Involvement of the Ras/MAPK Signaling Pathway in the Modulation of Urokinase Production and Cellular Invasiveness by Transforming Growth Factor-β_1 in Transformed Keratinocytes

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Transformed PDV keratinocytes respond to TGF-β_1 by stimulating cell motility and invasiveness concomitantly to enhancement of the urokinase-type plasminogen activator (uPA) expression/secretion. Depletion of extracellular signal-regulated kinase (ERK1, 2) proteins by treatment of PDV cells with antisense oligonucleotides reduced basal uPA production and abolished stimulation of uPA secreted levels and cell motility by TGF-β_1. PD098059, an inhibitor of mitogen-activated protein kinase (MAPK) kinase (MEK), decreased TGF-β_1-induced uPA mRNA expression, secreted activity in a dose-dependent manner, and abrogated TGF-β_1-stimulated cell motility and invasiveness. PDV-derived dominant-negative RasN17 cell transfectants secreted similar amounts of uPA and exhibited similar invasive abilities as the parental cells or control clones, but were unable to respond to TGF-β_1 for stimulation of uPA-secreted levels and invasiveness. These results suggest that a Ras/MAPK transduction pathway is involved in the invasive response of transformed keratinocytes to TGF-β_1. © 2000 Academic Press

Key Words: **TGF-β_1; uPA; keratinocytes; Ras; MEK; ERK; motility; invasiveness.**

The urokinase-type plasminogen activator (uPA) has been implicated in processes such as wound healing, angiogenesis and cancer (1, 2). uPA converts inactive plasminogen into the broad spectrum trypsin-like serine proteinase plasmin, which degrades several extracellular matrix components and activates other matrix metalloproteinases. Increased expression of uPA has been associated with tumoral invasiveness and metastasis (2). In addition, binding of uPA to its cell-surface receptor can stimulate epithelial cell motility by a mechanism independent of proteolysis (3).

uPA expression can be regulated by growth factors that bind to tyrosine kinase receptors and activate the Ras signaling pathway (4). This pathway involves the activation of the mitogen-activated protein kinase (MAPK) kinase, MEK, by c-Raf, which in turn activates extracellular signal-regulated kinase (ERK). Targets of ERK include transcription factors such as c-Myc and the Ets-family member Elk-1 that triggers expression of c-Fos (5). Alternatively, Ras can activate other c-Raf-independent pathways; for example, that involving the c-Jun N-terminal kinase (JNK, see Ref. 6).

We and others have reported that transforming growth factor-β_1 (TGF-β_1) stimulates the migration and invasiveness of transformed epithelial cells concomitantly to upregulation of uPA (7–9). TGF-β_1 exerts a dual function in carcinogenesis. It acts as a suppressor of tumor formation in early stages of carcinogenesis, but it can also stimulate invasiveness and metastasis of carcinoma cells (reviewed in Ref. 10). The effect of TGF-β_1 for stimulation of malignancy is direct on the cell and requires signaling through TGF-β cell-surface serine/threonine kinase receptors (11). In addition, TGF-β_1 needs to cooperate with a Ras oncogene for inducing progression to the invasive phenotype (10, 12).

The intracellular signals downstream of TGF-β receptors are still poorly understood, and several path-

Abbreviations used: uPA, urokinase-type plasminogen activator; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; ERK, extracellular signal-regulated kinase; TGF-β, transforming growth factor-β; TAK, TGF-β-activated kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase.

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ways might be involved. Proteins of the Smad family regulate several proliferative and differentiation responses to TGF-β and related factors (reviewed in Ref. 13). TAK1, a member of the MAPK kinase kinase family and potent activator of the p38 and stress-activated protein kinase (SAPK)/JNK pathways, is known to be involved in TGF-β-signaling (14). In addition, several reports have shown that TGF-β activates the Ras-MEK-ERK signaling pathway in epithelial cells (15, 16, our own results: Iglesias *et al.*, manuscript submitted for publication).

