We have raised specific antibodies to the second immunoglobulin-like domain of fibroblast growth factor receptors (FGFRs) and used these to investigate the expression and subcellular localization of FGFR-1, -2, -3, and -4 in breast epithelial cells. All four receptors classes could be detected in breast cell lines; however, FGFR-4 and FGFR-2 appeared to be expressed at a higher level in breast cancer cell lines than in normal epithelial cells. Surprisingly, FGFR-3 localized in the cell nucleus by immunofluorescence. A second antibody to a separate epitope confirmed this finding and showed that the form of FGFR-3 present must contain an intact kinase domain as well as the growth factor binding domain. Western analysis of fractionated cells revealed the presence of two forms of FGFR-3 of 135 and 110 kDa. The 110-kDa form was predominantly found in the nucleus, whereas the 135 kDa form was sometimes found in the nucleus. RT-PCR analysis of FGFR-3 mRNA showed the presence of a splice variant in which exons 7 and 8 are deleted. This results in the translation of FGFR-3 missing the transmembrane domain but with an intact kinase domain, which could be a soluble, intracellular receptor. Transfection experiments showed that FGFR-3 containing this deletion and no signal peptide gave an identical nuclear staining pattern to that seen in breast epithelial cells. We conclude that two forms of FGFR-3 are present in breast epithelial cells; a full-length 135-kDa receptor, which has a conventional membrane localization, and a novel soluble form of 110 kDa.

The fibroblast growth factor (FGF) family constitutes a family of nine structurally related polypeptides sharing 30–55% homology (Burgess and Maciag 1989). Acidic FGF (FGF-1) and basic FGF (FGF-2) are the best characterized members of the family, other members including products of the oncogenes int-2 (FGF-3) and hst/K-FGF (FGF-4), keratinocyte growth factor (FGF-7), and the recently discovered androgen-induced growth factor (FGF-8) and glial-activating factor (FGF-9) (Tanaka et al., 1992; Miyamoto et al., 1993). FGFs are involved in the control of a variety of biological functions including mitogenesis, chemotaxis, neuronal survival and neurite extension, mesoderm induction, angiogenesis, and wound healing (Burgess and Maciag, 1989). Biological responses to FGFs are mediated through specific high affinity receptors. Four structurally related genes encoding FGFRs have been identified (Jaye et al., 1992; Johnson et al., 1991; Keegan et al., 1991; Partanen et al., 1991). Each receptor contains an intracellular split tyrosine kinase domain and an extracellular domain containing up to three immunoglobulin-like domains. In addition, low affinity receptors for FGF are present on the surface of cells. These have been identified as heparin sulfate proteoglycans (Moscatelli, 1987). A ternary complex of FGF heparin sulfate proteoglycans and high affinity receptor appears to be required for biological activity (Yayon et al., 1991; Rapraeger et al., 1991; Roghani et al., 1994). Structural variants of the high affinity receptors can be generated by alternative splicing of their RNA transcripts (Hou et al., 1991). Ligand binding properties of the receptor are changed by the use of alternative third exons for receptors FGFR-1, FGFR-2, and FGFR-3 (Werner et al., 1992; Chellaiah et al., 1994), and the affinity of FGF binding may be affected by the loss of the first immunoglobulin-like domain (Johnson et al., 1990; Shi et al., 1993). Putative intracellular forms of FGF receptors may also be generated by alternative RNA splicing mechanisms by the substitution of a 267-base pair sequence encompassing the first Ig domain by an unrelated 144-base pair sequence (Hou et al., 1991). The 144-base pair insert contains stop codons leading to premature termination, but reinitiation of translation would lead to a receptor without signal peptide or acidic box having an intracellular localization. A rat analogue of FGFR-4 with this conformation has been reported (Horlick et al., 1992). FGF-1 and -2 have both been detected in the cell nucleus (Presta et al., 1993; Gualandris et al., 1993; Cao and Petterson, 1993; Cao et al., 1993; Amalric et al., 1994). When FGF2 is synthesized in a cell, only the higher molecular mass forms (24, 22.5, and 22 kDa) are detected in the nucleus (Bugler et al., 1993). However, exogenous 18-kDa basic FGF can enter the nucleus in the G1 phase of the cell cycle, where it is localized in the nucleolus (Baldin et al., 1990). The presence of FGFR-1 and FGFR-2 in the nucleolus is likely to be important in their biological function. It has been shown that internalization of FGFR-1 is essential for stimulation of cell division, and a dual model of action has been proposed (Wiedlocha et al., 1994). FGF-2 is bound to chromatin in the nucleolus (Gualandris et al., 1993), and it can affect gene transcription in cell-free systems (Nakanishi et al., 1992). FGF-2 taken into the nucleolus appears to increase the transcription of genes encoding ribosomal RNA (Bouche et al., 1987; Amalric et al., 1994). We report the production of specific antisera against the extracellular region of three FAF receptors and use these re-
agents to examine the intracellular localization of each receptor in breast epithelial cells. FGFR-1, -2, and -4 have a membrane-bound localization; however, in both breast cancer cells and normal breast epithelial cells, FGFR-3 was found in the nucleus.

