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Transcription of $\alpha 2$ Integrin Gene in Osteosarcoma Cells Is Enhanced by Tumor Promoters

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Integrin $\alpha 2\beta 1$ is a heterodimeric transmembrane receptor for collagens. In osteogenic cells the expression of $\alpha 2\beta 1$ integrin is induced by both Kirsten sarcoma virus and chemical transformation. The association of $\alpha 2$ integrin with transformed cell phenotype was studied further by testing the effects of two tumor promoters, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and okadaic acid (OA), on human MG-63 osteosarcoma cells. TPA, an activator of protein kinase C, increased the cell surface expression of $\alpha 2$ integrin and the corresponding mRNA levels. Nuclear run-on assays indicated that TPA activated the transcription of $\alpha 2$ integrin gene. TPA also slightly increased the expression of $\alpha 3$ integrin but had no effect on the transcription of $\alpha 5$, αv, or $\beta 1$ integrin subunits. OA, an inhibitor of serine/threonine phosphatases, increased $\alpha 2$ integrin gene transcription and mRNA levels, but in contrast to TPA, OA decreased $\alpha 3$ integrin expression. The increased expression of $\alpha 2$ integrin on TPA-treated MG-63 cells led to faster cell spreading on type I collagen. Our results link the enhanced transcription of $\alpha 2$ integrin gene to tumor progression and show the independent regulation of $\alpha 2$ integrin compared to other integrin genes.

Key Words: **integrins; cell adhesion; tumor promoters; collagen receptors.**

INTRODUCTION

Integrins are a large family of heterodimeric cell adhesion receptors, which have an important role as mediators of cell–matrix interactions [1, 2]. They also convey signals regulating cell migration, phenotype, differentiation, and gene expression. Integrins are composed of two distinct transmembrane glycoprotein subunits, α and β, which are noncovalently linked to each other. To date, 16 different α subunits and 8 β subunits have been identified [1, 2]. Integrins mediating cell adhesion to extracellular matrix are receptors for collagens, laminins, and fibronectin. The major cellular collagen receptors are $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin heterodimers, whereas the role of $\alpha 3\beta 1$ integrin as a putative collagen receptor is limited to some cell lines.

Our previous studies [3] have shown that the regulation of $\beta 1$ integrin heterodimer expression resembles that of the other multisubunit membrane protein complexes. Many cell lines, including fibroblasts and osteosarcoma cells, synthesize an excess of precursor $\beta 1$ integrin in the endoplasmic reticulum (ER) [3, 4]. In the ER a protein called calnexin is involved in the assembly of some $\beta 1$ integrins as well as in the retention of a pool of immature $\beta 1$ chains in the ER [5]. The size of the free pre-$\beta 1$ subunit pool is not stable, but can be regulated by growth and differentiation factors [3, 6] and might decrease during malignant transformation [7, 8]. The reduction in the size of the intracellular pre-$\beta 1$ integrin pool leads to its accelerated maturation and to the decelerated maturation of the pre-α integrins, whereas the number of integrin heterodimers on the cell surface remains the same [9]. The gene expression of integrin α subunits is rate limiting for the cell surface expression of $\beta 1$ integrin heterodimers, and the regulation of $\beta 1$ integrin gene might be less important. A similar model has been described for another subfamily of integrins, namely αv integrins [10].

Cellular integrin pattern changes during cell differentiation [11, 12], wound healing [13], and chronic inflammation [14]. Often, these phenomena are regulated by cytokines and growth factors, suggesting their role also in the regulation of integrin gene expression. The effects of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), bone morphogenetic protein-2 (BMP-2), platelet-derived growth factor, and transforming growth factor-β (TGF-β) on the expression of collagen-binding integrins has been previously described [3, 15–19]. IL-1β and TNF-α can turn on $\alpha 1$ integrin expression in MG-63 human osteosarcoma cells and increase its expression in fibroblasts [18] and endothelial cells [16]. BMP-2 down-regulates the expression of $\alpha 2$ integrin in HaCaT keratinocytes, but not in human

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osteogenic sarcoma cells [17]. In neural PC 12 cells, nerve growth factor induces the expression of α1 integrin [20]. In osteogenic cells and in fibroblasts the expression of α2 integrin is strongly enhanced by TGF-β [3]. This phenomenon might have important physiological consequences, explaining the contraction of collagenous matrix during tissue repair [21]. In a human osteogenic sarcoma cell line (HOS) both viral [8] and chemical transformation [8, 22] induce α2 integrin gene expression. The same integrin subunit is up-regulated in invasive melanoma cells [23–25].

Very little is known about the cellular mechanisms regulating integrin gene expression. The 5′-flanking region of several integrin genes, including α2 subunit [26], has been sequenced. Integrin α2 subunit promoter, as also α4, α7, αM, β3, and β7 integrin promoters, contains putative binding sites for AP-1 transcription factors [26–31]. These sites mediate transcriptional gene regulation by phorbol esters [32], serum [33], TNF-α [34], and TGF-β [35]. Recently the regulation of α2 integrin gene has been connected to nuclear factor-κB (NF-κB) rather than to AP-1 proteins [36].

Here we show that two tumor promoters, namely a phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and a serine/threonine phosphatase inhibitor okadaic acid (OA), can both enhance the transcription of the α2 integrin gene, whereas no significant increase was seen in the expression of other integrins. Thus the results connect the expression of α2 integrin with tumor progression of osteosarcoma cells.

