LIM Mineralization Protein-1 Potentiates Bone Morphogenetic Protein Responsiveness via a Novel Interaction with Smurf1 Resulting in Decreased Ubiquitination of Smads

Received for publication, October 11, 2005, and in revised form, March 13, 2006 Published, JBC Papers in Press, April 11, 2006, DOI 10.1074/jbc.M511013200

Sreedhara Sangadala, Scott D. Boden1, Manjula Vigeswarapu, Yunshan Liu, and Louisa Titus
From the Atlanta Veterans Affairs Medical Center and the Department of Orthopaedic Surgery, Emory University School of Medicine, Atlanta, Georgia 30329

Development and repair of the skeletal system and other organs is highly dependent on precise regulation of bone morphogenetic proteins (BMPs), their receptors, and their intracellular signaling proteins known as Smads. The use of BMPs clinically to induce bone formation has been limited in part by the requirement of much higher doses of recombinant proteins in primates than were needed in cell culture or rodents. Therefore, control of cellular responsiveness to BMPs is now a critical area that is poorly understood. We determined that LMP-1, a LIM domain protein capable of inducing de novo bone formation, interacts with Smurf1 (Smad ubiquitin regulatory factor 1) and prevents ubiquitination of Smads. In the region of LMP responsible for bone formation, there is a motif that directly interacts with the Smurf1 WW2 domain and can effectively compete with Smad1 and Smad5 for binding. We have shown that small peptides containing this motif can mimic the ability to block Smurf1 from binding Smads. This novel interaction of LMP-1 with the WW2 domain of Smurf1 to block Smad binding results in increased cellular responsiveness to exogenous BMP and demonstrates a novel regulatory mechanism for the BMP signaling pathway.

Bone morphogenetic proteins are critical regulators of osteoblast differentiation and bone formation (1). BMPs were originally identified as molecules that could induce ectopic bone and cartilage formation in rodents (2). Subsequently, various members of the BMP family have been shown to play a role in development of limb bud patterning, kidney, germ cells, nervous system elements, tendons/ligaments, and control of apoptosis (3). Several members of the BMP family are osteoinductive and able to induce the differentiation of mesenchymal cells into osteoblasts (4). The various activities of the BMPs are modulated through the control of antagonists, cell surface receptors, intracellular signaling proteins (Smads), and cross-talk with other signaling pathways (5, 6). Clinically, rhBMP-2 is being used to achieve spine fusion in patients with low back pain and avoids the morbidity of bone harvested from the pelvis (7, 8). Unfortunately, without improving the responsiveness to BMP-2, the high doses (milligrams) required in humans and the resultant cost are prohibitive for routine clinical use (9).

BMP responsiveness is dependent in part on the availability of intracellular signaling proteins. When activated, the type I BMP receptors phosphorylate intracellular mediators, the Smad proteins (10). The receptor-regulated Smads (R-Smads) are phosphorylated by the type I receptors upon ligand binding (11). Smad2 and Smad3 are involved in the TGF-β/activin pathway, whereas Smads 1, 5, and 8 act in response to BMPs (12). The phosphorylated R-Smads interact with the single common Smad, Smad4, forming a complex that translocates into the nucleus, associates with DNA, and is responsible for transcriptional regulation of target genes (13).

To examine strategies for improving cellular responsiveness to BMPs, we chose to study human mesenchymal stem cell (MSC) and osteoblast differentiation as a model. LMP-1 (LIM mineralization protein-1) was recently cloned and sequenced in our laboratory and is an intracellular protein that induces bone formation in vitro and in vivo (14, 15). LMP-1 overexpression induces BMP-2, -4, -6, and -7 expression in A549 embryonic lung cells and BMPs 4 and 7 in rabbit and human leukocytes during ectopic bone formation (16). The effects of LMP-1 reinforce the action of exogenously applied BMPs, and LMP activity is blocked by noggin, a BMP inhibitor (17).

