Interaction of type I collagen (COL(I)) with α2β1 integrin causes differentiation and transforming growth factor β (TGF-β) receptor down-regulation in osteoblastic cells (Takeuchi, Y., Nakayama, K., and Matsumoto, T. (1996) J. Biol. Chem. 271, 3938–3944). The TGF-β receptor down-regulation enables cells to escape from the inhibition of differentiation by TGF-β. To clarify how the cell-matrix interaction regulates these phenotypic changes, signaling pathways were examined in murine MC3T3-E1 cells. Attachment of cells to COL(I) stimulated tyrosine phosphorylation of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK), a mitogen-activated protein kinase (MAPK), and enhanced MAPK activity. Inhibition of tyrosine kinase by herbimycin A, destruction of focal adhesion by cytochalasin D, or overexpression of antisense FAK mRNA prevented the activation of ERK/MAPK and the increase in alkaline phosphatase (ALP) activity. Transient expression of a MAPK-specific phosphatase, CL100, also suppressed the elevation of ALP activity. In addition, introduction of a constitutively active MAPK kinase enhanced ALP activity in the absence of collagen production. TGF-β receptor down-regulation was abrogated by treatments that inactivate FAK, whereas the expression of CL100 had no effect. These results demonstrate that COL(I)-α2β1 integrin interaction facilitates differentiation and down-regulates TGF-β receptors via the activation of FAK and its diverse downstream signals. These signaling pathways may play an important role in the sequential differentiation of osteoblasts during bone formation.

Cells of osteoblast lineage exert various functions depending upon their differentiation stages to maintain bone formation.

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Production of type I collagen (COL(I))1 is one of the earlier events during this process, followed by sequential expression of alkaline phosphatase (ALP) and osteocalcin. Mineralization of newly formed matrix takes place thereafter (1). To form bones with structural integrity and physical strength, it appears to be of critical importance to maintain the sequential development of these multiple osteoblastic phenotypes.

Using osteoblastic cells from bone or of clonal origin, various hormones and cytokines are shown to affect the differentiation process and functional properties of these cells (1, 2). In addition to these factors, matrix COL(I) is required for the differentiation of osteoblasts (3, 4). We have demonstrated that the effect of COL(I) on the development of osteoblastic properties is mediated by the interaction of COL(I) with cell-surface α2β1 integrin and that the interaction also causes down-regulation of transforming growth factor (TGF-β) receptors without affecting the expression of the receptor mRNA (5), suggesting the translocation of TGF-β receptors from cell surface to intracellular compartments. Because TGF-β potently stimulates matrix protein synthesis but inhibits differentiation of osteoblasts (5), the down-regulation of its receptors may serve to facilitate further maturation of osteoblasts and mineralization of newly formed matrix. However, the mechanism and the intracellular pathways that control the differentiation and TGF-β receptor down-regulation of osteoblastic cells by COL(I)-α2β1 integrin interaction remain unclear.

Accumulating evidence indicates that stimulation of β1 integrin by matrix proteins initiates intracellular signaling pathways in many types of cells (6–9). One of the initial events triggered by the stimulation of β1 integrin is the association of its cytoplasmic domain with focal adhesion kinase (FAK), a cytosolic non-receptor tyrosine kinase, followed by tyrosine phosphorylation and activation of FAK (10). FAK-deficient mice display severe defects of mesodermal development in embryogenesis (11). The phenotype of FAK-deficient mice is strikingly similar to the fibronectin-deficient phenotype (12), suggesting that FAK uniquely mediates matrix-integrin interactions in cellular processes. Phosphorylated FAK associates with and activates several signal transduction molecules including Src and Grb2 (9), which may cause the activation of

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1 The abbreviations used are: COL(I), type I collagen; ALP, alkaline phosphatase; TGF, transforming growth factor; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; PI, phosphatidylinositol; ERK, extracellular signals-regulated kinase; BMP, bone morphogenetic protein; BSA, bovine serum albumin; MEM, minimum essential medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; mAb, monoclonal antibody.
mitogen-activated protein kinase (MAPK) via Ras (9), although MAPK can be activated in response to integrin ligation irrespective of FAK activation when an adaptor protein Shc is recruited and phosphorylated (13). FAK also activates diverse signaling molecules, including phosphatidylinositol (PI)-3 kinase (7).

MAPK plays diverse roles in the control of the proliferation and differentiation of various cells (14, 15), and extracellular signal-regulated kinase (ERK), a member of MAPK, is activated by integrin stimulation in fibroblasts (9, 16, 17). The activation of PI-3 kinase is associated with recycling via early endosomes (18) and endocytosis by activating Rho family proteins (19). Therefore, there is a possibility that the regulation of osteoblastic differentiation and the intracellular translocation of TGF-β receptors by the COL(I)-α2β1 integrin interaction may be mediated by these signaling cascades and that FAK is the immediate transducer of extracellular signals from α2β1 integrin. The present study was undertaken to identify signal transduction pathways for osteoblastic differentiation and TGF-β receptor down-regulation triggered by COL(I)-α2β1 integrin.

