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A novel form of FGF receptor-3 using an alternative exon in the immunoglobulin domain III

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Four distinct FGF receptors were cloned and characterized and it was demonstrated that the ligand binding site of FGF receptors is confined to the extracellular immunoglobulin-like (Ig)-domain 2 and 3. The Ig-domain 3 is encoded by two separate exons: exon IIIa encodes the N-terminal half, and the C-terminal half is encoded by either exon IIIb or IIIc in FGFR1 and FGFR2, whereas FGFR4 is devoid of exon IIIb. Alternative usage of exons IIIb and IIIc determine the ligand binding specificity of the receptor. To analyze the arrangement of these exons in FGFR3 we cloned the genomic sequence between exon IIIa and IIIc of FGFR3 and identified an alternative exon, corresponding to exon IIIb of the FGFR1 and FGFR2. The sequence of this exon shows Ig-domain hallmarks, 44% identity with exon IIIb of other FGF receptors and 36% identity with exon IIIc of FGFR3. Using this exon as a probe for mouse RNA as well as PCR analysis, demonstrated that exon IIIb encodes an authentic form of FGFR3 that is expressed in mouse embryo, mouse skin and mouse epidermal keratinocytes. The results demonstrate that the presence of alternative exons for Ig-domain 3 is a general phenomena in FGFR1, 2 and 3, and represents a novel genetic mechanism for the generation of receptor diversity.

FGF receptor; Alternative splicing; Ligand specificity

1. INTRODUCTION

Fibroblast growth factors (FGF) constitute a family of seven heparin-binding related polypeptides which are mitogenic for a wide variety of cells of epithelial, mesenchymal and neuronal origin and are important in early embryonal development (reviewed in [1,2]). FGFs act by binding and signal transduction through cell surface receptors. Four distinct FGF receptors (FGFR1–4) were identified and cloned from various species, including *Xenopus*, chicken, mouse and human. The four FGFRs were shown to belong to the receptor tyrosine kinase family. They share a basic structure of an extracellular ligand binding region consists of three immunoglobulin (Ig)-like domains, and a cytoplasmic split tyrosine kinase domain which are connected by a single transmembrane region (reviewed in [3–5]). A unique feature of this family of receptors is the multitude of structural variants including two and three Ig-domain receptors [6,7] secreted soluble receptors [8,9] and variants with truncated cytoplasmic domain [10]. Most of these forms can be explained by alternative splicing leading to deletion or termination [3]. The complexity of different FGFRs is further increased by differential usage of two alternative exons, denoted exon IIIb and IIIc, which encode the C-terminal half of Ig-domain 3. It has been shown that in FGFR2 this alternative exon usage results in a marked change of the receptor binding specificity [11–13]. Thus, FGFR2 and keratinocyte growth factor receptor (KGFR) are two receptors derived from the same gene by alternative usage of either exon IIIb (KGFR) or IIIc (FGFR2/bek). This represents a novel genetic mechanism for the generation of receptor diversity. The FGFR4 gene was shown to lack this gene arrangement of two alternative exons for Ig-3 domain [14], and to contain only exon IIIc but FGFR-1 was shown to possess both IIIb and IIIc exons [13,15]. To further analyze the generality of this unique gene arrangement we searched for alternative exons encoding for Ig-domain 3 in FGFR3. Previously we cloned the flg-2 gene [16] which was identified later to be the mouse FGFR3 [16,17]. We report here that the FGFR3 gene contains two alternative exons (IIIb and IIIc) for the C-terminal half of Ig-domain 3. We also report the cloning and sequence of exon IIIb and its expression in mouse tissues. The implication for binding specificity of FGFR3 is discussed.

