

Activation of Extracellular Signal-regulated Kinase 1/2 Inhibits Type I Collagen Expression by Human Skin Fibroblasts*

Received for publication, March 16, 2000, and in revised form, July 28, 2000

Published, JBC Papers in Press, August 3, 2000, DOI 10.1074/jbc.C000175200

Niina Reunanen^{‡§}, Marco Foschi[¶], Jiahuai Han^{||}, and Veli-Matti Kähäri^{‡§**}

From the [‡]Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, FIN-20520 Turku, Finland, [§]Department of Medical Biochemistry and Department of Dermatology, University of Turku, FIN-20520 Turku, Finland, [¶]Department of Medicine, University of Florence, Florence 50134, Italy, and ^{||}Department of Immunology, Scripps Research Institute, La Jolla, California 92037

Treatment with the lipid second messenger, ceramide, activates extracellular signal-regulated kinase-1/2 (ERK1/2), c-Jun N-terminal kinase, and p38 in human skin fibroblasts and induces their collagenase-1 expression (Reunanen, N., Westermarck, J., Häkkinen, L., Holmström, T. H., Elo, I., Eriksson, J. E., and Kähäri, V.-M. (1998) *J. Biol. Chem.* 273, 5137–5145). Here we show that C₂-ceramide inhibits expression of type I and III collagen mRNAs in dermal fibroblasts, suppresses pro α 2(I) collagen promoter activity, and reduces stability of type I collagen mRNAs. The down-regulatory effect of C₂-ceramide on type I collagen mRNA levels was abrogated by protein kinase C inhibitors H7, staurosporine, and Ro-31-8220 and potently inhibited by a combination of MEK1,2 inhibitor PD98059 and p38 inhibitor SB203580. Activation of ERK1/2 by adenovirus-mediated expression of constitutively active MEK1 resulted in marked down-regulation of type I collagen mRNA levels and production in fibroblasts, whereas activation of p38 by constitutively active MAPK kinase-3b and MAPK kinase-6b slightly up-regulated type I collagen expression. These results identify the ERK1/2 signaling cascade as a potent negative regulatory pathway with respect to type I collagen expression in fibroblasts, suggesting that it mediates inhibition of collagen production in response to mitogenic stimulation and transformation.

Fibrillar type I collagen is an abundant component of the extracellular matrix (ECM)¹ of various human connective tis-

sues. Type I collagen is a heterotrimeric molecule consisting of two α 1 chains and one α 2 chain. The expression of pro α 1(I) and pro α 2(I) collagen genes is coordinately regulated during tissue development, growth, and repair resulting in their synthesis in a 2:1 ratio (1). The expression of type I collagen in fibroblasts is stimulated by transforming growth factor- β (TGF- β) (2), interleukin-4 (3), and connective tissue growth factor (4) and inhibited by epidermal growth factor (5), tumor necrosis factor- α (TNF- α) (2), interferon- γ (2), glucocorticoids (6), and tumor promoters (7, 8) and by contact with three-dimensional collagen (9). Excessive deposition of type I collagen is observed *e.g.* in fibrosis of skin, lungs, and liver (see Ref. 1).

TNF- α is a proinflammatory cytokine that inhibits the formation of ECM by suppressing the expression of type I collagen and by inducing the production of matrix metalloproteinases (MMPs) by fibroblasts (2, 10). Binding of TNF- α to its 55-kDa cell surface receptor activates neutral sphingomyelinase, which hydrolyzes cell membrane sphingomyelin to the lipid second messenger, ceramide. We have recently shown that synthetic cell-permeable C₂-ceramide enhances fibroblast collagenase-1 (MMP-1) expression via coordinate activation of the following three distinct mitogen-activated protein kinase (MAPK) pathways: extracellular signal-regulated kinase-1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 (11).

Here we show that activation of ceramide signaling pathway potently inhibits the expression of type I collagen in human skin fibroblasts and that this involves coordinate activation of ERK1/2 and p38 MAPKs. In addition, we show that specific activation of ERK1/2 by adenovirus-mediated expression of constitutively active MEK1 results in potent suppression in type I and III collagen expression by normal skin fibroblasts. These results identify ERK1/2 as a potent negative regulatory pathway with respect to expression of type I collagen in fibroblasts, suggesting that it plays a role in the control of collagen deposition, *e.g.* in wound repair and tumor growth.

EXPERIMENTAL PROCEDURES

Materials—C₂-ceramide, H7, staurosporin, Ro-31-8220, PD98059, SB203580, and 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) were obtained from Calbiochem. Human recombinant transforming growth factor- β 1 (TGF- β 1) was from Sigma.

Cell Cultures—Normal human skin fibroblast cultures were established from punch biopsy of a voluntary healthy male donor (age, 23) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml of penicillin-G, and 100 μ g/ml of streptomycin. NIH-3T3 fibroblasts were obtained from

* This study was supported by grants from the Academy of Finland (Projects 30985 and 45996), the Sigrid Jusélius Foundation, the Cancer Research Foundation of Finland, and Turku University Central Hospital (Project 13336), by a research contract with Finnish Life and Pension Insurance Companies, by Turku Graduate School of Biomedical Sciences, and by personal grants (to N. R.) from Research and Science Foundation of Farnos, The Finnish-Norwegian Medical Foundation, The Finnish Medical Foundation, and the Turku University Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Turku Centre for Biotechnology, University of Turku, Tykistökatu 6B, FIN-20520 Turku, Finland. Tel.: 358-2-3338029; Fax: 358-2-3338000; E-mail: veli-matti.kahari@utu.fi.

