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Expression cDNA cloning of Fibroblast Growth Factor (FGF) Receptor in Mouse Breast Cancer Cells: A Variant Form in FGF-responsive Transformed Cells

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Summary: Expression cDNA library of fibroblast growth factor (FGF)-responsive mouse breast cancer cells (SC-3) was prepared and screened using chick FGF receptor (FGF-R) cDNA as a probe. Two positive clones were isolated. Sequence analysis revealed that two clones have an identical full open reading frame. Compared with sequence data on mouse brain FGF-R, SC-3 cells were found to contain FGF-R with 12 amino acids insertion near N-terminal region. This insertion was mainly composed of hydrophobic amino acids. Additionally, the deletion of two amino acids in extracellular domain and the substitution of one amino acid in C-terminal region were identified in SC-3 cell FGF-R. Transfection of this clone into CHO cells resulted in a significant increase in basic FGF (bFGF) binding.

The growth factors belonging to FGF family have been considered to play an important role in development (1). The successful cloning of FGF-related growth factors from malignant cells strongly suggests that these growth factors exert mitogenic and angiogenic actions on cancer cells in an autocrine or paracrine fashion (2, 3). Their growth-stimulating effects require the binding to cell surface receptors. Therefore, the molecular structure of receptors for FGFs or their related peptide should be clarified to obtain the clue for their action mode. Recently, cDNAs encoding FGF-R were cloned from chick embryo (4), mouse brain (5) and NIH 3T3 cells transformed with the K-FGF

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ABBREVIATIONS: FGF, fibroblast growth factor; FGF-R, fibroblast growth factor receptor; bFGF, basic fibroblast growth factor; CHO cell, chinese hamster ovary cell; FGF-R, fibroblast growth factor receptor; K-FGF, Kaposi sarcoma fibroblast growth factor; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle medium; MEM, Eagle minimum essential medium; DCC, dextran-coated charcoal; HMB medium, Ham F-12: MEM (1:1, v/v) containing 0.1% bovine serum albumin; BSA, bovine serum albumin; SSC, 0.18M NaCl, 0.015M sodium citrate, pH 7.0; Denhardt's solution, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% BSA; SDS, sodium dodecyl sulfate; D-PBS, Dulbecco's phosphate buffered saline.

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cDNA (6). Human homologues were also isolated (7-10). However, the structure of FGF-R in cancer cells, the growth of which is critically mediated through FGF-R-dependent process, remained to be clarified.

The growth of mouse mammary tumor (Shionogi Carcinoma 115) is strictly regulated by androgen (11). The cloned cells (SC-3) derived from Shionogi carcinoma 115 have been shown to be growth-stimulated by androgen-induced FGF-like growth factor in an autocrine fashion (12). The ability of androgen or this androgen-induced FGF-like growth factor to elicit the DNA synthesis in SC-3 cells was blocked by the addition of anti-bFGF antibody in the culture medium (13). In addition, the binding of bFGF to FGF-R on SC-3 cells was effectively inhibited by this growth factor (14), suggesting that ligand-induced activation of FGF-R is a crucial step for the proliferation of SC-3 cells. Actually, SC-3 cells have been demonstrated to express the FGF-R mRNA (15). Thus, we have decided to clone cDNA encoding FGF-R present in SC-3 cells.

MATERIALS AND METHODS

Cell lines. The methods for cloning and maintaining SC-3 cells were described previously (16). Chinese hamster ovary cells (CHO cells) (clone K-1) were maintained in 10% FCS-DMEM medium.

DNA synthesis. SC-3 cells were plated onto a 96-well plate (5 x 10^3 cells/well) containing 0.15 ml MEM supplemented with 2% DCC-treated FCS. On the following day, the medium was changed to 0.15 ml HMB medium [Ham F-12:MEM (1:1, v/v) containing 0.1% BSA] in the presence or absence of various concentrations of FGFs. After 24 h-culture, the cells were pulsed with [^{3}H]thymidine for 2 h at 37° C to qualify the DNA synthesis (13).

