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A Soluble Dominant Negative Fibroblast Growth Factor Receptor 4 Isoform in Human MCF-7 Breast Cancer Cells

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Fibroblast growth factor receptors (FGFRs) are receptor tyrosine kinases encoded by four closely related genes. FGFR 1, 2, and 3 have a number of isoforms derived by alternative splicing, alternative initiation and exon switching; however, FGFR4 has been reported to encode a single intact receptor with three extracellular immunoglobulin (Ig)-like domains, a transmembrane domain, and a split intracellular kinase. Here we describe a novel C-terminally truncated soluble isoform of FGFR4 expressed by human epithelial breast cancer MCF-7 cells. This isoform results from failure of splicing of intron 4 resulting in an mRNA species that encodes an in-frame premature stop codon. Cells transfected with the corresponding cDNA containing intron 4 express a truncated releasable protein that is identified in conditioned media. This soluble FGFR4 isoform (sFGFR4) abrogates the effect of FGF-1-induced MAPK phosphorylation and PRL gene activation. These findings represent the first description of an endogenous soluble C-terminally truncated FGFR4 isoform with FGF modulatory properties.

Key Words: **fibroblast growth factor receptor-4 isoforms; soluble dominant negative receptors; breast cancer; FGF.**

Fibroblast growth factor (FGF) signaling is mediated through one of 4 mammalian FGF receptor (FGFR) genes encoding a complex family of transmembrane receptor tyrosine kinases (1). Each prototypic receptor is composed of 3 immunoglobulin (Ig)-like extracellular domains, a single transmembrane domain, a split tyrosine kinase, and a COOH-terminal tail with multiple phosphorylation sites (1). Multiple forms of cell-bound or secreted forms of FGFR1, 2 and 3 have been characterized; these are generated by the same gene using alternative initiation sites, alternative splicing, exon switching, or variable polyadenylation (2, 3). FGFR4 has been reported to be expressed mainly outside the brain and nervous system, in adrenal, heart, lung, kidney, pancreas, muscle, and spleen (1, 4, 5). Whereas FGFR1, 2 and 3 are known to have multiple isoforms, FGFR4 was thought to be unique in that until only recently the intact full-length receptor was believed to be the sole isoform. We report here a soluble FGFR4 isoform that results from failure of splicing of intron 4, leading to an mRNA species with an in-frame premature stop codon. We demonstrate that this C-terminally truncated receptor isoform, that includes the signal peptide, the first Ig-like domain and acid box, is released in conditioned media and can functionally modulate FGF action.

MATERIALS AND METHODS

Cell culture. The MCF-7 human epithelial breast cancer cell line and the embryonic kidney HEK 293 cell line were propagated in DME with 10% FCS. The rat pituitary prolactin-producing PRL 235 cell line was cultured in Ham's F10 medium supplemented with 12.5% horse serum and 2.5% fetal calf serum with antibiotics.

Messenger RNA analysis by reverse transcription-PCR (RT-PCR). Total RNA was extracted by the guanidinium isothiocyanate method. One μg of DNase-treated RNA was used for reverse transcription. This was performed using 2.5 U/ml of Murine Leukemia Virus reverse transcriptase, 2.5 mM $MgCl_2$, 1 mM dNTP, 2.5 mM random hexamers, and 1 unit/ml of RNase inhibitor. The integrity of RNA from each sample was assessed by amplification of the PGK housekeeping gene as previously described (6). PCR analyses for FGFR4 were performed to examine FGFR4 expression and utilized several primer sets that were designed to span at least one intron (see Fig. 1) to specifically permit the exclusion of genomic DNA contamination. The identity of all PCR products was confirmed by Southern blotting hybridization and by sequencing.

Rapid amplification of complementary DNA (cDNA) ends (RACE). First strand cDNA was generated by RT from total RNA in the presence of the sense SMART II (Clontech Laboratories, Palo Alto, CA) oligonucleotide primer (5′-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3′) and the 5′-RACE cDNA synthesis primer (5′-CDS, Clonetech) followed by polymerase chain reaction (PCR) using sense

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primer (5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTAACA-ACGCAGAGT-3′) and anti-sense primer in exon 7 of FGFR4 (5′-GGCTCCGAAGCTGCTGCCGTTGATGAC-3′). PCR products were further amplified with an abridged internal universal amplification primer (5′-AAGCAGTGGTAACAACGCAGAGT-3′) and a nested antisense primer from exon 6 of FGFR4 (5′-CACAGCGTTCT-CTACCAGGC-3′) under the following conditions: 94°C for 20 s, 68°C for 30 s, and 72°C for 5 min. The PCR-generated products were cloned into the pCR-II vector as directed by the manufacturer (In-Vitrogen, Carlsbad, CA). Cloned inserts containing FGFR4 by Southern blot analysis were sequenced.

