Alternative Splicing in Fibroblast Growth Factor Receptor 2 Is Associated with Induced Epithelial-Mesenchymal Transition in Rat Bladder Carcinoma Cells

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We described previously that acidic fibroblast growth factor (aFGF), but not basic fibroblast growth factor (bFGF), can induce the rat carcinoma cell line NBT-II to undergo a rapid and reversible transition from epithelial to mesenchymal phenotype (EMT). We now find that NBT-II EMT is stimulated by keratinocyte growth factor (KGF) in cells grown at low density. Accordingly, a high-affinity receptor showing 98% homology to mouse FGF receptor 2b/KGF receptor was cloned and sequenced from NBT-II cells. Northern analysis indicated that mRNA for FGF receptor 2b/KGF receptor was drastically down-regulated within 1 wk in aFGF-induced mesenchymal NBT-II cells. This decrease coincided with an up-regulation of FGF receptor 2c/Bek, a KGF-insensitive, alternatively spliced form of FGF receptor 2b/KGF receptor. Functional studies confirmed that KGF could not maintain EMT induction on mesenchymal NBT-II cells. FGF receptor 1 and FGF receptor 2c/Bek could also support EMT induction when transfected into NBT-II cells in response to aFGF or bFGF. Such transfected cells could bind bFGF as well as aFGF. Therefore, EMT can be induced through different FGF receptors, but EMT may also regulate FGF receptor expression itself.

INTRODUCTION

Fibroblast growth factors (FGF) represent a well studied family of nine closely related mitogenic polypeptides (reviewed by Basilico and Moscatelli, 1992; Miyamoto *et al.*, 1993; Partanen *et al.*, 1993). Developmental in vivo as well as in vitro studies show that they support other functions, depending mainly on the context. The same factor may act as a cell proliferation factor in one system and as a differentiation factor in another system, or in the same system at a different time. This dual activity may reflect a diversity in cell receptor types or in intracellular signaling systems. Among them, kera-

tinocyte growth factor (KGF)¹ is mitogenic specifically on epithelial cells and is produced by stromal fibroblasts. It is apparently involved in normal keratinocyte proliferation (Rubin *et al.*, 1989; Aaronson *et al.*, 1991), migration (Tsuboi *et al.*, 1993), and differentiation (Guo *et al.*, 1993).

High-affinity FGF receptors constitute a complex family with at least four homologous groups (Ruta *et al.*, 1989; Dionne *et al.*, 1990; Hattori *et al.*, 1990; Mansukhani *et al.*, 1990, 1992; Reid *et al.*, 1990; Keegan *et al.*, 1991; Partanen *et al.*, 1991; Johnson *et al.*, 1993). These receptors share a highly homologous intracellular tyrosine kinase domain and an extracellular domain in-

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¹ Abbreviations used: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; EMT, epithelial-mesenchymal transition; KGF, keratinocyte growth factor.

cluding two or three immunoglobulin (Ig) domains. In FGF receptor groups 1 and 2 (FGFR1 and FGFR2), alternative splicing in the second half of the third Ig domain can generate several forms—a, b, or c—with distinct affinities for different members of the FGF family (Champion-Arnaud *et al.*, 1991; Johnson *et al.*, 1991; Miki *et al.*, 1992; Yayon *et al.*, 1992). FGFR2b, also termed KGF receptor (FGFR2b/KGFR), has been found to bind acidic FGF (aFGF) and KGF specifically. It is the only FGF receptor known to bind KGF with a high affinity (Miki *et al.*, 1991). On the other hand FGFR2c, originally named Bek (FGFR2c/Bek), recognizes both aFGF and basic FGF (bFGF). FGFR activation by ligands induces tyrosine autophosphorylation through receptor dimer formation and phosphorylation of associated transducing factors such as PLC γ (Mohammadi *et al.*, 1992) that bind to the tyrosine-phosphorylated receptor region through src-homologous domains termed SH2 domains.

We have described previously a carcinoma cell line, NBT-II, that is activated on two different pathways by the same factor, aFGF, depending on the confluency state of the cells (Vallés *et al.*, 1990b). When cultured at low confluency, cells are induced by aFGF to undergo an epithelial-mesenchymal transition (EMT) characterized by significant intracellular remodeling and by cell migration (Boyer *et al.*, 1989). In contrast, when they reach high confluency, only cellular mitogenic activity is activated. NBT-II cells show high affinity for aFGF but only low affinity for bFGF (Vallés *et al.*, 1990a). Tyrosine kinase activity has been found to be involved in the transduction of FGF activation in NBT-II cells in all cases. A cAMP-dependent pathway was also shown to be specifically involved during the activation of mitogenesis but not in the alternative EMT pathway (Boyer and Thiery, 1993).

In this paper, we examined the biological activity of KGF on NBT-II cells then cloned and sequenced two NBT-II FGF receptors whose expression was found to be modulated functionally and at the RNA level after induction of EMT, as well as by cell culture density. In addition, we analyzed the specificity of the FGFR involved in EMT-induction by transfecting and expressing FGFR1 and FGFR2c/Bek in NBT-II cells.