We are investigating the signaling events involved in the response of transformed epidermal keratinocytes (PDV cell line) to TGF-β_1. PDV cells contain an oncogenic mutation in the H-ras gene, and produce well differentiated squamous cell carcinomas upon injection in mice (17). In contrast to normal keratinocytes, PDV cells are refractory to TGF-β_1-induced terminal differentiation, although they are growth-inhibited at concentrations of the growth factor of 5–10 ng/ml. Under chronic exposure to TGF-β_1, PDV cells undergone an epithelial-mesenchymal conversion associated *in vivo* with the transition to a poorly differentiated tumor phenotype and increased metastatic abilities (18, 19). An early response of PDV cells to TGF-β_1 is enhanced expression/secretion of uPA and of its inhibitor PAI-1 (8). In this report, we examined the effect of interfering with the Ras/MAPK signaling pathway on the stimulation of uPA synthesis and cell migration/invasiveness by TGF-β_1.

MATERIALS AND METHODS

Cell culture and treatment conditions. MCA3D and PDV cells were cultured in Ham's F-12 medium supplemented with amino acids and vitamins in the presence of 10% fetal bovine serum (FBS) as described (18).

For TGF-β_1 treatments, human recombinant TGF-β_1 (Calbiochem–Novabiochem Intl., La Jolla, CA) was used. The growth factor was added to the cell cultures at a final concentration of 10 ng/ml.

PD098059 was obtained from Calbiochem–Novabiochem Intl. and added to PDV cell cultures 20 min prior to stimulation with TGF-β_1, at the concentrations indicated, in serum-free medium.

Transfection procedure. PDV cells cultured in Dulbecco's modified Eagle's medium (DMEM) were transfected with 5 μg of either the pMEXneo plasmid containing a dominant-negative H-ras gene (RasN17, see Ref. 20) or the empty vector, using Lipofectin (GIBCO Ltd.). After 18 h, cells were changed to fresh medium containing G418 (400 μg/ml) and 10% FCS, and 24 h later the medium was replaced by Ham's F-12 with 5% FCS and the same concentration of G418. After two weeks of selection, G418-resistant clones were isolated by cloning rings.

Oligodeoxynucleotide treatments. ERK antisense (5'-GCC GCC GCC GCC GCC AT-3') and sense (5'-ATG GCG GCG GCG GCG GC-3') phosphorothioate-modified oligodeoxynucleotides were synthesized and purified by Isogen Bioscience BV (Maarssen, The Netherlands). Cells (80% confluent) were treated for 48 h either with antisense or sense oligos, at a final concentration of 5 μM, in the presence of Lipofectin, as described by Sale *et al.* (21). After treatment, cultures were changed into serum-free medium, exposed to TGF-β_1 (10 ng/ml) for 24 h, and, thereafter, cells and conditioned media harvested. The uPA secreted activity was assayed in the conditioned media, as described below, and the expression of ERK1, 2 and p38 proteins determined in the cell lysates by Western immunoblotting.

For wound healing assays, treatments with antisense or sense oligos (5 μM) were only for 8 h, in the presence of Lipofectin. Thereafter, cells were washed, and assayed for motility in Ham's F-12 medium (5% FCS), as described below.

Immunofluorescence studies. Cells grown on glass coverslips were fixed and permeabilized in cold methanol. Coverslips were incubated with an anti-ERK1, 2 polyclonal antibody (K-23, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:50 dilution in PBS containing 2% BSA. As secondary antibody, anti-rabbit IgG coupled to rhodamine compound (Jackson ImmunoResearch Lab., West Grove, PA) was used. Coverslips were then mounted in Mowiol and examined with a fluorescence microscope (Axiophot; Carl Zeiss, OberKochen, Germany). Different fields of each preparation containing 30–60 cells were photographed. The percentage of cells with ERK predominantly or exclusively in the nucleus was determined counting 10 distinct fields from duplicates.

Northern and Western blot analysis. Northern blot hybridization experiments were performed with total RNA (10 μg) isolated from the cells by the guanidium thiocyanate procedure. The probe used for detection of uPA mRNA has been described elsewhere (8). 7S RNA probe was used as a control of RNA loading.

