cells were transfected as described above, pooled, and re-seeded the next day. Twenty-four hours after re-seeding, cells were washed twice in pulse-medium lacking [^{35}S]methionine and [^{35}S]cysteine (methionine-free Dulbecco's modified Eagle's medium, 0.5% dialyzed serum, 2 mM glutamine, 50 units/ml penicillin-streptomycin), and incubated for 15 min to deplete endogenous methionine. Cells were then incubated with the pulse-labeling medium containing [^{35}S]methionine and [^{35}S]cysteine (190 μCi/ml) for 2 h. After washing once with chase medium (pulse medium containing 150 mg/liter unlabeled methionine and cysteine), cells were then incubated with or without BMPs in the presence or absence of proteasome inhibitors for different time periods before cells were harvested. Cells were then lysed using the lysis buffer described above.

The obtained lysates were pre-cleaned with 50 μl of 50% protein G-Sepharose for 1 h before the supernatants were subjected to immunoprecipitation. The precipitated proteins were subjected to SDS-PAGE. The gel was fixed for 30 min in 10% acetic acid and 10% methanol, followed by washing three times, each for 10 min in water. The gel was then incubated for 20 min in Amplify (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom), and then vacuum-dried at 75 °C. The radioactive signals were visualized by autoradiography using BioMax™ MR film (Eastman Kodak Co.).

Separation of Proteins into Cytoplasmic and Nuclear Fractions—Cells were harvested as usual, then resuspended in 100 μl of ice-cold buffer A (10 mM Hepes-KOH, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, plus protease and phosphatase inhibitors). After incubation (shaking on ice) for 30 min, cells were sheared by aspirating 10 times through a 23-gauge needle syringe, centrifuged 2 min at 12,000 × g. The supernatant containing the cytoplasmic proteins was kept on ice, and the pellet containing nuclear proteins was resuspended in 50 μl of ice-cold buffer B (same as buffer A except containing 420 mM NaCl instead of 10 mM KCl) for 30 min on ice with constant shaking. Both fractions were centrifuged for 30 min at 12,000 × *g* to obtain supernatants containing the cytoplasmic and nuclear protein fractions.

RESULTS

Domain-specific Interaction between Smad1, HsN3, and Az in the Yeast Two-hybrid System—When Smad1 was used as the bait in a yeast two-hybrid screen, the ornithine decarboxylase Az, two ubiquitin fusion proteins (Uba52 and Uba80), and the proteasome β subunit HsN3 were isolated as Smad1 interactors[2] (37). The interaction between Smad1 and HsN3 in the yeast two-hybrid system is dependent upon the presence of the MH2 domain of Smad1, as shown in Fig. 1*A*. The MH2 domain of Smad1 is also required for Smad1 to bind Az (Fig. 1*B*, *panel 1*) as well as Ub fusion proteins (37). Because HsN3 and Az are both Smad1 interactors, we further tested the interaction between these two proteins. Domain-specific interaction was detected between HsN3 and Az (Fig. 1*B*, *panel 2*). Az is known to bind ODC via the C-terminal domain of Az. The successful targeting of ODC by Az is dependent upon the N-terminal 107 amino acids (38, 39). The proteasomal receptor(s) for Az/ODC complex is not known, but has been suggested to involve components in both the 20 S proteasome as well as in the 19 S regulator (26). Domain mapping analyses of the interaction between HsN3 and Az showed that the interaction requires the N-terminal 35 amino acids of HsN3 and the N-terminal 107 amino acids of Az, which overlaps with the known proteasome targeting signal on Az but is dispensable for ODC binding (Fig. 1*B*, *panels 2* and *3*). A scheme is presented in Fig. 1*C* to

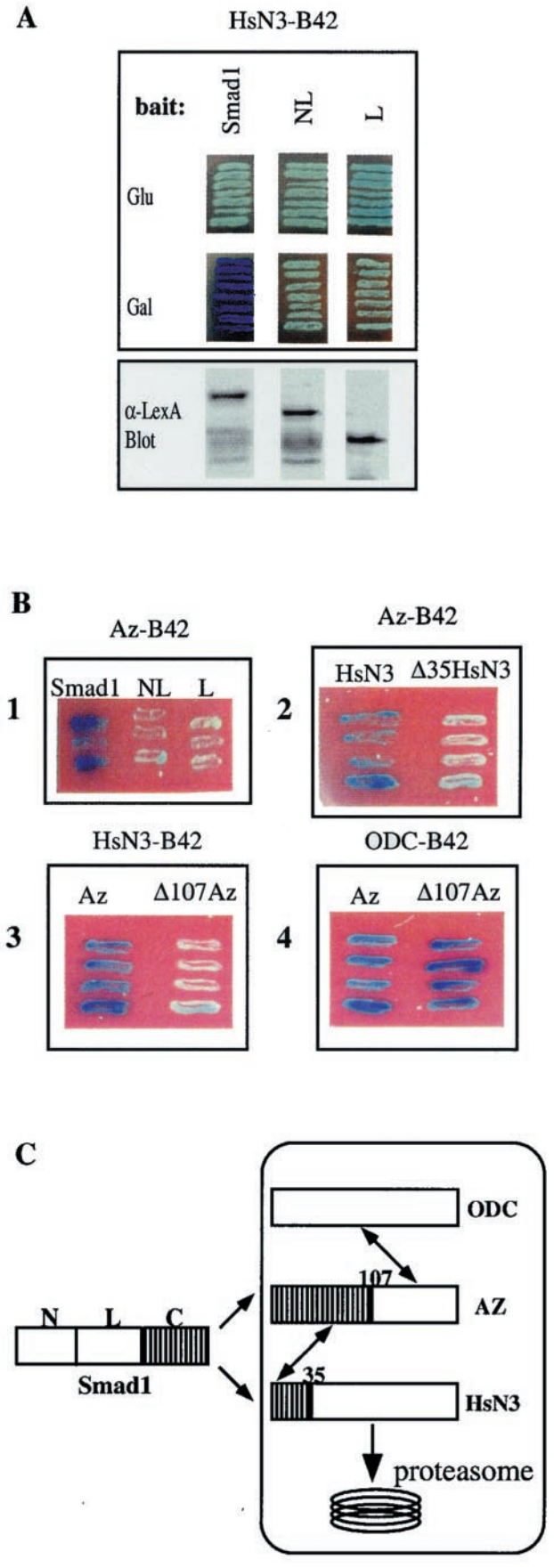

FIG. 1. **Domain-specific interaction between Smad1, HsN3, and Az in the yeast two-hybrid system.** *A*, the C-terminal domain of Smad1 is required for Smad1 binding to HsN3 in yeast. Yeast cells were transformed with B42-HsN3 and the indicated LexA fusion constructs of Smad1. *NL* (amino acids 1–271), Smad1 that lacks the MH2 domain; *L* (amino acids 147–271), Smad that lacks both the MH1 and MH2 domain. *Glu*, $U^-H^-W^-$ glucose X-gal plate; *Gal*, $U^-H^-W^-$ galactose X-gal plate. *Bottom panel* is Western blot with anti-LexA to detect the expression of the LexA fusion proteins. *B*, domain mapping of the interaction between Smad1 and Az and between Az and HsN3 using the yeast two-hybrid system. LexA fusion proteins are indicated *inside* each *box* immediately *above* the tested yeast transformants. *C*, a schematic summarizing the interaction between Smad1, Az, ODC, and HsN3 observed in yeast. The *hatched region* represents identified subdomains that are necessary for the indicated interactions. *N*, MH1; *L*, middle linker; *C*, MH2.

illustrate the known domain-specific interaction between ODC and Az, as well as the newly observed interactions between Smad1, Az, and HsN3. Because HsN3 is a proteasome component, whereas Az and Ub are both substrate targeting proteins, the ability of Smad1 to interact with these proteins suggest that Smad1, like ODC, could be targeted to proteasome for degradation because of its ubiquitination or Az interaction, or could itself play a role in the substrate targeting process, such as in regulating the delivery of ubiquitinated protein or Az-bound proteins to proteasome. Although studies to test the ability of Smad1 in regulating the proteasomal targeting of an Az-bound nuclear protein are reported elsewhere,[2] our studies of proteasomal degradation of Smad1 are described below.

