Vol. 178, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1991 Pages 8-15

Isolation from mouse fibroblasts of a cDNA encoding a new form of the fibroblast growth factor receptor (*flg*)

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Received May 22, 1991

Structural definition of the receptors for neurotropic and angiogenic modulators such as fibroblast growth factors and related polypeptides will yield insight into the mechanisms that control early development, embryogenesis, organogenesis, wound repair and neovessel formation. We isolated 3 murine cDNAs encoding different binding domains of these receptors (*flg*). Comparison of these ectoplasmic portions showed that two of the forms corresponded to previously described murine molecules whereas the third one had a different ectoplasmic portion generated by specific changes in two regions. Interestingly, expression of this third form seems to be restricted in its tissue distribution. Such modifications could influence the ligand specificity of the different receptors and/or their binding affinity. © 1991 Academic Press, Inc.

The fibroblast growth factor (FGF) family encompasses a large number of proteins that bind heparin, are angiogenic and mitogenic for many cell types of epithelial, mesenchymal and neural origin (for review, see 1, 2, 3). Like many other polypeptide growth factors, the acidic and basic FGFs (a- and b-FGFs) and probably several related proteins encoded by genes with transforming potential, including *int-2*, hst/K-FGF (Kaposi sarcoma growth factor), FGF-5, FGF-6 and KGF (Keratinocyte growth factor) (4) mediate their pleiotropic action by binding to

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Abbreviations: a-FGF, acidic fibroblast growth factor; b-FGF, basic fibroblast growth factor; bp, base pair(s); BSA, bovine serum albumin; cDNA, DNA complementary to RNA; dNTP, deoxyribonucleotide triphosphate; FGF-R, fibroblast growth factor receptor; Ig, immunoglobulin; kb, kilobase(s); kDa, kilodalton(s); nt, nucleotide(s); oligo, oligonucleotide(s); PCR, polymerase chain reaction; Pipes, 1,4-piperazine-diethanesulfonic acid; rRNA, ribosomal RNA; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na_3-citrate pH 7.6; tRNA, transfer RNA; UV, ultraviolet.

0006-291X/91 $1.50
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specific cell surface receptors (FGF-R) having an intrinsic protein tyrosine kinase activity (5) required for signal transduction and mitogenesis (6) and playing anessential role during embryogenesis (7,8).

Chick, mouse and human FGF-receptor (*flg*) cDNAs have been isolated and were shown to exhibit in their extracellular domain three repeats showing weak but significant homology with members of the immunoglobulin supergene family (9, 10, 11, 12, 13). In the mouse two different FGF-receptors (*flg*), generated by alternative splicing, have been isolated from endothelial cells (11). They have identical cytoplasmic segments but differ in their ectoplasmic portion by one Ig-like domain which is missing in one of the forms. This form with two Ig-like domains could be predominant in the human (14). Although such a difference could influence the interaction between a receptor and its ligand, it has been suggested, by analogy to the chicken FGF receptor, that both forms bind basic FGF and acid FGF (14).

We report here the isolation and characterization of three murine cDNA's encoding different FGF-R ectoplasmic segments which are probably generated at a posttranscriptional level by alternative splicing. Two forms are expressed in different tissues whereas the last one is restricted in its expression to mouse fibroblasts.

Materials and Methods

Cell culture: Mouse L cells and A431 epidermoid carcinoma cells were obtained from Dr. L. Kuhn (Swiss Cancer Research Institute) and from Dr. M. Das (University of Pennsylvania) respectively.

Enzymes and Bacterial procedures: Restriction and modification enzymes were purchased from Boehringer-Mannheim or Pharmacia and used according to supplier's protocols. Thermophilus aquaticus DNA polymerase was obtained from Cetus (Perkin Elmer). T7 DNA polymerase (Sequenase version 2.0) was purchased from United States Biochemicals.

