Signal Transduction Defect Appears to Be the Cause of Rat Prostate Cancer Cell Fibroblast Growth Factor Insensitivity

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Abstract
Using monolayer cultures of clonally isolated C3 and T5 rat prostate cancer cells, we determined that acidic (aFGF) and basic (bFGF) fibroblast growth factors profoundly enhanced T5 cell thymidine incorporation with half-maximum stimulation at 0.53 and 0.35 ng/ml, respectively. In contrast, aFGF or bFGF enhancement of C3 cell thymidine incorporation was about 5% of that of T5 cells, and effects were principally mitogen concentration independent. Saturation analyses and cross-linking studies established that both C3 and T5 cells contained high-affinity FGF receptors of 120 and 145 kilodaltons and that receptor content and $K_d$ of C3 and T5 cells were comparable. aFGF or bFGF stimulation of T5 cell thymidine incorporation profoundly decreased as cell plating density was reduced from $1.5 \times 10^5$ to $1.0 \times 10^5$ cells/well. The modest response of C3 cells to either aFGF or bFGF also decreased as cell plating density was reduced. Because heparin preserves FGF biological activity and enhances bFGF binding to high-affinity FGF receptors, we examined the effect of heparin on FGF stimulation of C3 cell thymidine incorporation. We found that changes in cell plating density and/or medium heparin concentration had variable, inconsistent effects. These were C3 cell plating density associated and included inhibition or modest enhancement of FGF effects. Binding analyses established that high-affinity bFGF binding of C3 and T5 cells immediately prior to assessing FGF-stimulated thymidine incorporation was comparable and independent of cell plating density, implying that C3 cell FGF insensitivity was not attributable to differences in C3 and T5 cell FGF receptor content at the time of mitogen stimulation. Our data show that C3 rat prostate cancer cell FGF insensitivity is not attributable to effects of cell plating density or mitogen instability during cell culture. C3 cell FGF receptor content and physical properties were essentially indistinguishable from those of FGF-responsive T5 rat prostate cancer cells, implying that C3 cell FGF insensitivity was not attributable to an abnormality of FGF binding to FGF receptor. The data suggest that C3 cell FGF insensitivity is due to defect(s) in FGF signal transduction.

Introduction
It has been long appreciated that high-affinity binding of affector to transmembrane receptors initiates the cascade of events culminating in growth factor or polypeptide hormone modulation of cell function (cf. Ref. 1). Whereas it is generally believed that affector binding to membrane receptors is diffusion limited (cf. Ref. 1), recent studies suggest that binding of FGF to FGF receptors may be a facilitated process. FGF modulation of cell function is mediated through specific, high-affinity ($K_d \approx 10^{-10}$ to $10^{-11}$ M) binding of mitogen to members of the FGF receptor family (2–6). Lower-affinity ($K_d \approx 10^{-8}$ M), high-capacity binding sites (7) that are HSPGs have been identified on the surface of FGF target cells (8). Prevention of bFGF binding to HSPGs by either removing surface HSPGs with heparitinase or blocking sulfation by chlorate treatment: (a) causes substantial reduction of bFGF binding to cell-surface FGF receptors, (b) blocks ability of bFGF to support 3T3 cell growth, and (c) blocks ability of bFGF to inhibit terminal differentiation of MM14 skeletal muscle cells (9). Additionally, wild-type CHO cells lack FGF receptors and contain low-affinity bFGF binding sites. Following transfection with an FGF receptor expression vector, these cells demonstrate high-affinity bFGF binding (10). In contrast, mutant CHO cells deficient in HSPG synthesis lack low-affinity bFGF binding sites and do not demonstrate high-affinity bFGF binding following transfection with an FGF receptor expression vector. Significantly, when these transfected mutant CHO cells are propagated on medium containing either heparin or heparin sulfate, high-affinity bFGF binding to FGF receptors is demonstrable (10). These studies suggest that low-affinity FGF binding to proteoglycans facilitates FGF binding to high-affinity FGF receptors.

