Inhibition of Growth of Malignant Rat Prostate Tumor Cells by Restoration of Fibroblast Growth Factor Receptor 2

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ABSTRACT

A loss of expression of fibroblast growth factor (FGF) receptor 2 IIIb (FGFR2IIIib), which responds to stroma-derived FGF, accompanies progression of premalignant androgen-responsive rat prostate tumor epithelial cells to the malignant phenotype. Concurrently, the level of FGFR2 gene expression is reduced and lost altogether in over 30% of cells, whereas all malignant cells abnormally express FGFR1, which is normally confined to stromal cells (S. Feng et al., Cancer Res., 57:5369-5378, 1997). To determine the relative roles of the FGFR2 and FGFR1 kinases in growth of malignant cells, we transfected malignant prostate epithelial cells with the wild-type FGFR2IIIib kinase and an artificial chimeric construct (FGFR2IIIib/R1) composed of the FGFR2IIIib ectodomain and the FGFR1 kinase domain. Population growth kinetics, in both the absence and presence of FGF-7, which binds only the FGFR2IIIib ectodomain, were then examined in the transfected cell populations. In contrast to the untransfected malignant tumor cells and those expressing the FGFR2IIIib/R1 chimeras, FGF-7 caused a dose-dependent net inhibition of the population growth rates of cells expressing the full-length FGFR2IIIib kinase. The results suggest that although the FGFR2 kinase can mediate positive mitogenic effects, it mediates a net restriction on the growth of prostate tumor epithelial cells relative to FGFR1. Highly malignant prostate tumor cells, which have lost the FGFR2 tyrosine kinase, retain the cellular response mechanisms to it. Restoration of the FGFR2 kinase to malignant tumors that are refractory to treatment may present a new avenue for gene therapy.

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy in men and ranks second as a cause of death among men after lung cancer in the United States (1). The clinical features of prostate cancers are for the most part androgen sensitive and respond to androgen therapies in the first treatment (2). However, they eventually progress to being resistant to androgen therapies and come to be highly malignant (2). For such relapsed or hormone-resistant prostate cancers, there is no effective therapy (2). Therefore, it is important to determine the causative changes that allow premalignant prostate cancers to progress to malignancy and how to reverse the process to develop new therapy for the refractory cancers.

Distinct changes occur in the FGF2 family in model rat prostate tumors during progression to malignancy that may Underlie progressive steps toward autonomic proliferation (3-5). The resident FGFR gene expressed in both normal prostate epithelial cells and those from premalignant differentiated, androgen-responsive, and slowly growing rat prostate tumors (type I) is FGFR2. Type I tumor epithelial cells express exclusively the FGFR2IIIib splice variant in the ectodomain, which recognizes stromal cell-derived FGF-7 (5-7) and possibly additional FGF-7 homologues. However, in anaplastic, hormone-insensitive, rapidly growing prostate tumors (type II), the FGFR2IIIb isoform disappears, concurrent with a progressive reduction and loss of FGFR2 gene expression in general and abnormal activation of the FGFR1 gene (5, 7). When premalignant type I tumor epithelial cells are implanted s.c. without homologous stromal cells, they initially differentiate into gland-like structures, but over time they progress to the malignant type II phenotype (5, 7). When implanted with homologous premalignant prostate tumor stromal cells, the resultant tumors retain the type I characteristics, including no change in FGFR2 or FGFR1 expression and no progression to malignancy (5). The exclusive expression of the FGFR2IIIib isoform in epithelial cells correlates with the response to homologous stromal cells, slow growth rate, and the differentiated, premalignant phenotype. In contrast, the complete loss of FGFR2IIIb and FGFR2 in some clones and the appearance of FGFR1 in epithelial cells is associated closely with emergence of the malignant phenotype. The introduction of FGFR1 by transfection in type I prostate tumor epithelial cells dramatically accelerates their progression to malignancy in vitro (7).

