Proteolysis of Latent Transforming Growth Factor-β (TGF-β)-binding Protein-1 by Osteoclasts

A CELLULAR MECHANISM FOR RELEASE OF TGF-β FROM BONE MATRIX*

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The binding of growth factors to the extracellular matrix (ECM) may be a key pathway for regulation of their activity. We have shown that a major mechanism for storage of transforming growth factor-β (TGF-β) in bone ECM is via its association with latent TGF-β-binding protein-1 (LTBP1). Although proteolytic cleavage of LTBP1 has been reported, it remains unclear whether this represents a physiological mechanism for release of matrix-bound TGF-β. Here we examined the role of LTBP1 in cell-mediated release of TGF-β from bone ECM. We first characterized the soluble and ECM-bound forms of latent TGF-β produced by primary osteoblasts. Next, we examined release of ECM-bound TGF-β by bone resorbing cells. Isolated avian osteoclasts and rabbit bone marrow-derived osteoclasts released bone matrix-bound TGF-β via LTBP1 cleavage. 1,25-Dihydroxyvitamin D₃ enhanced LTBP1 cleavage, resulting in release of 90% of the ECM-bound LTBP1. In contrast, osteoblasts failed to cleave LTBP1 or release TGF-β from bone ECM. Cleavage of LTBP1 by avian osteoclasts was inhibited by serine protease and metalloprotease (MMP) inhibitors. Studies using purified proteases showed that plasmin, elastase, MMP2, and MMP9 were able to cleave LTBP1 to produce 125–165-kDa fragments. These studies identify LTBP1 as a novel substrate for MMPs and provide the first demonstration that LTBP1 proteolysis may be a physiological mechanism for release of TGF-β from ECM-bound stores, potentially the first step in the pathway by which matrix-bound TGF-β is rendered active.

Recent evidence suggests that the binding of growth factors to the extracellular matrix (ECM) may be a major mechanism for regulation of growth factor activity (for review, see Ref. 1).

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§ This abbreviation is ECM, extracellular matrix; FRC, fetal rat calvarial cells; LTBP1, latent transforming growth factor-β-binding protein-1; MMP, matrix metalloproteinase; PTHrP, parathyroid hormone-related peptide; 1,25-D₃, 1,25-dihydroxyvitamin D₃; β-GP, β-glycophosphatase; PBS, phosphate-buffered saline; FBS, fetal bovine serum; αMEM, α-modified minimal essential medium; P/S, penicillin/streptomycin; Ab, antibody; FPLC, fast pressure liquid chromatography; LAP, latency-associated peptide; LG, l-glutamine; AP, aprotinin; TRAP, tartrate-resistant acid phosphatase; PL, plasmin; TIMP, tissue inhibitor of the matrix metalloproteinases; NC, no cells control; C, control.

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microfibrils (9, 10, 19, 20). Several studies have suggested that in addition to its role in storage of latent TGF-β in the ECM, LTBP1 may function as a vehicle for release of latent TGF-β from matrix-bound stores. Thus, purified proteases, such as plasmin and elastase, have been shown to release TGF-β from the matrix of fibroblasts and osteoblasts via cleavage of LTBP1 (9, 21). However, it is unclear whether this is a phenomenon associated with pathologically high concentrations of proteases or whether proteolytic cleavage of LTBP1 represents a true physiologic mechanism for release of ECM-bound TGF-β.

In the present study, we characterized the forms of latent TGF-β produced by primary bone cells and stored in the ECM. Next, by using osteoclast cell culture systems we investigated the molecular mechanism(s) by which TGF-β is released from bone ECM-bound stores during bone resorption. In particular, we examined whether proteolytic cleavage of LTBP1 may be a potential cellular mechanism for the release of TGF-β bound to bone ECM, and we determined whether proteases known to be important in osteoclast function may be involved in this process.

EXPERIMENTAL PROCEDURES

Reagents—Plasmin, elastase, aprotinin, leupeptin, and pepstatin A were purchased from Roche Molecular Biochemicals. Phenylmethylsulfonyl fluoride and E64 were purchased from Sigma. Matrix metalloproteinases (active MMP2 and MMP9) and the tissue inhibitor of the matrix metalloproteinases (TIMP1) were purchased from Oncogene Research Products (Cambridge, MA). 1,25-Dihydroxyvitamin D3 (1,25-D3) was purchased from Biomol (Plymouth Meeting, PA). Parathyroid hormone-related peptide (PTHrP) was purchased from Bachem (Torrance, CA).

Antibodies—Two different rabbit polyclonal antibodies against LTBP1 were used: AB-39 that recognizes mouse, rat, and human LTBP1 (kindly supplied by K. Miyazono, Japanese Foundation for Cancer Research, Tokyo, Japan), and a second antibody, termed the “LTBP1 hinge antibody,” raised against a synthetic peptide corresponding to residues 721–744 of the rat LTBP1 sequence (GenBank™ accession number M55431) that recognizes mouse and rat LTBP1. The specificity of these antibodies has been described elsewhere (10, 13, 20). A mouse monoclonal antibody against human LTBP1, as well as neutralizing antibodies to TGF-β1 and -2, a pan-specific TGF-β-neutralizing antibody, and a goat polyclonal antibody against latent TGF-β1 propeptide were purchased from R & D systems (Minneapolis, MN). The detection antibody for Western blotting was a peroxidase-conjugated donkey anti-rabbit (Amersham Biosciences).

