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Activities of MAP-Kinase Pathways in Normal Uroepithelial Cells and Urothelial Carcinoma Cell Lines

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It is often assumed that MAPK pathways drive proliferation of normal uroepithelial (UEC) and urothelial carcinoma (TCC) cells. To check this assumption, activities and inducibilities of promoters containing serum-response elements (SRE) or AP-1 binding sites were investigated in cultured UEC and seven TCC lines. Reporter plasmids dependent on SRE or AP-1 sites were highly active in UEC, but significantly less so in TCC lines. Reporter activity in TCC lines could be induced by constitutively active MEKK4 or TPA. Accordingly, phosphorylation of the MAPK pathway components MEK, ERK, and ELK1 was most pronounced in UEC and lower in TCC lines. MAPK-dependent promoter activities and bromodeoxyuridine incorporation decreased in UEC upon withdrawal of growth factors, but less so in TCC lines, in which serum diminution increased apoptosis. Likewise, E2F-dependent promoters responded to growth factors in UEC, but were more serum-independent in the TCC lines, which lack either RB1 or p16^{INK4A}. MEK inhibitors inhibited BrdU incorporation in UEC more strongly than in TCC lines. Thus, proliferation of normal uroepithelial cells is indeed associated with activation of MAPK pathways. However, autonomous proliferation of TCC lines—unexpectedly—appears much less dependent on MAPK activation and may rather be promoted by defects in cell cycle regulation.

INTRODUCTION

Increased proliferation is a characteristic property of tumor cells which is often caused by enhanced activity of intracellular signal transduction pathways [1]. MAP kinase (MAPK) pathways are thought to be particularly important [2]. In many cancers, they are constitutively activated by one of several mechanisms, such as mutations in *RAS* genes or overexpression of tyrosine receptor kinases, particularly ERBB1 or ERBB2. On the other hand, MAP kinase pathways are actively down-regulated in some cancer types [3, 4]. ERK and JNK MAPK pathways activate gene expression via promoters containing specific elements such as the serum-response element (SRE) or binding sites for activating protein-1 (AP-1). One important group of MAPK pathway target genes encodes proximate activators of the cell cycle such as c-MYC, D-cyclins, or CDK2 [5] that promote phosphorylation of the retinoblastoma protein RB, leading in turn to derepression of genes dependent on E2F transcription factors, e.g., *CCNE* and *MYC*. Of note, regulators of the cell cycle such as c-MYC and cyclins, on one hand, or pRB and the CDK inhibitor p16^{INK4a}, on the other hand, are themselves subject to activating or inactivating mutations, respectively, in many tumors [6].

It is commonly assumed that MAPK pathways are involved in the proliferation of transitional cell carcinoma of the bladder (TCC), a cancer derived from uroepithelial cells [7, 8]. The evidence is, however, indirect. Overexpression of epidermal growth factor receptor (EGFR) or ERBB2 [9–12] and mutations in Ha-RAS [13, 14] occur in TCC and could result in constitutive MAPK activation, as is commonly observed in squamous carcinomas as a consequence of EGFR overexpression [15]. Moreover, a significant number of papillary bladder carcinomas harbor activating mutations in the FGFR3 tyrosine receptor kinase [16, 17], which physiologically signals through MAPKs and STAT factors [18]. Actual measurements of MAPK activities or those of their ultimate promoter targets in TCC have, however, not been performed. By comparison, it is well established that almost all advanced bladder carcinomas lack either pRb or p16^{INK4a}, with cyclin D1 overexpression preferentially occurring in earlier stages [7, 8]. Accordingly, permanent TCC cell lines are defective in either pRb or p16^{INK4a} [19]. These cell lines resemble advanced TCC tissues in further respects, such as p53 mutations, cytokeratin patterns, and chromosomal aberrations. They are

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therefore generally regarded as representative of many advanced bladder cancers [20, 21].

Urothelial cancer lines can be compared to normal primary uroepithelial cells (UEC), which can be cultured in a chemically defined medium in which they proliferate spontaneously [22]. Their proliferation and survival are supported by supplementation with EGF, cholera toxin, and bovine pituitary extract (BPE). As for TCC cells, it is not known which intracellular pathways relay proliferation signals in their normal counterparts. Conceivably, MAP kinase pathways may be involved, since heparin-binding EGF, the growth factor most likely involved in autocrine growth stimulation [23], as well as the EGF and BPE supplements, act through tyrosine kinase receptors, i.e., EGFR and FGFRs.

If proliferation of normal uroepithelial cells involved activation of MAPK pathways, proliferation of TCC cells might be achieved by constitutive activation of these same pathways. Here, we present data that strongly suggest that proliferation ofnormal uroepithelial cells in primary culture is indeed associated with activation of MAP kinases. Surprisingly, however, typical TCC cell lines do not appear to depend on MAPK pathways for continuous proliferation.

MATERIALS AND METHODS

Plasmids. The following plasmids were kindly donated: pT109luc [24] was provided by Dr. S. K. Nordeen (Denver, CO); E2Fluc [25], provided by Dr. A. Harel-Bellan (Villejuif, France), contains an artificial promoter with three E2F-binding sites directing the firefly luciferase gene; c-mycP2luc [26], provided by Dr. A. Polack (Munich, Germany), contains the c-*MYC* P2 promoter in front of the luciferase gene; −3565/+263 ("cycEmaxluc"), provided by Dr. P. Jansen-Dürr (Innsbruck, Austria), contains 3.8 kb of the murine cyclin E promoter [27] in front of the luciferase gene. pTKRL and pGL3 were purchased from Promega Europe (Mannheim, Germany). SREluc, AP1luc, and pFC-MEKK were purchased from Stratagene (Heidelberg, Germany). Plasmid pLINEluc was constructed by insertion of bp −193 to +661 from the active LINE-1 element L1.2B (Dr. H. H. Kazazian, Philadelphia, PA) into the *Sma*I/*Bgl*II (*Bam*HI) sites of pGL3.