For Western immunoblotting, cell extracts (20 μg protein/lane) were resolved by 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA). ERK1, 2 and p38 proteins were detected with the rabbit polyclonal antibodies K-23 and H-147, respectively, both from Santa Cruz Biotechnology Inc., followed by exposure to peroxidase-conjugated secondary antibodies (Amersham Intl. Plc., Amersham Bucks, UK). The filters were developed using the enhanced chemoluminescent system (ECL, Amersham Corp., Arlington Heights, FL).

Migration and invasion assays. The capacity of the cells to migrate through Matrigel-coated filters was measured by using Transwell chambers (Costar Corp., Cambridge, MA) with 8-μm-pore polycarbonate filters coated with 30 μg of Matrigel (Collaborative Research, Bedford, MA), as described elsewhere (8). Cells were seeded on the upper compartment and incubated for 3 days in the absence or presence of TGF-β_1. The percentage of cells that migrated to the underside of the filter and to the bottom of the lower compartment was calculated respect to the total viable (nonmigrated and migrated) cells at the end of the incubation period.

Cellular motility was assayed by an *in vitro* wound model, as previously described (8). Confluent cell monolayers were gently scratched with a tip to produce a "wound." Then, cultures were allowed to grow for 24 h in Ham's F-12 medium containing 5% FCS with or without TGF-β_1.

Zymographic assay. uPA secreted activity of cell cultures was determined by caseinolytic zymography (8). Briefly, aliquots of conditioned serum-free medium, normalized for the same number of cells, were subjected to electrophoresis in 10% SDS–PAGE under nonreducing conditions. SDS was removed by extensive washing in 2.5% Triton X-100, the gels placed on 1% agarose gels containing 0.5% casein and 2 μg/ml of plasminogen, and incubated at 37°C for 24 h. Plasmin-dependent proteolysis was detected as a clear area in a white-blue field. Quantification of these areas was performed by densitometric analysis.

RESULTS

Effects of pretreatment with ERK antisense oligonucleotides. Transformed PDV keratinocytes express a certain basal amount of uPA mRNA and protein in

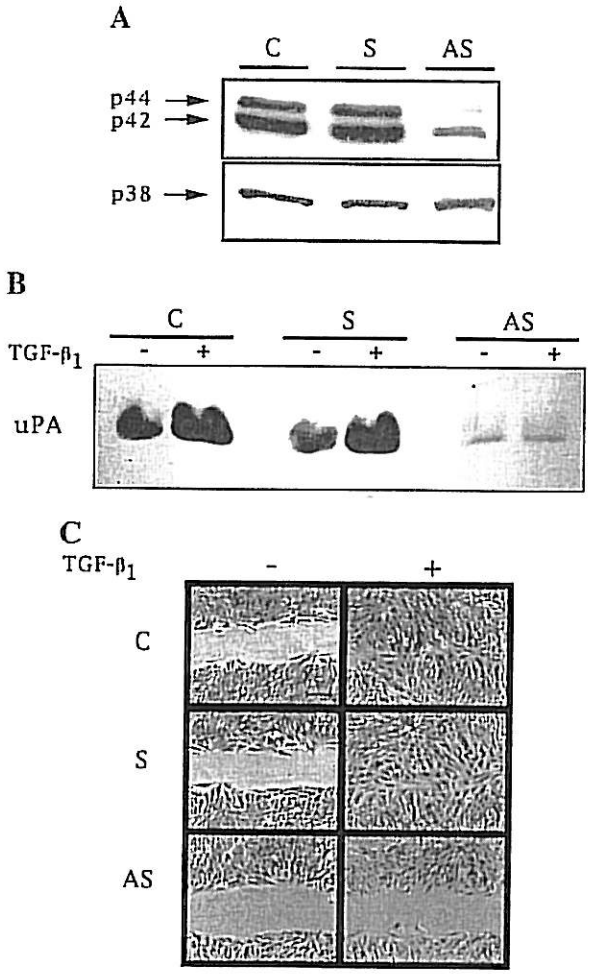

FIG. 1. Effect of ERK antisense pretreatment on TGF-β_1 stimulation of uPA secreted activity and cellular motility. (A) Western blot analysis of p44 (ERK1), p42 (ERK2) and p38 proteins synthesized by PDV cells (c) pretreated with ERK sense (s) or antisense (as) oligonucleotides. (B) Zymographic analysis of uPA secreted activity in the serum-free conditioned medium of cells unstimulated or stimulated with TGF-β_1 for 24 h under the different experimental conditions. (C) Wound healing assay in cell cultures pretreated with the sense and antisense oligos.