EXPERIMENTAL PROCEDURES

**Materials**—[γ-32P]dATP, enhanced chemiluminescence detection kit (ECL kit), including second antibodies and ERK/MAPK assay kit were purchased from Amersham, Japan. Mouse mAb against phosphotyrosine (4G10) and mouse mAb against FAK (2A7) were obtained from Upstate Biotechnology Inc. (Lake Placid, NY); mouse mAb against FAK (F15020) and ERK1 (MK12) were from Transduction Laboratories (Lexington, KY); rat mAb against mouse α2β1 integrin (HMα2) was from Sumitomo Denko Co. (Osaka, Japan); rat mAb against hamster IgG was from Pharmingene (San Diego, CA); ascorbic acid and L-azetidine-2-carboxylic acid were from Wako Pure Chemicals Ltd. (Osaka, Japan); Protein A-Sepharose (Pharmacia Biotech Inc., Tokyo, Japan) was from Iwaki Co. (Chiba, Japan); cytochalasin D and wortmannin was from Sigma; herbimycin A was from Koywa Medex Co. (Tokyo, Japan). MC3T3-E1 cells were kindly provided by Dr. H. Kodama (Ozu Dental College, Japan).

**Cell Culture and Cell Attachment**—MC3T3-E1 cells were cultured in MEM supplemented with 10% FBS, penicillin, and streptomycin with or without 50 mg/liter ascorbic acid. These cells were cultured red and was changed twice a week. L-azetidine-2-carboxylic acid (0.3 mM) in distilled water and 1 mg/ml herbimycin A, 1 mM cytochalasin D, and 1 mM wortmannin in MEM were added to the culture medium to give the indicated final concentrations.

Cells were detatched by trypsinization following by washing with MEM, 10% FBS to neutralize trypsin. Some cultures were treated with herbimycin A (0.1–0.4 mM/L) or wortmannin (10–100 mM) for 24 h before the trypsinside. The cells were washed twice with MEM containing 0.5% BSA, and cell suspensions were incubated in MEM, 0.5% BSA at 37 °C for 40 min in a water bath with agitation. Cells were then plated onto the dishes coated with or without rat tail COL(I) and incubated at 37 °C for the indicated times. Cells referred as suspended cells were held in suspension for an additional 60 min. In some experiments, cytochalasin D (1 μM) was added prior to agitation in the water bath. Dishes were coated with 1% BSA for 1 h at room temperature prior to plating the cells.

**Immunoprecipitation and Immunoblot Analysis**—Cells were washed with ice-cold PBS and lysed in modified immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 50 mM NaF, 0.5 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, and 4 μg/ml peptstatin A) on ice for immunoprecipitation (20), or in 10 mM Tris-HCl, 1% SDS, pH 7.4, with boiling for 5 min for immunoblot analysis. Whole cell lysates were centrifuged at 15,000 rpm for 15 min at 4 °C, and the supernatants were divided into aliquots with equal amounts of protein. Antibodies were added to the aliquots of lysates, and samples were rotated at 4 °C for 16 h. To eliminate the antibody–antigen complex, 4 μg of anti-mouse IgG antibody was added to the lysates followed by the addition of protein G-Sepharose (Pharmacia Biotech Inc., Tokyo, Japan), and the samples were rotated for 1 h. The immunoprecipitates were pelleted by microcentrifugation, washed three times with lysis buffer without SDS and deoxycholate, and then washed once with PBS. The pellets were boiled in equal volume of two times concentrated sample buffer and electrophoresed on 10% precast SDS-polyacrylamide gel electrophoresis gels (Daiichi Pure Chemicals, Co., Tokyo, Japan). After electrophoresis, proteins were transferred to Immobilon-P (polyvinylidene difluoride membrane from Millipore Corp., Bedford, MA). Phosphotyrosine-containing proteins were detected by incubation with anti-phosphotyrosine antibody followed by enhancement detection using horseradish peroxidase-conjugated anti-mouse IgG second antibody. To detect FAK and ERK, blots were probed with monoclonal anti-FAK and anti-ERK1 antibodies. Immunoreactive bands were visualized by the procedure mentioned in ECL™ kit.

**ALP Activity in Osteoblast Cell—**Osteoblastic cells were cultured in the absence of ascorbic acid until confluence. Cells were treated with either herbimycin A, cytochalasin D, or wortmannin in the presence of 50 μg/ml ascorbic acid for 3 days. Wortmannin was added to cultures every 12 h. Cells were washed twice with ice-cold PBS and scraped in 10 mM Tris-HCl containing 2 mM MgCl2 and 0.05% Triton X-100, pH 8.2. The cell suspension was homogenized using Pellet Pestle (Kontes, Vineyard, NJ) on ice after two cycles of freeze-thawing. Aliquots of supernatants were assayed for protein concentration with a Bio-Rad kit according to Bradford’s method and for ALP activity (21). In brief, the assay mixture contained 10 mM p-nitrophenyl phosphate in 0.1 M sodium carbonate buffer, pH 10, supplemented with 1 mM MgCl2, followed by an incubation at 37 °C for 30 min. After adding 0.1 M NaOH, the amount of p-nitrophenol liberated was measured by a spectrophotometer.