MATERIALS AND METHODS

Reagents

Human recombinant aFGF and bFGF were kindly provided by Dr. M. Jaye (Rhône Poulenc Rorer Central Research, King of Prussia, PA) and Dr. Prats (Toulouse, France), respectively. KGF was purchased from PeproTech (Rocky Hill, NJ). Mouse monoclonal antibodies against the desmosomal proteins desmoplakins I and II were a generous gift of Professor W.W. Franke (German Cancer Research Center, Heidelberg, Germany). Texas Red-coupled goat anti-mouse IgG (Immunotech, Marseille, France) was used as secondary antibody for indirect immunofluorescence.

Cell Culture

The rat bladder carcinoma NBT-II cell line was obtained from Professor Marc Mareel (University Hospital, Ghent, Belgium). Cells were cul-

tured in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine, antibiotics, and 10% heat-inactivated fetal calf serum (FCS) as previously described (Boyer *et al.*, 1989). Human keratinocyte primary culture YF29 (newborn foreskin, fourth passage) was grown by A. Rochat and Y. Barrandon (Ecole Normale Supérieure, Paris, France) in DMEM supplemented with glutamine, antibiotics, and 10% heat-inactivated FCS.

RNA Preparation and Polymerase Chain Reaction (PCR)

Total RNA was extracted from NBT-II cells, human keratinocytes, and Wistar rat lungs by the acid phenol method (Chomczynski and Sacchi, 1987). Poly (A)⁺ RNA was prepared from NBT-II cells, using the Fastrack kit (Invitrogen, San Diego, CA). Synthesis of cDNA was performed by Avian Myeloblastosis Virus reverse transcriptase as specified by the supplier (Stratagene, La Jolla, CA). The total volume for each reaction was 100 μ l for 4 μ g of RNA, using 5 μ l of cDNA as template, 0.4 μ g of each specific primer, 0.2 mM dNTPs, and 2 U of Taq polymerase in a buffer supplied with the enzyme (Perkin Elmer Cetus, Norwalk, CT). Annealing temperature was 46°C.

Primers

Specific primers were designed based on published FGFR sequences (Dionne *et al.*, 1990; Hattori *et al.*, 1990; Reid *et al.*, 1990). The locations are indicated in Figure 2A. The sequences were as follows: P1, GATTTTGTCAGTGAGAACAG; P2, ACACGGTTAATGTCTAGGA; P3, GCCGCCGGTGTAAACACCACGGAC; P4, TGGCAGACCTGTCAACCATGCAGA; P5, ATGGTCAG CTGGGGTCGTTTCATC; P6, TGTTTAACTGCGCTTTATGTG; P7, CACTCGGGGATAAATAGTTCCAAT; P8, TTGCTGTTTGGAGGACAGTGAG; and P9, ACCAAACCGTAGGCCTGTAGC.

Cloning and Sequencing

The 2.1-kilobase (kb) amplified fragment obtained with primers 5 and 6 (Figure 2A), the 1.3-kb amplified fragment obtained with primers 1 and 2 (Figure 2A), and the 150-base pair (bp) amplified fragment obtained with primers 3 and 4 (Figure 2A) were cloned directly into pCR 1000 vectors (Invitrogen) with T4 DNA ligase, following supplier instructions. Competent *Escherichia coli* cells (INV α F) were transformed by heat shock and grown in LB medium. Small scale plasmid preparations were performed using Qiagen (Chatsworth, CA) plasmid kits. Plasmids were sequenced in both directions by automated sequencing with an Applied Biosystems (Foster City, CA) DNA sequencer using M13 primers, as well as primers 1–8.

Transfection and Cell Selection

Constructs pFlg 24 and pGC 34 (Jaye *et al.*, 1988), kindly provided by M. Jaye, were cotransfected with 1 μ g of pAG60 vector coding for neomycin resistance (Colbère-Garapin *et al.*, 1981) into NBT-II cells by the calcium-phosphate method (Graham and van der Eb, 1973) using 10 μ g of DNA for each sample. After 24 h, precipitates were removed, and cells were treated by glycerol shock before another 24-h recovery period. Cells were then treated with 400 μ g/ml active G418 (GIBCO-BRL, Grand Island, NY) for 7 d. Surviving cells were cloned by limiting dilution.

Immunofluorescence Microscopy

Cells cultured on glass coverslips (12 mm) were stimulated with KGF (5 ng/ml), aFGF (10 ng/ml), or bFGF (10 ng/ml) 2 d after cell plating. Heparin (Choay Laboratories, Paris, France) at 10 μ g/ml was added to the culture medium to stabilize the biological activity of aFGF (Gospodarowicz and Cheng, 1986). Growth factors were renewed every other day for long term activation. Cells were processed for immunocytochemistry as described previously (Boyer *et al.*, 1989).