In this study, we show that restoration of the FGFR2IIIib kinase expression by transfection into highly malignant prostate tumor cell lines that have lost it and are expressing FGFR1 has a net inhibitory effect on both basal and FGF-stimulated population growth rates. In contrast, the FGFR1 intracellular domain fused to the same FGFR2IIIib ectodomain has no effect. The results suggest that the FGFR2 and FGFR1 kinases have quite different consequences on the growth of the malignant epithelial cells. Despite loss of the FGFR2 kinase and emergence of the extremely malignant phenotype, the type II tumor cells apparently retain internal growth-limiting response mechanisms to the FGFR2 kinase. Restoration of the FGFR2 kinase may present an alternative means of limiting growth rate of tumors that are refractory to androgen ablation and other treatments.

MATERIALS AND METHODS

Construction of FGFR cDNAs. Full-length FGFR2IIIbIIIb kinase (hereafter called R2IIIb/R2) was constructed by ligation of the cDNA coding for the extracellular domain of the rat FGFR2IIIbIIIb isoform (GenBank accession no. L19107; Refs. 8 and 9), and the cDNA coding for the intracellular kinase domain of rat FGFR2 (GenBank accession nos. L19109 and L19106; Ref. 8). Each was separately treated with ApaLl and PstI. The cDNA fragments were ligated at the ApaLl site and cloned into pcDNA3.1/Zeo expression vector (Invitrogen, Carlsbad, CA) at the PstI site (Fig. 1A). The chimeric FGFR2IIIb/R1 (hereafter called R2IIIb/R1) was prepared as follows: a cDNA fragment coding for the 12th amino acid residue of the third immunoglobulin-like loop through the transmembrane domain and eight residues into the intracellular juxtamembrane domain was generated by PCR with sense primer CAGTGGATCCACATGCGAAGAG and antisense primer GCCCTTCCTGAGCGTCCATTCC. The cDNA for the extracellular domain of the rat FGFR2IIIbIIIb isoform (7-9) was used as template to yield the KpnI site in the juxtamembrane region. The cDNA was cloned into pBlueScript SK vector (Stratagene, La Jolla, CA) for sequence analysis and then digested with DraI and KpnI. The cDNA for the extracellular domain of the rat FGFR2IIIbIIIb isoform (7-9) was digested with BamHI and DraI to yield the

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4 The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor.

1509

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DNA Synthesis and Cell Proliferation Assays. DNA synthesis was assessed by measuring the incorporation of [3H]thymidine into quiescent monoclonal cultures of transfected cells. Clonal cultures of AT3 cells were starved in serum-free RTT medium for 24 h, harvested, and distributed into $2 \times 10^5$ cell portions in 24-well plates in serum-free RTT medium. After the cells attached to the culture well, the medium was replaced with 1 ml of RTT serum-free medium containing 10 $\mu$g/ml heparin. Preliminary experiments indicated that 10 $\mu$g/ml of heparin was the optimum concentration resulting in the maximum stimulation of the mitogenic response of AT3 cells to FGF. Heparin yielded both maximum suppression of the background DNA synthesis in absence of FGF and potentiation of the activity of FGF at this concentration. The monoclonal cultures were treated with various concentrations (0–100 ng/ml) of FGF-1, FGF-2, or FGF-7 for 18 h and subsequently labeled with 1 $\mu$Ci of [3H]thyminidine (New England Nuclear, Boston, MA) for 6 h. After the labeling, the monoclonal cultures were washed with PBS, and then unincorporated [3H]thyminidine was washed out with 10% trichloroacetic acid. The monoclonal cultures were lysed with 0.5 N NaOH and counted by liquid scintillation counter (Packard Instrument, Meriden, CT).