By contrast, the rat parathyroid cell line PT-r contains aFGF receptors of 130 and 150 kDa (11). The 150 kDa receptor is a heparan sulfate proteoglycan that is distinct from the two major PT-r cell HSPGs, HPSGI and HSPGI1, which have mass greater than 235 kDa (11). When heparitinase digestion is performed following binding and cross-linking of radiolabeled aFGF to PT-r cells, the intensity of the 150 kDa receptor is diminished with a concomitant increase in the intensity of the 130 kDa receptor. Heparitinase digestion prior to incubation of PT-r cells with radiolabeled aFGF diminishes high-affinity

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1 The abbreviations used are: FGF, fibroblast growth factor(s); a-, acidic; b-, basic; CHO, Chinese hamster ovary; EGF, epidermal growth factor; FBS, fetal bovine serum; HSPGs, heparan sulfate proteoglycans; IGF-I, insulin-like growth factor I; iodo-, $^{125}$i-labeled; PDGF, platelet-derived growth factor; kDa, kilodalton(s).

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aFGF binding. These studies establish that the glycosaminoglycan moiety of the PT-r cell 150 kDa FGF receptor is critical for high-affinity aFGF binding (11). Together, the preceding studies imply that HSPGs facilitate FGF binding to high-affinity FGF receptors of some cells; however, this may not be a generalized property of FGF receptors.

In previous studies, we demonstrated that thymidine incorporation by members of the T-family of rat prostate cancer cells was profoundly modulated by aFGF, bFGF, and EGF (12). In contrast, thymidine incorporation by members of the C-family of rat prostate cancer cells was not enhanced by aFGF, bFGF, or EGF; however, FB5 caused significant enhancement of C-family cell thymidine incorporation (12). These initial studies suggested that the inability of aFGF, bFGF, or EGF to modulate C-family rat prostate cancer cell thymidine incorporation was not due to a generalized defect in cellular response mechanisms. More recently, we showed that C3 and T5 rat prostate cancer cells produce and secrete heparin-binding growth factors and that these include mitogens having immunological similarity to bFGF (13). These latter findings suggested that FGF insensitivity of C3 cells could be due either to down-regulation of C3 cells FGF receptors by elaborated heparin-binding growth factors or to the absence of FGF receptors. To address these possibilities, we performed detailed comparative analyses of rat prostate cancer cell FGF receptor content and physical properties and reexamined multiple aspects of aFGF and bFGF modulation of C3 and T5 prostate cancer cell thymidine incorporation. Results of those studies are detailed in this report.

Results

Fibroblast Growth Factor Modulation of C3 and T5 Rat Prostate Cancer Cell Thymidine Incorporation Differs. Using our standard titration protocol, we found that neither aFGF nor bFGF effectively modulated thymidine incorporation of growth-arrested C3 rat prostate cancer cells plated at 1.5 × 10^5 cells/well. FGF "stimulation" of thymidine incorporation by C3 cells plated at high density was modest and essentially independent of medium mitogen concentration (Fig. 1). In contrast, both aFGF and bFGF profoundly stimulated thymidine incorporation of identically plated and treated, growth-arrested T5 rat prostate cancer cells. The magnitude of effects in T5 cells was mitogen concentration dependent, and the maximum effect was about 20-fold greater than the maximum occurring with C3 cells (Fig. 1). When identical analyses were repeated using C3 and T5 cells derived from different frozen stocks, results comparable to those shown in Fig. 1 were obtained. Findings were independent of the cell passage or frozen stock used. The concentrations of aFGF and bFGF causing half-maximum stimulation of T5 cell thymidine incorporation, respectively, were 0.53 ± 0.10 ng/ml (mean ± SD; n = 3) and 0.35 ± 0.15 ng/ml (mean ± SD; n = 5).

Definition of aFGF and bFGF Binding by Rat Prostate Cancer Cells. To determine whether C3 cell insensitivity to aFGF and bFGF was attributable to the absence of FGF receptor binding activity, we performed comparative analyses of aFGF and bFGF binding by these rat prostate cancer cells. Preliminary analyses showed that both C3 and T5 rat prostate cancer cell monolayers were able to bind either iodo-aFGF or iodo-bFGF during incubation at 4°C and that maximum binding of 50 pM ligand was achieved during 4 h incubation. Specificity of radiolabeled mitogen binding was assessed by determining the ability of selected radioinert mitogens to inhibit iodo-bFGF binding to cell monolayers. These analyses showed (Table 1) that radioinert aFGF and bFGF were equally effective inhibitors of iodo-bFGF binding to prostate cancer cells. In contrast, other prototypic mitogens were ineffective inhibitors of iodo-bFGF binding.