**ERK/MAPK Activity in Osteoblast Cells—**MC3T3-E1 cells were plated onto dishes coated with or without COL(I) and maintained in MEM, 0.5% BSA for 3 h. The cells do not proliferate under this condition. Cells were lysed in 20 mM Hepes, pH 7.5, containing 1% Nonidet P-40, 10 mM EGTA, 40 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM dithiothreitol, 2.5 mM MgCl2, and protease inhibitors after indicated treatments. ERK/MAPK activity was measured in aliquots of cell lysates containing equal amounts of protein using an assay kit (Amersham, Japan) according to the manufacturer’s instructions. The substrate peptide used in the kit contains only one phosphorylation site, PLS/TP, that is more specific for ERK1 and ERK2 than commonly used myelin basic protein (22). Thus, it is not necessary to consider other kinase activities than ERK1/ERK2 when measured in quiescent cells. ERK/MAPK activation was expressed as a ratio of phosphorylated MAPK to unphosphorylated MAPK as determined by reduction mapping and confirmed by sequencing.

Exponentially growing MC3T3-E1 cells in 10-cm culture plates were washed with serum-free medium, and 10 μg of antisense FAK expression vector plasmid was added by calcium phosphate precipitation method (23). Cells were washed and fed with fresh medium containing 10% FBS. After 24 h, 300 μg/liter G418 was added to cultures for selection of G418 resistance. After 2 weeks, the G418-resistant cells were cloned and propagated. Cells expressing pcDNA3 without the FAK cDNA fragment were cloned in the presence of G418 as control cells.

**Transfection with Expression Vectors of CL100 and Constitutively Active MAPK Kinase—**MC3T3-E1 cells were trypsinized and resuspended at a concentration of 3 × 106 cells/ml in PBS. Five hundred μl of the cell suspension was electrophoresed at room temperature in a cuvette with 2 μg of the plasmid coding for either CL100 or a constitutively active mutant of Xenopus MAPK kinase (S218E/S222E (SESE)-MAPKK) (25) or a mock plasmid using a Life Technologies, Inc. Cell Porator. The electroporated cells adhered to 10-cm plastic dishes were replated onto 6-well plates without type I collagen coating in the presence and absence of 50 μg/liter ascorbic acid. ALP activity in cell extracts was assayed 72 h thereafter. To estimate transfection efficiency, cells were co-transfected with a plasmid of FLAG-tagged β-galactosidase gene. In every experiment, β-galactosidase enzymatic activity was essentially the same among samples to ensure that transfection efficiencies were constant. In parallel experiments, transfected MC3T3-E1 cells were expanded and fed with fresh medium containing 10% FBS. After 24 h, 300 μg/liter G418 was added to cultures for selection of G418 resistance. After 2 weeks, the G418-resistant cells were cloned and propagated. Cells expressing pcDNA3 without the FAK cDNA fragment were cloned in the presence of G418 as control cells.

**Transfection with Expression Vectors of CL100 and Constitutively Active MAPK Kinase—**MC3T3-E1 cells were trypsinized and resuspended at a concentration of 3 × 105 cells/ml in PBS. Five hundred μl of the cell suspension was electrophoresed at room temperature in a cuvette with 2 μg of the plasmid coding for either CL100 or a constitutively active mutant of Xenopus MAPK kinase (S218E/S222E (SESE)-MAPKK) (25) or a mock plasmid using a Life Technologies, Inc. Cell Porator. The electroporated cells adhered to 10-cm plastic dishes were replated onto 6-well plates without type I collagen coating in the presence and absence of 50 μg/liter ascorbic acid. ALP activity in cell extracts was assayed 72 h thereafter. To estimate transfection efficiency, cells were co-transfected with a plasmid of FLAG-tagged β-galactosidase gene. In every experiment, β-galactosidase enzymatic activity was essentially the same among samples to ensure that transfection efficiencies were constant. In parallel experiments, transfected cells were fixed and incubated with an anti-FLAG mAb (Eastman Kodak) and subsequently with a fluorescein isothiocyanate-conjugated anti-mouse IgG. Labeled cells with fluorescein isothiocyanate were routinely more than 90%.

**Affinity Cross-linking Analysis of Cell-surface TGF-β Receptors—**MC3T3-E1 cells in 10-cm culture dishes were washed twice with ice-
cold PBS. Binding of 100 pm 125I-TGF-beta (total radioactivity of approximately 200,000 cpm) was performed in a buffer containing 128 mM NaCl, 5 mM KCl, 5 mM MgSO4, 1.3 mM CaCl2, and 25 mM Hepes, pH 7.4 containing 0.3% BSA at 4°C for 4 h as reported previously (24). After washing the vesicles with ice-cold PBS, bound 125I-TGF-beta was chemically cross-linked in 2 ml of PBS containing 0.3 mM dithioerythritol (Pierce) at 4°C for 20 min. Chemical reaction was terminated by adding 200 μl of quenching buffer (100 mM Tris-HCl, pH 7.5, 200 mM glycine, and 20 mM EDTA), and cells were kept at room temperature for 1 min. After washing twice with PBS, cells were scraped into a tube containing 1 ml of PBS with 1% EDTA and protease inhibitors mixture of 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, and 10 μg/ml pepstatin A. Cells were then collected by centrifugation and solubilized in 40–100 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 1 mM EDTA, and protease inhibitors) for 40 min on ice. Lysates were centrifuged, and supernatants were subjected to SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel followed by autoradiography. Loading samples were adjusted to contain the same amount of protein. The same experiments were performed at least three times, and representative results from reproducible experiments are shown in the figures.