Near-confluent control and transfected AT3 cells were starved and seeded using the procedure described above. After the cells attached, the medium was replaced with 1 ml of medium containing 10 ng/ml FGF-1, FGF-2 or FGF-7, and 10 $\mu$g/ml heparin. Cells were counted on days 1, 3, 5, and 7 with a Coulter counter. An additional 10 ng/ml FGF-1, FGF-2, or FGF-7 were added on day 3. Cell population growth rates were expressed as doubling time of the cell population calculated during the logarithmic growth phase.

Radioreceptor Assays. Native FGF-1 was purified from bovine brain as described previously (13). Human recombinant FGF-2 was purchased from Upstate Biotechnology (Lake Placid, NY). Rat recombinant FGF-7 was prepared as described previously (14). FGF-1, FGF-2, and FGF-7 were labeled to specific activities of 1–4 $\times$ 10$^5$ cpm/ng with $^{125}$I-labeled sodium iodide by the chloramine T method (15).

The binding and covalent cross-linking of $^{125}$I-labeled FGF-1, FGF-2, and FGF-7 to receptors on transfected AT3 cells was performed as described (4, 15, 16).

RESULTS

Clonal Heterogeneity and Growth Properties of Malignant Dunning R3327AT3 Cell Populations. In a previous report, an analysis of over 50 clonal cultures derived from the Dunning R3327AT3 tumor revealed that although all cells ectopically express the normally mesenchymal cell-associated FGFR1, 30% fail to express the FGFR2 mRNA at all (7). The level of expression of functional transmembrane FGFR2 kinase in the remaining 70% of clonal cultures AT3-(R1,R2IIIc) that express both FGFR1 and exclusively FGFR2IIIC is dramatically reduced relative to levels of the FGFR2IIIB kinase in epithelial cells from their nonmalignant parent R3327PAP tumor (5–7). Clonal cultures from the malignant AT3 tumors expressing only FGFR1 were designated AT3-R1. Slopes or reciprocal of slopes of the curves shown in Fig. 2 indicated no significant difference in growth rate or population doubling times between the two types of clonal cultures of AT3 cells. As reported previously for the uncloned AT3 cell population (3, 4), the growth rate of both clonal populations was refractory to the addition of FGF-1 and FGF-2. However, an analysis of the stimulation of DNA synthesis in near-confluent cells brought to as near quiescence as possible revealed a difference in responsiveness between the two cell types. Both FGF-1 and FGF-2 stimulated DNA synthesis in the AT3-R1 cells in a dose-dependent manner, whereas the AT3-(R1,R2IIIC) cells were refractory (Fig. 3).

As expected, FGF-7 had no effect because all AT3 cells have lost the FGFR2IIIB ectodomain that binds it.

INHIBITION OF PROSTATE TUMOR GROWTH BY FGFR2

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Construction of FGFR2IIIB/R2 and FGFR2IIIB/R1 cDNAs. A, wild-type FGFR2IIIB ectodomain/R2 kinase. Left, the partial cDNA for the extracellular domain of rat FGFR2IIIB, consisting of two immunoglobulin-like disulfide loops, designated II and III. Bold, the exon IIIB sequence that comprises the second half of immunoglobulin loop III. Right, the full-length intracellular kinase domain of rat FGFR2; E, the two tyrosine kinase domains. The two cDNAs were generated, ligated, and cloned at the indicated restriction sites as described in detail in "Materials and Methods." B, chimeric FGFR2IIIB ectodomain/R1 kinase. Ligation sites of three cDNA fragments that were generated as described in detail in "Materials and Methods" are indicated. The KpnI site near the coding sequence for the transmembrane region of the rat FGFR2IIIB isoform was generated by PCR. The full-length cDNA was generated by ligation of the three fragments as indicated. SS, secretory signal sequence; AB, acidic box sequence; JM, transmembrane sequence; C, COOH-terminal sequence.
The ectodomain is absent in the tumor and derived cells and is specific for expression of the FGFR2 kinase on the two clonal variants in the AT3 tumor cell population. AT3-(R1,R2IIlc) and AT3-R1 cells were transfected with empty vector (data not shown) were detected. Lower expression of endogenous FGFR relative to the high expressers can be detected resulting from a proteolytic cleavage site upstream of the kinase domain, were a minor proportion compared to intact full-length kinase.