Motility Assays

Motility assays were performed using cells seeded on round coverslips (25 mm) and cultured for 2 d in standard medium. Coverslips were mounted in a Sykes-Moore chamber (Bellco Glass, Vineland, NJ) and time-lapse video cinematography was performed over 15 h after addition of growth factor. The average speed of locomotion was computed as total track length divided by the duration of the film, as described previously (Vallés *et al.*, 1990a).

Mitogenic Assay

Cells grown to confluency in 24-well plates in standard medium were rendered quiescent by culturing for 24 h in DMEM. aFGF, bFGF, and KGF were added at the indicated concentrations for 24 h, and [³H] thymidine (Amersham, Buckinghamshire, UK) (1 μ Ci/ml; 1 Ci = 37 GBq) was included during the last 4 h of incubation. Induction of DNA synthesis was measured by liquid scintillation spectrometry of acid-insoluble material.

Binding Studies

Acidic FGF and bFGF were labeled by a modification of the chloramine-T method as described (Hunter and Greenwood, 1962) to a specific activity of 90 000 cpm/ng and 300 000 cpm/ng, respectively. Equilibrium binding assays on NBT-II cells were carried out as described previously (Vallés *et al.*, 1990a). The affinity and number of receptors were determined according to Scatchard (Scatchard, 1949) using the LIGAND program (Munson and Rodbard, 1980).

Affinity-labeling Assay

Cross-linking of aFGF and bFGF to sparse cultures of NBT-II cells was carried out using the method described previously (Vallés *et al.*, 1990a). aFGF and bFGF were used at 2 and 10 ng/ml and competed with a 100-fold excess of unlabeled factor to determine specificity.

Northern Analysis

After separation on a 1.2% formaldehyde-containing agarose gel, poly (A)⁺ RNAs were transferred overnight to a nitrocellulose filter (PA 85, Schleicher and Schuell, Keene, NH). FGFR2 probes were prepared by PCR as described above using primers 3/4 and 7/8, incorporating ³²P dCTP. They were purified on NACS Prepac columns (Life Technologies, Grand Island, NY) then incubated overnight at 42°C with filters in the hybridization solution (5 $\times$ SSC, 5 $\times$ Denhardt's, 50% formamide and 50 μ g/ml salmon sperm DNA). After washing 10 min at 42°C in 0.25 $\times$ SSC and 0.1% sodium dodecyl sulfate (SDS), filters were dried and exposed for 2 d. FGFR1 and FGFR4 probes were prepared from plasmids and labeled by nick-translation.

Evaluation of FGFR2c/Bek RNA Expression

We used a method based on previous work from Champion-Arnaud *et al.* (1991). A FGFR2 1355-bp PCR fragment was synthesized using specific primers 1 and 2 as described above (also shown in Figure 2). This fragment was cloned and sequenced as described above. Because this region includes the alternatively spliced region, these primers can induce the amplification of FGFR2c/Bek as well as FGFR2b/KGFR cDNA fragments depending on the template DNA. After amplification and purification, the amplified one-half fragment was digested with *EcoRV*, then ³²P dCTP labeled, and analyzed on a 5% polyacrylamide gel. Because there is only one *EcoRV* restriction site in the FGFR2c/Bek 1/2 cDNA fragment and none in the FGFR2b/KGFR 1/2 cDNA fragment, the two smaller fragments identified by autoradiography represented digested FGFR2c/Bek. The amount of each fragment was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) then divided by its molecular weight. Relative proportions of the smaller fragment (cut) to the total amount of cDNA (cut + uncut) was calculated for each condition. The whole experiment was repeated twice starting from the first PCR step.

RESULTS

KGFR Induced EMT in Low Confluency NBT-II Cells

Low confluency and high confluency cultures of NBT-II cells were treated separately with KGF (5 ng/ml). Low confluency NBT-II cells were photographed after 48 h KGF activation (Figure 1, Ac). Low confluency NBT-II cells were also treated separately with bFGF (Figure 1, Aa) or aFGF (Figure 1, Ab) as negative and positive controls, respectively. Cell dissociation was used to quantify EMT and was measured by the extent of desmosome internalization as described previously (Boyer *et al.*, 1989) at different times after plating. As described previously for aFGF (Vallés *et al.*, 1990b), KGF readily induced EMT in low confluency NBT-II cells, even at lower concentrations than aFGF (1 or 2.5 ng/ml), but could not induce EMT in high confluency cells. The EMT induction in low confluency cells was total, but only transient, because after 3 d cells started reexpressing desmosomes (Figure 1B). After further time of treatment, most of the cells reverted to an epithelial phenotype. In contrast, KGF effectively induced mitogenesis in high confluency cells as estimated by thymidine incorporation (Figure 1C), as described previously for aFGF (Vallés *et al.*, 1990b), but at higher growth factor concentrations. To better understand the biological activities of KGF, we analyzed FGF receptor expression by NBT-II cells.