RNAse A protection assay: 20 µg of RNA were mixed with $1x10^6$ cpm of ^{32}P-labeled antisense RNA in 30 µl of hybridization solution (80% deionized formamide, 40 mM Pipes pH 6.4, 0.4 M NaCl, 1 mM EDTA). The solution was heated at 85°C for 5 min., immediately transferred to 50°C and incubated for 12 to 16 hours. After hybridization, 0.3 ml of a solution containing 40 µg/ml of RNAse A (Boehringer Mannheim) in 300 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl pH 7.5 were added. After 90 minutes at 33°C, the digestions were terminated by the addition of 20 µl of 10% SDS and 2.5 µl proteinase K (20 mg/ml; Boehringer Mannheim) and further incubated for 15 min. at 37°C. The samples were extracted with phenol-chloroform-isoamylalcohol (25:24:1) and ethanol precipitated in presence of 10 µg of carrier tRNA. The RNAs were resuspended in 10 µl of loading buffer (90% deionized formamide, 0.02% Bromophenol blue, 0.02% Xylen cyanol, 0.089 M Tris-HCl, 0.089 M Boric acid, 0.2 mM EDTA pH 8.0) heated at 85°C for 2 min. and cooled on ice. The samples were loaded on a 6% polyacrylamide/ 7M urea gel. Alternatively the samples were loaded on an 1.5% agarose gel in 1xTBE. Autoradiography was done on XAR-5 Kodak films in presence of an intensifying screen at -70°C.

cDNA synthesis: Double stranded complementary DNA was obtained by reverse transcription using the Invitrogen cDNA synthesis kit and RNA poly A^+ purified according to standard protocols (15).

Oligonucleotides: Synthesis of the oligonucleotides was done on an Applied Biosystems Model 380 B DNA synthesizer, or obtained from British Biotechnology Ltd, Oxford. The nucleotide seqences of the oligos are the following:

GC-1: 5'-CTTGGATCCCACCATGTGGGGCTGGAAGTGCCTC-3',
GC-2: 5'-TCCGGATCCCTACTCCAGGTTAGAGCGGTGAGGT-3',
GC-3: 5'GCTCTCCACCAGCTG-3',
GC-4: 5'-GCAGLGGGCACCGCATGC-3',
GC-5: 5'-AGATCGTTCCACGACGTC-3'.

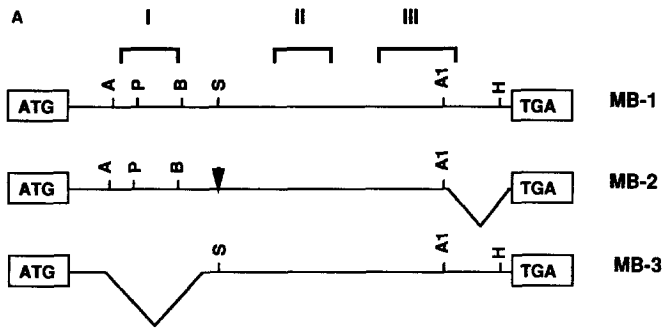
DNA sequencing: The FGF-R DNAs in pGem-1 were isolated by Bam H1 restriction digestion and inserted into M13 mp 19. Recombinant screening and preparation of the sequencing templates were done according to standard techniques (16). Sequencing of single stranded DNA was done with T7 sequenase version 2.0 (USB Biochemicals) according to supplier's protocol using the different oligonucleotides GC1 to GC-5 and the universal M13 sequencing primer (Biolabs).

