The fibroblast growth factor receptor, FGFR3, forms gradients of intact and degraded protein across the growth plate of developing bovine ribs

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Point mutations in the human fibroblast growth factor (FGF) receptor 3 gene (*Fgfr3*) produce a constitutively active receptor, which disrupts chondrocyte differentiation in the growth plate and results in skeletal dysplasias with severe shortening of the limbs. Alternative splicing of the Fgfr3 transcript gives rise to two isoforms, IIIc and IIIb, which vary in their specificity for FGF ligands. We examined the expression of these FGFR3 isoforms in the bovine fetal rib growth plate to determine whether levels of FGFR3 expression are zone-related. Transcripts for both Fgfr3 isoforms are expressed in rib growth plate, with maximum expression in the hypertrophic region and the least expression in the reserve zone. Fgfr3 IIIc is the predominant isoform in the growth plate. Western-blot analysis revealed the presence of full-length FGFR3 (135 kDa) for both isoforms in the reserve zone, a major 98 kDa fragment in all zones and smaller fragments primarily in the hypertrophic zone. Immunostaining localized FGFR3 to the pericellular region of reserve chondrocytes and to the extracellular matrix in the hypertrophic zone. These results suggest that the transmembrane form of FGFR3 increasingly undergoes proteolytic cleavage towards the hypertrophic zone to produce an extracellular-domain fragment of FGFR3, which is present in large amounts in the matrix of hypertrophic cells. These findings suggest a proteolytic regulatory mechanism for FGFR3, whereby Fgfr3 fragments could control availability of FGF for the intact receptor, and by which proteolysis could inactivate the receptor.

Key words: cartilage, development, extracellular matrix, proteolysis.

INTRODUCTION

Fibroblast growth factors (FGFs) constitute a family of structurally related polypeptides, which play a major role in mitogenesis, mesoderm induction, neuronal survival and neuritic extension [1], tumour angiogenesis [2] and atherosclerosis [3]. FGFs exert their influence by binding to cell-surface tyrosine kinase receptors. Four different FGF receptors (FGFRs) have been identified, which share 50–70 % overall amino acid identity. They share a basic structure of three glycosylated Ig-like domains, a single transmembrane domain and a cytoplasmic tyrosine kinase domain. Mutations in genes encoding *Fgfr1*, *2* and *3* cause different syndromes that interfere with bone growth and development [4]. Mutations in *Fgfr3*, in particular, are responsible for several clinically related forms of dwarfism in humans, including achondroplasia [5,6], hypochondroplasia [7] and thanatophoric dysplasia [8]. Ligands for FGFR1 and FGFR3 overlap in terms of specificity, but show opposite affinities for the receptors. Thus FGF1 and FGF9 bind more tightly to FGFR3 than FGF2, whereas FGFR1 is highly specific for FGF2 compared with FGF1 and FGF9 [9].

Mutations in FGFR3 exert their effects in the growth plates of developing long bones. Chondrocytes in the growth plate of developing long bones undergo proliferation, hypertrophy and cell death, enabling osteoblastic invasion of the tissue and subsequent bone formation. This process is acutely regulated such that only a few chondrocytes in the growth plate go through this differentiation process at a given time. FGFR3 mutations render the receptor constitutively active, suppressing chondrocyte proliferation, differentiation and maturation. Mice expressing a mutated FGFR3 (with a Glu^{380}-to-Arg achondroplasia mutation) exhibit a dominant dwarf phenotype, due to reduced proliferation and hypertrophy of chondrocytes in their growth plate [10], which is analogous to achondroplasia in humans [5,6]. Conversely, mice with a FGFR3-null mutation have skeletal overgrowth due to expansion of proliferative and hypertrophic zones. These observations indicate that FGFR3 is a negative regulator of bone growth [11]. Overexpression of FGF2 and FGF9 [12,13], which are ligands for FGFR3 in transgenic mice, result in a phenotype similar to FGFR3 achondroplasia, characterized by short and flat bones. This is due to the activation of the endogenous FGFR3 by these FGFs, which mimics the state of constitutively active FGFR3.

The role of FGFR3 is complicated further by the existence of two variants, IIIb and IIIc, which arise due to an alternative-splicing event in the third Ig-loop domain [14]. The IIIb splice variant encoded by exon 8 of the *Fgfr3* gene binds FGF1 preferentially and FGF9 to a lesser extent, and is the only FGFR3 isoform expressed in epithelial cells [15]. The IIIc variant is more promiscuous and binds a wide range of FGFs, including FGF1, FGF2, FGF4 and FGF9 [16–18].

The exact role of FGFR3 in the programmed progression of endochondral bone growth and how it is inactivated to produce proliferation and hypertrophy in a normal growth plate is not clear. In our efforts to elucidate this role, we examined the expression of FGFR3 isoforms in bovine fetal growth plate. Specifically, we wanted to determine whether the levels of FGFR3 expression could modulate receptor activity. Our findings suggest that the transmembrane form of FGFR3 undergoes proteolytic cleavage during its progression to the hypertrophic zone to

Abbreviations used: FGF, fibroblast growth factor; FGFR, FGF receptor; MBP, maltose-binding protein; IPTG, isopropyl β-D-thiogalactoside; MMP, matrix metalloprotease.

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produce the soluble extracellular form of FGFR3. This proteolysis of FGFR3 could regulate the rate of FGFR3 activation in a developing growth plate.

EXPERIMENTAL

All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise indicated. Plasmid MomFR3SV was provided by D. Ornitz, Washington University Medical School, St. Louis, MO, U.S.A.

Growth-plate cartilage isolation

Rib growth plates were isolated from third trimester bovine (*Bos taurus*) fetal calves and sectioned as described previously [19]. Growth plates devoid of connective tissue and perichondrium were sliced into 1 mm thick slices, starting at the hypertrophic zone and proceeding into the growth plate, using a vibratome. Slices for protein and RNA isolation were frozen in liquid nitrogen, pulverized on dry ice in a specialized steel mortar and pestle [20] and stored at −70 °C until needed.

Northern-blot analysis

Total RNA was isolated as described in [21]. Total RNA from a mixture of all zones was reverse-transcribed and amplified by PCR using primers specific for the *Fgfr3 IIIc* isoform (621 bp, forward, 5′-GCGTCGTGGAGAACAAGTTT-3′; reverse, 5′-TGTGTTGGAGCTCATGGATG-3′) or the *IIIb* isoform (310 bp, forward, 5′-GCACCGGCCCATCCTAC-3′; reverse, 5′-GTGAACACGCAGCCAAAA-3′; and 277 bp, forward, 5′-TGAGCGGGAAGCGGGAGAC-3′; reverse, 5′-GAGCGTGATGGGGGCGAGTA-3′). This was followed by a second PCR using the above PCR products as templates with the primers 5′-GGCACGCCCTACGTCACC-3′ (forward) and 5′-GGCTTCCACGAGCTCCTC-3′ (reverse) to obtain a 200 bp isoform-specific product. The PCR product was verified by sequencing.