FGFR2b/KGFR Was Found To Be a Predominant FGFR in Low Confluency NBT-II Cells

We designed several oligonucleotides to amplify specifically by the PCR method several members of the FGFR 1 and 2 groups, based on published mouse and human FGFR sequences (Figure 2A) (Dionne *et al.*, 1990; Hattori *et al.*, 1990; Reid *et al.*, 1990). These primers were used to amplify cDNA derived from low confluency rat NBT-II cells, rat lung, and human keratinocytes. Analysis of amplification products after 25 cycles are shown in Figure 2B. Primers specific for FGFR1, i.e., primers 9 and 10, resulted in a 1200-bp band using cDNA from rat lung but no band with NBT-II cells. Primers specific for FGFR2 (1/2 and 5/6) generated a 1350- and a 2100-bp band with NBT-II cells as well as rat lung. Primers specific for FGFR2b/KGFR-amplified fragments of $\sim$ 700 (5/8), 1400 (7/6), 600 (1/8), and 800 bp (7/2) in NBT-II cells or human keratinocytes. Overall, the size of amplified fragments was in accord with the expected size for FGFR2b/KGFR, suggesting that FGFR2b/KGFR was the main FGFR RNA present in low confluency NBT-II cells. Consistent with this conclusion, primer sets specific for FGFR2c/Bek (including primer 3 or 4) failed to amplify any DNA fragment in NBT-II cells as well as in keratinocytes, when the FGFR2c/Bek-specific set (3/6) detected a 1000-bp fragment in rat lung cells. However, when the PCR reaction was prolonged for 30 cycles, a 150-bp fragment

Figure 1. Activation of NBT-II cells with bFGF, aFGF, and KGF. (A) Cells were cultured at low density then activated for 2 d with (a) bFGF (10 ng/ml), (b) aFGF (10 ng/ml), or (c) KGF (5 ng/ml) before photography. (B) After trypsinization and replating, NBT-II cells were cultured without treatment (NT) or in the presence of aFGF or KGF. Cells were processed for immunofluorescence using anti-desmoplakin antibodies after 2, 3, or 4 d in culture. Cells showing at least one desmosome at a cell-cell border were counted as positive, and the proportion of positive cells was calculated. At least 500 cells were counted in each case. SE is indicated by a bar. (C) Mitogenic activities of confluent NBT-II cells were determined by thymidine incorporation after cell treatment with bFGF, aFGF, and KGF.

was obtained using a FGFR2c/Bek-specific set (3/6) in NBT-II cells, suggesting low-level expression. We compared the expression of the two other FGFR families described so far. According to PCR analysis, FGFR3 is

expressed by NBT-II cells (McEwen and Ornitz, personal communication). On the other hand, Northern and PCR analysis failed to detect FGFR4. Because neither of these two receptors bind to KGF (Vainikka *et al.*, 1992; Chel-

Figure 2. PCR amplification analysis of FGFR. General structure of FGFR is displayed in A to show the localization of oligonucleotide primers used to amplify specifically FGFR1, FGFR2b/KGFR, and FGFR2c/Bek. Using NBT-II cells, rat lung (lung), or human keratinocytes (Ker) cDNAs as template, PCR amplification was carried out for 25 cycles and then analyzed in B.

laiah *et al.*, 1994), we assumed they would not be involved in KGF-induced EMT and focused on FGFR2b/KGFR.

Sequence Analysis of NBT-II FGF Receptors

The 2.1-kb amplified fragment obtained with primers 5/6 (Figure 3A), the 150-bp amplified fragment obtained with primers 3/4 (Figure 3B), and two fragments obtained with primers 3/6 and 1/2 in lung cells were cloned directly into the PCR 1000 vector (Invitrogen).

The sequence of the fragment 5/6, corresponding to the full rat FGFR2b/KGFR, is reported in Figure 3A and is compared to the mouse sequence. It showed 98% identity with the mouse amino acid sequence. Virtually identical sequences were obtained from NBT-II cells and normal rat lung cells. The sequences showed 100% identity except for a short, two amino acid segment (valine-threonine) deleted in the NBT-II cell FGFR2b/KGFR, which was located in the intracellular juxta-membrane region (Figure 3A). According to a previous report (Johnson *et al.*, 1991), this site coincides with the border between exon 8 and exon 9.

The sequence of the short fragment 3/4 amplified from rat NBT-II cells after 30 cycles was compared to the mouse FGFR2c/Bek sequence published previously (Figure 3B). This fragment had 94% identity with the mouse amino acid sequence.

Induced EMT in NBT-II Cells Expressing Human FGFR1/Flg or FGFR2c/Bek

To assess the specificity of FGFR2b/KGFR as a mediator of EMT, we used a transfection approach to compare the activities of other FGFR in the EMT induction. Two cDNA clones containing the human FGFR1/Flg and the FGFR2c/Bek full length coding regions were cotransfected independently into low confluency epithelial NBT-II cells with a plasmid containing the neomycin-resistance gene. Geneticin-resistant clones were selected and tested for their ability to respond to both aFGF and bFGF. NBT-II parental cells or cells transfected with only the neomycin plasmid were used as negative controls. We selected two clones designated NBT-II.Flq and NBT-II.Bek for further study. Control untransfected cells (Figure 4, A-C) and cells transfected with either Flg (Figure 4, D-F) or Bek (Figure 4, G-I) were activated for 24 h with aFGF or bFGF before analysis by immunofluorescence. Untreated cells showed an epithelial phenotype with desmosomes located at cell-cell contact areas in both untransfected and transfected cells (Figure 4, A, D, and G). Treatment with aFGF induced rapid internalization of the desmosomes in all three cases (Figure 4, B, E, and H). Treatment with bFGF induced desmosome internalization