Plasmid constructs. Plasmids containing the corresponding region of human sFGFR4 cDNA were prepared by PCR derived from the 5′RACE products on MCF-7 cells and subcloned into pcDNA3.1/V5/His/Topo (InVitrogen). Full-length human FGFR3 and FGFR4 expression vectors were kindly provided by J. Henderson, McGill University, and J. Partanen, University of Toronto, respectively. The soluble chimeric dominant negative construct (KFGR-HFc; also referred to as dnFGFR in this report) represents a cDNA encoding individual immunoglobulin-like domains of the keratinocyte growth factor (KGF/FGF-7) receptor fused to the mouse immunoglobulin heavy chain Fc domain (HFc); this was kindly provided by Dr. W. LaRochelle (7). All plasmid inserts were sequenced to confirm sequence fidelity.

Western blot analysis. Protein concentrations were determined by the Bio-Rad method. Equal amounts of cell lysate protein (50 μg) were solublized in 2× SDS-sample buffer and separated on 8% SDS–polyacrylamide gels and transferred to nitrocellulose. Expression of FGFR3 or FGFR4 was detected using polyclonal affinity–purified rabbit antiserum directed against the carboxy terminus of human FGFR3 and FGFR4 (Santa Cruz). Expression of KFGR-HFc was detected by a goat anti-mouse IgG1 antibody (Sigma). Immunoprecipitation and immunoblotting with a monoclonal anti-His antibody (InVitrogen) was used to detect His-tagged-sFGFR4. To determine possible glycosylation, protein lysates were subjected to *N*-glycosidase F treatment according to the manufacturer's protocol (Boehringer Mannheim). Total and phosphorylated MAPK were detected by specific antisera (New England Biolabs) respectively. Protein loading was measured by detection of actin (Sigma). Protein bands were visualized using chemiluminescence (Amersham, Oakville, Ontario, Canada) and band intensities quantified by densitometry.

FGF stimulation. The effect of FGF on prolactin (PRL) promoter activity represents a well-characterized robust assay of FGF action in the pituitary (8, 9). We took advantage of this FGF effect to determine a potential functional role for sFGFR4 in modulating FGF action. Specifically, we examined the effect of FGF-1 on PRL promoter activity in PRL 235 pituitary cells in the absence or presence of 2 ml of serum-free conditioned media (SFCM) from HEK 293 cells transfected with sFGFR4. Media from the same cells transfected with empty vector served as controls. Stimulation of PRL by FGF-1 was analyzed in PRL 235 cells grown in 6-multiwell microtiter plates (5×10^4 cells/well), pre-incubated for 48 h in serum-free defined media (insulin [5 μg/ml], transferrin [5 μg/ml]), then treated with and without FGF-1 (Sigma, 50 ng/ml) and 10 U/ml of heparin in serum free medium for 24 h at 37°C. We selected FGF-1 as this FGF ligand has been previously shown to bind FGFR1-4 (10). PRL promoter activity was analyzed with reporter constructs pSV2A-rPRL-luc containing the 422-bp fragment of the rPRL promoter (kindly provided by H. Elsholtz, Toronto). To normalize for transfection efficiency variation within and between all experiments, 20 ng/well of pSV-β-Galactoside control vector (Promega, Madison WI) was included with each transfection. The results were normalized to β-galactosidase activity.

Statistical analyses. Data are expressed as mean ± SEM. Differences were examined by one-way ANOVA or Student's *t* test both with significance level of <0.05.

