Integrin $\alpha 2\beta 1$ Mediates Isoform-specific Activation of p38 and Upregulation of Collagen Gene Transcription by a Mechanism Involving the $\alpha 2$ Cytoplasmic Tail

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Abstract. Two collagen receptors, integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, can regulate distinct functions in cells. Ligation of $\alpha 1\beta 1$, unlike $\alpha 2\beta 1$, has been shown to result in recruitment of Shc and activation of the Ras/ERK pathway. To identify the downstream signaling molecules activated by $\alpha 2\beta 1$ integrin, we have overexpressed wild-type $\alpha 2$, or chimeric $\alpha 2$ subunit with $\alpha 1$ integrin cytoplasmic domain in human osteosarcoma cells (Saos-2) lacking endogenous $\alpha 2\beta 1$. The chimeric $\alpha 2/\alpha 1$ chain formed a functional heterodimer with $\beta 1$. In contrast to $\alpha 2/\alpha 1$ chimera, forced expression of $\alpha 2$ integrin resulted in upregulation of $\alpha 1$ (I) collagen gene transcription in response to three-dimensional collagen, indicating that the cytoplasmic domain of $\alpha 2$ integrin was required for signaling. Furthermore, signals mediated by $\alpha 2\beta 1$ integrin specifically activated the p38$\alpha$ isoform, and selective p38 inhibitors blocked upregulation of collagen gene transcription. Dominant negative mutants of Cdc42, M KK 3, and M KK 4 prevented $\alpha 2\beta 1$ integrin-mediated activation of p38$\alpha$. RhoA had also some inhibitory effect, whereas dominant negative Rac was not effective. Our findings show the isoform-specific activation of p38 by $\alpha 2\beta 1$ integrin ligation and identify Cdc42, M KK 3, and M KK 4 as possible downstream effectors. These observations reveal a novel signaling mechanism of $\alpha 2\beta 1$ integrin that is distinct from ones previously described for other integrins.

Key words: collagen • integrin • cytoplasmic domain • p38 MAPK

Integrin type receptors bind extracellular matrix (ECM) molecules and mediate cell adhesion, migration, and invasion during development, tissue repair, tumor invasion, and metastasis. They also act in concert with growth factor or cytokine receptors to regulate cell proliferation, differentiation, and survival (Yamada and Miyamoto, 1995). In the relatively short cytoplasmic domains of the integrin $\alpha$ and $\beta$ subunits do not have any intrinsic enzymatic activity, but integrin signaling is achieved by coupling signaling molecules, such as tyrosine kinases, to the cytoplasmic and transmembrane domains of the integrin subunits (Yamada and Miyamoto, 1995). The $\beta$ cytoplasmic domains share conserved regions, whereas the $\alpha$ cytoplasmic domains have highly divergent amino acid sequences (Laflamme et al., 1997). Integrins activate signaling pathways that are either common to all integrins or heterodimer specific (Giancotti, 1997). Early integrin-triggered signaling events seem to be mediated by the cytoplasmic domains of the $\beta$ subunits. These include the tyrosine phosphorylation of the focal adhesion kinase (FAK) (Schaller et al., 1994) and recruitment of components of the actin-based cytoskeleton, such as $\alpha$-actinin and talin (Horwitz et al., 1986; Otey et al., 1990). In the next step, a large number of proteins with enzymatic activity, like kinases and GTPases, are recruited to the sites of adhesion. A cification of these signaling proteins is mediated both by the $\beta$ subunits and specifically by individual $\alpha$ subunits (Howe et al., 1998). The functions of $\alpha$ cytoplasmic domains of various integrins are distinct. Cytoplasmic domains of $\alpha 5$ and $\alpha 4$ are not required for cell adhesion unlike those of $\alpha 2$ and $\alpha 4$. In the context of cell adhesion and migration, some of the $\alpha$ cytoplasmic domains seem to be interchangeable (Kassner and Hemler, 1993), whereas signaling events specific for each individual integrin heterodimer may be triggered...
by the α cytoplasmic domains (Z. Zhang et al., 1995; Sastry et al., 1996).

Four members of the integrin family are known to bind collagens, namely α1β1, α2β1, α3β1, and α10β1 (Camper et al., 1998). α1β1 and α2β1 are considered to be the two major collagen binding integrins, whereas α3β1 seems to function as an assisting receptor (DiPersio et al., 1995). The recently identified integrin α10β1 is expressed on chondrocytes, but little is known about its biology (Camper et al., 1998). α1β1 and α2β1 are concomitantly expressed by many cell types but they may regulate different functions. Three-dimensional ECM culture systems provide means to study cell–matrix interactions in a more natural environment than the traditional monolayer culture (Grinnell, 1994). The studies with three-dimensional collagenous matrices have shown that ligation of α2β1 triggers MMP-1 expression (Langholz et al., 1995; Riikonen et al., 1995a) and induces collagen gel contraction (Chan et al., 1992; Riikonen et al., 1995b). In fibroblasts, contact to collagen also leads to the activation of protein kinase (PK)-Cζ and NF-κB and that correlates with the upregulation of α2β1 integrin and MMP-1 gene expression (Xu and Clark, 1997), suggesting that these phenomena are mediated via α2β1. On the other hand, α1β1 integrin mediates downregulation of collagen α1(I) mRNA levels both in vitro and in vivo (Langholz et al., 1995; Riikonen et al., 1995; G ardner et al., 1999). A subset of integrins, including α1β1, have been shown to activate the mitogen-activated protein kinase (MAPK) extracellular signal-related kinase (ERK) via recruitment of Shc and activation of Ras (Wary et al., 1996; Mainiero et al., 1997; Pozzi et al., 1998), suggesting that α1β1 integrin is the collagen receptor that regulates cell proliferation in collagenous matrices. In contrast, α2β1 appears to be unable to recruit Shc and activate this specific signaling pathway.

We have previously observed that overexpression of α2β1 integrin prevents α1β1-mediated downregulation of collagen α1(I) mRNA levels when cells are brought into contact with collagen (Riikonen et al., 1995a), but the molecular mechanism of this phenomenon has been unclear. To study the structural requirements of signaling via the distinct collagen receptors, we constructed a chimeric collagen α1(I) mRNA levels is due to active signaling requiring the cytoplasmic domain of α2 subunit.

