Role of the $\alpha_2$-Integrin in Osteoblast-specific Gene Expression and Activation of the Osf2 Transcription Factor*

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Extracellular matrix molecules such as type I collagen are required for the adhesion, migration, proliferation, and differentiation of a number of cell types including osteoblasts. Matrix components often affect cell function by interacting with members of the integrin family of cell surface receptors. Previous work showed that collagen matrix synthesis, induced by addition of ascorbic acid to cells, precedes and is essential for the expression of osteoblast markers and induction of the osteocalcin promoter in murine MC3T3-E1 cells. This later response requires OSE2, the promoter element recognized by Osf2 (also called Cbfa1/AML3/PEBP2αA), a recently identified osteoblast-specific transcription factor. Osteoblasts express several integrins including $\alpha_2\beta_1$ which is a major receptor for type I collagen. This paper examines the role of the $\alpha_2$-integrin subunit in osteocalcin promoter activation and osteoblast differentiation. Disruption of $\alpha_2$-integrin-ECM interactions with a blocking antibody or DGEA peptide containing the cell-binding domain of type I collagen blocked activation of the mouse osteocalcin gene 2 promoter by ascorbic acid as well as induction of endogenous osteocalcin mRNA and mineralization. Furthermore, anti-$\alpha_2$-integrin blocking antibody or peptide reduced ascorbic acid-dependent binding of Osf2 to OSE2 without affecting levels of transcription factor mRNA. Time course studies revealed that ascorbic acid-dependent binding of Osf2 to OSE2 preceded increases in osteocalcin and bone sialoprotein expression and this increase in Osf2 binding was not accompanied by comparable changes in levels of transcription factor mRNA or protein. Taken together, these studies demonstrate that an $\alpha_2$-integrin-collagen interaction is required for activation of Osf2 and induction of osteoblast-specific gene expression. Furthermore, matrix signals may regulate Osf2 through a post-translational pathway or via an accessory factor.

As a cell primarily devoted to matrix production, the osteoblast must have the ability to monitor the composition of the extracellular matrix (ECM) to secret as well as adapt matrix composition to the changing mechanical needs of bone. Consistent with the concept that there is a dialogue between the osteoblast and its ECM, osteoblast precursors must secrete a collagenous matrix before they will differentiate. Inhibition of collagen synthesis by growing cells in the absence of ascorbic acid (AA) or through the use of specific inhibitors totally blocks osteoblast differentiation (1–6). In vivo, both bone formation and osteoblast differentiation, as assessed by expression of osteocalcin (OCN) and alkaline phosphatase mRNAs, are also severely reduced in vitamin C-deficient animals (7, 8). Thus, the ECM is an important, but poorly understood regulator of the osteoblast differentiation pathway.

Integrins are the principle mediators of the molecular dialogue between a cell and its ECM environment (for reviews, see Refs 9 and 10). These transmembrane receptors convey information from the ECM to the intracellular compartment utilizing several signal transduction pathways including those involving focal adhesion kinase and mitogen-activated protein kinases (MAPK) (11). The unique combinations of integrin subunits determines which ECM molecules will be recognized by a cell. For example, type I collagen interacts with $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_5\beta_1$-integrins while fibronectin binds $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_1\beta_1$ dimers. Osteoblasts express several integrin species including $\alpha_1$, $\alpha_2$, $\alpha_4$, $\alpha_5$, $\alpha_6$, $\beta_1$, $\beta_2$, and $\beta_3$ subunits (12–16) and their presence may be important in regulating the response of these cells to the ECM. Of particular interest, blocking $\alpha_2$-integrin-type I collagen interactions with specific peptides and antibodies prevents AA-dependent induction of alkaline phosphatase and differentiation-dependent down-regulation of transforming growth factor-β receptors in MC3T3-E1 preosteoblast cells suggesting that this integrin participates in the differentiation process (17).