β-Actin was amplified using the primers 5′-GTCGCCCTGGACTTCGAGC-3′ (forward) and 5′-GGTACATGGTGGTGCCGCCA-3′ (reverse), which produced a 265 bp PCR product. Bovine type-II collagen was amplified using the primers 5′-CTGGAGGCCATGAAGGTTTTCTG-3′ (forward) and 5′-AGTTTCCTGTCTCTGCCTTGAC-3′ (reverse), which produced a 796 bp PCR product. A 769 bp PCR product was obtained for bovine type-X collagen using the primers 5′-TCACTGTTATCCTCTCCAAAG-3′ (forward) and 5′-ATGACATCTCCCGAATGTTTC-3′ (reverse). PCR products were random-prime-labelled with [α-^{32}P]dCTP using a random-prime labelling kit (Roche, Nutley, NJ, U.S.A.). Total RNA (2 μg) was separated on a 1.1 % formaldehyde/agarose gel, transferred on to Biodyne nylon membrane (Pall Biosupport; Life Technologies, Rockville, MD, U.S.A.), stained with Methylene Blue and hybridized overnight at 45 °C (10^6 counts/ml) using ULTRAhyb hybridization buffer (Ambion, Austin, TX, U.S.A.). After two washes each with low- and high-stringency wash buffers, the blot was exposed to X-ray film. The blots were performed in triplicate and a representative blot is shown (see Figure 2, left-hand panel, below).

RNase-protection assay

A 356 bp region of *Fgfr3* cDNA, encoding part of exon 9 and exon 10, was cloned into the pCR II vector (Invitrogen, Carlsbad, CA, U.S.A.). The cloned vector was linearized with *Xba*I and *in vitro*-transcribed using the T7 promoter and [α-^{32}P]CTP (800 Ci/mmol; Dupont NEN, Boston, MA, U.S.A.). A 303 bp region of bovine *Fgfr1* was also cloned into the pCR II vector, linearized with *Xba*I and *in vitro*-transcribed using the SP6 promoter. Total RNA (5 μg) was hybridized at 37 °C overnight with 200000 c.p.m. of either the *Fgfr1* or *Fgfr3* probe. RNA hybrids were digested with RNase A and RNase T (Roche) at 37 °C for 45 min for *Fgfr3*, whereas samples with the *Fgfr1* probe were digested with RNase T1 at 30 °C for 30 min. The protected fragments were separated by electrophoresis on 6 % polyacrylamide/urea gels, analysed by autoradiography and quantified by densitometry.

Development of FGFR3 antibodies

Anti-FGFR3 antibodies were generated by immunizing rabbits (Genemed Synthesis, San Francisco, CA, U.S.A.) with the peptides CVVENKFGRIQQ (which is encoded by exon 6 and common to both isoforms), CTAGANTTDKELEVLS (in exon 9, specific to the IIIc isoform) or RLRLANVSERDGGEYLC (in exon 8, specific to the IIIb isoform), which were coupled to keyhole-limpet haemocyanin via cysteine. Peptides were selected to avoid cross-reactivity of the antibodies with FGFR1, the other FGFR present in the growth plate.

Cloning of murine and bovine *Fgfr3*

A 2.7 kb DNA fragment encoding part of mouse *Fgfr3 IIIb/IIIc* was excised from MomFR3SV [17] using *Eco*RI and *Xba*I, and was cloned in frame into pMal-c2 (New England Biolabs, Beverly, MA, U.S.A.). The plasmid was then transformed into competent Top10 *Escherichia coli* (Invitrogen) cells and induced with isopropyl β-D-thiogalactoside (IPTG). Maltose-binding protein (MBP)-IIIb/IIIc fusion protein was detected using Coomassie Brilliant Blue-stained SDS/polyacrylamide gels, and confirmed by Western immunoblotting using anti-FGFR3 antibodies.

A 350 bp region of bovine *Fgfr3*, encoding exons 6 and 7, was obtained by reverse transcription and PCR, subcloned into pCR II vector (Invitrogen) using TA cloning and sequenced to determine the reading frame relative to the multicloning restriction sites in the vector. The cloned vector was then linearized and *Xba*I linkers were added to adjust the reading frame to that of the pMal-c2 vector. This clone was then digested with *Xba*I and *Hin*dIII to excise the 425 bp fragment, which was then subcloned into pMal-c2 using *Xba*I and *Hin*dIII. The induced product was detected as described above.

Affinity chromatography of fusion protein R3-MBP (recombinant FGFR3 and MBP fusion protein)

A crude extract (in 20 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA, 1 mM azide and 10 mM β-mercaptoethanol) of induced bacterial cultures was loaded on to amylose resin columns (New England Biolabs). The bound fusion protein was eluted using 10 mM maltose, and the fractions with UV absorbance at 280 nm were pooled and concentrated using PALL filtron macrosep concentrators (Fisher Scientific, Fair Lawn, NJ, U.S.A.).

Affinity purification of FGFR3 antibodies

FGFR3 peptides or purified R3-MBP fusion proteins were immobilized on nitrocellulose membrane and blocked with 0.2 % I-Block (Applied Biosystems, Foster City, CA, U.S.A.) in Tris-buffered saline [50 mM Tris/HCl (pH 8) and 150 mM NaCl] for

3 h. They were then rinsed once in Tris-buffered saline with 0.1 % Tween-20, washed for 5 min with Immunopure gentle Ag/Ab binding buffer (Pierce, Rockford, IL, U.S.A.) and 0.1 % Tween-20, and incubated overnight at 4 °C in 10 % (v/v) antiserum made in Ag/Ab binding buffer and 0.1 % Tween-20. The blot was washed with binding buffer and the antibody was eluted from the membrane using Immunopure gentle Ag/Ab elution buffer (Pierce), and concentrated using PALL filtron macrosep concentrators.

Western-blot analysis for FGFR3

Total protein was extracted from each zone of growth plate using 8 vol. of 2 % (w/v) SDS in phosphate buffer (pH 7.0). In one instance the growth plate was extracted with 8 vol. of 3.4 M NaCl for 30 min at 4 °C, and then the residual cartilage pellet was extracted with 8 vol. of 2 M urea, 0.05 M sodium acetate (pH 6.0) and 1 % CHAPS for 3 h, 4 °C. The supernatants of the 3.4 M NaCl and the 2 M urea extracts were dialysed overnight against water at 4 °C. Growth-plate tissues were also extracted with 3.4 M NaCl and 2 M urea containing protease inhibitors (5 mM EDTA, 1 mM PMSF, 0.3 M amino hexanoic acid and 15 mM benzamidine) during extraction and dialysis. In both instances, protein was quantified using the Nano-Orange Protein quantification kit (Molecular Probes, Eugene, OR, U.S.A.). Total protein (15 μg) was then separated by SDS/PAGE (4–12 % Bis-Tris gels; Invitrogen) and transferred by trans-blot semi-dry transfer (Bio-Rad, Hercules, CA, U.S.A.) on to PVDF membranes (Millipore, Bedford, MA, U.S.A.). Membranes were blocked in 0.2 % I-Block and incubated with affinity-purified FGFR3 N-terminal-specific polyclonal antibody E6 (specific for FGFR3 exon 6), E8 (specific for FGFR3 exon 8) or E9 (specific for FGFR3 exon 9), or C-terminus-specific polyclonal antibody (C15sc123; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The blot was developed using a chemiluminescence system (ECL® Western-blot analysis system; Amersham Pharmacia Biotech, Picastaway, NJ, U.S.A.). All blots were performed in triplicate using extracts from different growth plates. Similar results were obtained and a representative blot is shown (see below).

Immunohistochemistry

Histology and immunohistochemistry were performed using standard procedures. Sections of tissue were de-parafinized, pretreated with 5 m-units of chondroitinase ABC (Seikagaku, Tokyo, Japan), in buffer containing 100 mM NaCl, 50 mM $CaCl_2$ and 50 mM Hepes (pH 7.0), for 3 h at 37 °C, rinsed with PBS, and incubated with anti-FGFR3 antibodies in PBS at 4 °C overnight. This was followed by a PBS wash and a 1 h incubation in a 1:200 dilution of biotinylated goat anti-mouse antibody in PBS. The biotinylated antibody was visualized by use of an avidin–biotin complex kit (Vectastatin ABC kit; Vector Laboratories, Burlingame, CA, U.S.A.). Slides were counterstained with haematoxylin.

