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Regulation of Urokinase-Type Plasminogen Activator Expression by an ERK1-Dependent Signaling Pathway in a Squamous Cell Carcinoma Cell Line

Ernst Lengyel, Rebecca Gum, Evan Stepp, Jose Juarez, Heng Wang, and Douglas Boyd

Department of Tumor Biology/Head and Neck Surgery, M.D. Anderson Cancer Center, Houston, Texas 77030

Abstract The urokinase-type plasminogen activator contributes to tissue remodeling by controlling the synthesis of the extracellular matrix-degrading plasmin. We undertook a study to determine the role of the extracellular signal-regulated kinases (ERKs) in the regulation of urokinase-type plasminogen activator expression in a squamous cell carcinoma cell line (UM-SCC-1) that contains a transcriptionally activated urokinase-type plasminogen activator gene. Transient transfection studies using a CAT reporter driven by the urokinase-type plasminogen activator promoter, which had progressive 5′ deletions or which had been point-mutated, indicated the requirement of binding sites for AP-1 (-1967) and PEA3 (-1973) for its maximal activation. Expression of a mutant *jun* protein, which lacks the transactivation domain, caused a dose-dependent repression of a CAT reporter driven by either the urokinase-type plasminogen activator promoter or three tandem AP-1 repeats upstream of a thymidine kinase minimal promoter indicating the importance of AP-1-binding transcription factor(s) in the regulation of urokinase-type plasminogen activator synthesis. Mobility shift assays with UM-SCC-1 nuclear extract revealed binding of *fos* and *jun*D proteins to an oligonucleotide spanning the AP-1 site at −1967. In-gel kinase assays indicated the constitutive activation of ERK1, which regulates *fos* synthesis via phosphorylation of $p62^{TCF}$, but not ERK2, in UM-SCC-1 cells. Moreover, the expression of a dominant-negative ERK1, but not ERK2, repressed urokinase-type plasminogen activator promoter activity. Similarly, interfering with the function of the c-*raf* serine-threonine kinase, which lies upstream of ERK1, by the expression of a kinase-inactive c-*raf* repressed the activity of a CAT reporter driven by either the urokinase-type plasminogen activator promotor or tandem AP-1 repeats. These data suggest that urokinase-type plasminogen activator expression in UM-SCC-1 cells is regulated partly by an ERK1, but not ERK2, -dependent signaling pathway.

Key words: urokinase-type plasminogen activator, ERK, MAPK, c-*raf*, AP-1

The ability of both normal and tumor cells to degrade the basement membrane and surrounding extracellular matrix is a prerequisite for tissue remodeling in both physiological and pathological processes including tumor cell invasion. This is accomplished through the actions of a variety of proteases including the urokinase-type plasminogen activator [Strickland and Reich, 1976; Andreasen et al., 1990; Ossowski and Reich, 1983], which controls the synthesis of the widely acting serine protease plasmin. Plasmin cleaves several extracellular matrix components, including laminin and fibronectin, and activates type IV collagenase [Liotta et al., 1981; Salo et al., 1982]. Urokinase-type plasminogen activator is synthesized and secreted as a 55-kDa proenzyme [Kasai et al., 1985; Nielsen et al., 1982] and subsequently activated by presently poorly defined mechanisms. Both the proenzyme and the active urokinase-type plasminogen activator can bind to a specific cell surface receptor [Stoppelli et al., 1986], which functions to increase the rate of plasmin generation as well as to internalize PAI-1-inactivated urokinase-type plasminogen activator [Ellis et al., 1991; Cubellis et al., 1990]. In resected squamous cell carcinoma, urokinase-type plasminogen activator is expressed predominantly by the tumor cells, as demonstrated by in situ hybridization studies [Sappino et al., 1991]. This plasminogen activator is required for the in vitro

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Address reprint requests to Douglas Boyd, Department of Tumor Biology, Box 108, M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030.

Dr. Lengyel is now at the Technical University Munich, Department of Obstetrics and Gynecology, 81675 Munich, Germany.

invasiveness of these cells, since a monoclonal antibody directed at the catalytic site of the enzyme attenuates the ability of cultured squamous cell carcinoma to penetrate an extracellular matrix-coated porous filter [Clayman et al., 1993].

Expression of the 6.4-kb urokinase-type plasminogen activator gene [Riccio et al., 1985] is regulated by 2.3 kb of 5′ flanking sequence [Verde et al., 1988], which contains binding sites for the transcription factors AP-1, PEA3, NF-κB, NF-1, SP1, and CREB [Nerlov et al., 1991; von der Ahe et al., 1990]. Transcription of the urokinase-type plasminogen activator gene yields a 2.5-kb message, the stability of which is determined by multiple sites in the 3′ untranslated region of the mRNA [Nanbu et al., 1995]. Regulation of urokinase-type plasminogen activator expression is controlled by a wide variety of agents, including phorbol ester, EGF, cAMP, and calcitonin [Grimaldi et al., 1986; Rossi et al., 1990; Rorth et al., 1990], and this requires intact binding sites for AP-1-, PEA3-, CRE, and NF-κB-binding proteins [Hansen et al., 1992; Rorth et al., 1990; Nerlov et al., 1992].

Although much effort has been focused on defining the transcriptional requirements for urokinase-type plasminogen activator expression, the signaling events farther upstream are poorly understood. Certainly, there is ample evidence to suggest that the activity and/or expression of transcription factors is controlled by multiple signal transduction pathways, including both *ras*-dependent and *ras*-independent pathways [Karin, 1994; Kolesnick and Golde, 1994; Stacey et al., 1991; de Groot and Sasso-Corsi, 1993; Deng and Karin, 1995]. One of the *ras*-dependent signaling pathways involves the extracellular signal-regulated kinase (ERK) subset of mitogen-activated protein kinase (MAPK), which induces gene expression by modulating the activity and/or amount of *ets* family members and c-*fos* [Gille et al., 1992; Rao and Reddy, 1993; Coffer et al., 1994]. The ERKs are activated by a dual-specificity mitogen-activated protein kinase kinase (MAPKK also referred to as MEK1), which is specific insofar as it does not activate the parallel *jun* N-terminal kinase pathway [Minden et al., 1994a]. MEK1, in turn, is stimulated by the protein serine/threonine kinase c-*raf* [Crews et al., 1992; Kyriakis et al., 1992; Jelinek et al., 1994] a cytoplasmic signal transducer that becomes activated in response to GTP-bound *ras* [Kolch et al., 1991; Bruder et al., 1992].

Since regulation of urokinase-type plasminogen activator synthesis by growth factors is mediated through transcription factors [Hansen et al., 1992; Rorth et al., 1990; Nerlov et al., 1992], which themselves are ERK substrates, we hypothesized that the expression of this protease is controlled by a signaling pathway involving these MAPK(s). We report herein that in a squamous cell carcinoma cell line, characterized as an avid secretor of urokinase-type plasminogen activator, regulation of promoter activity of this gene, which requires AP-1- and PEA3-binding factors, occurs via an ERK1-dependent signaling pathway.

