Characterization of FGFRL1, a Novel Fibroblast Growth Factor (FGF) Receptor Preferentially Expressed in Skeletal Tissues*

Beat Trueb‡, Lei Zhuang, Sara Taeschler, and Markus Wiedemann

From the ITI Research Institute, University of Bern, P. O. Box 54, CH-3010 Bern, Switzerland

Received for publication, January 10, 2003, and in revised form, May 16, 2003
Published, JBC Papers in Press, June 17, 2003, DOI 10.1074/jbc.M300281200

Clones for a novel transmembrane receptor termed FGFRL1 were isolated from a subtracted, cartilage-specific cDNA library prepared from chicken sternum. Homologous sequences were identified in other vertebrates, including man, mouse, rat and fish, but not in invertebrates such as Caenorhabditis elegans and Drosophila. FGFRL1 was expressed preferentially in skeletal tissues as demonstrated by Northern blotting and in situ hybridization. Small amounts of the FGFRL1 mRNA were also detected in other tissues such as skeletal muscle and heart. The novel protein contained three extracellular Ig-like domains that were related to the members of the fibroblast growth factor (FGF) receptor family. However, it lacked the intracellular protein tyrosine kinase domain required for signal transduction by transphosphorylation. When expressed in cultured cells as a fusion protein with green fluorescent protein, FGFRL1 was specifically localized to the plasma membrane where it might interact with FGF ligands. Recombinant FGFRL1 protein was produced in a baculovirus system with intact disulfide bonds. Similar to FGF receptors, the expressed protein interacted specifically with heparin and with FGF2. When overexpressed in MG-63 osteosarcoma cells, the novel receptor had a negative effect on cell proliferation. Taken together our data are consistent with the view that FGFRL1 acts as a decoy receptor for FGF ligands.

Most bones of the vertebrate skeleton are formed by a complex process termed endochondral ossification which involves a cartilage intermediate (1). This intermediate represents a highly specialized connective tissue. It consists of a single cell type, the chondrocytes, which are embedded in a rich extracellular matrix (2). Typically, this matrix makes up more than 90% of the cartilage volume and consists of collagens (types II, IX, X, and XI), proteoglycans (aggrecan, small leucine-rich proteins), and glycoproteins (matrilins, COMP).

During the first step of endochondral ossification, mesenchymal cells condense and differentiate into chondrocytes (3). These chondrocytes proliferate rapidly and lay down the cartilaginous model of the future bones. The chondrocytes undergo a complex series of distinct developmental stages, including proliferation, maturation, and hypertrophy. The hypertrophic cartilage is calcified and becomes vascularized. Finally, the calcified cartilage is invaded by osteoclasts and osteoblasts, which replace the cartilaginous tissue by bone.

Cartilage has become a popular tissue to study cell proliferation and differentiation in vitro (3). When cultivated on plastic dishes, chondrocytes rapidly dedifferentiate into fibroblast-like cells. In three-dimensional matrices, however, the chondrocytes undergo the ordered sequence of events observed during differentiation and maturation of cartilage in vitro. Three stages of chondrocyte differentiation have been defined in vitro: proliferative chondrocytes producing mainly collagen II, hypertrophic chondrocytes producing collagen X, and osteoblast-like cells producing collagen I and alkaline phosphatase.

Many vitamins, hormones, and growth factors are involved in the regulation of chondrocyte proliferation and differentiation (4). The mechanisms, how these substances act on gene expression, however, are not yet understood in detail. Vitamin D₃, ascorbic acid, and retinoic acid as well as the growth factors FGF,¹ TGF-β, bone morphogenetic protein (BMP), and insulin-like growth factor (IGF) play critical roles during endochondral ossification. Indian hedgehog (Ihh) and the parathyroid-hormone related peptide (PTHrP) constitute a paracrine feedback loop that regulates differentiation of proliferative chondrocytes into hypertrophic cells in the growth plate of long bones. At the level of gene expression, the transcription factors Sox9, L-Sox5, and Sox6 play an important role in the determination of the cartilage cell lineage (5). In particular, Sox9 appears to act as a key differentiation factor for chondrocytes analogous to the way that MyoD acts as a master gene during muscle differentiation.