In this study we report specific binding between LMP-1 and Smurf1 (Smad ubiquitin regulatory factor 1), an E3 ligase that ubiquitimates many molecules in BMP-2 signaling pathways, causing their degradation by proteosomes (18, 19). Changes in Smurf1 levels or activity have been shown to affect osteoblast function and bone formation in vitro and in vivo (18, 20–22). We show that LMP-1 competes with Smads 1/5 for Smurf1 binding and propose that LMP-1 interaction with Smurf1 enhances BMP-2 responsiveness by preventing the degradation of key signaling molecules.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cloning of cDNAs in Bacterial Expression Vectors—Escherichia coli XL1 blue and BL 21-codon plus (DE3)-RP (Stratagene) hosts were maintained on LB agar plates and grown at 37 °C in the presence of ampicillin at 100 mg/liter. All of the cloning methods were performed according to standard protocols. LMP-1, LMP-1t, LMP-2, LMP-3, Smad1, and Smad5 cDNAs were cloned into TAT-HA vector. LMP-1 mutants were generated using the following primers: hLMP1Mutant A forward primer, 5'-cgccccccgccccgacgacgtggtacac-3'; hLMP1Mutant A reverse primer, 3'-gtcagggtagtacctgctggtcagggccccg-3'; hLMP1Mutant B forward primer, 5'-ggccccgccc-
Expression and Purification of Recombinant Proteins—Bacterial cultures were grown at 37 °C until the A_600 reached 0.8. Isopropyl β-D-thiogalactopyranoside was added to 200 μM, and the culture was grown for another 8 h. The cells were harvested, and the pellets were suspended in ice-cold lysis buffer (20 mM phosphate buffer, pH 7.0, containing 50 mM Tris-HCl, pH 7.5, and 0.5 M NaCl). The uniform cell suspension was sonicated (Sonicator, model W-385, Heat Systems-Ultrasonics, Inc.) using 4 X 15 s bursts at minimum power output settings in ice with a 2-min interval between each burst. The lysate was centrifuged at 10,000 g at 4 °C, and the supernatant was applied to Sephacryl S-100/S-200 columns (HiPrep 16/60) using an AKTA fast protein liquid chromatography system with Unicorn 4.0 software (Amersham Biosciences) at a flow rate of 1 ml/min. Fractions (2–4 ml) were collected immediately after the void volume (35 ml). Aliquots from each fraction were assayed by slot blotting, SDS-PAGE, and Western blotting. The fractions, identified by Western blots, were pooled, dialyzed against 20 mM phosphate buffer, pH 7.5, containing NaCl (50 mM) and imidazole (20 mM), and applied to Ni2+ affinity resin (Probond, Invitrogen) previously equilibrated with 4 X 10 ml of buffer. Nonspecific proteins were washed off the column with 3 X 10 ml of 20 mM phosphate buffer, pH 6.0, containing NaCl (50 mM) and imidazole (20 mM), and applied to Ni2+ affinity resin (Probond, Invitrogen) previously equilibrated with 4 X 10 ml of buffer. Nonspecific proteins were washed off the column with 3 X 10 ml of 20 mM phosphate buffer, pH 6.0, containing NaCl (50 mM) and imidazole (20 mM). Affinity-bound proteins were eluted using three 10-ml washes with 20 mM phosphate buffer, pH 4.0, containing NaCl (50 mM). Fractions containing the desired protein were pooled (based on Western blot) and then concentrated and desalted using the centrprep devices (Amicon). The proteins were quantitated using Bio-Rad protein assay reagent. The yield of recombinant protein was routinely 0.5–1 mg of pure protein from every 2-liter culture.

Biotinylation of Protein Ligands—Purified protein ligands were prepared at 10 mg/ml in 50 mM sodium borate buffer, pH 8.5, 0.5 mM NaCl. Various amounts of sulfo-NHS-biotin (100 mM stock in Me2SO) was prepared at 10 mg/ml in 50 mM sodium borate buffer, pH 8.5, 0.5 mM NaCl. Various amounts of sulfo-NHS-biotin (100 mM stock in Me2SO) was exposed to x-ray film for signal detection.

Cell Culture—MSCs at passage 2 (Cambrex Bio Sciences) were grown at 37 °C in 5% CO2 in MSC basal medium supplemented with Singlequots (Cambrex Bio Sciences), split at confluence, and plated at 3 X 105 cells/well in 6-well dishes at passage 4 in these studies. The next day treatments were applied in the presence of 50 μM l-ascorbic acid 2-phosphate and 5 mM β-glycerol phosphate (Sigma-Aldrich). The medium was changed every 3–4 days with reapplication of treatments where appropriate. The cells were transduced for 30 min with adenoviral constructs in 0.3 ml of serum-free medium.

Preparation of Nuclear and Cytoplasmic Protein Fractions—Cell pellets were suspended in buffer A (20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.2% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml protease inhibitor mix (Sigma)), incubated on ice for 10 min, and centrifuged. Supernatants (cytoplasmic fraction) were collected, and nuclear pellets were suspended in high salt buffer B (buffer A plus 600 mM KCl, 20% glycerol), incubated on ice for 30 min, and centrifuged. Supernatants were collected as the nuclear fraction. The protein amounts were determined with Bio-Rad protein assay.

SDS-PAGE and Western Blotting—SDS-PAGE was performed using 10% gels and transferred to nitrocellulose membrane. The membrane was blocked with milk protein, incubated with specific antibody, washed with Tris-buffered saline containing 0.1% Tween 20 (TBST), incubated with anti-rabbit goat IgG-linked to horseradish peroxidase (PerkinElmer Life Sciences), and again washed with TBST. Chemiluminescent substrates were applied to the membrane, and the signal was detected by exposure to x-ray film. To demonstrate equal protein loading in each lane, a signal was developed for β-actin in all samples.

Biotin Transfer Assay for Detection of LMP-1-interacting Proteins—Sulfo-sulfosuccinimidyl-2-[6-(biotinamido)-2-[(p-azobenzenamido)-hexanoamido]ethyl-1,3'-dithiopropionate (Pierce), a trifunctional cross-linking agent was used to label LMP-1. The labeled protein was incubated as a bait with nuclear proteins and cross-linked to interacting proteins by UV (365 nm). Proteins that physically interact with LMP-1 retained the biotin group when suspended in SDS-PAGE reducing buffer. Biotin-containing target proteins were separated using neutravidin beads, detected by Western blotting with neutravidin-HRP, and the signal was developed with chemiluminescent substrate. Corresponding protein bands were in-gel digested with trypsin. Tryptic peptides were recovered and concentrated, and their mass profile was analyzed by MALDI-TOF at the Emory University Microchemical Facility.