MATERIALS AND METHODS

Cell lines. A human osteosarcoma cell line (MG-63) and a human fibrosarcoma cell line (HT-1080) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin-G, and 100 μg/ml of streptomycin. For the experiments, MG-63 cells were maintained in DMEM without FCS for 18 h and HT-1080 cells were maintained in DMEM with 1% FCS for 18 h. Thereafter, OA or TPA (both Sigma Chemical Co., St. Louis, MO) was added, and the incubation was continued for the indicated periods of time.

Immunoprecipitation. Polyclonal rabbit antisera against human β1 [3], α2, and α3 integrin subunits [8] were used in immunoprecipitation assays. Cell cultures were metabolically labeled with 50 μCi/ml of [^{35}S]methionine (Tran^{35}S-Label; ICN Biomedicals, Inc., Irvine, CA) for 18 h in methionine-free minimum essential medium. Cell monolayers were rinsed on ice with a solution containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM Tris–HCl (pH 7.4) and then detached by scraping. Cell pellets obtained by centrifugation at 500*g* for 5 min were solubilized in 200 μl of the same buffer containing 100 mM *n*-octyl-β-D-glucopyranoside (Sigma) on ice with occasional vortexing. Insoluble material was removed by centrifugation at 10,000*g* for 5 min at 4°C. Radioactivity in cell lysates was counted, and an equal amount of radioactivity was used in immunoprecipitation assays. Triton X-100 (0.5% v/v) and bovine serum albumin (0.5 mg/ml) were added to the supernatants, which were then precleared by incubation with 50 μl of packed protein A–Sepharose (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden). Supernatants were immunoprecipitated with anti-integrin antibodies for 12 h at 4°C. Immune complexes were recovered by binding to protein A–Sepharose and washing the beads four times with 25 mM Tris-buffered isotonic saline (pH 7.4) containing 0.5% Triton X-100 and 1 mg/ml bovine serum albumin and twice with 0.5 M NaCl and 25 mM Tris–HCl (pH 7.4). The immunoprecipitates were analyzed by electrophoresis on sodium dodecyl sulfate-containing 6% polyacrylamide gels under nonreducing conditions followed by fluorography. Integrin bands were quantified from fluorograms by the Microcomputer Imaging Device version M4 (Imaging Research, Inc., St. Catharines, Ontario, Canada).

Northern blot hybridizations. Total cellular RNA was isolated by using a guanidium thiocyanate/CsCl method [37]. RNAs were separated in formaldehyde-containing agarose gels, transferred to nylon membranes (Zeta-Probe; Bio-Rad Laboratories, Richmond, CA), and hybridized with ^{32}P-labeled (Amersham, UK) cDNA probes. The following cDNAs were used: human α2 integrin [38], human α3 [39], human *c-jun* [32], human *junB* [40], and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [41].

Flow cytometry. Cells were grown to early confluence and detached with trypsin–EDTA, and trypsin activity was inhibited by medium supplemented with serum. Cells were washed with PBS (pH 7.4) and then incubated with PBS containing 10 mg/ml bovine serum albumin (BSA), 1 mg/ml glycine, and 0.02% NaN_3 for 20 min at 4°C. Cells were collected by centrifugation, exposed to a saturating concentration of monoclonal antibody against α2 integrin (12F1 [42]) or α3 integrin (J143 [43]) in BSA/PBS (BSA concentration 1 mg/ml) containing NaN_3 for 30 min at 4°C, and stained with rabbit anti-mouse IgG coupled to fluorescein (1:20 dilution; Dacopatts, Denmark) for 30 min at 4°C. Cells were washed twice with PBS containing NaN_3 and suspended in the same buffer. In order to measure the amount of α2 and α3 integrin on the cell surfaces, the fluorescent excitation spectra were analyzed by using a FACScan apparatus (Becton–Dickinson). Control samples were prepared by treating the cells without primary antibodies.

Transcriptional nuclear run-on analyses. The cells were lysed with NP-40 (ICN) and the nuclei isolated by centrifugation (12,000*g*) for 3 s at 4°C. *In vitro* nuclear run-on analyses were performed with an equal number of isolated nuclei (10^7/reaction). The nuclei were incubated in the presence of 100 μCi of [α-^{32}P]UTP (3000 Ci/mmol; NEN) for 30 min at room temperature as described previously [44]. Radiolabeled RNA was hybridized with 2 μg of nitrocellulose-fixed plasmids: cDNAs for α2 [38], α3 [39], α5 [45], αv [46], and β1 integrin subunits [45]; MMP-1 [47]; GAPDH [41]; and pBluescript (Stratagene). The hybridization and washing conditions used were as described previously [48]. Quantitative analyses were done with a GS-250 molecular image system and results were corrected for levels of GAPDH transcripts in the same samples.

Plasmid constructs and transfections. The DNA fragment covering 999 bp of α2 integrin subunit 5′-flanking region [26] was generated with PCR in two parts using the Gene Amp PCR kit (Perkin–Elmer, Branchburg, NJ). Template DNA was isolated from human lymphocytes. The −1 to −999 fragment was amplified using two pairs of primers: (i) sense 5′-TTCTCGAGAGCAGATCTT CTTTCC-3′ and antisense 5′-TTAAGCTTGAGAGCAGGGAAAAGT-3′ and (ii) sense 5′-TTCTCGAGGATAATCATAACTTGTG-3′ and antisense 5′-TTAAGCTTCTAGAAGCTGTCCAG-3′. Forty cycles of PCR amplification were done in 5% DMSO and 1.25 mM $MgCl_2$ using the following protocol: denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 2 min at 72°C. The fragment was digested with *Xho*I/*Hin*dIII and subcloned into pBS0CAT [49] (named α2CAT) and pGL3 Basic (Promega) (named α2luc). Both strands of the subcloned PCR product were sequenced using an Applied Biosystems 377 automatic sequencer. pAPCAT2a (APCAT), which contains three AP-1 consensus sequences in front of the tk promoter in the

pBLCAT2 plasmid [50], was used as a control plasmid.