Polymerase chain reaction and cloning strategy: The FGF-R cDNAs were amplified by polymerase chain reaction. The 5' oligonucleotide (GC-1, Fig. 1) is complementary to FGF-R cDNA from nucleotide 58 to 78 in the reported sequence (11) and contains at its 5' end a BamH1 restriction enzyme recognition sequence. The 3' oligonucleotide (GC-2, Fig. 1) is complementary to FGF-R cDNA (11) from nucleotide 1165 to 1185. An additional feature of this oligonucleotide is the presence of a stop codon followed by a BamH1 restriction site which allows direct cloning into the BamH1 site of different cloning vectors. The PCR amplification was performed on mouse L cell fibroblast cDNA in 20 mM Tris HCl pH 8.3, 3 mM $MgCl_2$, 100 mM KCl containing 1 μM of oligomix (GC-1 and GC-2) and 0.25 mM dNTPs. PCR reaction was done on a Techno I apparatus. The samples were heat denatured at 90°C for 8 minutes, followed by 25 cycles of incubation at 90°C, 50°C and 70°C in the presence of 2.5 units of Taq polymerase per reaction. The material was digested by BamH1 and loaded on 1% low melting temperature agarose gel. The DNA fragments, were inserted into the BamH1 site of pGem-1 vector or M13 mp19.

Results and Discussion

To isolate the cDNAs coding for the FGF-R (*flg*) ectoplasmic domain, we used PCR to amplify specific nt sequences expressed in mouse L cell fibroblasts. We based the sequences of the two amplimers on the reported sequence (11). The 5' amplimer (GC-1) contained at its 3' terminus 21 nt coding for the first amino acids of the signal peptide. The 3' amplimer (GC-2) represented 21 nt, present just prior to the transmembranous region of the protein, followed by a stop codon. Restriction enzyme mapping showed that we isolated three different cDNAs (Fig. 1A). Two of them (MB-1 and MB-3) seemed to correspond to forms already described (11), whereas the third cDNA (MB-2) could be different as suggested by its intermediate size between MB-1 and MB-3 and the absence of the Sst I and Hind II restriction sites (Fig 1A).

M13 subcloning and sequencing of the three cDNA inserts (MB-1, MB-2 and MB-3) confirmed that MB-1 and MB-3 coded for the ectoplasmic domains of the two forms of the basic fibroblast growth factor receptor (Fig.1B). The difference between the sequence MB1 and MB-3 (removal of the first Ig-like domain) is located exactly at the same position as described (11) and is generated by alternative splicing (17). It does not seem, however, to have any effect on FGF binding since it was recently reported (14) that both acidic FGF and basic FGF bind with high affinity to receptors having only two Ig-like domains suggesting a different role, if any, for the missing Ig-like domain.



B

GC-1

MB1 — MWGWKCLLFWAVLVTATLCTARPAPTLPEQAQPWGVPVEVESLLVHPGDLLQLRCRLRDD 60
MB2 — MWGWKCLLFWAVLVTATLCTARPAPTLPEQAQPWGVPVEVESLLVHPGDLLQLRCRLRDD
MB3 — MWGWKCLLFWAVLVTATLCTARPAPTLPEQ----------

GC-3

MB1 — VQS I NWLRDGVQLVESNRTR I TGEEVEVRDS I PADSGLYACVTSSPSGSDTTYFSVNVSD 120
MB2 — VQS I NWLRDGVQLVESNRTR I TGEEVEVRDS I PADSGLYACVTSSPSGSDTTYFSVNVSD
MB3 — ----------D

GC-4

MB1 — ALPS SEDDDDDDDS S SEEKETDNTKPNRRPVAPYWTSPEKMEKKLHAVPAAKTVKFKCPS 180
MB2 — ALPS SEDDDDDDDS S SEEKETDNTKPN----PVAPYWTSPEKMEKKLHAVPAAKTVKFKCPS
MB3 — ALPS SEDDDDDDDS S SEEKETDNTKPNRRPVAPYWTSPEKMEKKLHAVPAAKTVKFKCPS

MB1 — SGTPNPTLRWLKNGKEFKPDHR I GGYKVRYATWS I IMDSVVPSDKGNYTCIVENEYGSIN 240
MB2 — SGTPNPTLRWLKNGKEFKPDHR I GGYKVRYATWS I IMDSVVPSDKGNYTCIVENEYGSIN
MB3 — SGTPNPTLRWLKNGKEFKPDHR I GGYKVRYATWS I IMDSVVPSDKGNYTCIVENEYGSIN