Smurf1-WW Domain and LMP-1 Interaction Assay (Ligand Blotting)—The proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane. The protein blots were blocked with 5% milk protein and preincubated with purified LMP-1 or LMP-2 protein (10 μM) or TBST buffer. The blots were incubated with Smurf antibody at 1:5000 dilution (rabbit antibody raised to WW domain peptide; Upstate Biotechnology, Inc, catalog number 07-249). After washes, the blots were incubated with HRP-labeled Anti-rabbit secondary antibody. The washed blots were then incubated with ECL substrate solution, and the membranes were exposed to x-ray film for signal detection.

Slot-Blot Assay—20 μl of purified Smurf1 (50 μg/ml) was blotted onto nitrocellulose in slot blot wells, and the wells were blocked with 0.5% Tween 20 in TBST for 30 min. The biotinylated ligand (LMP-1, LMP-2, LMP-3, Smad1, or Smad5) was mixed with varying concentrations of competing proteins or peptides and incubated in slot blot wells with Smurf1 for 90 min. The wells were washed, and the blots were blocked with TBST containing 0.5% Tween 20. Control wells contained LMP-1 hapten (an antigenic peptide from the c-terminal end of the polypeptide chain) as a competitor peptide. The blots were then incubated with HRP-labeled avidin for 1 h. After washing the blots were incubated with ECL substrate solution, and the membranes were exposed to x-ray film for signal detection.

Protein A-based Immunoprecipitation Assay—Protein A-agarose beads were incubated with LMP-1 antibody or Smurf1 antibody (H-60, Santa Cruz catalog number sc25510), washed three times, incubated with nuclear proteins, and washed again to remove unbound protein. The bound proteins were eluted by two washes in 0.1 M citric acid, pH 2.7. The eluates were neutralized with Tris base and concentrated by centrificon tubes (Ambicon) prior to SDS-PAGE and Western blotting.
Osteogenic Differentiation of hMSCs—hMSCs at passage 4 were seeded at $3 \times 10^4$ cells/well in a 6-well plate. The next day, the cells were infected with Ad35LMP-1 (1–10 pfu/cell) and incubated with and without BMP-2 (100 ng/ml). The medium was replaced every 3–4 days, and deposition of mineral was observed after 2 weeks. To assess mineralization, the cultures were washed with phosphate-buffered saline and fixed in a solution of ice-cold 70% ethanol for 2–3 h. The cultures were rinsed with water and stained for 10 min with 1 ml of 40 mM alizarin red (pH 4.1). The cultures were rinsed two or three times with phosphate-buffered saline to reduce nonspecific staining, air-dried, and photographed.

RNA Extraction—RNA was isolated from cells grown in 6-well plates using RNeasy mini kits (Qiagen). Briefly, the cells were disrupted in RNeasy lysis buffer (Qiagen) and passed over QiaShredder columns, and the eluate was brought to 35% EtOH and passed over RNeasy columns. The RNA was eluted from the membrane with water. All of the RNA samples were DNase-treated either using the Qiagen RNase-free DNase during the RNeasy procedure or after final harvest of the RNA using the Ambion DNA-free kit. After completion of the digestion, 5 μl of DNase inactivation buffer was added, and the samples were centrifuged for 1 min. The RNA containing supernatant was removed and stored at 4°C.

FIGURE 1. LMP-1 specifically interacts with Smurf1. a, LMP-1 is associated with an 85-kDa nuclear protein. Recombinant LMP-1 was labeled with sulfo- \textit{O}succinimidyl-2-(6-(\textit{N}′-\textit{N}′-\textit{O}xybenzamido)-2- \textit{p}-\textit{azidobenzamido)-hexanoamido}ethyl]-3′,4′-dithiopropionate-biotin transfer reagent and incubated with MSC nuclear proteins. Biotinylation of interacting proteins was accomplished as described under “Experimental Procedures.” Biotinylated proteins were enriched using neutravidin beads, separated by SDS-PAGE, and detected on Western blots using HRP-labeled neutravidin and ECL. Bands were excised for tryptic digestion and MALDI-TOF analysis. The smaller bands represent cytoskeletal proteins, whereas the 85-kDa band (*) was identified as Smurf1. In Western analysis of the 85-kDa LMP-1-interacting protein, Smurf1 antibody was used. Western analysis of the 85-kDa LMP-1-interacting protein was shown using antibody that binds Smurf1 (right panel). The doublet contains two protein bands, one running at 86 kDa and the other at 84 kDa (*). Both were immunoreactive and probably represent the two known splice variants of Smurf1 of similar molecular size. c, LMP-1 and Smurf1 co-immunoprecipitate from MSC nuclear extract with either LMP-1 antibody (Ab) or Smurf1 (H-60) antibody and protein A beads. Nuclear proteins were immunoprecipitated using LMP-1 antibody, Smurf1 (H-60) antibody, or preimmune serum (indicated at the top of the figure). The immunoprecipitated proteins were concentrated and analyzed on Western blots with LMP-1 and Smurf1 antibody separately (indicated at the bottom of the figure). The bands are the expected size for each of the proteins. d, Smurf1 binding to LMP-1 protein occurs at the WW domain(s) of Smurf1. Blots of SDS-PAGE resolved MSC nuclear proteins show the expected predominant band at 85 kDa when probed with Smurf1 WW domain antibody in Western blots (Control lane). Preincubation of recombinant LMP-1 (10 μM) with these blots inhibited Smurf1 antibody binding (LMP-1 lane), indicating that LMP-1 and the WW domain antibody interact with Smurf1 at the same site. e, purified non-denatured Smurf1 antibody binding (LMP-1 lane), indicating that the WW domain antibody interact with Smurf1 at the WW2 domain. Smurf1 was blotted onto nitrocellulose and incubated with Smurf1 (H-60) antibody or Smurf1 WW2 domain antibody in the absence (Control) or presence of LMP-1 (10 μM). LMP-1 blocks binding of the antibody raised against the WW2 domain (*) but has no effect on binding of (H-60) antibody that was raised against an epitope of Smurf1 that is distant from the WW2 domain. This result provides further evidence that LMP-1 binds Smurf1 at the WW2 domain.
−70 °C. Each sample consisted of RNA isolated from 2 wells of a 6-well plate.