HT-1080 and MG-63 cells were maintained in DMEM containing 10% FCS. Cells (3×10^5) were plated on a 30-mm dish and incubated for 18–24 h. The following constructs (4.5 μg of DNA) were used: α2CAT, APCAT, and α2luc. Transfections were performed with a calcium phosphate/DNA coprecipitation method [51]. Incubation time was 16 h for MG-63 cells and 4 h for HT-1080 cells. The glycerol shock was done for 3 and 2 min, respectively. TPA or OA was added to the cultures 20 h after the glycerol shock, and the incubations were continued for 48 h before assay of CAT activity as an index of promoter activity [52]. Transfection efficiency was monitored by cotransfection of the promoter construct with 3 μg of RSV/β-galactosidase construct and correction of the CAT activity for β-galactosidase activity [53] or for protein concentration (Bio-Rad). Luciferase activity was measured with the Luciferase Assay System kit (Promega) and luminescence was detected with Luminoskan (Labsystems).

Cell-spreading assays. The coating of a 96-well immunoplate (Maxi Sorp, Nunc, Denmark) was done by exposure to 0.2 ml of phosphate-buffered saline (pH 7.4) containing 0.1 μg/cm^2 type I collagen (from lathyric rat skin; Boehringer Mannheim) for 12 h at 37°C. Residual protein absorption sites in all wells were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at 37°C. Bovine serum albumin was also used to measure the nonspecific binding. Confluent cell cultures were treated with TPA for 18 h. Cells were detached by using 0.01% trypsin and 0.02% EDTA. Trypsin activity was inhibited by washing the cells with 1 mg/ml of soybean trypsin inhibitor (Sigma). In cell-spreading assays cells were suspended in DMEM with 50 μM cycloheximide (Sigma) and transferred into each well and incubated for 35 min at 37°C. The wells were washed with PBS and fixed with 8% formaldehyde and 10% sucrose in PBS for 30 min. The total number of cells attached per microscopic field and the percentage of spread cells was counted. A spread cell was characterized as one having a clearly visible ring of cytoplasm around the nucleus. A function-blocking antibody against α2 integrin (5E8 [54]) was used in some experiments.

RESULTS

TPA up-regulates the expression of α2 integrin on MG-63 cells. Previous studies have shown that $\alpha 3\beta 1$ heterodimer is the major β1 integrin on human osteosarcoma MG-63 cells. Small amounts of $\alpha 2\beta 1$ and $\alpha 5\beta 1$ integrins are also present [3, 55]. Here, we treated osteosarcoma cells with tumor promoters to see possible changes in integrin expression. At first the accumulation of radioactive integrins was measured after metabolic labeling with [^{35}S]methionine, immunoprecipitations with specific antibodies, and electrophoresis. Immunoprecipitations were done under conditions allowing the coprecipitation of α subunits in complex with β1 subunit when the β1-specific antibody was used. Similarly β1 subunit was coprecipitated in complex with α subunits when anti-α integrin antiserum was used. A relatively long (18-h) labeling time was selected because the accumulation of integrins in cells was to be studied. This allowed the estimation of the maturation rate of the integrin subunits as well. TPA (100 ng/ml) enhanced the amount of radiolabeled α2 integrin (in different experiments about 25- to 30-fold increase was seen) and simultaneously slightly increased the amount of α3 integrin (about 2-fold; Fig. 1A). No expression of a 190-kDa α1 integrin was detected in MG-63 cells before or after the treatment with TPA (not shown). TPA had a minimal effect on the amount of metabolically labeled β1 integrin subunit, whereas it seemed to accelerate the maturation of the pre-β1 integrin pool (Fig. 1A). This is in accordance with our previous observation that the maturation rate of the pre-β1 integrin subunit is dependent on the total number of α subunits available [3, 9].