Osteoblast-specific factor 2 (Osf2), also known as AML3 (18) or PEBP2aA1/Cbfa1 (19), is the bone-specific product of the Cbfa1 gene (20) and a possible mediator of the response of osteoblasts to ECM. Osf2 is one of three mammalian transcription factors related to the Drosophila protein runt (21). In vivo gene inactivation studies and in vitro and in vivo expression experiments indicate that Osf2 is a major regulator of the osteoblast phenotype and necessary for osteoblast-specific expression of the OCN gene (20, 22–25). We recently showed that AA increases mouse OCN gene 2 (mOG2) promoter activity approximately 20-fold in MC3T3-E1 preosteoblast cells. Significantly, this response, like the overall differentiation response of osteoblasts, requires collagen matrix synthesis and OSE2, ascorbic acid; OCN, osteocalcin; Osf2, osteoblast-specific factor 2; Cbfa, core binding factor α; mOG2, mouse osteocalcin gene 2; mAb, monoclonal antibody; DMEM, Dulbecco’s α-modified Eagle’s medium; PBS, fetal bovine serum; PBS, phosphate-buffered saline; BSP, bone sialoprotein; MAPK, mitogen-activated protein kinase; FITC, fluorescein isothiocyanate.

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§ The abbreviations used are: ECM, extracellular matrix; AA, ascorbic acid; OCN, osteocalcin; Osf2, osteoblast-specific factor 2; Cbfa, core binding factor α; mOG2, mouse osteocalcin gene 2; mAb, monoclonal antibody; DMEM, Dulbecco’s α-modified Eagle’s medium; PBS, fetal bovine serum; PBS, phosphate-buffered saline; BSP, bone sialoprotein; MAPK, mitogen-activated protein kinase; FITC, fluorescein isothiocyanate.
the downstream promoter-binding site for Osf2. Induction of matrix synthesis is also accompanied by a dramatic increase in the binding of a protein in nuclear extracts (presumably Osf2) to OSE2, suggesting that ECM synthesis up-regulates and/or activates Osf2 (26).

The present study was undertaken to evaluate the role of α2-integrin-type I collagen interactions in the control of osteoblast-specific transcription and differentiation. As will be shown, blocking integrin-type I collagen binding prevents activation of the OCN promoter by AA and suppresses binding of Osf2 to OSE2 DNA. Interestingly, the dramatic stimulation of Osf2 activity and osteoblast-specific gene expression by AA is not accompanied by comparable increases in transcription factor mRNA or protein suggesting that the ECM activates this factor through either a post-translational pathway or via an accessory factor.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Tissue culture medium and fetal bovine serum were obtained from HyClone (Logan, UT). [γ-32P]ATP and [α-32P]dTTP (3000 Ci/mmol) were purchased from Amer sham (Arlington Heights, IL). Pepsin (type II), trypsin (type II, BSS), and RGEs were purchased from Advanced Chem Tech (Louisville, KY) while the KDBGE peptide was synthesized by the core facility of the University of Michigan. Blocking monoclonal antibodies (mAbs) to the mouse α2-integrin subunit (clone Hm2) or α3 subunit (clone Hm5) and FITC-labeled anti-mouse α2-integrin antibody were purchased from Pharmingen (San Diego, CA). FITC-labeled IgG (Hamster) was purchased from KPL (Gaithersburg, MA). Normal hamster IgG was obtained from Sigma. Mouse Osf2 antisera was prepared in rabbits using a synthetic peptide having the following sequence: SFFFDPPSTRRSRFSPPS (amino acids 84–99 of the Osf2 sequence (20)). This antisera exhibited no detectable cross-reactivity with other members of the runt family of transcription factors on either gels retardation assays or Western blots (see Figs. 3C and 5A). All other chemicals were of analytical grade.