¹ The abbreviations used are: ECM, extracellular matrix; MMP(s), matrix metalloproteinase(s); TNF- α , tumor necrosis factor- α ; TGF- β , transforming growth factor- β ; MAPK(s), mitogen-activated protein kinase(s); MKK, MAPK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase; pfu, plaque-forming units; PKC, protein kinase C; FCS, fetal calf serum; CS, calf serum; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole;

DMEM, Dulbecco's modified Eagle's medium; kb, kilobase pair; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis.

ATCC (Manassas, VA) and cultured in similar medium except with 10% CS. For experiments, fibroblasts were maintained for 18 h in culture medium supplemented with 0.5% FCS. C_2 -ceramide was added alone or in combination with TGF- β 1, and the incubations were continued for 24 h. In experiments involving chemical signaling inhibitors, these were added 1 h prior to C_2 -ceramide. Cell viability was determined with trypan blue exclusion as described previously (8).

RNA Analysis—Total RNA was isolated from cells using the single-step method (12). Aliquots of total RNA were fractionated on gels, transferred to filters, and hybridized with cDNAs as described previously (11). The following cDNAs were used for hybridizations: a 0.7-kb human cDNA for pro α 1(I) collagen (13), a 1.2-kb human cDNA for pro α 2(I) collagen (14), a 0.7-kb human cDNA for pro α 1(III) collagen (15), a 2.0-kb human cDNA for collagenase-1 (MMP-1) (16), and a 1.3-kb rat cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (17). The [32 P]cDNA-mRNA hybrids were visualized by autoradiography, quantitated by scanning densitometry, and corrected for the levels of GAPDH mRNA in the same samples.

Transient Transfections and Chloramphenicol Acetyltransferase (CAT) Assays—Confluent NIH-3T3 fibroblast cultures were transiently transfected with 2 μ g of human pro α 2(I) collagen promoter/CAT construct -376 α 2(I)/CAT (18) (kindly provided by Dr. F. Ramirez, Mount Sinai School of Medicine, New York, NY). Transfections were performed by the calcium phosphate/DNA co-precipitation method followed by a 2-min glycerol shock (11). The cells were incubated with C_2 -ceramide (50 μ M) in DMEM and 1% CS for 40 h, and CAT activity was measured as described previously (11). The transfection efficiency was monitored by co-transfecting the cells with 2 μ g of the Rouse sarcoma virus/ β -galactosidase construct and correcting the CAT activities for β -galactosidase activity (11).

Determination of mRNA Stability—Confluent human skin fibroblasts in medium containing 1% FCS were incubated without or with C_2 -ceramide (50 μ M) for 6 h, RNA polymerase II inhibitor DRB (60 μ M) was added, and the cultures were harvested at 6-h intervals for RNA extraction and determination of pro α 1(I) and pro α 2(I) collagen and GAPDH mRNA abundance by Northern blot hybridizations.

Transduction of Fibroblasts with Recombinant Adenoviruses—Recombinant replication-deficient adenovirus RAdlacZ (RAd35), which harbors *Escherichia coli* β -galactosidase (*lacZ*) gene under the control of cytomegalovirus intermediate early promoter, and the empty adenovirus RAd66 were kindly provided by Dr. Gavin W. G. Wilkinson (University of Cardiff, Cardiff, Wales, United Kingdom) (19). Construction and characterization of recombinant adenoviruses harboring mutated, constitutively active MEK1 (RAdMEK1ca) (20), MKK3b (RAdMKK3bE) (21), and MKK6b (RAdMKK6bE) (21) genes driven by cytomegalovirus intermediate early promoter have been described previously. To determine the infection efficiency of normal human skin fibroblasts, cells in suspension were mixed with RAdlacZ at different multiplicities of infection, plated, incubated for 18 h in DMEM with 1% FCS, fixed, and stained for β -galactosidase activity (22, 23). In experiments, 5×10^5 cells in suspension were infected with RAd66, RAdlacZ, RAdMEK1ca, RAdMKK3bE, or RAdMKK6bE, at multiplicities of infection of 500 pfu/cell, which gives 100% transduction efficiency, plated, and incubated for 18 h. Culture medium with 10% FCS was replaced with one containing 1% FCS, the incubations continued for 24 h, and the cells were harvested for RNA extraction. In experiments involving PD98059, this was added to cultures at the time of infection.

Assay of MAPK Activation—Fibroblasts (5×10^5) were infected with RAdlacZ, RAdMEK1ca, RAdMKK3bE, or RAdMKK6bE, incubated, and lysed in 100 μ l of Laemmli sample buffer. The samples were sonicated, fractionated by 10% SDS-PAGE, and transferred to Hybond ECL membrane (Amersham Pharmacia Biotech). Western blotting was performed with phosphospecific antibodies for ERK1/2 and p38 (New England Biolabs, Beverly, MA), in 1:1000 dilution, as described previously (11, 23). As loading controls, the same samples were analyzed by Western blotting using antibodies specific for total ERK1/2 and p38 (New England Biolabs). Binding of primary antibodies was detected with peroxidase-conjugated secondary antibodies and visualized by ECL.