MATERIALS AND METHODS

Generation of Recombinant Domains of FGFR Receptors—PCR primers 5′-CGAGATTGCTTGCAGGATGGGCCGGTG-3′ (sense) and 5′-CAGATTGCCGTGGCAAGATGGGGCGGT-3′ (antisense) were used to generate PCR fragments encoding the second immunoglobulin domain of FGFR-1, FGFR-3, and FGFR-4. The conditions of the PCR reaction were as follows: 30 cycles of 1 min at 95 °C, 1 min at 45 °C, and 1 min at 72 °C followed by 10 min at 72 °C. The reaction mix contained 1 × reaction buffer (Peninsular), 1.25 mM MgCl₂, 200 μM dNTPs, 200 ng of each primer, 10 ng of FGFR receptor plasmid and 2 units of Taq polymerase (Peninsular). PCR fragments were purified and subcloned into pGEX-2T using the restriction sites present in the primers. Recombinant domains were expressed in JM101 strain of Escherichia coli and were purified by affinity chromatography using a glutathione-agarose column as described (Smith and Johnson, 1988).

Immunization of Rats—Rats were inoculated intramuscularly with 1 mg of purified receptor Ig2 domain-glutathione S-transferase fusion protein mixed with Hunter’s Titermax adjuvant, at monthly intervals for three months. Antiserum were prepared 4 weeks after inoculation.

Purification of Antibodies—An affinity column was prepared by coupling receptor Ig2 domains to CNBr-activated Sepharose (Pharmacia Biotech Inc.) following the manufacturer’s recommended protocol. 4 ml of antiserum were loaded onto each column and washed through with 10 column volumes of PBS. Bound antibodies were eluted with 3.5 M ammonium thiocyanate, 0.5 M ammonia, pH 11.2, and collected fractions were immediately neutralized with 1× phosphate buffer, pH 7.0. Fractions containing antibody were pooled, dialyzed against PBS, and concentrated using centrifugation 10 units.

Other Antibodies—Two additional antibodies were used in this study. A monoclonal antibody against FGFR-1 (Upstate Biotechnology) was raised against Pro22–His325 of the β-form of FGFR-1, corresponding to the second and third immunoglobulin-like domains. A second polyclonal antibody against FGFR-3 (Santa Cruz Biotechnology) was raised against amino acids 792–806 in the carboxyl terminus of FGFR-3. Both of these antibodies had been shown to be noncross-reactive with other FGFR receptors.

Cell Culture—Cell lines were grown in RPMI 1640 supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum.

Immunofluorescence—Cells were grown on glass coverslips for several days, rinsed briefly with PBS, and fixed in 3% formaldehyde in PBS for 10 min. The cells were permeabilized by treating with 0.2% Triton X-100 in PBS for 5 min, and cells were then treated with 50 mM ammonium chloride in PBS for 10 min. The cells were blocked with 10% goat serum in PBS for 1 h and then incubated with 1 μg/ml purified anti-FGFR antibody diluted in PBS containing 10% goat serum for 1 h. After washing in three changes of PBS, the cells were incubated with goat anti-rabbit IgG-fluorescein isothiocyanate (Pierce) or rabbit anti-mouse IgG-fluorescein isothiocyanate (Pierce) diluted 1:50 in PBS containing 10% goat serum for 1 h. The cells were washed extensively, mounted, and viewed using a fluorescence microscope.