RESULTS

Slices of the bovine growth plate were first examined using histology and Northern-blot analysis to differentiate the zones of the growth plate (Figure 1). Message levels for type-X collagen were observed only in the first zone of the growth plate, indicating that it represented the hypertrophic zone (Figure 1B). Transcripts for type-II collagen were observed in all zones, with higher levels in slices 3 and 4. Controls, such as 18 and 28 S bands and message levels of β-actin, helped in the comparison of total RNA levels between the different zones. Histological examination of haematoxylin- and eosin-stained sections of the growth-plate slices, along with the results of the Northern blots, helped determine that slices 1 and 2 of the growth plate represented the hypertrophic and prehypertrophic regions, that slices 3 and 4 represented the proliferating zones and that the reserve zone was represented by slices 5 and 6.

To determine the expression levels of FGFR3 in the growth plate, we first examined the message for the two isoforms of FGFR3 in the different zones. Primers based on the human *Fgfr3* nucleotide sequence were synthesized and used in reverse transcriptase PCR reactions with bovine growth-plate RNA to generate cDNA for a major portion of bovine *Fgfr3* (GenBank accession no. AF368288). The bovine sequence was used to design bovine primers and FGFR3-isoform-specific probes, which were radiolabelled with [^{32}P]P_i and used in Northern hybridization against total RNA isolated from different zones of bovine fetal rib growth plate. The Northern blot in Figure 2 (left-hand panel) shows the presence of transcripts of 4.5 kb for both *IIIb* and *IIIc Fgfr3* isoforms in all zones of the growth plate, with maximum expression in the prehypertrophic and hypertrophic zones (Figure 2, left-hand panel). The levels of Fgfr3 message for both isoforms decrease in the proliferating and reserve zones and create a gradient of Fgfr3 message in the growth plate.

The relative amounts of the message for isoforms of Fgfr3 present in the growth plate were examined by RNase-protection assay, using a probe that could detect both isoforms of Fgfr3 on the same gel, after hybridization and digestion with RNase A and RNase T. A PCR fragment of 345 bp encoding part of bovine *Fgfr3* exon 9 (137 bp) and exon 10 (217 bp) was cloned into the pCR II vector using TA cloning. The 465 bp radiolabelled probe generated by *in vitro*-transcription was hybridized in solution to total RNA isolated from the different zones of the growth plate. The protected fragment was 354 bp for the IIIc isoform and 217 bp for the IIIb isoform (which lacks the region of exon 9). Message levels for Fgfr1, the other FGFR in cartilage, were also examined using RNase-protection analysis. *In vitro* translation of a *Fgfr1* clone generated a 354 bp radiolabelled probe. Upon hybridization with growth-plate mRNA and digestion with RNase T, a 303 bp protected fragment was detected for Fgfr1. The relative levels of the receptors were quantified by densitometry and values were normalized to the percentage of the total pixel density across all six zones. Protected fragments for Fgfr1 and both isoforms of Fgfr3 were detected in all zones of the growth plate (Figure 2, right-hand panel). The IIIc isoform was the predominant isoform of Fgfr3 at the message level and both isoforms followed the same gradient seen in the Northern blot (Figure 2, left-hand panel). Absolute values for the Fgfr3 IIIb isoform were one-tenth of the values for the IIIc isoform. PhosphoImager analysis, which allowed band-density quantification, revealed that the ratio of the IIIc isoform to the IIIb isoform remained constant in all zones of the growth plate (results not shown). The message levels for Fgfr1 on the other hand remained relatively uniform in all zones of the growth plate (Figure 2, right-hand panel).

Three different antibodies were raised against the extracellular domain of bovine FGFR3 using peptides from exon 6 (E6, common to both isoforms), exon 8 (E8, IIIb-specific) and exon 9 (E9, IIIc-specific). Recombinant FGFR3 produced as fusion proteins coupled to MBP in the prokaryotic pMal-c2 system validated the specificity of the antibodies for FGFR3. Since the bovine sequence used for generation of the E8 and E9 antibodies