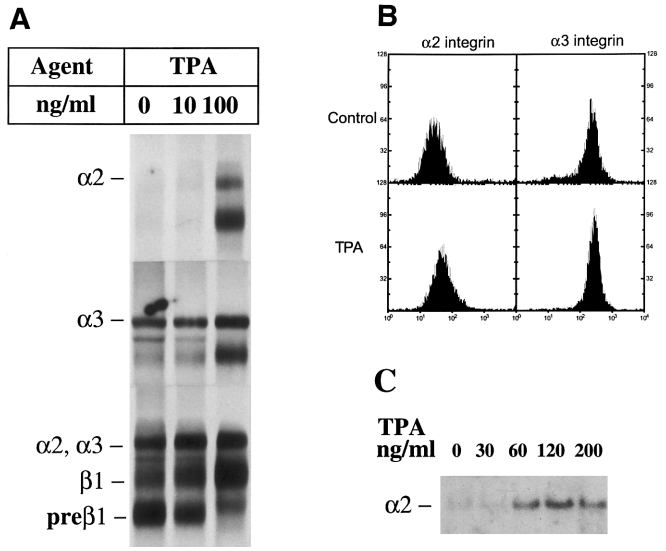

FIG. 1. Effect of TPA on the amount of metabolically labeled α2, α3, and β1 integrin subunits (A); the cell surface expression of α2 and α3 integrins (B); and the level of α2 integrin mRNA (C). Human MG-63 osteosarcoma cells were incubated for 36 h with different concentrations of TPA, the last 18 h with [^{35}S]methionine. Cellular integrins were immunoprecipitated with specific antibodies and analyzed by electrophoresis and fluorography (A). Expression of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins on cell surface was analyzed by flow cytometry. MG-63 cells were treated with TPA (100 ng/ml) for 24 h. Cells were detached by trypsin, enzyme activity was inhibited by a specific inhibitor, and cells were exposed to a saturating concentration of monoclonal antibody against α2 integrin (12F1) or α3 integrin (J143) and rabbit anti-mouse IgG coupled to fluorescein. The fluorescent excitation spectra were analyzed by using a FACScan apparatus (B). Integrin α2 mRNA levels were analyzed by Northern blot hybridization. Cells were treated with TPA for 24 h. Total cellular RNA was isolated, separated by gel electrophoresis, transferred to nylon membranes, and hybridized with ^{32}P-labeled α2 integrin-specific cDNA probe (C).

To analyze the cell surface expression of integrins MG-63 cells were exposed to saturating concentrations of monoclonal antibodies against α2 and α3 integrins and rabbit anti-mouse IgG coupled to fluorescein. The fluorescein excitation spectra were analyzed by using a FACScan apparatus. In all experiments TPA increased the cell surface expression of α2 integrin subunit (Fig. 1B), but had no effect on the amount of α3 integrin (Fig. 1B). The increase after 24 h was about 1.5-fold (not shown) and after 48 h up to 2.5-fold (Fig. 1B). After 48 h incubation with TPA the difference did not increase any more (not shown). The effect of TPA on α2

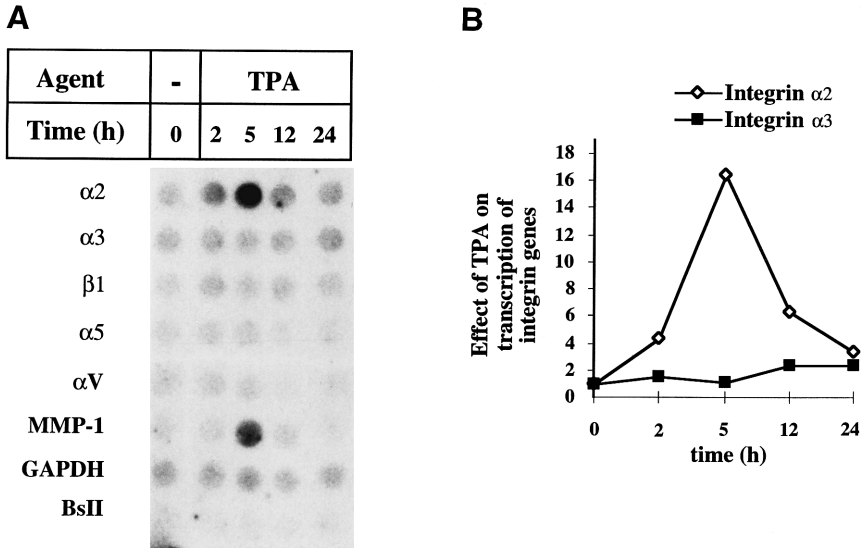

FIG. 2. Transcriptional regulation of $\alpha 2$, $\alpha 3$, $\alpha 5$, αv, and $\beta 1$ integrin and MMP-1 genes by TPA. Nuclear run-on assay was performed with nuclei from MG-63 cells treated with TPA (100 ng/ml) for 2, 5, 12, and 24 h prior to harvest. Nascent ^{32}P-labeled RNA was hybridized to nitrocellulose filter-immobilized cDNA probes (A). Quantitative analysis was done with an image analyzer system (B). The values for the integrin subunits were corrected to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcription in the same sample. Hybridization to Bluescript (BsII) was used to show the nonspecific background.

integrin mRNA expression was studied by Northern blot hybridizations. In all experiments TPA constantly increased $\alpha 2$ integrin mRNA levels (Fig. 1C). In different experiments the maximal effect varied between a 6- and a 16-fold increase. The TPA concentration needed was 60 ng/ml (maximal effect with 120 ng/ml; Fig. 1C).

Integrin $\alpha 2$ gene is regulated at transcriptional level. We studied the transcription rates of $\alpha 2$, $\alpha 3$, $\beta 1$, $\alpha 5$, and αv integrin and collagenase-1 (MMP-1) genes in MG-63 cells by nuclear run-on assays (Fig. 2). It was shown previously that MMP-1 is regulated at the transcriptional level by tumor promoters and therefore MMP-1 served as a positive control. MMP-1 transcription was induced by TPA at the 5-h time point (Fig. 2A). TPA enhanced the transcription of $\alpha 2$ integrin gene in 5 h (Figs. 2A and 2B). The maximal effects on $\alpha 2$ integrin transcription in two independent experiments were a 6- and a 17-fold increase, respectively. The transcription of $\alpha 3$ integrin was only slightly increased in 12 h (3-fold; Figs. 2A and 2B). Integrin αv, $\alpha 5$, and $\beta 1$ subunit genes were not significantly regulated by TPA (Fig. 2A).

The alterations in integrin expression were compared to changes seen in the expression of the immediate-early genes *c-jun* and *junB*. TPA up-regulated *junB* mRNA after 30 min, and the maximal effect was seen after 2 h (Figs. 3A and 3B). A similar effect was seen on *c-jun* expression (not shown). TPA up-regulated $\alpha 2$ integrin mRNA levels after 6 h (Figs. 3A and 3B) and increased only slightly the $\alpha 3$ integrin mRNA levels (2-fold; Fig. 3A). Thus induction of *junB* mRNA expression seemed to precede the activation of $\alpha 2$ integrin transcription.