Cell lines and culture. The bladder carcinoma cell lines VMCub1, VMCub2, 5637, HT1376, SW1710, and SD obtained from Dr. J. Fogh (Sloan-Kettering Institute, Rye, NY) and BFTC905 obtained from the DSMZ (Brunswick, Germany) (cf. Table 1 for properties of the cell lines) were routinely cultured in Dulbecco's minimal essential medium supplemented with 15% fetal calf serum and 100 μg/ml penicillin/streptomycin as described [19]. The hepatoma cell line HepG2 was maintained as described [28]. Normal uroepithelial cells were prepared from ureters of patients undergoing nephrectomy for nonurothelial renal disease as described by Southgate *et al.* [22]. They were routinely maintained in keratinocyte serum-free medium (Gibco) supplemented with BPE, EGF (5 ng/ml), and cholera toxin (30 ng/ml). All experiments involving normal uroepithelial cells were repeated with several independent cultures, each used after the first passage.

Transfection procedures. Cells were grown for 24 h in six-well plates before addition of DNA/FuGene (Roche, Mannheim, Germany) mixtures optimized as advised by the manufacturer. Luciferase activity was usually measured using the Dual Luciferase Reporter Assay System (Promega Europe) at 80% confluence, 48 h after transfection. Transfection efficiencies were adjusted by cotransfection of the *Renilla* luciferase plasmid pTKRL. Each experiment was performed in triplicate with at least three different passages of the cell lines and at least three independent cultures of normal uroepithelial cells.

TABLE 1

Some Properties of the TCC Lines Used

	p53 mutation	Rb (Western blot)	$p16^{INK4A}$	Doubling time (h)
VmCub1	Mutant exon 5	+	Mutant	20
VmCub2	Mutant exon 5	+	Deleted	22
5637	Mutant exon 8	−	+	37
HT1376	Mutant exon 7	−	+	37
SW1710	Mutant exon 8	+	Deleted	43
SD	Mutant exon 4	+	Deleted	28
BFTC905	Wild type (MDM2 ↑ ↑)	+	Deleted	23

Note. Molecular characterization of the TCC lines used in this study (cf. [19, 35, 36, 38].

Proliferation and apoptosis assays. Cell doubling times were determined by seeding cells into 6-well plates (10^4 cells/well) and counting after 24, 48, and 72 h in a Neubauer chamber. Bromodeoxyuridine (BrdU) incorporation assays were performed in 96-well plates using the *In Situ* Cell Proliferation ELISA (Roche). Apoptosis assays were performed using the Cell Death Detection $ELISA^{PLUS}$ (Roche).

Western blotting. Whole-cell lysates were prepared in RIPA buffer containing 50 mM Tris, pH 7.2, 150 mM NaCl, 40 mM NaF, 5 mM EGTA, 5 mM EDTA, 1 mM sodium orthovanadate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 10 μg/ml phenylmethylsulfonyl fluoride. Equal amounts (10 μg) of protein quantified by the Bradford method were separated by SDS–polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA). The membranes were then blocked with 10% nonfat milk powder and 0.1% Tween 20 in phosphate-buffered saline, overnight at 4°C. They were incubated successively with the primary antibodies anti-phospho-(Ser217/221)-MEK1/2 at a 1:1000 dilution, anti-MEK1/2 at 1:1000, anti-phospho-p44/42 MAPK (Thr202/Tyr204) at 1:2000 dilution (all purchased from Cell Signaling Technology, Bad Homburg, Germany), anti-MAPK1/2 (Erk1/2-CT) (Upstate Biotechnology, New York, NY) at 1:2000, B4 anti-phospho-ELK1 (Santa Cruz Biotechnologies, Santa Cruz, CA), and, as a control, anti-α-tubulin 3-5-1-2 (Sigma, St. Louis, MO) at 1:5000 for 1 h at room temperature. HRP-conjugated rabbit anti-mouse antibody (1:1000) was used as secondary antibody and binding was visualized by luminescence using the ECL Kit (Amersham–Pharmacia, Freiburg, Germany).

RESULTS

MAPK pathway activities were first studied in different TCC cell lines (VMCub1, VMCub2, HT1376, SD, 5637, SW1710, BFTC-905) under standard medium conditions (including 15% serum) and in normal uroepithelial cells proliferating in complete medium (including BPE, EGF, and cholera toxin). Important properties of the investigated TCC lines are compiled in