A

NBT-II KGFR: MVS^SGRPI^FL VLV^TMTATLSL ARPS^FSLVSD TLEP^EGA^PY WNT^EKMEKR
 Rat KGFR: -----
 Mouse KGFR: ---W---C- -----BP-----

LHA^VPAANTV KFRCPAGGNP TPTMRWLKNG KEFKQEHRIG GYKVRNQHWS LIME^SVVPSD

 KGN^YTCLVEN EYGSINHTYH LDVV^RRS^TQR PILQ^GGLPAN G^STVVGGDVE FVCKVYSDAQ

 PHIQWIKHVE KNGSKYGPDG LPYLKVLKHS GINSSNAEVL ALFNVTMDA GEYICKVSNY

 IQQANQSAWL TVLPKQAPV REKEITASPD YLEIAIYCIG VF^LLIACMVVT VIFCRMKTTT

 KKP^FSSQPA VHKLTKRIPL RRQ[.]VSABS SSSMNSNTPL VRITRLSST G^DTPMLAGVS

 EYELPEDPKM EFPRDKLT^LA KPLGEGCPGQ VVMABAVGID KDKPKE^QSTV AVKMLKDDAT

 EKDLSDLVSE MEMMKMIGKH KNIINLLGAC TQDGPLYVIV EYASKGNLRE YLRARRPFGM

 EYSYDINRVP BEQMTFKDLV SCTYQLARGM EYLASQKCIH RDLAARNVLV TENNVKMIAD

 FGLARDINNI DYYKKTNGR LPVKWMAPEA LFDRVYTHQS DVWSFGVLMW EIFTLGGSFY

 PGIPVEELFK LLKEGHRMDK PTNCTNELYM MMRDCWHAVP SQRPTFKQLV EDLDRILTLT

 TNEBYLDLTQ PLEQYSPSYF DTRSSCSSGD DSVFSPDPMP Y^DFC^LLPQYPH INGSVKT

 -----E-----

B

NBT-II FGFR2c: A^GVNTTD^KBIEVLYIRNVTPE^DAGE^YTYLAGNSIGISPHSAWLTVL
 Mouse FGFR2c: L-----R-----C-----

Figure 3. Sequence analysis of NBT-II FGFR2 receptors. (A) NBT-II PCR fragment 5/6 compared to rat lung PCR fragment 1/6 and to full length mouse FGFR2b/KGFR. Lines indicate stretches of amino acid sequence identity. (B) NBT-II PCR fragment 3/4, compared to mouse FGFR2c/Bek. Unique amino acids are elevated in the NBT-II sequences. The EMBL data library accession numbers for NBT-II FGFR2c, NBT-II FGFR2b, and rat lung FGFR2b are Z35137, Z35138, and Z35139, respectively.

only in the transfected cells and not in the untransfected cells (Figure 4, C, F, and I).

We found previously that cell migration was stimulated after inducing EMT in NBT-II cells. Because bFGF had no effect on migration in the parental cell line, we measured the induction of motility by bFGF in transfected cells. NBT-II.Bek cells migrated at an average speed of 62 μ m/h when activated for 15 h with aFGF and at a speed of 72.5 μ m/h when activated with bFGF. Similarly, NBT-II.Flg cells migrated at an average speed of 46 μ m/h when activated with aFGF and at a speed of 45 μ m/h when activated with bFGF. These values were similar to the average speed recorded with parental NBT-II cells, 30 μ m/h when aFGF activated (Vallés *et al.*

et al., 1990a). Nonactivated cells had a very low mobility, 3 μ m/h (Vallés *et al.*, 1990a).

Modulation of DNA Synthesis in FGFR-transfected Cells

We have previously shown that two different biological responses occur in activated NBT-II cells, depending on cell density (Vallés *et al.*, 1990b). We showed above that EMT can be induced by signaling through the FGFR1/Flg and FGFR2c/Bek receptors at low density. The question that followed was whether these two receptors could mediate DNA synthesis at confluency. Confluent NBT-II.Flg and NBT-II.Bek cells were analyzed for growth stimulation, as estimated by thymidine incorporation, after activation with acidic or basic FGF. In parental NBT-II cells, the dose-response curve stimulation of DNA synthesis by aFGF was bell shaped, whereas the stimulation of DNA synthesis produced by bFGF was a saturating curve (Vallés *et al.*, 1990a) (Figure 1C). In transfected NBT-II.Flg and NBT-II.Bek cells, stimulation with either factor also produced bell-shaped curves with peak at 1–5 ng/ml (Figure 5), suggesting the acquisition of ligand-binding abilities similar to those of parental cells for aFGF.