TPA could not activate the $\alpha 2$ integrin promoter construct in MG-63 cells (either CAT or luciferase construct; not shown). However, the transfection efficiency of MG-63 was very low and we wanted to confirm the results with another cell line. HT-1080 cells are human fibrosarcoma cells used previously to study the role of AP-1 in the regulation of MMP-1 [56]. TPA elevated the $\alpha 2$ integrin mRNA levels also in HT-1080 cells (about 13-fold; Fig. 4A). In the nuclear run-on assay TPA induced $\alpha 2$ integrin gene transcription (Fig. 4B). Furthermore, the AP-1-dependent transcription of the APCAT construct was increased by TPA (6-fold; Fig. 4C), whereas neither $\alpha 2$CAT (Fig. 4C) nor $\alpha 2$luc (not shown) was induced. Thus the data indicate that the 1-kb 5′-flanking region of the $\alpha 2$ integrin gene lacks fragments important in the activation of the reporter gene despite the fact that it contains two putative AP-1 binding sites [26].

OA activates transcription of the $\alpha 2$ integrin gene, but at a later time point than TPA. To test the effect of another tumor promoter, which acts with a protein kinase C-independent mechanism, we exposed MG-63 cells to OA. It increased the accumulation of metabolically labeled $\alpha 2$ integrin about 10-fold and concomitantly suppressed the amount of $\alpha 3$ integrin to 15% of that in control cells (Fig. 5A). The concentration of OA needed was 10 ng/ml (Fig. 5A). OA in concentrations larger than 5 ng/ml elevated in a dose-dependent man-

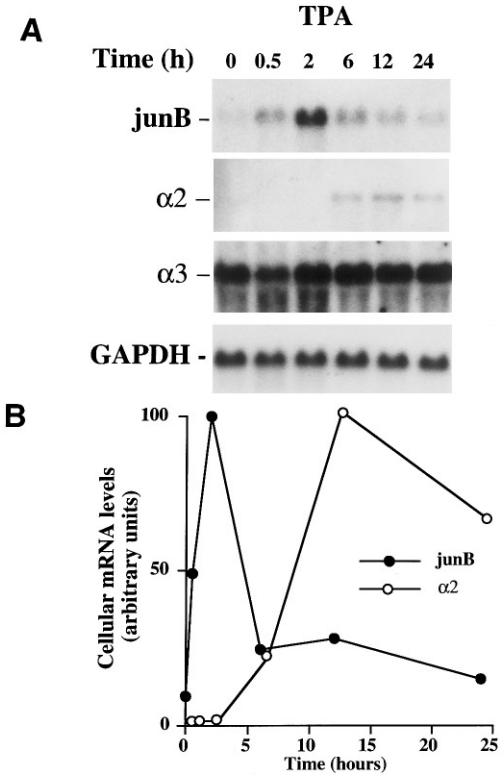

FIG. 3. Time scale of the effect of TPA on the mRNA levels of $\alpha 2$ integrin, $\alpha 3$ integrin, and *junB* proto-oncogene. MG-63 cells were incubated with TPA (100 ng/ml) for 0, 0.5, 2, 6, 12, or 24 h. Total cellular RNA was isolated from cells, separated by gel electrophoresis, transferred to nylon membranes, and hybridized with ^{32}P-labeled $\alpha 2$ and $\alpha 3$ integrin- and *junB*-specific cDNA probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control probe. (A) Autoradiograms and (B) quantitative analysis of the bands by an image analyzer system.

ner the mRNA levels of $\alpha 2$ integrin (4-fold effect with 15 ng/ml; Figs. 5B and 5C). OA-elicited reduction in $\alpha 3$ integrin expression was seen also in the corresponding mRNA levels (about 10% of control; Fig. 5C). OA up-regulated $\alpha 2$ integrin and down-regulated $\alpha 3$ integrin mRNA levels significantly later than TPA, that is, after 12 h (Fig. 5C). The transcription of the $\alpha 2$ integrin gene was increased later (12 h) than with TPA, as well (Fig. 6). In the experiment shown in Fig. 6 some increase in the transcription of the $\alpha 2$ integrin gene was seen already after 2 h when it was normalized to the transcription level of the control gene (GAPDH). However, this was less evident in other experiments. OA seemed at first (5 h) to slightly increase $\alpha 3$ integrin transcription but at a later time point (24 h) the transcription rate was below the control level. OA had no effect on the cell surface expression of $\alpha 2$ integrin but it reduced the expression of $\alpha 3$ integrin also on the cell surface (not shown). OA treatment decreased the precursor $\beta 1$ integrin pool to 15% of that in controls (Fig. 5A), without a concomitant increase in the number of mature $\beta 1$ subunits (as was seen with TPA in Figs. 1A and 4C). The decrease in the size of the precursor $\beta 1$ integrin pool in the ER may affect the cell surface expression of integrin α subunits [8, 9] and may partially explain why the increased synthesis of $\alpha 2$ integrin was not seen on the cell surface. The small change in the mobility of precursor $\beta 1$ integrin in OA-treated cells (Fig. 5A) may point to altered folding or glycosylation and therefore also to altered transport of $\beta 1$ integrins. The results indicate that integrin expression on the cell surface can also be regulated at the posttranslational level.