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2. MATERIALS AND METHODS

2.1. *DNA isolation, sequencing and PCR analysis*

A BALB/c mouse genomic library in EMBL4 was screened with a 170 bp *Pst*I fragment derived from FGFR3 (nucleotide) 957–1127, [16]). Positive plaques were isolated by three rounds of plating and probing. Oligonucleotides 1 (5′-CACACCGGCCCATCCTGCAGG) and 2 (5′-GAACCTCTAGCTCCTTGTCGG) derived from nucleotides 944–963 and 1144–1164 of FGFR3 [16], respectively, were used to amplify by PCR a 2 kbp fragment from positive phages. The isolated fragment was cloned in pBS (Bluescript) and sequenced by the automated DNA sequencer 373A (Applied Biosystem) using synthetic

oligonucleotides as primers and *Taq* dyedeoxy terminator cycle sequencing Kit according to the manufacturer's instructions. Oligonucleotides 3 (5'-AAGGTCCTCAAGTCCTGGATCAGTGAGAAT) and 4 (5'-GAGTATCACAGCTGCCA CC) were used to amplify by PCR a 0.4 kb fragment from mouse keratinocytes cDNA. Oligonucleotide 5 (5'-CGAAGATCTATCAGTTTCCATCAGCTCCTCCTCA-GCTGCTTGGGCCCGTG) which is located near the transmembrane region of FGFR3 (nucleotide 1295) and extends to exon IIIb was used together with oligonucleotide number 3 to amplify cDNA specific to exon IIIb from the above 0.4 kb PCR product Oligonucleotide 6 (5'-GGCGCTAACACCACCGAG) and oligonucleotide 7 (5'-TGGCAGCACCACCAGCCAC) were derived from FGFR3 sequence corresponding to exon IIIc and used as a control on the same template (see Fig 1). PCR was performed for 35 cycles of 1.5 min denaturation at 94°C, 2 min annealing at 56°C and 3 min extension at 72°C. The amplified DNA was extracted with phenol, precipitated with ethanol and analyzed by agarose gel electrophoresis followed by Southern blotting and probing with ^{32}P-labeled probes.

2.2. *RNA preparation and analysis*

Total RNA was extracted by the guanidine isothiocyanate method [18] and subjected to cDNA preparation by reserve transcriptase. For preparation of cDNA 5 mg of total RNA was incubated in 30 μl of reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 2.5 mM DTT, 10 mM $MgCl_2$), 1 mM dNTP, 15 u of RNAsin and 5 u of AMV super reverse transcriptase (Molecular Genetic resources) for 1 h at 42°C A portion of this reaction (10%) was used for the PCR reaction Hybridization to nitrocellulose filters was performed for 16 h at 42°C in 6 × SSC, 2 × Denharts' solution, 0.1 mg/ml salmon sperm DNA, 40% formamide and 0.1% SDS

3. RESULTS

3.1. *Gene structure of Ig-domain 3 of FGFR3*

In order to isolate a genomic clone containing exons encoding for the Ig-3 domain we used a 170 bp *Pst*I fragment derived from mouse FGFR3 (previously denoted flg-2 [16]) as a probe to screen a mouse gene library in EMBL4. This probe corresponds to exon IIIa, encoding the N-terminal half of Ig-domain 3. Some of the isolated clones were found to hybridize to the above probe as well as to a probe derived from exon IIIc of FGFR3. Amplification of the DNA between exons IIIa and IIIc by PCR, using primers 1 and 2 (see Section 2), yielded a 2 kbp fragment which was cloned into the *Eco*RV site of pBS. The DNA sequence of this fragment revealed the structure depicted in Fig. 1 and the partial sequence shown in Fig. 2. The only segment in the sequence between exons IIIa and IIIc which had typical Ig-domain features was found 1 kbp downstream of exon IIIa and is shown in Fig. 2. The sequence contains an open reading frame for 50 amino acids flanked by acceptor and donor splice sites in the correct orientation. It contains the sequence DXGXYXC which is typical for an Ig-domain near the second cysteine residue and this DNA segment may therefore be a candidate for exon IIIb of FGFR3.

3.2. *Expression of exon IIIb*

To determine whether the newly identified exon (IIIb) represents an authentic sequence of a new variant of FGFR3, we carried out PCR on cDNA prepared from

