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Differential Splicing in the Extracellular Region of Fibroblast Growth Factor Receptor 1 Generates Receptor Variants with Different Ligand-Binding Specificities

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We have cloned a genomic region of the murine fibroblast growth factor (FGF) receptor 1 (FGFR1) gene that includes three alternative exons for the third immunoglobulinlike domain in the extracellular region of the receptor. The mRNA of one of these splice variants encodes a secreted receptor that lacks transmembrane and cytoplasmic sequences as well as a portion of the third immunoglobulinlike domain. Highest levels of mRNA encoding this variant were found in brain, skeletal muscle, and skin. We expressed this form of FGFR1 in CHO cells and showed that the recombinant secreted protein binds acidic FGF. We also discovered a novel alternative exon in the third immunoglobulinlike domain that encodes part of a transmembrane FGFR1 mRNA. This exon is highly homologous to the corresponding region of the keratinocyte growth factor receptor. Transcripts including this exon were present at highest levels in the skin. We cloned an FGFR1 cDNA which includes this exon and expressed this receptor variant in L6 rat skeletal muscle myoblasts. The new receptor variant had a 50-fold-lower affinity for basic FGF than does the published FGFR1 variant, whereas both forms of receptor bound acidic FGF with high affinity. These results show that the third immunoglobulinlike domain plays an important role in determining the binding specificities for different FGFs. Our data provide the first evidence that differential splicing in the extracellular region of a receptor gene generates receptor variants with different ligand-binding specificities.

Four different fibroblast growth factor (FGF) receptors (FGFR1, FGFR2, FGFR3, and FGFR4) have recently been identified (4, 6, 8, 11, 12, 15, 16, 19). All FGF receptor cDNAs encode transmembrane protein tyrosine kinases with either two or three immunoglobulinlike (Ig-like) domains and a highly acidic region in the extracellular region. Characterization of FGFR1 and FGFR2 cDNAs of different species revealed divergent forms of receptor which differ in the number of extracellular Ig-like domains and in the type of Ig-like domain that is nearest to the membrane-spanning segment (2, 4, 9, 10, 19, 21). These forms arise from differential splicing (2, 10). Furthermore, a human FGFR1 cDNA which encodes a putative secreted form of FGFR1 has been cloned (9). This receptor differs from the transmembrane forms in the carboxy-terminal half of the third Ig-like domain and lacks transmembrane and cytoplasmic sequences. However, it has not been assessed whether this secreted receptor is actually expressed in vivo, and nothing is known about its binding properties or its biological activity.

To gain insight into the potential function of the different forms of FGFR1, we have cloned and analyzed a genomic region of the FGFR1 gene which contains three alternative exons for the third Ig-like domain. The arrangement of these exons is similar to that of the corresponding exons in the human gene (10). We showed that mRNAs encoding secreted forms of FGFR1 are expressed in vivo and that the encoded protein is functional and binds FGF. Finally, we discovered a novel exon encoding an alternative second half of the third Ig-like domain which is predominantly expressed in the skin. We provide evidence that the presence of this exon in a full-length FGFR1 mRNA changes the binding affinities of the encoded receptor for basic FGF (bFGF).

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MATERIALS AND METHODS

RNA preparation. Total cellular RNA was isolated from tissues of adult BALB/c mice as described previously (3). Polyadenylated RNA was isolated by affinity chromatography on oligo(dT)-cellulose (1). RNA was analyzed on agarose gels for integrity.

Northern (RNA) blot analysis. Northern blot analysis was performed as recently described (23). The amounts of RNA in each lane were estimated by staining of the membrane with methylene blue prior to hybridization. A nick-translated 137-bp DNA fragment encoding sequences specific for the IIIb form of murine FGFR1 (see below) was used as a hybridization probe.

RNase protection analysis. For RNase protection mapping of FGF receptor transcripts, DNA probes were cloned into the transcription vector pKS+ (Stratagene) and linearized. An antisense transcript was synthesized in vitro by using T3 or T7 RNA polymerase and [^{32}P]rUTP (800 Ci/mmol; Amersham). Samples of 50 μg of total cellular RNA were hybridized at 42°C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested for 40 min at 30°C with RNases A and T_1 as described previously (13). Protected fragments were separated on 5% polyacrylamide–8 M urea gels and analyzed by autoradiography. The same RNA samples were used for all protection assays. The results were reproduced with a different set of RNA preparations.

DNA templates for FGFR1. Three templates were used.