A comparable analysis using 125I-labeled FGF-1 confirmed the presence of an intense band of correct molecular mass in the high expressers (Fig. 4, Lanes 7 and 8). In contrast to FGF-7, the low levels of endogenous FGFR relative to the high expressers can be detected (Fig. 4). Although the increase of total radiolabeled FGF-2 bound to the high expressers was 20–25% that of FGF-7 and FGF-1, the differences in intensity of the radiolabeled complex of FGF-2 and the transfected FGFRIIIb isoforms were much less. This may reflect a superior efficiency in covalent affinity cross-linking of FGF-2 to the FGFRIIIb ectodomain relative to the other two FGFs.

Differential Effect of Expression of Recombinant FGFR2IIIb/R2 and FGFR2IIIb/R1 on Cell Population Growth Rates of AT3-(R1,R2IIlc) and AT3-R1 Cells. The basal and FGF-7-, FGF-1-, and FGF-2-stimulated growth rates expressed in cell population doubling times of the high expresser and control cell lines that were characterized above were determined. Generally, the population doubling time in the absence of FGF increased more than 1.5 times over that of control cell lines in all high expressing clones (H2–H4) of FGFR2IIb/R2 (Fig. 5A and B). Randomly selected low expresser clone L2 and high expresser clone H1 from the FGFR2IIb/R2 group were exceptions that may be clones with elongated doubling times unrelated to transfection or expression of FGFR2IIb/R2. Neither the clonal population L2 nor H1 was responsive to FGF-7. The addition of FGF-7 to the H cell lines expressing wild-type FGFR2IIb/R2 dramatically elongated the population doubling times by 3 to 7.5 times relative to controls (Fig. 5, A and B). In contrast, doubling times of all high expresser cell populations transfected with the chimeric FGFR2IIb/R1 containing the FGFR1 kinase were similar to those of control cell lines in the absence or presence of FGF-7.

Substitution of FGF-1 for FGF-7 in the same experiments described above resulted in a similar elongation of population doubling rates of the H clones expressing FGFR2IIb/R2 (Fig. 5, C and D). Low expresser clone L1 from the AT3-(R1,R2IIlc) population was an exception in which FGF-1 elongated the doubling time by 1.8-fold (Fig. 5C). Exposure to FGF-2 also elongated the doubling time of the same L1 clone (Fig. 5E). In contrast to FGF-7 and FGF-1, FGF-2 was generally much less effective in promoting elongation of population doubling rates in the high FGFR2IIb/R2 expresser cell lines showing a significant effect in only one line (H3). No lines transfected with the chimeric construct FGFR2IIb/R1 bearing the FGFR1 kinase exhibited a response to FGF-1 or FGF-2.

**Fig. 2.** Population growth kinetics of AT3 clonal cultures. Clonal cultures of AT3-(R1, R2IIlc) and AT3-R1 cells were established and analyzed for FGFR isotype and population growth rates determined as described in “Materials and Methods.” The indicated FGF was added on days 1 and 3. Cell population doubling times in h were calculated as the reciprocal of the slope of the linear part (logarithmic growth phase) of the indicated curves. Three independent experiments were performed with duplicate wells. The mean values of duplicate wells from one representative experiment are shown. The mean population doubling time in h and SD in h of three independent experiments for the AT3-(R1, R2IIlc) culture was 21.0 (SD, 0.1), 22.0 (SD, 0.0), 22.4 (SD, 0.9) and 21.0 (SD, 0.7) for none, FGF-1, FGF-2, and FGF-7, respectively. For the AT3-R1 clone, the comparable doubling times were 21.9 (SD, 2.0), 20.7 (SD, 1.6), 20.3 (SD, 1.7), and 21.9 (SD, 2.0).