Measurement of Fibroblast Collagen Synthesis—Cells were infected with adenovirus constructs RAd66, RAdMEK1ca, RAdMKK3bE, and RAdMKK6bE, as described above, and incubated for 18 h. The medium was then changed to glutamine-free DMEM with 2% FCS and 50 μ g/ml ascorbate, and the incubation was continued for 24 h. The medium was then replaced with a similar medium containing, in addition, [3 H]proline (20 μ Ci/ml) and β -aminopropionitrile (50 μ g/ml). After a 24-h incubation, the conditioned media were dialyzed against 1 mM EDTA, and aliquots were fractionated by SDS-PAGE in 10% polyacrylamide gel. The gel was processed for fluorography and exposed against Kodak

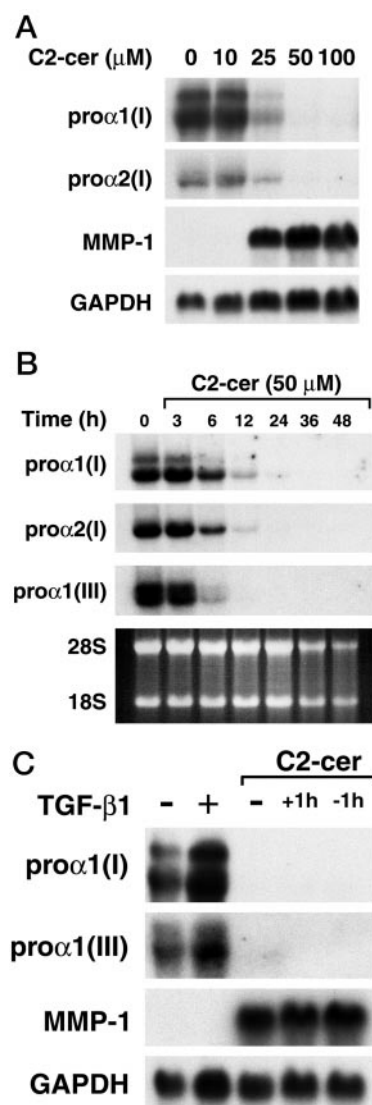


FIG. 1. C_2 -ceramide inhibits type I and III collagen gene expression in fibroblasts. A, normal human skin fibroblasts were incubated for 18 h in DMEM with 0.5% FCS, C_2 -ceramide (C_2 -cer) was added in concentrations indicated, and the incubations were continued for 24 h. B, human skin fibroblasts were incubated in the presence of C_2 -ceramide (C_2 -cer; 50 μ M) for different periods of time, as indicated. C, human skin fibroblasts maintained in DMEM with 0.5% FCS were incubated for 24 h with TGF- β 1 (5 ng/ml) alone or in combination with C_2 -ceramide (C_2 -cer; 100 μ M) added 1 h before or after TGF- β 1. A-C, after incubations, total RNA was isolated, and aliquots (15 μ g) were analyzed for the expression of pro α 1(I), pro α 2(I), and pro α 1(III) collagen, MMP-1, and GAPDH mRNA using Northern blot hybridizations. 28 S and 18 S rRNA were visualized by ethidium bromide staining.

X-Omat film as described previously (24). Collagenous polypeptides were quantified densitometrically.

RESULTS

C_2 -ceramide Inhibits Type I and III Collagen Gene Expression by Human Skin Fibroblasts—We have recently shown that the TNF- α -generated lipid second messenger, C_2 -ceramide, induces expression of MMP-1 and MMP-3 in human skin fibroblasts (11). As TNF- α also potently suppresses the expression of type I collagen by dermal fibroblasts, we wanted to examine the role of the ceramide signaling pathway in this respect. Treatment of human skin fibroblasts with C_2 -ceramide for 24 h resulted in marked suppression of the expression of mRNAs for type I collagen α 1 and α 2 chains noted with concentrations of 25 μ M, and maximal inhibition was observed at a 50 μ M con-