One was a 180-bp FGFR1 fragment encoding sequences specific for the secreted form of murine FGFR1. This fragment was generated by polymerase chain reaction (PCR) from the genomic clone, using 5'-TGGCTAGTTTGCTGG GAA-3' as a 5' primer and 5'-TGACTTATGGGTAA AGTT-3' as a 3' primer. The second template was a 137-bp cDNA fragment specific for the exon encoding the alternative third Ig-like domain of a membrane-spanning FGFR1 (IgIIIb). It was generated by PCR from the genomic clone, using 5'-CAGCATTCGGGAATTAATAG-3' as a 5' primer and 5'-GTGACAGTGAGCCACGCAGA-3' as a 3' primer. The third was a 361-bp *Aha*II-*Pvu*II fragment, corresponding to nucleotides 793 to 1154 of murine FGFR1 cDNA (19). It encodes the complete third Ig-like domain, including the sequences encoded by the IIIc exon.

Preparation of genomic DNA and cDNA. Total cellular DNA was isolated from Swiss 3T3 mouse fibroblasts as described previously (17). cDNA (first strand) was prepared from 3 μg of total cellular RNA, using Moloney murine leukemia virus reverse transcriptase (Promega) and oligo(dT) as a primer. The reaction was carried out as described by the manufacturer.

Cloning of genomic DNA and cDNA encoding a secreted form of FGFR1. Genomic DNA from Swiss 3T3 cells was amplified by using one primer corresponding to the coding sequences of the amino-terminal half of the third Ig-like domain of mouse FGFR1 (19) (amino acids 302 to 307; 5'-CCAGACAACTTGCCGTAT-3') and a 3' primer corresponding to the carboxy-terminal half of the third Ig-like domain (approximately amino acids 315 to 321; 5'-TTGTCG GTGGTATTAACTCCA-3'). A cDNA sequence containing the entire coding region of the secreted FGFR1 form was amplified by using a 5' primer hybridizing to sequences upstream from the initial methionine (5'-AACCGCAGAAC TGGGATG-3') and a 3' primer which hybridizes to 3' untranslated sequences of secreted FGFR1 (5-'TCACC AGCCTGTGGCCAGAT-3'). Reaction mixtures contained 20 ng of mouse genomic DNA or 10 ng of cDNA, 10 pmol of each primer, 200 μM each of the four deoxynucleoside triphosphates, and 1 U of *Taq* polymerase (Perkin Elmer-Cetus) in 30 μl of 10 mM Tris hydrochloride (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–100 ng of bovine serum albumin (BSA) per ml. Reactions were carried out in an Ericomp twin-block system. Twenty-five cycles, consisting of denaturation at 95°C for 30 s, annealing at 65°C for 1 min, and extension at 72°C for 1 min, were performed.

To subclone the amplified fragments, 1 U of Klenow polymerase was added to the reaction mixture and incubated for 1 h at 37°C. Amplified fragments were subcloned into the vector pKS(+) (Stratagene) and sequenced by the deoxychain termination method (20), using the Sequenase system (United States Biochemical Corp.)

Expression of a murine secreted FGFR1 in CHO cells. cDNA encoding a secreted form of murine FGFR1 with two extracellular Ig-like domains was inserted into the mammalian expression vector pBJ, in which expression of the secreted FGFR1 was driven by the SRα promoter (22), which is composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type I. CHOK1 cells (10^6 cells per 10-cm petri dish) were subsequently cotransfected with 10 μg of the expression plasmid and 1 μg of a vector containing the neomycin resistance gene (pSV2neo), using the lipofectin method (Bethesda Research Laboratories, Inc.) as instructed by the manufacturer; 48 h after transfection, cells were passaged 1:10 into selection medium (RPMI 1640, 10% fetal calf serum, 500 μg of geneticin [GIBCO] per ml).

Colonies of geneticin-resistant cells were isolated and analyzed for expression and secretion of the FGFR1 protein. For this purpose, the secreted protein was partially purified on wheat germ agglutinin-Sepharose (5) from 1 ml of serum-free RPMI 1640 medium conditioned by 10^5 cells for 48 h. Proteins bound to wheat germ agglutinin-Sepharose were analyzed for the presence of the secreted FGFR1 protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western immunoblotting using the polyclonal antiserum Ab15, which is directed against the extracellular region of FGFR1 (peptide NHTYQLDVVERSPHRPILQA, corresponding to amino acids 238 to 257 of the chicken FGFR1 [12]), and an alkaline phosphatase detection system (Promega).

Expression of a human FGFR1 cDNA clone containing exon IIIb. Sequences encoding the second half of the third Ig-like domain of the human FGFR1 (nucleotides 925 to 1068 [9]) corresponding to exon IIIc (10) were replaced by the human exon IIIb (10) by using standard cloning methods. The obtained recombinant DNA, which contains exon IIIb as the carboxy-terminal half of the third Ig-like domain, was cloned into the mammalian expression vector pSV7d (P. Luciw, Chiron Corp.) in which the FGFR1 cDNA is driven by the simian virus 40 early promoter. Rat L6 skeletal muscle myoblasts (ATCC CRL 1458) were subsequently cotransfected with this expression plasmid and pSV2neo as described above. Transfected colonies were assayed for expression of the FGF receptor by immunoblotting using polyclonal antibody 50, which is directed against peptide EYELPEDPRWELPRDR in the intracellular juxtamembrane region of the chicken FGFR1 (12).