We have recently shown that in normal dermal fibroblasts three-dimensional collagen activates three distinct classes of MAPKs, i.e., ERK, c-jun NH2-terminal kinase (JNK), and p38 (Ravanti et al., 1999). Ligation of α1β1 has been shown to activate the ERK kinase pathway, but α2β1 has not been previously linked to a specific MAPK kinase pathway. Here, we report that the α2 cytoplasmic domain–dependent regulation of type I collagen mRNA levels requires p38 activity. We also show that the ligation of α2β1 leads to the activation of p38 MAPK, especially its p38α isoform. The activation is dependent on the α2 chain cytoplasmic domain and the function of the downstream effectors Cdc42, M KK 3, and M KK 4 is required. These results indicate a crucial role for the p38 pathway in integrin α2β1 signaling and provide novel insight on molecular mechanisms of integrin-specific signal transduction.

### Materials and Methods

#### Reagents

Herbimycin A, bisindoylmaleimide, KT5720, KT5723, SB 203580, PD 98059, and SKF 8602 were obtained from Calbiochem-Novabiochem Corp. Cycloheximide was obtained from Sigma Chemical Co. (R)-(+)-perillyl alcohol (POH) was obtained from A drich and anisomycin was obtained from Boehringer Mannheim. α2 integrin cDNA corresponding to nucleotides 1–4559 in the published sequence (Takada and Hemler, 1989) and the X2C5FN Neo plasmid (Kawaguchi and Hemler, 1993) were provided by Dr. M. Hemler (Dana Farber, Boston, MA). The pA Wneo2 expression vector was provided by Dr. A. Weiss (University of California San Francisco, San Francisco, CA) (Ohashi et al., 1985). The oligonucleotides were used were purchased from K bor Lab. The glutathione-S-transferase (GST)–tagged ATF2 (residues 1–109) (Gupta et al., 1995), the pSV-MKK 3(αα) and pcDNA-MKK 4(αα) expression plasmids (Rain et al., 1996; Whitmarsh et al., 1997) were provided by Dr. R. Davis (University of Washington, Seattle, WA), and flag-tagged p38 isoform expression vectors (New et al., 1998) were provided by Dr. J. Han (Scripps Research Institute, La Jolla, CA). The pCEV29hoAαα and pCEV29hoBαα (Diederich et al., 1996) were provided by Dr. A. Weiss (University of California, San Diego, CA). Polyclonal antisera against α1 integrin cytoplasmic tail (A 81934) was purchased from Chemicon International, Inc., and antibody against α1 integrin used in flow cytometry, SR-84, was a gift from Dr. W. Retting (Boehringer Ingelheim, Germany).

#### Cell Culture

Human osteosarcoma cell line Saos-2 was obtained from the American Type Culture Collection. The cell cultures were maintained in DMEM supplemented with heat inactivated 10% FCS (GIBCO-BRL), 2 mM glutamine, 100 IU/ml penicillin-G, and 100 µg/ml streptomycin.

#### Plasmid Constructs and Stable Transfections

The α2 integrin expression construct was prepared as described previously (Riikonen et al., 1995a). The mutant α2 subunit, in which the cytoplasmic tail has been replaced with the corresponding α1 integrin sequence, was prepared in the following way: a silent point mutation (nucleotide 3488 in the published sequence; Takada and Hemler, 1989) that introduces a new NheI recognition site was made with the Altered sites II in vitro mutagenesis kit (Stratagene). Then, a synthetic oligo containing the correct sequences was inserted into both vectors and the correct orientation of the hinge region was confirmed. The correct orientation of the construct was checked by sequencing. Stable transfections were performed with the calcium polybrene/DNA method on confluent 60-mm dishes. Incubation with 5 µg DNA and 5 µg polybrene in 1 ml 10% FCS/DMEM per dish was carried out for 6 h, agitating the dishes once an hour. DM SO (30% in FCS) shock was done for 3 min, cells were washed twice with PBS, and culture medium was added. Neomycin analogue G418 (Life Technologies, Inc.) was added to the culture medium in a concentration of 400 µg/ml. G418-resistant cell clones were selected for 2–3 wk, isolated, and analyzed for their expression of α2 integrin. Control cells were transfected with the pA Wneo2 plasmid only.
Collagen Gels and Gel Contractions

Collagen gels were prepared from bovine dermal collagen, which contains 95% type I collagen and 5% type III collagen (Cellon). 8 vol of Cellon were mixed with 1 vol of 10% concentrated NaOH and 1 vol of 20% trypsin (0.2 M) and kept on ice. Cells were trypsinized, resuspended in 1/10 gel volume culture media DME supplemented with 10% FCS, mixed into neutralized Cellon solution, and transferred into 6-well plates. The collagen was allowed to polymerize for 2 h at 37°C, after which the culture media containing 10% FCS was added. The gels were detached from the sides of the wells, and incubation was continued for the times indicated. Cells were also cultured on plastic as a monolayer in culture media containing 10% FCS. In experiments involving inhibitors, the cells were pretreated with the inhibitor for 30 min at room temperature before mixing the cells with the neutralized Cellon solution, also supplemented with the inhibitor at the concentrations indicated. A freeze polymerization, culture media containing 10% FCS and the inhibitor was added and the gels were detached from the sides of the dishes. Incubation was continued for 48 h. When studying collagen gel contraction, cell culture wells were photographed after 48 h and the surface area of the gels were measured from the prints.