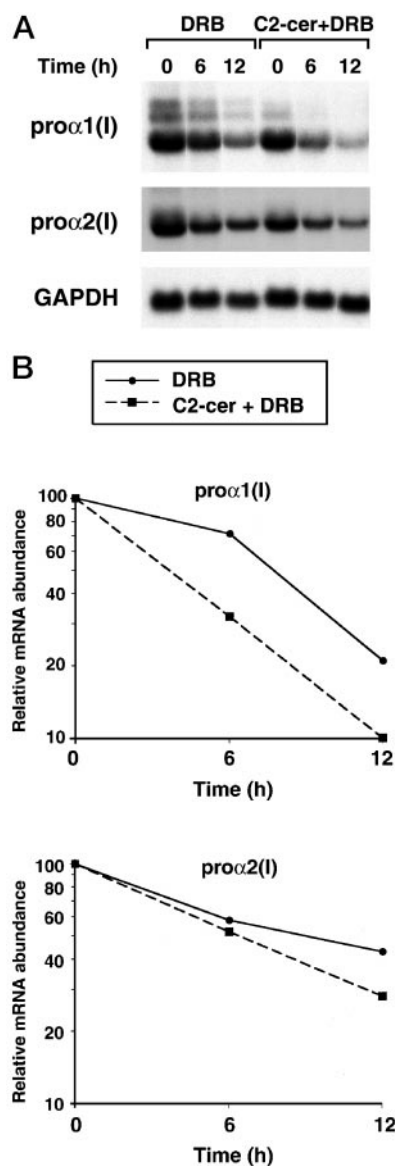


FIG. 2. C₂-ceramide reduces type I collagen mRNA stability in fibroblasts. A, confluent human skin fibroblasts in medium containing 1% FCS were treated with C₂-ceramide (C₂-cer; 50 μ M) for 6 h or left untreated, RNA polymerase II inhibitor DRB (60 μ M) was added, and the control (DRB) and C₂-ceramide-treated cultures (C₂-cer+DRB) were harvested at 6-h intervals for determination of pro α 1(I) and pro α 2(I) collagen and GAPDH mRNA abundance by Northern blot hybridizations. B, type I collagen mRNA levels in control (DRB) and C₂-ceramide-treated cultures (C₂-cer+DRB) were quantitated by densitometry, corrected for GAPDH mRNA levels, and are shown relative to the levels at the time of addition of DRB (100). Data from a representative experiment of two experiments with identical results are shown.

centration (Fig. 1A). Interestingly, induction of MMP-1 mRNA levels was also noted with C₂-ceramide treatment with a concentration of 25 μ M, and the maximal induction was obtained with 50 μ M (Fig. 1A). Suppression of type I and III collagen mRNAs was first noted at 6 h of incubation and was nearly maximal at the 12-h time point (Fig. 1B). Interestingly, the inhibition of type I and III collagen mRNA expression by C₂-ceramide was quite persistent, as it lasted at least 48 h (Fig. 1B). C₂-ceramide also entirely inhibited pro α 1(I) and pro α 1(III) collagen mRNA expression in the presence of TGF- β 1, a potent up-regulator of collagen expression in fibroblasts (Fig. 1C). Interestingly, TGF- β 1 had no effect on C₂-ceramide-elicited induction of MMP-1 mRNA levels, regardless of whether they

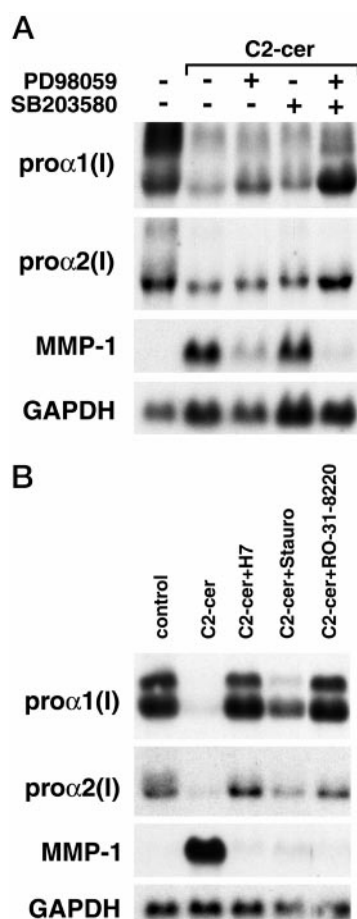


FIG. 3. Inhibition of type I collagen expression by C₂-ceramide is mediated by ERK1/2, p38, and protein kinase C. A, human skin fibroblasts were incubated for 18 h in DMEM with 0.5% FCS. C₂-ceramide (C₂-cer; 50 μ M) was added alone or in combination with MEK1,2 inhibitor PD98059 (40 μ M) or p38 MAPK inhibitor SB203580 (40 μ M), both added 1 h before C₂-ceramide, and the incubations were continued for 24 h. B, human skin fibroblasts were incubated for 18 h in DMEM with 0.5% FCS. C₂-ceramide (C₂-cer; 50 μ M) was added alone or in combination with protein kinase C inhibitors H7 (50 μ M) staurosporine (stauro; 25 nM) and Ro-31-8220 (1 μ M), all added 1 h before C₂-ceramide, and the incubations were continued for 24 h. A and B, total RNA was isolated, and aliquots (15 μ g) were analyzed for the expression of pro α 1(I) and pro α 2(I) collagen, MMP-1, and GAPDH mRNA with Northern blot hybridizations.

were added to cultures 1 h before or after C₂-ceramide (Fig. 1C).

C₂-ceramide Inhibits Pro α 2(I) Collagen Promoter Activity and Reduces Stability of Type I Collagen mRNAs—To determine whether the inhibitory effect of C₂-ceramide on type I collagen expression takes place at the transcriptional level, we transiently transfected NIH-3T3 fibroblasts with the human pro α 2(I) collagen promoter/CAT construct -376 α 2(I)/CAT and subsequently treated the cells with C₂-ceramide (50 μ M) for 40 h. Assay of CAT activity revealed that C₂-ceramide inhibited the activity of the pro α 2(I) collagen promoter by 55 \pm 9% (mean \pm S.E. of three independent transfections each performed in duplicate), as compared with the untreated control cells.