Binding and Cross-linking Analyses of FGFR in NBT-II.Flg and NBT-II.Bek Transfectants

The molecular species involved in these interactions with aFGF or bFGF were characterized by cross-linking and Scatchard analyses. When NBT-II.Flg cells were incubated with ¹²⁵I-labeled aFGF and cross-linked with a homobifunctional reagent, labeled complexes with molecular masses of 190 kDa, a broad band of ~170 kDa, and 130 kDa were obtained after analysis by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography (Figure 6A). These three bands were almost undetectable after incubation with an excess of unlabeled aFGF. The major broad band probably corresponds to the transfected receptor and the other two bands to the endogenous receptor, because affinity labeling of the parental cell line revealed three major bands of equal intensity. When incubated with ¹²⁵I-labeled bFGF, transfected NBT-II.Flg cells displayed a single band of 170 kDa. Likewise, when NBT-II.Bek cells were incubated with ¹²⁵I-labeled aFGF, labeled complexes with molecular masses of 190 kDa, a broad band of ~155 kDa, and 130 kDa were obtained (Figure 6B). Binding of ¹²⁵I-labeled bFGF to these cells revealed a single band of 155 kDa. After subtraction of the molecular mass of the ligand (~15 kDa), the sizes of the cross-linked bands in NBT-II.Flg and NBT-II.Bek cells were estimated to be ~160 and 130 kDa, respectively. Because parental NBT-II cells do not contain high affinity binding sites for bFGF (Figure 6B) (Vallés *et al.*, 1990a), we can assume that the 170- and 155-kDa bands correspond to the products of the transfected Flg and Bek genes.

Figure 4. Desmosome localization in NBT-II cells (A–C), NBT-II.Flg (D–F), and NBT-II.Bek (G–I). NBT-II cells formed epithelial clusters when cultured in standard medium. Desmosomes, visualized by immunofluorescence with antibodies to desmoplakin, were localized at cell-cell borders (A, D, and G). After induction with aFGF (20 ng/ml), all cells became scattered and spindle-shaped with internalized desmosomes (B, E, and H). Finally, after bFGF (20 ng/ml) induction, NBT-II.Flg and NBT-II.Bek also underwent an EMT characterized by desmosome internalization (respectively, F and I).

Scatchard analysis of the binding data indicated that NBT-II.Flg cells contain ~60 000 high affinity receptors per cell with a dissociation constant (K_d) of 90 pM for bFGF and 120 pM for aFGF. For NBT-II.Bek, Scatchard analysis showed 50 000 high affinity receptors per cell with a K_d of 25 pM for binding of bFGF.

EMT and Confluency-Induced FGFR2 Alternative Splicing in NBT-II Cells

Because we observed that KGF was only active for a limited period of time as an EMT inducer in low-confluency NBT-II cells, we investigated at the RNA level the expression of FGFR2b/KGFR by NBT-II cells cultured at different densities. Poly (A)⁺ RNA was extracted from nonactivated, low versus high confluency NBT-II cells, and compared to mesenchymal NBT-II cells previously activated with aFGF for 1 wk (5 ng/ml). Probes specific for FGFR2c/Bek and FGFR2b/KGFR, respectively, were obtained by PCR amplification using the primers 3/4 and 7/8 (Figure 2A). The results of hybridization are shown in Figure 7. In agreement with the PCR analysis (Figure 2), unactivated cells cultured at low density expressed mainly FGFR2b/KGFR RNA (Figure 7). Cells cultured at high density expressed mainly FGFR2c/Bek RNA and little FGFR2b/KGFR RNA. Mesenchymal NBT-II cells activated previously for 1 wk with aFGF showed a strong induction of

FGFR2c/Bek RNA expression, together with a down-regulation in FGFR2b/KGFR RNA.

To characterize the kinetics of this switch, we amplified by RT-PCR the FGFR2 fragment termed 1/2 that we have described (Figure 2B) and sequenced (Figure 3). This fragment was amplified from cDNAs derived from NBT-II cells treated for various times with aFGF. This particular set of primers amplifies both FGFR2 isoforms. We then determined the relative proportion of FGFR2c/Bek in the original cDNA populations by digesting the amplified 1/2 fragment with *EcoRV*. Only FGFR2c/Bek contains a *EcoRV* restriction site and is cut in two smaller fragments. After a ³²P dCTP labeling, the fragments were quantified by PAGE with a PhosphorImager (Figure 8). The proportion of FGFR2c/Bek rose from 15% in nonactivated NBT-II cells to ~70% in cells treated for 1 wk. The proportion of FGFR2c/Bek RNA doubled in the first 4 h, suggesting a process involving a rapid change in alternative splicing.