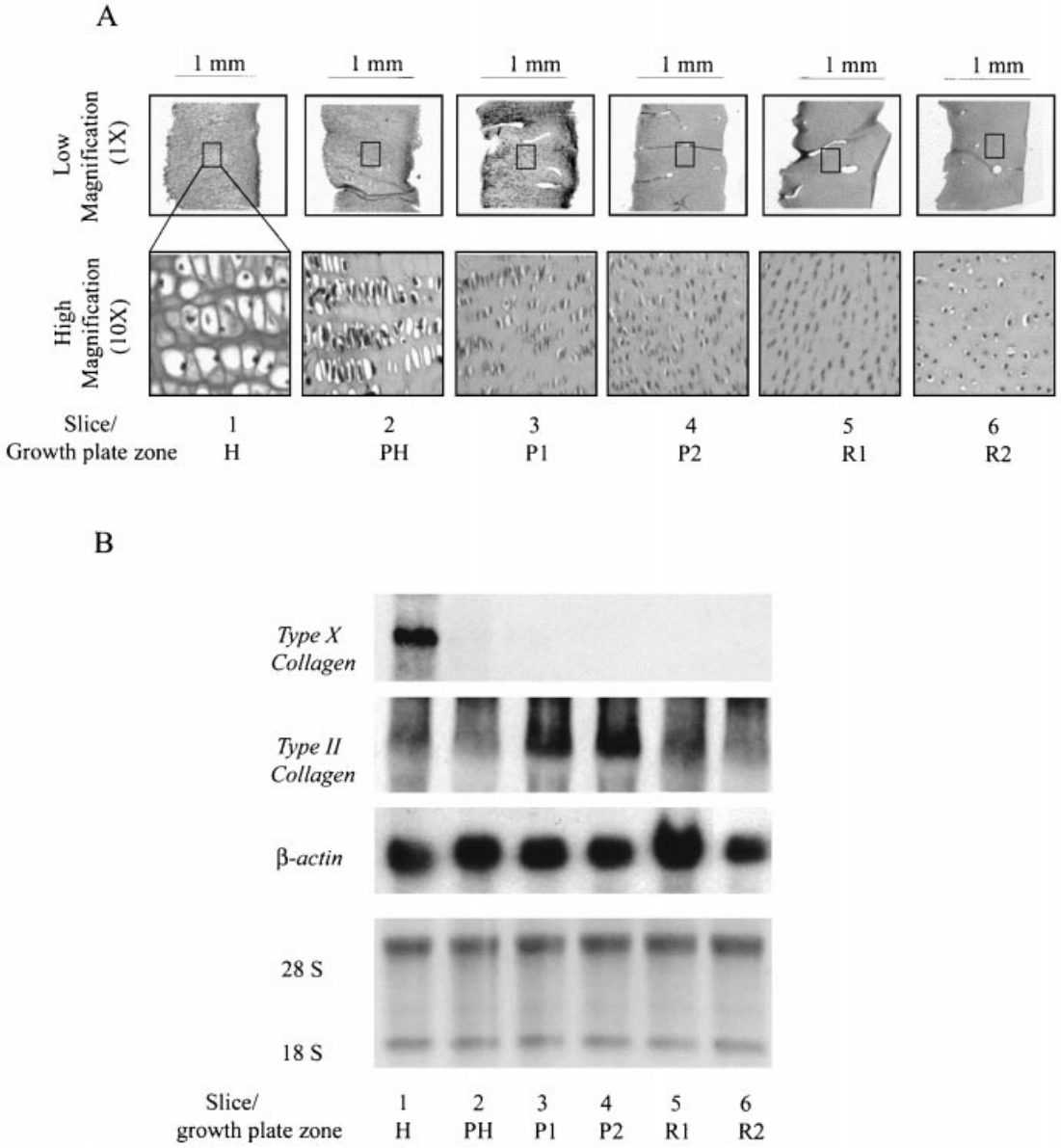

Figure 1 Analysis of 1 mm vibratome sections of bovine growth-plate zones

(**A**) Slices (1 mm) of bovine growth plate were paraffin-embedded, sectioned, stained with haematoxylin and eosin and photographed under low ($\times 1$) and high ($\times 10$) magnifications. (**B**) Total RNA isolated from the six slices of bovine growth plate was hybridized with random-prime labelled type-II collagen, type-X collagen and β-actin probes. Message for type-X collagen was observed only in slice 1, or the hypertrophic zone of the growth plate, while type-II collagen was expressed in all zones of the growth plate, with higher levels in the proliferating zones (slices 3 and 4). Message levels for β-actin acted as control, indicating relative levels of RNA between the different zones. The blot was stained with Methylene Blue (for 18 and 28 S RNA bands) after capillary transfer, and shows uniform transfer and loading of the RNA samples. Slice 1, H, hypertrophic zone; slice 2, PH, prehypertrophic zone; slices 3 and 4, P1 and P2, proliferative zones; slices 5 and 6, R1 and R2, reserve zones.

is 100 % homologous to the murine FGFR3 sequence, we excised regions of murine FGFR3 IIIb and IIIc sequences from MomFR3SV (two vectors, one each for IIIb and IIIc) [18] and ligated into pMal-c2. A bovine cDNA encoding a part of exon 6 and exon 7 of *Fgfr3* was used to produce another small recombinant *Fgfr3*, and this was used to test the immunoreactivity of antibody E6. After induction with IPTG, pMal-c2 produced a 50.8 kDa MBP. Plasmid E67R3-pMal (encoding a fusion protein of part of exons 6 and 7 of FGFR3 and MBP) produced a 62 kDa E6R3-MBP fusion protein, and plasmids E8R3-pMal and E9R3-pMal (encoding fusion proteins of FGFR3 exons 8 and 9, respectively, and MBP) generated 110 kDa E8R3-MBP and E9R3-MBP fusion proteins.

Affinity-purification of the E8R3-MBP and E9R3-MBP fusion proteins revealed lower-molecular-mass fragments of the fusion proteins, which indicated that these represent degradation products of the 110 kDa FGFR3-MBP protein (Figure 3). All three FGFR3 antibodies recognized the FGFR3 region of the fusion protein and did not detect MBP alone (Figure 4, top panel, compare induced pMal-c2, lanes 2, 6 and 10, with induced R3-pMal, lanes 4, 8 and 12). Absence of cross-reactivity of the isoform-specific E8 and E9 antibodies for the other isoform was

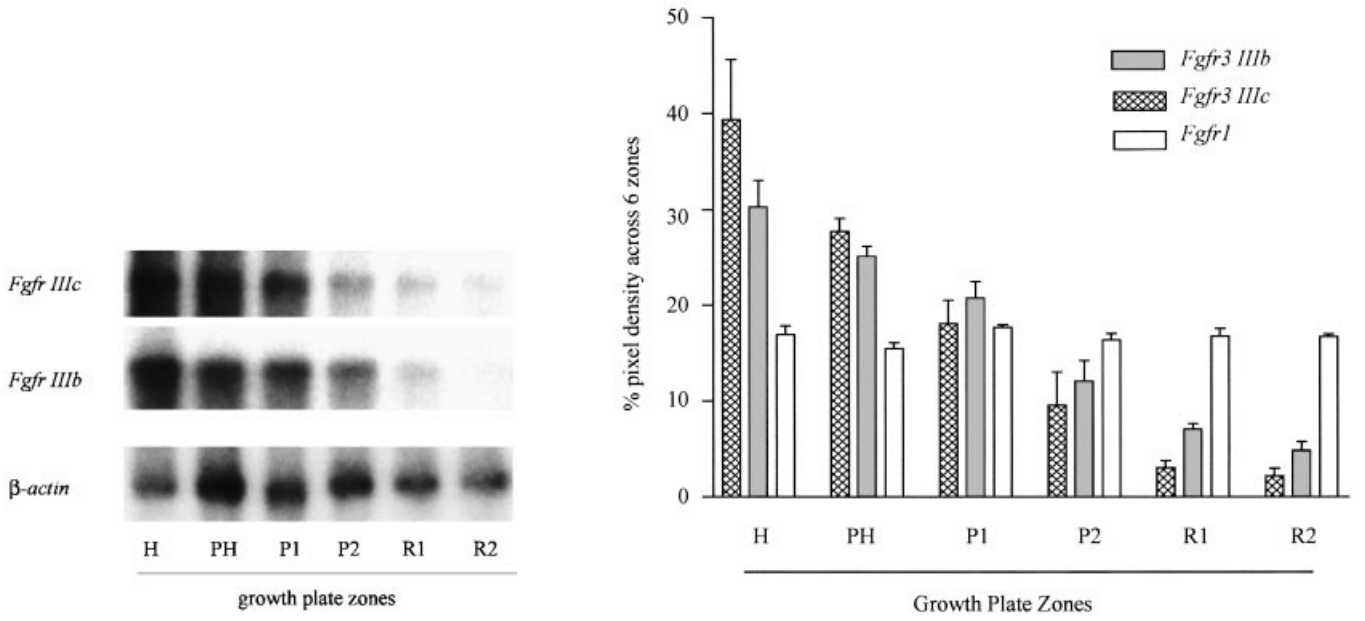

Figure 2 Message levels of Fgfr-3 isoforms in bovine fetal rib growth-plate zones

Left-hand panel: total RNA (2 μg) from each growth-plate zone was separated on a 1.1% formaldehyde/agarose gel, blotted and hybridized with random-prime labelled *Fgfr3 IIIc*, *Fgfr3 IIIb* or β-actin cDNA probes. A single mRNA transcript of 4.5 kb was detected for *Fgfr3 IIIb* and *IIIc* isoforms and a 2.0 kb transcript was detected for β-actin. The highest message level for Fgfr3 IIIb and IIIc isoforms was seen in the hypertrophic zone, which decreased towards the reserve zone. The blots were performed in triplicate and a representative blot is shown. Right-hand panel: 5 μg of total RNA from each zone of the growth plate was used in an RNase-protection assay for the Fgfr3 isoforms and Fgfr1. The protected fragments were separated on a 6% polyacrylamide/urea gel, analysed by autoradiography and quantified by densitometry. The values for the receptors were normalized to the percentage of the total absorbance across all six zones. The highest levels of Fgfr3 are observed in the hypertrophic zone, whereas the message level for Fgfr1 showed a uniform distribution throughout the growth plate. The absolute values for Fgfr3 IIIb were approx. 10% of the values obtained for Fgfr3 IIIc. Error bars indicate S.D. of four separate RNase-protection assays. For zone definitions, see Figure 1.