Transient Expression in COS-7 Cells—Ten million COS-7 cells were washed in 20 ml Hepes, pH 7.4, 140 mM NaCl, 5 mM KCl, 6 mM dextrose and then resuspended in 0.5 ml of the same buffer. 100 μg of human placental DNA (0.5 μg of pSG5-FGFR-3) was added and the cells were electroporated at 500 microfarads, 200 mV. The cells were cultured for 2 days, harvested, and resuspended in Læmmli sample buffer at a concentration of 30 million cells/ml. In immunofluorescence experiments, cells were fixed 3–7 days after electroporation.

Immunoblotting—7.5% polyacrylamide gels were run following the method (Laemmli, 1970) with 100 μg of protein loaded in each lane. Proteins were transferred onto nitrocellulose, which was blocked with 3% milk powder in PBS and probed with purified antibodies diluted to 1 μg/ml in PBS containing 0.1% Tween 20 and 0.3% milk powder (PBST). After washing in PBST, the nitrocellulose blots were incubated with anti-rabbit-horseradish peroxidase or anti-mouse horseradish peroxidase conjugate diluted in PBST containing 0.3% milk powder. After extensive washing, ECL reagents (Amersham Corp.) were used to detect bands.

Subcellular Fractionation—10 million cells were harvested, washed three times in PBS, and resuspended in 0.6 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 3 mM CaCl₂, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml chymostatin). The cells were left on ice for 30 min before homogenizing with 30 strokes of a tight fitting Dounce homogenizer. Nuclei and membranes were separated by spinning at 600 rpm for 5 min.

PCR Detection of Alternative Splice Variants of FGFR-3—Total RNA was extracted from 80% confluent cells using the modified RNAZOL protocol (Chomczynsky and Saachi, 1987). cDNA was made from 2 μg of RNA by adding to 200 units of Moloney murine leukemia virus reverse transcriptase, 1 mM dNTP, 10 mM dithiorthiol, 250 ng of random hexamers, 50 μM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, and incubating at 40 °C for 1 h followed by 5 min at 95 °C. PCR primers 5′-CGGACAGCTACACGGTG-3′ (sense) and 5′-GTGGTGTGTGGT-3′ (antisense) were used to generate PCR fragments encoding FGFR-3 from nucleotide 712 to 1310. One-tenth of the reverse transcriptase reaction product was added to a PCR reaction with the following incubation steps: 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C followed by 10 min at 72 °C. The reaction mix contained 1 × reaction buffer (Peninsular), 1.25 mM MgCl₂, 200 μM dNTPs, 200 ng of each primer, 1 μl of cDNA, and 2 units of Taq polymerase (Peninsular). PCR fragments were visualized on an agarose gel by ethidium bromide staining of Southern blot as described previously (Iocca et al.). PCR fragments were separated on a 1% agarose gel using a spin column DNA recovery system (FMC) and sequenced using deoxyrribonucleotide cycle sequencing (Applied Biosystems) and an Applied Biosystems model 373A DNA sequencer.

Generation of Variant Forms of FGFR-3—The transmembrane-deleted form of FGFR-3 was made by replacing the EcoRI-MluI (nucleotides 740–1283) restriction digested fragment of FGFR-3 with the same fragment from the smaller PCR product from a reaction on MCF-7 cDNA using primers 5′-CGGACAGCTACACGGTG-3′ (sense) and 5′-GTGGTGTGTGGT-3′ (antisense), as described above. The resultant construct was sequenced to show that no additional mutations had been introduced during the PCR reaction. The signal peptide was removed by deleting the first 5′-terminus of FGFR-3 up to the Small site at nucleotide 114. This would result in translation starting at the ATG codon at nucleotide 204. A construct lacking both signal peptide and transmembrane domain was made by combining both the above mutations. All forms of FGFR-3 were subcloned into a pSG5 vector for use in transfections.

RESULTS

Production of Antisera against FGFR-2, FGFR-3, and FGFR-4—We wished to raise antibodies that bound specifically to individual FGFR receptors with minimal cross-reactivity. We selected the second immunoglobulin domain as immunogen since it is present on most of the receptor variants and is less well conserved than several other domains within the receptor (Fig. 1A). PCR primers containing BamHI and EcoRI restriction enzyme sites were used to amplify the second immunoglobulin domain of FGFR-2, FGFR-3, and FGFR-4. The PCR fragments were subcloned into a pGEX-2T vector, and glutathione S-transferase fusion proteins were expressed and affinity-purified. As shown in Fig. 1B, pure fusion protein could be achieved for all three receptors. Rabbit antisera were raised against all three fusion proteins and were affinity-purified.