Next, we also examined whether the down-regulatory effect of C₂-ceramide on type I collagen mRNA expression also involves reduced stability of the corresponding mRNAs. A 6-h pretreatment of human skin fibroblasts with C₂-ceramide (50 μ M) followed by co-treatment in the presence of the RNA polymerase II inhibitor DRB revealed that the decay rate of

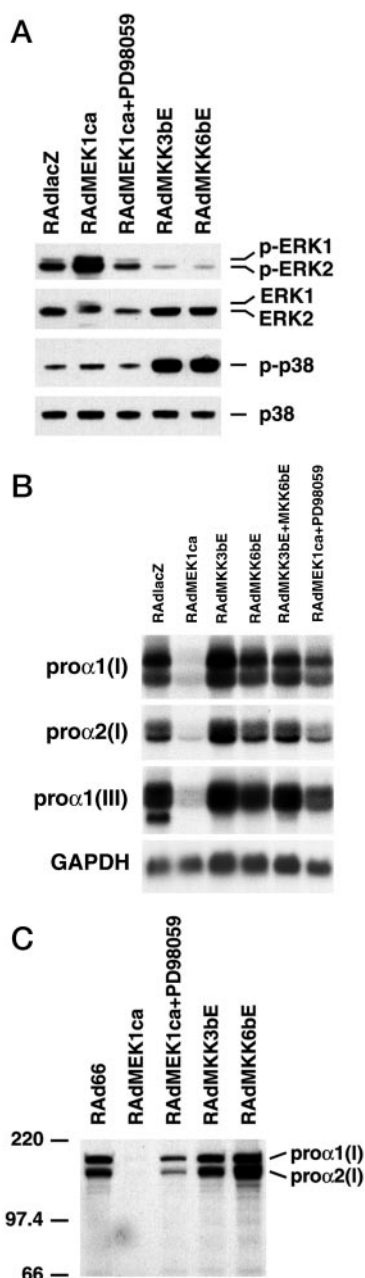


FIG. 4. Activation of ERK1/2 results in inhibition of type I collagen expression by human skin fibroblasts. *A*, human skin fibroblasts (5×10^5) were transduced with recombinant adenoviruses (500 pfu/cell) harboring β -galactosidase (RadlacZ), constitutively active MEK1 (RAdMEK1ca), constitutively active MKK3b (RAdMKK3bE), and constitutively active MKK6b (RAdMKK6bE). MEK1,2 inhibitor PD98059 ($40 \mu\text{M}$) was added to cultures indicated at the time of infection. After 18 h of incubation, medium was changed, fresh PD98059 was added, and the incubations were continued for 24 h. The levels of activated ERK1 and ERK2 (*p-ERK1* and *p-ERK2*) and activated p38 (*p-p38*) in cell lysates were determined by Western blot analysis using phosphospecific antibodies for these MAPKs. As controls, the levels of total ERK1/2 and p38 were determined in the same samples by Western blot analysis using specific antibodies. *B*, human skin fibroblasts were infected and incubated as in *A* and harvested, and aliquots ($9 \mu\text{g}$) of total RNA were analyzed for the levels of pro α 1(I), pro α 2(I), pro α 1(III) collagen, and GAPDH mRNA with Northern blot hybridizations. *C*, human skin fibroblasts were infected with 500 pfu/cell with empty control virus (RAd66) or with adenoviruses bearing constitutively active MEK1 (RAdMEK1ca), MKK3b (RAdMKK3bE), and MKK6b (RAdMKK6bE). MEK1,2 inhibitor PD98059 ($40 \mu\text{M}$) was added to cultures indicated at the time of infection. 16 h after infection, the cells were incubated for 24 h in glutamine-free medium containing ascorbate ($50 \mu\text{g/ml}$) and were subsequently labeled for 24 h with [^3H]proline in the presence of ascorbate and β -aminopropionitrile ($50 \mu\text{g/ml}$). Equal aliquots of conditioned media were fractionated by SDS-PAGE, and

pro α 1(I) and pro α 2(I) collagen mRNAs was accelerated in cells treated with C_2 -ceramide (Fig. 2, *A* and *B*).

Inhibition of Fibroblast Type I Collagen Production by C_2 -ceramide Is Mediated Coordinately by ERK1/2 and p38—We have recently observed that the maximal activation of fibroblast MMP-1 expression by C_2 -ceramide requires coordinate activation of ERK1/2, JNK, and p38 (11). To examine the roles of these MAPK pathways in the ceramide-elicited down-regulation of type I collagen expression, human skin fibroblasts were treated with C_2 -ceramide alone or in combination with the selective MEK1,2 inhibitor PD98059 and with a specific p38 MAPK inhibitor SB203580. As noted above, treatment with C_2 -ceramide ($100 \mu\text{M}$) resulted in nearly total inhibition of the expression of mRNA levels for pro α 1(I) and pro α 2(I) collagen in comparison with untreated control cells (Fig. 3*A*). Interestingly, treatment of cells with the combination of PD98059 and SB203580 markedly inhibited C_2 -ceramide-elicited down-regulation of pro α 1(I) and pro α 2(I) collagen mRNA abundance (Fig. 3*A*). PD98059 alone had a slight inhibitory effect, and SB203580 alone did not alter the effect of C_2 -ceramide on type I collagen mRNA levels (Fig. 3*A*). In comparison, the up-regulation of MMP-1 mRNA levels by C_2 -ceramide was potently inhibited (by 62%) by PD98059, as shown previously (11). Together, PD98059 and SB203580 entirely abrogated the induction of MMP-1 mRNA expression by C_2 -ceramide (Fig. 3*A*). Treatment with SB203580 or PD98059 had no effect on viability of the fibroblasts, as determined with trypan blue exclusion assay (not shown).