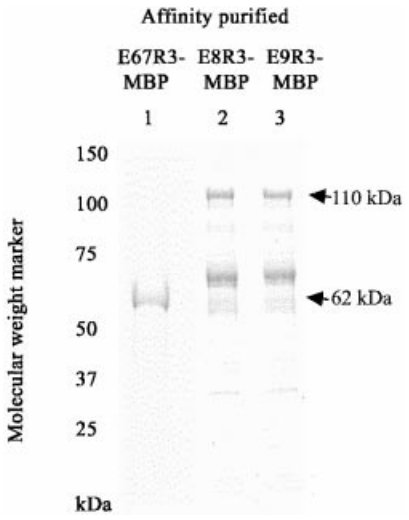

Figure 3 Affinity-purified FGFR3 fusion proteins

Coomassie Brilliant Blue-stained SDS/polyacrylamide gel of affinity-purified FGFR3-MBP fusion proteins using amylose resin. Cells transformed with R3-pMal and induced with IPTG produced FGFR3-MBP fusion proteins of 62 kDa (E67R3-MBP) and 110 kDa (E8R3-MBP and E9R3-MBP). The lower-molecular-mass bands of 60–70 kDa indicate degradation of the FGFR3-MBP proteins.

an indication of the specificity of the antibodies (Figure 4, bottom panel). This confirmed that the antibodies were able to recognize the peptide sequence in FGFR3 proteins, and this specificity provided a means to affinity-purify the antibodies from the antisera.

To examine whether the protein expression of FGFR3 in extracts from different regions of the growth plate follows the same pattern as its gene expression, Western blotting was carried out using the affinity-purified peptide antibodies. The expected full-length 135 kDa FGFR3 (Figure 5, arrow) was detected in the reserve and proliferative zones but was completely absent in the prehypertrophic and hypertrophic zones using all three FGFR3 antibodies (Figure 5). A 98 kDa (Figure 5, double arrowhead) form of FGFR3 was found to be the predominant species in both isoforms and was present in high amounts in the hypertrophic zone. Since the Northern-blot analysis detected only a single species of mRNA for the FGFR3 isoforms, it is unlikely that the 98 kDa form is a novel alternatively spliced isoform of FGFR3. The E8 antibody detected a 47 kDa species (Figure 5B, triple arrowhead) of FGFR3, which comprised most of the FGFR3 IIIb isoform seen in the hypertrophic zone (Figure 5C). The C-terminus-specific antibody (C15sc123) detected the 135 kDa FGFR3 in the reserve zone, the 98 kDa FGFR3 predominantly in the hypertrophic zone and shorter fragments in a gradient, with higher levels in the hypertrophic zone (results not shown).

The 98 kDa form of FGFR3, which was recognized by all the FGFR3 antibodies, appeared consistently. This form is susceptible to N-glycanase treatment, which indicates that it has N-linked oligosaccharides (results not shown) and is likely to be a form of the receptor truncated at the N-terminus. There was a gradual increase of lower-molecular-mass fragments of FGFR3 in the hypertrophic and prehypertrophic zones of the growth plate compared with the proliferating and reserve zones; these

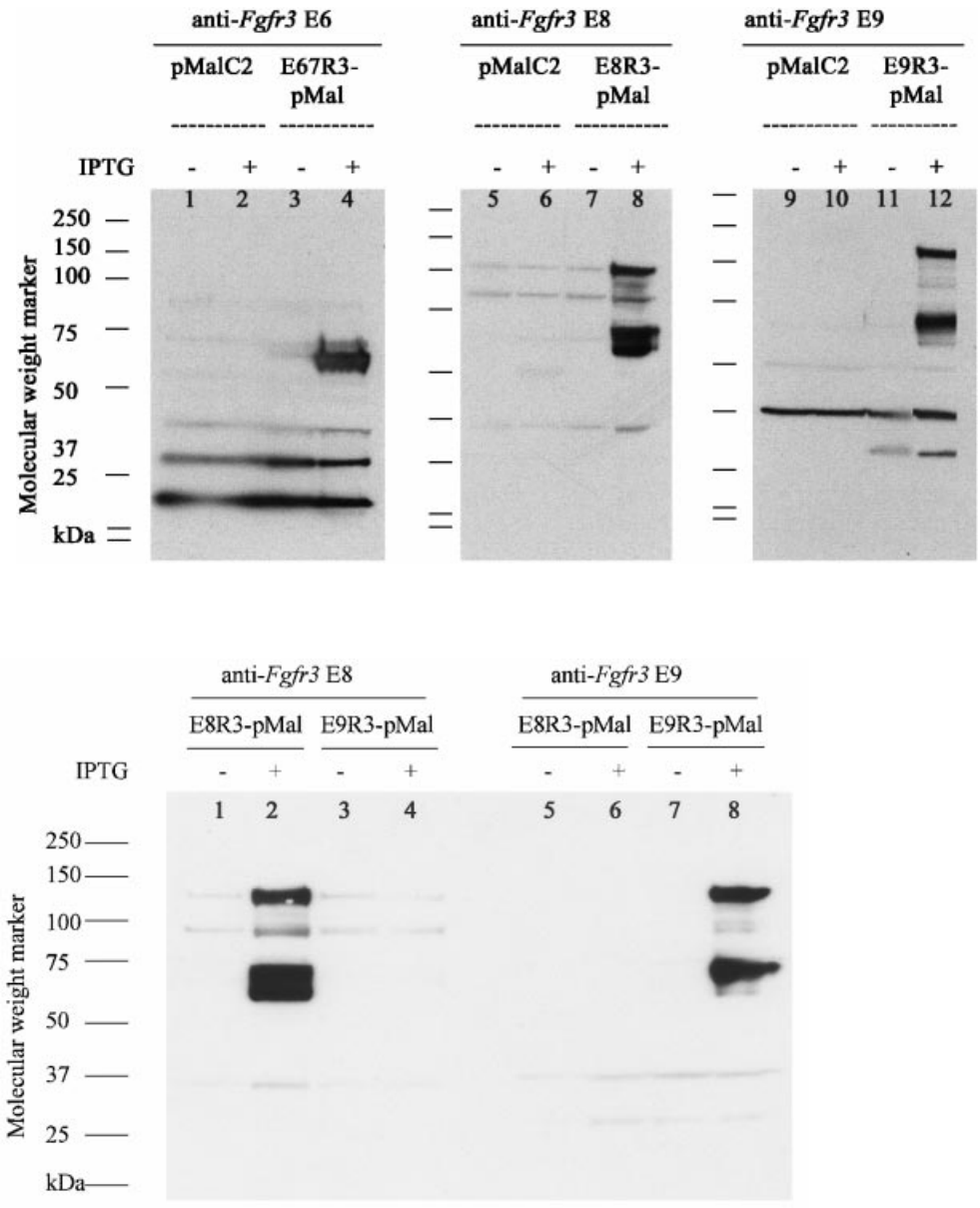

Figure 4 Western-blot analysis of FGFR3 fusion proteins

Top panel: an extract of *E. coli* transformed with vector pMal-c2 or R3-pMal was analysed using SDS/PAGE and Western blotting with antisera raised against peptide E6 (exon 6), peptide E8 (exon 8, IIIb-specific) and peptide E9 (exon 9, IIIc-specific). Antibody E6 recognized the 62 kDa E67R3-MBP fusion protein (lane 4), antibodies E8 and E9 recognized the 110 kDa E8R3-MBP and E9R3-MBP fusion proteins respectively (lanes 8 and 12) upon induction with IPTG. The antibodies did not recognize MBP (lanes 2, 6 and 10). Bottom panel: anti-FGFR3 E8 antibody recognized the E8R3-MBP fusion protein upon induction with IPTG (lane 2), but did not recognize the E9R3-MBP fusion protein (lane 4). Anti-FGFR3 E9 antibody recognized the E9R3-MBP fusion protein (lane 8) but not the E8R3-MBP protein (lane 6), indicating the specificity of the antibodies for the isoforms. Lower-molecular-mass proteins in lanes 2 and 8 indicate degradation of the 110 kDa R3-MBP fusion protein.

are probably products of proteolytic cleavage of the full-length receptor. All the FGFR3 antibodies detected these fragments, although there was a difference in their relative intensities. We examined the FGFR3 isoforms in fetal articular cartilage, which expresses high levels of message for Fgfr3, to determine whether FGFR3 undergoes cleavage as observed in the growth plate. Western-blot analysis using the two isoform-specific antibodies revealed that most of the FGFR3 in fetal articular cartilage is represented by the 135 kDa intact receptor (results not shown).