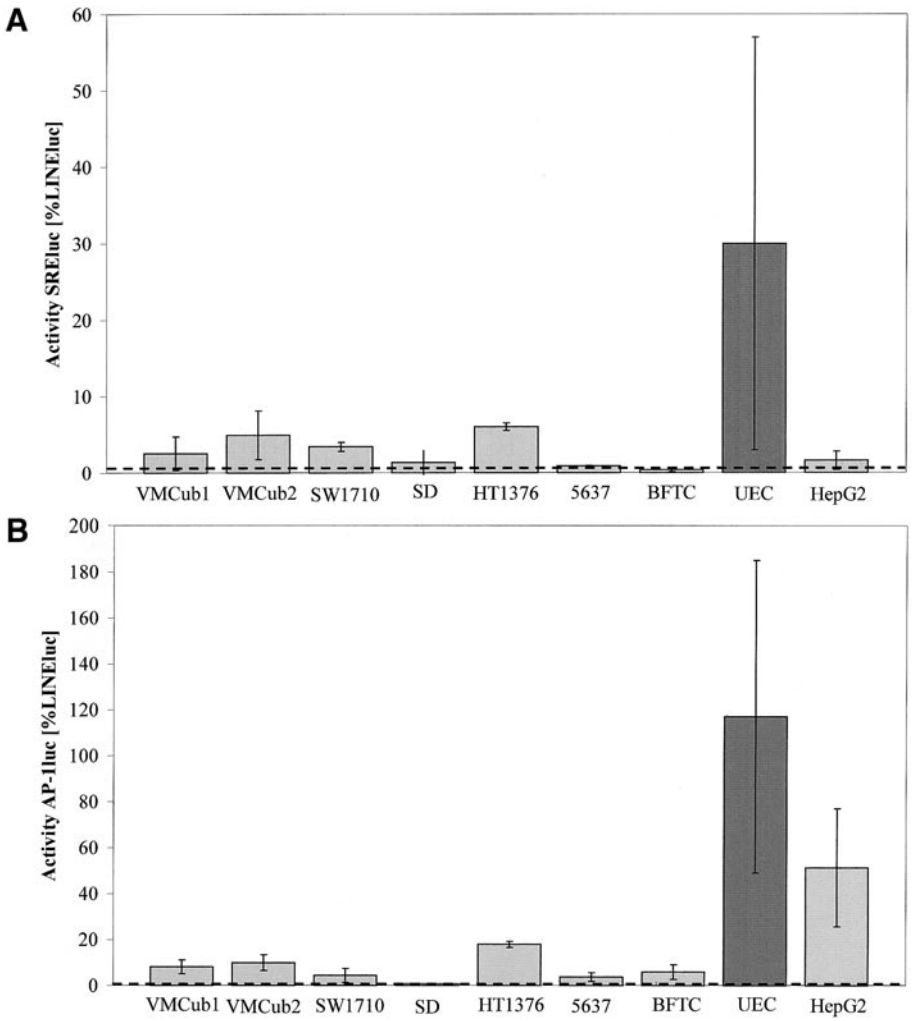

FIG. 1. Activities of SREluc and AP1luc reporter plasmids in TCC lines and normal uroepithelial cells. Mean ± SD activities of MAPK pathway reporter plasmids in the indicated TCC cell lines, in normal uroepithelial cells (dark column), and in HepG2 cells. Activities are corrected for transfection efficiencies as described under Materials and Methods and are expressed as percentage activity of the constitutively active pLINEluc plasmid. The broken line in each graph indicates the median activity level of the promoterless luciferase plasmid pGL3; the range in the individual cell lines was 0.2–1.1% of pLINEluc. (A) SREluc; (B) AP1luc.

Table 1. For comparison, the hepatoma cell line HepG2 was also included.

Activity of MAPK pathways should lead to increased activities of promoters containing SRE and/or AP-1-binding elements. Luciferase expression by the reporter plasmids SREluc and AP1luc specifically depends on these elements. These plasmids were transfected into TCC lines or normal uroepithelial cells and the resulting luciferase activity was measured 40 h later. Differences in transfection efficiencies were corrected for by cotransfection of a *Renilla* luciferase plasmid with a constitutively active promoter. Standard plasmids without a promoter (pGL3) or with a LINE-1 retrotransposon promoter equally active in each cell type (pLINEluc) were carried along in each experiment. Each experiment was repeated with several distinct passages of the TCC lines or with several independent primary cultures of normal uroepithelial cells.

Figure 1 shows the activities of SREluc and AP1luc in the various cells, expressed relative to the activity of the constitutively active LINE promoter. The results do not change qualitatively when the data are expressed relative to the background activity provided by the promoterless luciferase vector pGL3 (approximately 1% of pLINEluc in each cell line), indicated by the broken lines in Fig. 1. In the TCC lines SREluc activities ranged from 0.4 (BFTC905) to 6% (HT1376) of pLINEluc and were at most moderately higher than those of the promoterless control vector (Fig. 1A). Much higher activity was observed in normal uroepithelial cells that was almost fivefold stronger than in HT1376, showing the highest activity among the carcinoma cell

lines. The differences between normal uroepithelial cells and each of the TCC lines were statistically significant (*t* test: $P = 0.01–0.02$); the activity in HT1376 was significantly higher than in some other TCC lines. Activity of AP1luc (Fig. 1B) was more pronounced in several cell lines, ranging from 1% of pLINEluc in SD to 18% in HT1376 (HT1376 vs other cell lines: $P = 0.01–0.23$), but significantly higher activity ($P = 0.00002–0.0001$) was again observed in normal uroepithelial cells (Fig. 1B). In HepG2 cells AP1luc activity was elevated, even though SREluc was only weakly active. This is expected, because hepatocytes express several factors of the JUN family [29]. Among the different cells of urothelial origin, SREluc and AP1luc activities paralleled each other well ($R^2 = 0.96$, data not shown).

In all TCC lines, both AP1luc and SREluc could be induced by cotransfection of a constitutively activated MEKK4 or by the protein kinase C agonist 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Cotransfection of MEKK4 increased the activity of both reporters (Figs. 2A and 2B). The extent of activation in each cell type was in general inversely related to the basal activity measured. The only exception was observed in BFTC905, which was only weakly inducible. Notably, in normal uroepithelial cells very little additional activation was achieved by cotransfection of MEKK4, indicating that the activity of promoters depending on MAPK pathways was close to maximal under basal conditions. AP1luc and SREluc activities were also increased by treatment with 0.1 nM TPA in several cell lines displaying low basal activity (Fig. 2C).