Titration analyses of iodo-bFGF binding by C3 and T5 prostate cancer cells showed that these cells contained limited capacity, high-affinity binding sites. Scatchard analysis of the binding data (Fig. 2) yielded a rectilinear plot consistent with the presence of a single class of binding sites. Comparative analyses showed that T5 cell bFGF receptor site content was about twice that of C3 cells; however, the bFGF dissociation constant was comparable for these cell lines (Table 2). Saturation analyses also showed that T5 cell aFGF receptor content was

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Table 1 Mitogen specificity of FGF receptors of T5 rat prostate cancer cells

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Specific binding remaining (%)</th>
</tr>
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<tbody>
<tr>
<td>FGF</td>
<td>0</td>
</tr>
<tr>
<td>TGF-β</td>
<td>106</td>
</tr>
<tr>
<td>EGF</td>
<td>74</td>
</tr>
<tr>
<td>IGF-1</td>
<td>88</td>
</tr>
<tr>
<td>PDGF</td>
<td>88</td>
</tr>
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</table>

*Data are the means of duplicate determinations for which individual values differed from the mean by 5 to 7%. Nonspecific binding was about 48% of total bound radiolipote. Radiolabeled bFGF concentration was 0.1 nM, and radioinert competitor mitogen concentrations were 20 nM. Identical analyses performed with C3 cells gave comparable results. TGF-β, transforming growth factor β.*

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The "results" section discusses the findings of the study, focusing on the differences in the modulation of thymidine incorporation by FGFs in C3 and T5 cells. The study also examines the ability of these cells to bind to different FGFs, with specific analyses showing that aFGF and bFGF can bind effectively, while other prototypic mitogens are ineffective. The table provides specific binding values for different competitors, showing that the cells are sensitive to FGFs but not to other mitogens.

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Fig. 1. Fibroblast growth factor stimulation of rat prostate cancer cell thymidine incorporation. C3 or T5 cells were plated at 1.5 x 10^5 cells/well in 24-well plates and growth arrested by 24 h culture on serum-free medium. This medium was replaced with serum-free medium containing the indicated concentration of either aFGF or bFGF, and mitogen activity was quantified by the thymidine incorporation assay. Data are the mean specific incorporation (nonspecific incorporation subtracted) of duplicate determinations of a representative analysis. The analysis was repeated twice.
about 35% of bFGF receptor content, whereas T5 cell aFGF and bFGF dissociation constants were comparable (Table 2).

Characterization of Prostate Cancer Cell FGF Receptors Using Affinity Labeling. Having established that C3 and T5 prostate cancer cells contained limited capacity, high-affinity FGF binding sites, we used chemical cross-linking to affinity label iodo-aFGF and iodo-bFGF membrane binding components (Fig. 3). These analyses showed that both C3 and T5 cells contained iodo-aFGF binding components having mass of approximately 120 and 145 kDa. The intensity of iodo-aFGF labeling of the 120 and 145 kDa binding components of C3 and T5 cells was comparable (Fig. 3). C3 and T5 prostate cancer cells also contained membrane components that bound iodo-bFGF. Cross-linking analyses showed that the mass and relative content of C3 and T5 cell bFGF receptors were indistinguishable from those of C3 and T5 cell aFGF receptors (Fig. 3).

C3 and T5 Cell FGF Receptor Content Is Comparable at the Time of Mitogen Application to Growth-arrested Monolayers. To reliably assess mitogen-stimulated thymidine incorporation, it is necessary to growth arrest prostate cancer cells. The process of arresting C3 or T5 cell growth could differentially affect FGF receptor content. To examine this possibility, we assessed FGF receptor content of C3 and T5 cells that had been identically growth arrested and processed exactly to the stage of mitogen application as performed during the thymidine incorporation assay. These analyses (Table 3) revealed that the FGF receptor content of both C3 and T5 cells was significantly reduced as compared (Table 2) to cells subcultured for 24 h on FBS-containing medium. However, FGF receptor content of growth-arrested C3 and T5 cells was essentially identical and was independent of the number of cells initially plated per well (Table 3).

Cell Plating Density Affects FGF Stimulation of Prostate Cancer Cell Thymidine Incorporation. Multiple studies detail cell density-dependent effects on membrane receptor content and/or affecter modulation of cell function (14–20). Our preceding studies established that C3 and T5 prostate cancer cells contained FGF receptors (Tables 1 and 2; Fig. 2) and that FGF receptor content of growth-arrested C3 and T5 cells was comparable and independent of initial cell plating density (Table 3). These findings document the validity of the cell culture protocols for use in analyses of the effects of cell plating density on FGF-stimulated thymidine incorporation.