Statistical Analysis—Data were expressed as means ± S.E. and analyzed by one-way analysis of variance followed by Bonferroni’s method. p value of less than 0.05 was considered to be significant.

RESULTS

Attachment of Osteoblastic Cells to Type I Collagen Activates FAK and ERK/MAPK—Using non-transformed murine osteoblastic MC3T3-E1 cells, we have demonstrated that COL(I) is required for osteoblastic differentiation through its binding to α2β1 integrin (5). To clarify the mechanism whereby the interaction between COL(I) and α2β1 integrin causes osteoblastic differentiation, the effect of the attachment of MC3T3-E1 cells to COL(I) on tyrosine phosphorylation of intracellular signaling molecules was examined. As shown in Fig. 1A, Western blot analysis using an anti-phosphotyrosine antibody revealed that no significant tyrosine phosphorylation was observed in suspended cells. Only a weak phosphorylation was detected in cells on BSA-coated plastic dishes after culturing in serum-free medium for 1 h, when cells adhered at a small portion with spreading. When MC3T3-E1 cells were plated on dishes coated with COL(I), cells adhered with entire spreading after 1 h of culture even in the absence of serum, and several proteins were tyrosine-phosphorylated including pp125, pp120, pp80, and pp44 (Fig. 1A). Fig. 1B shows the time course of tyrosine phosphorylation of these proteins. Tyrosine phosphorylation was stimulated 20 min after the cellular attachment to COL(I) and was further enhanced until 120 min. When the same membrane was immunoblotted with anti-FAK and anti-ERK antibodies, anti-FAK antibody (F15020) was bound to pp125, and anti-ERK antibody was bound to pp44 (Fig. 1B). The anti-ERK antibody also detected 42-kDa protein that corresponds to ERK2, which is cross-reacted with this antibody. Among the two forms of ERK, ERK1 appears to be preferentially tyrosine-phosphorylated upon the attachment of cells to COL(I). Amounts of FAK and ERK proteins did not significantly change until 120 min. When FAK was immunoprecipitated from cell lysates by anti-FAK antibody (2A7), its tyrosine phosphorylation was enhanced with time when cells were cultured on COL(I) (Fig. 1C). Thus, FAK was included in pp125 and was activated in osteoblastic cells when they attached to COL(I). Results also suggest that ERK1 is activated on COL(I) with a similar time course to that of FAK.

It has been reported that ERK, a member of MAPK family, is activated by the attachment of cells to extracellular matrix (16) and that phosphorylation of tyrosine residues in ERK is required but is not sufficient for the activation of ERK (25). To examine whether tyrosine phosphorylation of ERK/MAPK was associated with an increase in its activity after attachment of cells to COL(I), MAPK activity was analyzed by phosphorylation.

Fig. 1. Effect of cellular attachment to type I collagen on tyrosine phosphorylation (A), and time course of tyrosine phosphorylation of pp125 and pp44 (B) and pp125MAPK (C) after attachment of osteoblastic MC3T3-E1 cells to type I collagen. A, MC3T3-E1 cells were plated onto plastic culture dishes coated with (lane 3) or without (lane 2) type I collagen and were incubated in MEM, 0.5% BSA for 1 h at 37°C. Cells were lysed in 10 mM Tris-HCl, 1% SDS, pH 7.4, and boiled for 5 min. Total protein concentrations in the supernatant of cell lysates were equalized before Western blot analyses. Samples were resolved on SDS, 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Immobile™-P), immunoblotted with anti-phosphotyrosine mAb (4G10), and visualized by enhanced chemiluminescence detection. Cells in suspension for 1 h at 37°C were similarly processed and analyzed ( lane 1). Molecular weight markers are indicated in the left margin. B, MC3T3-E1 cells were plated onto type I collagen-coated dishes and were incubated at 37°C for the indicated periods. Cell lysates were prepared as described above and were immunoblotted with anti-phosphotyrosine, anti-FAK, and anti-ERK1 antibodies. C, cells were prepared as described in B. Cell lysates were subject to an immunoprecipitation (IP) with anti-FAK antibody (2A7) and immunoblotted with anti-phosphotyrosine antibody (4G10).
FIG. 2. Activation of ERK/MAPK in MC3T3-E1 cells cultured on type I collagen. MC3T3-E1 cells were plated onto plastic culture dishes coated with (filled circles) or without (open circles) type I collagen and were incubated in MEM, 0.5% BSA until 180 min. At the indicated times, cells were lysed and processed as described in Fig. 1. Data are means ± S.E. for three determinations. #, significantly different from the activity in suspended cells (p < 0.01).

FIG. 3. Time course of the development of ALP activity in MC3T3-E1 cells cultured in the presence and absence of ascorbic acid. MC3T3-E1 cells were cultured in MEM, 10% FBS with (filled circles) or without (open circles) ascorbic acid on dishes without type I collagen coating for 7 days. ALP activity in cell lysates at the indicated times was assayed as described under "Experimental Procedures." Data are means ± S.E. for three determinations. *., significantly different from the values in the absence of ascorbic acid (p < 0.01).