**Real Time Reverse Transcription-PCR—**Two μg of total RNA was reverse transcribed in a 100-μl total volume containing 50 mM KCl, 10 mM Tris, pH 8.3, 5.5 mM MgCl₂, 0.5 mM each dNTPs, 0.125 μM random hexamer, 40 units RNase inhibitor, and 125 units MultiScribe (Applied Biosystems). In control samples the RNase inhibitor and MultiScribe were omitted. The samples were incubated for 10 min at 25 °C, 30 min at 48 °C, and then 5 min at 95 °C to inactivate the enzyme. Real time PCR was then performed on 5 μl of the resulting cDNA in a total volume of 25 μl containing 12.5 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems), and 0.8 μM each primer. The forward primer for osterix was 5′-tcagccccccagactc-3′, and the reverse primer was 5′-atggccagc-cagttgctctgag-3′ (PCR conditions: 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 95 °C for 15 s followed by 1 min at 62 °C). PCR was also performed on a 1:800 dilution of the cDNA with 18 S primers for normalization of the samples. Relative RNA levels were calculated using the ΔΔCₘ method (Applied Biosystems).

**siRNA Treatment of Cells—**MSCs were transfected with Oligofectamine (Invitrogen) transfection reagent and either irrelevant siRNA or specific siRNA for Smurf1 (5′-ccucagaagaaagacuuc-3′). Silencing of the gene and specificity was confirmed by real time reverse transcription-PCR analysis of specific Smurf1 mRNA with forward primer (5′-ccucagaggaaacgc-3′) and reverse primer (5′-tttgagttggcac-3′).

**Ubiquitination Reaction in Vitro—**Purified Smad1 (100 ng) was buffer-exchanged to ubiquitination buffer (50 μM Tris-HCl pH 7.8, 5 mM MgCl₂, 0.5 mM dithiothreitol, 2 mM NaF, and 3 μM okadaic acid). Smad1 was then combined with a mixture of purified E1 and E2 enzymes and incubated with Smurf1 (E3 ligase) in the presence or absence of recombinant LMP-1 or LMP-2 protein. The reaction mixture also contained 2 mM ATP, ubiquitin (150 μM), ubiquitin aldehyde (5 μM), and creatine kinase-ATP generating system (Boston Biochem). The ubiquitin aldehyde was included to prevent hydrolysis of polyubiquitin chains. The reaction mixture (40 μl) was incubated for 4 h at 37 °C. Aliquots at various time points were taken for SDS-PAGE and Western blotting using specific antibody for Smad1 and/or ubiquitin.

**Preparation of Peptides—**Peptides were synthesized with a protein transduction domain at the N-terminal end (rrrgtstklnk) (24).

**RESULTS**

**LMP-1 Interacts with Smurf1—**In an effort to understand the mechanism by which LMP-1 enhances osteoblast differentiation and possibly modulates BMP responsiveness, we elucidated proteins that interact with LMP-1 using a biotin transfer assay. Recombinant LMP-1 was labeled with sulfosuccinimidyl-1-[6-(biotinamido)-2-(p-azidobenzenamido)-hexanoamido]ethyl-1′-3′-dithiopropionate-biotin transfer reagent and incubated with nuclear proteins from MSCs. LMP-1 associated with four protein bands (Fig. 1a). The 85-kDa protein doublet matched the two isoforms of Smurf1 based on peptide mass profile. The other bands were identified as cytoskeletal proteins (tubulin, caldesmon, and meosin) and not likely related to the unique osteoinductive properties of LMP. Binding to cytoskeletal proteins has previously been observed for LMP and other proteins having both PDZ and LIM domains, as does LMP-1 (25, 26). Identity of the gel purified 85-kDa LMP-1-binding protein was verified by performing a Western blot using antibody that binds Smurf1 (Fig. 1b). Smurf1 is a member of the Hect family of E3 ligases and has been reported to interact with Smad1, Smad5, Runx2, Smad7, TGF-B1, and BMP receptors. (18–20, 27, 28) These interactions result in ubiquitination of the targeted protein and subsequent proteosomal degradation.