Cell Culture—Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). Rabbit calvarial osteoclasts (FRC) were isolated as described previously (9, 10). The cells were stored for up to 1 year using standard cryopreservation procedures and used for experiments after one further passage. UMR-106 cells were a gift from D. B. Rifkin (New York University, New York). Rabbit marrow cells were isolated using a modification of the method of Tezuka et al. (22). Femora, humeri, ulna, and radii of 11-day-old New Zealand White rabbits were removed. Connective tissues were dissected away, and the bones were minced in ω-modified minimal essential medium (αMEM). The bone pieces were vortexed to dissociate the cells, and bone particles were removed by sedimentation under gravity. The supernatant was centrifuged, and the cells were resuspended in αMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (LG), and 100 units/ml penicillin/streptomycin (P/S). The cells were then seeded into 12-well plates coated with 3%S-labeled bone ECM (see below).

Avian osteoclast precursors were isolated as described elsewhere from the medullary bone of egg-laying White Leghorn hens fed on a low calcium diet (23). The cells were plated in 150-mm Petri dishes as described elsewhere (23), and after overnight incubation, adherent cells were harvested by treatment with 2 mM EDTA in PBS for 5 min at 37 °C. The cells were then replated into 12-well plates coated with 3%S-labeled bone ECM (see below) in αMEM containing 2.5% FBS, 2.5% chicken serum, 2 mM LG, 100 units/ml P/S, and 6 μg/ml arabinos-β-d-xyloside. These cells have been shown to fuse and become multinucleated in the presence of 1,25-D3 (23) (see also Fig. 5, e and f).

The cells were cultured for up to 6 days, with or without 1,25-D, and/or protease inhibitors as described below. Culture media were changed every 2 days.

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Fast Pressure Liquid Chromatography (FPLC) Analysis—FPLC fractionation was performed as described previously in the cytotoxic assay. FRC cells were grown in 150-mm flasks in αMEM supplemented with 10% FBS, 2 mM LG, 100 units/ml P/S, and 30 μg/ml gentamycin. At confluence the media were changed to αMEM, supplemented as above but with the addition of 50 μg/ml ascorbic acid and 3 mM β-glycerophosphate (β-GP) and the reduction of the serum to 5%. Thereafter the media were changed every 3 days. Conditioned medium was harvested from four tissue culture flasks representing pre-confluent stages in the propagation of FRC cells to form mineralized bone-like nodules. The "pre-confluent" stage was from proliferating cultures at 90% confluence; the "early post-confluent" stage was from 2-day post-confluent cultures; the "nodule-forming" stage was from 6-day post-confluent cultures in which multilayered cellular nodules had formed, but were not yet mineralized; and the "mineralization" stage was from 14-day post-confluent cultures in which mineralized bone nodules were present (see Fig. 1, inset micrographs). For collection of conditioned media, phenol red-free Dulbecco’s modified Eagle’s medium was used containing 2 mM LG, 100 units/ml P/S, 0.1% bovine serum albumin, 50 μg/ml ascorbic acid. 25 ml of conditioned medium was collected per flask over a 48-h culture period. A total of 150 ml of conditioned medium per time point was concentrated 10-fold over a 50-ko cut-off membrane against a minicette concentrator (Millipore, Bedford, MA), and the samples were lyophilized and reconstituted with 1–2 ml of distilled water, pH 7.2. They were then dialyzed against 20 mM tris buffer, pH 7.2, and applied to an analytical Mono-Q anion exchange column (Amersham Biosciences). The column was eluted with a linear gradient of 0–0.5 M NaCl/20 mM tris buffer. Fractions were tested for TGF-β activity using the alkaline phosphatase microassay as described below.

TGF-β Measurement—TGF-β was measured as described previously by using either the ROS 17/2.8 microassay (24), which measures stimulation of alkaline phosphatase activity by TGF-β in ROS 17/2.8 osteosarcoma cells, or by using the mink lung epithelial cell luciferase bioassay (25), which measures stimulation of activity of the plasminogen activator inhibitor-1 promoter by TGF-β. To determine the total (active + latent) TGF-β levels, the samples were acidified to pH 2 using 1 M HCl and then reneutralized using 1 M NaOH immediately before addition to the assay plate. Latent TGF-β values were determined by subtracting active TGF-β measurements from total TGF-β.

Pulse-Chase Metabolic Labeling and Immunoprecipitation—To examine further the secreted and matrix-bound forms of latent TGF-β present in osteoclasts, FRC cells, or primary osteoclasts, metabolic labeling and immunoprecipitation were performed. Cells were plated into 12-well multwell plates at 10,000 cells/cm2 growth area. At 90% confluence, the cells were washed twice in PBS and incubated for 1 h in cysteine-free αMEM supplemented with 5% dialyzed FBS, 2 mM LG, 100 units/ml P/S, 50 μg/ml ascorbic acid. The cells were then labeled for 30 min using 100 μCi/ml [35S]cysteine (Amersham Biosciences) in cysteine-free αMEM supplemented with 5% dialyzed FBS and additives as above. This was followed by a "cold chase" in complete αMEM (containing 0.1 mg/ml l-cysteine), supplemented with 5% FBS and additives as above for a time course of 15 and 30 min, and 2, 6, 24, and 48 h.