**Selection and Analysis of Clonal Variants from the R3327AT3 Tumor Transfected with the FGFR2IIb Ectodomain Fused to the FGFR2 and FGFR1 Kinase Domains.** To determine the impact of expression of the FGFR2 kinase on the two clonal variants in the AT3 tumor cell population, we exploited the fact that the FGFR2IIb ectodomain is absent in the tumor and derived cells and is specific for FGF-7 (5–7, 17). This eliminated interference in quantitative assessment of transfected FGFR levels and biological response of the transfected product by the endogenous FGFR1 or FGFR2IIb ectodomain that binds both FGF-1 and FGF-2. Clonal variants of the AT3 cell population, AT3-(R1,R2IIlc) and AT3-R1, were transfected with wild-type FGFR2IIb/R2, chimeric FGFR2IIb/R1, and empty control vector (pcDNA3.1/Zeo). After selection in Zeocin, clonal cultures of transfecants were established and screened as described in “Materials and Methods.” Clones expressing FGFR2IIb/R2 or FGFR2IIb/R1 were screened for by radioreceptor assay with 125I-labeled FGF-7. Clonal cultures of high expressers that exhibited at least 10 times the binding and covalent affinity cross-linking to 125I-labeled FGF-7, FGF-1, and FGF-2 of transfected FGFR levels and biological response of the transfected product by the endogenous FGFR1 or FGFR2IIb ectodomain that binds both FGF-1 and FGF-2. Clonal variants of the AT3 cell population, AT3-(R1,R2IIlc) and AT3-R1, were transfected with wild-type FGFR2IIb/R2, chimeric FGFR2IIb/R1, and empty control vector (pcDNA3.1/Zeo). After selection in Zeocin, clonal cultures of transfecants were established and screened as described in “Materials and Methods.” Clones expressing FGFR2IIb/R2 or FGFR2IIb/R1 were screened for by radioreceptor assay with 125I-labeled FGF-7. Clonal cultures of high expressers that exhibited at least 10 times the background FGF-7-bound by untransfected AT3 cells or those transfected with the control vector were selected for further study (Table 1). Low expressing clones of AT3-(R1,R2IIlc) and AT3-R1 transfecants, which exhibited levels of FGF-7 binding near that of untransfected cells, were chosen randomly for additional controls. A randomly selected clone from each type of AT3 clone that was subjected to transfection and selection with empty control vector and the untransfected parent clonal cultures of the AT3 cells were also used as controls as indicated (Table 1). For comparison to FGF-7, the binding of radiolabeled FGF-1 and FGF-2 to clonal cultures selected for study was also examined.

To ensure that transfected AT3 lines exhibited the authentic recombinant FGFR2IIb connected to the intact intracellular kinase domain construct with correct GFG specificity that was indicated by the FGF-7 binding analysis, transfected cell lines were analyzed by binding and covalent affinity cross-linking to 125I-labeled FGF-7, FGF-1, and FGF-2 (Fig. 4). The results revealed the expected 120-kDa protein indicative of the complex of FGF-7 and the full-length wild-type FGFR2IIb/R2 or chimeric FGFR2IIb/R1 in all high expresser clones (Fig. 4, Lanes 3 and 4). No equivalent bands in untransfected (Fig. 4, Lane 1), low expresser transfecants (Fig. 4, Lane 2, L), or cells transfected with empty vector (data not shown) were detected. Lower molecular weight species, which are the truncated ectodomain resulting from a proteolytic cleavage site upstream of the kinase domain, were a minor proportion compared to intact full-length kinase.