Binding experiments. For ligand binding studies, acidic FGF (aFGF) was iodinated by using enzymobeads (Bio-Rad) as described by the manufacturer to a specific activity of 1 μCi/ng. Active ^{125}I-aFGF was subsequently separated from inactive ^{125}I-aFGF and free ^{125}I by chromatography on heparin-Sepharose. bFGF was iodinated to a specific activity of 0.25 μCi/ng by using iodobeads (Pierce) as described by the manufacturer and separated from free ^{125}I by chromatography on Sephadex G-25. Confluent monolayers of FGFR1-transfected L6 cells in 1-cm wells were incubated with 8,000 cpm of ^{125}I-aFGF or 15,000 cpm of ^{125}I-bFGF and increasing concentrations of unlabeled bFGF or aFGF at 4°C in binding medium (Dulbecco modified Eagle medium containing 0.2% BSA, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.1], and 15 U of heparin per ml). After 4 h, the cells were washed twice with cold phosphate-buffered saline and twice with phosphate-buffered saline containing 2 M NaCl. They were subsequently solubilized, and ^{125}I was quantified by gamma counting.

Cross-linking experiments. For cross-linking experiments, secreted FGFR1 protein was partially purified by wheat germ agglutinin affinity chromatography. Samples (50 to 100 ng) of the secreted receptor were subsequently allowed to bind ^{125}I-aFGF (40,000 cpm, 0.05 ng). Binding was performed in 30 μl of HEPES-buffered saline (10 mM HEPES [pH 7.4], 100 mM NaCl, 1 mM dithiothreitol) in the presence or absence of 50 or 150 ng of unlabeled basic or acidic FGF per ml, respectively, for 2 h at room temperature and 0.5 h at 37°C. The recombinant secreted protein was then cross-linked to the ligand with 1 mM bis(sulfosuccinimidyl)suberate (Pierce) at 4°C for 15 min. The reactions were stopped by the addition of Laemmli sample buffer (preheated to 95°C)

and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Nucleotide sequence accession number. The complete nucleotide sequence of the 2.02-kb mouse genomic fragment is in the GenBank data base under accession number M80363.

RESULTS

Cloning of mouse genomic DNA containing three alternative exons for half of the third Ig-like domain. A putative secreted form of FGFR1 was recently cloned from a human endothelial cell cDNA library (9). This form of FGFR1 differs from the membrane-spanning form in its carboxy-terminal half of the third Ig-like domain and lacks transmembrane and cytoplasmic sequences. Sequences encoding this carboxy-terminal half of the third Ig-like domain of secreted FGFR1 were found in the human FGFR1 gene immediately downstream of sequences encoding the amino-terminal half of the third Ig-like domain. In the human FGFR1 gene, the exon encoding the carboxy-terminal half of the third Ig-like domain of the published membrane-spanning receptor (labeled IIIc in reference (10) and a novel alternative exon for the third Ig-like domain (IIIb [10]) are located 3′ of the exon for the secreted variant.

Assuming that the genomic organization of the mouse FGFR1 gene might be similar to that of the human gene, we used specific amplifying primers corresponding to sequences encoding the amino-terminal and the carboxy-terminal halves (IIIc [19]) of the third Ig-like domain of murine FGFR1 to perform PCR on genomic DNA from Swiss 3T3 mouse fibroblasts (Fig. 1A). By using these primers, a single 2.02-kb fragment was amplified (Fig. 1B). Immediately downstream of the sequence encoding the amino-terminal half of the third Ig-like domain is a sequence coding for eight additional amino acids (drawn as a curvy line in Fig. 1B) followed by an amber stop codon (indicated with a Z). The absence of a hydrophobic stretch of amino acids suggests that this sequence is part of a secreted form of murine FGFR1. In contrast to the corresponding human fragment (9), these additional eight codons do not encode a complete Ig-like domain. However, we showed by cDNA cloning that this short open reading frame encodes the carboxy-terminal end of secreted forms of murine FGFR1 (see below).

In addition to the sequences encoding secreted forms of FGFR1, the 2.02-kb mouse genomic fragment contains an additional open reading frame (nucleotides 1048 to 1180 of the cloned DNA fragment) that encodes sequence elements (labeled IIIb in Fig. 1B) characteristic of Ig-like domains. The predicted amino acid sequence of exon IIIb shares a 42% identity with the carboxy-terminal half of the third Ig-like domain of the previously reported membrane-spanning murine FGFR1 (domain IIIc in reference 19; Fig. 1B). This receptor is otherwise identical to our PCR-generated sequences (for example, in the amino-terminal half of the third Ig-like domain). A sequence comparison of the new exon IIIb and the published exon IIIc is shown in Fig. 2A. Surprisingly, the sequence for exon IIIb is 84% identical with the corresponding sequence in the mouse keratinocyte growth factor (KGF) receptor (14) (Fig. 2B). This finding suggests that this sequence represents a newly discovered exon encoding an alternative second half of the third Ig-like domain. Expression of this novel exon was confirmed by RNase protection assay and Northern blotting (see below).