Construction and screening of cDNA library. Total cellular RNA from 4 x 10^8 SC-3 cells was prepared by the guanidium thiocyanate/cesium trifluoroacetate method (17). Poly(A) rich RNA was isolated by passing through oligo(dT)-cellulose column twice, and the aliquot (6 μg) was used to synthesize cDNA by a method of Gubler and Hoffman (18). Bst XI adaptors were ligated. cDNA with a molecular mass of more than 1.5 kb was collected after agarose gel electrophoresis, and inserted into the Bst XI site of pcDLSRα 296 expression vector (19). The constructed cDNA was transfected into E. Coli DH5α cells (17). This procedure provided us with 6 million independent colonies. These colonies (3 x 10^4 colonies per plate) were transferred to the nitrocellulose filter and hybridized with the random primed insert of chick FGF-R cDNA (4) at 6 x 10^5 cpm/ml in 5 x SSC, 5 x Dehardt's solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA at 55° C for 18h. The filters were washed three times in 0.2 x SSC, 0.1% SDS at 55° C for 20 min, and autoradiographed at -70° C. The positive clones were subjected to second screening, and finally subcloned into M13 mp18 or mp19 and sequenced by the dideoxy chain-termination method (20).

Transfection of FGF-R cDNA into CHO cells and [^{125}I]bFGF binding assay. CHO cells (5 x 10^5/100 mm-dish) were plated. On the following day, 20 μg of FGF-R cDNA cloned into the expression vector as described above was transfected by calcium phosphate precipitation method (21). After being cultured for 24 h, the transfected cells (10^5/well) were transferred onto 24-well plate and further cultured for 24 h in 10% FCS-DMEM. These cells were incubated with various concentrations of [^{125}I]bFGF ± 100-fold excess of radioinert bFGF at 0° C for 2 h in 1ml MEM containing 20 mM HEPES (pH 7.4), 0.15% (w/v) gelatin. At the end of the incubation, the cells were washed twice with cold D-PBS and twice with 2 M NaCl in 20 mM HEPES (pH 7.4). [^{125}I]bFGF bound to high affinity receptors was extracted by three subsequent washes with 2 M NaCl in 20 mM sodium acetate (pH 4.0).

Materials. The expression vector, pcDLSRα296 was kindly supplied by Dr. K. Arai. Chick FGF-R cDNA was also supplied by Dr. L.T. Williams. Unlabeled bovine brain bFGF, and aFGF were from R & D System Inc. (Minneapolis, MN). [^{125}I]bFGF (1000 Ci/mmol) was from Amersham Japan (Tokyo, Japan). The restriction enzymes were purchased from Toyobo Co. Ltd., (Tokyo, Japan). [^{32}P]dCTP and [methy-^{3}H]thymidine were obtained from Dupon New England Nuclear (Cambridge, MA). The other reagents were of analytical grade.

RESULTS AND DISCUSSION

To confirm our previous observation that the growth of SC-3 cells is remarkably stimulated by FGF-related growth factor, the cells used in the present experiments were exposed to FGFs (Fig. 1). Both aFGF and bFGF markedly enhanced the DNA synthesis in SC-3 cells. Compared with bFGF, aFGF showed the similar mitogenic activity (aFGF, ED_{50}=130 ± 34 pg/ml; bFGF, ED_{50}=105 ± 42 pg/ml(n=5). These growth factors have been proved to be associated with FGF-R (14), strongly suggesting that it is important to clarify the molecular structure of FGF-R. By screening our expression cDNA library (1.5 x 10^6 colonies), two positive clones were isolated. Both clones had 3.8 kb cDNA. Sequence analyses of these clones revealed that the 2.5 kb open reading frame has a methionine codon approximately 0.6 kb pairs downstream from the 5' end of these cDNAs and both clones have an identical open reading frame (Fig. 2). These cDNAs encode a protein of 832 amino acids and are very closely related to one of the recently published mouse brain FGF-R (5). The SC-3 cell FGF-R was found to have the same sequences in the signal peptide, three immunoglobulin domains, eight consecutive acidic amino acid

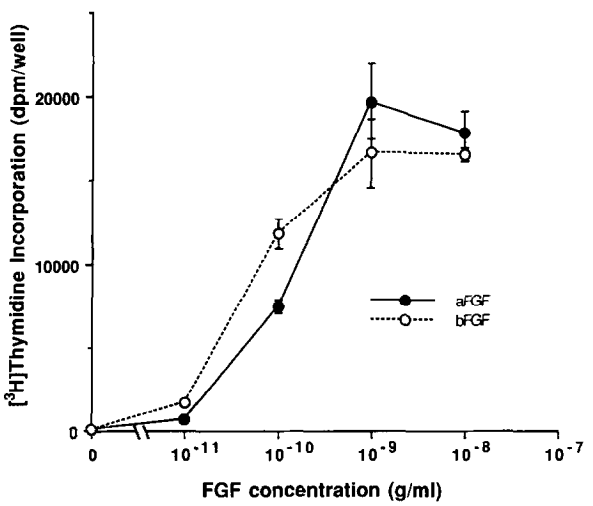

Fig. 1 *Effects of various concentrations of FGFs on DNA synthesis of SC-3 cells.*
SC-3 cells were plated and exposed to the various concentrations of aFGF(●----●) or bFGF(○——○) for 24h. DNA synthesis was then quantified as described previously(13). The data(mean±S.E.) were obtained by four replicate assays. Additional four separate experiments showed similar results.