To confirm the data from the promoter activity measurements, three components of the canonical MAPK pathway were investigated by Western blotting using antibodies specific for the active phosphorylated form. In normal urothelial cells under standard growth conditions, MEK, ERK and ELK1 were all substantially phosphorylated (Fig. 3). Interestingly, normal urothelial cells, like almost all others of uroepithelial origin, preferentially expressed the ERK2 p42 over the ERK1 p44 isoenzyme and this was the only phosphorylated form detectable (Fig. 3B). The only exception to this rule was HT1376, which expressed both isozymes and showed some phosphorylation of ERK1 as well. In accordance with the moderate promoter activity observed in HT1376, some phosphorylation of MEK and ELK1 was detectable. As expected, phosphorylation of all three components was increased by TPA as in all other TCC lines. Interestingly, BFTC905, which had also shown the weakest induction in the MEKK4 transfection experiment, also reacted weakly to TPA at the level of MEK phosphorylation. Moreover, the TCC lines SD and—to a lesser extent—VMCub1 and VMCub2 showed a substantial level of phosphorylated ERK2 under basal conditions (Fig. 3B) despite low SRE-dependent promoter activity (Fig. 1A). However, under normal growth conditions increased ERK phosphorylation was not accompanied by increased ELK1 phosphorylation, which could be strongly induced by TPA (Fig. 3C). Thus, while all TCC lines showed substantially less SRE-dependent promoter activity than normal uroepithelial cells, this difference was not in all cells reflected at each step of the MAPK cascade.

As expected, normal and transformed urothelial cells reacted differently to diminution of growth factor supply (Fig. 4). Withdrawal of EGF and cholera toxin from normal uroepithelial cell medium caused a decrease in BrdU incorporation (Fig. 4A) and protein content (Fig. 4B). In contrast, cultivation of the TCC lines in medium containing 0.5% serum did not affect BrdU incorporation. Protein content of the cultures decreased nevertheless (Fig. 4B), which can be ascribed to an increased apoptotic rate (Fig. 4C).

Table 2 shows a comparison of SREluc and AP1luc activities in several bladder carcinoma cell lines transferred after transfection from standard medium to medium with 0.5 or 20% serum. It is evident that the 40-fold difference in growth factor supply in most cases did not substantially affect the promoter activities. Typically, around 2-fold increases were observed with high serum that were not statistically significant. Likewise, the extent of induction by MEKK4 cotransfection did not change in most of the cell lines, with the exception of VMCub1, in which it increased in low-serum medium (data not shown). Withdrawal of growth factors from normal cells caused a decrease in SREluc activity from 27.3 ± 27.7 (relative to LINEluc) to 16.3 ± 7.6% and in AP1luc activity from 117.1 ± 68.4 to 75.6 ± 28.0%. The relatively high variation resulting from culture to culture differences masks the fact that in each single of the 9 or 13, respectively, investigated cultures from different donors a decrease in the activity of both reporters was observed upon growth factor withdrawal. Accordingly, induction by MEKK4 transfection was higher by 1.9-fold for SREluc and 4.8-fold for AP1luc in medium without supplements. In most TCC lines, SREluc activity was not significantly altered by addition of 5 ng/ml EGF to low-serum medium, except in the VMCub1 and SD cell lines, in which a moderate 2.5-fold increase was obtained (data not shown).

The same comparison between high and low serum or with/without the EGF and cholera toxin growth factors was also performed with three more reporter constructs containing promoters that essentially depend on E2F for their activities. E2Fluc is an artificial construct containing several E2F binding sites with an TATA box as the only other defined promoter element, whereas c-mycP2luc and cycEmaxluc are derivatives of natural promoters with crucial E2F binding sites. Under standard growth conditions all three constructs