The IIIc exon which is present in the published murine FGFR1 (19) is located at the 3′ end of the cloned genomic fragment (Fig. 1B).

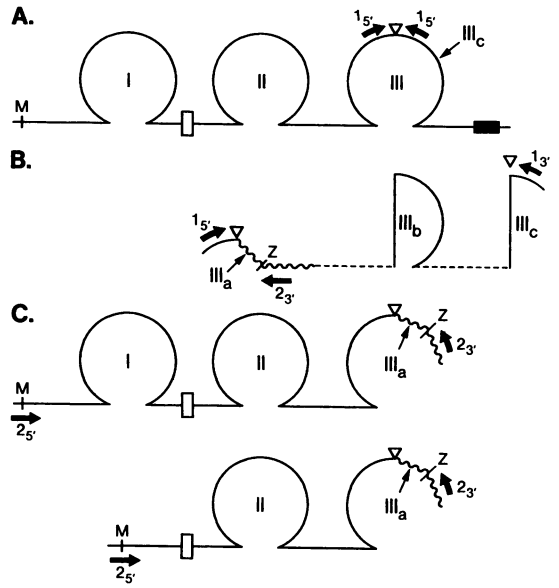

FIG. 1. Cloning of genomic DNA and cDNA encoding secreted forms of FGFR1. (A) The cDNA encoding a membrane-spanning form of mouse FGFR1 with three Ig-like domains (represented by the line). The coding sequences for the initiator methionine (M), the acid box (open box), the three Ig-like domains (I, II, and III) and the transmembrane region (solid box) are indicated. The two black arrows along domain III indicate oligonucleotide primers $1_{5'}$, and $1_{3'}$, which were used to amplify sequences encoding secreted forms of FGFR1 from genomic DNA. (B) A 2.02-kb genomic fragment which was amplified from genomic DNA of Swiss 3T3 mouse fibroblasts by using primers $1_{5'}$ and $1_{3'}$. The curvy line represents coding and noncoding sequences specific for the secreted form of FGFR1 (labeled IIIa and indicated with an arrow). Sequences encoding two alternative carboxy-terminal halves of the third Ig-like domain of membrane-spanning FGF receptors are labeled IIIb and IIIc. The IIIc sequence is the exon present in the published FGFR1 (19). Intron sequences are drawn as dotted lines. (C) cDNAs encoding secreted forms of mouse FGFR1 with two or three Ig-like domains. They were cloned by PCR from cDNA of total cellular RNA by using oligonucleotide primers $2_{5'}$, and $2_{3'}$. Sequences encoding the initiator methionine (M), Ig-like domains I, II, and III (I, II, and III), and the stop codon (Z) are indicated. The curvy line represents the sequences which are specific for the secreted forms of FGFR1 (IIIa; see Fig. 2B). The point of sequence divergence of membrane-spanning forms and secreted forms is indicated with a triangle.

Cloning of cDNAs encoding secreted forms of FGFR1. To identify and characterize secreted forms of mouse FGFR1, we performed PCR on cDNA from mouse skin RNA. Using a 5′ primer which includes the start codon and a 3′ primer complementary to sequences at the 5′ end of the cloned genomic fragment (IIIa) (Fig. 1B and C), we amplified two different fragments of approximately 1.0 and 0.7 kb which contain no intervening sequences. These fragments are cDNAs that encode putative secreted forms of FGFR1 with or without sequences for the first Ig-like domain (Fig. 1C). This result confirmed that the 5′ end of the cloned 2.02-kb genomic fragment (Fig. 1B) includes exon sequences for the carboxy-terminal portion of the two secreted forms of mouse FGFR1. The two secreted forms are identical to the corresponding membrane-spanning forms at their amino-terminal ends, including the Ig-like domains I and II and the amino-terminal half of the third Ig-like domain. They diverge from their corresponding membrane-spanning receptors after

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(A) FGFR1 IgIIIa    VLLTSFLG

    FGFR1 IgIIIb        SGINSSDAEVLTLFNVTEAQSGEYVCKVSNYIGEANQSAWLTV
                        ||| | ||    ||| |  | ||    ||||||
    FGFR1 IgIIIc    TAGVNTTDKEMEVLHLRNVSFEDAGEYTCLAGNSIGLSHHSAWLTV