**Cell Culture—**This study used two previously described subclones of a murine MC3T3-E1 preosteoblast cell line. Subclone 4 cells express high levels of osteoblast marker mRNAs and form a mineralized ECM after growth in AA-containing medium. Subclone 42 cells similarly exhibit a high differentiation/mineralization potential in the presence of AA and contain stably integrated copies of a 1.5-kilobase mouse osteocalcin gene 2 promoter fragment fused to a luciferase luciferase assay (30). Aliquots of these were subjected to SDS-PAGE and blotted with polyclonal anti-agarose-formaldehyde gel electrophoresis and transferred to nitrocellulose paper as described by Thomas (31). Mouse cDNA probes used for hybridization were obtained from the following sources: OCN (32) from Dr. John Wozney (Genetics Institute, Boston, MA), bone sialoprotein (BSP) (33) from Dr. Marion Young (NIDR, National Institutes of Health, Bethesda, MD), and mouse α2-integrin (34) from Dr. Samuel Santoro (Washington University, St. Louis, MO). The mouse Osf2 cDNA was recently described (20). All DNA inserts were excised from plasmid DNA with the appropriate restriction enzymes and purified by agarose gel electrophoresis before labeling with α-32PdCTP using a random primer kit (Boehringer-Mannheim). Hybridizations were performed as described previously using a Belco Autoblot hybridization oven (35) and quantitatively scanned using a Miniford InstantImager. All values were normalized for RNA loading by probing blots with cDNA to 18 S RNA (36).

**Luciferase and Mineralization Assays—**Subclone 42 cells were plated in 24-well plates and, after 2 days, cultures were treated with the indicated concentrations of antibodies or peptides in the presence or absence of AA. Medium was replenished every other day and cells harvested at day 6. Luciferase assays were performed using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) and reagents and protocols from Promega (Madison, WI). The DNA content of each sample was then measured for normalization (37).

**RESULTS**

**Requirement for the α2-Integrin Subunit in mOG2 Promoter Activation and Osteoblast Differentiation—**Previous work from this laboratory showed that cell-collagen interactions are crucial for osteoblast differentiation and tissue-specific gene expression. Induction of osteoblast markers by AA is blocked by collagen synthesis inhibitors or digestion of the ECM with purified collagenase (35). In a similar manner, AA-dependent mOG2 promoter activation also requires collagen synthesis (26). Little is known concerning the mechanism of this regulation. One possibility explored in the experiments described below is that osteoblast differentiation requires an α2-integrin type I collagen interaction. This integrin subunit is a component of the αβ1-integrin, a major binding site for collagens that...
has previously been detected in osteoblasts (14). As shown in Fig. 1, MC3T3-E1 cells clearly produce the \( \alpha_2 \)-integrin subunit as detected by both immunofluorescence (panel A) and Northern blotting (panel B). Levels of a 5-kilobase \( \alpha_2 \)-integrin mRNA increased with time in culture (result not shown), but were not substantially changed by AA treatment.

To determine whether an \( \alpha_2 \)-integrin-type I collagen interaction is required for activation of the osteocalcin promoter, specific peptides and antibodies were used to disrupt this and related interactions. These experiments used subclone 42 cells, a previously described, highly differentiating MC3T3-E1 subclone containing stably integrated copies of the mOG2 promoter driving expression of firefly luciferase. As previously reported, growth of these cells in the presence of AA induces OCN promoter activity as well as endogenous OCN and BSP mRNAs (26). Induction of osteocalcin promoter activity was selectively inhibited by a DGEA peptide (Fig. 2A) which is a component of the \( \alpha_2 \beta_1 \)-integrin-binding domain of type I collagen (39) or a specific \( \alpha_2 \)-integrin blocking antibody (Fig. 2B). Inhibition was dose-dependent and highly specific in that a control peptide with the sequence, KDGE, an RGDS peptide which mimics the cell-binding domain of fibronectin and related proteins, the inactive fibronectin cell-binding domain variant, RGES, or an anti-\( \alpha_5 \)-integrin blocking antibody were all totally inactive over the concentration range used for active inhibitors. Both the DGEA peptide and \( \alpha_2 \)-integrin antibody were also found to be highly effective and selective inhibitors of mineralization, the end point for osteoblast differentiation (Fig. 2C). Cells treated with inhibitors showed no signs of toxicity such as visible cell detachment or significant reduction in the amount of DNA per culture dish (control, 4.7 ± 0.2 \( \mu \)g of DNA/dish; 1.0 mM DGEA, 4.5 ± 0.1 \( \mu \)g of DNA/dish; 12 \( \mu \)g/ml of anti-\( \alpha_5 \) antibody, 4.4 ± 0.5 \( \mu \)g of DNA/dish).