When T5 cell plating density was varied between 1.0 x 10⁴ and 1.5 x 10⁵ cells/well, we found that the magnitude of either aFGF or bFGF enhancement of T5 cell thymidine incorporation was related to plating density (Fig. 4). Maximum mitogen stimulation of T5 cell thymidine incorporation was observed when 1.5 x 10⁵ cells were plated per well. When plating density was decreased 3-fold to 5 x 10⁴ cells/well, the magnitude of the maximum aFGF stimulation of T5 cell thymidine incorporation was decreased 1.5-fold, whereas the reduction in maximum bFGF-stimulated thymidine incorporation was 1.4-fold. Similarly, a 6-fold reduction in cell plating density to 2.5 x 10⁴ cells/well was associated with a 5.6-fold or 6.0-fold reduction, respectively, in maximum aFGF or bFGF stimulation of T5 cell thymidine incorporation (Fig. 4). Finally, a 15-fold reduction in cell plating density to 1.0 x 10⁴ cells/well was associated with a 19.8-fold reduction in maximum bFGF stimulation of T5 cell thymidine incorporation. aFGF stimulation of T5 cell thymidine incorporation could not be reliably assessed when 1.0 x 10⁴ T5 cells were plated per well (Fig. 4).

In contrast to the findings for T5 cells (Fig. 4), both the apparent mitogenicity of aFGF or bFGF and the magnitude of mitogen-mediated enhancement of C3 cell thymidine incorporation were sensitive to cell plating density (Fig. 5). As previously demonstrated (Fig. 1), neither aFGF nor bFGF showed consistent, concentration-dependent ability to significantly enhance C3 cell thymidine incorporation when 1.5 x 10⁵ cells were plated per well (Fig. 5). However, when plating density was decreased

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**Table 2.** FGF receptor content and dissociation constant of rat prostate cancer cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Receptor content (sites/cell)</th>
<th>Dissociation constant (pm)</th>
</tr>
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<tbody>
<tr>
<td>bFGF receptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>11,900 ± 2,045&lt;sup&gt;b&lt;/sup&gt;</td>
<td>210 ± 45</td>
</tr>
<tr>
<td>T5</td>
<td>22,260 ± 2,200</td>
<td>200 ± 90</td>
</tr>
<tr>
<td>aFGF receptors&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>8,070 ± 2,000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>170 ± 54</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are the mean ± SEM for four independent determinations performed in duplicate.

<sup>b</sup> Significantly different from the value for T5 cell receptor content, p < 0.05.

<sup>a</sup> aFGF receptor content of C3 cells was not determined.
Fig 3. Representative polyacrylamide gel characterization of solubilized preparations of 

\[ ^{125}\text{I}-\text{FGF} \] affinity-labeled rat prostate cancer cells. Subconfluent monolayers were incubated for 4 h at 4°C with either \[ ^{125}\text{I}-\text{aFGF} \] or \[ ^{125}\text{I}-\text{bFGF} \], 100 pm, in the absence \( a, b, c, d \) or presence \( a, b, c, d \) of 10 nm corresponding radiolabeled mitogen. FGFs were then cross-linked to receptors of washed monolayers by 15 min incubation at 4°C with buffer containing 0.25 mm disuccinimidyl suberate. Triton X-100-soluble extracts were prepared and analyzed by electrophoresis on 5.5% polyacrylamide gels, which were dried and autoradiographed at -80°C. The positions of migration of prestained myosin (205 kDa), phosphorylase b (103 kDa), and bovine serum albumin (67 kDa) are shown on the left. The slight differences in marker positions for the different representations reflect the fact that the analyses were not performed at the same time, and all samples were not electrophoresed on the same gel.