(B) FGFR1 IgIIIb        SGINSSDAEVLTLFNVTEAQSGEYVCKVSNYIGEANQSAWLTV
                        |||||| |||| ||||||  ||||||||||| |||||||||
    KGFR  IgIII         SGINSSNAEVLALFNVTEMDAGEYICKVSNYIGQANQSAWLTV
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FIG. 2. Amino acid sequence comparison of three alternative exons for half of the third Ig-like domain. (A) Amino acid sequence encoded by the three alternative exons IIIa, IIIb, and IIIc. IIIa represents the eight amino acids encoded by the exon for the secreted receptor, IIIc represents the sequence previously published for mouse FGFR1 (19), and IIIb represents a novel sequence for the carboxy-terminal half of the third Ig-like domain. (B) Amino acid sequence of IIIb in comparison with the corresponding sequence in the KGF receptor (KGFR) (14).

amino acid 312 (labeled with a triangle in Fig. 1). The secreted murine FGFR1 proteins are predicted to terminate at a stop codon eight amino acids after the point of sequence divergence in the third Ig-like domain and therefore do not contain a full Ig-like domain at their carboxy terminus.

Exons IIIa, IIIb, and IIIc are differentially expressed *in vivo*. To investigate the expression of the different FGFR1 splice variants in mouse tissues, we performed RNase protection assays with hybridization probes that specifically detect exons IIIa, IIIb, and IIIc, respectively. As shown in Fig. 3A, mRNA encoding secreted forms of FGFR1 was detected in brain, skeletal muscle, and skin. The size of the protected fragments determined on a sequencing gel was consistent with the presence of the stop codon eight amino acids after the point of sequence divergence in the third Ig-like domain (data not shown).

In addition to the IIIa exon which is present in the secreted FGFR1 cDNA, the 2.02-kb genomic fragment (Fig. 1B) contains the novel exon IIIb that has features characteristic of an Ig-like domain. This sequence is homologous to the carboxy-terminal half of the third Ig-like domain of the published murine FGFR1 (IIIc) and to the corresponding sequence in the KGF receptor (Fig. 2A and B). To determine whether this open reading frame is expressed as part of an FGFR1 mRNA, we amplified cDNA derived from mouse brain, using a 3′ primer hybridizing to this open reading frame and a 5′ primer derived from the amino-terminal half of the third Ig-like domain (19). Using these primers, we amplified a 135-bp fragment which contains no intronic sequences (data not shown). This result shows that the IIIb open reading frame is an authentic exon which encodes part of an FGFR1.

To investigate the expression of exon IIIb in different mouse tissues, we performed RNase protection assays by using a 137-nucleotide (nt) probe representing the unique sequences. As shown in Fig. 3B, the IIIb exon was primarily expressed in skin. Low expression levels of this exon were detected in brain, kidney, muscle, and placenta. In contrast, expression was not detected in liver, spleen, and testis. In Northern blot experiments with a IIIb-specific probe, we detected a major transcript of approximately 4.2 kb, which is consistent with the predicted size of a membrane-spanning form of FGFR1 (data not shown).

Whereas exons IIIa and IIIb have a limited tissue distribution, the IIIc exon was found in all tested tissues except liver. Using a 361-nt hybridization probe which is complementary to the coding sequences of the complete third