The Steady State Level of Smad1 Protein Is Reduced upon the Coexpression of the Activated BMP Type I Receptor ALK3Q233D—To explore a possible role of proteasome in degrading Smad1, we first tested the conditions required for the reduction of the steady state level of Smad1. Although increased expression of Az and HsN3 alone does not alter Smad1 level (data not shown), we observed a significant reduction of Smad1 level upon the activation of BMP type I receptor ALK3 (Fig. 2*A*). In these studies, 293 cells were transfected with Flag-Smad1 alone or together with the constitutively active BMP type I receptor ALK3Q233D that mimics the effect of BMP stimulation (40). The protein level of Smad1 was monitored by SDS-PAGE and Western blot (Fig. 2*A*, *top panel*). The detected Smad1 signals were then subjected to densitometry analyses, and the quantification results are presented as integrated optical density (IOD) of Flag-Smad1 (Fig. 2*A*, *bottom panel*). The IOD of Flag-Smad1 was reduced 68% upon ALK3Q233D coexpression. The ability of ALK3Q233D to phosphorylate Smad1 in this system was monitored by Western blot analysis with anti-Smad1-P, an antibody specific to the form of Smad1 phosphorylated at the C-terminal SSVS motif. As expected, ALK3Q233D expression led to Smad1 phosphorylation (Fig. 2*A*, *middle panel*). Thus, BMP type I receptor activation leads to both Smad1 phosphorylation at the C-terminal SSVS motif and a reduction of Smad1 protein level. These effects of the activated BMP type I receptor are dose-dependent, because increased expression of ALK3Q233D led to enhanced reduction of Smad1 protein level and enhanced increase of Smad1 phosphorylation (Fig. 2*B*). In this study, a constant amount of Smad1 expression construct was co-transfected with an increasing amount of ALK3Q233D into 293 cells. The change of Flag-Smad1 protein level in both the cytoplasm and the nucleus was monitored by separating the total proteins into cytoplasmic and nuclear fractions and then analyzed by SDS-PAGE and Western blot using anti-Flag antibody (Fig. 2*B*, *top panel*). The phosphorylated Smad1 protein level was detected by Western blot with anti-Smad1-P (Fig. 2*B*, *bottom panel*). To assist the analyses of Smad1 protein level changes, the IOD of Smad1 signals detected in the *top panel* of Fig. 2*B* were quantified, corrected according to equal nonspecific signals in each lane, and plotted. As shown in Fig. 2*C*, increased expression of ALK3Q233D led to a dose-dependent reduction of both cyto-

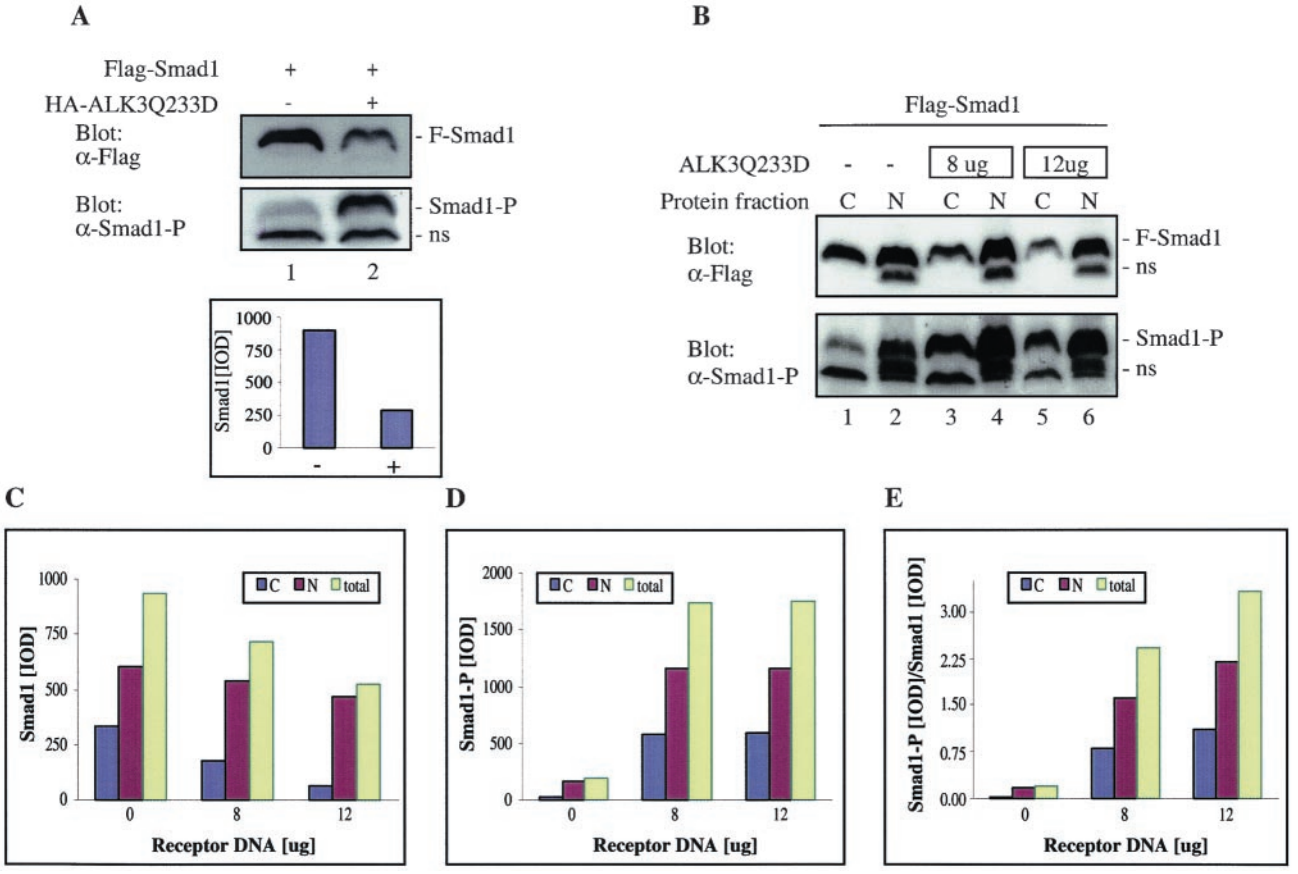

FIG. 2. **Coexpression of the constitutively activated BMP type I receptor ALK3Q233D and Smad1 leads to Smad1 phosphorylation and a decrease of Smad1 protein level.** *A*, ALK3Q233D induces the phosphorylation and the reduction of the protein level of Smad1. 293 cells were transfected with Flag-Smad1 alone (2 μg, *lane 1*) or together with the constitutively active BMP type I receptor HA-ALK3Q233D (4 μg, *lane 2*), and harvested after 24 h. Equal amount of total protein was analyzed by 15% SDS-PAGE and Western blot with anti-Flag (*top panel*), anti-Smad1-P (*middle panel*). *ns* refers to a nonspecific band in each panel. The Smad1 signal (*top panel*) was quantified, and the integrated optical density of Smad1 is represented as a *bar graph* (*bottom panel*). *B–E*, increased expression of ALK3Q233D leads to a dose-dependent reduction of Smad1 level accompanying an increase in Smad1 phosphorylation. *B*, Western blot analyses of the protein levels of Smad1 or Smad1-P in 293 cells expressing increased amount of ALK3Q233D. 293 cells were transfected with increasing amount of ALK3Q233D (0, 8, and 12 μg) and constant amount of Flag-Smad1 (4 μg). Transfected cells were lysed and cellular proteins were separated into cytoplasmic (*C*) and nuclear (*N*) fractions, which were analyzed by SDS-PAGE and Western blot. The *top panel* shows Smad1 protein level. The phosphorylation of Smad1 is shown on the bottom panel (*α-Smad1-P*). *C*, Smad1 signals in *top panel* of *B* were quantified using densitometry and presented as the IOD. *D*, Smad1-P signals in *bottom panel* of *B* were quantified using densitometry and presented as the IOD. Both sets of signals were corrected according to nonspecific signals in each lane. *E*, calculated arbitrary percentage of Smad1-P over total Smad1 using the data in *C* and *D*.

plasmic and nuclear Smad1 protein levels, although the reduction in the nuclear fraction was not as dramatic as that seen in the cytoplasmic fraction, possibly because of cytoplasmic to nuclear translocation of Smad1. The IOD of phosphorylated Smad1 (Smad1-P) was also quantified. As shown in Fig. 2*D*, transfecting 8 μg of ALK3Q233D construct significantly increased the level of Smad1-P, whereas transfecting 12 μg of ALK3Q233D construct did not lead to further increase of the level of Smad1-P. However, the relative percentage of Smad1-P over total Smad1 level exhibited a dose-dependent increase (Fig. 2*E*). These data together indicate that increased receptor activation leads to an enhanced reduction of total Smad1 protein level, accompanying an increase in Smad1 phosphorylation.