3-fold to 5 \( \times \) 10^4 cells/well, both aFGF and bFGF showed consistent, concentration-dependent enhancement of C3 cell thymidine incorporation (Fig. 5). Notably, the magnitude of mitogen-mediated enhancement of C3 cell thymidine incorporation was only about 5 to 7% of that achieved when 5 \( \times \) 10^4 T5 cells were plated per well (compare Figs. 4 and 5). A 2-fold reduction in C3 cell plating density to 2.5 \( \times \) 10^4 cells/well was associated with a 1.05-fold or 1.2-fold reduction, respectively, in the magnitude of the aFGF or bFGF maximally stimulated C3 cell thymidine incorporation (Fig. 5). Finally, a 5-fold reduction in cell plating density to 1.0 \( \times \) 10^4 cells/well was associated with a 2-3-fold or 3.3-fold reduction, respectively, in the magnitude of aFGF or bFGF maximally stimulated C3 cell thymidine incorporation.

**Heparin Modulation of C3 Cell FGF Sensitivity.** Culture of growth-arrested C3 cells on serum-free medium containing aFGF with or without heparin revealed that heparin variably affected aFGF stimulation of C3 cell thymidine incorporation (Fig. 6). When 1.5 \( \times \) 10^4 cells were plated per well, heparin consistently inhibited the minimal aFGF-mediated stimulation of C3 cell thymidine incorporation. In contrast, when cells were plated at lower densities, heparin was either without effect or only modestly enhanced aFGF stimulation of C3 cell thymidine incorporation. Heparin enhancement of aFGF-stimulated thymidine incorporation occurred only when medium mitogen concentration was 0.1 ng/ml or when C3 cells were plated at 1.0 \( \times \) 10^4/well (Fig. 6). Heparin also variably affected bFGF stimulation of growth-arrested C3 cell thymidine incorporation. In general, heparin caused less inhibition of bFGF-mediated C3 cell thymidine incorporation (Fig. 7). However, effects were modest and inconsistent, with the exception of a trend to increase bFGF-mediated thymidine incorporation by C3 cells plated at 2.5 \( \times \) 10^4/well (Fig. 7).

**Discussion**

Our studies establish that aFGF and bFGF modulation of C3 and T5 rat prostate cancer cell thymidine incorporation differs (Fig. 1). When we assessed the possible relation between FGF receptor content and C3 cell FGF insensitivity, we found that saturation analyses (Table 2; Fig. 2) indicated that C3 cell FGF receptor content was about 50% of that of T5 cells. However, the difference in prostate cancer cell FGF receptor content was less apparent when ligand was cross-linked to receptors (Fig. 3). This likely reflects inherent limitations of the cross-linking protocol to demonstrate small differences in receptor content due to confounding effects of cross-linking efficiency and recovery of cross-linked receptor from cell monolayers. The profoundly diminished ability of aFGF or bFGF to enhance C3 cell thymidine incorporation, relative to the ability of these mitogens to enhance T5 cell thymidine incorporation, does not appear attributable to the modest differences that characterized FGF receptors of these cells. First, there is neither a quantitative nor qualitative relation between the approximate 2-fold difference in receptor content of cells which have not been growth arrested (Table 2) and the nearly 20-fold difference (compare Figs. 1, 4, and 5) in FGF responsiveness of C3 and T5 cells. Moreover, the process of growth arrest eliminates differences in T5 and C3 cell FGF receptor content (Table 3). Secondly, T5 and C3 cell FGF receptor dissociation constants essentially are identical (Table 2). Additionally, other physical properties of aFGF and bFGF receptors of C3 and T5 prostate cancer cells appear comparable (Figs. 2 and 3). Moreover, physical properties of C3 and T5 prostate cancer cell FGF receptors are comparable to FGF receptors of other cells (21–24), including normal rat prostate epithelial and stromal cells (25) and tissues (26, 27). Finally, T5 cell FGF responsiveness, measured as the concentration of mitogen required to achieve half-maximum response, was comparable to that previously reported by us (12) and representative of that characteristic of mesoderm-derived cells (28, 29). Consequently, FGF insensitivity of C3 cells appears to

![Table 3](image-url)
represent a genuine failure of cellular response mechanisms activated by mitogen binding to receptor.

The process of growth arrest caused loss of C3 and T5 cell FGF receptors (compare Tables 2 and 3). However, FGF receptor content of these cells at the time of mitogen stimulation essentially was comparable and independent of initial cell plating density (Table 3). For T5 and C3 cells, 24 h culture on serum-free medium (growth arrest) results in an apparent 85% and 65% decrement, respectively, in FGF receptor content (compare Tables 2 and 3). These apparent values overestimate the actual decrease in receptor content. Based on the FGF receptor dissociation constants (Table 2), 500 pm ligand [the dose used for site content determination (Table 3)] would produce only 70% saturation of FGF receptor sites and thus would underestimate actual cell content (Table 3).