Inhibition of Type I Collagen Expression by C_2 -ceramide Is Dependent on Protein Kinase C Activity—Next, we further elucidated the signaling pathways mediating the inhibitory effect of the ceramide signaling pathway on type I collagen expression using chemical protein kinase C (PKC) inhibitors. Interestingly, the down-regulation of pro α 1(I) and pro α 2(I) collagen mRNA abundance by C_2 -ceramide was entirely abrogated by PKC inhibitors H7 ($50 \mu\text{M}$) and Ro-31-8220 ($1 \mu\text{M}$) and also potently by staurosporine (25 nM) (Fig. 3*B*). The induction of MMP-1 expression by C_2 -ceramide was also entirely inhibited by all three PKC inhibitors (Fig. 3*B*). Treatment of fibroblasts with PKC inhibitors H7 and Ro-31-8220 reduced the number of viable cells by 12%, whereas 24-h treatment with staurosporine resulted in reduced viability of nearly all cells, as determined with trypan blue exclusion assay (not shown).

Specific Activation of ERK1/2 Inhibits Type I and III Collagen Expression by Fibroblasts—To directly examine the role of ERK1/2 and p38 MAPK pathways in the regulation of the expression of the endogenous type I collagen genes, we used adenovirus-mediated gene delivery of constitutively active ERK1/2 and p38 MAPK pathways in the regulation of the expression of the endogenous type I collagen genes, we used adenovirus-mediated gene delivery of constitutively active MEK1, MKK3b, and MKK6b to human skin fibroblasts. Transduction of cells with 500 pfu/cell of recombinant adenovirus RAdMEK1ca, harboring constitutively active MEK1, resulted in specific activation of endogenous ERK1/2, but not p38, as compared with control virus (RAdlacZ)-infected cells (Fig. 4*A*). Interestingly, simultaneous treatment of RAdMEK1ca-infected fibroblasts with the MEK1/2 inhibitor PD98059 entirely inhibited activation of ERK1/2 (Fig. 4*A*). In parallel, transduction of cells with adenoviruses RAdMKK3bE and RAdMKK6bE bearing constitutively active MKK3b and MKK6b, respectively, resulted in specific activation of p38 and in reduction in the basal levels of phosphorylated ERK1/2 (Fig. 4*A*).

Interestingly, the activation of ERK1/2 by constitutively ac-

collagenous proteins were visualized by fluorography. The migration positions of pro α 1(I) and pro α 2(I) collagen chains are shown on the right. The migration positions of molecular mass markers (in kDa) are shown on the left.

tive MEK1 resulted in marked reduction in the endogenous mRNA levels for pro α 1(I) (85%), pro α 2(I) (89%), and pro α 1(III) (81%) collagen, as compared with cells transduced with control virus RAdlacZ (Fig. 4B). Inhibition of ERK1/2 activation with PD98059 almost completely restored the expression of type I and III collagen mRNAs in RAdMEK1ca-transduced fibroblasts (Fig. 4B). In contrast, adenovirus-mediated expression of constitutively active MKK3b slightly enhanced type I and III collagen mRNA abundance, and MKK6b alone had no marked effect (Fig. 4B).

Next, we also determined the effect of ERK1/2 and p38 activation on collagen production by dermal fibroblasts. For this, the cells were transduced with empty control adenovirus RAd66, or with RAdMEK1ca, RAdMKK3bE, and RAdMKK6bE, and subsequently labeled with 3 H-labeled proline for 24 h in the presence of ascorbic acid and β -aminopropionitrile, followed by fractionation of equal aliquots of conditioned media by SDS-PAGE. As shown in Fig. 4C, expression of constitutively active MEK1 in dermal fibroblasts entirely inhibited their pro α 1(I) and pro α 2(I) collagen production, and this inhibition was potently abrogated by simultaneous incubation of cells with the MEK1,2 inhibitor PD98059. Expression of constitutively active MKK3b had no marked effect on type I collagen production, and MKK6b even slightly (1.8-fold) up-regulated collagen production by fibroblasts.

DISCUSSION

MAPKs mediate extracellular signals, which regulate cell growth, differentiation, survival, and death (see Ref. 25). At present, the following three mammalian MAPK pathways have been characterized in detail: ERK1/2 pathway (Raf/MEK1, 2/ERK1,2), which is activated by mitogenic signals, JNK/stress-activated protein kinase (SAPK) (MEK kinase-1–4/MKK4, 7/JNK1–3), and p38 (MAPK kinase kinase/MKK3,6/p38 α , β) pathways, which are activated by inflammatory cytokines and cellular stress (see Ref. 25). Phosphorylation of the conserved threonine and tyrosine residues of MAPKs by specific upstream dual-specificity kinases (MAPK kinases) results in activation and nuclear translocation of MAPKs, which in turn phosphorylate and activate nuclear protein kinases, *e.g.* MAPK-activated protein kinases 1, -2, and -3 or transcription factors including Elk-1, c-Jun, and activating transcription factor-2 (see Ref. 25). It has been shown that activation of the ERK1/2 cascade by constitutively active Raf-1 or MEK1 results in transformation of fibroblasts (26, 27) and that the ERK1/2 pathway is activated in renal and breast carcinomas *in vivo* (28, 29). In addition, blocking the ERK1/2 pathway with specific MEK1,2 inhibitor reverses the transformed phenotype of colon carcinoma cells *in vitro* and arrests their growth *in vivo* (30). Recent observations also provide evidence that ERK1/2, JNK, and p38 regulate the proteolytic capacity of normal fibroblasts and malignant cells (9, 11, 23, 31–33).