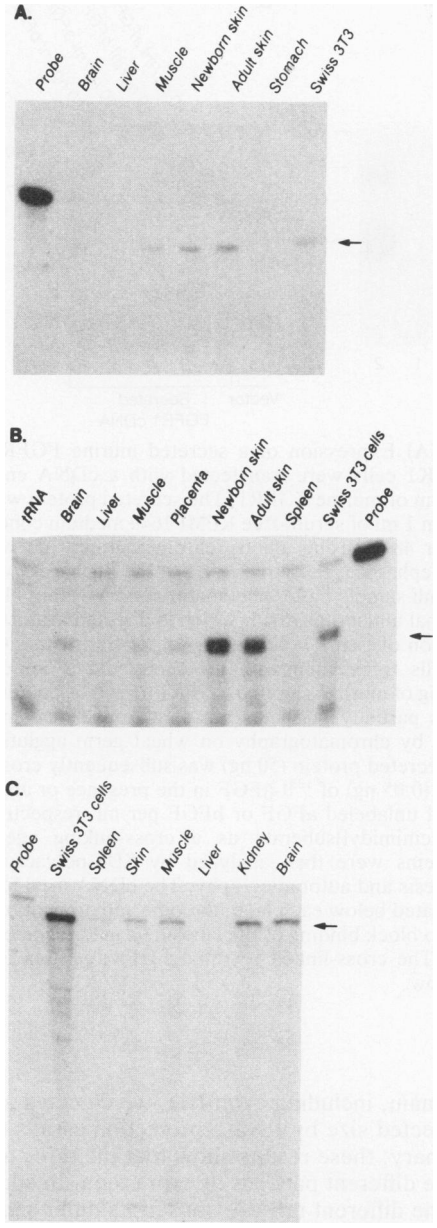

FIG. 3. mRNA expression of exons IIIa, IIIb, and IIIc. (A) Total cellular RNA (50 μg) from mouse tissues was analyzed by RNase protection assay using a 180-nt probe complementary to 3′ coding and noncoding sequences of the murine secreted FGFR1 mRNA. This sequence is unique to the secreted variants of murine FGFR1 and is not present in transmembrane forms (see Materials and Methods). The gel was exposed for 4 days. The major protected fragment is indicated with an arrow. (B) Total cellular RNA (50 μg) from mouse tissues was analyzed by RNase protection assay using a 150-nt probe complementary to sequences encoding the alternative second half of the third Ig-like domain of FGFR1 (exon IIIb). This probe is specific for the FGFR1-IIIb form (see Materials and Methods). The gel was exposed for 48 h. The major protected fragment is indicated with an arrow. The upper band results from incompletely digested hybridization probe. (C) Total cellular RNA (50 μg) from mouse tissues was analyzed by RNase protection assay using a 361-nt probe complementary to the coding sequences of the complete third Ig-like domain of the published FGFR1 sequence (19) which contains exon IIIc. The gel was exposed for 8 h. The major protected fragment which is expected for IIIc-containing transcripts is indicated with an arrow.

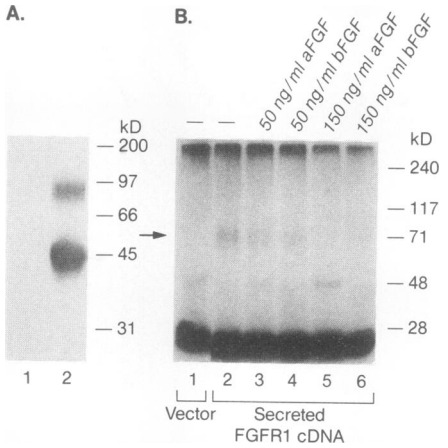

FIG. 4. (A) Expression of a secreted murine FGFR1 in CHO cells. CHOK1 cells were transfected with a cDNA encoding the secreted form of murine FGFR1. The secreted protein was partially purified from 1 ml of serum-free RPMI 1640 medium conditioned by 10^5 cells for 48 h, using affinity chromatography on wheat germ agglutinin-Sepharose. Proteins bound to the Sepharose were eluted with Laemmli sample buffer and analyzed by Western blotting with the polyclonal antiserum Ab15, which is directed against the extracellular region of FGFR1. Lanes: 1, vector-transfected CHO cells; 2, CHO cells transfected with the secreted FGFR1 cDNA. (B) Cross-linking of murine secreted FGFR1 to aFGF. Secreted FGFR1 protein was partially purified from conditioned medium of transfected cells by chromatography on wheat germ agglutinin-Sepharose. The secreted protein (50 ng) was subsequently cross-linked to 40,000 cpm (0.05 ng) of ^{125}I-aFGF in the presence or absence of 50 or 150 ng of unlabeled aFGF or bFGF per ml, respectively, using bis(sulfosuccinimidyl)suberate as a cross-linking agent. Cross-linked proteins were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The cDNA used for transfection is indicated below each lane; the type and amount of unlabeled FGF used to block binding of the labeled ligand are indicated above each lane. The cross-linked secreted FGFR1 protein is indicated with an arrow.

Ig-like domain, including exon IIIc, we detected a fragment of the expected size by RNase protection assays (Fig. 3C).

In summary, these results show that the three alternative exons have different patterns of expression. In addition, the levels of the different mRNA transcripts differ significantly. This conclusion is based on the facts that the protection assay shown in Fig. 3A (IIIa probe) was exposed to film for 4 days, the protection assay shown in Fig. 3B (IIIb probe) was exposed for 2 days, and the assay shown in Fig. 3C (IIIc probe) was exposed for only 8 h. Furthermore, a 201-bp protected fragment which would be expected with the IIIc probe for exon IIIa- or IIIb-containing transcripts could not be detected after the short exposure time. This result shows that FGFR1 transcripts containing exon IIIc are predominant in all tissues.

A secreted form of murine FGFR1 binds FGF. There have been no studies of the function of secreted forms of FGF receptors. We have therefore expressed a secreted murine FGFR1 protein with two extracellular Ig-like domains in CHO cells. The conditioned medium of the cells transfected with the secreted FGFR1 cDNA contained a protein of the expected size (45 kDa) that was recognized by FGFR1 antibodies (Fig. 4A, lane 2). No FGFR1 protein was detected in conditioned medium of vector-transfected cells (lane 1). The high-molecular-weight band which was also detected by FGFR1 antibodies most likely represents a dimeric form of the secreted protein. To assess whether the recombinant protein can bind FGF, this protein was partially purified by chromatography on wheat germ agglutinin-Sepharose, incubated with ^{125}I-aFGF, and covalently cross-linked. A protein cross-linked to ^{125}I-aFGF migrated on SDS-polyacrylamide gels at about 65 kDa (Fig. 4B, lane 2). The size of this protein corresponds to the expected size of a 1:1 complex of secreted FGFR1 with ^{125}I-aFGF. This ^{125}I-aFGF-cross-linked protein was not visualized in the presence of an excess (150 ng/ml) of unlabeled aFGF or bFGF (Fig. 4B, lanes 5 and 6). At 50 ng of either ligand per ml, only a partial competition was observed (lanes 3 and 4). The inhibition of ^{125}I-aFGF binding by unlabeled bFGF shows that the secreted form of murine FGFR1 is also a receptor for bFGF. No ^{125}I-aFGF-cross-linked protein was detected in control protein preparations from conditioned medium of vector-transfected cells (lane 1). An additional band of 48 kDa which was present in all lanes most likely results from oxidized FGF.