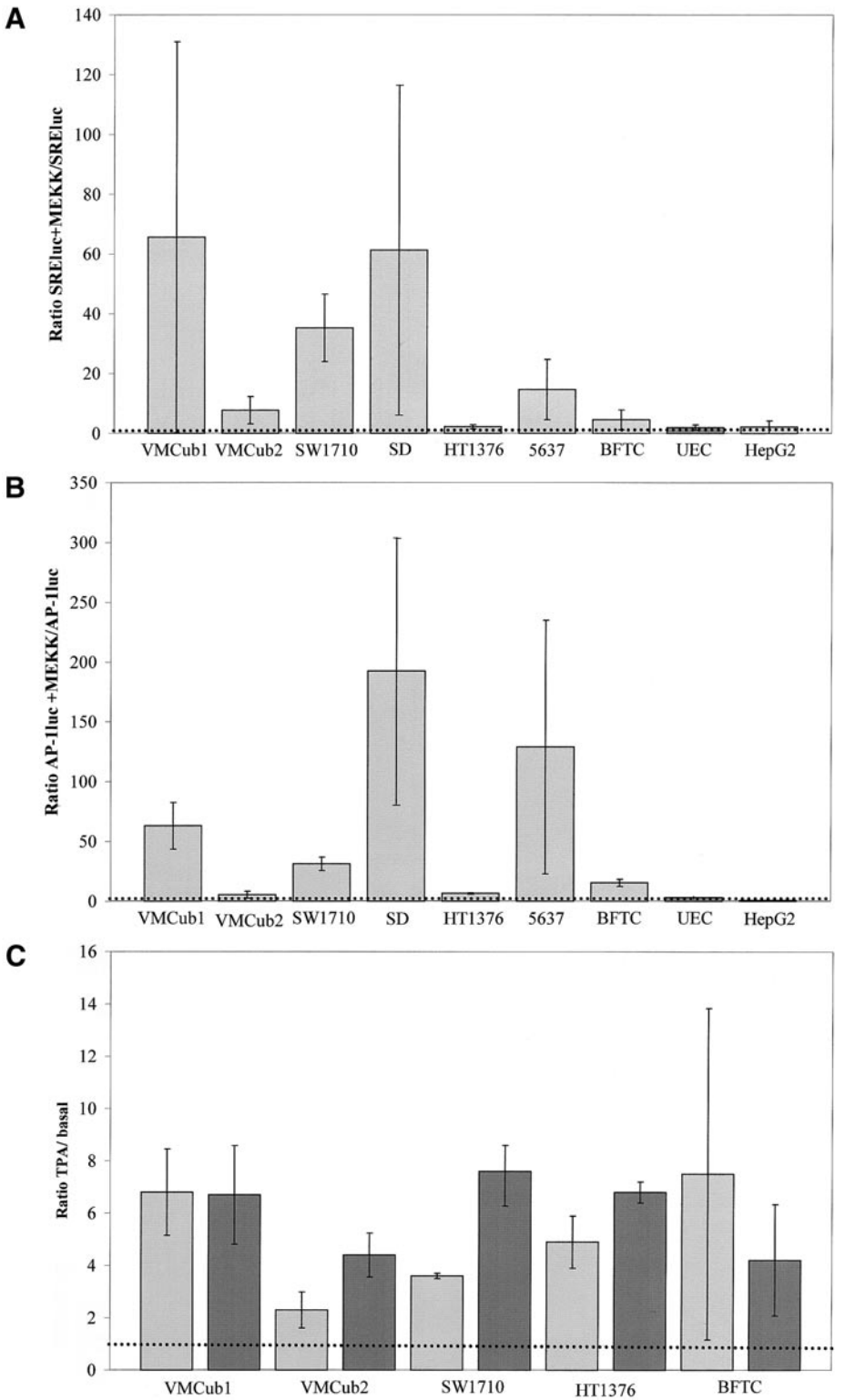

FIG. 2. Activation of SREluc and AP1luc reporter plasmids by MEKK4 and TPA in TCC lines and normal uroepithelial cells. Induction of MAPK pathway reporter plasmid activities by cotransfection of constitutively active MEKK4. Columns for (A) SREluc and (B) AP1luc show the mean ratios ± SD of activities after MEKK4 cotransfection to basal activities for each indicated TCC cell line, normal uroepithelial cells (dark column), and HepG2 cells. Inducibilities in UEC were significantly lower than in VMCub1, SW1710, SD, and 5637 cell lines (*t* test: $P < 0.05$). (C) Mean ratios ± SD of TPA-induced vs basal SREluc (light columns) and AP1luc (dark columns) activities in five TCC cell lines. In each graph the dotted line marks a ratio of 1, i.e., no induction.