Conditioned media were harvested and protease inhibitors added (1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 1 μM pepstatin A, 10 μM leupeptin). The cells + matrix were washed twice with ice-cold PBS and then lysed in ice-cold radioimmunoprecipitation buffer (RIPA) (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate). The deoxycholate-insoluble material (essentially matrix) was washed twice more in ice-cold PBS, transferred to a 1.5-ml tube, and centrifuged for 5 min at 14,000 rpm. The pellet was then digested for 2 h at 37 °C on an end-over rotator in 500 μl of a solution consisting of 0.2 units/ml plasmin in plasmin digestion buffer (see under "Protease Digestions"). This digestion releases any LTBP1 that may still be bound to the ECM (9, 21).

LTBP1 and TGF-β in the supernatant and in the plasmid digest of the matrix were determined by immunoprecipitation followed by SDS-PAGE and autoradiography as described previously (9, 24) using a rabbit polyclonal antibody specific for LTBP1 or using a goat polyclonal antibody specific for LAP.

Preparation of Bone ECM—To prepare bone ECM for culturing with osteoclasts, FRC cells were plated into 12-well plates as described above. At confluence, the medium was changed to αMEM supplemented with 5% FBS, 2 mM LG, 100 units/ml P/S, 30 μg/ml gentamycin, 50 μg/ml ascorbic acid, and 3 mM β-GP. Thereafter, the culture media were changed every 3 days. Cultures were maintained for 10–14 days, by
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RESULTS

Characterization of Secreted Latent TGF-β Complexes Produced by Primary Bone Cell Cultures—As matrix laid down by primary osteoblast cultures was to be used to examine release of bone matrix-bound TGF-β by osteoclasts, it was first necessary to characterize the latent TGF-β complexes produced by the osteoblast cultures and determine what proportion of the latent TGF-β was complexed to LTBP1. Fig. 1 shows results from Mono-Q FPLC analysis of conditioned media samples from FRC cultures at the pre-confluent, early post-confluent, nodule-forming, and mineralizing stages (see “Materials and Methods”). The insets in Fig. 1 show the appearance of the cultures at each of these stages. Two major peaks of latent TGF-β activity were observed, one eluting at 0.22 M NaCl (peak II), which is the expected elution position for the 100-kDa small latent TGF-β complex (16, 24), and the other eluting at 0.3 M NaCl (peak III), which corresponds to the 290-kDa large latent TGF-β complex, containing LTBP1 (16, 24). The pre-confluent and early post-confluent cultures produced predominantly the small latent TGF-β complex (peak II). However, with maturation in culture, the cells switched to producing larger amounts of the 290-kDa (LTBP1-containing) complex (peak III), which made up ~40% of the total latent TGF-β secreted. In addition, a minor peak eluting at 0.05 M NaCl (peak I) was also observed in nodule-forming and mineralizing cultures. At present the nature of this peak is unknown. Antibody neutralization studies indicated that the latent TGF-β activity was predominantly TGF-β1 in all three peaks with smaller amounts of TGF-β2 (data not shown). In the pre-confluent and early post-confluent cultures, peak III showed ~20–30% TGF-β2 activity, in contrast to the nodule-forming and mineralizing cultures, which showed no detectable TGF-β2 activity in this peak (data not shown).

Analysis of Matrix-bound Latent TGF-β in Primary Bone Cell Cultures—LTBP1 has been shown to be cross-linked in the ECM via the action of transglutaminase (28) and is highly insoluble. Previous studies have shown that proteases such as plasmin and elastase can release proteolytic fragments of LTBP1 from the ECM (9, 21) and that plasmin can activate latent TGF-β (29, 30). These serine proteases were therefore used to examine the ECM-bound forms of latent TGF-β in primary bone cell cultures (see Fig. 2). Treatment of 35S-labeled bone ECM with plasmin or elastase resulted in release of radiolabeled products, which could be immunoprecipitated with antibodies against LTBP1 (see Fig. 2a). Plasmin released a doublet at ~110–130 kDa (black arrowhead), corresponding
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S. L. Dallas, unpublished observations.

To analyze further the production of LTBP1-bound forms of latent TGF-β in primary bone cell cultures, pulse-chase immunoprecipitation experiments were performed (see Fig. 3). Immunoprecipitation analysis, using a 30-min pulse of [35S]cysteine labeling, indicated that significant amounts of LTBP1 were secreted into the culture media of FRC cells after only 15 min of chase (Fig. 3a). A major band at 170–190 kDa was observed (black arrowhead), corresponding to free LTBP1. This band represented about 90% of the immunoprecipitable LTBP1. In addition a minor band at ~290 kDa was observed (white arrowhead), representing LTBP1 complexed to TGF-β. These bands increased in intensity up to 6 h. However, by 24 and 48 h an additional lower molecular weight band was evident at ~100 kDa (gray arrow), which presumably represents a proteolytically processed form of LTBP1.

To detect LTBP1 that is cross-linked in the ECM, the matrix was digested with plasmin (see above). This resulted in release of proteolytic fragments of free LTBP1 of 110–130 kDa (Fig. 3c, black arrowheads), and a band representing cleaved LTBP1 complexed to TGF-β at 230 kDa (Fig. 3c, white arrowhead). These experiments indicated that not only was LTBP1 rapidly secreted but it was also rapidly incorporated into the matrix, with significant amounts of free LTBP1 and large latent complex detected in the ECM after only 15 min of chase. Again the intensity of these bands increased up to 6 h in culture, after which no significant further increase was observed.