We have recently set out to identify and characterize novel cartilage proteins by a subtractive cDNA cloning approach (6, 7). Special emphasis was put on regulatory proteins that might play a role during chondrocyte proliferation and differentiation. Two cDNA libraries were constructed with mRNA from cartilage and subtracted with mRNA from skin or skeletal muscle. These libraries comprised many clones for known cartilage-specific proteins. In addition, our libraries contained several novel clones whose sequences have not yet been stored in public data banks. Some of the novel clones coded for a structural protein that was highly related to the family of the matrilins (6, 7). Another set of clones was found to code for a novel transmembrane protein related to members of the fibroblast growth factor receptor family. Here we describe the characterization of this novel membrane protein and demonstrate that it represents a new player in the FGF signaling system.

The abbreviations used are: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FGFRL, fibroblast growth factor receptor-like; GFP, green fluorescent protein, IL-1, interleukin-1; TGF-β, transforming growth factor β.

* This work was supported by the Swiss National Science Foundation (Grants 31-61296.00 and 31-102251), the Swiss Cancer Liga (Grant OCS 1211-02-2002), and by the Swiss Foundation for Research on Muscular Diseases. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ535114 and AJ536020.

1 To whom correspondence should be addressed: ITI Research Inst., University of Bern, P.O. Box 54, CH-3010 Bern, Switzerland. E-mail: beat.trueb@iti.unibe.ch.

This paper is available on line at http://www.jbc.org

33857
Characterization of FGFR1

EXPERIMENTAL PROCEDURES

RNA Extraction and Northern Blotting—Total RNA was isolated from various sources by the guanidinium thiocyanate method (8) utilizing RNAzol reagent (Biogenesis, Beverly, MA). A tissue kit from Qiagen (Hilden, Germany) was used for the preparation of embryonic or adult tissues. The RNA was precipitated in lysis buffer and extracted once with an equal volume of phenol/chloroform. Following isolation on RNase-free columns, the purified RNA was resuspended in 10 mM NaCl and tested for the presence of 1% formaldehyde and transferred to nylon membranes by vacuum blotting. Radioactively labeled cDNA probes were hybridized at 42°C to the membranes in a buffer containing 50% formamide as described (9, 10). Following stringent washing, the membranes were exposed to x-ray film.

Subtracted cDNA Libraries—Poly(A) RNA was purified from total RNA by chromatography on oligo(dT)-Sepharose (Amersham Biosciences). Two subtracted cDNA libraries were constructed by the biotin/streptavidin/phenol method (10, 11). In brief, the poly(A) RNA isolated from embryonic chicken sterna and from embryonic chicken control tissues (skin and skeletal muscle) was transcribed separately into double stranded cDNA and provided with specific adapters. An adapter with an internal SfiI restriction site was used for the cDNA from sterna, whereas a biotinylated adapter without restriction site was used for the cDNA from the control tissues. The cDNAs were amplified by PCR with primers designed according to the adapter sequences. The cDNA preparation from sterna was then hybridized overnight at 68°C with a 10-fold excess of biotinylated cDNAs from the control tissues. All hybrids containing at least one biotinylated strand were purified by extraction with phenol in the presence of streptavidin. After a second round of hybridization and extraction, the material remaining in the aqueous phase was amplified by PCR to repopulate the cDNAs from cartilage. The entire procedure was repeated a total of three times and then the products were ligated into the SfiI restriction site of the cloning vector pUC13. The plasmids were transfected into competent bacteria and plated onto selective agar plates. The inserts from clones of interest were radioactively labeled and used as probes to screen a conventional cDNA library, which had been generated from the cartilage of embryonic chicken sterna (6, 9). The DNA sequences of the inserts were determined by the dideoxy chain termination method. All sequences were analyzed with the software computer package of the genetics computer group at the University of Wisconsin.