To determine whether the Smurf1/LMP-1 interaction occurs between the endogenous proteins in cells, nuclear protein extracts of MSCs were incubated with an LMP-1-specific antibody, immunoprecipitated, and analyzed in Western blots with LMP-1 and Smurf1 antibody, separately. The expected size bands showing both endogenous LMP-1 (50 kDa) and Smurf1 (85 kDa) are present in the complex immunoprecipitated with the LMP-1 antibody (Fig. 1c). This observation was confirmed by detection of LMP-1 when Smurf1 (H-60) antibody was used for the immunoprecipitation (Fig. 1c).

We next determined that LMP-1 protein inhibits Smurf WW domain antibody from binding to Smurf1, suggesting that LMP-1 interacts with Smurf1 via its WW domains, which are also required for interaction with Smads 1/5 (Fig. 1d) (18). Further, we observed that purified nonadenatured LMP-1 binds purified nonadenatured Smurf1 at the WW domain. LMP-1 substantially reduces binding of the Smurf1 WW domain antibody but has no effect on binding of antibody raised to a different Smurf1 epitope (Fig. 1e). These findings raise the possibility that LMP-1 could increase cellular responsiveness to BMPs by blocking Smurf1 from ubiquitinating Smads 1/5 and thereby increasing the available levels of R-Smads (21).

**Determination of the Domains of LMP-1 and Smurf1 That Are Involved in Mutual Binding—**To better understand the interaction of LMP-1 with Smurf1, we determined the specific region of LMP-1 most likely to interact with Smurf1 by domain and motif analysis. Three bone-inducing LMP variants (LMP-1, LMP1t, and LMP-3) each contain a unique 45-amino acid peptide sequence that includes two putative Smurf1 WW domain interaction sites (A and B) (Fig. 2a) (29). The WW domain-interacting sites are absent in the nonosteogenic LMP variant (LMP-2). Two LMP isoforms (LMP-1t and LMP-3) are truncated at the C terminus and do not contain the LIM domains but do induce bone formation. Thus, it is the 45-amino acid osteoinductive region and not the LIM domains that are required for bone formation (30). We next prepared slot blots with recombinant Smurf1 and used biotin-labeled LMP variants to demonstrate that only the LMP variants with the WW domain interaction sites were able to bind to Smurf1 (Fig. 2b).

To determine which of the two WW domain interaction sites were required for the binding of LMP with Smurf1, we prepared two mutant forms of recombinant LMP proteins by substituting alanines for prolines in the putative binding sites. We found that only WW domain interaction site B is required for the interaction with Smurf1 (Fig. 2c). We further demonstrated that LMP-1 was no longer able to bind to a mutant form of Smurf1 that had its WW2 domain deleted, suggesting that the Smurf1 WW2 domain is required for LMP-1 binding (Fig. 2d).

To determine whether the LMP/Smurfl interaction is of sufficient binding affinity to displace the normal binding partners of Smurf1, we studied the competitive binding of LMP-1, Smad1, and Smad5 to Smurf1 and found that the relative binding affinity of LMP-1 for Smurf1 is similar to that of Smad1 and Smad5, suggesting that the LMP-1 interaction with Smurf1 is physiologically relevant (2e).

**LMP-1 Peptides Containing WW Domain-interacting Site B Bind Smurf1—**To further confirm the identity of the LMP motif that interacts with Smurf1 and to determine whether the activity of the full-length LMP protein could be replicated by a small peptide, we synthesized small peptides comprising various portions of the 45-amino acid osteoinductive region containing one, two, or none of the putative WW domain-interacting sites (Fig. 3a). We studied the competitive binding affinity of these LMP peptides and found that the two peptides that contained intact WW domain-interacting Site B were able to compete with full-length LMP-1 (Fig. 3b) and with Smad1 and...
FIGURE 2. Determination of the domains of LMP-1 and Smurf1 that are involved in mutual binding. a, the putative osteoinductive region of LMP variants contains two motifs (sites A and B) that are computationally predicted to interact with Smurf1 WW domains. Nonosteoinductive LMP-2 does not contain these motifs. b, only LMP variants containing the two putative WW domain-interacting sites bind to Smurf1 on slot blots. Purified Smurf1 was blotted onto nitrocellulose in slot blot wells as described under “Experimental Procedures.” After blocking, biotinylated LMP variants were incubated with the blots for 90 min. The wells were washed and incubated with HRP-labeled avidin for 1 h. The washed blots were incubated with ECL substrate and exposed to x-ray film for signal detection. The lower panel shows the signal obtained with the aliquots of biotinylated LMPs, demonstrating normalized specific activity of biotin incorporation as determined by 2-hydroxyazobenzene-4-carboxylic acid assay. c, mutation of WW domain-interacting site B abolishes Smurf1 binding to LMP-1. Purified LMP-1 or LMP-1 mutated within either WW domain-interacting site A or site B were run on gels, blotted onto nitrocellulose, and incubated with biotinylated Smurf1. Binding was detected using HRP-labeled avidin/ECL and exposure to x-ray film (left panel). The right panel demonstrates the purity and integrity of input proteins. The site A mutant of LMP-1 exhibited comparable binding to that of wild type (wt). d, deletion of the Smurf1 WW2 domain results in loss of LMP-1 binding to Smurf1. Mutant Smurf1 without WW domain 2 (Smurf1 ΔWW2) was prepared as described under “Experimental Procedures,” and either the mutant or wild type Smurf1 (Smurf1 wt) was blotted onto nitrocellulose in a slot blot apparatus. After blocking, biotinylated LMP-1 or LMP-2 was incubated with the membrane and detected as described above. Failure of LMP-1 to bind the mutated Smurf1 suggests that the WW2 domain is required for LMP-1 binding. As expected, LMP-2 does not bind either wild type or mutated Smurf1. In the lower panel of each doublet, aliquots of Smurf1 wild type or mutant protein were blotted onto nitrocellulose. These proteins were detected using Smurf1 (H-60) antibody and HRP-labeled second antibody to demonstrate equal loading of the Smurf proteins. e, labeled LMP-1, Smad1, and Smad5 exhibit comparable binding to Smurf1 on slot blots. Purified Smurf1 was blotted onto nitrocellulose membrane in a slot blot apparatus. After blocking, biotinylated ligand (LMP-1, Smad1, or Smad5) was mixed with varying concentrations (0–10 μM) of competing proteins and incubated in slot blot wells with Smurf1 for 90 min. Binding of biotinylated ligands was detected as previously described. The unlabeled LMP-1, but not LMP-2, shows similar binding competition with all three labeled ligands.