In the present study, we show that activation of ceramide signaling pathway potentially inhibits type I collagen gene expression in fibroblasts at the level of transcription and mRNA stability and that this effect requires PKC activity, as well as coordinate activation of ERK1/2 and p38 MAPKs. Furthermore, induction of MMP-1 mRNA expression by C₂-ceramide was entirely abrogated by blocking both ERK1/2 and p38 MAPK pathways, as well as inhibition of PKC activity. Together with our previous observations (11), these results show that activation of the ceramide signaling pathway results in potent suppression of type I collagen expression and in induction of MMP-1 and MMP-3 expression in dermal fibroblasts, suggesting that in these cells ceramide pathway plays an important role in mediating the inhibitory effects of TNF- α on ECM deposition.

To examine the specific roles of ERK1/2 and p38 pathways in

the regulation of type I collagen expression we utilized adenovirus-mediated gene delivery of the constitutively active ERK1/2 activator MEK1 and p38 activators MKK3b and MKK6b to normal human skin fibroblasts. The results of these experiments clearly identify the ERK1/2 cascade as a potent negative regulatory pathway with respect to type I and III collagen expression. In contrast, p38 appears to serve as a weak positive regulator of type I and type III collagen expression, possibly because of reduction in the basal levels of activated ERK1/2 in cells expressing constitutively active MKK3b or MKK6b. These observations are in accordance with previous studies showing that blocking the ERK1/2 pathway by dominant negative Raf-1 enhances α 1(I) collagen promoter activity in transiently transfected hepatic stellate cells (34) and that transformation of fibroblasts with oncogenic Ras, the upstream activator of Raf-1, results in suppression of type I collagen gene expression (35, 36). However, the effects of ERK1/2 activation on type I collagen expression appear to be cell-specific, as in human osteoblastic cells the ERK1/2 pathway mediates the stimulation of type I collagen gene expression by TGF- β and bone morphogenetic protein-2 (37) and hypergravity (38). In addition, blocking the ERK1/2 pathway with PD98059 in part inhibits the up-regulation of type I collagen gene expression by insulin-like growth factor-1 in hepatic stellate cells (39).

Controlled deposition of collagenous ECM is an important feature of normal tissue development, growth, and repair, whereas excessive collagen accumulation results in destruction of normal tissue architecture and function, as detected *e.g.* in lung fibrosis, liver cirrhosis, keloids, and hypertrophic scars (1). The results of this study show that activation of mitogen-responsive ERK1/2 MAPK is alone sufficient to inhibit type I and III collagen expression in normal human skin fibroblasts. Together with our recent observations showing that activation of ERK1/2 in human gingival (23) and dermal fibroblasts² results in induction of MMP-1 and stromelysin-1 (MMP-3) expression and suppresses their decorin production (40), the results of the present study suggest that the ERK1/2 signaling pathway plays an important role in mediating the inhibitory signals on fibroblast ECM deposition, *e.g.* during wound repair and tumor growth.

Acknowledgments—The expert technical assistance of Hanna Haavisto and Tarja Heikkilä is gratefully acknowledged. We also thank Drs. E. Vuorio, E. Bauer, and P. Fort for plasmids.

REFERENCES

- Prockop, D. J., and Kivirikko, K. I. (1995) *Annu. Rev. Biochem.* **64**, 403–434
- Kähäri, V.-M., Chen, Y. Q., Su, M. W., Ramirez, F., and Uitto, J. (1990) *J. Clin. Invest.* **86**, 1489–1495
- Postlethwaite, A. E., Holness, M. A., Katai, H., and Raghov, R. (1992) *J. Clin. Invest.* **90**, 1479–1485
- Frazier, K., Williams, S., Kothapalli, D., Klapper, H., and Grotendorst, G. R. (1996) *J. Invest. Dermatol.* **107**, 404–411
- Laato, M., Kähäri, V.-M., Niinikoski, J., and Vuorio, E. (1987) *Biochem. J.* **247**, 385–388
- Hämäläinen, L., Oikarinen, J., and Kivirikko, K. I. (1985) *J. Biol. Chem.* **260**, 720–725
- Goldstein, R. H., Fine, A., Farnsworth, L. J., Poliks, C., and Polgar, P. (1990) *J. Biol. Chem.* **265**, 13623–13628
- Westermarck, J., Ilvonen, E., and Kähäri, V.-M. (1995) *Biochem. J.* **308**, 995–999
- Ravanti, L., Heino, J., López-Otín, C., and Kähäri, V.-M. (1999) *J. Biol. Chem.* **274**, 2446–2455
- Westermarck, J., Häkkinen, L., Fiers, W., and Kähäri, V.-M. (1995) *J. Invest. Dermatol.* **105**, 197–202
- Reunanen, N., Westermarck, J., Häkkinen, L., Holmström, T. H., Elo, I., Eriksson, J. E., and Kähäri, V.-M. (1998) *J. Biol. Chem.* **273**, 5137–5145
- Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Mäkelä, J. K., Raassina, A., Virta, A., and Vuorio, E. (1988) *Nucleic Acids Res.* **16**, 349
- Mäkelä, J. K., Vuorio, T., and Vuorio, E. (1990) *Biochim. Biophys. Acta* **1049**, 171–176
- Sandberg, M., Mäkelä, J. K., Multimäki, P., Vuorio, T., and Vuorio, E. (1989)