BMP7 Induces the Reduction of Smad1 Steady State Protein Level in a Time-dependent Fashion—The above studies demonstrated the ability of a constitutively active BMP type I receptor (ALK3Q233D) to induce the reduction of Smad1 level in a dose-dependent fashion. We next examined the time course of the reduction of Smad1 protein level in response to BMP type I receptor activation. To do so, we reconstituted a BMP-responsive system in 293 cells by transiently transfecting the cells with BMP7-responsive type I receptor (R1) and the common BMP type II receptor (bRII) together with Flag-Smad1. Cells were treated with BMP7 for different time periods as indicated. Additionally, we transfected a second set of cells with the kinase-deficient mutant form of R1, R1K235R, which lacks the kinase activity as a result of the abolishment of its ATP binding site (41). This serves as the control group to determine the role of the type I receptor kinase activity in the change of Smad1 phosphorylation and protein level upon receptor binding to BMP7. The protein levels of Smad1 and Smad1-P were detected by SDS-PAGE and Western blot (Fig. 3*A*). A time-dependent reduction of Smad1 steady state level was detected in R1/bRII-transfected cells but not in R1K235R/bRII group (Fig. 3*A*, *top panel*, compare *lanes 1–4* with *lanes 5–8*; IOD percentage shown in *bottom panel*). Thus, BMP7 induces the reduction of the steady state level of Smad1 in a time-dependent fashion, and the reduction is dependent upon the activation of the BMP type I receptor kinase activity. A similar experiment was carried out by replacing R1 with ALK3, which is the BMP2/4 type I receptor. A time-dependent reduction of Smad1 level was also observed (Fig. 3*B*). Thus, BMP7, -2, and -4 can all induce time-dependent reduction of the steady state level of Smad1.

We noted that Smad1 phosphorylation at its C-terminal SSVS motif was detected in R1/bRII transfected cells even

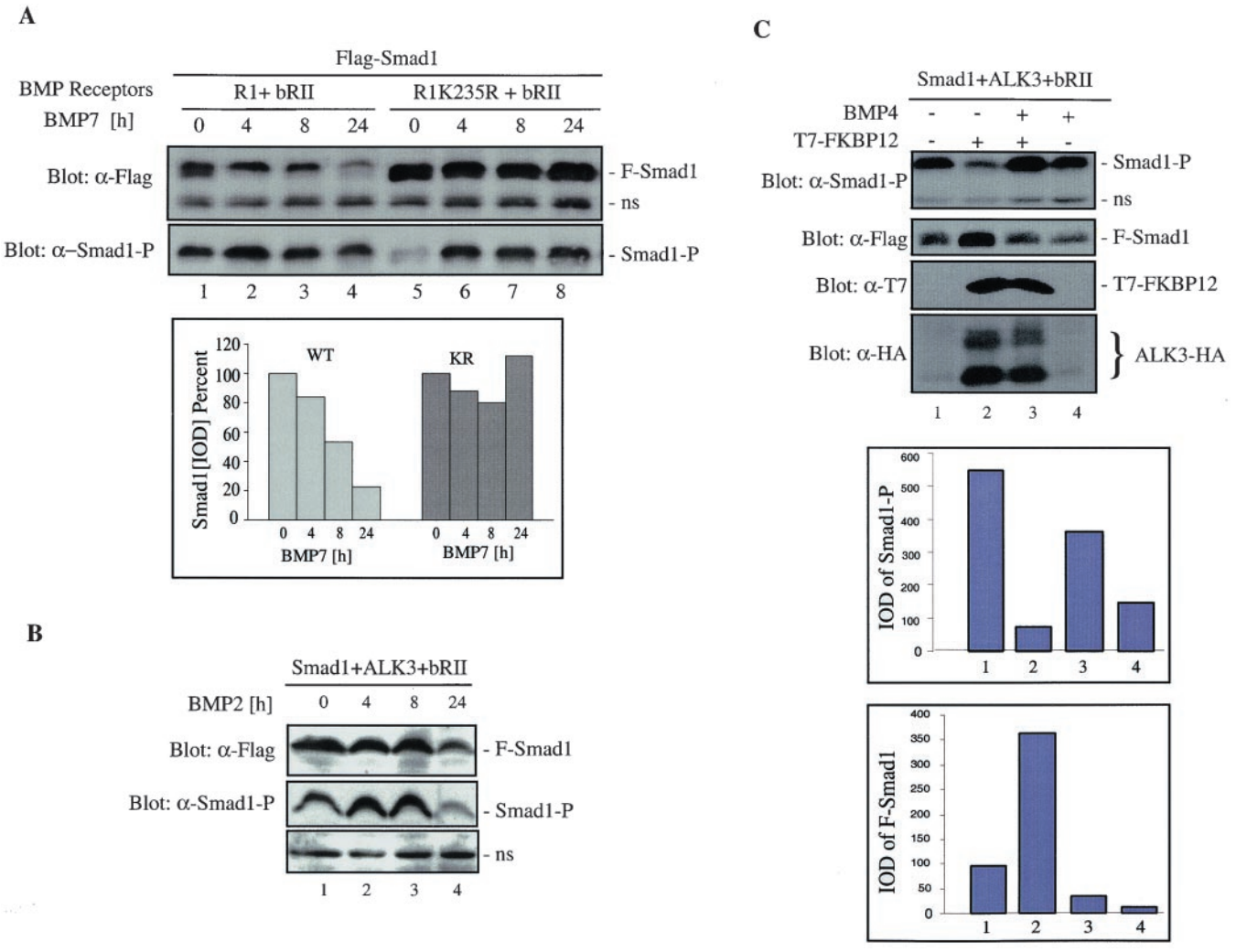

FIG. 3. **BMPs induce a reduction of Smad1 protein level in a time-dependent fashion.** *A*, BMP7 induces a time-dependent reduction of Smad1 protein level. 293 cells were transfected with Flag-Smad1 together with either the wild type BMP7 type I (*R1*) and type II (*bRII*) receptors (*lanes 1–4*) or the kinase-dead BMP7 type I (*R1K235R*) and type II (*bRII*) receptors (*lanes 5–8*). Cells were treated with 250 ng/ml BMP7 for the indicated periods and then harvested together 36 h after transfection. The *top panel* shows Western blot of F-Smad1; the *middle panel* shows Western blot of Smad1-P. The IOD of F-Smad1 corresponding to each time point was quantified, divided by the IOD of zero time points and presented as percentage of IOD in the *bottom panel*. *B*, BMP2 also induces a reduction of Smad1 level in a time-dependent fashion. 293 cells were transfected with ALK3 and bRII, treated with 400 ng/ml BMP2 for the indicated time periods, and the levels of F-Smad1 and Smad1-P were analyzed by Western blot. *ns* represents a nonspecific band to serve as an internal protein level control. *C*, FKBP12 blocks receptor leakiness in the 293 overexpression system. 293 cells were transfected with Flag-Smad1, wild type ALK3 type I receptor, and BMP type II receptor (*bRII*) in the presence or absence of T7-FKBP12 (FKBP12: 6 μg, others 4 μg each). Cells were treated with 400 ng/ml BMP4 for 8 h and then harvested and analyzed by SDS-PAGE and Western blot using anti-Flag and anti-T7 antibody, as indicated. The *top panel* shows the level of Smad1-P, the *second panel* shows the level of total Smad1, the *third panel* shows the level of FKBP12 and the fourth panel shows the steady state level of transfected HA-tagged ALK3. The higher levels of HA-ALK3 in lanes 2 and 3 reflect a stabilization effect of FKBP12 on ALK3 (Gruendler C., *et al.*, unpublished data).

before BMP7 treatment (Fig. 3*A*, *middle panel*, *lane 1*). These data suggest that R1 was constitutively activated independent of BMP7. In other words, the overexpressed R1 exhibited leaky signaling. Similar leakiness was also observed for ALK3 (see below). We also noted that Smad1 phosphorylation was induced by BMP7 in cells overexpressing the signaling defective R1K235R (Fig. 3*A*, *bottom panel*, *lanes 6–8*). This appears to be caused by the presence of 293 cell endogenous BMP type I and type II receptors, whose activation by exogenous BMP7 led to the observed phosphorylation of Smad1 (data not shown).