Specificity of Anti-FGFR Receptor Antibodies—In order to test the cross-reactivity of the three purified antisera and a FGFR-1 monoclonal antibody (Upstate Biotechnology) against the second and third immunoglobulin domains (Pro22–His325 of β form), the four classes of FGFR receptor were transiently expressed in COS-7 cells and lysates made of the expressing cells (the second antibody against the C terminus of FGFR-3 was not tested for specificity since it had already been shown to be specific for FGFR-3). Fig. 2 shows the results of Western blot analysis with each antibody failing to detect an endogenous receptor band in control COS-7 cells but detecting receptor expression specifically in the lane transfected with its respective receptor. In each case the band size detected is consistent with expression of the full-length cDNA construct (130 kDa for
FGFR-1, 130 kDa for FGFR-2, 135 kDa for FGFR-3, and 120 kDa for FGFR-4). Lower bands seen for FGFR-2 and FGFR-3 may represent under-glycosylated forms of the receptors.

Localization of FGF Receptors in Transfected Cells—Full-length constructs of FGF receptors under an SV40 promoter were introduced into COS-7 cells by electroporation. After 48 h the cells were fixed and analyzed by immunofluorescence to find the localization of the transiently expressed receptors. As shown in Fig. 3, A–D, all four receptors gave staining patterns.

Fig. 1. Domains of FGFRs to which antisera were raised. A, structure of FGFR, position of PCR primers, and domains to which antisera bind. I, II, and III refer to immunoglobulin domains, TM to the transmembrane domain, J M to the juxtamembrane domain, TK to the tyrosine kinase domain, KI to the kinase insert domain, and CT to the carboxyl-terminal tail. B, Coomassie Blue-stained SDS-polyacrylamide gel of purified glutathione S-transferase fusion proteins.

Fig. 2. Western analysis to show the cross-reactivity of anti-FGFR antisera. Lysates of COS cells transfected with FGFR-1, -2, -3, or -4 or untransfected (lanes M, 1, 2, 3, and 4, respectively) were run on a 7.5% polyacrylamide gel and transferred to nitrocellulose. The blots were probed with antisera against FGFR-2, FGFR-3, or FGFR-4 or a monoclonal antibody against FGFR-1 (UBI) by Western analysis.

Fig. 3. Immunofluorescent staining of COS cells transfected with FGFR-1 (A), FGFR-2 (B), FGFR-3 (C), and FGFR-4 (D) and stained with antibodies against the transfected receptor. E and F show COS-7 cells transfected with FGFR-3 and treated with conditioned medium from breast epithelial cells (E) or 20 ng/ml acidic FGF (F) for 24 h before immunofluorescence detection of FGFR-3. G, H, I, and J show the basal levels of immunofluorescence of mock-transfected COS cells detected with antibodies against FGFR-1 (G), FGFR-2 (H), FGFR-3 (I), and FGFR-4 (J).
consistent with a plasma membrane localization. In each case, no staining was seen in the cell nucleus, but cytoplasmic vesicles stained brightly, especially around the nucleus. In all cases the antibodies were able to produce high levels of immunofluorescence in expressing cells and negligible staining of the nonexpressing cells, showing that all the antibodies were able to recognize native, glycosylated receptor protein. Fig. 3, G-J show untransfected COS cells stained with antibodies against FGFR-1, -2, -3, and -4. Indistinct pale stain was seen using all antibodies, with those against FGFR-2 and -3 showing some stain in the nucleus as well as the cytoplasm. We conclude that the nonspecific bands seen in Western blot experiments (Fig. 2) do not contribute significantly to immunofluorescence.

Intracellular Localization of FGFR-3—A range of normal and cancer breast cell lines were used to find the cellular localization of the different classes of FGFR-3 by immunofluorescence. The results are shown in Figs. 4 and 5 and are summarized in Table I. Different FGFR-3 gave staining patterns, indicating that they occupy different sites within the cell. Antibodies against FGFR-3 gave very faint cytoplasmic staining indistinguishable from background levels, indicating that the level of expression of FGFR-3 is quite low (Fig. 4, E and F). The antibodies against FGFR-2 and FGFR-4 gave staining consistent with a plasma membrane localization, with vesicular staining in the cytoplasm and a faint ring of stain around the edge of some cell types (Fig. 4, A–D). For each of these antibodies, higher levels of staining were seen in breast cancer cells compared with normal breast epithelial cells.