Smad5 (Fig. 3c) for binding with Smurf1. This result is consistent with the mutational analysis above establishing Site B as the more critical site in LMP.

LMP-1 Interaction with Smurf1 Blocks Smurf1-induced Ubiquitination of Smad1 and Smad5 and Increases Cellular Responsiveness to BMP-2—The most relevant physiologic question is whether these mechanisms are active in undifferentiated MSCs, which are the initiating cells in adult osteogenesis. In these cells, we investigated whether the LMP-1 interaction with Smurf1 results in an increased cellular response to BMP-2. We measured the levels of Smad1 that were phosphorylated in response to BMP-2 treatment (100 ng/ml) and found a significantly greater increase in the presence of LMP-1 (Fig. 4a). This response could be observed after 4 or 24 h (data not shown) but was most prominent 8 h after treatment. In a separate experiment we also demonstrated a similar increase in the type 1A BMP receptor in cells treated with LMP-1 and BMP-2 (data not shown). To confirm that this observation could be due to LMP-induced reduction of Smurf1-mediated ubiquitination of Smad1, we performed ubiquitination assays. Less ubiquitinated Smad1 was observed in the presence of LMP-1 (Fig. 4b), demonstrating that LMP-1 can inhibit this critical function of Smurf1. To further suggest that the LMP-1 effects on BMP responsiveness are conveyed by negating the effect of Smurf1, we demonstrated that the expression of small interference RNA to Smurf1 resulted in a similar increase in phosphorylated Smad1 upon BMP-2 exposure as seen with LMP-1 (Fig. 4c).
We report a novel interaction between LMP-1 and the ubiquitin E3 ligase, Smurf1, in cell lysates in vitro. The ubiquitin-proteasomal pathway is recognized as the major intracellular mechanism for regulated degradation of many short-lived proteins. Proteasomal protein degradation involves a cascade of enzymatic reactions catalyzed by the E1 ubiquitin-activating enzymes, the E2 ubiquitin conjugating enzymes, and the E3 ligases that have a critical role in defining substrate specificity and subsequent protein degradation by the 26 S proteasomes. It is the E3 ligase that determines which molecules will be targeted by ubiquitination for proteasomal degradation. Smurf1 is a member of the Hect family of E3 ligases and has been reported to interact with Smad1 and Smad5, as well as Runx2, Smad7, and TGFβ1 and BMP receptors (18–20, 27). These interactions result in ubiquitination of the target protein and subsequent degradation by proteasomes. Thus, Smurf1 interaction with Smads 1 and 5 reduces Smad1 and 5 protein levels in cultured cells and affects embryonic patterning by BMP signals in Xenopus embryos. The degradation of Smad1 and Smad5 by interaction with Smurf1 occurs independent of BMP receptor activation, indicating that Smurf1 not only functions downstream of activated Smads to turn off BMP signals but may also adjust the basal levels of Smads available for BMP signaling. Further, enhanced Smurf1 levels have been shown to simultaneously reduce cellular responsiveness to Smad1/5 (BMP) signaling and enhance sensitivity to the Smad2 (activin/TGF-β1) pathway (18). Smurf2 shares 83% homology with Smurf1 and interacts more broadly than Smurf1 with R-Smads, allowing interference with both the BMP and TGF-β1 pathways (32, 33).

Rapidly emerging research has focused on the probability that osteoblast differentiation and bone formation can be regulated by controlling degradation of proteins, especially proteins that are involved in BMP signaling. A recent study showed that inhibition of 26 S proteasomal activity stimulates bone formation in vitro and in vivo (34). This report suggested that future drugs designed to induce bone formation might focus on inhibiting protein degradation. Far more specific studies of the importance of protein degradation in bone formation have focused on the role of Smurf1, the E3 ligase that regulates the levels of Smads 1 and 5, setting the level of cellular responsiveness to BMPs. These studies have shown that overexpression of Smurf1 reduces osteoblast differentiation and bone formation in vitro and in vivo in transgenic animals (21, 22). Further, expression of a dominant negative Smurf1 that binds proteins, but cannot ubiquitinate them, mimics the effect of BMP-2 to induce bone formation in pleuripotent myoblasts. Cells stably expressing the dominant negative Smurf1 are even more responsive to BMP-2 treatment, presumably because of the enhanced levels of Smad1 and Smad5 (20, 21).