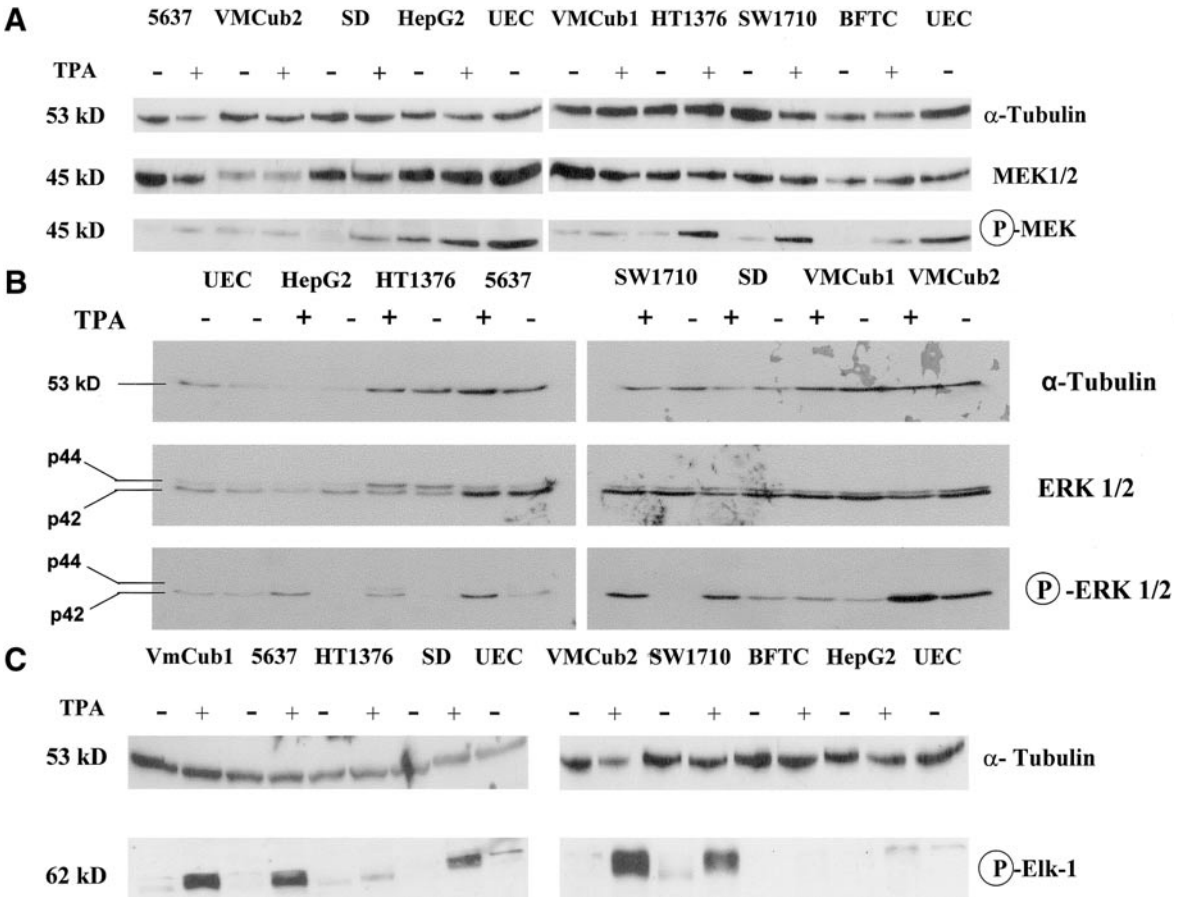

FIG. 3. Western blot analysis of MEK, ERK, and ELK-1 phosphorylation in TCC lines and normal uroepithelial cells. Cell lysates from the indicated cells untreated (−) or pretreated with 0.1 μM TPA for 15 min (+) were run on a polyacrylamide gel, blotted, and probed successively with antibodies directed against phosphorylated MEK1/2 and total MEK1/2 (A), phosphorylated ERK1/2 and total ERK1/2 (B), and phosphorylated ELK-1 (C) as indicated. In each experiment, α-tubulin was employed as a loading control.

have been found to be more active in TCC cells defective in RB regulation than in normal uroepithelial cells (Steinhoff *et al.*, 2002). Here, we determined how their activity would be affected by growth factor withdrawal. In normal uroepithelial cells, all three promoters reacted to withdrawal of growth factors by decreasing activity (Fig. 5). In the TCC lines, diminishing the serum concentration to 0.5% in general had less effect, with slight decreases in some cell lines, particularly in E2Fluc activity (Fig. 5A), but unchanged or even increased activity in most.

The MEK inhibitor PD98059 at 20 or 50 μM significantly inhibited BrdU incorporation into normal uroepithelial cells grown with BPE as the only supplement (Fig. 6). Inhibition was alleviated by additional supplementation with EGF and cholera toxin. A slight inhibition of BrdU incorporation by higher PD98059 concentrations was also observed in HT1376 cells. In all other TCC lines, in low- or high-serum medium, no significant inhibition was observed (data not shown); instead, occasionally a small increase was seen, as in SD cells shown in Fig. 6. Similarly, the MEK inhibitor UO126 at 5 μM significantly affected BrdU incorporation in normal cells, but not in TCC cell lines, although phosphorylation of ERK2 was diminished at this concentration (data not shown).

DISCUSSION

The main finding of the present study is that MAPK pathways leading to activation of SRE and AP-1 promoter elements exhibit very high activity in proliferating normal uroepithelial cells, but are much less active in established urothelial carcinoma cell lines. Thus, surprisingly, proliferation of TCC cell lines is not driven by constitutive activation of MAPK signaling pathways. Our data therefore do not support the idea that TCC lines typically achieve independence from external growth factors by constitutively activating the MAPK pathways that are important in their normal counterparts.

Nevertheless, several experiments in this study fit

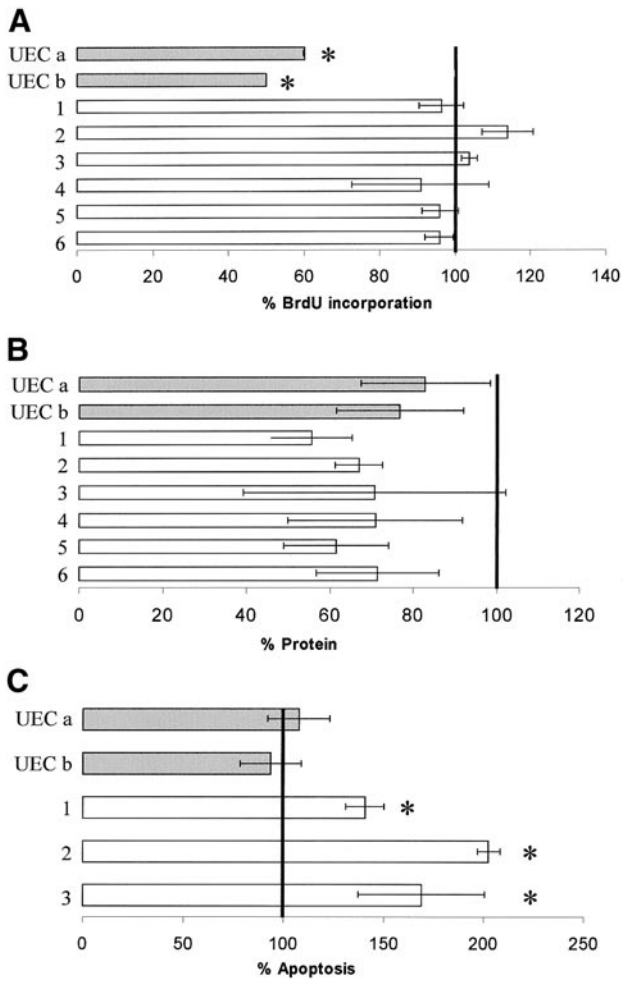

FIG. 4. Effects of growth factor withdrawal on growth of TCC lines and normal uroepithelial cells. Changes in BrdU incorporation (A), total protein (B), and apoptotic rates (C) after 48 h of cultivation in medium without EGF and cholera toxin (normal cells, UEC, shaded bars) or in medium supplemented with 0.5% instead of 15% fetal bovine serum (TCC lines, light bars). The TCC lines in this experiment were 1, VMCub1; 2, VMCub2; 3, 5637; 4, HT1376; 5, SD; 6, SW1710. Values are expressed as percentages of those in standard medium set as 100% (indicated by the thick vertical line). Significant differences ($P < 0.05$) compared to standard medium conditions are indicated by asterisks.