comparison with normal cultured keratinocytes (8). uPA is secreted into the extracellular medium where it can be determined by plasmin-dependent caseinolytic zymography of the serum-free conditioned medium. To study the possible involvement of ERK in basal as well as TGF-β_1-stimulated production of uPA, we synthesized oligodeoxynucleotides directed against both the p42 (ERK2) and p44 (ERK1) isoforms of ERK. Pretreatment of PDV cells with these antisense oligonucleotides (5 μM) resulted in about 90% depletion of ERK1, 2 proteins, while the sense oligonucleotides had none effect on ERK expression (Fig. 1A). As a control, we analyzed the expression of the p38 MAPK, which acts in a different signaling pathway from ERK regulating inflammatory responses (22), and found no changes in the p38 protein levels in cells pretreated with the ERK sense or antisense oligos (Fig. 1A). PDV cells pretreated with the sense oligos responded to TGF-β_1 by a 2- to 3-fold elevation in uPA secreted levels, as did the parental cell line (Fig. 1B). Conversely, in cells pretreated with the antisense oligos the amount of secreted uPA was highly reduced (by about 95%) and the response to TGF-β_1 completely blocked (Fig. 1B). Furthermore, cells treated with the antisense oligonucleotides did not respond to TGF-β_1-induced motility, while untreated cells or cells pretreated with the sense oligos were stimulated to close a wound area made 24 h before addition of the growth factor (Fig. 1C).

Effect of PD098059 on the invasive response of PDV cells. We show in a separate report that TGF-β_1 induces a rapid and transient activation of the Ras-MEK-ERK signaling pathway in PDV cells. PD098059, an inhibitor of MEK activity, blocks TGF-β_1-induced phosphorylation and nuclear accumulation of ERK in a dose-dependent manner, at concentrations of 10–50 μM (Iglesias *et al.*, manuscript submitted for publication). Therefore, we used the same range of concentrations to analyze the effect of PD098059 on the stimulation of uPA expression/secretion by TGF-β_1. PD098059, at 10 μM, blocked induction of uPA mRNA expression by a treatment of 24 h with TGF-β_1. At higher concentrations of the inhibitor (25–50 μM), uPA messages in stimulated cells were decreased below the basal level (Fig. 2A). In unstimulated cells, treatment with PD098059 highly diminished constitutive uPA mRNA expression (Fig. 2A). We also analyzed the effect of PD098059 on uPA secreted activity, and again a dose-dependent inhibition was observed (Fig. 2B). However, in this case, a little inhibitory effect was observed at 10 μM, and a higher concentration of 25–50 μM was required for PD098059 to block stimulation of uPA secreted activity by TGF-β_1. A possible explanation for the differences observed between the results of the Northern blot and zymographic analysis might be a high stability of the uPA mRNA in PDV cells, as found in other carcinoma cell lines (23).

Next, we studied whether PD098059 inhibited the migratory and invasive responses of PDV cells to TGF-β_1. As shown in Fig. 2C, PD098059 at concentrations of 25-50 μM significantly inhibited TGF-β_1-induced recolonization of a wound area made in confluent cells, and, at 50 μM, reduced (by 60–70%) stimulation of PDV cell invasiveness through the reconstituted basal membrane Matrigel after a 3 days treatment with TGF-β_1 (Fig. 2D). Concentrations of PD098059 lower than 50 μM had no significant effect on the invasion assay (not shown). Unstimulated PDV cells were invasive compared with nontransformed MCA3D keratinocytes, used in this experiment as a negative control (see Fig. 2D). Treatment of MCA3D cells with TGF-β_1