To investigate whether the fragments of FGFR3 are a result of cleavage of the receptor close to the membrane on the extracellular surface, the growth plate was extracted with 3.4 M NaCl followed by treatment with 2 M urea. The shorter fragments detected by antibodies to the extracellular domain were easily extracted with 3.4 M NaCl (Figure 6, lanes 1, 3 and 5), whereas the 78, 98 and 135 kDa forms of FGFR3 detected by all four antibodies required more stringent extraction with 2 M urea (Figure 6, lanes 2, 4, 6 and 8). The shorter fragments of FGFR3

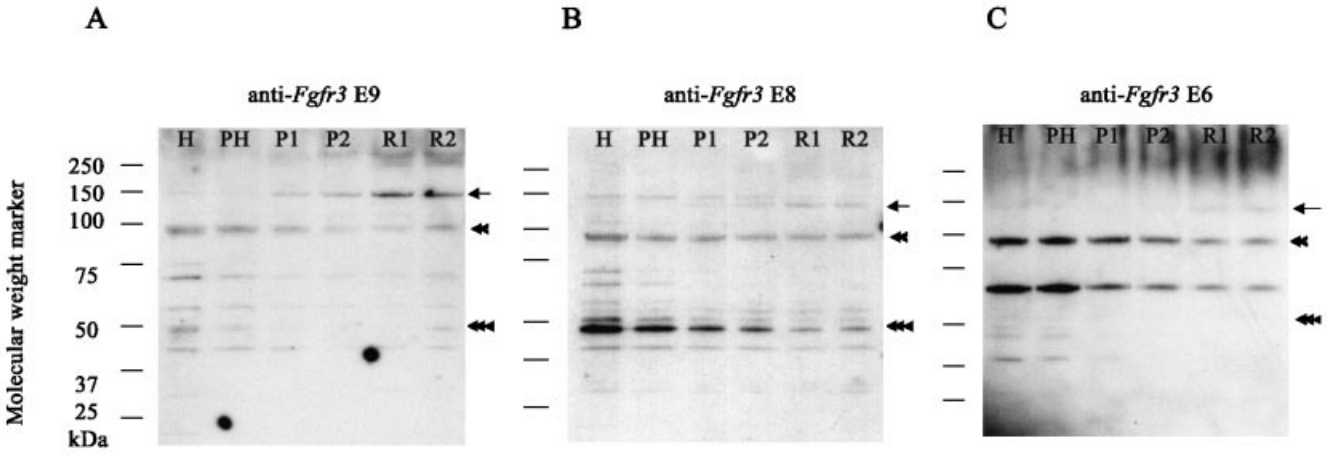

Figure 5 SDS/PAGE and Western-immunoblot analysis of FGFR3 protein expression in rib growth plate

Total protein (15 μg) extracted from each zone of the growth plate was analysed by Western blotting on 4–12% Bis-Tris gels. Anti-FGFR3 antibodies (E6, E8 and E9, affinity-purified using FGFR3-MBP fusion proteins) against the extracellular region of FGFR3 were used to detect the total FGFR3 protein content in growth-plate samples. Full-length FGFR3 receptor (135 kDa, arrow) was observed in the reserve and proliferating zones as a gradient, with undetectable levels in the hypertrophic zone. Several lower-molecular-mass proteins, including a major 98 kDa form (double arrowhead) and a 48 kDa form (triple arrowhead) increased in a gradient from the reserve to the hypertrophic zone. See Figure 1 for zone definitions.

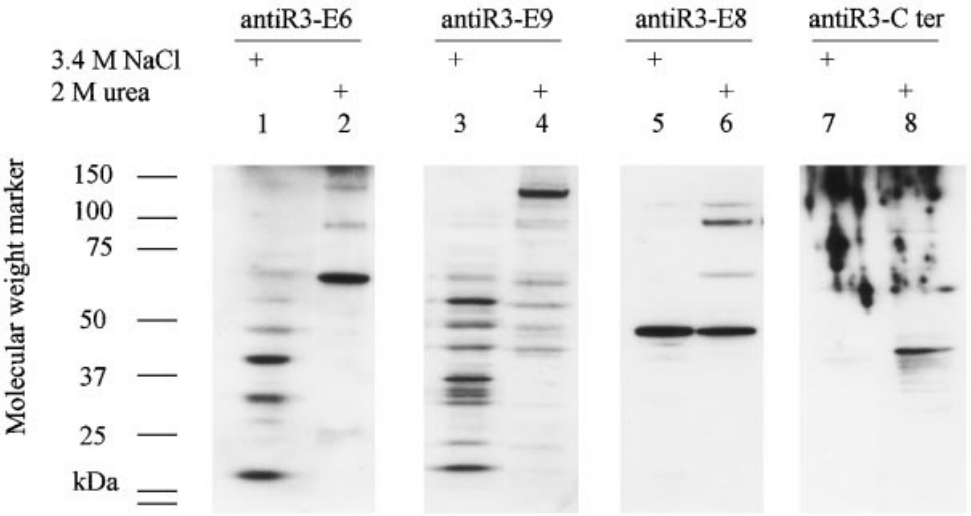

Figure 6 SDS/PAGE and Western-blot analysis of FGFR3 in 3.4 M NaCl and 2 M urea extracts of the growth plate

Total protein (15 μg) from 3.4 M NaCl and 2 M urea extracts were analysed by Western blotting using anti-FGFR3 antibodies. Shorter fragments of FGFR3 are detected in the 3.4 M NaCl extract with antibodies against the extracellular domain of FGFR3 (lanes 1, 3 and 5) and in the 2 M urea extract with the intracellular FGFR3-specific antibody (lane 8). The 135, 98 and 70 kDa fragments of FGFR3 were observed specifically in the 2 M urea extracts and were detected by all antibodies (lanes 2, 4, 6 and 8).

that were detected by the C-terminus-specific antibody were resistant to extraction by 3.4 M NaCl and were extracted only with 2 M urea (Figure 6, lane 8). Extractions using protease inhibitors gave similar results. NaCl extracts of the hypertrophic zone showed higher amounts of FGFR3 fragments, as compared with extracts of the reserve zone (results not shown). This suggests that the lower-molecular-mass fragments of FGFR3 extracted with 3.4 M NaCl are soluble and are released into the extracellular matrix after cleavage of the membrane-embedded receptor. This is supported by the observation that these fragments were detected by the antibodies against the extracellular domain but not by the C-terminus-specific antibody. The short fragments could only be detected using the C15sc123 antibody when extracted with 2 M urea, which suggests that these might be the transmembrane fragments left after cleavage of the extracellular domain of the receptor. These results indicate that the full-length FGFR3 is present only in the reserve and proliferating zones of the growth plate and that it is cleaved increasingly towards the hypertrophic zone.