\( \alpha_2 \)-Integrin Is Necessary for OSE2 Activation—In an earlier study, we showed that AA-dependent activation of the OCN promoter requires OSE2, the cis-acting element in this promoter that is regulated by Osf2. Furthermore, nuclear extracts from AA-treated cells were found to contain more OSE2 binding activity (presumably Osf2) than controls (26) (also see Fig. 5, below). Because peptide or antibody blocking of \( \alpha_2 \)-integrin function inhibited osteoblast differentiation and transcriptional activity of the OCN promoter, we considered it important to examine the requirement for this integrin species in Osf2 activation as measured by its ability to bind OSE2 (Fig. 3). This and subsequent experiments used subclone 4 MC3T3-E1 cells grown for various times in the presence or absence of AA. Like the stably transfected subclone 42 cells described above, this subclone also readily differentiates after treatment with AA (26). Cells were grown for 3 days in the presence of AA and the indicated peptides or antibodies before RNA or nuclear extracts were prepared. DGEA peptide or anti-\( \alpha_2 \)-integrin antibody selectively suppressed expression of endogenous OCN mRNA (panel A) without affecting the Osf2 message. In contrast, binding of nuclear extracts to OSE2 was clearly inhibited by both of these treatments (panel B). Control peptide, IgG, and anti-\( \alpha_2 \)-integrin antibody were all devoid of inhibitory activity.

The shifted species generated when subclone 4 nuclear extracts were reacted with OSE2 did, in fact, contain Osf2 because it was supershifted by an anti-Osf2 antiserum (panel C). The specificity of the anti-Osf2 antiserum was confirmed by demonstrating that it was not able to interact with the shifted species generated between a Jurkat cell nuclear extract and OSE2 (also see Fig. 5 for further information on the specificity of this antiserum). Jurkat cells contain AML1 and -2, two related mammalian runt domain proteins that are also capable of binding the OSE2 core sequence (21). Taken together, these experiments demonstrate that AA-dependent induction of the OCN gene, differentiation/mineralization of MC3T3-E1 preosteoblast cells, and stimulation of Osf2 transcription factor binding to OSE2 all require an \( \alpha_2 \)-integrin-collagen interaction.

Relationship Between Osf2 Levels, Osteoblast Marker mRNA Expression, and OSE2 Binding Activity—As noted above, although inhibition of \( \alpha_2 \)-integrin binding to collagen blocked osteoblast-specific gene expression and binding of Osf2 to OSE2, these treatments did not affect Osf2 mRNA levels. Assuming Osf2 mRNA and protein levels are related, this suggests that an integrin-mediated signal increases the transcriptional activity of Osf2 without affecting its actual levels. This
hypothesis was explored further in the experiments shown in Figs. 4 and 5 which compared the temporal relationship between Osf2 mRNA and protein with OSE2 binding activity and osteoblast marker mRNA expression during AA-induced differentiation. As previously reported, several days were required for full induction of OCN and BSP mRNAs (3, 26, 35) (Fig. 4). The first detectable increase in BSP message was seen after 2 days while 4 days were required for induction of the OCN mRNA. At day 6, AA treatment increased OCN and BSP mRNA levels approximately 12-fold (Fig. 4B). Interestingly, levels of Osf2 mRNA were only slightly affected by AA treatment over the same time period. After 4 and 6 days, AA increased Osf2 mRNA only 1.5–2-fold.