Release of LTBP1 and LTBP1 Complexed with Latent TGF-β by Rabbit Marrow Cells—To determine whether proteolytic cleavage of LTBP1 could be a potential cellular mechanism for release of matrix-bound latent TGF-β, experiments were performed...

**FIG. 2.** Release of LTBP1 and large latent TGF-β complex from bone matrix by digestion with plasmin (0.1 units/ml for 2 h at 37 °C) or elastase (1 μg/ml for 2 h at 37 °C), as shown by immunoprecipitation using anti-LTBP1 and anti-LAP antibodies. a, autoradiograph showing cleaved LTBP1 (black arrowhead) and large latent TGF-β complex containing cleaved LTBP1 (white arrowhead) immunoprecipitated from plasmin (PL) and elastase (EL) digests of 35S-labeled bone ECM. Samples precipitated with antiserum specific for LTBP1 (Ab) or with control rabbit serum (C) are indicated above the lanes. b, autoradiographs showing cleaved LTBP1 (black arrowheads) and large latent TGF-β complex (white arrowhead) immunoprecipitated from plasmin digests of 35S-labeled bone ECM (0.1 unit/ml plasmin for 2 h at 37 °C). Samples were precipitated with anti-LTBP1 or anti-LAP to confirm co-migration. The controls for these antibodies are normal rabbit serum (RS) and goat immunoglobulin (GT IgG), respectively. Molecular mass markers (kDa) are indicated on the right of the gels. c and d, histograms showing TGF-β concentrations in plasmin or elastase digests of bone ECM, as measured by bioassay; c, latent TGF-β; d, active TGF-β. Samples are as follows: C, control without proteases; PL, plasmin (0.1 unit/ml for 2 h at 37 °C); EL, elastase (1 μg/ml for 2 h at 37 °C). Note that plasmin releases 15-fold more latent TGF-β than active and that elastase releases only latent TGF-β, with undetectable levels of active TGF-β. Values are the means ± S.E. from triplicate samples. The dashed line indicates the detection limit of the assay. * signifies significantly different from control (p < 0.05) using analysis of variance, followed by Student Newman Keuls method of multiple comparisons.

of the total TGF-β released. In contrast to plasmin, elastase did not release any detectable active TGF-β from bone ECM (Fig. 2d). The doses of plasmin and elastase used are sufficient to abolish completely the LTBP1 fibrillar staining in bone matrix preparations from fetal rat calvarial cells (9).2

2 S. L. Dallas, unpublished observations.
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Fig. 4. Release of LTBP1 and large latent TGF-β complex from 
35S-labeled bone ECM by rabbit marrow cells as demonstrated 
by immunoprecipitation using anti-LTBP1 antibodies. The 
samples are as follows: NC, control with no cells; C, rabbit marrow cells; 
PTHrP, rabbit marrow cells treated with 100 ng/ml PTHrP; AP, rabbit 
marrow cells treated with 50 µg/ml aprotinin; PTHrP + AP, rabbit 
marrow cells treated with 100 ng/ml PTHrP and 50 µg/ml aprotinin. 
Gels a–c show samples immunoprecipitated with antiserum specific for 
LTBP1, and gels d–f show corresponding control samples immunopre-
cipitated with non-immune rabbit serum. a shows cleaved LTBP1 
(black arrowhead) and large latent TGF-β complex (white arrowhead) 
released into the culture medium by rabbit marrow cells during 0–24 h 
of culture. b shows cleaved LTBP1 and large latent TGF-β complex 
released into the culture medium during 24–48 h of culture. c shows 
LTBP1 and large latent TGF-β complex bound in the matrix at the end 
of the 48-h culture period (the matrix was treated with plasmin to 
release the bound LTBP1 and LTBP1/TGF-β, hence a doublet is seen for 
free LTBP1, see black arrowheads). Note the release of LTBP1 into the 
media by rabbit marrow cells with a corresponding decrease in matrix-
bound LTBP1. This release is blocked by aprotinin.

formed using cultures of neonatal rabbit marrow cells. These 
cultures consist of a mixed population of cells that is highly 
enriched for mature osteoclasts but also contain some stromal 
cells and cells in the osteoblastic lineage, which can support 
and activate the osteoclasts. When neonatal rabbit marrow cells 
were cultured on 35S-cysteine-labeled bone ECM, specific 
radioabeled products were released into the culture medium, 
which could be immunoprecipitated with antibodies against 
LTBP1. Fig. 4, a and b, shows radiolabeled fragments released 
into the culture medium during 0–24 h and 24–48 h of culture. 
In control cultures (NC) in which no cells were seeded onto 
the labeled matrix, a very small amount of LTBP1 was detected 
in the medium, presumably representing passive diffusion of 
LTBP1 from the ECM and/or low level degradation by serum 
components in the medium. In contrast, when rabbit marrow 
cells were cultured on the labeled ECM (C), a major band of 
~130 kDa, corresponding to a cleaved fragment of LTBP1 
(black arrowhead), and a minor band of ~230 kDa, correspond-
ing to cleaved LTBP1 complexed to small latent TGF-β (white 
arrowhead), were released into the culture medium. Release 
was approximately equivalent during the 0–24- and 24–48-h 
culture periods. Treatment with PTHrP, a stimulator of oste-
oclast activity through its action on osteoblasts and stromal 
cells, produced a slight stimulation in release of LTBP1 and 
LTBP1 complexed to latent TGF-β, which was most evident in 
the first 24-h culture period. The serine protease inhibitor, 
aprotinin (AP) completely blocked release of LTBP1 under both 
control and PTHrP-stimulated conditions. As expected, release 
of LTBP1 and LTBP1/TGF-β by rabbit marrow cells into the culture 
medium was concomitant with a reduction in LTBP1 bound in the matrix (see Fig. 4c).