TPA increases MG-63 cell spreading on type I collagen. To study the effect of TPA on the attachment of MG-63 cells to type I collagen we performed cell-spreading assays. Integrin $\alpha 2\beta 1$ was considered the collagen receptor of MG-63 cells, since anti-$\alpha 2$ integrin antibody could inhibit the MG-63 cell adhesion on type I collagen, but anti-$\alpha 3$ integrin antibody had no effect (not shown). TPA enhanced the spreading of MG-63 cells on type I collagen (Table 1). The increased $\alpha 2$ integrin expression on cell surface was associated with altered cell behavior, since after TPA treatment anti-$\alpha 2$ integrin antibody could almost completely block cell spreading (Table 1).

DISCUSSION

In many cell lines $\alpha 2\beta 1$ integrin is the major collagen receptor. The conformation of $\alpha 2\beta 1$ integrin can be regulated, and under certain conditions it also functions as a laminin-1 receptor [57]. In skin and mucosa $\alpha 2$ integrin is located between keratinocytes, suggesting its participation in cell–cell adhesion [58]. Integrin $\alpha 2\beta 1$ has an important role in the cell migration and in the reorganization of collagenous matrix [21, 24, 59]. In HOS cells $\alpha 2$ integrin expression is induced after both chemical and viral transformation [8, 22, 60]. Here we provide further evidence that in osteosarcoma cells $\alpha 2\beta 1$ integrin is associated with tumor progression by showing that tumor promoters TPA and OA can both increase the transcription of the $\alpha 2$ integrin gene, but not the genes of other integrins.

TPA is a phorbol ester and an activator of protein kinase C, whereas OA is an inhibitor of serine/threonine protein phosphatases 1 and 2A [61]. The effect of OA on integrin expression has not been reported previously. TPA is known to regulate $\alpha 5\beta 1$ integrin function in CHO cells without modifying its number or phosphorylation [62]. TPA can also induce several cell lines, including mononuclear phagocytes and K562 erythroleukemia cells, to differentiate and concomitantly there are alterations in their integrin pattern [63, 64]. These changes seem to reflect the altered cell phenotype more than the direct effects of TPA. In

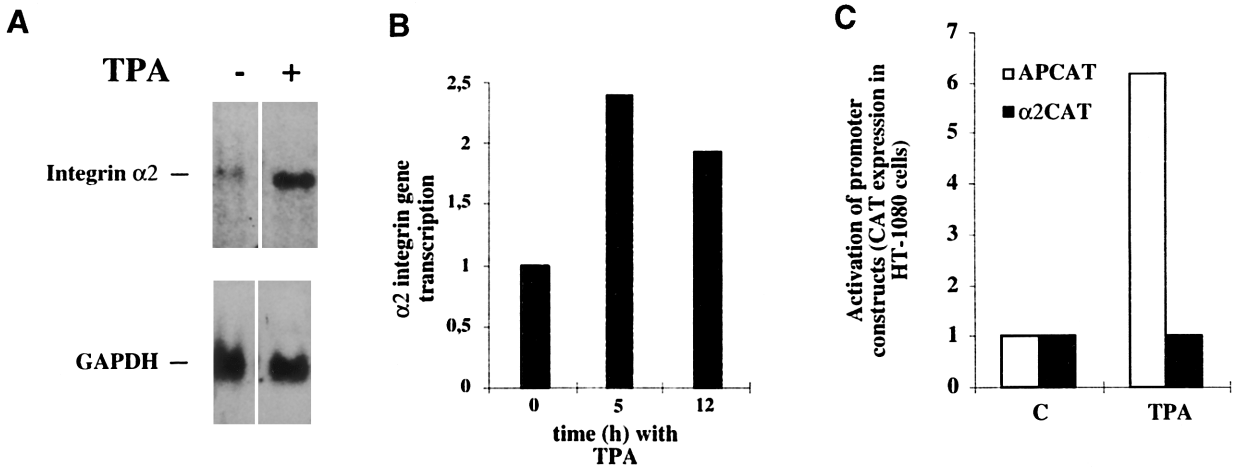

FIG. 4. TPA (60 ng/ml) elevates α2 integrin mRNA in human HT-1080 fibrosarcoma cells (A). TPA increases the transcription of the α2 integrin gene (B). TPA induces the expression of APCAT construct but has no effect on the expression of an α2 integrin promoter containing the CAT construct (α2CAT) (C). In (A) total cellular RNA from the HT-1080 cell line was separated by gel electrophoresis, transferred to nylon membrane, and hybridized with ^{32}P-labeled α2 integrin-specific cDNA probe. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as a control probe. In (B) nuclear run-on assay was performed with nuclei from HT-1080 cells treated with TPA (100 ng/ml) for 0, 5, and 12 h prior to harvest. Nascent ^{32}P-labeled RNA was hybridized to nitrocellulose filter-immobilized cDNA probes. Quantitative analysis was done with an image analyzer system. The values for α2 integrin subunits were corrected to GAPDH transcription in the same sample. In (C) transfections were performed with the calcium phosphate/DNA coprecipitation method. TPA was added to the cultures 20 h after the glycerol shock, and the incubations were continued for 48 h before assay of CAT activity as an index of promoter activity. Transfection efficiency was monitored by correcting the CAT activity for protein concentration in cell lysates.

melanoma cells TPA increases $\alpha 2\beta 1$ integrin expression [65].