Immunofluorescence

The cells grown on immunofluorescence glass slides (CML) covered or uncovered with collagen film (Cellon) were rinsed with PBS and fixed with methanol at −20°C for 5 min. The cells grown inside collagen gels were excised from culture wells, embedded in OCT compound (Tissue Tek; Miles Scientific), and frozen in isopentane chilled with liquid nitrogen. Sections of 10-μM thickness were cut and picked up onto microscope slides and treated as described above. The slides containing the fixed samples were incubated in 2% BSA in PBS and monodonal anti–CD 49b antibody (Chemicon International Inc.) was added to the solution and incubated 30 min at room temperature. A freeze polymerization, the cells were incubated with anti–mouse–FITC conjugate (Dako A/S) for 30 min and mounted above observation and photography. For the staining of the actin filaments, plastic coverslips (Nunc) were coated with PBS containing 16 μg/ml type I collagen overnight at 4°C and blocked with 1% BSA in PBS for 1 h at 37°C. Cells were allowed to adhere and spread on collagen coated coverslips for 24 h in DME. Coverslips were washed once with PBS and fixed with 2% paraformaldehyde, permeabllized with 0.5% Triton X-100 in PBS for 10 min, and incubated with rhodamine-conjugated phallolidin (Sigma Chemical Co.) 1:1,000 for 30 min and mounted above observation and photography. For the staining of the actin filaments, plastic coverslips (Nunc) were coated with PBS containing 16 μg/ml type I collagen overnight at 4°C and blocked with 1% BSA in PBS for 1 h at 37°C. Cells were allowed to adhere and spread on collagen coated coverslips for 24 h in DME. Coverslips were washed once with PBS and fixed with 2% paraformaldehyde, permeabllized with 0.5% Triton X-100 in PBS for 10 min, and incubated with rhodamine-conjugated phallolidin (Sigma Chemical Co.) 1:1,000 for 30 min and mounted above observation and photography.

Flow Cytometry

Cells were grown to early confluence, 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 BSA, 1 mg/ml glycine, and 0.02% NaN3 for 20 min at 4°C. Cells were collected by centrifugation, exposed to saturating concentration of mAb against α2 integrin (12F1) or αv integrin (SR-84) in BSA/PBS (BSA concentration 1 mg/ml) containing NaCl for 30 min and +4°C, and stained with rabbit anti–mouse IgG coupled to fluorescein (1:20 dilution; Dako A/S) for 30 min at 4°C. Cells were washed twice with PBS containing NaN3 and suspended in the same buffer. To measure the amount of α2 integrin on the cell surfaces, the fluorescent excitation spectra were analyzed by using a FACSscan apparatus (Becton Dickinson). Control samples were prepared by treating the cells without primary antibodies.

Immunoprecipitation of Integrin from Metabolically Labeled Cells

Cells were metabolically labeled with 100 μCi/ml of [35S]methionine (Tran35S]-label, ICN Biomedicals Inc.) for 16 h in methionine-free minimum essential medium. Cell monolayers were rinsed on ice with a solution containing 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 25 mM Tris-HCl, pH 7.4, and then detached by scraping. Cell pellets were obtained by centrifugation at 50,000 g for 5 min. Cells were homogenized in 10% of the same buffer containing 100 mM N-octyl-p-D-glucopyranoside (Sigma Chemical Co.) 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% vol/vol) and BSA (0.5 mg/ml) were added to the supernatants, which were preclarified by incubation with 50 μl of packed protein A–Sepharose (Pharmacia LKB Biotechnology Inc.). Supernatants were immunoprecipitated with antiintegrin antibody (12 F1 or A B1934) for 12 h at 4°C followed by incubation with secondary antibody (rabbit anti–mouse, DAKO), when 12F1 was used. 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 BSA, and twice with 0.5 M NaCl and 25 mM Tris-HCl, pH 7.4. The immunoprecipitates were analyzed by electrophoresis on SDS-containing 6% polyacrylamide gels under nonreducing conditions followed by fluorography.

Transcriptional Nuclear Run-on Analyses

The cells were lysed with NP-40 (ICN) and the nuclei were isolated by centrifugation (12,000 g for 3 s at 4°C). The nuclei were incubated in the presence of 100 μCi of [α-32P]UTP (3,000 Ci/mm, NEN) for 30 min at room temperature as described previously (Banerji et al., 1984). Radiolabeled RNA was hybridized with 2 μg of nitrocellulose-fixed plasmids: cDNA’S for human α1(I) collagen, human α2(I) collagen, GA PDH, and pBluescript (Stratagene). The hybridization and washing conditions used were as described previously (Sistonen et al., 1992). Quantitation was performed with GS-250 Molecular Image System (Bio-Rad Laboratories) and the results were corrected for the levels of GA PDH transcriptions in the same samples.

Cell Spreading Assays

The coating of a 96-well immunoplate (Maxi Sorp; Nunc) was done by exposure to 0.2 ml of PBS, pH 7.4, containing 0.1 μg/cm2 (1.64 μg/ml) 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% BSA in PBS for 1 h at 37°C. BSA was also used to measure the nonspecific binding. 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 Chemical Co.). In cell spreading assays, cells were suspended in DME with 50 mM cycloheximide (Sigma Chemical) 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 one microscopic field and the percentage of spread cells were counted. A spread cell was characterized as one having a clearly visible ring of cytoplasm around the nucleus.

Assay of MAPK Activation

The activation of ERK 1 and 2, JNK/SA PK, and p38 MAPK, was determined by Western blotting using antibodies specific for the phosphorylated, activated forms of the corresponding MAPKs (New England Biolabs). The control blots for the total (phosphorylated and nonphosphorylated forms) protein levels were done by using antibodies recognizing the corresponding MAPKs (p38, ERK2, New England Biolabs; JNK1, Santa Cruz Biotechnology). Saos cell clones were either grown in monolayer for 24 h or seeded in collagen gels as described earlier. Once polymerized, the gels were incubated from the dish and incubated for the times indicated. The cells were released from the gels as described above and lysed in 100 μl of Lammli sample buffer. Cells grown in monolayer were washed once with warm PBS and lysed in 100 μl of Lammli sample buffer. The positive control treatment for the JNK Western blot was done by treating cells in suspension with 10 μg/ml anisomycin (Boehringer Mannheim). The samples were sonicated, fractionated by 10% SDS-PAGE, and transferred to

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In Vitro p38 and JNK Kinase Assay and Western Blot Analysis of Flag-tagged Protein

Subconfluent Saos cell clones plated on 60-mm dishes were transfected using 4 μl of FuGene 6 transfection reagent (Boehringer Mannheim) and 2 μg of either eukaryotic expression vector alone (pcDNA3; Invitrogen Corp.) or the same vector containing the tag-flagged p38 isoform (New et al., 1998). Cotransfection of dominant negative signaling proteins was done by using 12-16 μg of FuGene 6, 3-8 μg of empty expression vector or the same vector containing the effector mutant and 2 μg of flag-tagged p38. 36 h after transfections, the cells were treated with collagen gel for 3 h as described earlier or left untreated. Cells were solubilized with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 0.5 mM DTT, 1 mM PM SF in PBS, pH 7.4) supplemented with leupeptin, antipain, and pepstatin, 2 μg/ml each. The extracts were centrifuged 3,000 rpm for 15 min at 4°C. 1 μg of M2 antibody (Eastman Kodak Co.) conjugated to protein G-Sepharose (Pharmacia-LKB Biotechnology) was used for immunoprecipitation. The immunoprecipitates were washed twice with RIPA buffer, once with LiCl wash buffer (500 mM LiCl, 100 mM NaCl, pH 8.0) and diluted so that equal number of cells from both clones were used. The cells were either lysed immediately, treated with anisomycin for 10 min or seeded inside collagen for 3 h.