Osteoclasts but Not Osteoblasts Release LTBP1 from Bone 
ECM—To rule out that osteoblasts and/or stromal cells in the 
rabbit marrow cultures were releasing LTBP1 from bone ECM 
and to verify that osteoclasts were responsible for release of 
LTBP1 and large latent TGF-β complex, experiments were 
performed using pure populations of osteoblast-like cells fol-
lowed by the use of relatively pure populations of osteoclasts 
derived from avian osteoclast precursors. Primary cultures of 
fetal rat calvarial osteoblasts as well as the rat osteosarcoma 
cell line, UMR-106, failed to release LTBP1 and large latent 
TGF-β complex from bone matrix, either under control or 
PTHrP-stimulated conditions (data not shown).

In contrast, when avian osteoclast precursors were cultured 
on radioabeled bone ECM, cleaved fragments of LTBP1 were 
released into the culture medium (see Fig. 5, a–d). These avian 
osteoclast precursors fuse in culture over a 6-day period to form 
highly purified populations of multinucleated cells (>95% 
pure), which are positive for tartrate-resistant acid phospha-
tase (TRAP) and are capable of resorbing bone (8, 23). The 
avian osteoclast cultures released LTBP1 under control, un-
stimulated conditions (C) (see Fig. 5a, black arrowheads). This 
release occurred at a low level throughout the entire 6-day 
culture period and was most evident as a dramatic decrease in 
the amount of ECM-bound LTBP1 and LTBP1/TGF-β at the 
end of the culture period (Fig. 5b). Treatment of the avian 
osteoclast cultures with 1,25-D3, which enhances fusion of 
precursors and stimulates osteoclastic activity (23), resulted in 
a marked stimulation in release of LTBP1 and LTBP1/TGF-β 
from bone ECM. Release was maximal between days 3 and 4 of 
culture and resulted in the release of essentially all the ECM-
bound LTBP1 and LTBP1/TGF-β into the culture medium by 
the end of the 6-day culture period (Fig. 5b). For subsequent 
experiments, the media samples were therefore collected over 
days 1–4 of culture. Fig. 5, e and f, shows photomicrographs of 
the avian osteoclasts formed after 6 days of culture on bone 
ECM under control culture conditions (Fig. 5e) and in the 
presence of 1,25-D3 (Fig. 5f). Note that in the presence of 
1,25-D3, fusion of the precursors to form multinucleated oste-
oclasts is enhanced, and TRAP staining is much more intense.

To confirm that the avian osteoclasts were releasing TGF-β 
from the bone matrix, the TGF-β content of the bone ECM was 
measured after culturing with avian osteoclasts for 6 days (see 
Fig. 5g)). Bone ECM that had been cultured with avian oste-
oclasts showed an ~60% reduction in TGF-β content compared 
with controls cultured without cells. Stimulation of the avian 
osteoclasts with 10−8 M 1,25-D3 further enhanced this release, 
resulting in the loss of ~90% of the matrix-bound TGF-β.

Dose-response experiments indicated a dramatic stimulation 
in release of ECM-bound LTBP1 by avian osteoclast precursors 
treated with doses of 1,25-D3 as low as 10−10 M compared with 
unstimulated controls (see Fig. 5, h and i). Maximal release 
was seen between 10−8 and 10−7 M and subsequent experi-
ments were performed using the 10−8 M dose of 1,25-D3.

Effects of Protease Inhibitors on Release of Bone Matrix-
bound LTBP1 and LTBP1 Complexed to Latent TGF-β by 
Avian Osteoclasts—The osteoclast culture systems we have 
used produce complex mixtures of proteases including cathep-
sins, plasminogen activators, and matrix metalloproteinases 
(MMPs) (reviewed in Ref. 31). In particular, osteoclasts express 
high levels of cathepsin K (32) and MMP9 (33, 34). To investi-
gate which protease(s) were responsible for cleavage of LTBP1, 
a number of protease inhibitors were examined (Fig. 6).
Under basal, unstimulated conditions (C), only a small amount of LTBP1 was released from radiolabeled bone ECM by avian osteoclasts when compared with matrix cultured without cells (NC). This low level release was blocked by the serine protease inhibitor, AP. The other protease inhibitors tested did not have any notable effect on basal release of LTBP1/TGF-β from bone ECM. These included the serine protease inhibitor leupeptin, the cysteine protease inhibitor pepstatin A, and the tissue inhibitor of matrix metalloproteinases-1 (TIMP1). In contrast, under 1,25-D3-stimulated conditions, essentially all the matrix-bound LTBP1 and large latent TGF-β complex were released into the culture media. Both the serine protease inhibitor, aprotinin, and the matrix metalloproteinase inhibitor, TIMP1, blocked release of LTBP1 and LTBP1/TGF-β almost to the level seen in unstimulated control cells. The serine protease inhibitor leupeptin also partially blocked release of LTBP1 and LTBP1/TGF-β. In contrast, the aspartic (acid) protease inhibitor, pepstatin A, was without effect. In separate experiments we have also found that the cysteine protease inhibitor E64 is without effect in this assay (data not shown).