A comparable analysis using 125I-labeled FGF-1 confirmed the presence of an intense band of correct molecular mass in the high expressers (Fig. 4, Lanes 7 and 8). In contrast to FGF-7, the low levels of endogenous FGFR relative to the high expressers can be detected (Fig. 4). Although the increase of total radiolabeled FGF-2 bound to the high expressers was 20–25% that of FGF-7 and FGF-1, the differences in intensity of the radiolabeled complex of FGF-2 and the transfected FGFRIIIb isoforms were much less. This may reflect a superior efficiency in covalent affinity cross-linking of FGF-2 to the FGFRIIIb ectodomain relative to the other two FGFs.
These results indicate that restoration of recombinant FGFR2 kinase increases the doubling rate of populations of both clonal subtypes of highly malignant AT3 cells, which are otherwise refractory to or the growth rate of which is stimulated by FGF-1 or FGF-2.

Characterization of the FGFR2IIIb/R2-mediated Inhibition of AT3 Cell Population Growth Rates. Fig. 6 demonstrates that the FGF-stimulated elongation of the cell population doubling rates of clones expressing high levels of FGFR2IIIb/R2 kinase is dose-dependent, with a maximum at 10–100 ng/ml FGF. Time curves using high expressers H3 and H4 expressing FGFR2IIIb/R2 indicated that the exponential expansion in cell number declines after 3 days of exposure to FGF-7 and FGF-1, whereas the effect of FGF-2 was less pronounced (Fig. 7). A kinetic analysis of the effect of FGF-7 on DNA synthesis on clone H3 [AT3-(R1, R2IIIc)] expressing high levels of FGFR2IIIb/R2 kinase revealed that FGF-7 actually stimulated DNA synthesis by nearly 2-fold when thymidine incorporation was measured after 18 h of exposure, whereas a depression of nearly 3-fold was observed after 56 h of exposure to FGF-7 (Fig. 8). Both responses were dose-dependent and reached a maximum of about 10 ng/ml. This suggests that the response of DNA synthesis to FGF-7 mediated by the FGFR2IIIb/R2 kinase is bi-directional with respect to time of application of the FGF. The long-term net effect of FGF-7, as illustrated above by the effects on cell division and net growth rates of the cell populations, is to limit expansion of the cell population.

Fig. 9 shows that the depression of population growth rates of the H3 clone of AT3-(R1, R2IIIc) cells expressing recombinant FGFR2IIIb/R2 by FGF-7 is reversible, eliminating the possibility that the inhibition was simply due to massive cell death or cytotoxicity. After removal of FGF-7 after 3 or 5 days of exposure, the cell population resumed an exponential rate of proliferation.

Finally, we wanted to eliminate the possibility that the longer-term inhibitory effect on cell population growth rates was due to a time-dependent accumulation of a truncated ectodomain, which could act as a dominant-negative feedback inhibitor of endogenous FGFR action (11). We analyzed the transfected FGFR2IIIb/R2 product after the 3-day incubation during which the inhibition of FGF-7 on DNA synthesis and elongation of doubling rate was apparent. Immunochemical analysis with an antibody against the FGFR2 ectodomain revealed only the 120-kDa species, which was the size of a complex of FGF-7 and full-length FGFR2IIIb1 kinase (Fig. 10). No lower molecular bands could be detected. This suggested that the inhibition of the AT3 cell growth rate mediated by the transfected FGFR2IIIb is likely due to the FGFR2 kinase.

DISCUSSION

The transplantable Dunning R3327AT3 rat tumor is a selected extreme prototype of malignant and metastatic prostate tumors (type II) that develop after orchiectomy or antiandrogen therapy and are refractory to current treatments (12). In contrast to their nonmalignant parent type I tumors, which are composed of well-differentiated and interactive epithelial and stromal compartments, the AT3 tumors consist of a relatively homogenous and undifferentiated epithelial cell type that is unresponsive to stroma and most other environmental controls (3–8). A clonal analysis of expression of FGFR mRNA in AT3 cells revealed that although 100% of cells ectopically express FGFR1, 30% of the AT3 cells no longer express the FGFR2 gene at all (7). The remaining 70% exclusively express the FGFR2IIIb isoform of FGFR2 at decreased levels of transmembrane product (7). This is in contrast to their nonmalignant parent, in which 100% of epithelial cells express exclusively the FGFR2IIIb splice variant and no FGFR1. Thus, there is a strong correlation between the loss of FGFR2IIIb expression, the reduction in total expression of functional FGFR2 kinase, the activation of the FGFR1 gene, and the emergence of the malignant phenotype.