Hect domain E3 ligases contain a conserved cysteine at the C terminus of the Hect domain that can form a thio-ester bond with ubiquitin. Another motif often found in this family of E3 ligases is the WW domain, a region of about 30 amino acids containing two highly conserved tryptophans and a conserved proline. The WW domains have a secondary structure of the Hect domain that can form a thio-ester bond with ubiquitin. A recent study showed that inhibition of 26 S proteasomal activity stimulates bone formation in vitro and in vivo (34). This report suggested that future drugs designed to induce bone formation might focus on inhibiting protein degradation. Far more specific studies of the importance of protein degradation in bone formation have focused on the role of Smurf1, the E3 ligase that regulates the levels of Smads 1 and 5, setting the level of cellular responsiveness to BMPs. These studies have shown that overexpression of Smurf1 reduces osteoblast differentiation and bone formation in vitro and in vivo in transgenic animals (21, 22). Further, expression of a dominant negative Smurf1 that binds proteins, but cannot ubiquitinate them, mimics the effect of BMP-2 to induce bone formation in pleuripotent myoblasts. Cells stably expressing the dominant negative Smurf1 are even more responsive to BMP-2 treatment, presumably because of the enhanced levels of Smad1 and Smad5 (20, 21).
of changes in the rest of the polypeptide conformation. This is most likely because of fewer residues being involved and the disruptive nature of proline residues on the secondary structure. It should be noted that in addition to the defined sites, there can be other regions of Smurf1 and LMP-1 polypeptides that may assist or determine the binding characteristics of Smurf1-LMP-1 interaction.

We prepared several chemically synthesized peptides from the osteogenic region of LMP-1 to test their ability to bind and/or compete with other binding peptides. Although we observed binding with site B-containing peptides, it is quite possible that short peptides may assume an entirely different configuration in comparison with corresponding regions in the native protein and exhibit binding properties that are different from the native protein. However, based on our observations, we conclude that the binding selectivity of peptides in the osteogenic region supports our working hypothesis that the LMP osteogenic region determines the binding specificity toward Smurf1 and results in protection of osteogenic Smads from ubiquitination.

Recent studies have revealed a considerable degree of cross-talk between the BMP pathway and the TGF-β/H9252/activin, Wnt, Ca/calmodulin, vitamin D, extracellular signal-regulated kinase (Erk)-MAPK, p38-MAPK, and JAK-STAT pathways (6, 36). This cross-talk adds considerable complexity to the network of regulation of the BMP signaling cascade. In addition, Smurf1 has been shown to control proteasomal degradation of MEKK2, RhoA, and Runx2 (20, 35, 37), presumably through binding of these proteins to its WW domains. Based on our work we predict that LMP-1 will also protect these factors from Smurf1-mediated proteasomal degradation. Regulation of MEKK1 and RUNX2 degradation affect osteoblast function by modulating AP-1 activity, suggesting that LMP-1 may also enhance BMP-2 induction of bone formation by Smad-independent mechanisms.
In conclusion, we have elucidated a novel interaction between LMP-1 and Smurf1 that represents a powerful control mechanism over BMP signaling and responsiveness. The LMP-1 interaction requires the Smurf1 WW2 domain, it is dependent on a specific motif in LMP-1, and it can be mimicked by a small peptide containing only that motif. Further, LMP-1 competitively binds to Smurf1, probably preventing ubiquitin-mediated proteasomal degradation of Smads, contributing to an enhanced cellular responsiveness to BMP-2.

Taken collectively, the current report defines the LMP-1 interaction with Smurf1 for the first time and attempts to define the physical regions in binding partners. Our efforts also point to interaction of Smurf1 and LMP-1 and its physiological relevance in potentiating cellular responsiveness to BMP stimulation, which would make clinical translation much easier.

Acknowledgments—The authors thank Chantrice Rogers, Liping Zhu, Maggie Bargouti, Mesfin Teklemariam, Vishali Velaga, Dorota Lyszczowicz, and Sabita Saldanha for technical assistance. We also thank Dr. Imamura (Japanese Foundation for Cancer Research Cancer Institute, Tokyo) for providing cDNAs for Smads and Smurf1.