Unexpectedly the antibody against FGFR-3 gave a nuclear staining pattern with no stain in the nucleoli but good stain over the rest of the nucleus (Fig. 5). The Ig2-glutathione S-transferase protein against which the anti-FGFR-3 antibody was raised was used to block specific binding of these antibodies by preincubating the diluted antibody with 10 μg/ml fusion protein. As shown in Fig. 5, B and D, such treatment could completely block the nuclear immunofluorescence of anti-FGFR-3. A second antibody against the C-terminal tail of FGFR-3 (Santa Cruz Biotechnology) was used to confirm the localization of FGFR-3. This antibody was reported to bind specifically to FGFR-3, and in our hands it bound to FGFR-3 but not to FGFR-1, -2, or -4 by Western analysis. The same staining pattern was found in both the normal and malignant cell lines, with anti-FGFR-3 giving nuclear staining with exclusion from the nucleoli (Fig. 5, C and F). Similar levels of staining for FGFR-3 were seen in normal and malignant breast cell lines.

Effect of Growth Factor Treatment on the Localization of FGFR-3—Our results showed different localizations for FGFR-3 depending on whether we transfected the full-length cDNA into COS cells or studied the localization of native FGFR-3 in epithelial cells. There are several possibilities why this discrepancy might occur, such as translation of different forms of FGFR-3 in the two systems, differences in the quantity of FGFR-3 being made, or differences in the environment of the two cell types. Previous studies have shown that breast epithelial and cells synthesize FGF1 and FGF2 so that autocrine stimulation of these cells is possible (Gomm et al., 1991; Luqmani et al., 1992). We tested whether growth factor stimulation could change the localization of transfected FGFR-3 by transiently expressing FGFR-3 in COS-7 cells and treating with 20 ng/ml FGF1 or FGF2 or with conditioned medium from the breast epithelial cells MCF-7 and HBR-SV-161 and the myoepithelial cell line HBL-100. None of these treatments was able to change the localization of FGFR-3. The same cytoplasmic stain was seen with perinuclear vesicle staining but no staining within the nucleus (Fig. 3, E and F).

Subcellular Localization of FGFR-3 by Immunoblotting—Cells were separated into nuclear and membrane fractions, which were analyzed by Western blotting to find the localization of FGFR-3. In the case of FGFR-1, FGFR-2, and FGFR-4 receptors, bands of 130, 130, and 120 kDa, respectively, were seen in the membrane fractions of MCF-7 cells and HBR-SV-161 cells but not in the nuclear fractions. In the case of FGFR-3 bands of 110 and 135 kDa were seen, and there was evidence for nuclear localization. Using the antibody against the ligand binding domain of FGFR-3, both bands appeared in the nuclear fraction with very little FGFR-3 present in the cytoplasm. The second antibody against the C-terminal tail of FGFR-3 showed some of the FGFR-3 in the nucleus and some in the cytoplasm. The 110-kDa form of FGFR-3 was predominantly in the nucleus, whereas the 135-kDa form was present in both fractions (Fig. 6). The localization of 135-kDa FGFR-3 varied between being fully nuclear to being fully cytoplasmic, with different batches of cells giving differing results. Localization of this form of FGFR-3 may be dependent on stimulation of the cell in a manner analogous to that described for FGFR-1 (Prudovsky et al., 1994). These results confirm the observation that FGFR-3...
FGFR-3 (Santa Cruz).

Sera against the C-terminal tail of are probed with a second purified antibody to which it was raised; with the glutathione S-transferase fusion protein to which it was raised; C and F are probed with a second purified antiserum against the C-terminal tail of FGFR-3.

The second immunoglobulin domain of FGFR-3 is probed with purified antisera against the N terminus of FGFR-3, but after preincubation with the glutathione S-transferase fusion protein to which it was raised. The second immunoglobulin domain and the transmembrane domain.