The second half of the third Ig-like domain confers the binding specificity of FGFR1 toward aFGF and bFGF. In addition to the IIIa exon which is part of a secreted FGFR1 mRNA, both the human and the murine FGFR1 genes contain two alternative exons encoding the carboxy-terminal half of the third Ig-like domain of membrane-spanning receptors (exons IIIb and IIIc; Fig. 1) (10). To compare the binding properties of these membrane-spanning FGFR1 variants that differ in their third Ig-like domain, the IIIc exon of the human FGFR1 was replaced by the human exon IIIb which is 100% homologous to the murine exon IIIb at the amino acid level. The constructed cDNA (FGFR1-IIIb) encodes a receptor with two extracellular Ig-like domains, a highly acidic region, a transmembrane region, and a full cytoplasmic domain. It differs from the published human FGFR1 (FGFR1-IIIc) only in the second half of its third Ig-like domain. L6 rat skeletal muscle myoblasts which lack endogenous FGF receptors were transfected with the constructed FGFR1-IIIb cDNA. The binding properties of the FGFR1-IIIb expressing cells were compared with those expressing the FGFR1 protein that contains the IIIc domain (FGFR1-IIIc) (9). As shown in Fig. 5A and B, both aFGF and bFGF competed for the binding of ^{125}I-aFGF to FGFR1-IIIc and FGFR1-IIIb. However, whereas ^{125}I-aFGF binding to FGFR1-IIIc transfectants was competed for more efficiently by unlabeled bFGF than by unlabeled aFGF (50% inhibition at 4 ng of bFGF and 25 ng of aFGF per ml, respectively), 20- to 25-fold more bFGF was required to compete for ^{125}I-aFGF binding to the FGFR1-IIIb transfectants (50% inhibition at 200 ng of bFGF and 10 ng of aFGF per ml, respectively). Therefore, there was an approximately 50-fold difference in the relative affinities of bFGF for the two receptors. This result was reproduced in four independent experiments. The low affinity of FGFR1-IIIb for bFGF was confirmed when ^{125}I-bFGF was used as a tracer. As shown in Fig. 5C and D, specific binding of radiolabeled bFGF was obtained with FGFR1-IIIc-transfected cells (Fig. 5C), whereby ^{125}I-bFGF was equally well competed for by aFGF and bFGF. In contrast, no specific binding of ^{125}I-bFGF to FGFR1-IIIb-transfected L6 cells was observed (Fig. 5D). These results show that the carboxy-terminal half of the third Ig-like domain determines the specificity for bFGF binding.

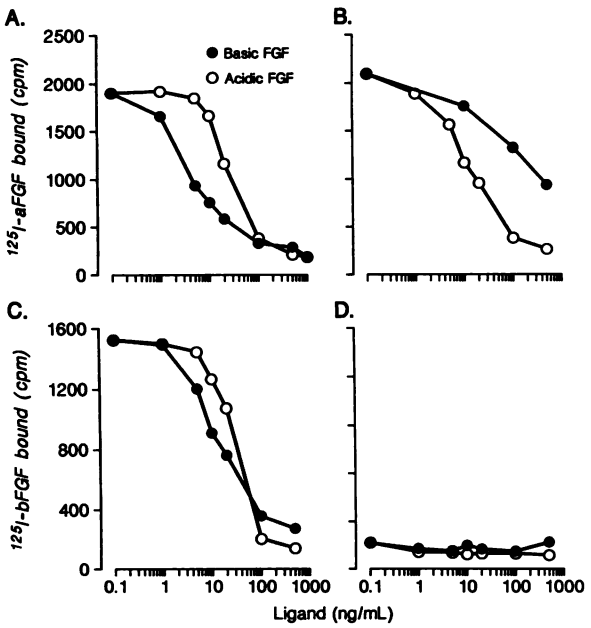

FIG. 5. Specific binding of radiolabeled aFGF and bFGF to L6 skeletal muscle myoblasts transfected with FGFR1-IIIb or FGFR1-IIIc. The binding of ^{125}I-aFGF (A and B) and ^{125}I-bFGF (C and D) to L6 skeletal muscle myoblasts transfected with either FGFR1-IIIc (A and C) or FGFR1-IIIb cDNA (B and D) at 4°C was determined in the presence of increasing concentrations of unlabeled aFGF or bFGF. The binding of labeled ligand in the presence of the indicated concentrations of unlabeled FGF is expressed as counts per minute bound per well. The cell number per well was identical for FGFR1-IIIb and FGFR1-IIIc transfectants. Binding experiments with FGFR1-IIIb- and FGFR1-IIIc-transfected cells were carried out in parallel, using the same dilutions of unlabeled ligand. Values shown are the means of triplicate samples and are representative of at least three experiments.