FIG. 4. Effects of ascorbic acid (A), L-azetidine-2-carboxylic acid, and anti-α2β1 integrin antibody (B) on tyrosine phosphorylation of pp125FAK in MC3T3-E1 cells. A, MC3T3-E1 cells were plated onto plastic dishes without type I collagen coating in MEM, 10% FBS in the presence or absence of ascorbic acid. At the indicated time, cells were lysed and processed as described in Fig. 1. In the upper column, pp125FAK was immunoblotted with anti-phosphotyrosine mAb (4G10) and was visualized by enhanced chemiluminescent detection kit (arrow heads). In the lower column, cell lysates prepared as described above were immunoprecipitated (IP) by anti-FAK mAb (2A7), and the immunoprecipitates were immunoblotted by the anti-phosphotyrosine antibody. B, cells were plated as above and were cultured in MEM, 10% FBS in the absence of ascorbic acid for 72 h. Then, 50 μg/liter ascorbic acid was added to cultures with 0.3 μg/ml L-azetidine-2-carboxylic acid or 10 μg/liter anti-α2β1 integrin blocking antibody. At the indicated times, cells were lysed for Western blot analyses using the anti-phosphotyrosine antibody as described in Fig. 1.

Stable Expression of Antisense FAK mRNA Impairs Development of ALP Activity—To verify further the involvement of FAK activation in the development of ALP activity in osteoblastic cells, we cloned MC3T3-E1 cells expressing antisense FAK mRNA. These cells contained less FAK protein estimated...
Effects of the inhibition of tyrosine kinases by herbimycin A and disruption of focal adhesion by cytochalasin D on ALP activity in osteoblastic cells

Cells were treated with herbimycin A, cytochalasin D, or wortmannin, and ALP activity was measured as described under "Experimental Procedures." Data are means ± S.E. for three determinations.

Table I

| Ascorbic acid | Herbimycin A | Cytochalasin D | Wortmannin | ALP activity |
|---------------|--------------|----------------|------------|--------------|
| mg/liter      | µM           | µM            | nM         | nmol/min/µg protein | % control |
| 50            | 0            | 0             | 0          | 1.40 ± 0.02  | 100 ± 1  |
| 50            | 0.1          | 0             | 0          | 0.90 ± 0.02   | 64 ± 1   |
| 50            | 0.4          | 0             | 0          | 0.61 ± 0.05   | 44 ± 2   |
| 50            | 0            | 0.3           | 0          | 0.90 ± 0.06   | 64 ± 4   |
| 50            | 0            | 1             | 0          | 0.64 ± 0.08   | 46 ± 4   |
| 50            | 0            | 0             | 10         | 1.57 ± 0.13   | 112 ± 9  |
| 50            | 0            | 0             | 100        | 1.50 ± 0.15   | 107 ± 11 |
| 0             | 0            | 0             | 0          | 0.69 ± 0.04   | 49 ± 3   |

* Significantly different from the control with ascorbic acid alone (p < 0.01).

Fig. 5. Effects of herbimycin A, cytochalasin D, and wortmannin on tyrosine phosphorylation of pp125Yck and pp44ERK (A), and ERK/MAPK activity (B) after attachment of MC3T3-E1 cells to type I collagen. A, MC3T3-E1 cells were plated onto culture dishes with type I collagen coating and were incubated in MEM, 0.5% BSA at 37 °C for 2 h in the presence of 0.4 mg/liter herbimycin A, 1 µM cytochalasin D, or 100 nM wortmannin. Cell lysates were prepared and processed as described in the legend to Fig. 1. Immunoblot analysis with anti-phosphotyrosine antibody (4G10) was performed using either whole cell lysates (upper column) or after immunoprecipitation (IP) with anti-FAK antibody (2A7) (lower column). Molecular weight markers are indicated in the left margin. B, cells were processed as above in the presence of 0.4 mg/liter herbimycin A, 1 µM cytochalasin D, or 100 nM wortmannin, and ERK/MAPK activity was measured as described in the legend to Fig. 2. Data are expressed as activities over the control values in cells on dishes without type I collagen coating and are means ± S.E. for three determinations. *, significantly different from the activity in cells on dishes without type I collagen coating (p < 0.01).

Fig. 6. Expression and tyrosine phosphorylation of FAK in MC3T3-E1 cells expressing antisense FAK mRNA. MC3T3-E1 cells stably expressing antisense FAK mRNA were cloned in the presence of 300 mg/liter G418. Cells transfected with the expression plasmid alone was also cloned in the presence of G418. Cells were plated onto type I collagen-coated dishes and were incubated at 37 °C for 2 h. Whole cell lysates (upper and middle panels) were immunoblotted with anti-FAK antibody (1F5020) (upper panel) and anti-phosphotyrosine antibody (4G10) (middle panel). Immunoprecipitates with anti-FAK antibody (2A7) were also immunoblotted with anti-phosphotyrosine antibody (4G10) (lower panel) to confirm defective tyrosine phosphorylation of FAK in cells expressing antisense FAK mRNA.