Such an mRNA would produce FGFR-3 missing half of the final immunoglobulin-like domain. An FGFR-3 variant without a signal peptide and transmembrane domain gives an identical, nuclear localization to that seen in breast epithelial cells—In order to investigate whether the exon-deleted form of FGFR-3 could account for the nuclear localization seen in epithelial cells, a series of FGFR-3 variants were made and transfected into COS-7 cells. A variant missing the signal peptide was made by removing 120 nucleotides from the beginning of FGFR-3 so that translation would start at the second ATG at codon 69, resulting in an almost complete FGFR-3, which would lack the signal peptide. A second variant was made that contained the usual N terminus of FGFR-3 but in which the observed transmembrane deletion was included. A third variant combined the lack of signal peptide with the transmembrane deletion. All three variants together with full-length FGFR-3 were transiently expressed, under an SV40 promoter, in COS-7 cells. After 7 days the cells were fixed and stained for FGFR-3 by immunofluorescence. As shown in Fig. 8, full-length FGFR-3 gave a cytoplasmic staining pattern, as did the variants that were missing either the signal peptide or the transmembrane domain, the latter presumably in secretory vesicles. The variant missing both the signal peptide and the transmembrane domain gave a staining pattern very similar to that seen in epithelial cells (Fig. 5), with FGFR-3 being present in the nucleus but being excluded from the nucleolus. Translation of this variant of FGFR-3 in breast epithelial cells may account for the observed nuclear localization.

**DISCUSSION**

We have raised specific antibodies to the second immunoglobulin-like domain of FGFR-2, FGFR-3, and FGFR-4 and

**Table 1**

| Cell line  | Anti-FGFR-1          | Anti-FGFR-2         | Anti-FGFR-3          | Anti-FGFR-4          |
|------------|----------------------|---------------------|----------------------|----------------------|
| MCF-7      | Background cytoplasmic| Cytoplasmic vesicles| Nuclear              | Cytoplasmic vesicles |
| T47D       | Background cytoplasmic| Cytoplasmic vesicles| Nuclear              | Cytoplasmic vesicles |
| MDA-MD-231 | Background cytoplasmic| Cytoplasmic vesicles| Nuclear              | Cytoplasmic vesicles |
| MCF-10A    | Background cytoplasmic| Cytoplasmic vesicles, paler stain| Nuclear + plasmalemma| Cytoplasmic vesicles, paler stain |
| HBR-SV161  | Background cytoplasmic| Cytoplasmic vesicles, paler stain| Nuclear              | Cytoplasmic vesicles |
| HBL-100    | Background cytoplasmic| Cytoplasmic vesicles| Nuclear              | Cytoplasmic vesicles |

*a MCF-7, T47D, and MDA-MB-231 are breast cancer cell lines; MCF-10A and HBR-SV161 are breast epithelial cell lines; and HBL-100 is a breast myoepithelial cell line.*

**FIG. 5.** Immunofluorescent staining of breast epithelial cells with antisera to FGFR-3. A, B, and C show staining of HBR-SV161 cells, while D, E, and F show staining of MCF-7 cells. A and D are probed with purified antisera against the second immunoglobulin domain of FGFR-3. B and E are probed with the same antibody but after preincubation with the glutathione S-transferase fusion protein to which it was raised; C and F are probed with a second purified antiserum against the C-terminal tail of FGFR-3 (Santa Cruz).

Molecular Structure of FGFR-3 in Breast Epithelial Cells—The exclusion of full-length transfected FGFR-3 from the nucleus of COS-7 cells suggests that a different form of FGFR-3 may be responsible for the nuclear staining pattern observed in the nucleus. The presence of a 110-kDa form of FGFR-3 in the nuclear fraction of epithelial cells supports this idea, so we were interested in detecting a splice variant of FGFR-3 that might account for our results. Reverse transcriptase-PCR was performed on RNA purified from a range of nonmalignant and malignant breast epithelial cells. Using primers toward the N terminus of FGFR-3, we were unable to detect variant forms; however, using primers that would amplify across the transmembrane domain, we found a previously undescribed form of FGFR-3 (Fig. 7A). The full-length receptor would be expected to give a 598-base pair fragment; however we detected a smaller fragment of 262-base pair as well as the expected fragment (Fig. 7C). DNA sequencing of the smaller fragment showed that it encoded a form of FGFR-3 with two exons deleted (Fig. 7B). Such an mRNA would produce FGFR-3 missing half of the final immunoglobulin-like domain and the transmembrane domain. No frameshift would occur, so the kinase and carboxyl-terminal tail would be intact. Such a protein would no longer be attached to membranes and would be either intracellular or secreted, depending on whether a signal peptide was translated. The exon-deleted form of FGFR-3 appears to be present in significant quantities in breast epithelial cells at the mRNA and protein level. In HBR-SV161 (nonmalignant breast epithelial) cells there are equal quantities of the two forms, and in MCF-10A (nonmalignant breast epithelial) cells the exon deleted form is the more abundant.