RESULTS

MCF-7 Cells Express a Novel FGFR4 Isoform Which Fails to Splice Intron 4

To characterize FGFR4 expression in MCF-7 cells, primers were designed to identify by RT-PCR the secretable first Ig-like domain with its signal peptide (I_1, exons 2–3) the second Ig-like domain (I_2, exons 5–6) and the third Ig-like loop (I_3) with the transmembrane and kinase domains (K) (exons 6–11) of FGFR4 (Fig. 1). We identified expression of all domains of FGFR4 in MCF-7 cells (11). However, RT-PCR of exons 2–11 identified the predicted 1500-bp mRNA encoding the full-length FGFR4 as well as a product that migrated at approximately l00 bp larger than the expected species.

To precisely define the 5′ terminus of MCF-7-derived FGFR4 cDNA and to characterize potential insertions responsible for a larger FGFR4 isoform in MCF-7 cells, we adopted a rapid amplification of cDNA ends (RACE) approach. RACE-derived products were cloned into TA vectors and screened by hybridization with full-length FGFR4 cDNA. At least 10 clones from each RACE-derived cDNA sample were examined. Multiple clones contained intron 4 sequence; there were no intronic sequences other than intron 4 identified, thus excluding the possibility of genomic DNA contamination. All exon-intron boundaries conformed to the GT/AG rule. Sequence analysis of these clones revealed that transcription initiation occurs in exon 1 of the human FGFR4 cDNA. Intron 4 analysis revealed an in-frame TAG stop codon at nucleotides 5061–5063 (Fig. 1c). The presence of this FGFR4 isoform in MCF-7 cells was further verified by independently performed RT-PCRs using primers flanking intron 4. Specifically, primers spanning exon 2 through 6 revealed the expected species as well as a larger product of 110 bp more than the expected, consistent with inclusion of only intron 4 but not intron 3 (Fig. 1d). The fidelity of this product to FGFR4 was confirmed by sequencing.

These analyses indicated the presence of an mRNA species derived from failure of splicing of intron 4, which includes an in-frame TAG. This premature stop codon would be expected to yield a truncated soluble-FGFR4 isoform that contains only the signal peptide, the first Ig-like domain, and the acid box. We have named this putative MCF-7 breast cancer-derived soluble-FGFR4 isoform (sFGFR4).

sFGFR4 Is a Soluble Isoform of FGFR4

To characterize sFGFR4 protein, we transfected HEK 293 cells with the corresponding sFGFR4 sequence, wild type full length FGFR4, or empty vector. As a control for interference with FGFR action, we used a potent engineered chimeric dominant negative