the expectation that proliferation of TCC cells is to a large extent independent of external signals. Most importantly, they did not react to substantial diminution of the serum concentration (to 0.5%) by a decrease in DNA synthesis. The apoptotic rate increased upon serum diminution, indicating that serum factors are required for survival, but do not regulate proliferation. Serum independence was also reflected at the level of promoter regulation through SRE and AP-1 sites and largely extended to E2F sites. Since growth autonomy does not appear to be established by genetic or epigenetic aberrations causing constitutive activation of MAPK pathways, activation of other signaling pathways could be responsible, e.g., of the Wnt pathway important in colon cancer [30], of the Hedgehog pathway as in basal cell carcinoma of the skin [31], or of STAT factors as in squamous cell carcinoma of the head and neck [15]. These pathways are currently being investigated in our laboratory.

More likely, growth autonomy may be related to the alterations in the *RBI* or *CDKN2A* genes that are present in all TCC cell lines (Table 1), as they are in almost all advanced TCC tissues. An important component in stimulation of cell proliferation by MAPKs is activation of cell cycle regulators that promote phosphorylation of RB, prominently by promoting transcription of cyclin D1. Conceivably, the requirement for MAPK activity to drive proliferation is diminished in TCC cells directly by the mutations in cell cycle regulators. Deletion of RB has been shown to partially obliterate the requirement for an activated RAS in transformation of mouse fibroblasts [32]. In accordance with this notion, E2F-dependent promoters are two- to fivefold more active in TCC lines lacking RB or $p16^{INK4A}$ than in proliferating normal uroepithelial cells (Steinhoff *et al.*, submitted for publication) and—as shown here—respond only slightly or not at all to diminution of the serum concentration. From this point of view, MAPK activation in TCC lines might be largely unnecessary for cell proliferation. Moreover, it has to be considered that MAPK activation does not always promote cell proliferation, but can induce differentiation or even apoptosis in some cell types. For instance, in cultured keratinocytes MAPK activation can be associated with proliferation or differentiation, depending on its kinetics [33]. In the same manner, MAPK activation can induce differentiation or proliferation of PC12 neuroblastoma cells, depending on the kinetics of stimulation [34]. Therefore, down-regulation of the MAPK pathway in TCC cells might be necessary to maintain a permanent proliferative state.

While all TCC lines showed substantially lower activities of MAPK-dependent promoters than normal urothelial cells, the phosphorylation of MEKs and ERK2 in particular was not always decreased to the same extent. For instance, the TCC line SD displayed low MAPK-dependent promoter activity despite phosphorylated ERK levels that were not substantially lower than in normal proliferating UEC. ERK phosphorylation did not result in ELK1 phosphorylation in this cell line, indicating that a further regulation step is active in preventing ERK phosphorylation and promoter activation. ERK activity in these cells could well affect properties such as motility. Importantly, however, in none of the TCC lines, including SD, was cell proliferation sensitive to MEK inhibitors. Therefore, although individual kinases in the canonical MAPK pathway may be active in some TCC lines, MAPK-

TABLE 2

Effect of Growth Factor Withdrawal on SREluc and AP1luc Reporter Plasmid Activities in TCC Lines

	VMCub1	VMCub2	SW1710	SD	HT1376	5637
SREluc						
0.5% FCS	0.4 ± 0.2	1.9 ± 1.0	1.6 ± 0.2	0.7 ± 0.7	12.3 ± 5.0	0.3 ± 0.1
n	9	9	12	9	12	6
20% FCS	2.5 ± 2.2	4.9 ± 3.2	3.4 ± 0.6	1.3 ± 1.6	6.0 ± 0.5	0.9 ± 0.03
(% of 0.5% FCS)	(684%)	(259%)	(210%)	(202%)	(49%)	(270%)
n	9	9	9	12	9	6
AP1luc						
0.5% FCS	3.1 ± 2.0	1.6 ± 0.1	9.6 ± 0.9	0.6 ± 0.02	17.0 ± 11.2	2.2 ± 0.8
n	9	9	6	6	9	6
20% FCS	8.1 ± 3.0	9.9 ± 3.4	6.5 ± 0.5	0.8 ± 0.2	17.9 ± 1.3	3.7 ± 1.9
(% of 0.5% FCS)	(258%)	(616%)	(68%)	(137%)	(105%)	(171%)
n	12	6	6	6	6	6

Note. Mean ± SD activities of SREluc and AP1luc reporter plasmids in the indicated TCC cell lines transferred to 0.5% serum or 20% serum after transfection. Activities were corrected for transfection efficiencies as described under Materials and Methods and are expressed as percentages of pLINEluc. Each experiment was performed in triplicate with two to four independent passages.