Involvement of ERK/MAPK Activity in the Elevation of ALP Activity—Tyrosine phosphorylation of FAK may be involved in the activation of ERK/MAPK via the Grb2-Sos-Ras pathway (9). Therefore, we next examined the effect of activation and inactivation of ERK/MAPK on the development of ALP activity. For the inactivation, MC3T3-E1 cells were transiently transfected with the expression plasmid for a constitutively active mutant of MAPKK (SESE-MAPKK) (23). The expression of CL100 significantly suppressed the increase in ALP activity in the presence of ascorbic acid but not in its absence (Table III). In parallel with the suppression of ALP activity, the transfection of CL100 significantly suppressed ERK/MAPK activity in the presence of ascorbic acid (35 ± 22 and 70 ± 7 cpm/µg protein with and without expression of CL100), respectively, p < 0.01) but did not affect tyrosine phosphorylation of FAK (data not shown). In addition, the expression of active MAPKK significantly increased the ALP activity in the absence of ascorbic acid but not in its presence (Table III). These results demonstrate that ERK/MAPK is an essential mediator for the development of ALP activity induced by ascorbic acid. Thus, it is suggested that the ERK/MAPK signaling pathway in association with FAK activation after COL(I)-α2β1 integrin interaction is required for osteoblastic differentiation.

Intracellular Signals Involved in Down-regulation of TGF-β Receptors—We have reported (5) that the COL(I)-α2β1 integrin interaction causes a reduction in the cell-surface expression of TGF-β receptors along with the development of ALP activity. To clarify the signaling pathway involved in the down-regulation of TGF-β receptors following FAK activation, the effects of wortmannin, herbimycin A, cytochalasin D, and CL100 were...
These results are consistent with the assumption that the three or four determinations present studies demonstrate that the attachment of MC3T3-E1 affects the differentiation of osteoblasts has been unclear. The mechanism whereby cell-matrix interaction affects the expression of COL(I) with cell-surface receptors using 125I-TGF-β receptors was performed as described under “Experimental Procedures.” The importance of the present results lies in the observation that this signaling cascade is required for the differentiation of osteoblastic cells.

Thus, disruption of the cascade at any point curtails the stimulation of ALP activity as follows: the first step is the COL(I)-α2β1 integrin interaction, and blockade of the interaction by an anti-α2β1 integrin antibody (5) or by suppressing collagen synthesis (3, 5) inhibited the elevation of ALP activity. Although α1β1 and α3β1 integrins are also receptors for COL(I), α2β1 integrin appears to be the functional receptor for COL(I) in osteoblastic cells (5). The second step is tyrosine phosphorylation of FAK triggered by the COL(I)-α2β1 integrin interaction. Inhibition of tyrosine kinase activity by herbimycin A or destruction of the integrity of focal adhesion by cytochalasin D (8, 20) prevented tyrosine phosphorylation of FAK as well as the increase in ALP activity. The essential role of FAK activation in this signaling cascade for osteoblastic differentiation is further corroborated by the fact that the expression of antisense FAK mRNA in osteoblastic cells prevented the increase in ALP activity. The third step is the activation of ERK/MAPK by transient expression of a MAPK-specific phosphoprotein phosphatase, CL100 (22, 23), suppressed the elevation of ALP activity by COL(I)-α2β1 integrin interaction, and its activation by a constitutively active MAPK (SESE-MAPKK) stimulated the ALP activity, suggesting that ERK/FAK signaling pathways play important roles in osteoblastic differentiation.

**Table II**

| ALP activity (nmol/min/µg protein) | MAPK activity (cpm/µg protein) |
|-----------------------------------|--------------------------------|
| Mock-transfected control          |                                 |
| Antisense FAK/clones 1            |                                 |
| Antisense FAK/clones 2            |                                 |

* Significantly different from the mock-transfected control (p < 0.01).

**Table III**

| Ascorbic acid (mg/L) | Wortmannin (nM) | Herbimycin A (mg/L) | Cytochalasin D (µM) |
|----------------------|-----------------|---------------------|---------------------|
| 50                   | 10              | 0.25                | 0.01                |
| 50                   | 100             | 0.25                | 0.01                |
| 50                   | 1000            | 0.25                | 0.01                |

* Significantly different from the activity in mock-transfected cells in the absence of ascorbic acid (p < 0.01).

* Significantly different from the activity in mock-transfected cells in the presence of ascorbic acid (p < 0.01).

molecules. These results are in agreement with the previous observations in fibroblasts that aggregation of β1 integrin family via attachment to extracellular matrix triggers localized intracellular accumulation of 20 signal transducing molecules, including Ras, Raf, MAPKK, ERK/MAPK, and Rhoα, followed by the activation of ERK/MAPK (8). The effect of wortmannin, herbimycin A, cytochalasin D (A), and CL100 (B) on the down-regulation of cell-surface TGF-β receptors in the presence of ascorbic acid in MC3T3-E1 cells. A, MC3T3-E1 cells were cultured in the absence of ascorbic acid for 4 days. Cells were then treated with the indicated concentrations of wortmannin, herbimycin A, or cytochalasin D in the presence of 50 mg/liter ascorbic acid for 3 days. Affinity cross-linking of cell-surface TGF-β receptors using 125I-TGF-β was performed as described under “Experimental Procedures.” TGF-β receptors of type I and type II are indicated by arrowsheads. B, cells were transiently transfected with CL100 expression plasmid in the presence of ascorbic acid. Affinity cross-linking of TGF-β receptors was performed 3 days after transfection. Molecular weight markers are indicated in the margin of each figure.