GC-5

MB1 — HTYQLDVVERSPHRPHPSAGLPANKTVALGS NVEFMCKVYSDPQPHI QWLKH I EVNGSK 300
MB2 — HTYQLDVVERSPHRPHPSAGLPANKTVALGS NVEFMCKVYSDPQPHI QWLKH I EVNGSK
MB3 — HTYQLDVVERSPHRPHPSAGLPANKTVALGS NVEFMCKVYSDPQPHI QWLKH I EVNGSK

MB1 — IGPDNLPYVQI LKTAGVNTTDKEMEVLH LRNVSFEDAGEYTCLAGNSIGLSHHSAWLTVL 360
MB2 — IGPDNLPYVQI LKTAGVNTTDKEMEVLH LRNVSFEDAGEYTCLAGNSI----------
MB3 — IGPDNLPYVQI LKTAGVNTTDKEMEVLH LRNVSFEDAGEYTCLAGNSIGLSHHSAWLTVL

GC-2

MB1 — EALEERPAVMTSPLYLE
MB2 — - ALEERPAVMTSPLYLE
MB3 — EALEERPAVMTSPLYLE

Figure 1. cDNA structures and deduced amino acid sequences of the different mouse FGF-receptor ectoplasmic domains.
(A) Restriction maps of the three FGF-R cDNAs (MB-1, MB-2, MB-3) flanked by two boxes representing the two amplimers GC-1 and GC-2 containing the codons ATG and TGA respectively. The broken lines correspond to the deleted segments in MB-2 and in MB-3 and the inverted triangle to the two arginines at position 148 and 149. Symbols used correspond to: A, Ava II; AI, AccI; B, BglI; H, Hind II; P, Pvu II; S, Stu I; I, II, III represent the three different Ig-like domains.
(B) Amino acid sequence of the different mouse FGF-receptors. The respective positions of the oligonucleotides are shown (GC-1, GC-2, GC-3, GC-4 and GC-5). The differences between the different sequences are shown by gaps in MB-3 and MB-2.

Absence of Sst I restriction site in the third form (MB2) (Fig. 1A) led us to the conclusion that a second deletion could be present. We confirmed this change (deletion of two arginines residues 148 and 149). This alteration had already been reported (11) and could also be due to alternative splicing. The variability observed in this region is interesting. Changes localized in the same region have been found in

the human and the chicken cDNAs. In the human sequence, the two arginine residues are either missing or replaced by arginine and methionine, whereas in the chicken, one arginine is missing, the other one being substituted by a glutamine residue. The biological significance of these alterations is not known. The third form (MB-2) was then identical to MB-1 up to amino acid 348 (Fig. 1). Past this point, 39 nt were missing, generating in the protein a deletion of 13 amino acids. Both sequences were again identical up to the 3' amplimer. This difference between L cells FGF-receptors and other reported sequences present in other tissues is positioned just upstream of the transmembrane segment. Deletion of this segment removes from the protein a stretch of mainly hydrophobic amino acids localized at the limit of the third Ig like domain. No functional role, up to now, can be assigned to this region and if this deletion influences the third Ig-like domain is known. Other features such as signal peptide coding sequence and presence of 8 putative glycosylation sites were not affected.

The coding potential of the cDNA fragments we isolated was also verified using specific mRNAs which were generated by in vitro transcription of *flg* DNA (in pGem-1) and translated in rabbit reticulocyte lysate (data not shown). The 3 RNAs directed the synthesis of polypeptides of 45, 42, 33 kDa in size corresponding to FGF-Rs polypeptides predicted from the 1128, 1083 and 961 nt open reading frames in MB-1, MB-2 and MB-3 DNA fragments.