In order to investigate whether our N-terminus-specific antibodies would detect FGFR3 fragments in the extracellular matrix of the chondrocytes, we used these antibodies on growth-plate

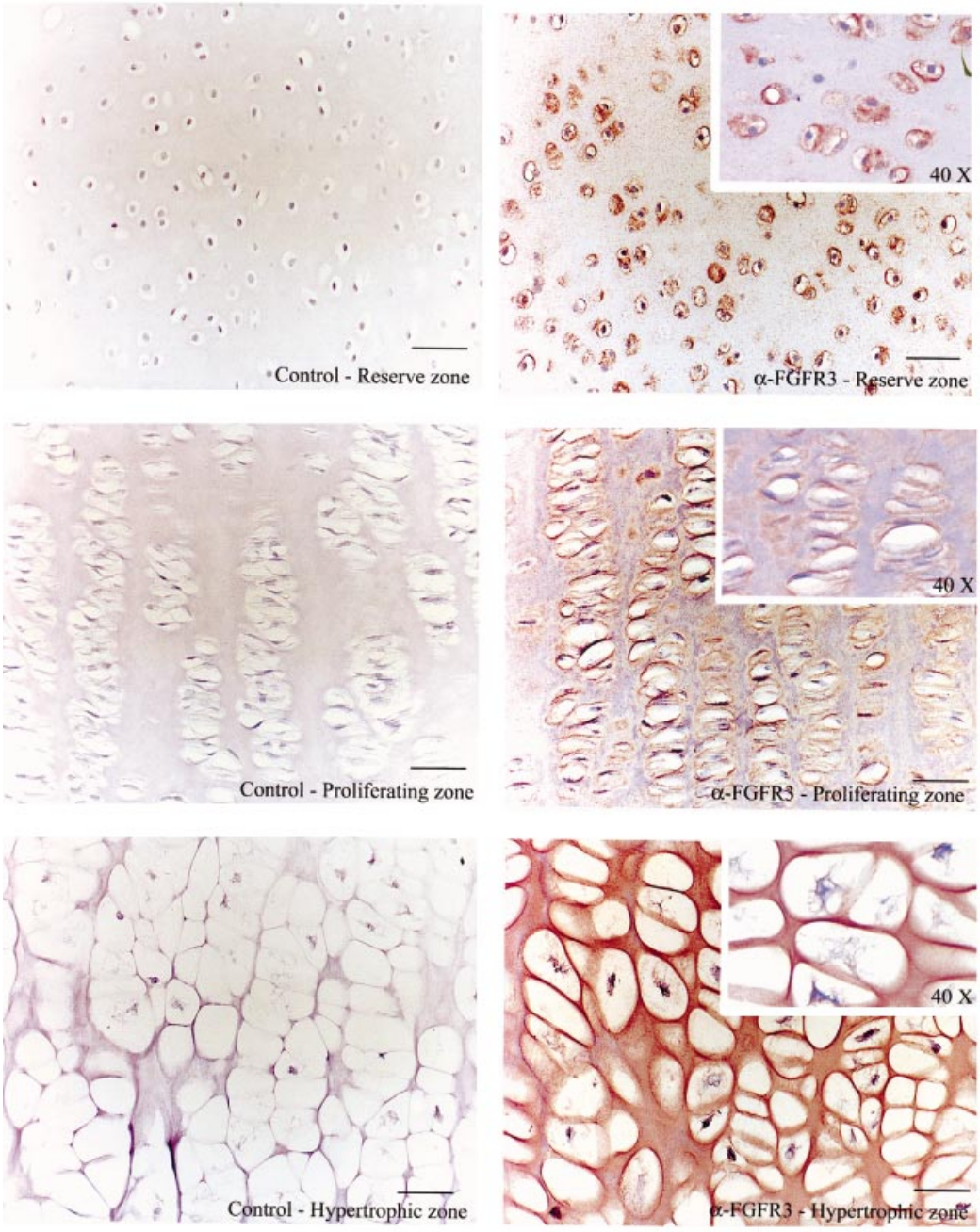

Figure 7 Immunohistochemistry for localization of *Fgfr3* in bovine rib growth plate

De-paraffinized longitudinal sections of growth plate were treated with 5 m-units of chondroitinase ABC, for 3 h at 37 °C. Sections were then incubated overnight with either pre-immune serum or serum from rabbits immunized with FGFR3 (common to both isoforms), followed by incubation with biotinylated goat anti-mouse antibody and then visualized using a avidin–biotin complex kit. Sections were counterstained with haematoxylin. Localization of antigen (FGFR3) in the section was visualized by appearance of a red colouration. Control slides were incubated with pre-immune serum. Higher magnification ($\times$ 40) in the insets shows the difference in the cellular versus matrix distributions of FGFR3 in the growth plate. FGFR3 was localized primarily in the matrix of the hypertrophic zone, indicating the presence of a soluble extracellular region of FGFR3, whereas the reserve zone had greater staining in the pericellular region, indicating high amounts of intact transmembrane receptor. The proliferative zone showed FGFR3 in the pericellular region as well as in the matrix surrounding the cells (scale bars, 10 μm).

sections in immunohistochemical experiments. The de-paraffinized sections were pretreated with chondroitinase ABC in order to degrade the chondroitin sulphate chains on aggrecan and to expose the epitopes for the antibodies. All three antisera detected a similar pattern of FGFR3 expression in tissue sections. Intense immunoreactivity was observed in the pericellular region of the reserve zone of the growth plate (Figure 7), as compared with the control, which is an indication of membrane localization of the bound receptor. The proliferative cells showed pericellular staining along with diffuse staining in the extracellular matrix. The hypertrophic zone showed strong immunostaining in the matrix surrounding the cells. The staining was specific for

chondrocytes and the matrix surrounding the chondrocytes and did not stain the epithelial cells lining the blood capillaries (results not shown). Pre-immune sera did not stain the tissue sections.

DISCUSSION

Our results indicate that the mRNA for both isoforms of Fgfr3 is found in all zones of the growth plate. The IIIc isoform of Fgfr3 comprises the major portion of the Fgfr3 message. The mRNA for Fgfr3 is expressed as a gradient, with lower levels in the reserve zone and higher levels in the hypertrophic and prehypertrophic zones. This is consistent with the recent observation that high levels of Fgfr3 message are expressed in the hypertrophic and proliferating zones of growth plates of developing mouse embryos and human fetuses [22,23]. Examination of protein levels for FGFR3 confirms that both FGFR3 isoforms are translated and expressed in the growth plate. Furthermore, like the mRNA levels, the protein products are expressed at a higher level in the hypertrophic zone, particularly when detected with E8 and E9 antibodies. However, there is also considerable fragmentation of the receptor in the hypertrophic zone.

Western-blot analysis indicates that there are two distinct gradients for FGFR3 across the zones of the growth plate (Figure 5). The first gradient is observed for the full-length receptor, which is seen to be expressed at the highest level in the reserve zone but decreases through the growth plate and is undetectable in the hypertrophic zone. The second gradient consists of the fragments of FGFR3, which progressively increase from the reserve to the hypertrophic zone. The net result of the two gradients suggests that FGFR3 is produced at high levels in the hypertrophic zone, but that the receptor undergoes rapid degradation to produce 98 and 70 kDa forms of the receptor. Increased degradation is observed in the prehypertrophic and hypertrophic zones. This produces fragments from the extracellular region of the receptor that are released from the cell surface into the extracellular matrix. These results are consistent with those obtained for immunostaining, with more full-length receptor observed on the cell surface of reserve cells and higher levels of immunoreactive material in the extracellular matrix of hypertrophic cells (Figure 7). The message for Fgfr1, the other FGFR in cartilage, indicates that FGFR1 is expressed uniformly in all zones of the bovine rib growth plate (Figure 2, right-hand panel). Earlier reports [24], which indicate Fgfr1 message levels only in the hypertrophic zones of developing mouse embryos, could be due to developmental differences between the two species. However, relative levels of FGFR1 and FGFR3 in the growth plate could not be compared. At the level of expressed sequence tags, *Fgfr3* predominates in fetal cartilage (P. J. Neame, unpublished work). This also confirmed that the antibodies raised were specific to FGFR3 and ruled out the possibility of their binding to FGFR1.