The BMP Type I Receptor Leakiness Can Be Blocked by FKBP12 in the 293 Overexpression System—Although a time-dependent effect of BMPs on Smad1 protein level was detected in the above studies, we were concerned by the observed signaling leakiness of overexpressed R1. Because the immunophilin FKBP12 has been shown to play an important role in keeping the type I receptor inactive before ligand stimulation (7–9), we suspect that the lack of sufficient endogenous FKBP12 in 293 cells may contribute to the observed leaky signaling of the overexpressed type I receptors. Thus, we overexpressed FKBP12 together with the BMP type I receptor (ALK3) and the BMP type II receptor (bRII) in 293 cells, which were either treated with BMP4 or untreated. Smad1-P level was monitored by Western blot (Fig. 3*C*, *top panel*). A high level of Smad1-P was detected by simply coexpressing both the type I and type II receptors in the absence of any BMP4, indicating the leaky signaling of the overexpressed ALK3 (Fig. 3*C*, *top panel*, *lane 1*). Upon the coexpression of FKBP12, Smad1 phosphorylation resulted from leaky signaling was greatly decreased (Fig. 3*C*, *top panel*, *lane 2*) but was induced upon BMP4 treatment in the presence or absence of FKBP12 (Fig. 3*C*, *top panel*, *lanes 3* and *4*). Thus, co-expression of FKBP12 reduces Smad1 phosphorylation resulted from leaky signaling from the overexpressed type I receptor but still allows an inducible

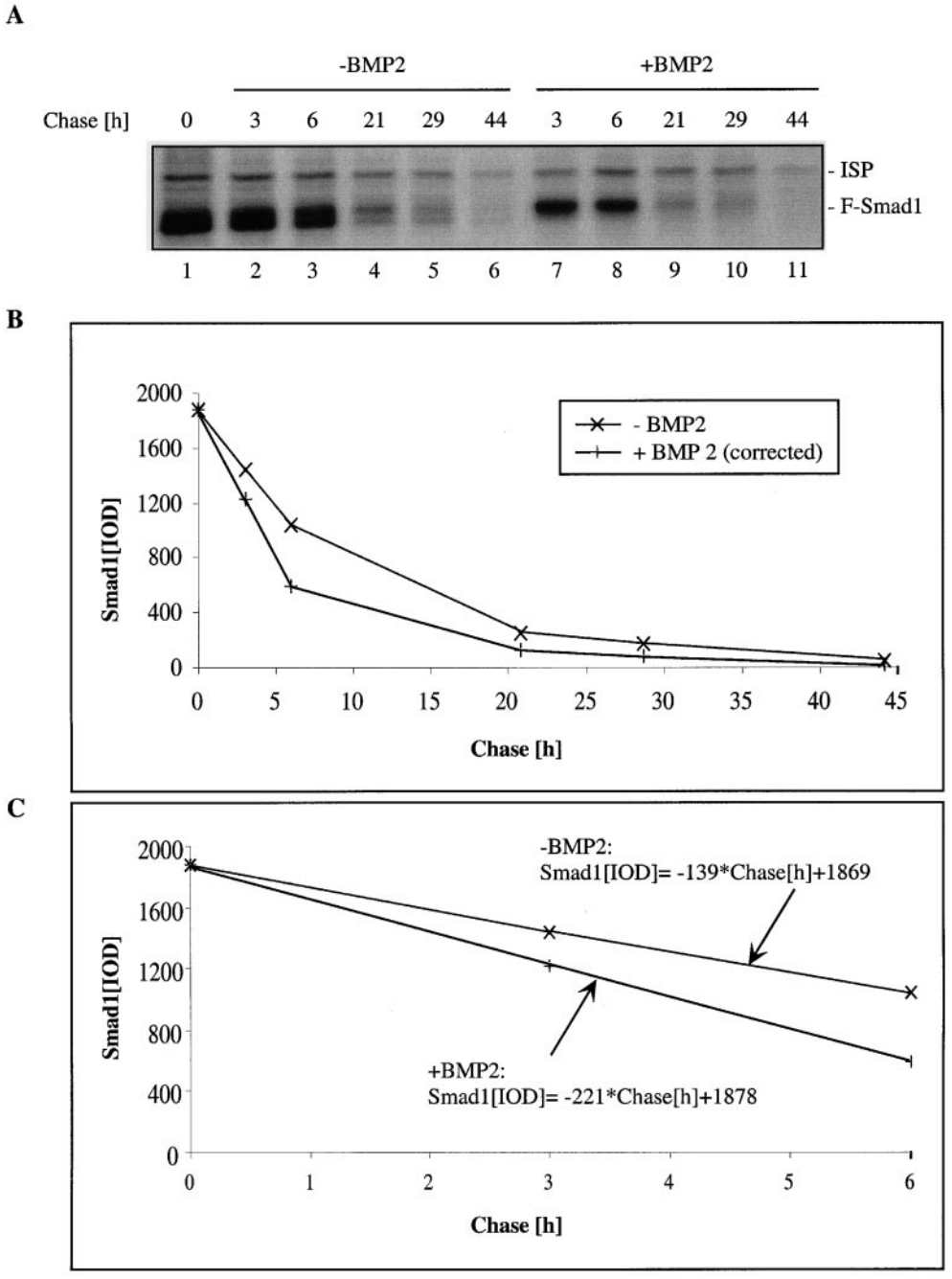

FIG. 4. **The reduction of Smad1 level induced by BMPs is caused by a reduction of Smad1 half-life.** *A*, analyses of Smad1 level change in the presence or absence of BMP2 using pulse-chase assays. 293 cells were transfected with Flag-Smad1, BMP type I and II receptors (ALK3, bRII) (4 μg each), and T7-FKBP12 (6 μg). Cells were pulsed for 2 h in [^{35}S]methionine and [^{35}S]cysteine followed by chasing in the presence or absence of 400 ng/ml BMP2 for the indicated time points. Cells were lysed, and Smad1 was immunoprecipitated, separated on SDS-PAGE, and detected by autoradiography. *ISP*, a nonspecific band used as an internal standard protein for quantification shown in *B*. *B*, quantification of Smad1 signals in *A* using densitometry. Correction of Smad1 signals was carried out based upon the signals of the nonspecific band (internal standard protein). *C*, calculation of the half-life of Smad1 based upon the linear decrease of Smad1 signals within the first 6 h of chase.

phosphorylation of Smad1 by BMP4.

The steady state level of Smad1 was also monitored by Western blot with anti-Flag (Fig. 3*C*, *second panel*). Coincide with the ability of FKBP12 to inhibit leaky signaling-induced Smad1 phosphorylation, FKBP12 also restored the steady state level of Smad1 (Fig. 3*C*, *second panel*, compare *lanes 1* and *2*). Coexpression of FKBP12 also allowed the detection of BMP4-induced reduction of the steady state level of Smad1 (Fig. 3*C*, *second panel*, compare *lanes 2* and *3*). The absence of FKBP12 allowed a further reduction of Flag-Smad1 signal (*second panel*, *lane 4*). Therefore, FKBP12 blocks leaky signaling-induced Smad1 phosphorylation and the reduction of Smad1 protein level.

Pulse-Chase Analysis of the BMP-induced Reduction of Smad1 Protein Level Reveals a Reduction of Smad1 Stability upon BMP Treatment—After solving the problem of receptor leakiness of ALK3 in the overexpression system, we then tested the change of Smad1 half-life in this system to further determine the nature of the BMP-induced reduction of Smad1 level. The Smad1 protein level was subjected to a pulse-chase analysis (Fig. 4). 293 cells were transfected with Flag-Smad1, BMP type I (ALK3) and type II (bRII) receptors, and FKBP12. The