METHODS

Cell Culture

UM-SCC-1 cells derived from a squamous cell carcinoma of the oral cavity were obtained from Dr. Thomas Carey at the University of Michigan. All experiments made use of UM-SCC-1 cells prior to passage 25. NIH OVCAR-3 cells were provided by Dr. M. Schmitt (Technical University, Munich, Germany). The OVCAR-3 cell line was derived from a malignant ascites of a patient with progressive adenocarcinoma of the ovary [Hamilton et al., 1983]. All cells were maintained in McCoy's 5A medium containing 10% FBS. Conditioned medium was collected in McCoy's 5A medium supplemented with 4 μg/ml transferrin, 5 μg/ml insulin, and 10 ng/ml EGF (serum-free medium).

DNA Constructs

The TAM 67 vector, encoding a c-*jun* protein lacking the transactivation domain (amino acids 3–122 absent) of the molecule, has been described elsewhere [Brown et al., 1993; Domann et al., 1994]. The c-*raf* C4 expression vector encodes a mutant c-*raf* lacking the kinase domain of the serine-threonine kinase [Bruder et al., 1992]. The ERK1 and ERK2 constructs contain the coding region of these MAPKs in which the conserved lysine involved in phosphate transfer (codons 71 and 52 for ERK1 and ERK2, respectively) has been changed to arginine, thus impairing the catalytic efficiency [Frost et al., 1994]. The 3X AP1 pBLCAT construct consists of 3 AP-1 tandem repeats upstream of a thymidine kinase minimal promoter-CAT reporter (pBLCAT) [Angel et al., 1988]. The 5′ deletion

urokinase-type plasminogen activator promoter fragments and mutated urokinase-type plasminogen activator promoter-CAT reporter constructs have been described elsewhere [Verde et al., 1988; Nerlov et al., 1992].

Chloramphenicol Acetyltransferase Assays

UM-SCC-1 cells were co-transfected at 70% confluency with urokinase-type plasminogen activator promoter-chloramphenicol acetyltransferase (CAT) reporter constructs and 5 μg of an expression vector bearing the β-galactosidase gene. DNA dissolved in 1 ml of HBS buffer (25 mM Hepes, 1 μM $MgCl_2$, 0.1 μM $CaCl_2$, 0.1 M NaCl, 5 μM KCl, and 0.7 μM Na_2HPO_4 pH 7.44) was mixed with 50 μg hexadimethrine bromide (Polybrene, Aldrich Chemicals, WI) and added to the cells in 10% fetal bovine serum (FBS). After 5 h, the cells were shocked for 4 min with 33% dimethylsulfoxide (DMSO) and cultured for an additional 60 h. The cells were then harvested and lysed by repeated freeze–thaw cycles in a buffer containing 0.25 M Tris-HCl pH 7.8. Transfection efficiencies were determined by assaying for β-galactosidase activity. CAT activity was subsequently measured by incubating cell lysate (normalized for transfection efficiency) at 37°C for 6 h with 4 μM [^{14}C]-chloramphenicol and 1 mg/ml acetyl coenzyme A. After 3 h, the acetyl Coenzyme A (CoA) was replenished. The acetylated products were extracted with ethyl acetate and subjected to thin-layer chromatography (TLC) using chloroform/methanol (95:5) as the mobile phase.

Nuclear Run-on Experiments

Approximately 6×10^7 cells were scraped into cold phosphate-buffered saline (PBS) and resuspended in a hypotonic buffer containing 10 mM Hepes (pH 7.9), 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5% NP-40. The cells were lysed with a Dounce homogenizer and, following centrifugation, the nuclear pellets resuspended in transcription buffer, consisting of 150 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM Hepes (pH 7.9), 10% glycerol and 5 mM, DTT. Nuclei were incubated at 20°C for 30 min in the presence of 1 mM NTP and 250 μCi [^{32}P]UTP, after which they were collected by centrifugation. Nuclei were treated with DNase 1 and proteinase K and subsequently with phenol/chloroform and the RNA precipitated. Treatment with DNase I/proteinase K followed by phenol/chloroform extraction was repeated for a second time. Newly transcribed RNA (6.6×10^7 cpm/ml per reaction) was incubated for 48 h at 42°C with Nylon-immobilized cDNAs (5 μg/mm^2) corresponding to the *Sma*I/*Bam*H1 fragment (1,321 bp) from the 5′ end of the urokinase-type plasminogen activator cDNA [Axelrod et al., 1989], a housekeeping mRNA (GAPDH), or the linearized vector. After hybridization, the filter was washed at 65°C with 0.1 × SSC in the presence of 1% sodium dodecyl sulfate (SDS) and exposed to X-ray film.

Mobility Shift Assays

Nuclear extract was prepared essentially as described by Dignam et al. [1983]. UM-SCC-1 nuclear extract (8 μg) was incubated for 20 min in a buffer (25 mM Hepes buffer pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl, and 2.5% glycerol) with 2 μg of poly dI/dC and 5 fmoles (2×10^4 cpm) of a Klenow end-labeled (^{32}P-ATP) 30-mer oligonucleotide (TTCTTTGTCCAGGAGGAAATGAAGTCATCTG) which spans the AP-1 binding site at position −1967 of the urokinase-type plasminogen activator promoter in the absence or presence of a 100 fold excess of the wild type or mutated (TTCTTTGTCCAGGAGGAAATc̄c̄AGāgATCTG) AP-1 competitor sequence. After this time 1 μg of the indicated antibody was added and incubation continued for 15 more min. The reaction mixture was electrophoresed in a 6% polyacrylamide gel, containing 5% glycerol using 0.5 × TBE (89 mM Tris, 89 mM boric acid, and 1 mM EDTA) running buffer. The gel was rinsed with water, dried, and exposed to X-ray film overnight.

MAPK Assays

These were carried out essentially as described elsewhere [Morino et al., 1995] but with minor modifications. Briefly, cells were extracted with buffer A (1% NP-40, 25 mM Tris–HCl, pH 7.4, 25 mM NaCl, 1 mM sodium vanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 nM okadaic acid, 0.5 mM EGTA, and 1 mM PMSF). Insoluble material was removed by centrifugation at 14,000 rpm and the supernate assayed for protein. Cell extracts were incubated at 4°C for 1 h simultaneously with 0.5 μg of a polyclonal antibody that recognizes human and murine ERK1 and ERK2 (Santa Cruz Biotechnology #93) and Protein-A agarose beads. The beads were washed with buffer A, resuspended

in 3X sample buffer containing DTT and the immune complexes electrophoresed in an SDS–polyacrylamide gel containing 0.5 mg/ml myelin basic protein (MBP). SDS was removed from the gel with two changes of 20% 2-propanol in 50 mM Tris–HCl (pH 8.0) and then with 50 mM Tris–HCl containing 5 mM 2-mercaptoethanol. The enzyme was first denatured by treating the gel with two changes of 6 M guanidine HCl and then renatured overnight with 50 mM Tris–HCl (pH 8.0) containing 0.04% Tween 40 and 5 mM 2-mercaptoethanol. After renaturation, the gel was preincubated at 25°C for 1 h with a 40 mM HEPES (pH 8.0) solution containing 2 mM dithiothreitol, 0.1 mM EGTA, and 5 mM $MgCl_2$. Phosphorylation of MBP was carried out by incubating the gel at 25°C for 1 h in the same buffer, supplemented with 40 μM ATP, and 25 μCi of [γ^{32}P]ATP. After incubation, the gel was washed in a solution containing 5% trichloroacetic acid (TCA) and 1% sodium pyrophosphate until radioactivity became negligible and then dried and autoradiographed.