The pattern of FGFR3 fragments detected by the E6 antibody, which recognizes both isoforms of FGFR3, did not appear to be the intermediate of those patterns seen with the E8 and E9 antibodies. This may be due to the higher expression levels of the IIIc isoform over the IIIb isoform in the growth plate (compare Figure 5C with 5B). This also could be attributed to the fact that the E6 antibody recognizes FGFR3 fragments with higher affinity than the E9 antibody. When the fragments were enriched, as seen in the 3.4 M NaCl extract, the FGFR3 fragments detected by the E6 and E9 antibodies appeared to be similar (Figure 6, lanes 1 and 2), thus indicating that the IIIc isoform is the predominant form of FGFR3 in the growth plate.

The data in the present study make it possible to speculate that the full-length 135 kDa receptor undergoes proteolytic degradation/cleavage soon after it is synthesized by the chondrocytes in the growth plate. This cleavage is probably in the most N-terminal Ig-like domain of the receptor (Figure 8), as the 98 kDa receptor still has N-linked oligosaccharides and is not extracted by 3.4 M NaCl (Figure 6). The 98 and 70 kDa forms of FGFR3 are truncated transmembrane forms of the full-length

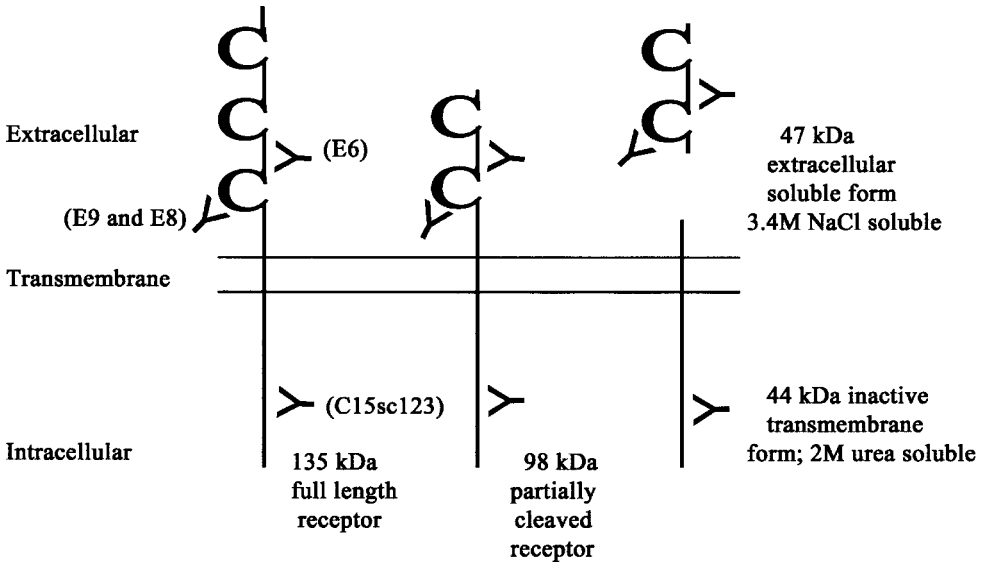

Figure 8 Probable structure of FGFR3 on chondrocytes of the growth plate and the location of the peptides used for raising FGFR3-specific antibodies

Antibody E6 was raised against the peptide from exon 6 located in the region between Ig loops 2 and 3, and antibodies E8 and E9 were raised in the third Ig-like domain encoded by exons 8 (IIIb-specific) and 9 (IIIc-specific) of FGFR3. The C-terminal antibody was directed towards a peptide in the intracellular region of the growth plate. The different forms of the receptor in the growth plate include the 135 kDa FGFR3 with three Ig-like loops, the 98 kDa clipped FGFR3 with a partial loss of the Ig-like domain, the 47 kDa soluble extracellular form of FGFR3 and the 44 kDa transmembrane C-terminal fragment left behind on the cell surface after proteolytic cleavage of the receptor.

receptor, because they can be detected by the C-terminus-specific antibody. The receptor undergoes further proteolysis close to the transmembrane domain, in the extracellular domain of the receptor, releasing 48 kDa fragments into the extracellular matrix of the chondrocytes. This leaves behind the transmembrane and cytoplasmic C-terminal region of the receptor on the chondrocytes, which was detected by the C-terminus-specific antibody (Figure 6, lane 8). Cleavage of the receptors to produce soluble receptor fragments has been observed with FGFRs in other tissues. There are several reports of soluble forms of the extracellular domains of FGFR1 in extracellular matrix of vascular endothelial cells, blood, human vitreous fluid and human cerebral spinal fluid. It has been proposed that these soluble fragments of the receptor play a regulatory role, perhaps in controlling biological availability of FGFs, in these tissues [25–28].

Mouse models developed with null mutations in matrix metalloproteases (MMPs) have been shown to exhibit abnormal patterns of skeletal growth-plate development [29–31]. MMP-2, -9 and -13 have been shown to be associated with growth-plate cartilage and MMP-9 and -13 are mainly involved in apoptosis and angiogenesis [30,32]. There do not appear to be any MMP-2 sites in FGFR3 or FGFR1, similar to the FFG sequence recognized in, for example, aggrecan [33]. However, the growth plate might contain other potential proteases that could target the receptor for cleavage.

Comparison of the amino acid sequence of the extracellular domains of FGFR3 and FGFR1 indicates a high degree of homology. For the extracellular domain of the receptor to be released *in vivo*, the cleavage site is most likely to occur between the ligand-binding domain and the cell membrane, rather than within the globular domains. The major soluble fragment is 48 kDa, suggesting that this is the extracellular domain. There is a potential glutamate-rich cleavage site near the membrane-insertion site in FGFR3 (VVLPAEEELVEA) which is not conserved in FGFR1. Further analysis of this could be achieved by preparation of modified recombinant forms of the receptor.

Whereas there are no data to support this, we propose that the soluble forms of FGFR3 in the developing growth plate may play a role in sequestering the ligand, thus reducing the activation of the receptor and transmitting signal across the cell membrane. The second and third Ig-like domains of FGFRs are responsible for receptor–ligand interaction [34–36]. Since the receptor in the reserve and proliferating zones is primarily the full-length form or the 98 kDa form, both can still bind FGFs. However, in the prehypertrophic and hypertrophic zones of the growth plate very little receptor is intact. We have observed FGF2 in NaCl and urea extracts of the reserve zone (results not shown), indicating that FGFs are available and may interact with soluble receptors in the extracellular matrix. The increase in the soluble extracellular ligand-binding fragments of FGFR3 could trap FGFs in the matrix, thus controlling availability of the FGFs to the receptor on the cell surface. This would mean that increased levels of FGF could overcome this regulatory mechanism. Indeed, it has been observed that overexpression of FGF1 and FGF9 (other ligands for FGFR3) in transgenic mice [12,13] produces a phenotype similar to the FGFR3 mutations. These mouse models mimic FGFR3 mutation by saturating the receptor with excess ligand. Recently, disruption of perlecan (a heparan sulphate proteoglycan) [23] in mice has also been reported to cause a phenotype similar to mice expressing FGFR3 mutations in achondroplasia and thanatophoric dysplasia. It has been hypothesized that perlecan might play a role in binding and sequestering FGFs in the cartilage matrix, thus regulating activation of FGFRs. The FGFR3 fragments could play a similar role and might be involved in modulation of receptor activation, thus allowing for precisely controlled proliferation and hypertrophy in normal developing growth plate. The interplay of intact receptors of different types, their matrix-associated fragments, their relative affinities and the abundance of ligands may result in complex and subtle regulation of cell phenotype.

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