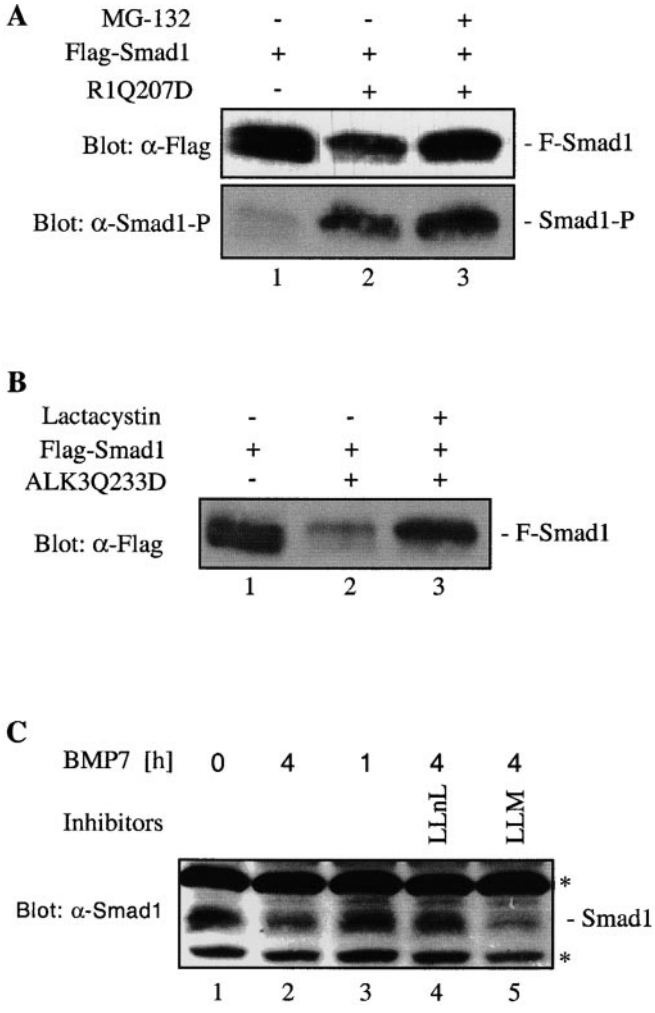

FIG. 5. **The BMP-induced reduction of Smad1 involves proteasome-mediated degradation of Smad1.** *A*, the reduction of Smad1 protein level upon the activation of BMP type I receptor R1 is sensitive to proteasome inhibitor MG-132. 293 cells were transfected with Flag-Smad1 (4 μg) and with or without the activated BMP type I receptor R1Q207D (4 μg). Cells were treated for 8 h with 50 μM MG-132 before cell lysates were made and subjected to SDS-PAGE and Western blot analyses using anti-Flag (*top panel*) and anti-Smad1-P (*bottom panel*). *B*, the reduction of Smad1 protein level upon the activation of BMP type I receptor ALK3 is sensitive to proteasome inhibitor lactacystin. 293 cells were transfected with Flag-Smad1 (4 μg) and with or without the activated BMP type I receptor ALK3Q233D (6 μg). Cells were treated for 8 h with 25 μM lactacystin before harvesting, SDS-PAGE analysis and Western blot. *C*, BMP7 causes a reduction of the steady state level of Smad1 in P19 cells. P19 cells either were not exposed to BMP7 (*lane 1*) or were treated with BMP7 (250 ng/ml) for 1 h (*lane 3*) or for 4 h (*lanes 2*, *4*, and *5*) in the absence of any drugs (*lane 2*) or in the presence of 50 μM LLnL (*lane 4*) or LLM 50 μM (*lane 5*). Cell lysates with equal amount of total protein were separated on SDS-PAGE. Smad1 was detected by Western blot using an affinity-purified anti-Smad polyclonal antibody 367 (43), which recognizes Smad1 as well as other Smads. The two proteins (marked by *asterisks*) could be other Smads that cross-reacted with antibody 367.

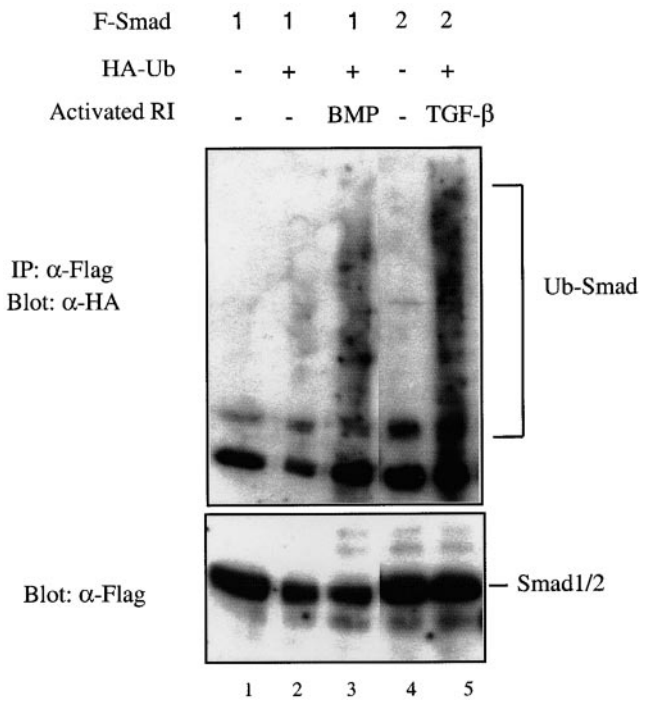

FIG. 6. **Smad1 is ubiquitinated upon BMP type I receptor activation.** 293 cells were transfected with the indicated plasmids. Cell lysates were prepared and denatured by SDS and boiling as described under "Experimental Procedures." Flag-tagged Smad1 and Smad2 were precipitated with monoclonal anti-Flag antibody. The immunoprecipitates were then blotted with a monoclonal anti-HA antibody (*top panel*). The expression level of Smad1 and Smad2 were analyzed by Western blot (*bottom panel*). The high molecular weight Smad Ub-conjugates in *lanes 3* and *5* are marked by a *bracket*.

transfected cells were pulsed for 2 h in medium containing [^{35}S]methionine and [^{35}S]cysteine and then chased for the indicated time periods in the presence or absence of BMP2. Smad1 protein was immunoprecipitated by anti-Flag, separated on SDS-PAGE, and detected by autoradiography (Fig. 4*A*). The signal of Smad1 was quantified and corrected according to the signals of a nonspecific protein, which served as the internal standard protein (Fig. 4*A*, *top band*). The result of the corrected IOD of Smad1 signal is plotted in Fig. 4*B*. Within the first 6 h of chase, an enhanced linear decrease of Smad1-IOD in cells treated with BMP2 was detected. After 6 h the decrease was no longer linear, possibly because of the synthesis of new, and thus unlabeled, Smad1 proteins that compete with labeled Smad1 for degradation. We thus calculated the half-life of Smad1 based upon the change of Smad1 IOD during the first 6 h, as shown in Fig. 4*C*. The half-life of Smad1 was 6.7 h in the absence of BMP2 stimulation, whereas BMP2 treatment decreased its half-life to 4.2 h. Two additional experiments were carried out and yielded similar percentages of reduction of Smad1 half-life upon BMP2 treatment (data not shown). These data point out that the reduction of Smad1 protein level upon receptor activation involves an enhanced Smad1 degradation.

Proteasome Is Involved in the Receptor Activation-induced Smad1 Degradation—Because our studies of the protein level change of Smad1 were initiated by the observation that Smad1 has a physical link with proteasome-mediated degradation pathways, we tested whether the enhanced Smad1 degradation involves the 26 S proteasome. 293 cells were transfected with Smad1 alone or together with the constitutively active BMP type I receptors (R1Q207D or ALK3Q233D) in the presence or absence of specific proteasome inhibitors MG-132 or lactacystin. The reduction of Smad1 protein level upon the activation of BMP type I receptors was blocked by both inhibitors (Fig. 5, *A* and *B*, *lane 3*). The involvement of proteasome in destabilizing receptor-activated Smad1 was further demonstrated by the ability of the proteasome inhibitors lactacystin and LLnL to block the receptor activation-induced half-life decrease of Smad1 in a pulse-chase experiment (data not shown). These data thus indicated that the reduction of Smad1 half-life is the result of proteasomal degradation of Smad1.