M[1] W G W K C L L F W A V L V T A T L C T[20] A R P A P T L P E Q G S S S
ATGTGGGGCTGGAAGTGCCTCCTCTTCTGGGCTGTGCTGGTCACAGCCACTCTCTGCACTGCCAGGCCAGCCCCAACCTTGCCCGAACAAGGCTCTTCCTCC
W P L W V A[40] A A A Q P W G V P V E V E S L L V H P G[60] D L L Q L R C R
TGGCCCTTGTGGGTGGCTGCTGCAGCTCAGCCCTGGGGAGTCCCTGTGGAAGTGGAGTCTCTCCTGGTCCACCCTGGCGACCTGCTACAGCTTCGCTGTCGG
L R D D V Q S I N W L R[80] D G V Q L V E S N R T R I T G E E V E V[100] R D
CTTCGCGATGATGTGCAGAGCATCAACTGGCTGCGGGATGGGGTGCAGCTGGTGGAGAGCAACCGTACCCGCATCACAGGGGAGGAGGTGGAGGTGCGGGAC
S I P A D S G L Y A C V T S S P S G[120] S D T T Y P S V N V S D A L P S
TCCATCCCCGCTGACTCTGGCCTCTACGCTTGCGTGACCAGCAGCCCCTCTGGCAGCGATACCACCTACTTCTCCGTCAATGTCTCAGATGCACTCCCATCC
S E D D[140] D D D D D S S S E E K E T D N T K P N[160] V A P Y W T S P E K
TCGGAAGATGATGACGACGACGATGACTCCTCCTCGGAGGAGAAAGAGACGGACAACACCAAACCAAACCCTGTAGCTCCCTACTGGACATCCCCAGAGAAA
M E K K L H A V P A[180] A K T V K F K C P S S S G T P N P T L R W[200] L K N G
ATGGAGAAGAAACTGCATGCGGTGCCCGCTGCCAAGACGGTGAAGTTCAAGTGCCCGTCGAGTGGGACACCCAACCCCACTCTGCGCTGGTTGAAAAATGGC
K E F K P D H R I G G Y K V R Y[220] A T W S I I M D S V V P S D K G N Y
AAAGAGTTTAAGCCTGACCACCGAATTGGAGGCTACAAGGTTCGCTATGCCACCTGGAGCATCATAATGGATTCTGTGGTGCCTTCTGACAAGGGCAACTAC
T C[240] I V E N E Y G S I N H T Y Q L D V V E R[260] S P H R P H P S A G L P
ACCTGCATCGTGGAGAATGAGTATGGGAGCATCAACCACACCTACCAGCTTGACGTCGTGGAACGATCTCCGCACCGACCCCATCCTTCAGCAGGGCTGCCT
A N K T V A L G[280] S N V E F M C K V Y S D P Q P H I Q W L[300] K H I E V N
GCCAACAAGACAGTGGCCCTGGGCAGCAATGTGGAGTTCATGTGTAAGGTGTACAGCGATCCGCAGCCTCACATTCAGTGGCTGAAGCACATCGAGGTGAAC
G S K I G P D N L P Y V Q I[320] L K T A G V N T T D K E M E V L H L R N[340]
GGGAGTAAGATCGGGCCAGACAACTTGCCGTATGTCCAGATCCTGAAGACTGCTGGAGTTAATACCACCGACAAGGAAATGGAGGTGCTTCATCTACGGAAT
V S F E D A G E Y T C L A G N S I G L S[360] H H S A W L T V L E A L E E
GTCTCCTTTGAGGATGCGGGGGAGTATACGTGCTTGGCGGGTAACTCTATCGGACTCTCCCATCACTCTGCATGGTTGACCGTTCTGGAAGCCCTGGAAGAG
R P A V M T[380] S P L Y L E I I I Y C T G A F L I S C M[400] L G S V I I Y K
AGACCAGCTGTGATGACCTCACCGCTCTACCTGGAGATCATTATCTACTGCACCGGGGCCTTCCTGATGTCCTGCATGTTGGGCTCTGTCATCATCTATAAG
M K S G T K K S D F H S[420] Q M A V H K L A K S I P L R R Q V T V S[440] A D