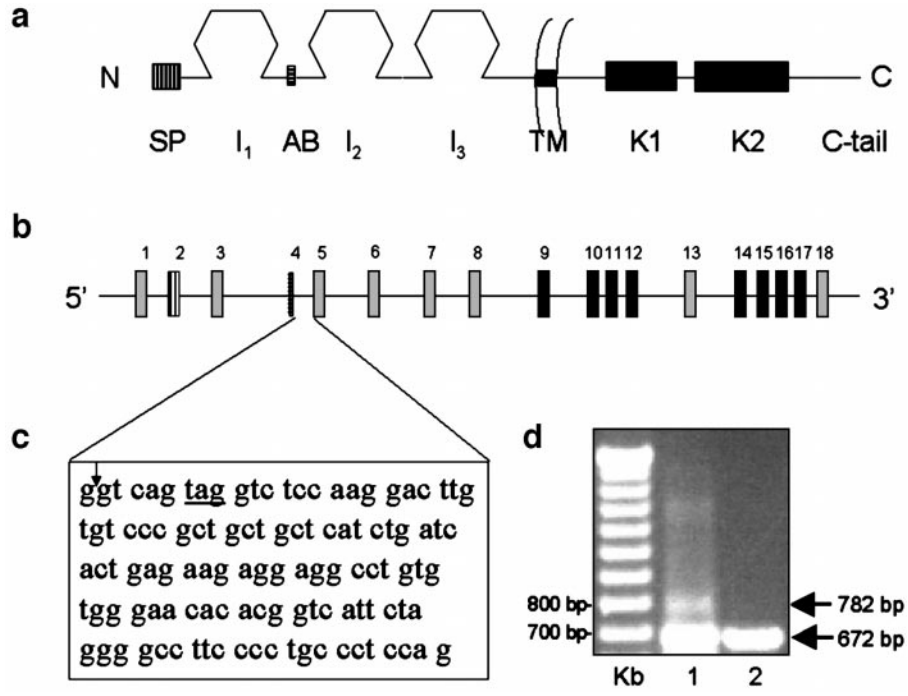

FIG. 1. FGFR4 protein and gene structure and RNA analysis. (a) The FGFR4 protein is a transmembrane tyrosine kinase receptor with three extracellular immunoglobulin (Ig)-like loops. (b) The FGFR4 gene contains 18 exons numbered above the line. Exon 1 is untranslated. Exon 2 encodes the signal peptide (SP). Exon 3 encodes the first Ig-like domain (I_1). Exon 4 encodes an acid box. Exons 5 and 6 encode the second Ig-like domain (I_2). Exons 7 and 8 encode the third Ig-like domain (I_3). Exon 9 encodes the transmembrane domain (TM). Exons 10–12 and 14–17 encode the split kinase (K1, K2). (c) 5′ rapid amplification of cDNA ends (RACE) from MCF-7 RNA yielded products that included intron 4 sequence. The intron 4 sequence is shown with the putative in-frame TAG *stop codon* underlined. The arrow indicates the exon 4/intron 4 junction. (d) RNA extracted from MCF-7 cells was further examined by reverse transcribed (RT)-PCR using primers spanning exons 2 through 6. The expected 672-bp product (bottom arrow) is clearly demonstrated MCF-7 cells (lane 1) as well as in HEK 293 cells transfected with full-length FGFR4 as controls (lane 2). An additional 110-bp larger product (top arrow) is noted in the MCF-7 cells (lane 1) consistent with failure of splicing of intron 4. The integrity of RNA and adequacy of the RT reaction of all samples was documented by the presence of RNA for the housekeeping gene PGK-1 (not shown). The size of PCR products was determined by comparison with size markers (kb) and the identity of all PCR products was confirmed by sequencing.

FGFR construct (7). As the first Ig-like domain is highly conserved among all FGFRs (1), antibodies directed against this region of FGFR4 cannot readily distinguish between the different members of the FGFR family. To overcome this problem, we used a tagged expression vector system for expression of sFGFR4. We immunoprecipitated cell lysates and corresponding SFCM from sFGFR4-transfected cells and immunoblotted the resultant complexes with anti-His antibody. Whole cell lysates from sFGFR4-, FGFR4-, and empty vector-transfected cells yielded no signals recognizable by the anti-His antibody (data not shown). In contrast, SFCM from sFGFR4-transfected HEK 293 cells yielded a protein product which migrated at ~32 kDa size (Fig. 2a). Pretreatment of this protein with *N*-glycosidase F resulted in a decrease to ~14 kDa consistent with the known N-linked glycosylation of the first Ig-like domain of FGFR4 (12). SFCM from HEK 293 cells transfected with dnFGFR yielded the expected chimeric protein of the expected 80 kDa size (Fig. 2b).

FGFR4 Antagonizes FGFR Activation

Multiple lines of evidence support the importance of MAPK in mediating FGFR4 signaling to the nucleus

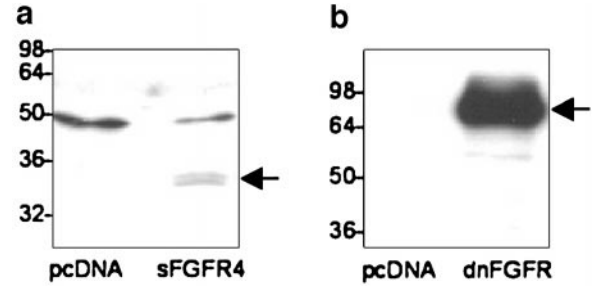

FIG. 2. Characterization of sFGFR4 protein in conditioned media. (a) Immunoprecipitation with anti-His of protein from serum-free conditioned media (SFCM) of HEK 293 cells transfected with sFGFR4 identifies a protein of ~34 kDa not present in cells transfected with empty vector (pcDNA). (b) Immunoprecipitation with anti-IgG1 of SFCM from cells transfected with dnFGFR identifies a product of ~80 kDa consistent with the chimeric protein composed of the ligand binding domain and HFc.