Comprehensive research effort has been focused on the regulation of extracellular matrix genes. Very little is known about the gene regulation of their receptors. The cellular signal transduction pathways regulating the expression of α2 integrin are mostly unknown. In fibroblasts and in osteogenic cells TGF-β is a potent inducer of the synthesis of mRNA levels of α2 integrin [3, 15]. TGF-β regulates α2 integrin gene at the transcriptional level (J. Heino *et al.*, unpublished results). The mechanisms used by TGF-β to regulate gene expression include c-Jun, JunB, and accordingly the activation of AP-1 [66]. AP-1 mediates, for example, the autoinduction of the TGF-β1 gene [35]. Both TPA and OA share the ability to activate AP-1 with TGF-β [56, 67, 68]. Some previously published results as well as some of our experiments give indirect evidence suggesting that AP-1 may also participate in the regulation of the α2 integrin gene: (i) the induction of *junB* mRNA expression preceded the induction of α2 integrin gene transcription in MG-63 cells (Fig. 3), (ii) glucocorticoid dexamethasone, a potent inhibitor of AP-1 function, prevented the effects of TGF-β, TPA, and OA on α2 integrin expression (L. Nissinen *et al.*, unpublished), and (iii) the previously described 1-kb fragment of the α2 integrin promoter contains two putative AP-1 binding sites [26] and JunB is a potent activator of promoters containing more than one AP-1 element [40, 69]. To study the function of the first 1-kb

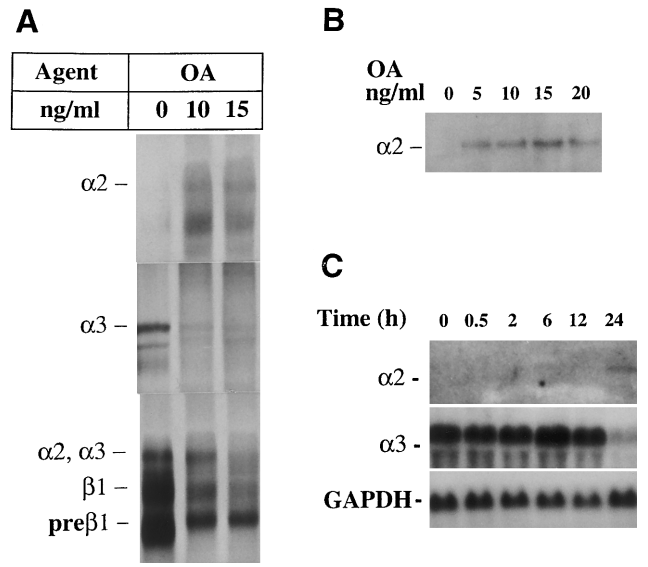

FIG. 5. Effects of okadaic acid (OA) on the amount of metabolically labeled α2, α3, and β1 integrin subunits (A) and the expression of α2 integrin mRNA (B and C). In (A) MG-63 human osteosarcoma cells were incubated for 36 h with different concentrations of OA, the last 18 h with [^{35}S]methionine. Cellular integrins were immunoprecipitated with specific antibodies and analyzed by electrophoresis and fluorography. In (B and C) total cellular RNA was isolated from cells, separated by gel electrophoresis, transferred to nylon membranes, and hybridized with ^{32}P-labeled α2 and α3 integrin-specific cDNA probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control probe.

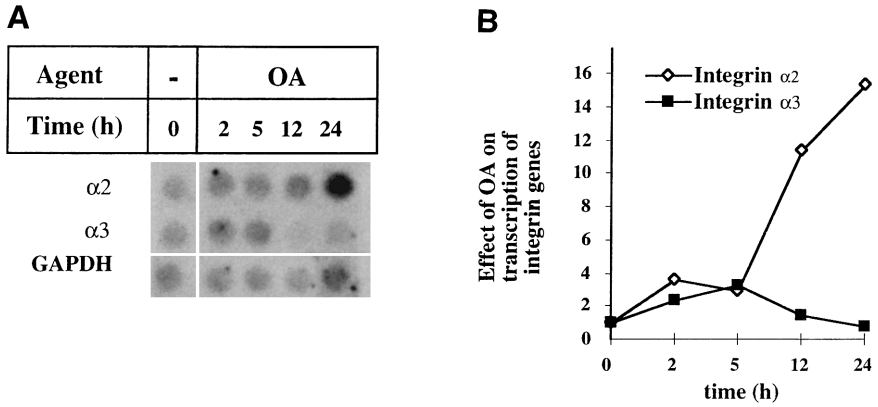

FIG. 6. Effects of okadaic acid (OA) on the transcription of $\alpha 2$ and $\alpha 3$ integrin genes. In (A) nuclear run-on assay was performed with nuclei from MG-63 cells treated with OA (10 ng/ml) for 2, 5, 12, and 24 h prior to harvest. Nascent ^{32}P-labeled RNA was hybridized to nitrocellulose filter-immobilized cDNA probes. In (B) the quantitative analysis was done with an image analyzer system. The values for integrin subunits were corrected to GAPDH transcription in the same sample.

fragment of the 5′-flanking area in the $\alpha 2$ integrin gene we transiently transfected MG-63 cells with the $\alpha 2$CAT construct, but we were not able to show its activation after TPA treatment. Because of the low transfection efficiency of MG-63 cells we also used another cell line, HT-1080. In these cells TPA elevated $\alpha 2$ integrin mRNA levels and enhanced the transcription of $\alpha 2$ integrin gene, as well. Despite the fact that TPA activated the APCAT construct the 1-kb 5′-flanking region of the $\alpha 2$ integrin gene could not activate the marker gene reading, suggesting that the putative AP-1 binding sites are not active or not alone sufficient for $\alpha 2$ integrin gene activation. Phorbol esters are also known to activate other transcription factors, including NF-κB [70]. Recently it was suggested that PKC-ζ, a protein kinase C isoform, and NF-κB as its downstream target are involved in the regulation of $\alpha 2$ integrin after stimulation by collagenous matrix [36]. Another transcription factor, Sp1, has been indicated as participating in the down-regulation of $\alpha 2$ integrin gene expression [71].