Western blots were next used to assess Osf2 protein levels in whole cell extracts (Fig. 5). In agreement with a previous report (18), our anti-Osf2 antibody detected a major band at 65 kDa with minor species migrating slightly above and below this.
band on 10% SDS gels (panels A and B). Antibody specificity was confirmed by the observation that no signal was detected in extracts from the following nonbone-derived cell lines: 3T3/L1 adipocytes, F9 teratocarcinoma cells, C2/C12 myoblasts, C3H10T1/2 mesenchymal cells, or Jurkat cells (panel A). As noted above (Fig. 3C), this antibody also failed to interact with the OSE2-binding species in Jurkat cells (i.e. AML1 and AML2). AA treatment had only minor effects on the amount of total immunoreactive Osf2 at each of the time points examined (panel B); the ratio of total immunoreactive material in AA-treated versus control samples as determined by densitometry of Western blots was 1.3, 1.4, 1.2, and 1.7 for days 1, 3, 5, and 7, respectively. In contrast, AA increased the relative abundance of the fastest migrating immunoreactive band, particularly at days 1, 5, and 7 when AA/C ratios for this species were, respectively, 2.1, 1.8, and 3.6.

The small effect of AA on Osf2 mRNA and protein levels is to be contrasted with its ability to rapidly and dramatically stimulate binding of nuclear extracts to OSE2 as measured by gel retardation assays (Fig. 5C). OSE2 binding activity was detected as early as 24 h after AA addition, before any detectable change in Osf2 mRNA, and continued to increase up to day 7. In summary, the dramatic increase in osteoblast marker mRNAs, OCN promoter activity, and binding of nuclear extracts to OSE2 is not accompanied by parallel changes in levels of the Osf2 transcription factor. These results suggest that AA increases DNA binding and transcriptional activity of Osf2 without having major effects on expression of this protein.

**DISCUSSION**

This study examined the role of the α2-integrin subunit, a component of one of the major collagen receptors, in Osf2 activation, osteoblast-specific gene expression, and differentiation. We used either a specific anti-mouse α2-integrin blocking antibody or a DGEA peptide that mimics the cell-binding domain of type I collagen to inhibit the interaction of cells with type I collagen via α2-subunit-containing integrins. Our studies demonstrate: 1) that an α2-integrin-collagen interaction is necessary for activation of the OCN promoter, osteoblast differentiation, and binding of Osf2 to OSE2 and 2) that collagen matrix-dependent signals dramatically increase binding of Osf2 to DNA while having only modest effects on the cellular
concentrations of this transcription factor. This is the first study to link a specific integrin species to the activation of osteoblast-specific transcription by Osf2.

Several recent studies have postulated roles for integrins in osteoblast differentiation. Particular emphasis has been placed on those integrin species interacting with type I collagen and fibronectin. Using the same antibodies and peptides evaluated in the present study, Takeuchi and co-workers showed that binding of $\alpha_2$-integrins to type I collagen, rather than $\alpha_5$-integrin binding, is necessary for AA-dependent induction of alkaline phosphatase and down-regulation of transforming growth factor-$\beta$ receptors in MC3T3-E1 cells (17). In addition, a peptide containing the RGD cell-binding domain of fibronectin was reported to inhibit both bone formation and resorption in a mineralizing organ culture system (40). Similarly, long-term culture of calvarial osteoblasts with anti-fibronectin antibodies or fibronectin peptides containing the RGD cell-binding domain has also been reported to suppress differentiation (41). The present work is consistent with the study by Takeuchi and co-workers (17) which linked $\alpha_2$-integrins and collagen synthesis to osteoblast differentiation. In contrast, we were unable to obtain evidence for involvement of either the RGD cell-binding domain of fibronectin or the $\alpha_2$-integrin in MC3T3-E1 differentiation, at least over the range of concentrations found to be inhibitory for the DGEA peptide and anti-$\alpha_2$ antibody. One possible explanation for this discrepancy is related to the choice of cell populations used in these studies. Our experiments used homogeneous subclonal populations of MC3T3-E1 cells that had been selected for ability to express osteoblast markers and mineralize after exposure to AA. These cells are relatively mature in that they express Osf2 even under conditions where they are not secreting an ECM or expressing osteoblast markers (i.e., in the absence of AA, see Figs. 4 and 5). Our results, therefore, likely represent effects of ECM on the later stages of osteoblast differentiation. In contrast, primary cultures or calvarial explants contain osteoblasts/osteoblast precursors in various stages of maturation as well as nonosteoblastic cells. Some of the earlier steps in the maturation of osteoblast precursors may require RGD/$\alpha_2$-integrin binding for cell growth or differentiation, but this requirement is apparently lost in more mature cells.