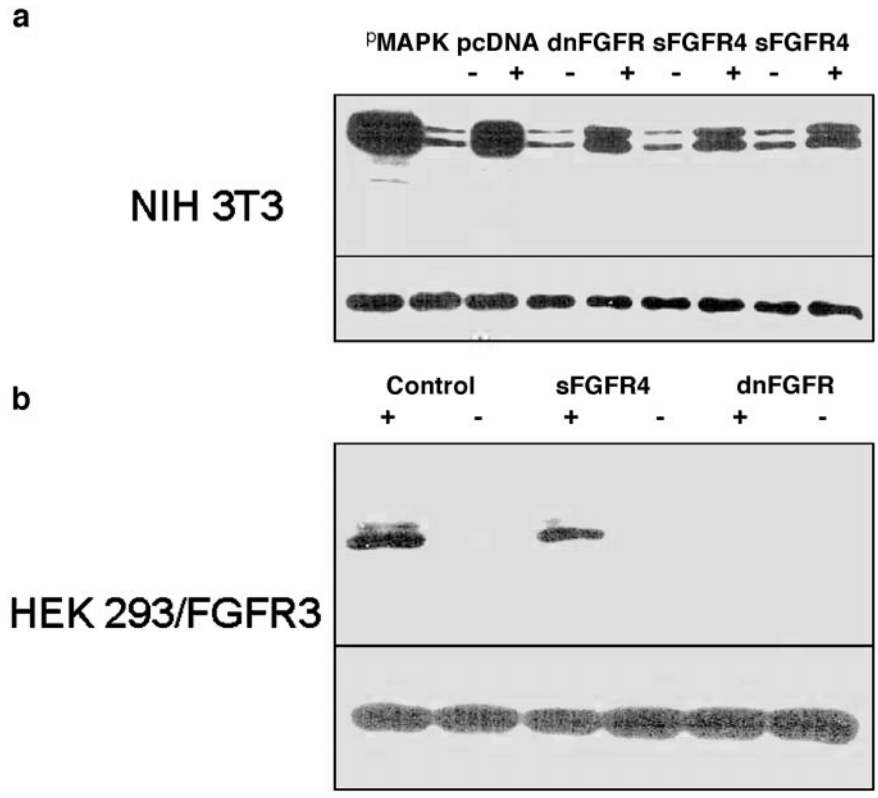

FIG. 3. sFGFR4 Modulates FGF-1-induced MAPK activation. (a) NIH 3T3 cells treated without (−) or with (+) FGF-1 (50 ng/ml) for 30 min 24 h after transfection with empty vector (pcDNA), dnFGFR or sFGFR4 as illustrated. The upper panel demonstrates the phospho-specific MAPK activity with positive lysate control (^{P}MAPK). The lower panel depicts total MAPK levels. Note that sFGFR4-transfected cells demonstrate attenuated MAPK stimulation similar to that seen in dnFGFR-transfected cells. (b) HEK 293 cells were transiently transfected with FGFR3 and treated 24 h later with FGF-1 in the presence or absence of 2 ml of SFCM from cells transfected with either sFGFR4 or dnFGFR as indicated. Note that FGF-1 treatment results in brisk MAPK phosphorylation in control FGFR3-transfected control cells. The presence of sFGFR4 reduces basal as well as FGF-1-induced MAPK activation. More complete inhibition of MAPK is noted in the presence of dnFGFR-containing media, consistent with the much higher levels of dnFGFR (see Fig. 2). Total MAPK levels, shown immediately below in the lower panel, are unaffected by treatments.

(13). We thus examined the effect of FGF-1 on MAPK in the presence and absence of sFGFR4. To address this question, FGF-1-induced MAPK activation was investigated in NIH 3T3 cells endogenously expressing FGFR4, or in HEK 293 cells transfected with FGFR3. In NIH 3T3 cells, transfection of sFGFR4 or dnFGFR markedly abrogated MAPK phosphorylation compared with control FGF-1 treated cells (Fig. 3a). In HEK 293 cells transiently transfected with FGFR3, the addition of SFCM enriched with sFGFR4 resulted in some attenuation of basal as well as FGF-1-induced MAPK phosphorylation (Fig. 3b). This attenuation, however, was not as marked as that noted in the presence of SFCM enriched with dnFGFR. This difference may be in part related to the significantly higher levels of dnFGFR in SFCM (Fig. 2).