Immunoprecipitation of FAK and Western Blot Analysis

The cells were brought into suspension, counted, and diluted so that equal number of cells from both clones were used. The cells were then either lysed immediately, treated with anisomycin for 10 min or seeded inside collagen for 3 h.

Results

Chimeric α2/α1β1 Integrin Functions like the Wild-type α2β1 Receptor in the Formation of Focal Contacts

Initially, we made stable transfectants expressing wild-type α2 or chimeric α2/α1 subunit using Saos-2 cells, which have endogenous α1β1 but no α2β1 (Vihinen et al., 1996). Flow cytometric analysis using anti-α2 mAb (Fig. 1 B) confirmed that both the wild-type and the chimeric receptor were expressed on the surface of the transfected cells. Based on the flow cytometric measurements, we selected cell clones with similar expression levels of wild-type α2 or chimeric α2/α1 integrin to be used in the experiments (Fig. 1 B). A iso, the cell clones used in the experiments and not shown in the Fig. 1 B were tested. In addition, we measured the expression levels of α1β1 from both mock-transfected and α2-transfected cells to ensure that the α2 CDNA transfections did not alter the expression of the endogenous α1β1. The average levels of α1 integrin on four independent single cell clones tested were 60.8 ± 3.8 (arbitrary units) in mock-transfected cells and 68.3 ± 8.6 in α2-transfected cells. The corresponding values for α2 integrin were 13.5 ± 0.7 (represents negative background level fluorescence) and 362 ± 54. Immunoprecipitation experiments were performed to confirm that the chimeric α2/α1 subunit was associated with the endogenous β1 subunit to form a typical heterodimeric αβ complex. The chimeric structure of the heterodimer was verified using an antibody recognizing the α1 cytoplasmic tail (not shown).

Deletion of the α2 cytoplasmic domain has been shown to result in ligand-independent recruitment of the integrin to preformed focal contacts (Kawaguchi et al., 1994). To study if swapping of the α2 cytoplasmic domain to the significantly shorter α1 tail results in indiscriminate integrin recruitment into focal adhesion sites formed by other integrins, we studied both wild-type α2 and chimeric α2/α1 in Saos-2 cells spread on serum proteins. Both clones showed a diffuse staining pattern indicating that the recep-
tors do not localize to focal contacts ligand independently (Fig. 2 A, a and b). When these clones were allowed to adhere to and spread on collagen in serum-free conditions both wild-type and chimeric receptors were able to form focal contacts (Fig. 2 A, c and d) indicating that the a2 chain cytoplasmic tail can be replaced with the corresponding a1 sequence without affecting cellular localization of the receptor. Also, the formation of actin stress fibers on collagen was similar in both clones (Fig. 2 B). In cells cultured inside three-dimensional collagen, no aberrant clusters of integrins were seen and both receptors showed a diffuse staining pattern (Fig. 2 A, e and f).

**a2 Cytoplasmic Tail Is Required for Fast Spreading on Collagen and Collagen Gel Contraction**

Cell adhesion to type I collagen was studied to determine the effect of swapping the tails on the adhesive properties of the integrin. Adhesion mediated by the chimeric a2/a1 receptor was found to be equivalent to that of the wild-type receptor (Fig. 3 A). Both the wild-type a2-transfected cells and the a2/a1 chimera-transfected cells adhered to collagen at the early time point studied (1 h) more efficiently than the mock-transfected cells. When studying focal contact formation by a2 wild-type and chimeric a2/a1-transfected clones, we noticed that even though cell adhesion to collagen was not influenced by the replacement of the a2 cytoplasmic tail with the a1 tail, the efficiency of the clones to spread on collagen was somewhat different. The cells expressing a2 wild-type receptor spread faster than cells expressing a2/a1 chimeric receptor, so that after 30 min on collagen 61.5 ± 6% of adherent a2-transfected cells had spread, whereas only 43 ± 8.3% of adherent a2/a1-transfected cells had spread (Fig. 3 B). At this early time point, mock-transfected cells expressing only a1b1 had just begun to adhere and virtually no spreading was seen. At later time points (i.e., 2 h and later), also the mock-transfected cells spread efficiently on collagen. Since both a2 wild-type and a2/a1 chimeric receptor were expressed at similar levels on the cell surface (Fig. 1 B), the small difference seen in cell spreading may indicate that the cytoplasmic tail of a2 functions in linking the integrin to the cytoskeleton in a manner promoting spreading on collagen. Therefore, we wanted to study the ability of a2 cytoplasmic tail to mediate cytoskeleton-dependent events and assayed the ability of a2- and a2/a1-transfected cells to contract collagen gels. As expected, vector control cells, having endogenous a1b1 but no a2b1, showed very weak contraction during the 72-h experiment (area of the gel 1.8 ± 0.35-fold reduced), whereas wild-type a2b1-expressing cells contract the gel efficiently (area of the gel 4.0 ± 1.0-fold reduced) (Fig. 3 C). The cy-