Because the above studies were carried out in overexpression systems, we further tested whether proteasomal degradation of Smad1 is also induced by BMPs under physiological conditions. We used P19 cells, a BMP7-responsive cell line with detectable

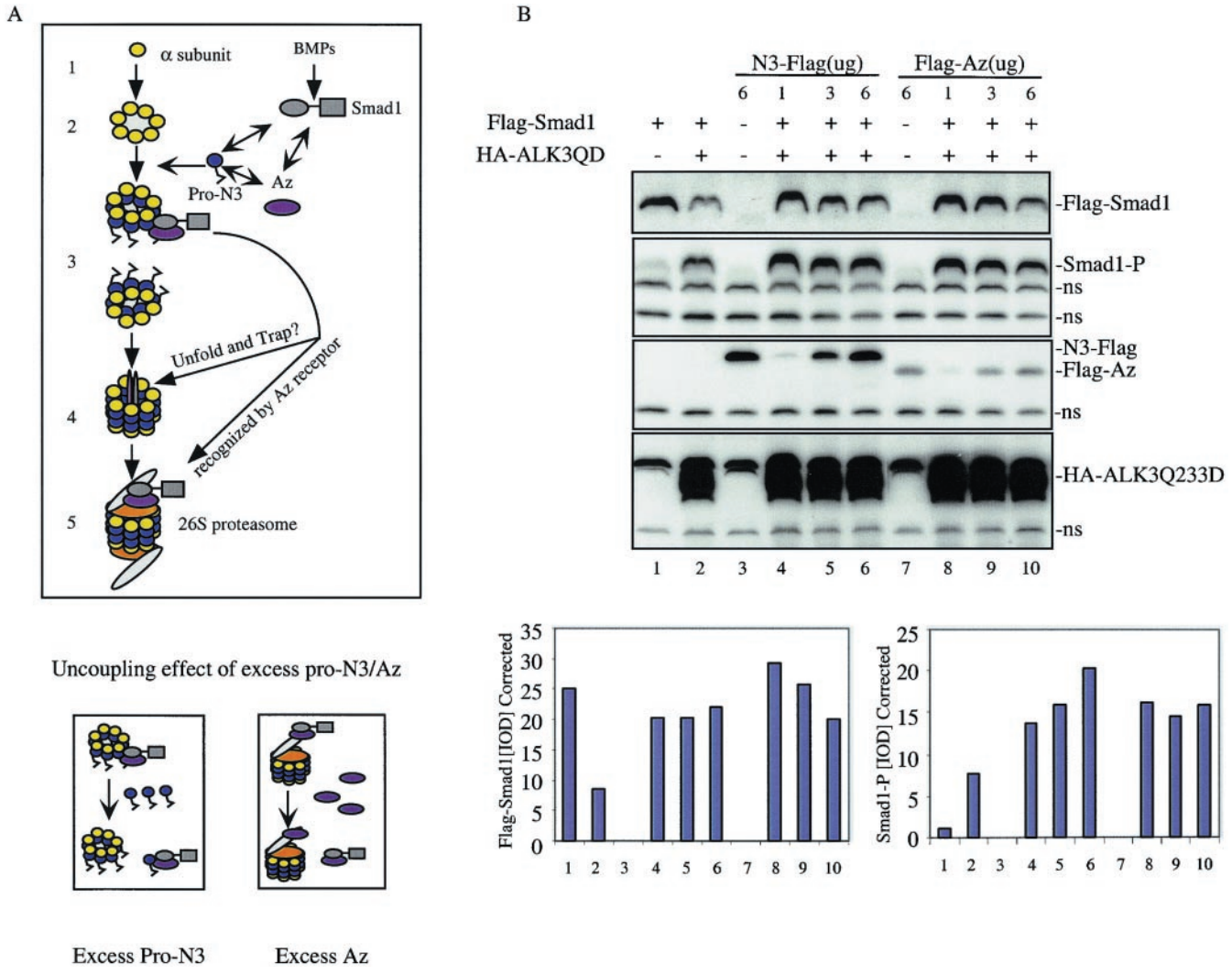

FIG. 7. **Smad1 degradation induced by BMP type I receptor activation also involves Az and HsN3.** *A*, *top panel* illustrates the observed interaction between Smad1, HsN3, and Az[2] along the proteasome assembly pathway. *Bottom panels* illustrate possible uncoupling effects caused by excess Az and prosequence-containing HsN3. See text for details. *B*, overexpression of HsN3 or Az interferes with Smad1 degradation. 293 cells were cotransfected with Flag-Smad1 (2 μg) or ALK3Q233D (4 μg) in the presence or absence of increasing amount of Flag-HsN3 or T7-Az as indicated. The steady state levels of Flag-Smad1 (*top panel*) and Smad1-P (*second panel*) were detected by Western blot and quantified using densitometry. As a result of toxicity effect of excess HsN3 and Az, total protein levels were also reduced, as reflected by the reduction of the signals of nonspecific bands (*ns*) in the *second panel*. Thus, the signals of Smad1 (IOD) were corrected based upon the signals of the nonspecific bands (*ns*) and shown in the *bottom panels*.

quantities of endogenous Smad1. The BMP7-responsive P19 cell line was treated with or without BMP7 for either 1 or 4 h. Cells exposed to BMP7 for 4 h were either not pretreated or pretreated with proteasome inhibitors. Two types of inhibitors were used: LLnL, which is a potent peptidyl aldehyde inhibitor of proteasome as well as a calpain I inhibitor, and LLM, which is a very weak proteasome inhibitor but strong calpain II inhibitor (42). Cell lysates containing equal amount of total proteins were analyzed by Western blot using a polyclonal anti-Smad antibody 367 that was raised against Smad1 but cross-reacts with other Smads (43). As shown in Fig. 5*C*, the steady state protein level of Smad1 was reduced in cells exposed to BMP7 for 4 h (Fig. 5*C*, *lane 2*). The reduction is time-dependent, because no reduction was observed after BMP7 exposure for 1 h (Fig. 5*C*, *lane 3*). The decrease of Smad1 level can be effectively blocked by LLnL but not by LLM (Fig. 5*C*, *lanes 4* and *5*). The selective change of the steady state level of Smad1 but not the two cross-reacted proteins (as marked by an asterisk), which are likely other Smads, indicates the specificity of the decrease of Smad1 in response to BMP7. The selective sensitivity of the decrease of Smad1 to LLnL but not to LLM suggests the role of proteasome instead of lysosomal degradation in this process.

BMP Type I Receptor Activation Induces Polyubiquitination of Smad1—Because most of the known proteasome substrates are ubiquitinated, we tested whether Smad1 is ubiquitinated in response to BMP type I receptor activation. As shown in Fig. 6, polyubiquitinated Smad1 was detected only upon BMP type I receptor activation. In the same experiment, Smad2 polyubiquitination upon TGF-β type I receptor was used as a positive control, as reported previously (31).

Receptor Activation-induced Smad1 Degradation Also Involves the Targeting Role of Antizyme and HsN3—Smad1 interacts with the proteasome subunit HsN3 and the proteasome substrate targeting protein Az, as shown in Fig. 1. Additional studies, as detailed in a separate report,[2] demonstrated that Smad1 forms a complex with Az and HsN3 before HsN3 is incorporated into the 20 S proteasome. Although the molecular details involved in Smad1 targeting to proteasome are not mapped out, the observed physical interaction between Smad1, HsN3, and Az suggests that Az and HsN3 may play an important role in this process. Because HsN3 is rapidly assembled