**Cleavage of LTBP1 by Purified MMPs**—The above experiments suggested the involvement of serine proteases and/or matrix metalloproteinases in release of LTBP1 and large latent TGF-β complex from bone ECM by avian osteoclasts. To confirm that MMPs were able to cleave LTBP1, experiments were performed using the purified proteases. LTBP1 was immunoprecipitated from the conditioned media of CHO-L76 cells which stably overexpressed human LTBP1 using a mouse monoclonal antibody against LTBP1. The immunoprecipitates were then digested with plasmin, MMP2, or MMP9 and analyzed by immunoblotting using a rabbit polyclonal antibody against LTBP1 (see Fig. 7a). LTBP1 immunoprecipitates incubated with digestion buffer alone (C) gave a single LTBP1 immunoreactive band at -190 kDa (black arrow). Digestion
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FIG. 6. Immunoprecipitation showing release of LTBP1 (black arrowhead) and large latent TGF-β (white arrowhead) from [35S]cysteine-labeled bone ECM by avian osteoclasts and inhibition by protease inhibitors. a shows release of LTBP1 and large latent TGF-β complex into the culture medium over the 4-day culture period; b shows LTBP1 and large latent TGF-β complex bound in the ECM at the end of the 4-day culture. Samples are as follows: NC, control without osteoclasts; C, avian osteoclasts; AP, avian osteoclasts treated with apotinin (50 μg/ml); leu, avian osteoclasts treated with leupeptin (10 μmol/l); pep, avian osteoclasts treated with pepstatin A (1 μmol/l); TIMP, avian osteoclasts treated with TIMP-1 (1.5 μmol/l). Samples treated with 10−4 M 1,25-D3 are denoted +D3. Note the release of LTBP1 and LTBP1/TGF-β by avian osteoclasts stimulated with 1.25-D3. Release was blocked to control levels using inhibitors of serine proteases (aprotinin and leupeptin) and by an inhibitor of MMPs (TIMP-1) but not by an inhibitor of aspartic (acid) proteases (pepstatin A).

FIG. 7. a, Western blot showing cleavage of LTBP1 by plasmin (PL), MMP2, and MMP9. LTBP1 was immunoprecipitated from the conditioned media of CHO-L76 cells stably transfected with human LTBP1, using a monoclonal antibody specific for human LTBP1. The immunoprecipitate was digested with plasmin, MMP2, or MMP9 (25 μg/ml) for 6 h at 37 °C and then electrophoresed in 4–20% gradient SDS-PAGE gels under non-reducing conditions. The samples were immunoblotted using a polyclonal antibody to LTBP1 (Ab39). The samples are as follows: IgG, sample immunoprecipitated with non-immune IgG; C, sample immunoprecipitated with LTBP1 monoclonal antibody and incubated in digestion buffer alone; PL, MMP2, and MMP9, sample immunoprecipitated with anti-LTBP1 monoclonal antibody and then digested with the indicated protease. Note cleavage of immunoprecipitated LTBP1 by plasmin, MMP2, and MMP9 to give fragments in the range 125–165 kDa. b, Western blot showing LTBP1 fragments released by digestion of ECM from fetal rat calvarial cells with plasmin (PL), MMP2, and MMP9. ECM preparations were digested with plasmin, MMP2, or MMP9 (25 μg/ml) for 6 h at 37 °C and then electrophoresed in 4–20% gradient SDS-PAGE gels under non-reducing conditions. The samples were immunoblotted using a polyclonal antibody to LTBP1 (Ab39). Note that plasmin and MMP2 released cleaved LTBP1 fragments; however, MMP9 failed to release LTBP1 fragments from the ECM. c, immunofluorescent images of ECM from fetal rat calvarial cells following digestion with PL, MMP2, and MMP9. Note the well organized fibrillar network in control cultures (C), the reduced staining intensity following MMP2 digestion, and virtual absence of fibrillar staining following plasmin treatment. MMP9 treatment failed to reduce the fibrillar staining significantly compared with undigested controls (bar, 25 μm).

We also examined the ability of MMPs to cleave LTBP1 in its ECM-bound form, which is localized to 10 nm matrix microfibrils in primary osteoblast cultures (10). Treatment of osteoblast-derived ECM with either plasmin or MMP2 resulted in the release of cleaved fragments of LTBP1 into the digest supernatant in the range 125–150 kDa (see Fig. 7b). In contrast, MMP9 failed to release significant amounts of LTBP1 from the ECM. Activity of the MMP2 and MMP9 preparations was confirmed by gelatin zymogram analysis (data not shown). Western blot data were confirmed by parallel immunofluorescent staining for LTBP1 in matrix preparations that were digested with plasmin, MMP2, or MMP9 (see Fig. 7c). Fibrillar LTBP1 staining was observed in control cultures (C). Treatment with MMP2 dramatically reduced fibrillar LTBP1 immunostaining, and treatment with PL almost completely abolished LTBP1 staining. In contrast, treatment with MMP9 produced only a slight reduction in staining intensity.