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**Figure 2.** Characterization of the cellular location of a2 and a2/a1 integrin and formation of actin stress fibers. Stable Saos-2 transfectants were either grown in DME 10% FCS on immunofluorescence glass slides, plated on collagen coated glass slides, and allowed to adhere for 4 h in DME (serum-free) or grown inside a three-dimensional collagen gel for 48 h. (A) After incubation, the cells were fixed and stained for a2 integrin by using mAb followed by FITC-labeled rabbit anti-mouse antibody. Cells were washed, mounted, and examined on a microscope: (a) a2 clone 47 and (b) a2/a1 clone 18 on serum-derived fibronectin and vitronectin; (c) a2 clone 47 and (d) a2/a1 clone 18 on collagen; (e) a2 clone 47 and (f) a2/a1 clone 18 inside collagen gel; and (g) vector clone as a negative control. (B) Cells were stained for actin using fluorescein-conjugated phalloidin (a) a2 clone 47 and (b) a2/a1 clone 18 on collagen.
The gels were photographed and surface areas of the gels were measured. The age of spread cells was counted. (C) The ability of various collagen receptor integrins to mediate collagen gel contraction was tested using stable transfected clones of human osteosarcoma Saos-2 cells (mock-, α2-, or α2α1-expressing clones). 5 × 10^4 cells were seeded inside a collagen gel, the polymerized gel was detached from the sides of the dish and the cells were cultured for 3 d in DME 10% FCS. The gels were photographed and surface areas of the gels were measured.

toplasmic tail of α2 seemed to be essential for linkage of the integrin to the cytoskeletal machinery since the cells expressing chimeric α2α1 failed to contract the collagen gel (Fig. 3 C).

Integrin α2 Cytoplasmic Tail Is Required for the Upregulation of Collagen α1(I) and α2(I) Gene Expression at the Transcriptional Level

Overexpression of the wild-type α2 in Saos-2 cells resulted in upregulation of the mRNA levels of both collagen α1(I) (1.4–4.3-fold) and α2(I) (3.3-fold) in response to three-dimensional collagen matrix. To test the generality of this observation, all together three independent single cell clones overexpressing α2β1 were tested (not shown). In contrast, vector control cells showed a downregulation ranging from 0.8 to 2.4-fold for collagen α1(I) mRNA (Fig. 4, A and B). Interestingly, the chimeric receptor failed to mediate upregulation of the collagen mRNA s in response to three-dimensional collagen matrix as seen with the Northern blot hybridization of total RNA isolated from two single cell clones pooled together. The mRNA levels of collagen α1(I) in cells expressing the chimeric receptor were downregulated by 1.5–1.8-fold in response to collagen compared with the average 3.6-fold upregulation seen in cells expressing the α2 wild-type receptor (Fig. 4, A and B). Also, expression of collagen α2(I) was downregulated in the α2α1-transfected cells. These results were again confirmed to be reproducible with RNA from a third independent single cell clone (not shown). Together, cells transfected with the chimera responded to collagen identically to the mock-transfected cells. Integrin α1β1 is known to function as a negative regulator of collagen (Langholz et al., 1995; Riikonen et al., 1995a). The transmembrane region of this receptor has been suggested to signal through interaction with caveolin (Wary et al., 1996), whereas the possible signaling function of α1 cytoplasmic domain cannot be excluded. In the α2α1-transfected cells, no further reduction of collagen levels was seen when compared with the mock-transfected cells having endogenous α1β1 integrin, suggesting that the short α1 cytoplasmic tail is not alone sufficient to regulate collagen gene expression. To test this further, we transfected Saos-2 cells with the X2C5PFNeo plasmid coding for α2 subunit with α5 cytoplasmic tail (Kawaguchi and Hemler, 1993). A gain, similarly as both the mock-transfected and the α2/ α1-transfected cells, collagen mRNA levels were decreased in response to collagen (not shown).

To assess the contribution of increased transcription of collagen α1(I) on this elevation seen in mRNA levels, we performed nuclear run-on experiments. Nuclei were isolated from α2- or α2α1-transfected cells grown either in monolayer or in three-dimensional collagen gel for 48 h. The rate of the collagen gene transcription was compared with that of GAPDH. A 1.8-fold increase of transcription was seen in α2-transfected clones cultured in three-dimensional collagen when compared with the rate of transcription in cells grown in monolayer (Fig. 4 C). In contrast, cells transfected with the chimeric α2α1 chain showed a 1.5-fold decrease in transcription rate (Fig. 4 C). Even though the increased transcription rate of collagen α1(I) gene accounted for most of the upregulation seen in α2β1-overexpressing clones, we also wanted to investigate the stability of the mRNA s. We used actinomycin D to block transcription in cell clones grown in monolayer and in collagen gels. Type I collagen mRNA s seem to have relatively long half-lives (>8 h) in Saos-2 cells transfected with wild-type α2, but no obvious difference was seen between
the various culture conditions. Both in monolayer and in three-dimensional matrix collagen, mRNA levels were reduced by ~50% after 8 h treatment with actinomycin D (Fig. 4 D).

**α2β1 Integrin-mediated Upregulation of Collagen α1(I) Expression in Three-Dimensional Collagen Requires p38 MAP Kinase Pathway**

To identify the downstream components of α2β1-mediated upregulation of collagen gene expression, we tested specific inhibitors at concentrations sufficient to inhibit various signaling kinases (Alessi et al., 1995; Dudley et al., 1995; Broberg and Heino, 1996). The cells were exposed to inhibitors before seeding them inside collagen. Of the various compounds tested, the p38 inhibitor, SB203580, showed the most potent inhibition of collagen α1(I) gene expression (5.5-fold) in α2β1-overexpressing cells grown in three-dimensional collagen gel (Fig. 5 A). The compounds that showed some inhibition (about twofold) included tyrosine kinase inhibitor herbimycin A, PKA inhibitor KT5823, PKA inhibitor KT5720, and Ras farnesylation inhibitor (R)-(+)-Perillyl alcohol (POH), whereas MEK inhibitor PD98059 had no effect. High concentrations of the PKC inhibitor bisindolylmaleimide resulted in downregulation of collagen mRNA levels (twofold at 5 μM and 4.1-fold at 20 μM concentrations), but this effect was seen in both α2- and mock-transfected cells and was therefore considered to be nonspecific. In addition, phosphatidylinositol-3-kinase (PI-3K) inhibitor, wortmannin treatment resulted in a slight reduction of collagen α1(I) mRNA in α2-transfected cells (not shown) and this effect was smaller in vector-transfected cells (Fig. 5 A). The small effects of various inhibitors suggest that corresponding signaling proteins might participate in integrin signaling, but this hypothesis was not studied further. Another specific inhibitor targeting the p38 MAP kinase pathway, SKF86002, was tested to confirm the result obtained with SB203580. Treatment with this compound also resulted in concentration-dependent inhibition of α2β1-mediated upregulation of collagen α1(I) mRNA levels (6.1-fold at 10 μM and 9.5-fold at 20 μM). 20 μM SB203580 was a potent inhibitor of collagen mRNA levels in α2-transfected cells inside collagen (Fig. 5 B), but it had no effect on mRNA levels in mock-transfected cells inside collagen (Fig. 5 A) or α2-transfected cells in monolayer (Fig. 5 C), excluding the possibility that the compound could function as a general downregulator of collagen gene expression.