Western Blotting

These were carried out as described previously [Lengyel et al., 1995a]. Briefly, conditioned medium from equal numbers of cells or cell extracts generated in a PBS buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (RIPA), was denatured in the absence of reducing agent and electrophoresed in a 12.5% SDS–PAGE gel. The resolved proteins were transferred to a nitrocellulose filter. The filter was blocked with 3% bovine serum albumin (BSA) and incubated with a polyclonal antibody to urokinase-type plasminogen activator #389 (American Diagnostica, Greenwich, CT) or with a polyclonal antibody (Santa Cruz Biotechnology #93) cross-reacting with human and murine ERK1 and ERK2. Subsequently, the blot was incubated with horseradish peroxidase (HPO) conjugated antirabbit IgG and immunoreactive bands visualized by ECL as described by the manufacturer (Amersham, Arlington Heights, IL).

RESULTS

The Urokinase-Type Plasminogen Activator Gene Is Transcriptionally Activated in UM-SCC-1 Cells

UM-SCC-1 cells are avid secretors of urokinase-type plasminogen activator (Fig. 1A) and this accounts, in part, for the ability of these cells to penetrate an extracellular matrix-coated porous filter [Clayman et al., 1993]. To determine the contribution of transcriptional activation to the synthesis of urokinase-type plasminogen activator in these cells, nuclear run-on experiments were carried out. Nuclei from UM-SCC-1 cells and from OVCAR-3 cells (used as a negative control), previously characterized as urokinase-type plasminogen activator-deficient [Lengyel et al., 1995a], were isolated and incubated with radioactive UTP. Newly synthesized radioactive RNA was subsequently extracted and hybridized with nylon filter-immobilized urokinase-type plasminogen activator, GAPDH and the vector DNAs (Fig. 1B). Hybridization of the radioactive RNA from UM-SCC-1 cells with the urokinase-type plasminogen activator *c*DNA gave a strong signal in comparison to the negative control cell line characterized by its weak secretion of the plasminogen activator (Fig. 1A). Quantitation of the signal with a Betascope 603 indicated that the urokinase-type plasminogen activator mRNA signal from UM-SCC-1 cells was 10 times higher than that of the negative control cell line after correcting for loading inequalities (as determined by hybridization of the RNA with the GAPDH probe). These data indicated that the high level of steady-state urokinase-type plasminogen activator mRNA [Clayman et al., 1993] and protein in these cells (Fig. 1A) could be accounted for by a high rate of transcription of the urokinase-type plasminogen activator gene.

Constitutive Activation of the Urokinase-Type Plasminogen Activator Promoter Requires Intact Binding Sites for AP-1 and PEA3

To identify the region of the urokinase-type plasminogen activator promoter required for the constitutive expression of the urokinase-type plasminogen activator gene in UM-SCC-1 cells, we used a CAT reporter construct driven by 5′ deletion fragments of the promoter or, as a positive control, the RSV LTR (Fig. 2A). A basal CAT activity, presumably reflecting the presence of minimal promoter elements including a TATA and CAAT-like boxes and a Sp1 binding site [Verde et al., 1988], was evident with the reporter driven by 72 bp of the urokinase-type plasminogen activator promoter. This activity appeared to be somewhat diminished with longer 5′ stretches of the promoter up to 1,870 bp this possibly reflecting the presence of negative *cis*-

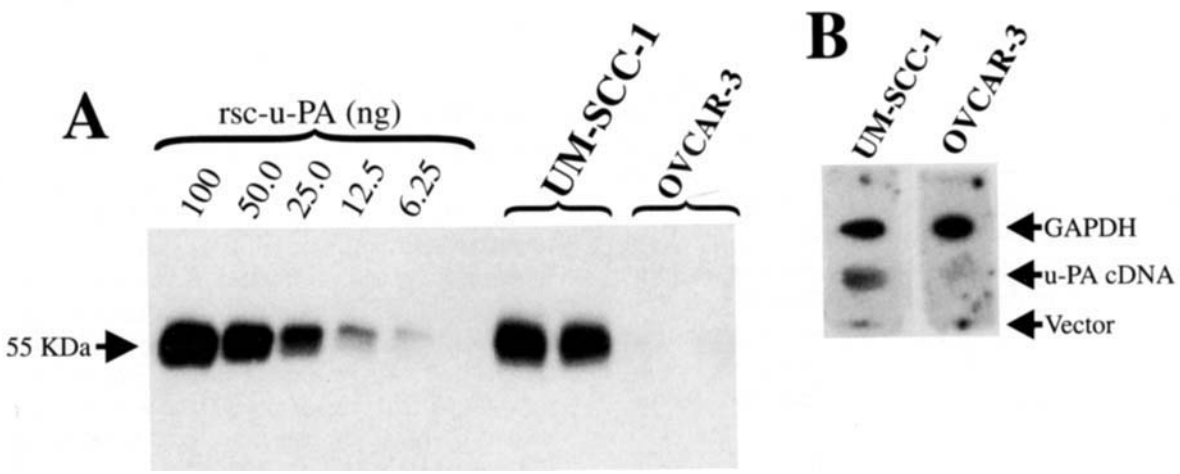

Fig. 1. Transcriptional activation of the urokinase-type plasminogen activator gene in UM-SCC-1 cells that are avid secretors of the plasminogen activator. **A:** UM-SCC-1 and OVCAR-3 cells (used as a negative control), were grown to 80% confluency in the presence of 10% FBS and changed to serum-free medium. After an additional 16 hours, the conditioned medium was harvested and clarified, and the cells were counted. Conditioned medium, normalized to cell number, and recombinant single-chain urokinase-type plasminogen activator (rsc-u-PA), was denatured, in the absence of reducing agent, and electrophoresed in a 7.5% gel. Electrophoresed proteins were transferred to a nitrocellulose filter. The filter was blocked with 3% BSA and incubated with a polyclonal antibody against human urokinase-type plasminogen activator. Reactive proteins were visualized by ECL. **B:** At 90% confluency, cells were harvested and nuclei isolated. The nuclei were incubated with radioactive UTP and finally dissolved in guanidinium isothiocyanate. Purified radioactive RNA (6.6×10^7 cpm/ml) was incubated at 42°C for 48 h with nylon-immobilized cDNAs corresponding to urokinase-type plasminogen activator (u-PA cDNA), GAPDH, or the urokinase-type plasminogen activator plasmid vector (Vector). Filters were washed at 65°C using 0.1 × SSC/1.0% SDS and exposed to X-ray film. Radioactivity on the blot was quantified using a 603 Betascope.

acting sequences [Verde et al., 1988]. By contrast, a strong activation of the CAT reporter, which was not further augmented with longer stretches of the 5′ flanking sequence, was evident with 2,109 bp of 5′ flanking sequence. These data indicate that a region of the urokinase-type plasminogen activator promoter residing between −1870 and −2109 is required for optimal expression of the urokinase-type plasminogen activator gene in a cultured squamous cell carcinoma cell line.