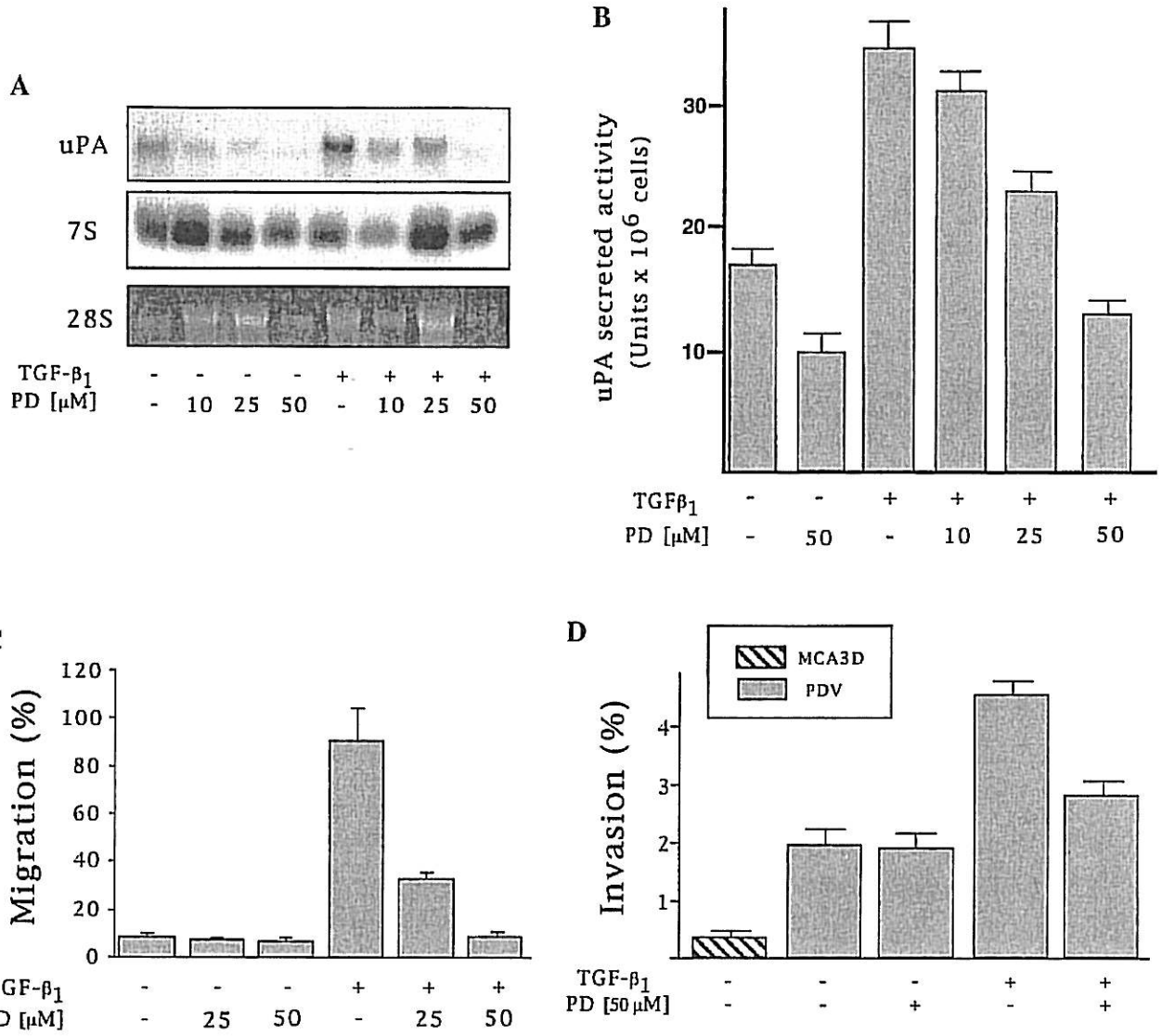

FIG. 2. Effect of PD098059 on TGF-β_1-stimulated uPA expression/secretion and cell migration/invasiveness. (A) Analysis of uPA mRNA expression in PDV cells unstimulated or stimulated with TGF-β_1 for 24 h in the absence of serum. PD098059 was added at the indicated concentrations. The intermediate and bottom panels show hybridization with a 7S RNA probe and the ethidium bromide stain of the gel corresponding to the 28S RNA, respectively, to normalize the loading. (B) uPA secreted activity was determined in the conditioned serum-free media of PDV cells unstimulated or stimulated with TGF-β_1 for 24 h, in the absence or presence of PD098059 at the indicated concentrations. Each bar represents the mean (±SD) of triplicates. (C) Wound healing assay. Cells invading the wound area made 24 h before were counted in five different fields as those of micrographs shown in Fig. 1. Mean values (±SD) are the percentages respect to the number of migrated cells in cultures treated with TGF-β_1 alone (100%). (D), Cell invasion assay through Matrigel-coated filters. The percentage of migrated cells was calculated respect to the total viable cells at the end of the incubation period (3 days). Where indicated, PD098059 was added at 50 μM. Immortalized MCA3D keratinocytes were used as a negative control. Each bar represents the mean (±SD) of triplicates.