In Situ Hybridization—In situ hybridization studies were performed essentially as described (9, 12) utilizing labeled RNA probes. Samples from 17-day-old mouse embryos were embedded in paraffin and cut into serial sections. Riboprobes were transcribed from the mouse FGFR1 cDNA sequence (nucleotides 681—1417), which had been cloned into the vector pGK, with RNA polymerase T7 (Pharmacia) or SP6 (sense) in the presence of 5’-32P-CTP or 5’-32P-UTP. The probes were purified by gel filtration and separated on a 5% polyacrylamide gel to confirm size and purity. The tissue sections were digested with proteinase K and hybridized with the probes at a concentration of ~5 × 107 dpm/ml for 18 h at 60°C. Following hybridization, the slides were treated with RNAse A and washed in 0.1 x standard saline citrate at 65°C for 2 h. The slides were then coated with NTB-2 emulsion, exposed for 3 days at 4°C, and developed with D-19 developer and fixer (Eastman Kodak Co.). Following staining with hematoxilin and eosin, the slides were inspected under a Nikon Eclipse E1000 microscope equipped with dark field optics.

Green Fluorescent Protein (GFP) Fusion Protein Expression—MG-63 and COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown under an atmosphere of 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 9% fetal bovine serum. The cDNA sequence of mouse FGFR1 corresponding to amino acid residues 1–468 was subcloned into the SacII/BamHI restriction site of the GFP expression vector pEGFP-N1 (Clontech). Likewise, the sequence for the intracellular domain (residues 493–1355) was ligated into the HindIII/KpnI site of the expression vector pEGFP-C3. The reading frame of the resulting constructs was verified by DNA sequencing. The plasmids (1 μg/well) were mixed with Opti-MEM 1 (Invitrogen) containing 3 μl of FuGENE 6 reagent (Roche Diagnostics) and added to cultivated cells that were growing on circular cover slips placed into 6 well plates (13). One and two days after transfection, the slides were inspected under a Zeiss LSM 410 confocal microscope. Analogous experiments were also performed with the human FGFR1 sequence.

Protein Expression in Insect Cells—The extracellular portion of the human and the chicken FGFR1 protein was expressed in insect cells utilizing the BacVector transfection kit from Novagen. The cDNA sequences corresponding to amino acid residues 25–355 of the chicken or 36–368 of the human protein were ligated into the EcoRI/HindIII or BamHI/SacI restriction site, respectively, of the transfer plasmid pBAC-3. This transfer plasmid harbors the polyhedrin promoter and the sequence for the signal peptide of the baculovirus envelope protein gp64 to direct expression of proteins into the secretory pathway of infected insect cells. Furthermore, the fusion protein is expressed together with a His-tag that allows the purification of the recombinant protein on affinity columns. Authenticity and reading frame of the resulting constructs were verified by DNA sequencing. The transfer plasmids (500 ng) were transfected together with the BacVector-3000 Triple Cut virus DNA (100 ng) into Sf9 cells that had been cultivated in Grace Medium (Invitrogen) with 9% fetal bovine serum. Three days after transfection, viral particles of the supernatant were purified to homogeneity by the agarose overlay technique and amplified to a viral titer >107/ml. Recombinant protein was produced by infection of fresh Sf9 cells growing in 75-cm2 flasks with this viral stock utilizing a multiplicity of 1–4 plaque forming units/cell. Two days after infection, the supernatant of the insect cells was harvested, and the expressed proteins were purified by chromatography on nickel agarose (His-select HC nickel affinity gel, Sigma) and/or heparin-Sepharose (Amersham Biosciences) as suggested by the supplier.

Interactions of the Recombinant Protein—Recombinant protein (50 μg) was loaded onto a small heparin-Sepharose column (bed volume: 0.5 ml, Amersham Biosciences) and eluted with 0.5 M NaCl, 0.2% Triton X-100, 50 mM sodium phosphate, pH 8.0. The column was extensively washed with the same buffer, and bound protein was eluted with a linear gradient of 0.15–1.35 M NaCl in a total volume of 22 ml. Fractions of 0.5 ml were collected and checked for their NaCl concentration by measuring the conductivity with a WTW LF330 conductivity meter (Weilheim, Germany). Aliquots (32 μl) from every third fraction were separated on a 10% SDS-polyacrylamide gel, transferred to a nylon membrane, and detected by immunoblotting with an antibody directed against the His-tag of the expressed protein.