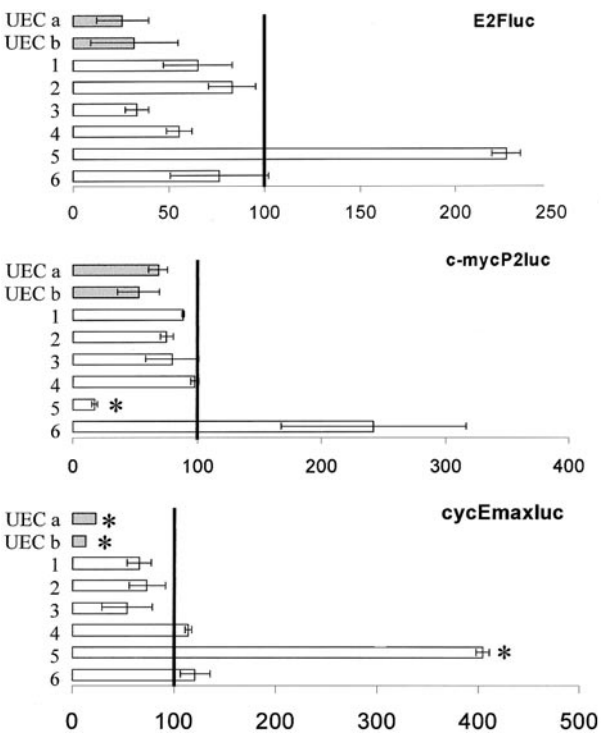

FIG. 5. Effect of growth factor withdrawal on E2F-dependent promoters in TCC lines and normal uroepithelial cells. Activities of the E2F-dependent reporter plasmids E2Fluc, c-mycP2luc, and cycEmaxluc in normal uroepithelial cells in medium without EGF and cholera toxin (UECa); medium without EGF, cholera toxin, and BPE (UECb); or medium supplemented with 0.5% serum only (TCC lines 1–6). The TCC lines in this experiment were 1, VMCub1; 2, VMCub2; 3, 5637; 4, HT1376; 5, SD; 6, SW1710. Values are expressed as percentages of those in standard complete medium set as 100% (indicated by the thick vertical line). Significant differences compared to standard medium conditions are indicated by asterisks.

dependent promoter activities are low and this pathway does not appear to influence cell proliferation.

Of note, all experiments in this study were performed in continuously growing cells. In theory, activation of MAPK pathways might be important for TCC cells to reenter the cell cycle from a state of quiescence. However, they do not establish a quiescent state easily, as demonstrated by their behavior following serum withdrawal. Since in almost all TCC lines (with the partial exception of BFTC905) SREluc and AP1luc activity as well as phosphorylation of MEK, ERK, and ELK1 could be induced by MEKK4 or TPA, all essential components of the MAPK pathways would be present.

Compared to keratinocytes, uroepithelial cells have been little studied. Normal uroepithelial cells proliferate spontaneously when cultured in a chemically defined medium, but proliferation and survival are enhanced by growth factors such as EGF and the mixture contained in BPE [22]. This study demonstrates for the first time that very high MAPK activity is present in these cells during proliferation. This activity is almost maximal in the presence of growth factors, since little further stimulation could be achieved by MEKK4 cotransfection. Remarkably, substantial activity was retained after withdrawal of growth factors.

Autocrine stimulation of uroepithelial cell proliferation, probably by HB-EGF [23], is well established. Presumably because of this autocrine component, various studies have reported differences in the degree to which proliferation is stimulated by EGF [22, 23]. In our hands, withdrawal of EGF decreased BrdU incorporation by 30–50% (cf. Fig. 4). In the absence of EGF, uroepithelial cells became much more sensitive to the MEK inhibitor PD98059. This indicates that MAPK

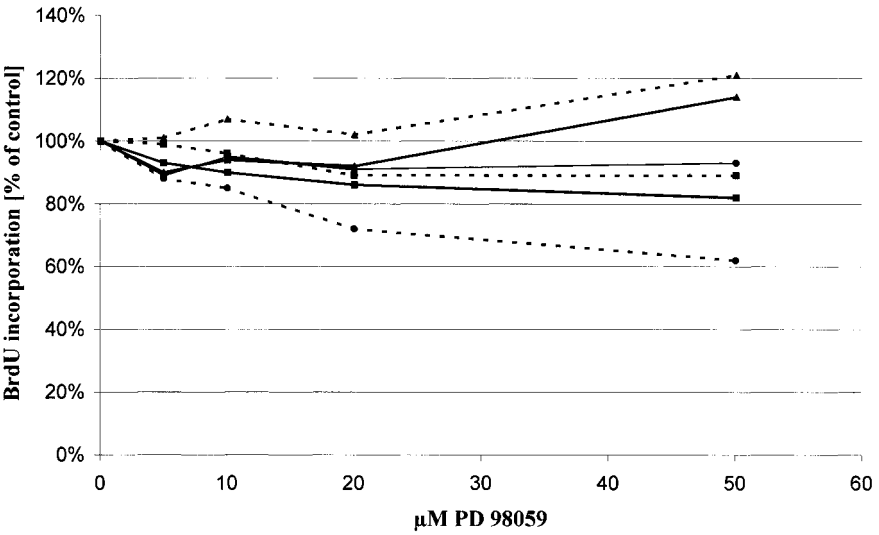

FIG. 6. Effects of PD98059 on DNA synthesis in TCC lines and normal uroepithelial cells. DNA synthesis over 24 h was measured by BrdU incorporation after preincubation with and in the presence of the indicated concentrations of PD98059. Data points reflect the means of at least three independent experiments in eight independent culture wells each. Circles, UEC; squares, HT1376; triangles, SD; continuous lines, complete medium; broken lines, BPE only (UEC), 0.5% serum (TCC).

activation is not only associated with, but also necessary for, proliferation. Like in other cell systems [34], addition of EGF appears to induce a robust activation of the MAPK pathway that cannot entirely be blocked by the inhibitor.

Over the past years, inhibitors of many signaling pathways, including MAPK inhibitors like PD98059 and UO126, have been developed and clinical trials with related compounds have begun. Therefore, our data carry substantial clinical implications in predicting that many advanced TCC, possibly all those with defects in RB or $p16^{INK4A}$, may not respond to inhibitors of MAPK pathways, or at least less than normal proliferating urothelial cells. Targets for drug therapy of advanced TCC may need to be sought elsewhere.

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