The region of the urokinase-type plasminogen activator promoter residing between −1870 and −2109 contains binding sites for AP-1 and PEA3 shown to be required for the stimulation of several inducible genes including keratin 8 and collagenase [Auble and Brinckerhoff, 1991; Pankov et al., 1994]. To determine the role of these transcription factor binding sites in the constitutive expression of the urokinase-type plasminogen activator gene, we transiently transfected UM-SCC-1 cells with a CAT reporter driven by the wild-type or point-mutated urokinase-type plasminogen activator promoter (Fig. 2B). Mutation of the AP-1 binding site at −1967 (mutant B) and to some extent at −1885 (mutant G), resulted in a reduction of the constitutive activation of the urokinase-type plasminogen activator promoter. Likewise, mutation of a PEA3 (mutant A) motif at −1973, which has been shown to be important for the regulation of urokinase-type plasminogen activator gene transcription by macrophage colony-stimulating factor [Stacey et al., 1995], impaired the activity of the urokinase-type plasminogen activator promoter in UM-SCC-1 cells. A region of the urokinase-type plasminogen activator promoter shown to be necessary for the maximal stimulation by phorbol ester [Nerlov et al., 1992] was also required for optimal promoter activity as evidenced by a lower CAT conversion with mutant I.

Repression of Urokinase-Type Plasminogen Activator Promoter Activity by Co-expression of a Transactivation Domain-Lacking c-*jun*

To determine the role of AP-1-binding transcription factors in the regulation of urokinase-type plasminogen activator expression in UM-SCC-1 cells, we made use of a dominant negative c-*jun* expression vector (TAM-67). This construct encodes a mutant c-*jun* that lacks the entire transactivation domain of this transcription factor and that inhibits AP-1-mediated pro-

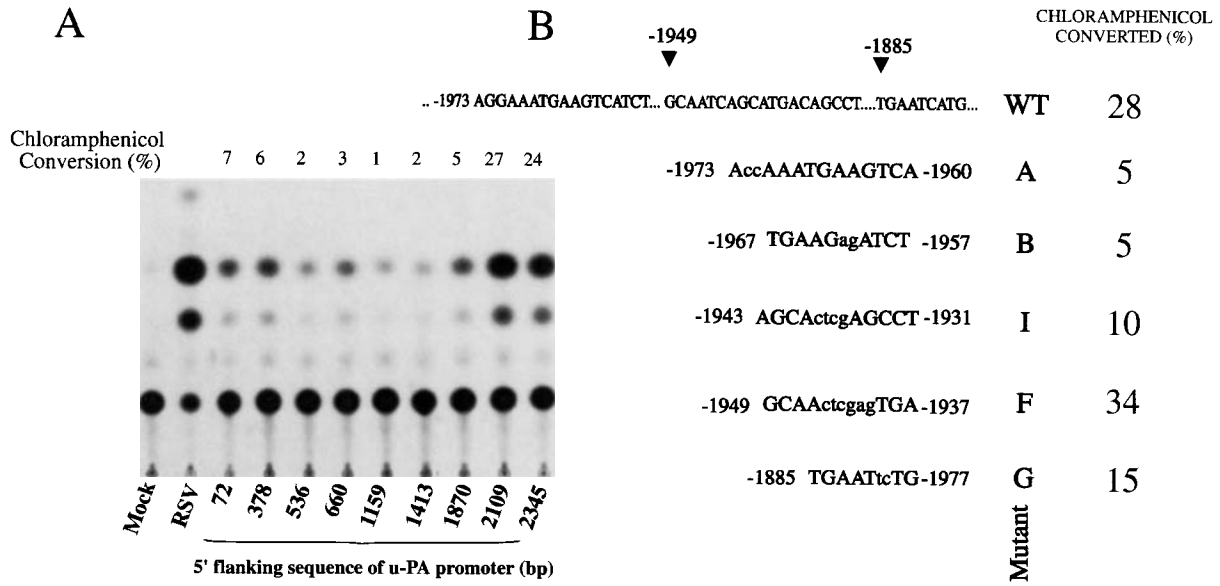

Fig. 2. The constitutive activation of the urokinase-type plasminogen activator promoter in UM-SCC-1 cells requires AP-1 and PEA3 binding sites at −1967 and at −1973, respectively. **A:** UM-SCC-1 cells were transiently transfected at 80% confluency with an equimolar amount (equivalent with 10 μg of the full-length urokinase-type plasminogen activator promoter) of a CAT reporter driven by the 5′ deletion fragments of the urokinase-type plasminogen activator promoter. All transfections were performed in the presence of 5 μg of a β-galactosidase-expressing vector. As a control, cells were transfected with no DNA (Mock) or with a CAT reporter driven by the RSV LTR as a positive control (RSV). After 5 h, the medium was changed and cells cultured for an additional 60 h. The cells were harvested and assayed for β-galactosidase activity. Cell extracts, corrected for differences in transfection efficiency, were incubated with [^{14}C]-chloramphenicol for 4 h. The mixture was extracted with ethyl acetate and subjected to thin layer chromatography. **B:** Transfections were carried out as described above. The mutated nucleotides in context of 2,345 bp of 5′ flanking sequence of the urokinase-type plasminogen activator promoter (constructs A, B, I, F, G) are indicated in lower case. The position of the sequence is indicated relative to the transcriptional start site. Conversion of [^{14}C]-chloramphenicol was determined with a 603 Betascope. Wt, wild-type urokinase-type plasminogen activator promoter. The data are typical of four separate experiments.

cesses through a quenching mechanism by inhibiting the function of endogenous *fos* and *jun* proteins [Brown et al., 1993; Domann et al., 1994]. UM-SCC-1 cells were transiently transfected with a CAT reporter driven by 2109 bp of the urokinase-type plasminogen activator 5′ flanking sequence and the TAM-67 expression vector. A dose dependent reduction in CAT activity was apparent with increasing amounts of the c-*jun* mutant (Fig. 3A). By contrast, co-transfection of the urokinase-type plasminogen activator construct with equivalent amounts of the empty vector (CMV vector) did not reduce urokinase-type plasminogen activator promoter activity. To verify that this construct was indeed inhibiting AP-1-controlled gene expression, UM-SCC-1 cells were co-transfected with a CAT reporter driven by three tandem AP-1 repeats upstream of a thymidine kinase minimal promoter and an amount of TAM-67 which repressed urokinase-type plasminogen activator promoter activity. These experiments (Fig. 3B) indicated that the activity of the AP-1-driven promoter (3X AP-1 pBL-CAT), which was strongly activated in UM-SCC-1 cells, was repressed by the co-expression of the mutant c-*jun*. By contrast, the activity of the CAT reporter driven by the thymidine kinase minimal promoter alone (pBL-CAT) was low to begin with and was entirely unaffected by expression of TAM-67. These data suggest that constitutive urokinase-type plasminogen activator expression does indeed depend on AP-1-binding transcription factors.