REFERENCES

1. Liu, Y., Titus, L., Barghouthi, M., Viggieswarapu, M., Hair, G., and Boden, S. D. (2004) Bone 35, 673–681
2. Wrenn, J. M. (1989) Prog. Growth Factor Res. 1, 267–280
3. Dacey, P., and Karsenty, G. (2000) Kidney Int. 57, 2207–2214
4. Cheng, H., Jiang, W., Phillips, F. M., Haydon, R. C., Peng, Y., Zhou, L., Luu, H. H., An, N., Breyer, B., Vanichakarn, P., Sztakowski, J. P., Park, J. Y., and He, T. C. (2003) J. Bone Joint Surg. Am. 85-A, 1544–1552
5. Peng, Y., Kang, Q., Cheng, H., Li, X., Sun, M. H., Jiang, W., Luu, H. H., Park, J. Y., Haydon, R. C., and He, T. C. (2003) J. Cell Biochem. 90, 1149–1165
6. von Bahrnolf, A., and Cho, K. W. (2001) Dev. Biol. 239, 1–14
7. Goulet, J. A., Sennunas, L. E., DeSilva, G. L., and Greenfield, M. L. V. H. (1997) Clin. Orthop. 339, 76–81
8. Boden, S. D., Kang, J. D., Sandhu, H. S., and Heller, J. G. (2002) Spine 27, 2662–2673
9. Martin, G. J., Boden, S. D., Morone, M. A., and Moskovitz, P. A. (1999) J. Spinal Disord. 12, 179–186
10. Hoodless, P. A., Haerry, T., Abdollah, S., Stapleton, M., O’Connor, M. B., Attisano, L., and Wrana, J. L. (1996) Cell 85, 489–500
11. Lo, R. S., Chen, Y. G., Shi, Y., Pavletich, N. P., and Massague, J. (1998) EMBO J. 17, 996–1005
12. ten Dijke, P., Fu, J., Schaap, P., and Roelen, B. A. (2003) J. Bone Joint Surg. Am. 85-A, Suppl. 3, 34–38
13. Miyazono, K. (2000) Cytokine Growth Factor Rev. 11, 15–22
14. Boden, S. D., Liu, Y., Hair, G. A., Helms, J. A., Hu, D., Racine, M., Nanes, M. S., and Titus, L. (1998) Endocrinology 139, 5125–5134
15. Boden, S. D., Titus, L., Hair, G., Liu, Y., Viggieswarapu, M., Nanes, M. S., and Boden, S. D. (1998) Spine 23, 2486–2492
16. Minamidle, A., Boden, S. D., Viggieswarapu, M., Hair, G. A., Oliver, C., and Titus, L. (2003) J. Bone Joint Surg. Am. 85-A, 1030–1039
17. Yoon, S. T., Park, J. S., Kim, K. S., Li, J., Attallah-Wasif, E., Hutton, W. C., and Boden, S. D. (2004) Spine 29, 2603–2611
18. Zhu, H., Kavsak, P., Abdollah, S., Wrana, J. L., and Thomsen, G. H. (1999) Nature 400, 687–691
19. Izzu, L., and Attisano, L. (2004) Oncogene 23, 2071–2078
20. Zhao, M., Qiao, M., Oyajobi, B. O., Mundy, G. R., and Chen, D. (2003) J. Biol. Chem. 278, 27939–27944
21. Zhao, M., Qiao, M., Harris, S. E., Oyajobi, B. O., Mundy, G. R., and Chen, D. (2004) J. Biol. Chem. 279, 12854–12859
22. Ying, S. X., Hussain, Z. J., and Zhang, Y. E. (2003) J. Biol. Chem. 278, 39029–39036
23. Green, N. M. (1975) Adv. Protein Chem. 29, 85–133
24. Mi, Z., Mai, J., Lu, X., and Robbins, P. D. (2000) Mol. Ther. 2, 339–347
25. Guy, P. M., Kenny, D. A., and Gill, G. N. (1999) Mol. Biol. Cell 10, 1973–1984
26. Vallenius, T., Luukko, K., and Makela, T. P. (2000) J. Biol. Chem. 275, 11100–11105
27. Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001) J. Biol. Chem. 276, 12477–12480
28. Murakami, G., Watabe, T., Takaoka, K., Miyazono, K., and Imamura, T. (2003) Mol. Biol. Cell 14, 2809–2817
29. Nakayama, T., Gardner, H., Berg, L. K., and Christian, J. L. (1998) Genes Cells 3, 377–394
30. Liu, Y., Hair, G. A., Boden, S. D., Viggieswarapu, M., and Titus, L. (2002) J. Bone Min. Res. 17, 406–414
31. Diefenderfer, D. L., Osyczka, A. M., Garino, J. P., Leboy, P. S. (2003) J. Bone Joint Surg. Am. 85-A, Suppl. 3, 19–28
32. Zhang, Y., Chang, C., Gehling, D. J., Hemmati-Brivanlou, A., and Derynck, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 974–979
33. Lin, X., Liang, M., and Feng, X. H. (2000) J. Biol. Chem. 275, 36818–36822
34. Garrett, J. R., Chen, D., Gutierrez, G., Zhao, M., Escobedo, A., Rossini, G., Harris, S. E., Gallwitz, W., Kim, K. B., Hu, S., Crews, C. M., and Mundy, G. R. (2003) J. Clin. Investig. 111, 1771–1782
35. Yamashita, M., Ying, S. X., Zhang, G. M., Li, C., Cheng, S. Y., Deng, C. X., and Zhang, Y. E. (2005) Cell 121, 101–113
36. Hassel, S., Schmitt, S., Hartung, A., Roth, M., Nohe, A., Petersen, N., Ehrlich, M., Henis, Y. I., Sebald, W., and Knaus, P. (2003) J. Bone Joint Surg. Am. 85-A, Suppl. 3, 44–51
37. Wang, H. R., Zhang, Y., Ozdamar, B., Ogumijimi, A. A., Alexandrova, E., Thomsen, G. H., and Wrana, J. L. (2003) Science 302, 1775–1779