² N. Reunanen, M. Ahonen, M. Foschi, J. Han, C. López-Otín, and V.-M. Kähäri, submitted for publication.

- Matrix* **9**, 82–91
16. Goldberg, G. I., Wilhelm, S. M., Kronberger, A., Bauer, E. A., Grant, G. A., and Eisen, A. Z. (1986) *J. Biol. Chem.* **261**, 6600–6605
17. Fort, P., Marty, L., Piechaczyk, M., El Sabrouly, S., Dani, C., Jeanteur, P., and Blanchard, J. M. (1985) *Nucleic Acids Res.* **13**, 1431–1442
18. Boast, S., Su, M.-W., Ramirez, F., Sanchez, M., and Avvedimento, E. V. (1990) *J. Biol. Chem.* **265**, 13351–13356
19. Wilkinson, G. W. G., and Akrigg, A. (1992) *Nucleic Acids Res.* **20**, 2233–2239
20. Foschi, M., Chari, S., Dunn, M. J., and Sorokin, A. (1997) *EMBO J.* **16**, 6439–6451
21. Wang, Y., Huang, S., Sah, V. P., Ross, J., Brown, J. H., Han, J., and Chien, K. R. (1998) *J. Biol. Chem.* **273**, 2161–2168
22. Ahonen, M., Baker, A. H., and Kähäri, V.-M. (1998) *Cancer Res.* **58**, 2310–2315
23. Ravanti, L., Häkkinen, L., Larjava, H., Saarialho-Kere, U., Foschi, M., Han, J., and Kähäri, V.-M. (1999) *J. Biol. Chem.* **274**, 37292–37300
24. Kähäri, V.-M., Vuorio, T., Nantö-Salonen, K., and Vuorio, E. (1984) *Biochim. Biophys. Acta* **781**, 183–186
25. Westermarck, J., and Kähäri, V.-M. (1999) *FASEB J.* **13**, 781–792
26. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) *Cell* **77**, 841–852
27. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. (1994) *Science* **265**, 966–970
28. Oka, H., Chatani, Y., Hoshino, R., Ogawa, O., Kakehi, Y., Terachi, T., Okada, Y., Kawaichi, M., Kohno, M., and Yoshida, O. (1995) *Cancer Res.* **55**, 4182–4187
29. Sivaraman, V. S., Wang, H., Nuovo, G. J., and Malbon, C. C. (1997) *J. Clin. Invest.* **99**, 1478–1483
30. Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Teclé, H., Barrett, S. D., Bridges, A., Przybranowski, S., Leopold, W. R., and Slatiel, A. R. (1999) *Nat. Med.* **5**, 810–816
31. Westermarck, J., Holmström, T., Ahonen, M., Eriksson, J. E., and Kähäri, V.-M. (1998) *Matrix Biol.* **17**, 547–557
32. Simon, C., Goepfert, H., and Boyd, D. (1998) *Cancer Res.* **58**, 1135–1139
33. Johansson, N., Ala-aho, R., Uitto, V.-J., Grénman, R., Fusenig, N. E., López-Otin, C., and Kähäri, V.-M. (2000) *J. Cell Sci.* **113**, 227–235
34. Davis, B. H., Chen, A., and Beno, D. W. A. (1996) *J. Biol. Chem.* **271**, 11039–11042
35. Slack, J. L., Parker, M. I., Robinson, V. R., and Bornstein, P. (1992) *J. Biol. Chem.* **12**, 4714–4723
36. Andreu, T., Beckers, T., Thoenes, E., Hilgard, P., and von Melchner, H. (1998) *J. Biol. Chem.* **273**, 13848–13854
37. Palcy, S., and Goltzman, D. (1999) *Biochem. J.* **343**, 21–27
38. Gebken, J., Lüders, B., Notbohm, H., Klein, H. H., Brinckmann, J., Müller, P. K., and Bätge, B. (1999) *J. Biochem.* **126**, 676–682
39. Svegliati-Baroni, G., Ridolfi, F., Di Sario, A., Casini, A., Marucci, L., Gaggiotti, G., Orlandoni, P., Macarri, G., Perego, L., Benedetti, A., and Folli, F. (1999) *Hepatology* **29**, 1743–1751
40. Laine, P., Reunanen, N., Ravanti, L., Foschi, M., Santra, M., Iozzo, R. V., and Kähäri, V.-M. (2000) *Biochem. J.* **349**, 19–25