Nuclear Extract from UM-SCC-1 Cells Contains AP-1-Binding Transcription Factors

Mobility shift assays were undertaken to identify transcription factors, which were binding to the AP-1 site at −1967 of the urokinase-type plasminogen activator promoter. UM-SCC-1 nuclear extract was incubated with an oligonucleotide containing the −1967 AP-1 site in the presence, or absence, of the indicated reagent and the mixture electrophoresed in a 6% acrylamide gel. The mobility of the AP-1-contain-

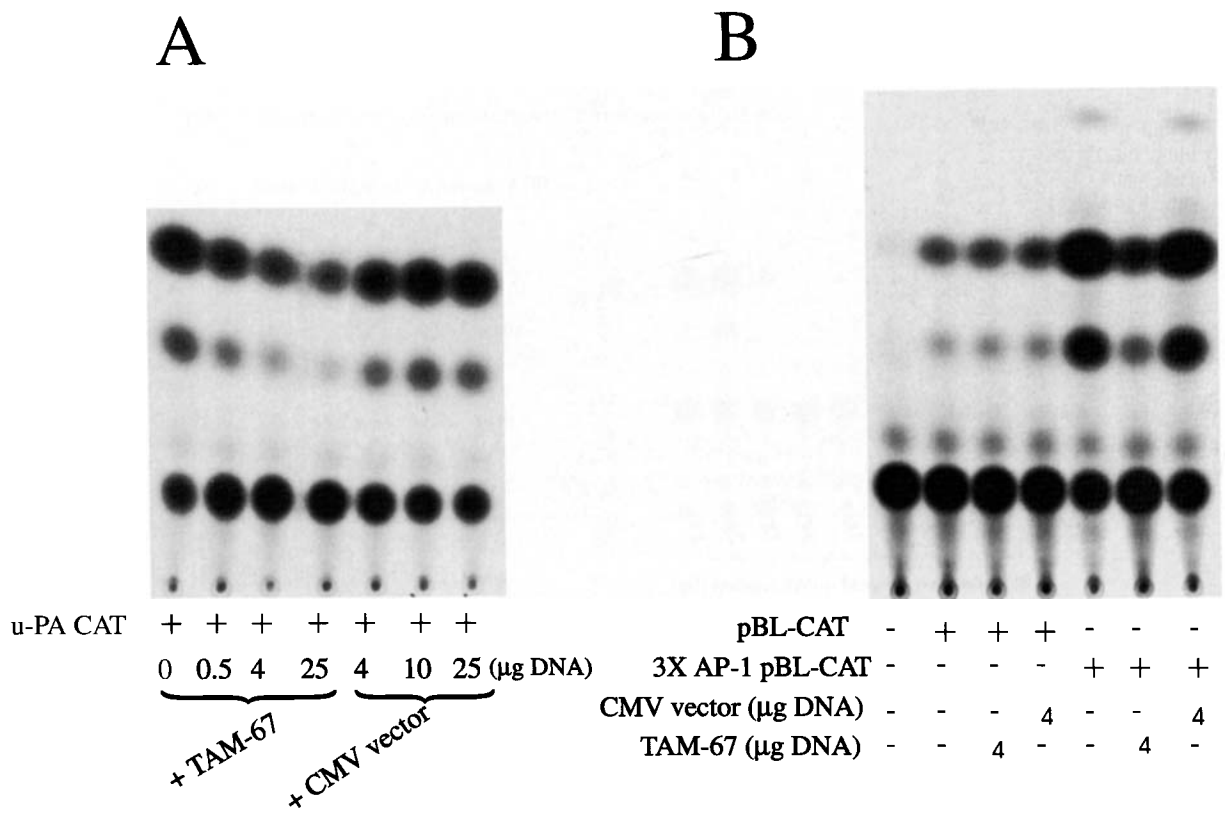

Fig. 3. Repression of urokinase-type plasminogen activator promoter activity by co-expression of a transactivation domain-lacking c-*jun*. **A:** UM-SCC-1 cells were transiently transfected with 10 μg of a CAT reporter driven by the wild type urokinase-type plasminogen activator promoter (u-PA CAT) and the indicated amounts of an expression vector encoding a transactivation domain-lacking c-*jun* protein (TAM-67) or the indicated amounts of the empty vector (CMV vector). An equal amount of cell lysate protein was incubated for 6 h with [^{14}C]-chloramphenicol, extracted with ethyl acetate and the acetylated products resolved by thin-layer chromatography. **B:** UM-SCC-1 cells were co-transfected with 10 μg of a CAT reporter driven by three tandem AP-1 repeats upstream of a thymidine kinase minimal promoter (3X AP-1 pBL-CAT) or the thymidine kinase minimal promoter (pBL-CAT) with, or without, 4 μg of TAM-67 or an equimolar amount of the empty vector (CMV vector). Reporter activity was determined by incubation of an equal amount of extracted cell protein with [^{14}C]-chloramphenicol for 6 h. After this time, the cell lysate was extracted with ethyl acetate and the acetylated products resolved by thin-layer chromatography. The data are representative of three separate experiments.

ing oligonucleotide was reduced (arrow) in the presence of UM-SCC-1 nuclear extract (Fig. 4). The occurrence of a wide band is consistent with the ability of different members of the AP-1 family, as homo- or heterodimers, to bind to the same consensus sequence. The specificity of the AP-1-binding interaction was indicated by the ability of an excess of unlabelled oligonucleotide (specific comp.) to compete for the binding whereas an oligonucleotide (mutant comp.), which had been mutated in the AP-1 sequence, was unable to compete for the binding.