**Three-dimensional Collagen Gel Induce Isoform Specific Activation of p38α in Saos-2 Cells and the Efficient Activation Requires α2 Cytoplasmic Tail**

The ability of the selective p38 inhibitors, SB203580 and
The expression level of collagen was examined with mock-transfected cells and no activation of p38 \(\alpha 2\) was detected. To study whether \(\alpha 2\beta 1\) cytoplasmic tail could specifically activate some isoform of p38, we overexpressed various forms of flag-tagged p38 kinases (New et al., 1998) in Saos cell stable clones expressing either wild-type \(\alpha 2\) or chimeric \(\alpha 2/\alpha 1\). The transfected cells were seeded inside a collagen gel, after 3 h the cells were collected, flag-tagged p38 was immunoprecipitated, and an in vitro kinase assay was performed.

As seen in Fig. 6 E, p38\(\alpha x\) isoform was activated efficiently in \(\alpha 2\)-transfected cells (activity 0.47 units; arbitrary units = densitometric units – background), whereas in \(\alpha 2/\alpha 1\)-transfected cells the activity was 0.03. No activation of p38\(\beta 2\) was detected (\(\alpha 2\) clone 45 = 0.04 and \(\alpha 2\alpha 1\) clone 12 = 0.02). p38\(\gamma\) showed high activity in both cell clones (\(\alpha 2\) clone 45 = 0.24 and \(\alpha 2\alpha 1\) clone 12 = 0.32) and p38\(\delta\) activity was high in both clones (\(\alpha 2\) clone 45 = 0.64 and \(\alpha 2\alpha 1\) clone 12 = 0.23). The results were confirmed with two individual \(\alpha 2\)- or \(\alpha 2\alpha 1\)-expressing clones (Fig. 6 F). The expression levels of the transiently transfected kinases in various clones were equal as shown by Western blot analysis done by using antibody against the flag-tag.

**The Effect of p38 Inhibitors SB203580 and SKF86002 on the Various p38 Isoforms Expressed in \(\alpha 2\)-Transfected Saos-2 Cells**

As seen in Fig. 6 E and F, p38\(\alpha x\) was activated only in \(\alpha 2\)-transfected cells and no activation of p38\(\beta 2\) was seen.
However, equally high activity of p38γ was seen in both α2- and α2/α1-transfected cells and the p38β activity was higher in α2 cells than in α2/α1 cells. To study which of these activated kinases are relevant to the α2-mediated upregulation of collagen, shown to be inhibited by chemical inhibitors (SB203580 and SK F86002), we tested the effect of these inhibitors in an in vitro kinase assay. α2-transfected Saos-2 cells were transiently transfected with flag-tagged p38 isoforms (α, β2, γ, or δ), the cells were treated with collagen and the kinase activity of each iso-
form was measured in the presence or absence of the inhibitory compound. In accordance with previously published experimental data and recent structural evidence (Kumar et al., 1997; Whitmarsh et al., 1997; Lisnock et al., 1998) the inhibitor SB 203580 had no effect on the γ and δ isoforms of p38 (Fig. 7). Previously, SKF 86002 has been shown to inhibit p38α (Lee et al., 1994) and we show here that it has no effect on the γ and δ isoforms. From these results we can conclude that it is the p38α isoform that is essential in the α2β1 integrin–dependent upregulation of collagen.

Collagen Gel Induces Transient Activation of ERK1 and 2 but not JNK/SAPK in Both the α2- and α2/α1-Transfected Cells

We have recently shown that seeding dermal fibroblasts inside three-dimensional collagen gels results in activation of ERK1 and 2, JNK/SAK, and p38 MAPks (Ravanti et al., 1999). Therefore, we examined also the activation of ERK1 and 2 and JNK/SAK in both α2- and α2/α1-transfected Saos-2 cells by Western blot analysis of cellular proteins, using phosphospecific antibodies to detect activated forms of these MAP kinases. The levels of activated ERK1 and 2 were increased 2 h after seeding the cells inside collagen (3-fold in α2 and 1.5-fold in α2/α1 cells) and they increased further (up to 4-fold in α2 and 7-fold in α2/α1 cells) at 6-h time point. This activation in response to the collagen gel was transient in both clones since no phosphorylated ERK1 or 2 was detected at 12-h time point. Protein levels of ERK2 remained constant at all time points, as shown with the antibody recognizing all forms of ERK2 (Fig 8 A). Low levels of ERK1 were also detected at 2-, 4-, and 6-h time points. No induction in the levels of phosphorylated JNK/SAK was seen in these cells in response to three-dimensional collagen. Some activated JNK/SAK was seen at 0-h time point, immediately after trypsinization but no active protein was detected inside collagen gel. Treatment with anisomycin was used as a positive control for JNK activation. Protein levels of JNK1 remained constant at all time points, as shown with the antibody recognizing all forms of JNK1 (Fig. 8 B). The results with the phosphospecific antibodies were confirmed with a JNK in vitro kinase assay. Endogenous kinase was immunoprecipitated with anti-JNK1 antibody recognizing also JNK2 and 3 and recombinant c-Jun protein was used as a substrate (Fig. 8 C).

Three-dimensional Collagenous Matrix Fails to Activate FAK

Ligation of integrins leads to activation of FAK. To check whether FAK would play a role in the activation of p38 in response to three-dimensional collagen, we allowed both α2- and α2/α1-transfected cells to interact with collagen gels for 1, 2, or 3 h. (Fig. 9 or not shown). The polymerization of the collagen gel takes place in 1 h, so shorter time points could not be studied. No phosphorylated FAK was detected in cells treated with collagen, in contrast to cells from both clones adhering to fibronectin (Fig. 9). Some phosphorylation of FAK was seen in cells lysed immediately after trypsinization. The experiment was repeated with similar results by using antibodies recognizing all phosphorylated forms of FAK (Fig. 9). In the lower panel of Fig. 9 phosphorylated FAK is the upper band. The lower band seen in all samples could not be recognized by anti-FAK antibody and its identity is unknown.