To test for a potential interaction with FG, human recombinant basic fibroblast growth factor was radiolabeled to a specific activity of 2000 Ci/mmol (100 μCi/ml, Amersham Biosciences) with 125I (carrier-free) and purified to homogeneity. A 150-μl aliquot of the expressed protein (5 μg) was incubated for 2 h at room temperature with 2.5 μl of labeled FG2 and 25 μl of nickel-agarose (Sigma) in 1 ml of 300 mM NaCl, 0.2% Triton X-100, 2 mg/ml bovine serum albumin, 50 mM sodium phosphate, pH 8.0. In some experiments, the binding reaction was competed with 5 μg of recombinant basic fibroblast growth factor (Roche Applied Science) or with 10 μl of fetuin bovine serum. The beads were collected by centrifugation and washed three times with 1 ml of 300 mM NaCl, 0.2% Triton X-100, 50 mM sodium phosphate, pH 8.0. Bound proteins were eluted with 40 μl of hot SDS sample buffer containing 2% β-mercaptoethanol and analyzed on a 15% SDS-polyacrylamide gel, followed by autoradiography.

Cell Proliferation Assay—A cell proliferation assay kit from Roche Applied Science was used to analyze the effect of FGFR1 on cell growth. MG-63 cells were grown on cover slips to 30% confluence. The cDNA sequences for chicken and mouse FGFR1 were ligated into the eukaryotic expression vectors pcDNA3.1(+).+ and pcDNA3.1(−).− and transfected into the cells as described above. Following transfection, the cells were synchronized by starvation in medium lacking any fetal bovine serum. After 24 h the cells were stimulated to proliferate again by the addition of insulin (0.5 μg/ml), FG2 (5 ng/ml), and heparin (300 ng/ml) in Dulbecco’s modified Eagle’s medium. After 18 h, the cells were labeled with bromodeoxyuridine and fixed with cold glycine ethanol buffer as described by the supplier of the kit. Proliferating cells were visualized by indirect immunofluorescence utilizing anti-bromodeoxyuridine antibody and a secondary, fluorescein-labeled anti-mouse Ig antibody. Cell growth was determined by counting immunoreactive cells in relation to the total cell number.

RESULTS

Cloning of Chicken FGFR1—Our cDNA libraries that had been constructed with mRNA from chicken cartilage and substracted with mRNA from chicken skin and muscle comprised more than 500 clones. About 300 individual clones were analyzed with respect to their insert size (200–600 bp), their DNA sequence, and their hybridization pattern. One-third of these clones showed the expected behavior on a Northern blot: they were expressed in sternal cartilage but not in skeletal muscle (Fig. 1). A high redundancy was observed as the majority of the
latter clones coded for type II collagen, the predominant colla-
gen of cartilage. The remaining cartilage-specific clones coded
for type IX collagen, type XI collagen, aggrecan, link protein,
chondromodulin, and matrilin-3 (Fig. 1). Finally, seven clones
were found to code for short fragments of a novel protein.

The novel clones were utilized as probes to identify overlapping
clones in a conventional cDNA library prepared from
chicken cartilage. Our efforts led to the isolation of 14 cDNA
clones that altogether spanned a cDNA of 6175 nucleotides
(GenBank™ accession number AJ535114). This cDNA con-
tained an open reading frame of 1461 nucleotides that could be
translated into a novel protein of 487 amino acids with a
molecular mass of 54,000 Da. Utilizing the information of the
chicken sequence and various expressed sequence tags, we
were able to clone the homologous proteins from man (Gen-
Bank™ accession number AJ277437), mouse (GenBank™ ac-
cession number AJ293947), and rat (GenBank™ accession
number AJ536020). The sequences for the human and the
mouse protein have already been published as short sequence
papers (14, 15). A strong conservation of the amino acids was
observed when the four protein sequences were compared (Fig.
2). The chicken amino acid sequence shared 74% sequence
identity with the human and 72% identity (78% similarity) with the rat sequence. The mouse sequence
resembled with the human and the chicken sequence. Thus, verte-
brates from fish to man contain a novel, homologous protein
that belongs to the FGFFR family. Lower animals, including
insects and nematodes, do not appear to possess this protein.