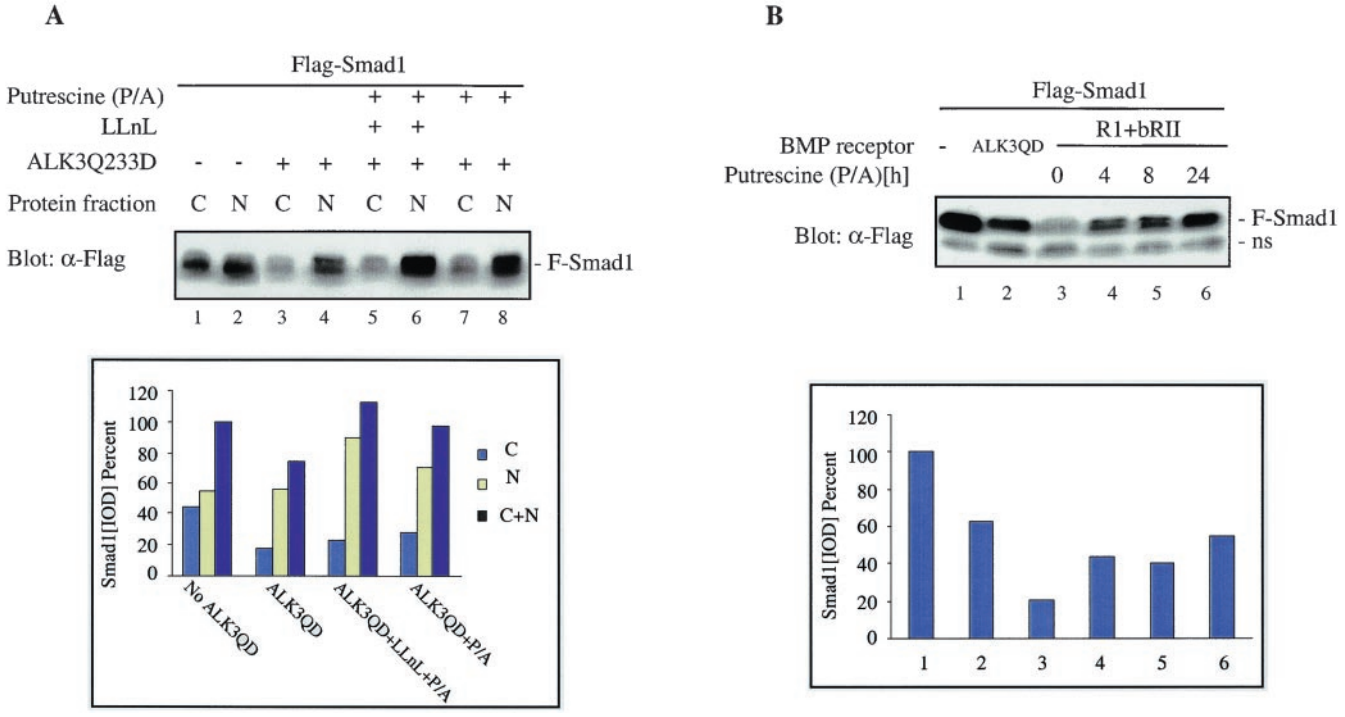

FIG. 8. **The polyamine inducer putrescine inhibits BMP type I receptor-induced Smad1 degradation.** *A*, high levels of putrescine stabilize nuclear Smad1 level in cells expressing activated BMP type I receptor. 293 cells were transfected with Flag-Smad1 (4 μg) and ALK3Q233D (8 μg). Cells were treated for 8 h with 25 mM putrescine in the presence of 2.5 mM aminoguanidine (which reduces cellular toxicity of putrescine), harvested, and cell lysates fractionated into cytoplasmic and nuclear fractions. The fractionated lysates were then analyzed by 15% SDS-PAGE and Western blot using anti-Flag. The levels of F-Smad1 was quantified by densitometry and illustrated as percentage of IOD at *right*. *B*, high levels of putrescine block time-dependent reduction of Smad1 level induced by leaky signaling from overexpressed BMP type I and type II receptors. 293 cells were transfected with Flag-Smad1 (4 μg) and the indicated receptors (8 μg total). Cells expressing both ALK2 (R1) and bRII were exposed to 25 mM putrescine and 2.5 mM aminoguanidine for different periods of time as indicated and then harvested at the same time. Cell lysates were analyzed by 15% SDS-PAGE and Western blot using anti-Flag. The levels of F-Smad1 was quantified by densitometry and illustrated as percentage of IOD at *right*.

into the 20 S proteasome (21), the complex formation between Smad1, HsN3, and Az may either lead to the trapping of Smad1 inside of the proteasome for degradation or allow HsN3 to dock Az-bound proteins such as Smad1 to proteasome complex for degradation. The docking of Az-bound Smad1 to proteasome likely involves protein-protein interactions between HsN3 and other proteasome components during HsN3 assembly and between Az and additional proteasome receptors, as illustrated in *top panel* of Fig. 7. Therefore, excess HsN3 and Az should compete with such interactions to uncouple the targeting/docking process, thereby blocking Smad1 targeting to proteasome, as illustrated in Fig. 7*A* (*bottom panels*). Consistent with such a prediction, ALK3Q233D-induced reduction of Smad1 was inhibited upon the expression of excess HsN3 (Fig. 7*B*, *top panel*, *lane 4*) or excess Az (Fig. 7*B*, *top* panel, *lane 8*). The slight reduction of Smad1 levels upon further increased expression of HsN3 and Az (*lanes 5*, *6*, *9*, and *10*) may be partially caused by toxicity effects of these proteins, because we observed extensive cell death under these conditions (data not shown). We also monitored the levels of phosphorylated Smad1 under these conditions (Fig. 7*B*, *second panel*). Increased levels of Smad1-P were detected in cells expressing excess HsN3 and Az (Fig. 7*B*, *second panel*, *lanes 4–7* and *8–10*). The role of Az in targeting Smad1 to proteasome was further tested by directly altering the endogenous levels of Az in 293 cells by treating cells with putrescine, a polyamine known to enhance Az translation (44). As shown in Fig. 8*A*, putrescine treatment efficiently stabilized the nuclear Smad1 in cells expressing activated BMP type I receptor (Fig. 8*A*, compare *lanes 4* and *8*), whereas the proteasome inhibitor LLnL further protected Smad1 from degradation (Fig. 8*A*, *lane 6*). Putrescine treatment also efficiently blocked the decrease of Smad1 level in response to leaky signaling of the BMP type I receptor, as shown in Fig. 8*B*. These data, together with the observed physical interaction between Smad1, HsN3, and Az,[2] strongly support novel targeting roles of Az and HsN3 in the proteasomal degradation of Smad1 induced by BMP type I receptor activation.

DISCUSSION

The BMPs are well known for their abilities to induce ectopic bone formation as well as their roles in early embryogenesis and tissue and organ morphogenesis. Smad1 has been shown to be a key signal transducer of BMPs. Our studies reported here have revealed an interesting aspect of the regulation of Smad1, *i.e.* the BMP-induced proteasomal targeting and the subsequent degradation of Smad1. The studies were based upon an observation made in 1996, during the search of Smad1 interactors using the yeast two-hybrid system. We uncovered the novel ability of Smad1 to bind the proteasome β subunit HsN3 and two types of proteasomal substrate targeting proteins: Ub and Az. Although initial studies were focused upon the functional roles of these Smad1 interactors in the signaling process of Smad1, which will be reported separately,[2] here we have presented our detailed studies of Smad1 itself as a proteasomal substrate and the regulatory factors involved in this novel event.

We first demonstrated in the 293 overexpression system that the steady-state level of Smad1 is significantly reduced upon the co-expression of a constitutively active BMP type I receptor.

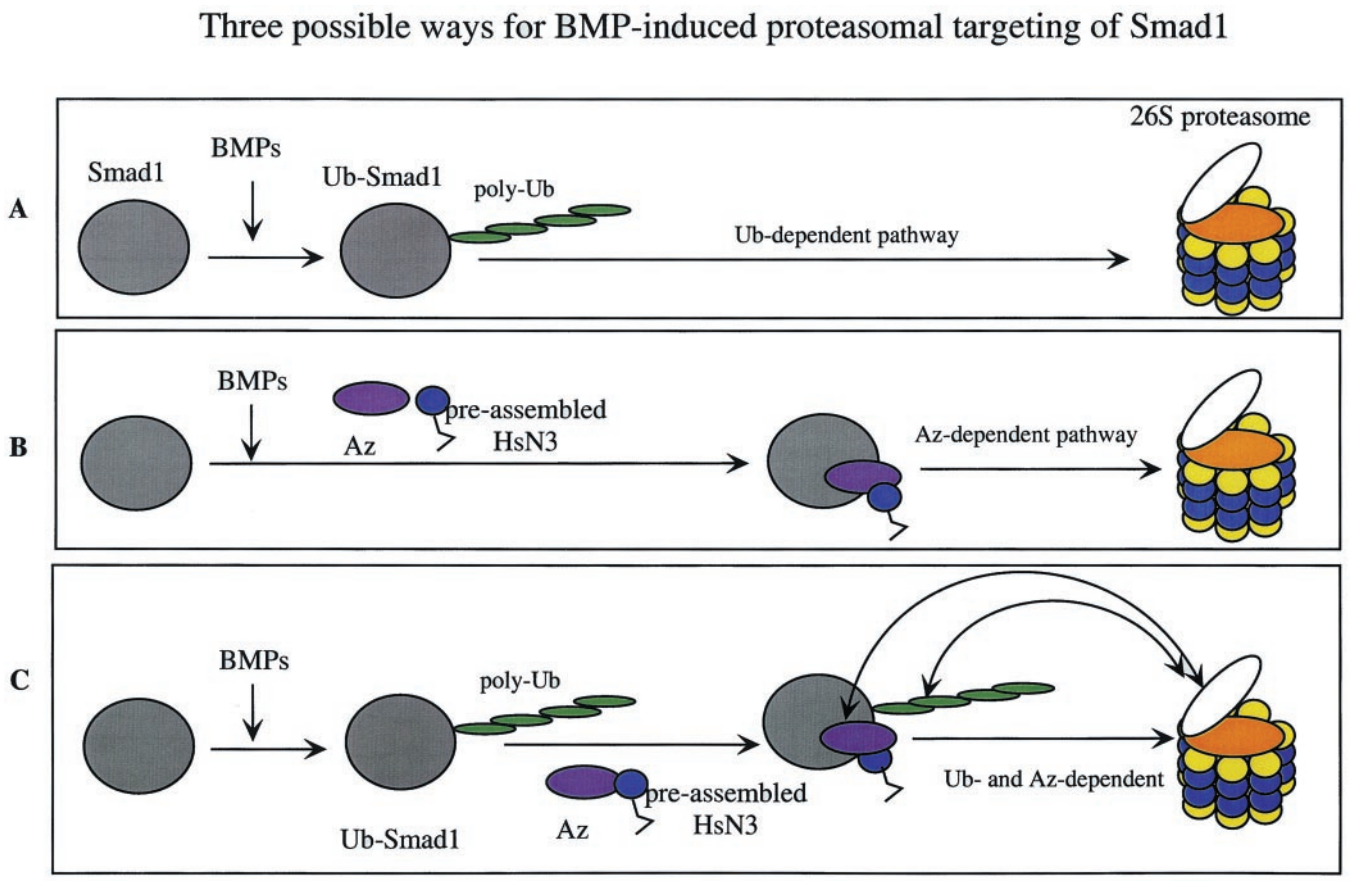

FIG. 9. **Three possible mechanisms of BMP-induced proteasomal targeting of Smad1.** See text for details.