When observed data are corrected for the degree of receptor saturation, the actual growth arrest-associated decrement in T5 and C3 cell FGF receptor content, respectively, was 80% and 55%.

Our studies do not address potential causes for prostate cancer cell FGF receptor loss during growth arrest. However, it is likely that the decrement in FGF receptor content is at least partially due to inability or diminished ability of growth-arrested cells to replace receptors down-regulated (24, 25) by prostate cancer cell binding of elaborated heparin-binding mitogens. In previous studies, we showed that medium conditioned by C3 or T5 prostate cancer cells contained mitogens that stimulated prostate cancer cell proliferation (12). More recently, we established that (a) heparin-binding mitogen production by prostate cancer cells was about 1000-fold greater than that of normal rat prostate cells, and (b) prostate cancer cell-associated and secreted mitogens included polypeptides having immunological similarity with and/or identity to bFGF (13). Thus, C3 and T5 prostate cancer cells produce large quantities of mitogens that are expected to bind to FGF receptors and promote receptor down-regulation. The fact that the profound growth arrest-related decrements in T5 prostate cancer cell FGF receptor content do not cause complete loss of FGF responsiveness implies FGF receptor excess.

The magnitude of maximum bFGF enhancement of T5 cell thymidine incorporation was related to cell plating density (Fig. 4). When fold change in maximum T5 cell thymidine incorporation was plotted versus fold change in cells plated per well, a linear (three points) relationship was obtained. Because these data are quite limited, they should be considered preliminary. Nonetheless, a qualitative relation between cell plating density and maximum bFGF enhancement of T5 cell thymidine incorporation was indicated (Fig. 4). This result, and the fact that T5 cell FGF receptor content is independent of initial cell plating density (Table 3), implies that the observed effects of plating density on maximum bFGF-mediated thymidine incorporation principally are attributable to cell number. Because T5 cell response to aFGF was less than that to bFGF (Fig. 4), comparable maximum aFGF response data are more limited (two points). However, the data obtained for the relation between plating density and maximum aFGF-mediated T5 cell thymidine incorporation essentially were identical to those for bFGF (Fig. 4). These results imply that cell-cell interactions minimally affect T5 cell FGF responsiveness.

When C3 cells were plated at high, subconfluent density, 1.5 x 10⁵ cells/well, FGFs failed to enhance thymidine incorporation following growth arrest (Figs. 1 and 5). As plating density was decreased, FGFs enhanced growth-arrested C3 cell thymidine incorporation; how-
ever, response was poorly related to cell plating density (Fig. 4) and was about 20-fold less than that of T5 cells (compare Figs. 4 and 5). Consequently, FGF insensitivity of C3 cells is only modestly affected by plating density, and FGF receptor content of growth-arrested C3 cells is plating density independent (Table 3). Therefore, the essential absence of plating density effects on C3 cells distinguishes these cancer cells from other cells in which plating density affects mitogen receptor content and responsiveness (14–20).

The diminished responsiveness of C3 cells, relative to T5 cells, was not attributable to FGF instability because inclusion of heparin, which is known to protect both aFGF and bFGF from inactivation (30), failed to significantly and consistently enhance C3 cell FGF responsiveness (Figs. 6 and 7). In fact, heparin principally caused inhibition of thymidine incorporation by C3 cells plated at 1.5 × 10⁵ cells/well on aFGF-containing medium (Fig. 6), caused variable inhibition of thymidine incorporation by C3 cells plated on bFGF-containing medium (Fig. 7), and elicited moderate elevation of aFGF or bFGF enhancement of thymidine incorporation only when cells were plated at low densities (Figs. 6 and 7). Significantly, these modest effects of heparin were poorly related to medium heparin concentration (Figs. 6 and 7). Additionally, the fact that specific FGF binding by growth-arrested FGF-responsive T5 cells was comparable to that of C3 cells (Table 3) shows that C3 cell FGF insensitivity is not attributable to differences in cell content of FGF:receptor complexes at the time of mitogen stimulation. Consequently, the findings that neither changes in cell plating density nor medium heparin concentration (Figs. 6 and 7) was able to enhance FGF effectiveness in C3 cells implies that the inability of heparin to enhance FGF mitogenicity for C3 cells is not attributable to failure of these cells to effectively bind FGFs.