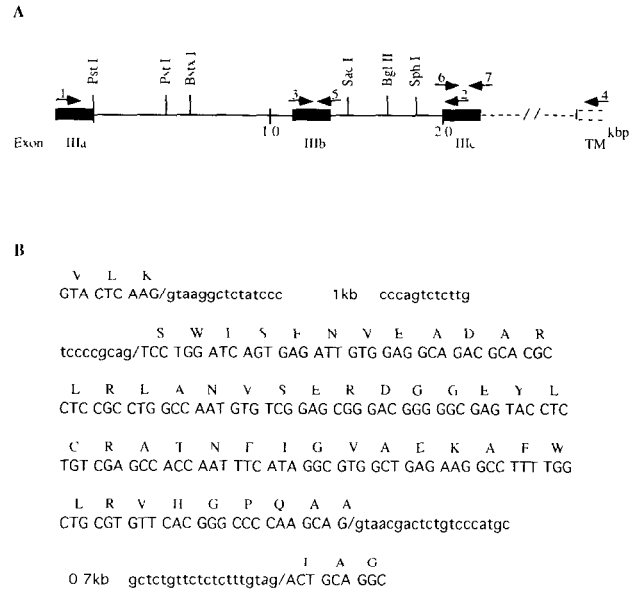

Fig. 1. Genomic structure of the murine FGFR3 domain III. (A) Physical map of the exons encoding domain III The map was derived from DNA sequence of the 2 kbp fragment described in the text The transmembrane encoded Exon TM (broken line) is shown to illustrate the position of oligonucleotide 4. Numbered arrows show the position of oligonucleotides used for PCR (see Section 2). (B) Partial sequence of the 2 kb genomic fragment showing the sequence of exon IIIb and the exon–intron junctions of exons IIIa, IIIb and IIIc. DNA sequence of the exons is shown in capitals. Deduced amino acids are shown in a single-letter code.

RNA derived from mouse epidermal keratinocytes, using primers upstream and downstream (oligo 1 and oligo 4 in Fig. 1) to the putative new open reading frame. These primers where chosen so that only amplification of authentic cDNA would yield fragments of the expected size while amplification of genomic DNA (a possible contaminant of the RNA preparation) would yield fragments containing intronic sequences. A 420 bp DNA fragment was obtained as expected from the sites of the primers. This product was then used as a template for PCR using a primer specific for the new exon (oligo 5 in Fig. 1) and a second primer derived from the N-terminal half of Ig domain III and encoded by exon IIIa in FGFR3 (oligo 1 in Fig. 1) giving rise to a 400 bp DNA fragment (Fig. 2A, lane 1). Using two primers within exon IIIb (oligos 3 and 5 in Fig. 1), the only noticeable product was a 200 bp fragment as expected from exon IIIb (Fig. 2A, lane 2). As a control, oligos 6 and 7 (Fig. 1), specific to exon IIIC, were used giving a 154 bp fragment (Fig. 2A, lane 3). Furthermore, the 400 bp product and the tentative exon IIIb PCR products, but not exon IIIc derived product, hybridized with exon IIIb specific probe (Fig. 2B, lanes 1, 2 and 3, respectively). A DNA probe containing a large fragment of the authentic FGFR3 hybridized to both the 400 bp product and to exon IIIc but not to exon IIIb

(Fig. 2C, lanes 1, 3 and 2, respectively). Hybridization with exon IIIc specific probe gave a single positive band with exon IIIc derived PCR product only (Fig. 2D, lane 3). These results clearly demonstrate that the identified new exon, IIIb, encodes an alternative carboxy terminal half of Ig-domain III of FGFR3. PCR analysis with exon IIIb specific primers gave a positive signal as determined by Southern blot when performed on cDNA derived from a 17.5-day mouse embryo and an adult mouse skin but not on cDNA derived from an undifferentiated teratocarcinoma cell line (data not shown). Subcloning and sequence analysis of the amplified 0.4 kb fragment (Fig. 2, lane 1) confirmed its identity as an authentic novel cDNA of FGFR3 as well as the predicted exon–intron borders (Fig. 1B).

4. DISCUSSION

The ligand-binding site of FGFRs was localized previously to Ig domains 2 and 3 [3–5,19]. Each of these domains is encoded by two exons, one for the N-terminal half (IIIa) and the other for the C-terminal half [3,11,15]. The C-terminal half of Ig-domain 3, containing approximately 50 amino acids, may be encoded by either of the two alternative exons IIIb or IIIc. We and others have shown previously that this confined variable region confers ligand specificity on FGFR2 [11–13] and that the ligand binding site of FGFRs is confined to Ig-domains 2 and 3 [19]. A similar arrangement of alternative exons was also described for FGFR1 [13] but not for FGFR4 [14]. In this report we provide evidence that FGFR3 also contains two alternative exons for the C-terminal half of Ig-domain 3 similar to FGFR1 and FGFR2. We searched for exon IIIb of FGFR3 by sequencing the entire DNA between exons IIIa and IIIc and we have identified a segment with Ig-domain characters flanked by consensus splice sites.