The EMT Response Is Differentially Modulated by aFGF and KGF in the NBT-II Cell System

Because FGFR2b/KGFR is the only FGFR known to bind to KGF with high affinity, we investigated whether KGF could maintain EMT activation in long term activated mesenchymal NBT-II cells that expressed only low

Figure 5. Mitogenic activity in NBT-II.Flg and NBT-II.Bek. Confluent NBT-II.Flg (A) and NBT-II.Bek (B) cells were activated with aFGF or bFGF then analyzed for growth stimulation, as estimated by thymidine incorporation.

amounts of FGFR2b/KGFR RNA. NBT-II cells were activated for 1 wk with aFGF (5 ng/ml), which resulted in a 100% mesenchymal phenotype as judged by the total disappearance of desmosome-positive cells. These cells were then passaged and cultured for ≤ 3 d in medium with or without aFGF (5 ng/ml) or with KGF (2.5 ng/ml). Cells cultured for 2 d in the presence of KGF became insensitive to the growth factor and progressively regained their epithelial organization (Figure 9Ac) similar to cells cultured without growth factor (Figure 9Aa). This reversion is in contrast with the response of cells replated in the presence of aFGF, which maintained a mesenchymal phenotype (Figure 9Ab).

Desmosome-positive cells were counted after 1, 2, and 3 d (Figure 9B). As described earlier (Boyer *et al.*, 1989), in the absence of aFGF, mesenchymal cells rapidly reverted to an epithelial phenotype with desmosomal intercellular junctions reappearing in 24 h. The rate of desmosome formation was slightly delayed in comparison to trypsinized epithelial nontreated NBT-II cells (Figure 1B). Cells treated with aFGF showed no

Figure 6. Crosslinking of [125 I] FGFs to NBT-II.Flg and NBT-II.Bek. (A) [125 I] aFGF or (B) [125 I] bFGF was covalently cross-linked to subconfluent parental NBT-II, NBT-II.Flg, or NBT-II.Bek cells in the presence (+) or absence (-) of 100-fold molar excess of unlabeled competitor, either aFGF (A) or bFGF (B).

desmosome staining at any time, whereas cells treated with KGF presented a mixed population of immunoreactivity. The large standard error reflects the heterogeneity that was observed because of the presence of both epithelial and mesenchymal cells. After 4 d, cul-

Figure 7. Northern analysis of NBT-II FGF receptors. NBT-II cells were cultured at low confluency (LC), high confluency (HC), or activated for 1 wk with 10 ng/ml aFGF (Act); the latter converting them totally to a mesenchymal phenotype. After poly (A)⁺ RNA was analyzed on a formaldehyde-agarose gel and transferred to nitrocellulose, blots were hybridized separately with probes prepared against FGFR2b/KGFR, FGFR2c/Bek, and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Figure 8. Kinetics of induced FGFR2 alternative splicing. The pair of primers 1 and 2 was chosen to amplify together the two FGFR2 isoforms from NBT-II cDNA. The PCR fragment 1/2 consisting of a mixture of both FGFR2c/Bek and FGFR2b/KGFR isoforms was treated with *EcoRV*, which specifically cuts the FGFR2c/Bek form. The resulting DNA fragments were then radiolabeled, separated on a polyacrylamide gel, and quantified using a PhosphorImager. The FGFR2c/Bek-derived fragment was cut into two fragments. The proportion of the cut to the total (cut + uncut) fragment was calculated with corrections for their respective molecular weight at specific times after aFGF activation. Bars indicate SE. *, significant difference ($p < 0.01$) between untreated and treated cells.

tures showed large areas of epithelial cells surrounded by mesenchymal cells. A large proportion of these cells expressed desmosomes, indicating a general but not complete reversion from mesenchymal to epithelial phenotype.

DISCUSSION

This study has explored the activation pathway leading to FGF-induced EMT in a rat bladder carcinoma cell line. We first demonstrated that KGF can induce an EMT in low confluency NBT-II cells. We then cloned and sequenced FGFR2b/KGFR from normal rat lung cells and NBT-II cells as an EMT mediator in this model. We also analyzed how distinct transfected FGF receptors could also convey the EMT signal when activated by their specific ligands. Finally, we described the modulation of FGF receptors after EMT. When NBT-II cells were activated for 1 wk with aFGF and had fully converted to a mesenchymal phenotype, they were found to have switched from expressing FGFR2b/KGFR to FGFR2c/Bek RNA. This switch was confirmed by a functional study, which showed that KGF had only limited activity on mesenchymal NBT-II cells.

The rat receptor we cloned from low confluency NBT-II cells and from rat lung shows extensive homology with the FGFR2b/KGFR previously described in mouse and human (Hattori *et al.*, 1990; Miki *et al.*, 1992). Like the mouse FGFR2b/KGFR, it did not include the first

Ig domain and the acidic box that have been described in some cases for the human FGFR2b/KGFR (Hattori *et al.*, 1990; Miki *et al.*, 1992). The function of the first Ig domain and of the acidic box are still unclear. In *Xenopus* FGFR1, the acidic box was found to be involved in ligand binding (Amaya *et al.*, 1991), but several forms of FGF receptors already characterized do not include it (Miki *et al.*, 1992). The only difference found between the rat lung FGFR2b/KGFR and the NBT-II FGFR2b/KGFR was a deletion of the two-amino acid stretch valine-threonine. Interestingly, this deletion was reported in other tumors (Hattori *et al.*, 1990; Houssaint *et al.*, 1990; Hou *et al.*, 1991) and normal cells (Yazaki *et al.*, 1993), in FGFR2b/KGFR as well as in FGFR1b, the equivalent spliced variant from FGFR1. The location of this region at the border between exons 8 and 9 suggests that the deletion could be linked to the RNA splicing process. According to previous reports (Feramisco *et al.*, 1980; Glass *et al.*, 1986), the RRQVT sequence present in FGFR2 represents a potential cAMP or cGMP protein kinase phosphorylation site.