FGF activation of the PRL promoter is a robust biological assay dependent on MAPK activation (8). We therefore used this model to confirm that sFGFR4 modulates FGF-mediated MAPK activation. After stimulation with FGF-1, PRL-luciferase activity and PRL content of PRL 235 cells treated in the presence of SFCM from HEK 293 cells transfected with empty-vector increased by approximately 5-fold (Fig. 4). By comparison, the response of PRL 235 cells to the same FGF-1 treatment in the presence of SFCM from HEK 293 cells transfected with sFGFR4 was significantly attenuated by approximately 50% compared with control cells (Fig. 4).

DISCUSSION

We have identified a novel C-terminally truncated isoform of FGFR4 in the human breast cancer MCF-7 cells, and we have named this isoform sFGFR4. This novel splice variant results from failure of exclusion of intron 4 and leads to expression of a truncated receptor containing an intact signal peptide and the first Ig-like FGFR4 domain as well as the acid box. sFGFR4 is released as a soluble isoform and can be identified in the conditioned media of transfected cells. Conditioned media from cells with sFGFR4 attenuates FGF-1 sig-

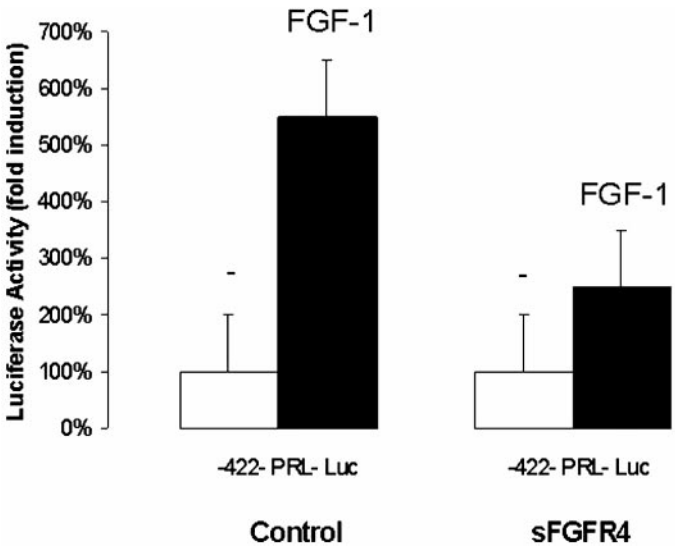

FIG. 4. sFGFR4 abrogates the effect of FGF on PRL gene activation. Pituitary PRL 235 cells were transiently cotransfected with a −422 PRL-luciferase reporter and 24 h later treated with FGF-1 (50 ng/ml) for 9 h in the presence of SFCM from empty vector (control) or sFGFR4-transfected HEK 293 cells as indicated. The effect of FGF-1 in the presence or absence of sFGFR4 is expressed as fold induction of light emission directed by the promoter activity integrated over 15 s compared with empty vector transfected cells. Each value represents the mean fold change in three wells compared with an equal number of wells of cells transfected with empty vector and all experiments were performed in triplicate. PRL-luciferase activity shows significant ($P < 0.05$) increase in response to FGF-1 stimulation. This effect was significantly abrogated in the presence of sFGFR4 enriched SFCM.

naling, an effect evidenced by abrogation of FGF-1-induced MAPK phosphorylation and prolactin promoter activation.

Whereas FGFR1, 2, and 3 are known to have multiple isoforms, FGFR4 is somewhat unique in that until relatively recently the intact full-length receptor was believed to be the sole isoform. The genomic structure of FGFR4 is composed of 18 instead of 19 exons found in the other FGFRs (14). The additional exon in other FGFRs is located between exons 8 and 9 of FGFR4 and by alternative splicing, encodes isoforms of the third Ig-like domain. Although FGFR4 has no reported third Ig-like domain variants, several other isoforms have recently been described. Human intestinal epithelial cells have been shown to express an FGFR4 transcript where exon 9 is displaced by intron 9 leading to loss of the transmembrane domain (12). Alternative splicing of intron 17 in the mouse leads to a truncated FGFR4 with a shorter intracellular tail (15). The functional significance of these two FGFR4 splice variants is unknown. Our current findings represent the first report of alternative splicing leading to a soluble C-terminally truncated FGFR4 isoform (sFGFR4) lacking the transmembrane and entire intracellular domains.