Type I collagen-$\alpha_2\beta_1$-integrin interactions regulate many cellular activities including activation of collagenase (42), stimulation of collagen gel contraction (43), and kidney tubule formation (44), although in no case has the entire integrin-mediated pathway been delineated. Integrins convey information about the extracellular environment by serving as both a direct physical link between the ECM and actin cytoskeleton via the proteins, talin, and vinculin, and by functioning as signal transduction molecules to stimulate tyrosine phosphorylation cascades linked to MAPK and other pathways (45–47). Recently, Takeuchi and co-workers (48) provided compelling evidence for the involvement of focal adhesion kinase and MAPK in AA/collagen-dependent induction of alkaline phosphatase, an osteoblast differentiation marker. Inhibition of tyrosine kinase, destruction of focal adhesions, or overexpression of antisense focal adhesion kinase mRNA prevented AA-dependent activation of extracellular signal-regulated kinase and MAPK as well as stimulation of alkaline phosphatase. In addition, forced expression of CL100, a MAPK-specific phosphatase, inhibited alkaline phosphatase activity while introduction of a constitutively active MAPK enhanced alkaline phosphatase in the absence of AA. Although it is not currently known whether these treatments also inhibit the expression of other osteoblast marker genes, these results suggest that integrin activation of MAPK or related pathways is an important component of the cellular response to ECM.

Evidence presented in this study suggests that an $\alpha_2$-integrin-mediated signaling cascade is necessary for activation of the osteoblast-specific transcription factor, Osf2, and gene expression. Of particular interest, the dramatic AA/ECM-dependent increase in OCN promoter activity and Osf2 binding to OSE2 was not accompanied by parallel changes in levels of transcription factor mRNA or protein. In contrast, previous reports of Osf2 regulation by 1,25-dihydroxyvitamin D$_3$ and BMP7 indicated that these factors acted at the mRNA level (20, 49).

There are at least two possible ways Osf2 binding to OSE2 might be enhanced. First, all members of the runt domain family of transcription factors function as heterodimers of $\alpha$ and $\beta$ subunits thereby making the availability of both subunits a potential site for regulation. Osf2 is one of three $\alpha$ subunits identified in humans and mice. The common ubiquitously expressed $\beta$ subunit, Cbf, exists in two splice forms in the mouse, designated $\beta_1$ and $\beta_2$ (50). Thus, regulation of amount or splicing of the $\beta$ subunit is one potential route for ECM regulation. Phosphorylation of Osf2 represents a second, and, perhaps, most direct method for regulation by an integrin-mediated signaling cascade. In a recent study, Tanaka and co-workers (51) showed that AML1/Cbfa2 is phosphorylated in a hematopoietic cell line in vivo on two serine residues within the proline-, serine-, threonine-rich region of the molecule. In vitro addition of ERK also phosphorylated the same sites. Furthermore, ERK-dependent phosphorylation was only able to potentiate the transactivation ability of AML1 when the phosphorylation sites were kept intact. Of particular interest, these serine residues are conserved in Osf2 (20). As was shown in the present study, Osf2 Western blots detected at least three species with apparent molecular masses near 65 kDa which might represent different phosphorylated states of this transcription factor (Fig. 5). Interestingly, AA treatment appeared to increase the relative abundance of the fastest migrating species and this is consistent with the relative abundance of the fastest migrating species. However, additional studies will be required to determine whether Osf2 can actually be phosphorylated. Ongoing studies in this laboratory are attempting to resolve which of these possible mechanisms explain how integrin-ECM interactions activate osteoblast-specific gene expression.

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