DISCUSSION

FGF receptors are among the few known growth factor receptors for which alternatively spliced variants have been found (4, 7, 9, 10, 18, 19). Prior to this study, the expression of these splice variants was inferred from cDNA clones, but there were no data on whether these variants are actually expressed at detectable levels in vivo or whether they are biologically active. In an attempt to gain insight into the potential function of the splice variants, we have identified and characterized a region of the mouse FGFR1 gene that contains alternative exons for the third Ig-like domain. We examined the in vivo expression pattern of transcripts that include these exons and analyzed the binding properties of the encoded receptor variants.

To investigate the expression pattern of FGFR1 forms which differ in the carboxy-terminal half of the third Ig-like domain, we have cloned a genomic region that includes three alternative exons for this domain. Exon IIIa, which is part of secreted FGFR1 mRNAs, is located at the 5′ end of this genomic sequence. Exons IIIb and IIIc, which are part of transmembrane FGFR1 mRNAs, are located in the middle or at the 3′ end of the cloned genomic fragment, respectively. We demonstrated that the three alternative exons in the third Ig-like domain have a differential pattern of expression in vivo. The expression of the secreted FGFR1 mRNA (exon IIIa) in brain, skeletal muscle, and skin represents the first report on in vivo expression of these FGFR1 forms and suggests that the encoded proteins are functionally important. Exon IIIb was predominantly expressed in skin, indicating that the encoded receptor plays a specific role in this tissue. Whereas exons IIIa and IIIb have a limited tissue distribution, the published variant of the third Ig-like domain (IIIc [19]) was expressed in all tissues except liver (Fig. 3C). We showed that some tissues, such as brain and skin, simultaneously express all three forms of the third Ig-like domain, whereas only exon IIIc was found in other organs. In all tissues that express all three exons, exon IIIc had the highest level of expression.

The striking differences in the expression patterns of the three exons suggest that they are functionally different. We have therefore investigated their ligand binding specificities. Our data show that the secreted murine FGFR1 is a functional protein which binds aFGF and bFGF. This is especially remarkable since it lacks almost the complete second half of the third Ig-like domain. The secreted form of murine FGFR1 differs in this respect from the human form which has a complete third Ig-like domain. Although we do not know the role of either the mouse or human secreted form of receptor, our findings show that these proteins are potentially functional in vivo.

One of the most striking findings of our study was the different ligand binding specificities of transmembrane receptors which differ in the second half of the third Ig-like domain. We demonstrated that the replacement of the IIIc exon by the IIIb exon reduces the relative affinities of the receptor for bFGF approximately 50-fold (50% inhibition at 4 and 200 ng of bFGF per ml, respectively), whereas the affinity for aFGF was not affected (50% inhibition at 25 and 10 ng of aFGF per ml). This finding suggests that the second half of the third Ig-like domain confers the specificity for binding to bFGF. Differential splicing within the third Ig-like domain therefore provides a mechanism to change the affinity of the receptor for different ligands. This finding also provides an explanation for observations from several groups who identified FGFR2 variants which also differ in the second half of the third Ig-like domain (2, 4, 6, 8, 14). Characterization of the human FGFR2 genomic sequence that includes these exons revealed that the alternative exons correspond to exons IIIb and IIIc of FGFR1 (10). Whereas the *bek* form of FGFR2 includes exon IIIc, exon IIIb is present in the K-*sam* form of FGFR2 (10). Furthermore, exon IIIb is present in the receptor for KGF, which is a splice variant of FGFR2 (10, 14). This receptor was found to bind aFGF and KGF with high affinity but exhibits only low-affinity binding for bFGF, whereas the *bek* form of FGFR2 binds both aFGF and bFGF with high affinity (4, 14). The differences in the affinities of these forms of FGFR2 for bFGF have a pattern similar to that found for binding of bFGF to FGFR1-IIIb and FGFR1-IIIc. By analogy, one might expect that the FGFR1-IIIb form is another receptor for KGF. This hypothesis is further supported by our finding that the murine FGFR2-IIIb exon which is present in KGF receptor is 84% homologous to the murine FGFR1-IIIb exon (Fig. 2B).

In summary, these results provide evidence that the second half of the third Ig-like domain confers the specificity of FGF receptors for bFGF, KGF, and possibly other FGF-like molecules. Differential splicing within this domain therefore represents a novel mechanism to generate receptor variants with different ligand-binding specificities.

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