Effect of Inhibitors of GTPases and MAPKKs on α2β1-Mediated Activation of p38α

To investigate possible downstream effectors of α2β1 integrin in the activation of p38α, we used dominant negative mutants of the Rho family GTPases and the p38 upstream kinases, MKK3 and MKK4. The effector mutants or an empty vector in control cells were cotransfected with the flag-tagged p38α into the α2-expressing cells and, 36 h after transfection, the cells were exposed to three-dimensional collagen and an in vitro p38 kinase assay was performed. Of the Rho family GTPases Cdc42 seemed essential for α2β1-mediated signaling since dominant negative Cdc42 constantly resulted in an inhibition of p38α activity (Fig. 10 B). Similar inhibition was not seen when the cells were transfected with wild-type Cdc42, used as a control. In three separate experiments, 4 µg/plate dominant negative Rac slightly decreased p38α activity (75 ± 12% of control) and mutant RhoA was only somewhat more effective (70 ± 27% of control). The experiment was also repeated with higher plasmid concentration (8 µg/plate) and p38α activity was unaltered in dominant negative Rac transfected cells (116% of control). Mutant RhoA showed some inhibition (66% of control). A gain, dominant negative Cdc42 was the most efficient (17% of control). The dominant negative forms of the MAPK kinases known to function upstream of p38, namely MKK3 and MKK4, both had an inhibitory effect. Dominant negative MKK3 inhibited p38α activity by 90–91% and dominant negative MKK4 by 76–90% (Fig. 10). These results indicate that the activity of Cdc42 and the MAPK kinases MKK3 and MKK4 are necessary for the α2β1 integrin-mediated p38α activation.

Discussion

The integrins provide a physical linkage between the cy-

Figure 7. The effect of p38 inhibitors SB 203580 and SKF 86002 on the various p38 isoforms. Equal numbers of stable transfected Saos-2 cells were transiently transfected with flag-tagged p38 isoforms (α, β2, γ, or δ). 36 h after transfection, the cells were detached from the plate and seeded inside collagen gel. A 2-h incubation with inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added. Levels of phosphorylated ATF2 protein as a substrate to the kinase reaction the inhibitors or DMSO (control) were added.
Integrin 
subunit–specific interactions with other cellular proteins are of special interest because they may explain the distinct signaling functions of integrin heterodimers sharing a common β1 subunit (Werb et al., 1989). The two collagen receptors, α1β1 and α2β1 integrins, have distinct effects on cellular signaling and gene expression (Langholz et al., 1995; Rikonen et al., 1995a; Ravanti et al., 1999). Here, the function of their α cytoplasmic domains was studied by swapping the α1 tail into α2 and expressing the chimeric integrin in cells negative for α2 integrin. The swapping of the α1 and α2 subunit cytoplasmic domains did not affect the localization to focal adhesions or the ability to mediate cell adhesion to collagen. This is in agreement with previously published data where the specific sequence of the α tail seemed less important than the number of residues present. Four to seven residues after the conserved GFFKR sequence were needed to be included for optimal adhesive activity (Kassner et al., 1994). Here, fast cell spreading and the ability to contract three-dimensional collagen gels were impaired if the cells expressed chimeric α2α1 instead of the α2 integrin. Previously, α2, α4, and α5 cytoplasmic tails have been shown to be interchangeable with respect to their positive contri-

Figure 8. Regulation of ERK and JNK MAPKs in response to collagen gel. Cells from two separate α2 or α2α1 single cell clones (45, 47 and 2, 12) were pooled together and either lysed immediately (0 h sample) or seeded in collagen gel and incubated for different periods of time, as indicated. (A) The levels of activated ERK1 and 2 (ERK1-P and ERK2-P) were determined by Western blot analysis using a phosphospecific antibody for ERK1 and 2, and an antibody recognizing all forms of JNK1 was used as a control. The positive control treatment (+) was 10 min anisomycin (10 μg/ml). For the kinase assay, equal numbers of stable transfected Saos-2 cells (α2 or α2α1) were either lysed immediately after trypsinization, treated with collagen for 3 h or anisomycin (10 μg/ml) for 10 min, and the JNK kinase activity was assayed.

Figure 9. Three-dimensional collagenous matrix fails to activate FAK in Saos-2 cells. Cells from two separate α2 or α2α1 single cell clones (45, 47 and 2, 12) were pooled together and either lysed immediately (0 h sample), seeded in collagen gel, or allowed to adhere to fibronectin for 1 h. FAK was immunoprecipitated and immunoblotted with an antiphosphotyrosine antibody (4G10). Alternatively, cell lysates were fractionated on SDS-PAGE and immunoblotted with phosphospecific FAK antibody. The blots were reprobed with anti-FAK antibody to show the levels of FAK protein. Note that in the lower panel the anti-phospho FAK antibody recognizes two bands, but it is the upper one that comigrates with the band recognized with the anti-FAK antibody. The origin of the lower band is unknown.
Figure 10. A possible role for RhoA, Cdc42, M K K 3 , and M K K 4 in integrin α2β1-mediated activation of p38α. Equal numbers of stable α2-transfected Saos-2 cells were transiently cotransfected with flag-tagged p38α (2 μg) and the dominant negative form of the protein indicated (4 μg). The cells were treated and assayed for p38 kinase activity. The possible effect of the cotransfected plasmid on p38α-flag expression was checked with Western blot analysis of the cell lysate using flag-tag recognizing antibody M2. A autoradiogram of a representative experiment is shown. (B) Various concentrations of dominant negative (DN) Cdc42 were tested in cotransfections with 2 μg of p38α-flag. Each experiment was repeated 2 to 4 times and mean values ± SE M are shown. Transfection of wild-type (wt) Cdc42 was used as a control.
which the cytoplasmic domain of α2 is replaced with the corresponding sequence in α1, is not able to upregulate collagen synthesis in response to three-dimensional matrix. Second, regulation of collagen gene transcription was shown to require p38 MAPK activity based on the use of two p38 kinase inhibitors, SB 203580 and SK F 86002. Third, contact with three-dimensional collagen results in only a transient activation of ERK1 and 2, no evident activation of p38-like kinase. A c- tivation of p38 MAPK requires intact α2 subunit since levels of activated p38 were significantly lower in cells expressing chimeric α2/α1 receptor. Finally, using transient transfections of flag-tagged isoforms of p38 and dominant negative signaling proteins, we were able to show that α2 cytoplasmic domain specifically activates the α isoform and has no effect on the p38β2 isoform. We also show that the activity of Cdc42 and the MAPK kinases M KK 3 and M KK 4 is necessary for the α2β1 integrin-mediated p38α activation.