**Figure 7.** Effects of wortmannin, herbimycin A, cytochalasin D (A), and CL100 (B) on the down-regulation of cell-surface TGF-β receptors in the presence of ascorbic acid in MC3T3-E1 cells. A, MC3T3-E1 cells were cultured in the absence of ascorbic acid for 4 days. Cells were then treated with the indicated concentrations of wortmannin, herbimycin A, or cytochalasin D in the presence of 50 mg/liter ascorbic acid for 3 days. Affinity cross-linking of cell-surface TGF-β receptors using 125I-TGF-β was performed as described under “Experimental Procedures.” TGF-β receptors of type I and type II are indicated by arrowsheads. B, cells were transiently transfected with CL100 expression plasmid in the presence of ascorbic acid. Affinity cross-linking of TGF-β receptors was performed 3 days after transfection. Molecular weight markers are indicated in the margin of each figure.
MAPK mediates the signal for osteoblastic differentiation.

Receptor and non-receptor tyrosine kinases are shown to play an important role in the regulation of the proliferation and differentiation of various types of cells via the activation of MAPK. However, it is as yet unclear how the same signaling pathway controls such diverse cellular events (26). For instance, EGF receptor tyrosine kinase activates cell growth, whereas nerve growth factor receptor tyrosine kinase initiates differentiation of neuronal cells (14, 27). Although both kinases activate MAPK, the time course of its activation by the former is rapid and transient, while that by the latter is slow and sustained. Similar to these observations, basic fibroblast growth factor stimulates its receptor tyrosine kinase and MAPK cascade in a rapid and transient time course (28) and strongly inhibits ALP activity but stimulates proliferation in osteosarcoma cells (29) and in osteoblastic MC3T3-E1 cells. In contrast, MAPK activation by COL(1)-o2b1 integrin interaction in osteoblastic cells is slow and sustained. These different processes of MAPK activation may cause discrete nuclear localization of the active kinase and may explain diverse effects of the single kinase (14).

Although the present studies demonstrate that the signaling pathway from β1 integrin to ERK/MAPK via FAK is required for osteoblastic differentiation, it may not be sufficient for stimulating differentiation. In fact, the β1 integrin-FAK-ERK/MAPK cascade is widely present among mesenchymal cells (8, 9, 16). Focal adhesions induced by integrins are reported to cause co-localization of signaling molecules including growth factor receptors (26), which may facilitate the interactions of these molecules (6). Several members of the bone morphogenic protein (BMP) family are potent stimulators of osteoblastic differentiation (30, 31). Our recent observations demonstrate that MC3T3-E1 cells possess types I and II BMP receptors and express BMP-2 mRNA, and extracellular matrix synthesized by these cells exhibit immunostaining with anti-BMP-2 antibody. Therefore, the possibility remains that these molecules (6) may be involved in the internalization of BMP receptors on osteoblastic cells to generate additional signals for promoting osteoblastic differentiation. Alternatively, there may be a cross-talk between these intracellular signaling pathways. The inter-relationship and the relative roles of these pathways in the regulation of osteoblastic differentiation remain to be clarified.

The down-regulation of TGF-β receptors induced by COL(1)-o2b1 integrin interaction was prevented by herbimycin A or cytochalasin D, and was abrogated in cells expressing anti-FAK mRNA. The receptor down-regulation was also prevented by the inactivation of ERK/MAPK with transient expression of CL100. In contrast, the elevation of ALP activity was prevented by the inactivation of ERK/MAPK but not by wortmannin. These results suggest that the down-regulation of TGF-β receptors by COL(1)-o2b1 integrin interaction is mediated via pathways susceptible to wortmannin but not MAPK and that the elevation of ALP activity is not mediated via the former pathways but the latter one after FAK activation. FAK is directly associated with and activates PI-3 kinase (7, 32), and integrin-mediated signaling pathways also include phospholipase A2 and C (7). Wortmannin not only inhibits PI-3 kinase but also affects these phospholipases (33). Because FAK activation was not affected and ERK/MAPK activity was only partially suppressed by wortmannin in our experiments, it is possible that FAK activation generates several distinct signaling pathways to cause diverse effects in osteoblastic cells. The fact that the down-regulation of TGF-β receptors is susceptible to wortmannin is intriguing if it occurs through translocation of the receptors, because PI-3 kinase binds to platelet-derived growth factor receptor through its SH2 domain and directly mediates the receptor trafficking (18). PI-3 kinase is also involved in clathrin-independent endocytosis via activation of small GTP-binding proteins of the Rho family (19). In addition to PI-3 kinase, other wortmannin-sensitive signaling molecules, phospholipase A2 and C, are also involved in cytoskeletal changes (7). Thus, these molecules may be implicated in the internalization of TGF-β receptors to cause their down-regulation. However, the cytoplasmic domain of TGF-β receptors lacks SH2 binding domains, and the mechanism that causes their down-regulation has yet to be elucidated.