Subsequently, the oligonucleotide containing the AP-1 site at −1967, was incubated with nuclear extract and antibodies directed at *fos* family members (Santa Cruz Biotechnology #SC 253X), c-*jun* (Santa Cruz Biotechnology #SC 822X), *jun*B (Santa Cruz Biotechnology #SC 46X) and *jun*D (provided by Dr. M. Yaniv, Institute Pasteur, Paris). A supershifted species was detected (Fig. 4) when the nuclear extract was incubated with a polyclonal antibody (pan-*fos* Ab), which cross-reacts with multiple *fos* family members. Similarly, a supershifted complex was observed following incubation of the nuclear extract with a polyclonal anti-*jun*D antibody. The different position of the supershifted bands presumably is a consequence of the interaction of the antibody with the complex; this may be disruptive for the binding of other factors which form the complex. In contrast, we saw no evidence of ATF-2 in the AP-1-binding complex a finding at variance with a report indicating the urokinase-type plasminogen activator promoter

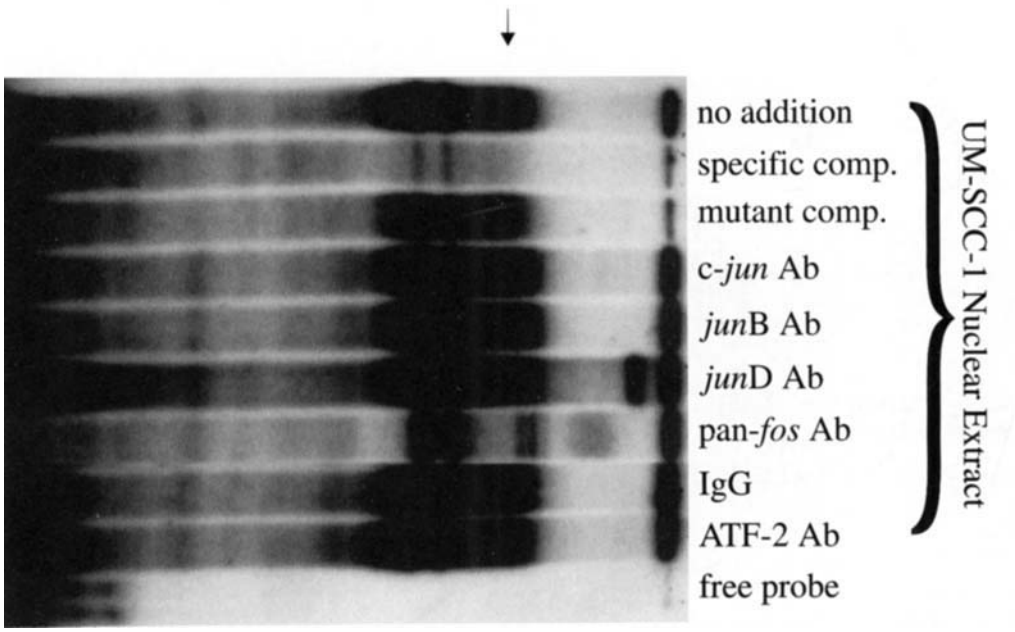

Fig. 4. Nuclei from UM-SCC-1 cells contain AP-1 binding transcription factors. UM-SCC-1 nuclear extract (8 μg) was incubated with 5 fmol of a Klenow end-labeled (^{32}P-ATP) 30-mer oligonucleotide which spans the AP-1 binding site at position −1967 of the urokinase-type plasminogen activator promoter in the presence, or absence, of a 100-fold excess of oligonucleotide competitors. Antibodies (Ab) to the indicated transcription factors or control IgG were added 20 min later and incubated for an additional 15 min. The reaction mixtures were electrophoresed in a 6% polyacrylamide gel. The experiment was carried out 3 times. The arrow indicates a specific binding complex of UM-SCC-1 nuclear factor(s) with an oligonucleotide spanning the AP-1 motif at −1967 of the urokinase-type plasminogen activator promoter.

to be activated by an ATF-2/c-*jun* heterodimer in HepG2 cells [De Cesare et al., 1995]. This difference may very well reflect differences in the regulation of urokinase-type plasminogen activator expression between the two cell lines used in the separate studies.

Inhibition of Urokinase-Type Plasminogen Activator Promoter Activity by a Dominant-Negative ERK-1 Mutant

Since our data indicated that urokinase-type plasminogen activator promoter activity in UM-SCC-1 cells was regulated in part by an AP-1-dependent mechanism, we undertook experiments to determine the role of the ERKs in urokinase-type plasminogen activator expression since AP-1-binding factors, including *fos,* are regulated by this MAPK subset [Gille et al., 1992]. UM-SCC-1 and NIH-3T3 (used as a negative control for kinase activity [Lengyel et al., 1995b]) cells were extracted and subjected either to Western blotting (Fig. 5A) or to an in-gel kinase activity assay (Fig. 5B). Western blotting using a polyclonal antibody, which cross-reacts with both human and murine ERK1 and ERK2, revealed the presence of proteins which were indistinguishable in size to that of ERK1 (44 kDa) and ERK2 (42 kDa) in both UM-SCC-1 cells and in NIH3T3 cells. A band, which was slightly smaller than 44 kDa may represent a hypophosphorylated form of ERK1 or a proteolytic fragment of the MAPK. Assaying for substrate (MBP) phosphorylation following immunoprecipitation of the cell extract with the polyclonal anti-ERK antibody indicated the presence of a 44-kDa kinase activity in UM-SCC-1 cells, but not in the NIH 3T3 cells (Fig. 5B). These data suggested that UM-SCC-1 cells, which express urokinase-type plasminogen activator partly through AP-1-dependent mechanisms, contain a constitutively activated ERK1.

To determine whether urokinase-type plasminogen activator promoter activity was controlled by the constitutively activated ERK1, we employed expression vectors encoding dominant-negative ERK1 and ERK2 [Frost et al., 1994]. A dose-dependent inhibition of a urokinase-type plasminogen activator promoter-driven CAT reporter was achieved by the cotransfection of the dominant negative ERK1 expression vector (Fig. 5C). [^{14}C]-chloramphenicol acetylation was reduced from 66% to 21% in the presence of 2 μg