ATGAAGAGCGGCACCAAGAAGAGCGACTTCCATAGCCAGATGGCTGTGCACAAGCTGGCCAAGAGCATCCCTCTGCGCAGACAGGTAACAGTGTCAGCTGAC
S S A S M N S G V L L V R P S R L S[460] S S G T P M L A G V S E Y E L P
TCCAGTGCATCCATGAACTCTGGGGTTCTCCTGGTTCGGCCCTCACGGCTCTCCTCCAGCGGGACCCCCATGCTGGCTGGAGTCTCCGAATATGAGCTCCCT
E D P R[480] W E L P R D R L V L G K P L G E G C F G[500] Q V V L A E A I G L
GAGGATCCCCGCTGGGAGCTGCCACGAGACAGACTGGTCTTAGGCAAACCACTTGGCGAGGGCTGCTTCGGGCAGGTGGTGTTGGCTGAGGCCATCGGGCTG
D K D K P N R V T K[520] V A V K M L K S D A T E K D L S D L I S[540] E M E M
GATAAGGACAAACCCAACCGTCTGACCAAAGTGGCCGTGAAGATGTTGAAGTCCGACGCAACGGAGAAGGACCTGTCGGATCTGATCTCGGAGATGGAGATG
M K M I G K H K N I I N L L G A[560] C T Q D G P L Y V I V E Y A S K G N
ATGAAAATGATTGGGAAGCACAAGAATATCATCAACCTTCTGGGAGCGTGCACACAGGATGGTCCTCTTTATGTCATTGTGGAGTACGCCTCCAAAGGCAAT
L R[580] E Y L Q A R R P P G L E Y C Y N P S H N[600] P E E Q L S S K D L V S
CTCCGGGAGTATCTACAGGCCCGGAGGCCTCCTGGGCTGGAGTACTGCTATAACCCCAGCCACAACCCCGAGGAACAGCTGTCTTCCAAAGATCTGGTATCC
C A Y Q V A R G[620] M E Y L A S K K C I H R D L A A R N V L[640] V T E D N V
TGTGCCTATCAGGTGGCTCGGGGCATGGAGTATCTTGCCTCTAAGAAGTGTATACACCGAGACCTGGCTGCTAGGAACGTCCTGGTGACCGAGGATAACGTA
M K I A D F G L A R D I H H[660] I D Y Y K K T T N G R L P V K W M A P E[680]
ATGAAGATCGCAGACTTTGGCTTAGCTCGAGACATTCATCATATCGACTACTACAAGAAAACCACCAACGGCCGGCTGCCTGTGAAGTGGATGGCCCCTGAG
A L F D R I Y T H Q S D V W S F G V L L[700] W E I F T L G G S P Y P G V
GCGTTGTTTGACCGGATCTACACACACCAGAGCGATGTGTGGTCTTTTGGAGTGCTCTTGTGGGAGATCTTCACTCTGGGTGGCTCCCCATACCCCGGTGTG
P V E E L F[720] K L L K E G H R M D K P S N C T N E L Y[740] M M M R D C W H
CCTGTGGAGGAACTTTTCAAGCTGCTGAAGGAGGGTCATCGAATGGACAAGCCCAGTAACTGTACCAATGAGCTGTACATGATGATGCGGGACTGCTGGCAT
A V P S Q R P T F K Q L[760] V E D L D R[766] I V A L T S N Q E Y L D L S[780] I P
GCAGTGCCCTCTCAGAGACCTACGTTCAAGCAGTTGGTGGAAGACCTGGACCGCATTGTGGCCTTGACCTCCAACCAGGAGTATCTGGACCTGTCCATACCG
L D Q Y S P S F P D T R S S T C S S[800] G E D S V F S H E P L P E E P C
CTGGACCAGTACTCACCCAGCTTTCCCGACACACGGAGCTCCACCTGCTCCTCAGGGGAGGACTCTGTCTTCTCTCATGAGCCGTTACCTGAGGAGCCCTGT
L P R H[820] P T Q L A N S G L K R R[832]
CTGCCTCGACACCCCACCCAGCTTGCCAACAGTGGACTCAAACGGCGCTGA