In this report, we show that expression by transfection of the FGFR2IIIb1 kinase into clonal cultures of AT3 cells expressing

### Table 1 Selection of stable transfectants expressing FGFR by radiolabeled FGF-7, FGF-1, and FGF-2 binding

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*Total cell surface binding of the indicated radiolabeled FGFs was used to screen for clonal cultures of high expressers of the transfected cDNA as described in "Materials and Methods." Transfected clonal populations that exhibited radiolabeled FGF binding (in units of cpm per 10^5 cells) more than 10-fold higher than those of the untransfected AT3 cell populations were designated H for "high expressers." Clonal cultures exhibiting a level of FGF binding in cpm per 10^5 cells that was less than twice that of untransfected AT3 cell cultures were designated L for "low expressers."*

*Clones transfected with pCDNA3.1/Zeo without an insert.*
either FGFR1 and FGFR2 [AT3-(R1,R2IIlc)] or only FGFR1 (AT3-R1) has a net effect of elongating both the basal and FGFinduced population doubling rate in both malignant cell types. This was in contrast to the same FGFR2IIlb ectodomain fused to the FGF1 kinase that had no effect. This suggests that the FGFR1 and FGFR2 kinases are different and do not elicit identical signals. From the current results and due to lack of knowledge about how FGFR kinases elicit signals, the mechanism underlying the elongation of population doubling rates of the transfected AT3 populations by, specifically, the FGFR2 kinase remains to be established. The FGFR2IIlb kinase may simply be acting as a dominant-negative inhibitor of dimerization and activation of the endogenous FGFR1 kinase that is supporting AT3 cell growth. The basal population growth rate might be dampened by FGF-independent heterodimerization of the minority resident FGFR1 with the high level transfected FGFR2. However, the inhibition of cell growth is significantly stimulated by FGF-7 that does not bind to FGFR1. Either the FGFR1 partner of heterodimers of resident FGFR1 and transfected FGFR2IIlb kinases does not need to be occupied by a FGF, but the FGF2 partner does, or the inhibitory effect is due to unique activity of the FGFR2IIlb kinase by FGF-7, independent of endogenous FGFR. The facts that FGF-7 stimulates DNA synthesis in 18-h cultures and then inhibits it in 56-h cultures and that the transfected FGFR2IIlb gene product is largely the intact kinase at all times further support the conclusion that it is FGFR2 kinase activity that mediates the dual effect of FGF-7 in transfected AT3 cells. At present, the positive effect of FGF-1 and FGF-2 on DNA synthesis in the untransfected AT3-R1 clonal cultures relative to the refractory AT3-(R2,R2IIlc) clones remains to be explained. Clonal cultures derived from both cell types exhibit similar basal

![Figure 5](image-url) Differential effect of transfected wild-type FGFR2IIlb/R2 and chimeric FGFR2IIlb/R1 kinases on AT3 cell population doubling rates. Basal growth rates and the effect of FGF-7 (A and B), FGF-1 (C and D), and FGF-2 (E and F) were determined in three independent experiments with duplicate wells as described in Fig. 2. The indicated mean population doubling times in h was calculated from the three experiments. Bars, SD. Cell lines were those described in Table 1 either untransfected, transfected with vector with no insert, or transfected [low (L) and high (H) expressers] with the FGFR construct indicated.