Cytoplasmic RNA isolated from mouse L cells or from a human epidermoid carcinoma cell line A431, which was reported to be negative for FGF-R expression (18), were examined by Northern blot analysis (data not shown). Expression of the FGF-R gene was confirmed by the presence of a 4.5 kb mRNA transcript in L cells but not in A431. This transcript corresponds in size to *flg* mRNA expressed in the mouse brain and kidney (11). No transcript was visible in liver or spleen cells. To confirm the existence of the MB-2 transcript in L cells we analysed cytoplasmic RNAs by an RNase A protection assay. MB-1 and MB-2 cDNAs differ at their 3' end by 39 nt which are present only in MB-1. Such a difference is detectable only by an RNase protection assay and its detection would confirm that MB-2 cDNA isolation is the result of the presence of a specific MB-2 transcript in L cells. As probe in this assay, we chose an antisense RNA containing 567 nt (Fig. 2A). A hybrid between the probe and MB-1 RNA should be resistant to RNase digestion and a protected fragment of 525 nt should be detected. If the MB-2 RNA is present, a second protected molecule of a length comprised between 439 and 478 nt due to the presence of RNase A preferential cleavage sites in the RNA loop (19) should be observed. As seen on a non denaturing gel, two bands (P1, P2) were visible when the antisense probe was hybridized to cytoplasmic RNA from L cells (Fig. 2B, lane d). No signal was observed when tRNA or A431 cytoplasmic RNA was used (Fig. 2B, lanes b and c, respectively). This protective pattern was also visible on a denaturing gel (Fig. 3, lane d) and clearly demonstrates that MB-2 RNA is present

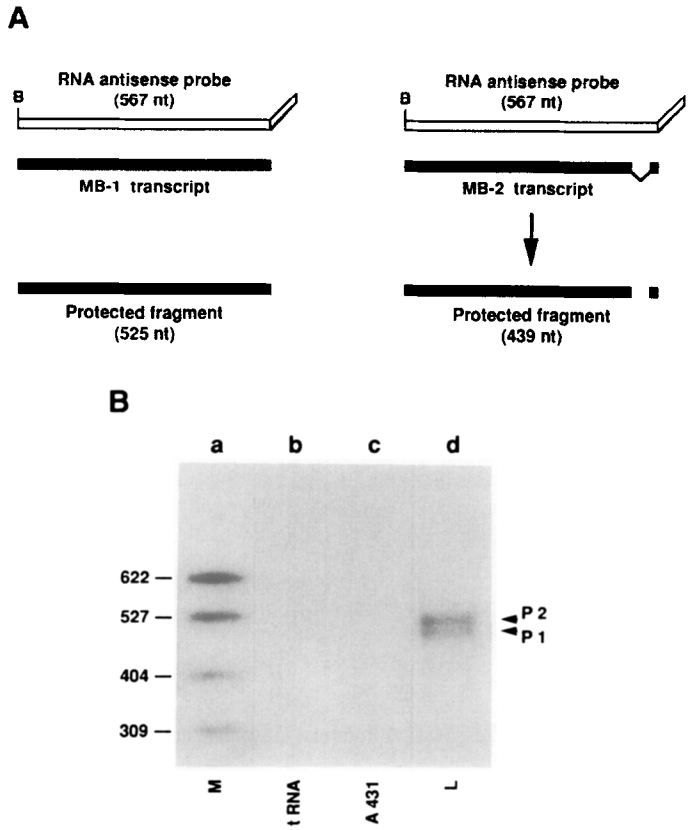


Figure 2. RNAse A protection assay on mouse L cells RNA.
(A) Schematic representation of the hybrids between MB-1 and MB-2 RNAs (open boxes) and the RNA antisense probe (dark bars) extending to the Bst X1 site (B). Sizes of the specific protected fragments are indicated in nucleotides (NT).
(B) 20 µg of cytoplasmic RNA from A431 cells (lane c), L cells (lane d) or control tRNA (lane b) were hybridized with FGF-R Bst X1 antisense probe and the RNAse A resistant fragments were analysed on a 1.5% non denaturing agarose gel in 1 x TBE. Protected fragments P1 and P2 are shown by arrows. End labeled DNA molecular weight markers correspond to pUC9 digested with Hpa II and end-labeled with T4 DNA polymerase (16).

in mouse L cells. Presence of the MB-2 transcript in L cells was also detected when another antisense probe was used in the RNase A assay (data not shown).