Fig. 2 Nucleotide Sequence of the open reading frame of SC-3 cell FGF-R cDNA and deduced amino acid.

Numbers above amino acids refer to the amino acid sequence. Heavily underlined region indicates the position of 12 amino acids insertion, compared with mouse brain bFGF-R sequence. The arrow head and the asterisk also indicate the position of 2 amino acid deletion and one amino acid alteration, respectively. The presumed signal peptide and transmembrane regions are also indicated by the thin underline. The eight consecutive acidic amino acid domain is also marked(∿∿∿∿).

residues, the transmembrane region and the split intracellular tyrosine kinase domain as those of mouse brain FGF-R. The most noticeable difference between two FGF-R sequences is that the SC-3 cell FGF-R contains an insertion of 12 amino acids into amino acids 31-42. This inserted sequence is mainly composed

of the hydrophobic amino acids. Two forms of the mouse brain FGF-R sequences have been reported (5). Compared with a longer FGF-R in mouse brain, a shorter one has a deletion of 89 amino acids from amino acids 31-118. This deletion has been proposed to be arisen by a different splicing mechanism (5). Interestingly, 12 amino acids insertion detected in the SC-3 cell FGF-R occurs at the splicing point proposed in the mouse brain FGF-R. Thus, one may speculate that this insertion in the SC-3 cell FGF-R is created by a splicing mechanism uniquely operating in SC-3 cells. In line with this consideration, Southern blot analyses using DNAs from SC-3 cells and NIH 3T3 cells did not show any significant difference in restriction enzyme digestion patterns (data not shown). Additionally, the identical sequences in two independent clones would eliminate the possibility that this insertion is arisen by the cloning artifact.

Two other minor differences in the SC-3 cell cDNA compared with the mouse brain are the deletion of 2 amino acids in the region between the first and second immunoglobulin-like domain, and a conversion from His (CAC) to Arg (CGC) near the C-terminal. The latter sequence was identified in the human placenta (9) and xenopus embryo (22) FGF-R.

Taking advantage of the expression cDNA library, the isolated FGF-R cDNA clone was directly transfected into CHO cells. Although CHO cells (subclone K-1) used here were found to contain some amounts of cell surface FGF-R, CHO cells transfected with FGF-R cDNA contained a 3-fold higher numbers of FGF-R without a change of the affinity toward bFGF in comparison with those in mock-transfected cells (Fig. 3). These results confirmed that expression cDNA library prepared in this study is suitable for cloning of cDNA encoding the functional proteins.

The biological significance of 12 amino acids insertion and 2 amino acid deletion identified in this study remains to be obscure. Reid et al. (5) proposed that a shorter FGF-R (two immunoglobulin domains in the extracellular domain) is a receptor for aFGF while a longer one (three immunoglobulin domains in the extracellular domain) is a receptor for bFGF. This proposal would be based upon the finding that aFGF binds with higher affinity to a receptor of 125K Da that is approximately 20K Da smaller than the bFGF-R (23). According to these considerations, FGF-R isolated from SC-3 cells is a receptor for bFGF. When SC-3 cells were stimulated with bFGF or aFGF, however, their mitogenic potency has been observed to be similar(see Fig. 1). To stimulate the proliferation of nontransformed mesoderm-derived cells, however, aFGF is 100-fold less potent than bFGF (24). These differences may be related to the unique sequence present in the SC-3 cell FGF-R. More important question is whether or not the unique sequences in the SC-3 cell FGF-R can elicit the cell transformation. To obtain the clue for this

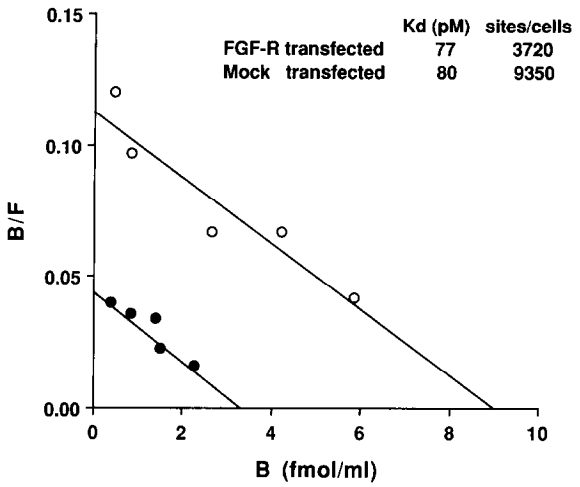

Fig. 3 Enhancement of [^{125}I]bFGF binding in CHO cells transfected with SC-3 cell FGF-R cDNA.
CHO cells were transfected with the SC-3 cell FGF-R cDNA(○——○) or vector alone(mock transfection)(●——●). [^{125}I]labeled bFGF bound to high-affinity receptor was quantified. Two additional experiments showed similar results.

important issue, more data on the structure of FGF-R in the FGF-responsive malignant cells are definitely required. Also, the transforming activity of the SC-3 cell FGF-R cDNA should be addressed. Our current studies are directed toward dissolving these interesting questions.

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