Expression of FGFRL1—The expression of FGFRL1 was an-
alyzed on Northern blots containing RNA from various chicken
tissues (Fig. 3). Two bands of similar intensities corresponding
to mRNA species of 7 and 4 kb were detected. The size of the
larger band is consistent with the total length of our cDNA
sequence. The shorter band might represent a mRNA species
that was generated by utilization of an alternative polyadeny-
lation site at position 3416 of our cDNA sequence. This notion is
consistent with the fact that a radioactively labeled probe de-
hybridized with the individual cDNA clones that had been radioactively labeled. The clones coded for fragments of the polypeptide chains from collagens II, IX, XI, aggrecan (AGC1), link protein (LP), chondromodulin (ChM1), matrix Gla protein (MGP), matrilin-3 (Matn3), and the novel FGFRL1 protein as
demonstrated by DNA sequencing. A control blot that was hybridized with radioactively labeled cDNA for 18 S RNA is included.

FGFRL1 during Evolution—A peculiar observation was
made with the murine FGFRL1 sequences. Compared with the
chicken and the human sequences, the rat sequence diverged in
the intracellular, histidine-rich region at residue 475 and
stopped after 54 unrelated residues (Fig. 2). If a single nucle-
otide would be deleted at this position, the reading frame would
change to a highly similar sequence that would end after 21
residues with the motif Y-Q-C as found in the chicken and the
human protein. Nevertheless, the frameshift is real, since it
was confirmed in the mouse sequence. It is therefore likely that
the murine FGFRL1 genes have sustained a frameshift mutation
relatively late during evolution.

The complete genomic sequences of several organisms have
recently been elucidated and deposited in public data banks.
We therefore checked whether other species may also contain a
receptor similar to FGFRL1. Neither the fruit fly Drosophila
melanogaster nor the roundworm Caenorhabditis elegans pos-
sessed any gene that would give rise to a transmembrane protein with three related Ig-like repeats. A similar sequence,
however, was identified in the genome of the pufferfish Fugu
rubripes. This fish contained a gene with six exons that could
be transcribed into a mRNA of ~3000 nucleotides and trans-
lated into a protein of ~500 residues (Fig. 2). The putative fish
protein shared 67–73% sequence identity with FGFRL1 from
chicken, rat and man. It should be noted that the fish sequence
also ended with the peculiar histidine-rich region and the motif
Y-Q-C as the human and the chicken sequence. Thus, verte-
brates from fish to man contain a novel, homologous protein
that belongs to the FGFFR family. Lower animals, including
insects and nematodes, do not appear to possess this protein.

Expression of FGFRL1—The expression of FGFRL1 was an-
alyzed on Northern blots containing RNA from various chicken
tissues (Fig. 3). Two bands of similar intensities corresponding
to mRNA species of 7 and 4 kb were detected. The size of the
larger band is consistent with the total length of our cDNA
sequence. The shorter band might represent a mRNA species
that was generated by utilization of an alternative polyadeny-
lation site at position 3416 of our cDNA sequence. This notion is
consistent with the fact that a radioactively labeled probe de-
hybridized with the very 3‘ end of the total cDNA sequence hybrid-
ized with the 7-kb mRNA species but not with the 5-kb species
(not shown).