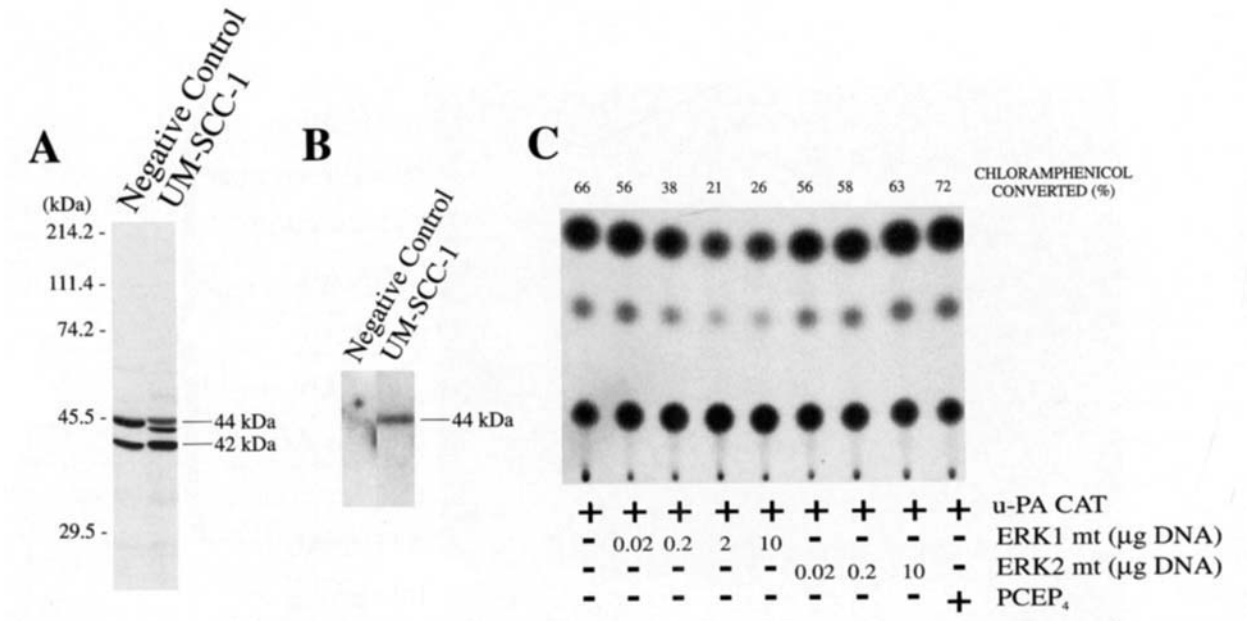

Fig. 5. A dominant negative ERK1 expression vector represses urokinase-type plasminogen activator promoter activity in UM-SCC-1 cells which contain a constitutively activated ERK1. **A:** UM-SCC-1 and NIH3T3 cells (Negative Control) were extracted with RIPA buffer and supernates from a 14,000-rpm centrifugation assayed for protein. Equal amounts of protein were subjected to SDS–PAGE, and the resolved proteins transferred to nitrocellulose. The filter was probed with 0.1 μg/ml of a polyclonal antibody (Santa Cruz Biotechnology #93), which crossreacts with human and murine ERK1 and ERK2, and the bands visualized by ECL. Molecular-weight markers are indicated to the left. **B:** For the in-gel kinase assays, cells were extracted with buffer A, and centrifuged at 14,000 rpm to clear cellular debris. Equal amounts of protein in the cell supernates were reacted for 3 h at 4°C with 0.5 μg of the polyclonal anti-ERK1/ERK2 antibody and protein-A-Sepharose, and the precipitate electrophoresed in an SDS–PAGE gel containing 0.5 mg/ml myelin basic protein (MBP). SDS was subsequently removed from the gel by washing with 20% 2-propanol. The enzyme was denatured with 6 M guanidine HCl at room temperature and renatured with 50 mM Tris–HCl (pH 8.0) containing 0.04% Tween 40 and 5 mM 2-mercaptoethanol. Phosphorylation of MBP was carried out by incubating the gel in the presence of 40 μM ATP and 25 μCi [γ^{32}P]-ATP. After incubation, the gel was washed with 5% TCA and 1% sodium pyrophosphate and subjected to autoradiography. Panel C; UM-SCC-1 cells were transiently transfected and assayed for CAT activity as described in the legend to Figure 2. Briefly, cells were co-transfected with 10 μg of a CAT reporter driven by the wild type urokinase-type plasminogen activator promoter and increasing amounts of a dominant-negative ERK1 (ERK1 mt), ERK2 (ERK2 mt) or an amount of the empty expression vector ($pCEP_4$) equivalent with 10 μg of the ERK1 mutant. The data are typical of three separate experiments.

of the ERK1 mutant expression vector (ERK1 mt). By contrast, the urokinase-type plasminogen activator promoter was not inhibited in UM-SCC-1 cells co-transfected with the dominant negative ERK2 construct (ERK2 mt) or the empty expression vector ($pCEP_4$). The lack of effect of the ERK2 mutant is consistent with the in-gel kinase results which indicate ERK1, but not ERK2, to be constitutively activated in UM-SCC-1 cells.

Expression of a Dominant-Negative c-*raf* Blocks Constitutive Urokinase-Type Plasminogen Activator Expression

Since the ERKs lie downstream of the c-*raf* serine-threonine-kinase we undertook experiments to determine if urokinase-type plasminogen activator promoter activity could be repressed by the co-expression of a dominant negative c-*raf*. UM-SCC-1 cells were transiently co-transfected with a urokinase-type plasminogen activator promoter-driven CAT reporter and increasing amounts of an expression vector encoding a kinase-inactive c-*raf* (*raf* C4). The encoded molecule contains the N-terminal 257 amino acids of c-*raf* and thus lacks the kinase domain of the serine-threonine kinase [Bruder et al., 1992]. Co-transfection of the *raf*C4 mutant into UM-SCC-1 cells led to a dose-dependent reduction in the activity of the urokinase-type plasminogen activator promoter-driven CAT activity (Fig. 6A). By contrast, the expression vector lacking the *raf* C4 coding sequence (Vector) failed to repress the activity of the

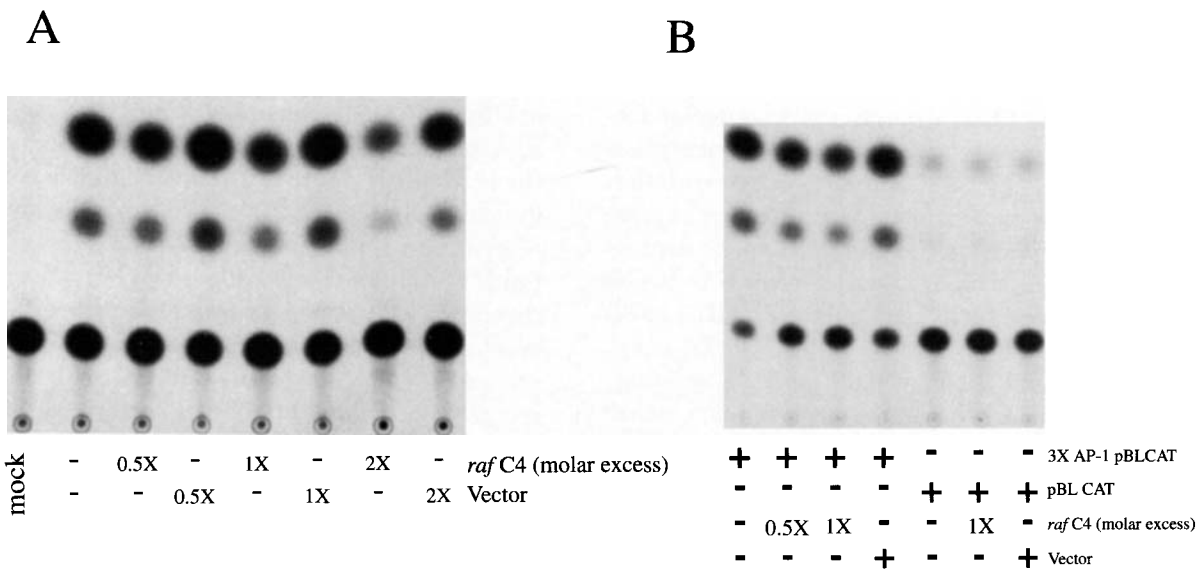

Fig. 6. Repression of the urokinase-type plasminogen activator promoter-driven CAT reporter by co-expression of a kinase-inactive c-*raf*. UM-SCC-1 cells were co-transfected, as in the legend to Figure 3, with a CAT reporter driven by the full-length urokinase-type plasminogen activator promoter (**A**) or a thymidine kinase minimal promoter (pBL CAT) flanked by three tandem AP-1 repeats (3X AP-1 pBL CAT) (**B**) in the presence, or absence, of an equimolar amount (1X) of the mutant c-*raf* expression vector (*raf* C4) or the empty expression vector (Vector). In **B**, the amount of vector used is equimolar with 1X of the c-*raf* C4. Reporter activity was determined by incubation of the cell extract, adjusted for variation in transfection efficiency, with [^{14}C]-chloramphenicol for 6 h. After this time, the cell lysate was extracted with ethyl acetate and the acetylated products resolved by thin-layer chromatography. Mock; no DNA. The experiment was repeated twice.

urokinase-type plasminogen activator promoter-driven CAT reporter.