We previously showed that C3 cells are responsive to the mitogenic effects of FBS (12). That finding and the results of our present studies imply that C3 prostate cancer cell FGF insensitivity is due to defect(s) in signal transduction occurring subsequent to mitogen binding to receptor. A similar finding recently has been reported for some human prostate cancer cells (31). DU 145 prostate cancer cells elaborate bFGF-like mitogens, contain FGF receptor transcripts, and are FGF responsive. In contrast, PC 3 prostate cancer cells elaborate bFGF-like mitogens, contain FGF receptor transcripts, and are not FGF responsive (31). Therefore, DU 145 human prostate cancer cells appear to have properties similar to those of T5 rat prostate cancer cells, whereas properties of PC 3
human prostate cancer cells appear similar to those of C3 rat prostate cancer cells. An initial attempt to assess a potential cause of PC 3 cell FGF insensitivity by defining c-myc induction yielded ambiguous results (31). bFGF does not increase PC 3 cell c-myc transcript content. However, because growth-arrested PC 3 cells expressed high levels of c-myc, results may reflect consequences of high, basal c-myc expression rather than postreceptor defect(s) in signal transduction. In contrast, although c-myc transcripts were demonstrable in growth-arrested DU 145 cells, content was markedly less than that of PC 3 cells, and bFGF treatment caused a transient increase in c-myc transcript content (31). It appears that detailed characterization of multiple aspects of the signal transduction pathway will be required to dissect the cause of FGF insensitivity of human and rat prostate cancer cells.

Materials and Methods

Cell Lines and Cell Culture. Morphological and biochemical properties of clonally derived AXC/SSh C3 and T5 rat prostate cancer cell lines have been detailed (12, 13). Culture and subculture of these cells from low-passage stocks were as previously described (12).

Other Materials. FBS was from HyClone Laboratories (Logan, UT). Other materials for culture of prostate cancer cells were obtained and used as previously described (12, 32). [methyl-3H]Thymidine (6.7 Ci/mmol) was obtained from NEN/DuPont (Boston, MA). 125I-labeled aFGF (200 μCi/μg). 125I-labeled bFGF (200 μCi/μg), bovine aFGF, and human recombinant bFGF were from R & D Systems, Inc. (Minneapolis, MN). Human recombinant IGF-I was a Collaborative Research (Bedford, MA) product. Recombinant EGF and PDGF were obtained from Amgen Biologicals (Thousand Oaks, CA). Radioreceptor thymidine, aprotinin, pepstatin A, phenylmethylsulfonyl fluoride, ovalbumin, and porcine heparin (sodium salt) were from Sigma Chemical Co. (St. Louis, MO). Leupeptin was purchased from Calbiochem (La Jolla, CA). Radioreceptor aFGF and bFGF were diluted into 10 mM sodium phosphate, pH 7.5, containing 100 mM NaCl and 0.5 mg/ml ovalbumin prior to use. Concentrated stock solutions of radioreceptor EGF, IGF-I, and PDGF were prepared and stored as previously described (32). Disuccinimidyl suberate was purchased from Pierce (Rockford, IL), and reagents for electrophoresis were from Bio-Rad (Richmond, CA). Culture media and solutions were prepared in water that had been purified by reverse osmosis and then distilled from glass.

Saturation Analysis of Fibroblast Growth Factor Binding. Prostate cancer cells, propagated on cell line-specific parental medium (12), were subcultured on phenol red-free parental medium for 72 h. On the day before initiating saturation binding assay, these cells were harvested, and 105 cells/well were subcultured on 24-well plates on phenol red-free parental medium. To initiate the binding assay, culture medium was removed and replaced with 1.0 ml binding medium (40 mM HEPES and 1 mg/ml BSA in phenol red-free bicarbonate containing modified Eagle's minimum essential medium, pH 7.4) prewarmed to 37°C. Following 10 min incubation at 37°C, binding buffer was removed, and monolayers were washed a second time by incubation with fresh binding buffer at 37°C. The plates were cooled to 4°C at the conclusion of the second 37°C incubation. Binding buffer was then replaced with 200 μl of 4°C binding buffer containing 50 to 500 pm radiolabeled aFGF or bFGF. Nonspecific binding was quantified by incubating parallel cultures with radiolabeled aFGF or bFGF in the presence of a 100-fold molar excess of the corresponding radioreceptor FGF. Plates containing triplicate determinations were placed in sealed bags and incubated at 4°C for 4 h. At conclusion, binding buffer was removed by aspiration, and monolayers were washed four times at 4°C with 1 ml of 4°C binding buffer. Monolayers were then washed with 1.0 ml of 25 mM HEPES, pH 7.5, containing 1.6 mM NaCl and 0.1% BSA. Monolayers were then solubilized by covering with 750 μl of 20 mM HEPES, pH 7.4, containing 1% Triton X-100 and 10% glycerol, followed by 30 min incubation at 37°C. Isotope content of appropriate aliquots was determined by γ-scintillation spectrometry.