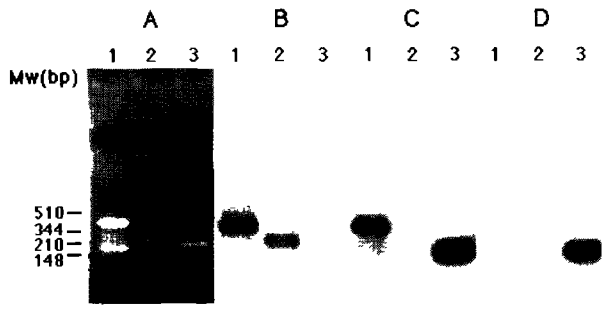

Fig. 2. PCR of exon IIIb and IIIc of FGFR3. cDNA derived from keratinocyte-RNA was used for PCR followed by gel electrophoresis and Southern blot. Oligonucleotides 1 and 5 (lane 1), 3 and 5 (lane 2) and 6 and 7 (lane 3) were used to amplify different mouse keratinocytes cDNA. (A) PCR-amplified products as seen by ethidium-bromide. (B) Southern-blot of (A) hybridized with exon IIIb specific probe. (C) Southern blot of (A) hybridized with *Eco*RI fragment (780–2880 bp) of FGFR3. (D) Southern blot of (A) hybridized with exon IIIc specific probe.

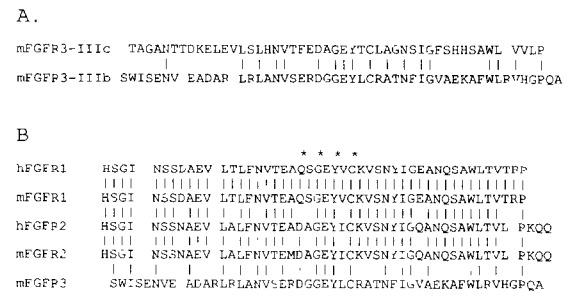

Fig. 3. Comparison of the amino acid sequences of exon IIIb of FGFR3. (A) Comparison of IIIb and IIIc from FGFR3. (B) Comparison of IIIb from various FGF receptors. Asterisks indicate the common Ig sequence DXGXYXC. Amino acids are given in the single-letter code.

Furthermore, we demonstrated that this sequences is expressed in mouse tissues. The candidate for exon IIIb showed an open reading frame of 50 codons flanked by acceptor and donor splice sites at the 5′ and 3′ respectively (Fig. 1). In Fig. 3 we compare the amino acid sequences of the known exons IIIb from FGFR1, 2 and 3. It is shown that whereas the sequence of IIIb from FGFR1 and 2 are highly homologous, the sequence of IIIb from FGFR3 is markedly different from that of FGFR1 and 2. Exon IIIb confers upon FGFR2 high affinity binding of KGF [11,12]. The sequence dissimilarity between exons IIIb and IIIc from FGFR3 (Fig. 3) suggests that exon IIIb may also confer on FGFR3, a different ligand binding specificity. It is however not very likely that this will endow FGFR3 with KGF binding because of the marked differences between the sequence of IIIb reported here and that of KGFR ([11,12], Fig. 3). In the case of FGFR2 it has been shown that the alternative use of exons IIIb and IIIc is mutually exclusive in several cell lines [12]. Furthermore, the use of exon-specific probes of either IIIb or IIIc of FGFR2 for in situ hybridization demonstrated that the tissue localization of the two FGFR2 variants differs markedly [20]. Alternative splicing between these two exons in FGFRs may therefore increase receptor diversity, determine differential tissue expression, and allow cells to respond selectively to environmental changes such as changes in available FGF ligands. Such a response may be important in developmental processes, pathological disorders or tumorigenesis. The analysis of these phenomena with FGFR3 variants described here may help further our understanding of the receptor-ligand relationship in this complex family.

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