To determine the effect of the kinase-deficient c-*raf* on AP-1-dependent gene expression, UM-SCC-1 were transiently co-transfected with the mutant c-*raf* expression vector and a CAT reporter driven by 3 AP-1 tandem repeats upstream of a thymidine kinase minimal promoter (Fig. 6B). The strong activation of this reporter construct (3X AP-1 pBLCAT) in UM-SCC-1 cells was effectively repressed by the co-expression of amounts of the c-*raf* mutant (but not by the empty expression vector-Vector), which we found to reduce the activity of the urokinase-type plasminogen activator promoter-driven CAT reporter. By contrast, a CAT reporter driven by only the thymidine kinase minimal promoter (pBL CAT) was weakly activated in UM-SCC-1 cells and this did not appear to be altered by the dominant negative c-*raf* (Fig. 6B).

DISCUSSION

While the invasiveness of a number of different cell types, including squamous cell carcinoma, has been ascribed, in part, to the proteolytic action of the urokinase-type plasminogen activator, the signaling events that regulate the expression of this protease have not been determined. We report herein, that the expression of the urokinase-type plasminogen activator in at least one squamous cell carcinoma cell line is regulated by a constitutively activated ERK1-dependent pathway. To our knowledge, this is the first report demonstrating the role of this signaling kinase in the constitutive expression of this protease.

The ERK1-dependent pathway required for urokinase-type plasminogen activator expression in UM-SCC-1 cells may very well be identical to the *ras*-signaling pathway described by Minden et al. [1994a] in which the sequential activation of c-*raf*, MAPKK and the ERK group of the MAPKs leads to the increased synthesis or activation of several DNA-binding proteins including c-*fos* and *ets*-related transcription factors [Gille et al., 1992; Rao and Reddy, 1993; Coffer et al., 1994; Crews et al., 1992; Kyriakis et al., 1992; Jelinek et al., 1994]. Indeed, several observations by this and other groups provide indirect support for the contention that this pathway is involved in the regulation of urokinase-type plasminogen activator expression in

UM-SCC-1 cells. First, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (PMA), which, via protein kinase C, is a potent activator of c-*raf* [Sozeri et al., 1992] strongly activates urokinase-type plasminogen activator gene transcription [Stoppelli et al., 1986]. Moreover, the regulation of urokinase-type plasminogen activator expression by this agent is, like the constitutive expression of the plasminogen activator in UM-SCC-1 cells, mediated partly through the PEA3 (−1973) and AP-1 (−1967, −1885) motifs [Nerlov et al., 1992]. Second, EGF, which upregulates urokinase-type plasminogen activator expression [Grimaldi et al., 1986] induces ERK activity [Minden et al., 1994b]. Third, the stimulation of urokinase-type plasminogen activator gene expression by *ras* [Bell et al., 1993] can be abrogated by a dominant-negative MAPKK expression vector [Lengyel et al., 1995a]. However, not all studies indicate a common pathway in the regulation of urokinase expression. Thus, using A431 cells, Kessler and Markus [1993] demonstrated that the induction of urokinase-type plasminogen activator synthesis by EGF and phorbol ester utilized separate pathways in A431 cells, since downregulation of protein kinase C with phorbol ester had little effect on the stimulation of this protease by EGF. Whether this divergence reflects the different cell lines used is unclear at the present time. Certainly, however, the fact that multiple pathways can be used by the same cells as evident in the study by Kessler and Markus [1993] and others [Botteri et al., 1990; Degen et al., 1985] would suggest that urokinase-type plasminogen activator expression can be controlled by multiple mechanisms, any one of which may dominate, depending on the conditions and cell type.

The sensitivity of urokinase-type plasminogen activator expression in UM-SCC-1 cells to the c-*raf* dominant negative expression vector suggests that the regulation of protease synthesis, at least, in this cell line is dependent on a stimulus at or upstream of c-*raf*. One possibility is that c-*raf* is activated by *ras* itself or by protein tyrosine kinases which mediate their effects through the GTP-binding protein [Bruder et al., 1992; Kolch et al., 1991]. In either case, *ras* should be activated in UM-SCC-1 cells. However, metabolic labeling of UM-SCC-1 cells with ^{32}P, followed by the elution of GTP and GDP from immunoprecipitated *ras* indicated a high GDP/GTP ratio suggesting that this GTP-binding protein was essentially inactive in our cells (data not shown). Thus, it is unlikely that in UM-SCC-1 cells the initial stimulus for urokinase-type plasminogen activator expression is at the level of *ras*. More probable is that the activity of c-*raf* in UM-SCC-1 cells is altered by gene rearrangement [Teyssler et al., 1986; Storm and Rapp, 1993] or by regulators of this serine-threonine kinase such as protein kinase C [Sozeri et al., 1992] or membrane-associated protein phosphatases [Dent et al., 1995]. It is interesting to note that a similar situation exists in renal cell carcinoma in that a constitutively activated ERK1 is observed in the absence of a mutated H-, K-, or N-*ras* [Miyajima et al., 1995].

While the sensitivity of urokinase-type plasminogen activator expression in UM-SCC-1 to the kinase-deficient c-*raf* and dominant negative ERK1 expression vectors argues for a role of this MAPK in the regulation of urokinase-type plasminogen activator expression, we cannot currently exclude a role for the c-*jun*-regulating JNKs in this process. The activation of JNK by *ras* occurs through MEKK and is independent of c-*raf* [Minden et al., 1994; Derijard et al., 1995; Hibi et al., 1993], leading to increased AP-1 activity subsequent to c-*jun* phosphorylation. However, any speculation as to the role of JNKs in the regulation of urokinase-type plasminogen activator expression must take into account our observations that interfering with c-*raf* and ERK1 function with appropriate dominant-negative expression vectors effectively suppresses urokinase-type plasminogen activator promoter activity. Thus, either the JNKs play only a minor role in the constitutive expression of urokinase-type plasminogen activator by UM-SCC-1 cells or, alternatively, both ERKs and JNKs are concurrently required for the stimulation of this promoter.

In conclusion, our data indicate that, for at least one squamous cell carcinoma cell line, urokinase-type plasminogen activator expression is driven by an ERK1-dependent signaling pathway. This raises the interesting possibility that targeting of this kinase may represent a novel means of repressing the inappropriate synthesis of urokinase-type plasminogen activator in pathological conditions such as tumor cell invasion.

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