An FGFR-3 Variant without a Signal Peptide and Transmembrane Domain Gives an Identical, Nuclear Localization to That Seen in Breast Epithelial Cells—In order to investigate whether the exon-deleted form of FGFR-3 could account for the nuclear localization seen in epithelial cells, a series of FGFR-3 variants were made and transfected into COS-7 cells. A variant missing the signal peptide was made by removing 120 nucleotides from the beginning of FGFR-3 so that translation would start at the second ATG at codon 69, resulting in an almost complete FGFR-3, which would lack the signal peptide. A second variant was made that contained the usual N terminus of FGFR-3 but in which the observed transmembrane deletion was included. A third variant combined the lack of signal peptide with the transmembrane deletion. All three variants together with full-length FGFR-3 were transiently expressed, under an SV40 promoter, in COS-7 cells. After 7 days the cells were fixed and stained for FGFR-3 by immunofluorescence. As shown in Fig. 8, full-length FGFR-3 gave a cytoplasmic staining pattern, as did the variants that were missing either the signal peptide or the transmembrane domain, the latter presumably in secretory vesicles. The variant missing both the signal peptide and the transmembrane domain gave a staining pattern very similar to that seen in epithelial cells (Fig. 5), with FGFR-3 being present in the nucleus but being excluded from the nucleolus. Translation of this variant of FGFR-3 in breast epithelial cells may account for the observed nuclear localization.

**FIG. 5.** Immunofluorescent staining of breast epithelial cells with antisera to FGFR-3. A, B, and C show staining of HBR-SV161 cells, while D, E, and F show staining of MCF-7 cells. A and D are probed with purified antisera against the second immunoglobulin domain of FGFR-3. B and E are probed with the same antibody but after preincubation with the glutathione S-transferase fusion protein to which it was raised; C and F are probed with a second purified antiserum against the C-terminal tail of FGFR-3 (Santa Cruz).
have used these to examine the intracellular localization of FGFRs in normal breast epithelial cells and breast cancer cells. All the antibodies used were able to recognize native, glycosylated FGF receptors as seen by their ability to immunofluorescently stain transfected COS-7 cells. When used to stain breast cancer cells, the anti-FGFR-1 monoclonal gave levels of staining similar to background. However, Western analysis using the same antibody was able to detect FGFR-1 in these cells. These results indicate that FGFR-1 is present in breast epithelial cells but at relatively low levels at which the monoclonal antibody has difficulty at detecting it by immunofluorescence. Higher levels of FGFR-2, FGFR-3, and FGFR-4 were seen in immunofluorescence experiments. These results are consistent with reports of the amount of FGFR mRNA present in breast cancer cells where FGFR-3 and FGFR-4 could be detected easily by Northern analysis, whereas FGFR-2 and FGFR-1 could barely be detected (Lehtola et al., 1992; Ron et al., 1993). We notice higher expression of FGFR-4 and FGFR-2 in breast cancer cell lines than in normal breast epithelial cells. The higher levels of FGFR-4 expression are consistent with the report of FGFR-4 and FGFR-2 gene amplification in breast cancers (Jaakkola et al., 1993; Adnane et al., 1991).