The data presented clearly show that the p38α isoform is essential in the α2β1 integrin-dependent upregulation of collagen expression. The facts to support this area follows. First, the p38α isoform is activated in response to collagen in the α2-transfected but not the α2/α1-transfected cells. Second, the p38β2 isoform is not activated in these cells. Third, even though p38γ showed high activity in both clones and p38βγ was more efficiently activated in the α2-transfected cells, these isoforms cannot be responsible for the upregulation of collagen gene expression since the p38 inhibitors used have no effect on these kinases (Fig. 7). Together, these findings demonstrate that the cytoplasmic sequence of α2 integrin subunit regulates the ability of α2β1 integrin to activate p38 kinase in an iso- form-specific manner and suggest a novel signaling mecha- nism for α2β1.

An issue that arises from the data is the mechanism by which α2β1 integrin activates the p38 pathway. Integrins are known to activate the Rho family of GTPases. Integrin ligation to the ECM leads to the activation of Cdc42 that subsequently activates Rac; Rho, on the other hand, has been shown to be activated independently of Cdc42 by integrin ligation (Price et al., 1998). Of the GTPases Cdc42 has been shown to activate both the p38 and JNK pathways and Rac1 has been shown to activate p38 (Bagrodia et al., 1995; Coso et al., 1995; M inden et al., 1995; S. Zhang et al., 1995). Cdc42 and Rac1 have shown to induce integrin-mediated cell motility on collagen and invasiveness through collagen gels (K eely et al., 1997), suggesting a role for these small GTPases in the inside-out signaling regulating the function of the collagen binding integrins. Here we identify Cdc42 as an important upstream effector of the α2β1 integrin-activated signaling pathway. Cdc42 has been shown to activate both the MAPK kinases M KK 3 and M KK 4 may be involved.

The signaling molecules downstream of Cdc42 and upstream of the MAPK kinases remain to be clarified in further studies. The various p38 isoforms seem to be differentially activated by upstream MAPK kinases: M KK 3, M KK 4, and M KK 6 (Cohen et al., 1997; J iang et al., 1997), which in turn are activated by M A PK KS (D enhardt, 1996). MAPK K KS have been shown to be effective in the p38 pathway involve A SK and TA K 1 (transforming growth factor-activated kinase) (D erijard et al., 1995; Y amaguchi et al., 1995; W. Wang et al., 1997) but little is known about their upstream effectors. PI-3K has been shown to function in integrin-mediated cell migration and invasion and it may function upstream of Rac1 and Cdc42 (K eely et al., 1997). In fact, a lipid product of PI-3K has been shown to interact with Rac1 (M issy et al., 1998). A recent study shows that Cdc42 controls integrin-dependent activation of Akt (Clark et al., 1998). Very recently a mechanism was suggested in which PI-3K activates NF-κB (B eraud et al., 1999), a transcription factor that has been shown to be activated in fibroblasts in response to collagen gel (X u and Clark, 1997). In our experiments, the PI-3K inhibitor, wortmannin, slightly reduced α2β1-mediated upregulation of collagen α1(II) thereby leaving open the possibility that PI-3K may be one of the upstream effectors of the signaling pathway described here.

Other candidates for upstream effectors that could mediate α2β1-related activation of p38 include p21 activated kinases (PA K s), the best characterized effectors of Cdc42 and A CK s (activated Cdc42-binding kinases) recently shown to be activated by cell adhesion via integrin β1 (Y ang et al., 1999). In a study published by B ourdoulos et al. (1998), alteration in integrin occupancy by fibronectin led to upregulation of A CK and p38 MAPK while a concomitant inhibition of PA K and JNK/S A K was seen. This is interesting from the point of view of this discussion since similar effects on the MAPK s were seen in the α2-transfected Saos cells in response to collagen. In addition, T A K 1 might be an interesting candidate, since it is activated by TGF-β and has been shown to activate both M KK 3 and M KK 6 (Y amaguchi et al., 1995; E nslen et al., 1998). On the other hand, TGF-β has many effects on the cell, one of them being the upregulation of collagen levels (M assagué, 1990). Ho w is integrin signaling then transduced to these molecules, especially to Cdc42? One attractive model for α2β1 signaling would be the phosphorylation of the α2 cytoplasmic domain. Phosphorylation in response to integrin ligation could generate a binding site for an effector kinase inside the cell. H owever, our preliminary data have failed to convincingly show α2 phosphorylation in response to binding to collagen (Ivaska, J., and J. H eino, unpublished results).

It is evident that the two collagen receptors studied here function in close collaboration to regulate cell behavior in response to collagenous matrix. Impaired regulation of collagen turnover may lead to pathological conditions. For example, skin fibroblasts from scleroderma patients show upregulated collagen synthesis and concomitantly reduced expression of α1β1 (I varsson et al., 1993). A striking example of correlated functions of integrins α1β1 and α2β1 is seen in α1 null mice, in which the absence of α1β1 leads to enhanced collagen synthesis in skin. However, simultaneously collagenase-3 expression is increased, possibly via increased α2β1 ligation, leading to a situation in which the collagen accumulation is seen only if the degradation of collagen is prevented (G ardn er et al., 1999). Previous studies have shown the regulation of specific MAPK pathways by α1β1 integrin (W ary et al., 1996) and also introduced the dual role of MAPK pathways in the regulation of collagen production (D avis et al., 1996). Here, we provide new information which connects α2β1 integrin to the regu-
lation of p38α via Cdc42 and MAPKKks, MKK3 and MKK4, and show how α2β1 functions in the regulation of the delicate balance of collagen accumulation in tissues.

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