We next detected a dose-dependent decrease of Smad1 protein level in response to increased expression of the activated BMP type I receptor. The temporal effect of receptor activation on Smad1 protein level was then studied using a reconstituted BMP7-responsive system in 293 cells. During the use of this 293 overexpression system, we noted receptor leakiness as a result of overexpressing both the type I and type II receptors of BMPs. We then identified a way to block the observed receptor leakiness, by overexpressing the cytoplasmic inhibitor FKBP12. Using such a reconstituted BMP-responsive system containing the receptors and FKBP12, we detected the reduction of Smad1 protein half-life in response to BMPs, thus indicating a destabilization effect of BMPs on Smad1. The role of proteasome in the destabilization of Smad1 upon receptor activation was then tested and confirmed by using proteasome inhibitors.

The detection of the involvement of the 26 S proteasome in BMP-induced destabilization of Smad1 raised the question of which proteasome targeting mechanism is utilized for Smad1 degradation. So far we have detected high molecular weight ubiquitin conjugates of Smad1 only in cells expressing activated BMP type I receptor, suggesting that the activation of BMP type I receptor induces Smad1 polyubiquitination, which could be a mechanism for targeting Smad1 to proteasome. We also tested the role of the Smad1 interactors, HsN3 and Az, in the BMP receptor-induced Smad1 degradation. Az is a well known proteasome targeting protein for ODC (26). HsN3 has also been implicated in a proteasome targeting process (22). Our separate studies of the physical interaction between Smad1, Az, and HsN3 in mammalian cells suggest the formation of a ternary complex between Smad1, Az, and HsN3 during the assembly process of HsN3.[2] The targeting role of HsN3 for Smad1 was tested by transiently overexpressing HsN3 to uncouple the assembly of HsN3 from Smad1 interaction with HsN3. Consistent with a targeting role of HsN3, excess HsN3 efficiently blocked receptor-induced Smad1degradation. Similarly, excess Az also blocked the receptor-induced proteasomal degradation of Smad1, suggesting an important targeting role of Az for receptor-activated Smad1. This observation, together with the observation of Az-dependent degradation of Smad1 interactor SNIP1,[2] points out a novel and important functional role of Az in targeting Smad1 and Smad1 interactors to proteasome along the BMP signaling pathways. Until now ODC has been the only protein known to be targeted to proteasome via its interaction with Az. Thus, our observations extend the targeting role of Az to proteins other than ODC and suggest that Az is a general targeting protein.

The involvement of Az in targeting Smad1 to proteasome also suggests that BMP-induced Smad1 degradation is subjected to multilevel regulations, because the protein level of Az is highly sensitive to cellular polyamine levels, which change during cell proliferation and differentiation and in response to extracellular stimuli and stress. Furthermore, if the targeting of Smad1 is dependent upon the ternary complex formation of Smad1, HsN3, and Az, very different responses are expected in cells that have different levels of endogenous Az. For example, in a cell line that has very low levels of endogenous Az, increased expression of Az may even enhance Smad1 degradation, whereas a different cell line that has high levels of endogenous Az would exhibit an opposite response caused by the uncoupling effect of excess Az. Thus, the targeting role of Az in BMP-induced Smad1 degradation indicates the complexity of Smad1 regulation and suggests a new mechanism that contributes to the well observed diversity of BMP responses in different cell types and tissues.

Our current data suggest three possible mechanisms for

proteasomal targeting of Smad1 upon the activation of BMP type I receptors. As illustrated in Fig. 9, BMPs could induce the targeting of Smad1 to proteasome through increased Smad1 polyubiquitination and the direct targeting of polyubiquitinated Smad1 to the 26 S proteasome via the conventional ubiquitin-dependent pathway (Fig. 9*A*). A second mechanism, as shown in Fig. 9*B*, involves an ubiquitin-independent and Az-dependent targeting event. In this case, there is a complex formation between Smad1, HsN3, and Az upon BMP stimulation. The complex is formed along the assembly pathways of HsN3, whose assembly may assist the final docking of Smad1 to proteasome. The final docking of Smad1 into the degradation chamber may involve the interaction between Az and additional proteasomal proteins ("receptors") at the 19 S regulators, as suggested by recent observations (26). These two mechanisms could simultaneously co-exist or operate separately in different cell types as a result of differential expression of these targeting proteins or ubiquitination machinery. The third possible mechanism is that Smad1 ubiquitination and the targeting role of Az and HsN3 could be coupled steps along the same targeting process of Smad1 (Fig. 9*C*). In this case, ubiquitination of Smad1 alone is not sufficient to target Smad1 to proteasome but further requires additional targeting role of Az and HsN3 after the ternary complex formation between Ub-Smad1, Az, and HsN3. Future studies will be aimed toward the dissection of these different mechanisms and their relationships.

Concomitant with our studies, others have reported proteasomal degradation of Smad1 and Smad2. Smad1 has been shown to undergo proteasomal degradation upon its ubiquitination by a Hect family ubiquitin-protein isopeptide ligase, Smurf1 (30). However, Smurf1-regulated Smad1 degradation is not induced by BMPs and has been suggested to adjust the basal level of Smad1 (30). Similar to BMP-induced Smad1 degradation reported here, the TGF-β-induced proteasomal degradation of Smad2 has been reported and shown to involve receptor-induced Smad2 ubiquitination (31). Our recent studies have suggested that Az and HsN3 also play a role of proteasomal targeting of Smad3 upon TGF-β stimulation (data not shown). Because Smad3 forms a complex with Smad2 in response to TGF-β, it is possible that Az and HsN3 are also involved in the reported Smad2 degradation regulated by TGF-β receptor. What is the functional role of receptor-induced proteasomal targeting of R-Smads? One obvious function, as suggested for Smad2 degradation in TGF-β pathway, is to serve as a negative feedback mechanism to turn off activated R-Smads. However, we also observed that the targeting of Smad1 to proteasome is accompanied by the targeting of multiple Smad1 interactors (data not shown). One such interactor, SNIP1, is a nuclear repressor of the master transcription activator CBP/p300.[2] Thus, Smad1 targeting to proteasome may serve as means to bring Smad1 interactors to proteasome for degradation, thereby playing a critical role in Smad-mediated signaling events.

In conclusion, the studies reported here establish a novel aspect of Smad1 regulation in the signaling pathways of BMPs and also provide new directions for future characterization of the molecular mechanisms involved in this important aspect of Smad regulation and signaling.

Acknowledgments—We thank S. Guedes for technical assistance and W. Weber for data quantification, Dr. J. Massagué for ALK3Q233D and BMPRII constructs, Drs. M. Treies and D. Bohmann for Ub-HA construct, Dr. C. Heldin for ALK3 construct, Dr. A. Roberts for Smad1 construct and polyclonal anti-Smad1, Dr. P. Coffino for cDNAs of antizyme and ODC, Dr. M. Yasuko for valuable advice on antizyme studies, Dr. E. J. Corey for lactacystin, Drs. K. Sampath and H. Dorai at the Creative BioMolecule Inc. for BMP7, and Dr. A. Celeste from the Genetic Institute Inc. for BMP2.

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PROTEIN SYNTHESIS
POST-TRANSLATION MODIFICATION
AND DEGRADATION:

Proteasomal Degradation of Smad1 Induced by Bone Morphogenetic Proteins

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J. Biol. Chem. 2001, 276:46533-46543.
doi: 10.1074/jbc.M105500200 originally published online September 24, 2001