The induction of integrin gene transcription by tumor promoters was limited to the $\alpha 2$ integrin gene. Neither of the factors used in this study could induce the expression of another collagen receptor, $\alpha 1\beta 1$ integrin, which is not normally detectable in MG-63 cells but can be induced by proinflammatory cytokines [18]. Neither was the transcription of the $\alpha 5$, αv, and $\beta 1$ integrin genes altered by TPA. The only other integrin regulated by tumor promoters was $\alpha 3$. OA, like TGF-β [15], could down-regulate $\alpha 3$ expression, whereas in most experiments TPA slightly up-regulated its synthesis, even though the effect was often marginal. Integrin $\alpha 3\beta 1$ is a laminin-5 receptor in some osteosarcoma cells [17] and very little is known about the molecular mechanisms regulating its expression. TGF-β has an opposite effect on $\alpha 3$ integrin expression in fibroblasts compared to osteosarcoma cells [3]. BMP-2 is also a down-regulator of $\alpha 3$ integrin expression in osteosarcoma cells [17]. In the sarcoma virus-induced transformation of several murine cell lines $\alpha 3$ was the only integrin the expression of which was not down-regulated [72]. All these data indicate that the individual integrin genes are independently regulated.

TABLE 1

Effect of TPA and Anti-$\alpha 2$ Integrin Antibody (5E8) on the Spreading of MG-63 Cells on Type I Collagen

	Percentage of spread cells
Experiment 1	
Collagen/control	77
Collagen/TPA	89
BSA	—[a]
Experiment 2	
Collagen/control	69
Collagen/TPA	84
BSA	—[a]
Experiment 3	
Collagen/control	48 ± 12
Collagen/TPA	66 ± 4*
Experiment 4	
Collagen/TPA	16 ± 4
Collagen/TPA+5E8 antibody	2 ± 0.5

Note. Confluent cell cultures were treated with TPA for 18 h and cells were detached by using trypsin. Trypsin activity was inhibited by washing the cells with soybean trypsin inhibitor. 10,000 cells were suspended in DMEM with 50 μM cycloheximide, then transferred into wells precoated overnight with type I collagen, and incubated for about 30 min at 37°C. The wells were washed with PBS and cells were fixed. The percentage of spread cells was counted. 9–18 microscopic fields (on average 300 cells) were analyzed. Experiments 3 and 4 show results (means ± SD) from five parallel experiments (cells from five parallel culture plates).

[a] No adherent cells detected.

* t test, $P = 0.04$.

It is evident that tumor promoters can increase the transcription of the $\alpha 2$ integrin gene, but still other regulatory mechanisms may also be involved. In MG-63 cells the gene activation could alone explain the increased mRNA levels, but especially in HT-1080 cells the participation of the regulation of mRNA half-life could not be excluded. OA increased the amount of metabolically labeled $\alpha 2$ integrin in the cells but could not increase its amount on cell surfaces, suggesting that integrin transport to cell surface is regulated, as well. The amount of $\beta 1$ subunit may be one of the factors regulating the surface expression of the α integrins [3, 8, 9]. Despite the fact that the increase in cell surface expression of $\alpha 2$ integrin was sufficient to change the behavior of cells the increase was much smaller than could have been predicted from the large increase in the accumulation of radiolabeled $\alpha 2\beta 1$ integrin. This is further evidence suggesting that factors other than the $\alpha 2$ integrin synthesis rate may regulate the number of $\alpha 2\beta 1$ integrins on the cell surface. It is, for example, possible that TPA simultaneously decreases the half-life of integrins on the cell surface or increases the intracellular pool of integrins. However, the radiolabeled pool of integrins represents probably only a fraction of the total cellular pool of integrins. Therefore the changes in the total amount of integrins can be less dramatic than predicted from the changes in the amount of radiolabeled integrins. The fact that in different experiments there was variation in the effect of TPA on $\alpha 2$ integrin mRNA levels (from 6- to 16-fold increase in different experiments) and on $\alpha 2$ integrin transcription rate (from 6- to 17-fold increase in different experiments) may suggest that cell culture conditions, e.g., the confluence of the monolayers, may modify the effect of tumor promoters.

Our data associate $\alpha 2$ integrin expression with malignant transformation [8, 60] and tumor progression of osteogenic sarcoma cells. Integrin $\alpha 2$ is also overexpressed in aggressive melanoma-derived cell lines [23–25]. Here, in the presence of TPA MG-63 cells showed increased adhesion and spreading on type I collagen. We have previously shown that overexpression of $\alpha 2$ integrin in osteosarcoma cells also leads to increased cell migration on collagen and faster invasion through collagenous matrix [60]. In rhabdomyosarcoma cells the expression of $\alpha 2$ integrin has been connected to invasion and formation of metastasis [73]. The fact that $\alpha 2\beta 1$ integrin-related signals can induce the expression of MMP-1 [74, 75] can be important for cancer cell invasion.

To conclude, the results indicate that the regulation of individual integrin genes is independent and that enhanced transcription of the $\alpha 2$ integrin gene is a common consequence of cell stimulation by either TPA or OA. In osteosarcoma cells the observations link the enhanced transcription of the $\alpha 2$ integrin gene to tumor progression, whereas a similar connection with the expression rate of the other integrin genes could not be shown.

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