Fibroblast Growth Factor Single-Dose Ligand-binding Assays. Prostate cancer cells, propagated as detailed above, were harvested and subcultured on 24-well plates on parental medium. These cells were grown arrested exactly as previously detailed by culture on serum-free medium (12, 13). To initiate the binding assay, plates were cooled to 4°C, and serum-free medium was removed and replaced with 500 μl of Dulbecco's phosphate-buffered saline cooled to 4°C. The phosphate-buffered saline wash was repeated once, and the final wash was replaced with 200 μl of binding buffer (20 mM HEPES and 0.1 mg/ml BSA in bicarbonate containing modified Eagle's minimal essential medium, pH 7.4) containing 500 μM radiolabeled bFGF in the absence or presence of 50 μM radioreceptor bFGF. Plates containing triplicate determinations were placed in sealed bags and incubated at 4°C for 2 h. Preliminary analyses established that maximum binding occurred during 2 h incubation of monolayers with 500 pm ligand.) At conclusion, binding buffer was removed by aspiration, and monolayers were washed twice at 4°C with 500 μl of binding buffer, followed by a single wash with 500 μl of 25 mM HEPES, pH 7.5, containing 1.6 mM NaCl and 0.1% BSA. The monolayers were then extracted by covering with 500 μl of 20 mM sodium acetate, pH 4.0, containing 1.6 mM NaCl. Isotope content of appropriate aliquots of these fractions was quantified by γ-scintillation spectrometry. Greater than 80% of specific binding was in the pH 4.0 sodium acetate fraction. These data were used to calculate FGF receptor content.

Affinity Labeling and Polyacrylamide Gel Electrophoretic Analysis of FGF Receptors. Affinity labeling of FGF receptors was achieved by incubating parallel monolayers with 100 pm radiolabeled aFGF or bFGF in the absence or presence of 10 μM radioreceptor aFGF or bFGF, using methods detailed for saturation analysis of FGF receptors. After 4 h incubation at 4°C, binding buffer was removed, and monolayers were washed three times at 4°C using 2 ml of fresh binding buffer. Washed monolayers were covered with fresh binding buffer, and sufficient freshly prepared disuccinimidyl suberate in dimethyl sulfoxide was added to achieve 0.25 mM disuccinimidyl suberate and 1% dimethyl sulfoxide. The cross-linking reaction was stopped after 15 min incubation at 4°C by removing the incubation buffer and washing the monolayers at 4°C with collection buffer. Collection buffer composition was 10 mM Tris-Cl, pH 7.0, containing 250 mM sucrose and 1 mM EDTA. Immediately prior to use, collection buffer was supplemented to contain the
following protease inhibitors: phenylmethylsulfonyl fluoride, 0.5 mM; pepstatin, 1 μg/ml; leupeptin, 1 μg/ml; and aprotinin, 2.5 μg/ml. Cells were scraped into protease inhibitor containing collection buffer and collected by 5 min centrifugation at 12,000 × g at 4°C. Cell pellets were extracted by resuspension and incubation in a minimum volume of protease inhibitor-supplemented collection buffer containing 1% Triton X-100. Affinity-labeled proteins in the Triton X-100 extract were separated by polyacrylamide gel electrophoresis, performed essentially as detailed by Laemmli (33). Radiolabeled proteins were visualized by autoradiography of dried gels at −80°C.

Other Methods. C3 and T5 prostate cancer cell FGF responsiveness was quantified by using the [3H]thymidine incorporation assay previously described by us (12, 13). Significance of differences was assessed by multway analysis of variance (34).

References