Full-length FGFR4 is known to be a high affinity receptor for both acidic FGF (aFGF; FGF-1) and basic FGF (bFGF; FGF-2) (16). In Xenopus embryos, the expression of a dominant negative form of FGFR4 lacking the transmembrane and cytoplasmic domain blocks the effect of FGF-8 on neurogenesis (17). Interestingly, endogenous neurogenesis was blocked more efficiently by xFGFR-4a than a similarly designed dominant-negative FGFR-1 construct (17), consistent with the preferential dependence of FGF-8 signaling through FGFR-4. In general, truncated membrane-bound FGFRs that lack a functional tyrosine kinase domain have been shown *in vitro* to disrupt FGFR signaling of multiple receptor isoforms by competing for ligand binding and forming inactive heterodimers with endogenous FGFRs (18). *In vivo,* this dominant-negative approach has been used to show that FGFs are required for *Xenopus* gastrulation (19) and also for epidermal organization, differentiation and wound healing (20), and branching morphogenesis of the lung (21). The potency of defective membrane-bound receptors has been limited by the need to be greatly overexpressed in order to compete effectively for ligand binding with native receptors at the cell surface. To circumvent this limitation, a secreted soluble dominant-negative receptor (dnFGFR) which can bind a specific subset of FGFs extracellularly and disrupt signaling of virtually all FGFR isoforms was developed (7). In the current study, the endogenous sFGFR4 was shown to display qualitatively similar effects to those of the potent engineered chimeric dnFGFR, particularly on MAPK activation. Further studies are required however using the various FGFs, including the most selective FGFR4 ligand FGF-19 (22), to accurately characterize ligand interactions with sFGFR4. Moreover, the functional significance of the secretable first Ig-like domains of FGFR4 in primary breast cancer cells remains to be established. This isoform may act to disrupt some or all of the various FGFRs expressed in breast cancer cells (11).

FGFR expression in the breast may not always be deleterious. Immunoblotting of normal human breast identifies a 115 and shorter 106-kDa isoforms of FGFR1. The C-terminally truncated 106-kDa isoform appears to be the major form in breast fibroblasts and myoepithelial cells, whereas epithelial cells contain equal amounts of the 115- and 106-kDa forms. In contrast, breast cancer cells contain mostly the intact 115-kDa form (23). Multivariate analyses reveal that FGFR1 expression was associated with longer relapse-free survival and improved overall survival (24). Similarly two isoforms of FGFR3 have been identified with the shorter 110 kDa form predominantly found in the nucleus. Unlike full-length 135 kDa FGFR3, the 110-kDa isoform results from splicing of exons 7 and 8 with deletion of the transmembrane domain but with an intact kinase domain, which could be a soluble, intra-

cellular receptor (25). Transient transfection of individual FGFRs into cos-7 cells reveals that only FGFR4 but not FGFR-1, FGFR-2 and FGFR-3 was capable of mediating breast cancer cell membrane ruffling (26).

As with other tyrosine kinase receptors, FGFRs are activated by dimerization resulting in autophosphorylation and subsequent recruitment of intracellular signaling proteins, namely phospholipase C-γ (PLC-γ) (1, 27). A 28-amino-acid peptide containing Tyr-766 of FGFR1 has been identified as the major binding site for PLC-γ; the analogous residue is conserved in all FGFRs with Tyr-754 being the putative site in FGFR4. Activated FGFR1 and FGFR4 homodimers interact with PLC-γ (28). DNA synthesis and cell proliferation are equally induced by FGFR1 and FGFR4 (28). However, unlike FGFR1, intact FGFR4 phosphorylates and activates PLC-γ weakly and does not phosphorylate Shc (29) nor does it associate with Sos (30). More recent studies examining mutations in the activation loop of kinase domains of FGFR1, FGFR3, and FGFR4 reveal that all 3 receptor domains can equally transform NIH 3T3 cells, induce neurite outgrowth in PC12 cells, and phosphorylate Shp2, PLC-gamma, and MAPK (13). Thus, we elected to determine the potential modulatory effect of this isoform on FGFR4 and other FGFR signaling by examining downstream effects on MAPK phosphorylation. Indeed, conditioned media from sFGFR4-transfected cells effectively abrogated FGF-induced MAPK activation, consistent with an important modulatory role for this isoform in regulating FGF signaling. Our current data also point to a dominant negative FGFR4 isoform that may play a role in modulating other FGFRs, which are frequently expressed by the same cells.

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