The RNase A protection assay was also used to analyse the presence of a similar transcript in another mouse tissue. Using the BstXI probe (Fig. 2A), two protected fragments (P1 and P2) could be seen in L cells (Fig. 3, lane d). P1 protected fragment could also be seen in expressing tissues such as brain (Fig. 3, lane e) but not in non expressing tissues (Fig. 3, lane c) or in yeast tRNA used as control (Fig. 3, lane b). Although the P2 protected fragment was heterogenous in size, which is due to presence of preferential RNase A cleavage sites, it migrates in the size range expected for a fragment protected by an MB-2 transcript. It can be observed only in L cells. No other fragment of the size of P2 could be seen in the brain (Fig. 3, lane e). This suggests that MB-2 could be restricted in its expression. We cannot,

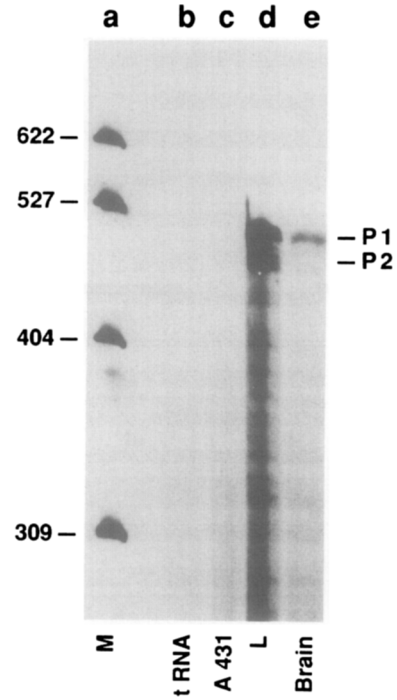


Figure 3. Comparison of MB-2 expression in mouse fibroblast and brain by RNAse A protection assay.
RNA isolated from mouse L cell fibroblast or brain has been hybridized to the same RNA antisense probe described in Fig. 2A. The samples were analysed on a 4.5% denaturing polyacrylamide gel and loaded as follows: DNA molecular weight marker in base pairs corresponding to pUC9 digested with Hpa II (lane a); control yeast tRNA (lane b); A431 RNA (lane c); mouse L cell RNA (lane d); brain RNA (lane e). Abbreviations : P1 and P2, protected fragments, shown by arrows.

however, exclude that the amount of MB-2 RNA is too low to be detected. To exclude that the MB-2 cDNA sequence is due to an allelic polymorphism present in L cells but not in mouse tissue, Southern blot analysis of mouse L cell and liver genomic DNAs for Hind II polymorphism (Fig. 1A) were performed to detect any alteration in the mouse L cell DNA. No difference between the two genomes was observed (data not shown). Taken together, our data suggest that alternative splicing plays an essential role in the generation of diverse FGF-Rs. In 3T3 fibroblasts expression of a fibroblast growth factor cDNA confers growth advantage and promotes tumorigenesis (20, 21) or autonomous cell growth in baby hamster kidney-derived cells (22). If these phenomena are linked to the presence of different forms of *flg* at the surface of these cells, they can now be investigated at the protein and at the RNA processing level. It could help in defining binding specificities and presence of specific forms of the FGF receptor (*flg*).

Acknowledgments

We thank V. Müller for providing the mouse RNAs. The work was supported by grant # 31-26320.89 from the Swiss National Foundation for Scientific Research to

C.B., grant FOR. 383.90.2, from the Swiss Cancer League to N.F. and by a senior international fellowship grant FOG TW 01557 to G.H.C. from the Forgarty International Center.

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