for 3 days neither stimulated invasiveness nor uPA production (8).

TGF-β_1 stimulation of uPA secreted activity and cellular invasiveness are inhibited by expression of a dominant-negative RasN17 mutant gene. To investigate the role of Ras in the invasive response of PDV cells to TGF-β_1, a vector containing a dominant-negative RasN17 mutant gene was transfected into PDV cells. RasN17 transfectant and control clones (designated as RN and CE, respectively) were isolated. Two RN clones (−2 and −7), that did not respond to TGF-β_1-induced nuclear translocation of ERK proteins (Figs. 3A and 3B), were selected for the studies presented here.

Treatment of control CE-1 and CE-6 clones with TGF-β_1 for 24 h resulted in a 2-fold enhancement of

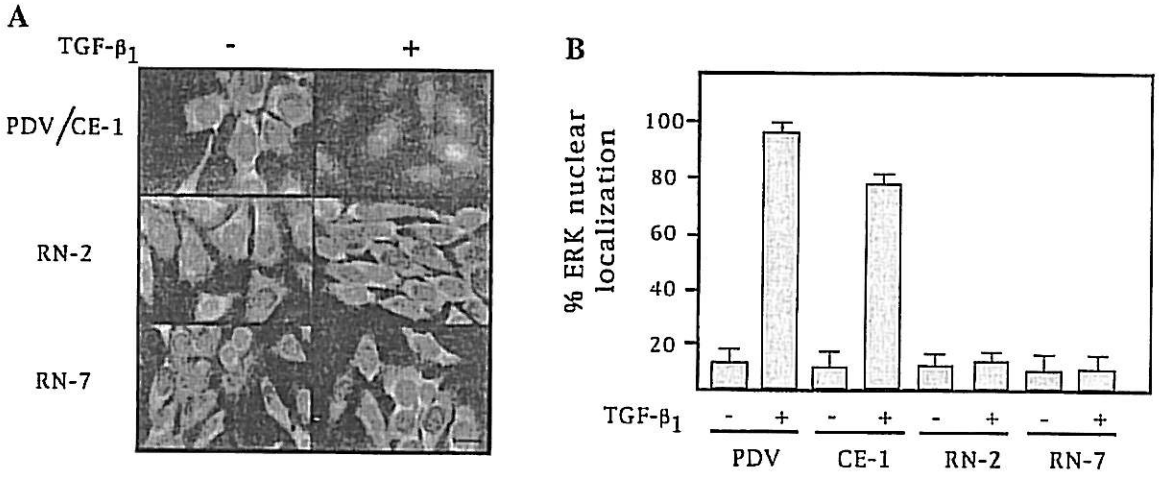

FIG. 3. TGF-β_1-induced ERK1, 2 nuclear translocation is inhibited in PDV cells transfected with a dominant-negative RasN17 mutant. (A) Representative micrographs of control (CE) and RasN17 (RN) transfected cell clones showing immunofluorescence staining of ERK1, 2 proteins before and after a 30-min stimulation with TGF-β_1. (B) Percentage of cells with ERK1, 2 localized predominantly or exclusively into the nucleus. Values represent the mean (±SD) of counts in 10 different fields, each containing a total of 30–60 cells, by duplicates, from three independent experiments.