![Figure 6](image-url) Dose-dependent effect of FGF-1, FGF-2, and FGF-7 on population growth rates of high expressers of the FGFR2IIlb/R2 kinase. The indicated amounts of the indicated FGF on population doubling time (A) and total cell number on day 7 (B) of high expresser lines H1, H2, H3, and H4 (Table 1, R2IIlb/R2) was determined by methods described in the legend to Fig. 2. Results from representative FGFR2IIlb/R2-transfected clonal line H3 are shown. Two independent experiments were performed with duplicate wells. Data are mean of duplicate wells from one representative experiment.

![Figure 7](image-url) Time curve of effect of FGF-1, FGF-2, and FGF-7 on growth of high expressers of R2IIlb/R2. Growth curves of high expresser clones H3 and H4 (Table 1) from the two types of AT3 cells were determined in absence (none) and presence of the indicated FGF. The results are representative of three independent experiments with duplicate wells.

![Figure 8](image-url) DNA synthesis of high expressers of FGFR2IIlb/R2 after 18 and 56 h of exposure to FGF-7. High expresser clones H1, H2, H3, and H4 of FGFR2IIlb/R2 (Table 1) in serum-free medium were exposed for 24 h to 10 ng/ml FGF-7 and 10 μg/ml heparin for 18 or 56 h and then exposed to [3H]thymidine for 6 h, and the thymidine incorporation was determined. Two independent experiments were performed with triplicate point determinations each. The data shown was from clone R2IIlb/R2-H3. Data are the mean and SD of triplicate wells.
experiments were performed with duplicate determinations. The mean value of duplicate content of FGF-7 was changed on days 3 and 5 in all cultures. Two independent or day 5 (A) was compared to the growth rate of clonal cultures of high expresser cell lines populations of FGFR2IIIb/R2. The effect of withdrawal (arrows) of FGF-7 on day 3 (D)

Fig. 9. Effect of withdrawal of FGF-7 on the proliferation rate of high expresser populations of FGFR2IIIb/R2. The effect of withdrawal (arrows) of FGF-7 on day 3 (□) or day 5 (○) was compared to the growth rate of clonal cultures of high expresser cell lines H1, H2, H3, and H4 in absence of FGF-7 (○). The complete medium with the appropriate content of FGF-7 was changed on days 3 and 5 in all cultures. Two independent experiments were performed with duplicate determinations. The mean value of duplicate wells from one representative experiment is shown.

Fig. 10. Covalent cross-linking analysis of FGFR2IIIb/R2 under conditions where population growth rates, phenotypic parameters when introduced in vivo (7) and similar behavior when transfected with the FGFR2IIIb/R2 or FGFR2IIIb/R1 constructs used in this study. In conclusion, the current study confirms that the FGFR2 kinase has a net growth-controlling role in epithelial cells in addition to any roles in stimulation of cell multiplication, which is quite different from the FGFR1 kinase in the same context. In the rat Dunning tumor model used here, the loss of the FGFR2IIIb ectodomain, which mediates response to stromal-derived FGF-7 and the FGFR2 kinase in general, is a hallmark of onset of malignancy (5–7). The fact that restoration of the FGFR2IIIb kinase to extremely malignant AT3 cells has a net effect of dampening cell population growth rates suggests that restoration of the gene to refractory human prostate tumors might elicit the same result. In a previous report, we showed that restoration of the FGFR2IIIb kinase to the malignant AT3 cells also markedly reduced the size and restored stromal-responsiveness and an appearance of the differentiated phenotype in resultant tumors in vivo (7). Whether the effect of the FGFR2 kinase on reduction in cell population growth rate observed in the current study in vitro underlies or is related to the tumor phenotype in vivo remains to be established. The above changes in AT3 cell phenotype have not been observed in vitro. The mechanism of how the FGFR2 kinase dampens cell proliferation also remains to be established. Recent results suggest that the FGFR1 and FGFR3 kinases in proper cell context may indirectly inactivate cell cycle promoter p34cdc2 and activate cell cycle inhibitor p21WAF/CIP1, respectively, by activation of p13ink4a and Stat1 or their homologues (18–21).

REFERENCES

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