The two mRNA species were detected in RNA preparations
from the cartilaginous sterna of 16-day-old chicken embryos
(Fig. 3, left). Very faint bands that became clearly visible after
prolonged exposure were also noticed with RNA preparations
from embryonic femur, skeletal muscle, and heart. In contrast,
RNA preparations from skin, gizzard, liver, and brain did not
reveal any signal. Fairly strong bands were also detected with
RNA preparations from adult chicken sterna (Fig. 3, right). In

Fig. 1. Analysis of cDNA clones from the subtracted cDNA libraries. Total RNA from sternal cartilage (C) and skeletal muscle (M) of
16-day-old chicken embryos was separated on agarose gel and transferred to nylon membranes. The membranes were hybridized with the
individu cDNA clones that had been radioactively labeled. The clones coded for fragments of the polypeptide chains from collagens II, IX, XI,
aggrecan (AGC1), link protein (LP), chondromodulin (ChM1), matrix Gla protein (MGP), matrilin-3 (Matn3), and the novel FGFRL1 protein as
demonstrated by DNA sequencing. A control blot that was hybridized with radioactively labeled cDNA for 18 S RNA is included.
this case, the signal obtained with the cranial portion of the sternum, which is known to contain many hypertrophic chondrocytes in a mineralized matrix, barely differed from that of the caudal portion, which contains proliferative and resting chondrocytes in a non-mineralized matrix. All the other tissues investigated from the adult animal (brain, gizzard, skeletal muscle, calvaria) revealed faint bands that became clearly visible after prolonged exposure. Thus, the FGFRL1 gene is expressed at fairly high level in cartilage and at very low level in many other tissues.

**Tissue Distribution**—The tissue distribution of the FGFRL1 mRNA was further investigated by in situ hybridization on whole body sections of 17-day-old mouse embryos (Fig. 4). Our antisense probe hybridized specifically with a mRNA in all cartilaginous structures. A relatively strong signal was observed in the nasal cartilage, the ribs, and the sternum as well as in the cartilaginous rudiments of developing bones such as the vertebrae and the pelvic bone. Strong expression was also observed in some muscular tissues, including the muscles of the tongue and the diaphragm. In contrast, no signal was detected in the eye, the brain, and the spinal cord. Moreover, the lung and most of the inner organs, including liver, stomach, intestine, and colon, showed very low signal. Hybridization of a consecutive tissue section with our sense probe showed very low background signal, demonstrating the specificity of our probe (Fig. 4).

The cartilaginous vertebrae of a 17-day-old embryo was investigated in greater detail (Fig. 5). Relatively strong expression of FGFRL1 mRNA was observed in the developing vertebral bodies. No differences were noted between the cranial and the caudal portion of the vertebrae. Regions of mineralizing cartilage containing hypertrophic cells showed substantially reduced signal. Likewise, the nucleus pulposus that would later give rise to the intervertebral disc revealed only a weak signal. All the tissues adjacent to the vertebrae, including the spinal cord at the dorsal part and the inner organs at the ventral part, reacted only weakly with our probe. Nevertheless, the signal at these locations appeared to be slightly stronger than the background observed with the sense probe (Fig. 5). These results are consistent with the view that FGFRL1 is
expressed at fairly high level in all cartilaginous tissues of the skeleton as well as in a few specialized muscles and at very low level in several other tissues.

Subcellular Localization—As a receptor for growth factors, the FGFRL1 protein should be located at the plasma membrane. To study the subcellular distribution, we fused the cDNA sequence for mouse and human FGFRL1 to the sequence for GFP and transfected the resulting constructs into human (MG-63) and monkey (COS-1) cells. When inspected by confocal microscopy, the majority of the signal emitted from GFP was found to be distributed along the plasma membrane (Fig. 6). Some signal could also be detected at compartments of the secretory pathway (Golgi, secretory vesicles), but virtually no signal was detected in the nucleus or the cytoplasm. Thus, the novel receptor is faithfully expressed from our constructs and inserted into the plasma membrane where it could theoretically interact with ligands.

A similar experiment was performed with the cytoplasmic portion of the FGFRL1 protein fused to GFP. After transfection of the corresponding construct into MG-63 or COS-1 cells, the fusion protein was found to be distributed all over the cytoplasm and the nucleus in a very diffuse fashion (not shown). The distribution could not be distinguished from that obtained with cells that had been transfected with a construct for GFP alone. Thus, the cytoplasmic tail of the FGFRL1 protein does not appear to interact with proteins of any specific subcellular site.