uPA secreted levels (Fig. 4A), the same effect produced by TGF-β_1 on the parental cell line (see Fig. 1B). This response was blocked in RN-2 and RN-7 clones, although the basal level of uPA secreted by the cells was not affected (Fig. 4A). Interestingly, both control and RasN17 clones exhibited similar basal invasive abilities as the parental cell line (Fig. 4B). However, while the control and parental cells responded to TGF-β_1 for a 2-fold increase in invasiveness, the growth factor was unable to stimulate invasion of RN-2 and RN-7 cell clones (Fig. 4B).

Since *in vitro* invasiveness and basal uPA production of RN clones were closely similar to that of PDV and control clones, we compared the *in vivo* tumorigenic properties of the cell lines by intradermal injection (10^6 cells) into athymic nude mice (18). RN-2 and RN-7 clones produced tumors with similar efficiencies as PDV and CE-1 cell lines. The estimated latency periods to reach a tumor size of 0.5-cm^2 were 4–5 weeks. Moreover, all tumors were histologically typed as well differentiated squamous cell carcinomas, and no significant differences were observed among the distinct cell lines.

DISCUSSION

Transformed PDV keratinocytes respond to TGF-β_1 by increasing their migration and invasiveness, concomitantly to stimulation of uPA expression/secretion (8, this paper). In this report, we show that signaling through the Ras/MAPK transduction pathway is involved in the invasive response of PDV cells to the growth factor.

PDV cells depleted of ERK 1, 2 proteins by treatment with ERK antisense oligonucleotides were unable to respond to TGF-β_1 for enhanced secretion of uPA and stimulation of cell motility (Fig. 1). This treatment also inhibited uPA levels secreted by the cells in basal conditions, suggesting that a MAPK cascade is also involved in the regulation of constitutive synthesis of uPA. Basal expression/secretion of uPA in PDV cells is likely due to the presence of a codon 61 mutated H-Ras oncogene (17), as oncogenic Ras activates uPA transcriptional activity through a MAPK signaling pathway (24).

The MEK inhibitor PD098059 was also an effective inhibitor of uPA synthesis in PDV cells. It decreased both basal and TGF-β_1-stimulated uPA expression/secretion in a dose-dependent manner (Fig. 2). To significantly inhibit stimulation of uPA secreted levels by a 24 h treatment with TGF-β_1, concentrations of PD098059 of 25–50 μM were required, the same range of concentrations that inhibited TGF-β_1-stimulated cell migration and invasiveness (Fig. 2). These findings further support our contention for an involvement of uPA in the invasive response of PDV cells to the growth factor, as synthetic peptides antagonizing the binding of uPA to cell-surface receptors inhibited TGF-β_1 stimulation of PDV cell migration and invasiveness (8). However, it is unlikely for uPA to be the only contributing factor to these responses, as multiple genes are activated by TGF-β_1 that might be involved in the invasive response of transformed keratinocytes; for example, extracellular matrix proteins and other proteinases (25), that could be regulated by signaling through MAPKs.

Transfection of PDV cells with a dominant-negative RasN17 mutant construct abolished the ability of TGF-β_1 to stimulate uPA secreted activity and cellular invasiveness (Fig. 4), suggesting a role for Ras in the