DISCUSSION

Although LTBP1 was originally identified as a component of the large latent TGF-β complex (13, 14), it is now clear that the LTBP1s 1–4 are members of the fibrillin superfamily that have...
multiple functions as both structural ECM proteins and as regulators of TGF-β availability (11, 12). Our data, showing release of ECM-bound LTBP1 and LTBP1 complexed to latent TGF-β by osteoclasts, represent the first demonstration that proteolytic cleavage of LTBP1 may be a physiological mechanism for the release of latent TGF-β from ECM-bound stores. Although previous studies (9, 20, 21) using purified proteases have implied a proteolytic mechanism for release of latent TGF-β from ECM through cleavage of LTBP1, the amounts of proteases used in these studies are high relative to physiological concentrations. Our studies using osteoclast populations show that these cells, which are actively involved in matrix turnover, are able to release cleaved fragments of LTBP1 and LTBP1 complexed to latent TGF-β from matrix that is laid down by osteoblasts.

In the present studies we have also characterized the forms of latent TGF-β produced by primary osteoblasts and incorporated into the ECM. The major secreted forms were the small latent complex containing TGF-β1 and large latent complexes containing predominantly TGF-β1, with smaller amounts of TGF-β2. Previously, we reported characterization of the forms of latent TGF-β produced by osteosarcoma cell lines (24). Whereas one cell line (UMR-106) produced exclusively the small latent TGF-β complex, and one (MG63) produced exclusively the large latent TGF-β complex, a third cell line (ROS 17/2.8) produced both complexes and therefore resembled most closely the primary bone cells examined in the present studies. Pulse-chase immunoprecipitation studies demonstrated that the large latent complex containing LTBP1 is rapidly secreted and rapidly incorporated into the ECM of primary osteoblasts, similar to data reported using a human fibroblastic cell line (19). However, in contrast to the fibrosarcoma cells, the major proportion of the LTBP1 produced by primary osteoblasts remained in the culture medium. This suggests that either the osteoblast cells produce an excess of LTBP1 over that which is required for matrix incorporation or that the mechanisms controlling matrix incorporation may be different in these two cell types. Interestingly, only a minor fraction (<10%) of the LTBP1 produced by primary osteoblasts was complexed to TGF-β, with the remaining 90% occurring in a free, uncomplexed form. In various cell types examined, including fibroblasts (19, 24), osteoblasts, (9, 24) and breast cancer cells, the amount of free LTBP1 can vary between 50 and 95%. This suggests functions for LTBP1 that are independent of its role in TGF-β regulation. In previous studies (9, 10) we have demonstrated that bone matrix-bound LTBP1 is present as part of an organized microfibrillar network, which also contains fibrillin-1. These observations, together with the high degree of homology of LTBP1 with the fibrillins, support an important independent role for LTBP1 as an extracellular matrix protein.

Release of bone matrix-bound TGF-β by resorbing osteoclasts would be expected to have important consequences for bone cells, as TGF-β is capable of regulating all the steps in the bone remodeling cascade (reviewed in Ref. 5). TGF-β has been shown to inhibit osteoclast activity, both by stimulating osteoclasts to undergo apoptosis and by inhibiting formation of osteoclasts from their precursors. TGF-β is also a powerful chemoattractant and mitogen for osteoblast precursors (5). Therefore, TGF-β released from bone matrix could attract osteoclasts to the site of bone resorption and stimulate them to proliferate. The effect of TGF-β on mature osteoblasts is then to inhibit proliferation and stimulate production of bone ECM proteins, including type I collagen, fibronectin, and osteocalcin (reviewed in Ref. 35). In addition to stimulating these ECM proteins, TGF-β1 also induces expression of its own message as well as expression of LTBP1 (24, 35).

Interestingly, 1,25-dihydroxyvitamin D₃ stimulated release of LTBP1 from bone ECM by avian osteoclasts. This effect is most likely due to 1,25-D₃ enhancing resorptive activity through stimulating fusion of osteoclast precursors to form mature resorbing cells. However, a direct effect of 1,25-D₃ on protease activity in osteoclasts is also possible.

Our studies using protease inhibitors suggested the involvement of serine proteases and/or MMPs in release of ECM-bound LTBP1 and LTBP1 complexed to latent TGF-β by avian osteoclasts and rabbit bone marrow cells. These findings are consistent with our biochemical observations that plasmin, MMP2, and MMP9 can cleave LTBP1. Our data using inhibitors of cysteine and aspartic proteases (e.g. cathepsins B, L, D, and K) suggest that this group of proteases are not involved in cleavage of LTBP1 by avian osteoclasts. This is in agreement with the biochemical data of Taipale and co-workers (21) showing that cathepsins B and D were negative or showed a very limited ability to cleave LTBP1 from the ECM of epithelial cells.