Interactions of the FGFRL1 Protein—To investigate a possible interaction of FGFRL1 with putative ligands, chemical amounts of the FGFRL1 protein were required. We therefore cloned the cDNA for FGFRL1 into an expression vector and expressed the extracellular domain of FGFRL1 in *Escherichia coli*. Although we were able to isolate high amounts of fusion proteins from inclusion bodies, the purified proteins did not fold in a correct way as demonstrated by SDS-polyacrylamide gel electrophoresis. In the absence of reducing agents, the proteins formed large aggregates linked by disulfide bonds although there should be no free sulfhydryl group available after formation of the correct disulfide bridges in each of the three Ig loops (not shown).
Characterization of FGFR1

We therefore utilized a baculovirus system and produced the extracellular domain of FGFR1 in insect cells. The signal peptide of the envelope protein gp64 as well as a stretch of 6 histidine residues were included in the expression construct to facilitate purification of the secreted protein by affinity chromatography on nickel columns. With this system, we were able to isolate small amounts of the pure protein, which migrated on an SDS-polyacrylamide gel with an apparent molecular mass of 53 kDa (Fig. 7). This value is in good agreement with the calculated molecular mass of the fusion protein (54 kDa after glycosylation). The mobility of the expressed protein barely changed prior to and after reduction of disulfide bonds (Fig. 7), indicating that the cysteine residues did not participate in the formation of any unwanted intermolecular disulfide bond. It is therefore likely that the protein expressed in insect cells had adopted a more native conformation than the misfolded protein expressed in bacteria.

FGF receptors are known to interact with heparin or heparan sulfate chains. This interaction is believed to be crucial for the dimerization and subsequent signaling of the receptors (16–18). We therefore investigated whether the chicken FGFR1 protein might also interact with heparin. For this purpose, the recombinant protein was loaded onto a heparin-Sepharose column and eluted with a linear gradient of salt. As seen in Fig. 8, the protein bound specifically to the column and eluted as a relatively broad peak at an ionic strength corresponding to 600 mM NaCl. Analogous results were obtained with the human protein. Thus, there is a specific interaction of FGFR1 with heparin.

Next, we studied the putative interaction of FGFR1 with FGF ligands. FGF2, which occurs in virtually all tissues and which is known to interact with all conventional FGF receptors (16, 17), was iodinated and analyzed on a polyacrylamide gel. Consistent with its amino acid sequence, the radioactively labeled growth factor migrated with an apparent molecular mass of 18,000 Da (Fig. 9). Our preparation contained a relatively high amount of low molecular material that had probably been changed prior to and after reduction of disulfide bonds (Fig. 7), which is known to interact with all conventional FGF receptors (16, 17). As a matter of fact, this interaction has been used to isolate the receptors by affinity chromatography (19). Binding to heparin or heparan sulfate is believed to be crucial for the function of the FGFs, since it induces the dimerization of the receptors (16–18). (ii) The domain structure of FGFR1 is highly related to the four members of the FGFR family that also contain three extracellular Ig-like domains (22.5 kDa).

We therefore investigated whether our novel receptor might have any influence on the proliferation of mesenchymal cells. MG-63 cells were chosen for this experiment, because these osteosarcoma cells had shown the correct expression and segregation of the new receptor to the plasma membrane (see Fig. 6). The sequences of chicken and mouse FGFR1 were cloned in sense or antisense orientation into a eukaryotic expression vector and transfected into MG-63 cells. After synchronization by starvation, the cells were stimulated to proliferate by the addition of insulin, FGF2, and heparin. Finally, the stimulated cells were labeled with bromodeoxyuridine and analyzed by immunofluorescence using an antibody against bromodeoxyuridine. Under these conditions, about half of the cells had started to proliferate as detected by the incorporation of bromodeoxyuridine into newly synthesized DNA (Fig. 10). When the cells were transfected, prior to stimulation, with our FGFR1 sense construct, there was a pronounced reduction in the ratio of cells undergoing DNA duplication. No reduction was observed with cells that had been transfected with our antisense construct or with the empty expression vector (Fig. 10). These results suggest that FGFR1 has a negative effect on DNA synthesis and proliferation of living cells.