The most striking effect of TGF-β in osteoblasts is the stimulation of matrix protein synthesis including COL(1). TGF-β also enhances the expression of o2b1 integrin in osteoblastic cells (34), which is expected to facilitate the interaction of osteoblasts with COL(1). In contrast, TGF-β strongly inhibits the differentiation of osteoblasts (35, 36). The observations presented herein suggest that the signaling cascades initiated by COL(1)-o2b1 integrin interaction can directly facilitate osteoblastic differentiation on the one hand via the FAK-MAPK pathway, and, at the same time, enable osteoblasts to escape from the inhibitory effect of TGF-β on the differentiation of osteoblasts by the down-regulation of TGF-β receptors via FAK-mediated pathway(s) sensitive to wortmannin. These observations illustrate the importance of cell-matrix interactions in the regulation of the sequential development of osteoblast phenotypes during the bone formation process.

REFERENCES

1. Stein, G., and Lian, J. B. (1993) Endocr. Rev. 14, 424–442
2. Centrella, M., Horowitz, M. C., Wozney, J. M., and McCarthy, T. L. (1994) Endocr. Rev. 15, 27–39
3. Franceschi, R., Iyer, B. S., and Cui, Y. (1994) J. Bone Miner. Res. 9, 843–854
4. Quarles, L., Yokoyama, T., and Wenzel, R. M. (1993) J. Bone Miner. Res. 8, 1359–1368
5. Takeuchi, Y., Nakayama, K., and Matsumoto, T. (1996) J. Bone Miner. Res. 11, 791–805
6. Chao, M. V. (1992) J. Biol. Chem. 267, 2139–2142
7. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
8. Parsons, J. T., Schaller, M. D., Hildebrand, J., Khavari, P. A., and Schlessinger, J. (1994) J. Cell Biol. 125, 129–139
9. Takeuchi, Y., Nakayama, K., and Matsumoto, T. (1996) J. Bone Miner. Res. 11, 791–805
10. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
11. Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. (1995) J. Cell Sci. 118, 135–144
12. Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. (1995) J. Cell Sci. 118, 135–144
13. Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. (1995) J. Cell Sci. 118, 135–144
14. Marshall, C. J. (1995) Cell 80, 179–185
15. Nishida, E., and Gotto, Y. (1993) Trends Biochem. Sci. 18, 128–131
16. Morino, N., Nimura, T., Hamasaki, K., Tatekara, R., and Kikuchi, K. (1995) J. Biol. Chem. 270, 269–273
17. Zha, X., and Assoian, R. K. (1995) Mol. Biol. Cell 6, 273–282
18. Schmalzing, G., Richter, H. P., Hansen, A., Schwarz, W., Just, I., and Aktories, K. (1996) J. Cell Biol. 133, 191–200
19. Vuori, K., and Ruoslahti, E. (1995) J. Biol. Chem. 270, 22259–22262
20. Masuyama, N., Suzuki, A., Ueno, N., and Nishida, E. (1995) EMBO J. 14, 233–239
21. Takeuchi, Y., Kudama, Y., and Matsumoto, T. (1994) J. Biol. Chem. 269, 32634–32639
22. Ehrlich, P. A., and Cooper, J. A. (1992) Science 255, 219–225
23. Plopper, G. E., McNamee, H. P., Dike, L. E., and Ingber, D. E. (1995) Mol. Biol. Cell 6, 1349–1356
24. Chao, M. V. (1992) Cell 70, 955–977
25. Cao, Y., and Cooper, J. A. (1996) Science 271, 212–215
26. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
27. Takeuchi, Y., Nakayama, K., and Matsumoto, T. (1996) J. Bone Miner. Res. 11, 791–805
28. Chao, M. V. (1992) J. Biol. Chem. 267, 2139–2142
29. Takeuchi, Y., Nakayama, K., and Matsumoto, T. (1996) J. Bone Miner. Res. 11, 791–805
30. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
31. Takeuchi, Y., Nakayama, K., and Matsumoto, T. (1996) J. Bone Miner. Res. 11, 791–805
32. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
33. Takeuchi, Y., Nakayama, K., and Matsumoto, T. (1996) J. Bone Miner. Res. 11, 791–805
34. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
35. Takeuchi, Y., Nakayama, K., and Matsumoto, T. (1996) J. Bone Miner. Res. 11, 791–805
36. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
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29. Rodan, S. B., Wesolowski, G., Yoon, K., and Rodan, G. A. (1989) J. Biol. Chem. 264, 19934–19941
30. Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988) Science 242, 1528–1533
31. Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J. M., Rosen, V., Wang, E. A., Kahn, A. J., Suda, T., and Yoshiki, S. (1991) J. Cell Biol. 113, 681–687
32. Chen, H.-C., and Guan, J.-L. (1994) J. Biol. Chem. 269, 31229–31233
33. Cross, M. J., Stewart, A., Hodgkin, M. N., Kerr, D. J., and Wakelam, M. J. O. (1995) J. Biol. Chem. 270, 25352–25355
34. Heino, J., and Massague, J. (1989) J. Biol. Chem. 264, 21806–21811
35. Harris, S., Bonewald, L. F., Harris, M. A., Sabatini, M., Dallas, S., Feng, J. Q., Ghosh-Choudhury, N., Wozney, J., and Mundy, G. R. (1994) J. Bone Miner. Res. 9, 855–863
36. Wrana, J. F., Maeno, M., Hawrylyshyn, B., Yao, K. L., Domenicucci, C., and Sodek, J. (1988) J. Cell Biol. 106, 915–924