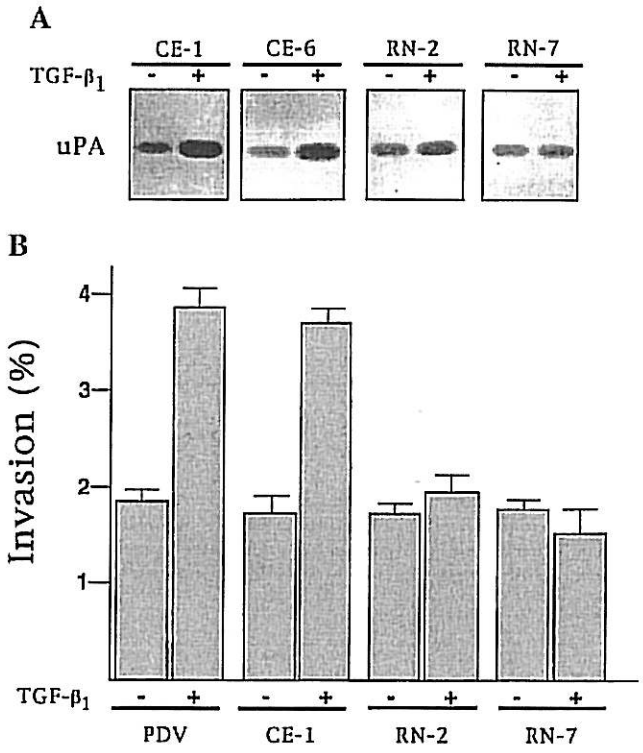

FIG. 4. Stimulation of uPA secreted activity and cellular invasiveness in response to TGF-β_1 is blocked in PDV cells transfected with a dominant-negative RasN17 mutant. (A) Zymographic analysis of secreted uPA in control (CE) and RasN17 transfected (RN) clones unstimulated or stimulated with TGF-β_1 for 24 h. (B) Invasion assay through Matrigel-coated filters of the cell lines unstimulated or stimulated with TGF-β_1 for 3 days. The percentage of invasive cells was calculated as in the legend of Fig. 3.

invasive response of transformed keratinocytes to the growth factor. However, clones expressing the RasN17 construct secreted similar amounts of uPA, and exhibited similar invasive abilities, as unstimulated PDV cells or control clones. This observation would indicate either that basal uPA expression in PDV cells is regulated by a Ras-independent signaling mechanism or, more likely, that the expression of dominant-negative Ras proteins, which have a preferential affinity for GDP (20), is not enough to inactivate permanently activated codon 61-mutated proteins, which retain high affinity binding for GTP (26). In fact, although RasN17 cell clones exhibited slower rates of proliferation than parental cells or control clones when cultured in the presence of serum (data not shown), they produced tumors in nude mice with similar efficiencies and histological characteristics.

Recently, it has been demonstrated that a cross-talk between the Ras/MAPK and Smad signaling pathways exists (reviewed in Ref. 27). We show in a separate report (Iglesias *et al.*, submitted for publication) that disruption of Smad signaling by transfection of PDV cells with a dominant-negative Smad4 cDNA construct, while reducing the antiproliferative response of PDV cells to TGF-β_1, does not inhibit uPA production. On the contrary, PDV derived dominant-negative Smad4 transfectants showed increased basal secretion of uPA to levels comparable to those induced by TGF-β_1 in the parental cells, and this increase was associated with a constitutive hyperactivation of Ras-ERK signaling and with progression to a poorly differentiated tumor phenotype, in contrast to PDV-derived dominant-negative RasN17 cells.

Taken together, our results suggest that Ras/ERK signaling is necessary for TGF-β_1 to stimulate uPA production and cell migration/invasiveness in transformed PDV keratinocytes. This assumption is in line with other observations demonstrating that activation of ERK regulates epithelial cell motility and migration across the extracellular matrix (28, 29), and that the MEK inhibitor PD098059 reduces concomitantly uPA expression and invasiveness in carcinoma cell lines characterized as avid secretors of uPA (30). Nevertheless, other Ras downstream transduction pathways, such as those leading to activation of p38 and JNK, might also be involved in the response of transformed keratinocytes to the growth factor. We have found that TGF-β_1 activates p38 in PDV cells (unpublished results), and both p38 and JNK MAPK cascades appear to be involved in TGF-β signaling (14, 31–33). On the other hand, p38 has been recently found to regulate uPA mRNA stability in carcinoma cells (34), and JNK was shown to mediate uPA gene transcriptional activation (35). Studies aimed to evaluate the involvement of stress-activated MAPKs on the response of PDV cells to TGF-β_1 are currently in progress.

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