Transfection experiments with FGFR1/Flg and FGFR2c/Bek indicated that different FGFR could convey the signal for EMT induction when activated specifically. The FGFR actually available on the target population appears to be responsible for the ligand specificity of induction. The FGFR2b/KGFR has only been described so far in epithelial cell types (Hattori *et al.*, 1990; Miki *et al.*, 1992). Our observations agree with this cell-phenotype restriction, because we found FGFR2b/KGFR was essentially expressed only in NBT-II cells with an epithelial phenotype. The distribution of FGFR2c/Bek and FGFR1 are more diversified, including epithelial as well as mesenchymal cell phenotypes (Reid *et al.*, 1990; Bernard *et al.*, 1991; Korhonen *et al.*, 1992; Luqmani *et al.*, 1992; Peters *et al.*, 1992).

Very few cases of induced FGFR regulation have been described so far. Differentiation of rat mammary epithelial to myoepithelial-like cells in culture was shown to be followed by bFGF receptor expression (Fernig *et al.*, 1990). More recently, a switch between FGFR2b/KGFR and FGFR2c/Bek was described to follow the growth of a tumor in vivo that was associated with the acquisition of the malignant phenotype (Yan *et al.*, 1993). We describe here a switch associated with an induced EMT, which is characterized by its reversibility, by the very short induction period and by the well defined target population. We also found that coinciding with this switch most NBT-II cells could not maintain a mesenchymal phenotype when treated with KGF, whereas aFGF remained fully efficient. The fact that some cells still expressed a mesenchymal phenotype after 4 d of KGF treatment (Figures 1B and 8B) can probably be explained by the relative heterogeneity of the NBT-II cells. As seen in Figure 7 and Figure 8, the RNA switch is not total, and some cells probably express enough FGFR2b/KGFR to remain induced and maintain

Figure 9. EMT stability under KGF treatment. (A) Low confluency NBT-II cells were activated for 1 wk with 5 ng/ml aFGF, converting them to a mesenchymal phenotype. To determine whether this phenotype could be maintained by various FGFs, cells were then passed to new culture dishes and cultured in standard medium (a), medium containing 5 ng/ml aFGF (b), or medium containing 2.5 ng/ml KGF (c), for 2 d before they were photographed using phase contrast microscopy. (B) In parallel, cells were processed for desmosome immunolabeling after 1, 2, or 3 d of culture under the same conditions to calculate the percentage of desmosome-positive cells. Cells showing at least one desmosome at a cell-cell border were counted as positive, and the proportion of positive cells was calculated. At least 500 cells were counted for each condition. Bars indicate SE.

a mesenchymal phenotype. It is also possible that some cells "oscillate" between the epithelial and mesenchymal phenotype, in accordance with the alternate expression of FGFR2b/KGFR and FGFR2c/Bek. This hypothesis is currently under investigation. Little is known about the mechanisms controlling FGFR2 alternative splicing. Phenotype-specific repressors and modulation of the pre-mRNA secondary structure appear to be involved in the control of the FGFR2 alternate splicing (Gilbert *et al.*, 1993).

During embryonic development, a linkage between FGFR expression and differentiation process involving phenotype transition may also occur. In accord with this concept, it has been observed that distinct FGFR are expressed at specific stages in cells expressing epithelial or mesenchymal phenotypes in the same organ (Heuer *et al.*, 1990; Reid *et al.*, 1990; Wanaka *et al.*, 1990; Peters *et al.*, 1992; Patstone *et al.*, 1993). Also, during nervous system development in the chick embryo, FGF receptors alternate sequentially with NGF

receptors on differentiating neural cells (Heuer *et al.*, 1990). Accordingly, bFGF was found to enhance the promoter activity of the NGF receptor gene in neuroblastoma cells (Taiji *et al.*, 1992). Such mechanisms can represent an induction for growth, differentiation, or migration depending on the spatio-temporal program. Clearly, the signal transduction machinery must also adapt to changes in growth factor receptor modulation. For example, a defect in signal transduction was reported to abrogate FGF activation of a rat prostate cancer cell despite the presence of functional high-affinity FGFR (Shain *et al.*, 1992).

In this study, we discovered that cell culture density can modulate the expression of FGFR. Cell culture density has been described previously to modulate other growth factor receptor expression (Kornilova *et al.*, 1992), as well as growth hormone release *in vitro* (Sugimoto *et al.*, 1991). The significance of these observations to *in vivo* situations remains to be explored. However, they appear likely to be especially relevant

to mechanisms of development and tumor progression.

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