Our studies identify LTBP1 as a new substrate for MMPs. Interestingly, both MMP2 and MMP9 were able to cleave the soluble form of LTBP1; however, only MMP2 cleaved the ECM-bound form of LTBP1. This suggests that the cleavage sites used by MMP9 may be unavailable when LTBP1 is incorporated into the ECM. Thus, efficient cleavage of soluble forms of LTBP1 by a protease may not necessarily imply that the same protease will cleave LTBP1 once it is assembled into ECM microfibrils. Another possibility is that degradation of other collagenous or non-collagenous ECM components may be required prior to LTBP1 cleavage to expose cryptic proteolytic cleavage sites on LTBP1. Thus, a cascade of proteases that are involved in the degradation of bone ECM by osteoclasts may be required for release of LTBP1 and TGF-β from bone ECM.

Recent studies (36, 37) have shown that other members of the fibrillin superfamily, such as fibrillins 1 and 2, are also substrates for MMPs. Breakdown of microfibrillar proteins by MMPs may be important in pathological conditions and may contribute to the disease phenotype in inherited disorders such as Marfan’s and related syndromes. Interestingly, fibrillin-1 mutations associated with Marfan’s syndrome and ectopia lentis have been shown to result in the production of mutant protein, which is more susceptible to degradation by proteases including MMPs (36, 37). This effect appears to be through disruption of calcium binding and apparent exposure of cryptic protease cleavage sites. Breakdown of fibrillin-containing microfibrils that also contain LTBP1 would be expected to result in the release and activation of matrix-bound TGF-β, which has been strongly implicated in a number of fibrotic diseases and could play a role in scleroderma, another disease for which the fibrillin-1 gene has been implicated (reviewed in Ref. 38).

Our studies showing release of TGF-β from bone matrix by plasmin and elastase suggest that these proteases are much more efficient at cleaving LTBP1 and releasing latent TGF-β from bone matrix than they are at activating the latent TGF-β released. Thus, concentrations of plasmin that can cleave essentially all the LTBP1 and abolish LTBP1 immunoreactivity in the ECM are capable of activating only a small proportion of the latent TGF-β released. Although several studies (29, 30) have implicated plasmin as an activator of TGF-β, these studies have been performed using conditioned medium containing predominantly the 100-kDa small latent TGF-β complex, which may be more readily activated than the large latent complex, or samples in which the latent TGF-β complexes are not defined. Other studies (reviewed in Ref. 39) have been performed using co-culture systems, where again the form of latent TGF-β is not defined and may involve release from ECM-bound stores. Our
data support the notion that there may be several sequential steps in the process by which ECM-bound TGF-β is rendered active, as suggested by Rifkin and co-workers (40). Proteases such as plasmin and MMPs may be important initially in release of latent TGF-β from ECM-bound stores. Activation may then take place by cell surface-localized mechanisms, perhaps involving interactions with mannose 6-phosphate receptors (40), integrins (41), or thrombospondins (42). A recent report (43) has also implicated surface-bound forms of MMP2 and MMP9 in activation of the small latent TGF-β complex. Further studies are therefore clearly warranted to clarify the steps in the activation pathway.

This study has focused on one potential pathway for release of latent TGF-β from bone ECM. However, other mechanisms for TGF-β storage and release in bone ECM may also play an important role. For instance, many growth factors can bind to matrix via heparan sulfate-containing proteoglycans and other members of the proteoglycan family. Mature TGF-β can bind to heparin (44) and our own unpublished studies indicate that LTBP1 itself binds to heparin.3 Studies by Tiedemann et al. (45) have demonstrated the importance of heparan sulfate-containing proteoglycans in assembly of fibrillin-1 into the ECM. Thus heparan sulfate-containing proteoglycans may play a similar role in LTBP1 (and by implication TGF-β) incorporation. At present the potential role of proteoglycan-degrading enzymes, such as heparanase, in release of TGF-β from bone ECM remains to be determined. However, Taipale et al. (21) reported that various glycosidases, including heparinas I and III and chondroitinase ABC as well as a combination of all three, were unable to cleave LTBP1 from the ECM of epithelial cells.

Mature TGF-β is also known to bind to proteoglycans such as decorin and biglycan, which may provide an alternative pathway for storage and release of TGF-β in bone ECM (46). These latent TGF-β complexes could be viewed as “secondary” complexes because it is the mature TGF-β homodimer (i.e. after activation) that is bound. In contrast, the LTBP1-bound TGF-β can be thought of as a “primary” latent TGF-β complex, because this latent complex is assembled inside the cell prior to secretion (17). Although our studies have demonstrated a likely role for LTBP1 in both the storage and release of latent TGF-β in bone ECM, other secondary latent TGF-β complexes, such as the decorin and biglycan-bound forms, may also be released and activated during bone resorption.

In summary, the data presented here, using osteoclast culture systems, provide the first demonstration that cell-mediated release of ECM-bound latent TGF-β can occur via proteolytic cleavage of LTBP1. LTBP1 is known to regulate TGF-β activity at multiple levels including enhancing secretion of the latent TGF-β complex, facilitating storage of the latent TGF-β complex in the ECM and modulating activation of latent TGF-β. The involvement of LTBP1 in the cell-mediated release of latent TGF-β from ECM-bound stores confirms an additional regulatory role for this ECM protein and may provide an important pathway for communication between cells and the extracellular matrix.

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3 S. L. Dallas, unpublished observations.
Proteolysis of Latent Transforming Growth Factor-β (TGF-β)-binding Protein-1 by Osteoclasts: A CELLULAR MECHANISM FOR RELEASE OF TGF-β FROM BONE MATRIX

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