The localization of most of the receptors was as expected with FGFR-1, FGFR-2, and FGFR-4 giving a cytoplasmic vesicle staining pattern consistent with internalized membranes and associating with the membrane rather than the nuclear fraction of fractionated cells. However, by both of these tests, FGFR-3 appears to be associated with the nucleus. Anti-FGFR-3 antisera give a blockable staining pattern in which it is excluded from the nucleolus but present throughout the rest of the nucleus. Confirmatory experiments showed that a second antibody against a separate region of FGFR-3 gave an identical staining pattern. This experiment also shows that the FGFR-3 isoform being detected will contain both the growth factor binding domain and the C terminus of the receptor, so it will have a complete tyrosine kinase domain as well as an antibody binding domain. The staining pattern seen suggests that much of the nuclear FGFR-3 may be soluble since confocal microscopy showed FGFR-3 staining evenly distributed throughout the nucleus rather than concentrated around the edge. Several nonspecific bands appear on Western blots probed with antibodies against FGFR-3. However, we do not believe that these are responsible for the nuclear staining pattern seen in epithelial cells since untransfected COS-7 cells gave pale, background immunofluorescent staining patterns with both cytoplasmic and nuclear components and failed to give the clear nuclear stain seen in breast epithelial cells, which expressed higher levels of FGFR-3. In addition, we expect FGFR-3 to be present in breast epithelial cells due to our Western data (Fig. 6) and reports of FGFR-3 mRNA in breast epithelial cells (Lehtola et al. 1992; McLeskey et al., 1994). Two forms of FGFR-3 are
expressed in breast epithelial cells as seen by immunoblotting of fractionated cells. These correspond to the full-length 135-kDa form and a smaller form of 110 kDa. Of these the 110-kDa form is predominantly associated with the nucleus of fractionated cells. The 135-kDa form was found in different compartments when different batches of cells were used. This could reflect differences in the stimulatory state of those cells. There is evidence that FGF2 expression is regulated by cell density (Bost and Hjelmeland, 1993), so autocrine stimulation by FGFs may differ in the different cell batches studied. There is evidence for FGFR-1 being translocated to a perinuclear localization upon interaction with its ligand during G1 period of the NIH 3T3 cell cycle (Prudovsky et al., 1994). In the case of the 135-kDa form of FGFR-3, the nuclear and cytoplasmic localizations seen on Western blots of fractionated cells could reflect differences of localization caused by growth factor stimulation. However, such a migration to a perinuclear position would not give the staining pattern throughout the nucleus that we have observed in epithelial cells but would give a perinuclear staining pattern (Prudovsky et al., 1994).

COS-7 cells transfected with the full-length FGFR-3 cDNA under an SV40 promoter gave a membrane staining pattern rather than the nuclear staining pattern seen in breast epithelial cells. There are several possible explanations for this discrepancy: (a) the COS cell transfection system leads to overexpression of proteins and the quantities of FGFR-3 produced are too high for correct localization; (b) only the full-length form of FGFR-3 is being expressed, whereas the 110-kDa form may be giving the nuclear localization in epithelial cells; or (c) there may be differences in growth factor production in the different cell types leading to translocation of the receptor. We have attempted to distinguish between these possibilities and find that growth factor stimulation is unlikely to account for differences in localization. Expression of an alternative variant of FGFR-3 is more likely to account for differences in localization since a smaller form of FGFR-3 was seen in the nucleus of breast epithelial cells. We used reverse transcriptase-PCR to amplify mRNAs transcribed from the FGFR-3 gene and detected an exon-deleted form of FGFR-3 that would lack the second half of the third immunoglobulin domain and the transmembrane domain. This splice variant of FGFR-3 would have the usual kinase domain and carboxyl-terminal tail, since no frameshift occurs as a consequence of the deletion. It would be missing 12 kDa of encoded protein sequence, including two N-linked glycosylation sites, which would account for much of the observed size decrease (Fig. 6). In addition, the loss of the transmembrane domain explains the soluble appearance of FGFR-3. It is not localized exclusively on the nuclear membrane but appears to be distributed throughout the nucleus. This would require the use of an alternative ATG codon at either codon 69 or codon 159 of FGFR-3. This would generate an intracellular rather than a secreted form of FGFR-3, which would be more consistent with our findings of 110-kDa FGFR-3 within the nucleus. Transfection of such a construct into COS-7 cells gave the nuclear staining pattern observed in epithelial cells, so this variant is a good candidate for the nuclear FGFR-3. In the case of FGFR-1, variants lacking a signal peptide and predicted to have an intracellular localization have been described (Jaye et al., 1992). The deletion of exons 7 and 8 represents a new splice variant of FGFR-3. FGFR-3 has been studied less fully than several other FGFRs, and the only splice variants described to date have used alternative forms of exon III (Avivi et al., 1993; Chellaiah et al., 1994).

The function of a nuclear FGF receptor can only be speculated on at present; however, both FGFR1 and FGFR2 have been found in the nucleus and probably have functions within it (Gualandris et al., 1993; Wiedlocha et al., 1994). FGFR-3 is able to bind both FGFR1 and FGFR2 (with lower affinity), so it could have a role in the nuclear function of both these growth factors (Chellaiah et al., 1994). A nuclear FGF receptor could have a role either in the transport of FGF to the nucleus or in the storage of FGF in an inactive form within the nucleus. Alternatively, it could have a more active role in mediating the nuclear function of FGF. The presence of a complete tyrosine kinase domain opens the question of whether the activated receptor can phosphorylate nuclear targets. Further experimentation will be required before a functional role for FGFR-3 in the nucleus of epithelial cells can be determined.

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FGF Receptor Localization
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