**DISCUSSION**

Utilizing a subtractive cDNA cloning approach, we have identified and cloned a novel cell surface receptor from chicken, mouse, rat, and man, which we have termed FGFR1. Several lines of evidence suggest that this receptor represents a new member of the FGF receptor family: (i) the extracellular domain of FGFR1 exhibits up to 48% sequence similarity with members of the FGFR family. In contrast, the sequence similarity with other surface receptors is minimal. (ii) The domain structure of FGFR1 is highly related to the four members of the FGFR family that also contain three extracellular Ig-like repeats and a hydrophilic box separating the first and the second Ig-like repeat (16, 17). (iii) When expressed by insect cells, FGFR1 is able to interact with heparin. A similar interaction has been observed with all FGF receptors (16, 17). As a matter of fact, this interaction has been used to isolate the receptors by affinity chromatography (19). Binding to heparin or heparan sulfate is believed to be crucial for the function of the FGFs, since it induces the dimerization of the receptors (16–18). (iv) FGFR1 interacts with FGF2. This interaction is specific as it can be competed with an excess of the unlabeled ligand. Based on the specific radioactivity of our FGF2 preparation and on the amount of bound ligand, we estimated the dissociation constant \( K_D \) of the FGF2-FGFR1 complex to be \( 0.6 \times 10^{-8} \) M. Compared with the \( K_D \) of the FGF2-FGFR2 complex (10\(^{-10}\) M), this value seems rather low. The actual affinity, however, might be higher under physiological conditions in the absence of any detergent. In our experiments we had to include 0.2% Triton X-100 to prevent the loss of the recombinant protein by unspecific adsorption to plastic and glass surfaces. The affinity might also be higher in the natural, trimeric complex consisting of receptor, ligand, and heparin (18), since heparin will interact with both, FGF2 and FGFR1. Nevertheless, we cannot rule out the possibility that FGF2 as used in our study does not represent the natural ligand for FGFR1 and that the physiological ligand would have a higher

**Fig. 7.** SDS-polyacrylamide gel electrophoresis of the recombinant FGFR1 protein. The extracellular domain of chicken FGFRL1 was expressed in insect cells utilizing a baculovirus system and purified from the cell culture supernatant by affinity chromatography. About 5 µg of purified protein was resolved on a 10% polyacrylamide gel in the absence (– βSH) or presence (+ βSH) of 2% β-mercaptoethanol. The migration positions of three globular protein markers are included (albumin, 67 kDa; ovalbumin, 45 kDa; cytochrome c, 22.5 kDa).
affinity. At any rate, plasmon resonance experiments will be required to conclusively determine the $K_D$ value.

Two other research groups have recently described the cloning and sequencing of the same receptor-like molecule, which they have termed FGFR5 (20, 21). Kim et al. (20) used the polymerase chain reaction in combination with degenerate primers to isolate clones for the novel FGFR5 from man. These authors provided evidence that FGFR5 is specifically expressed in the human pancreas. Sleeman et al. (21) identified clones for murine FGFR5 from an expressed sequence tag data bank. These authors demonstrated expression of FGFR5 in many different tissues from mouse and man, including kidney, brain, muscle, lung, and liver. It is difficult to compare these results with our expression data, because neither of the two groups included any samples from cartilage in their studies. We have tried to reproduce these findings with commercial as well as homemade Northern blots. In relation to the high expression of FGFR1 in cartilage, we observed only weak expression in pancreas, kidney, and lung (14). It is possible that differences in the sensitivity of the hybridization protocol applied may explain part of the apparent discrepancies. Sleeman et al. (21) used probes of high specific radioactivity prepared by the polymerase chain reaction, whereas we used conventional probes labeled by random oligonucleotide priming. It should also be

---

**Fig. 8. Binding of FGFRL1 to heparin.** Recombinant FGFRL1 protein was loaded onto a heparin-Sepharose column and eluted with a linear gradient of 0.15–1.35 M NaCl. Individual fractions were collected and checked for their NaCl concentration by measuring the conductivity (bottom). Proteins from every third fraction were separated on a polyacrylamide gel and analyzed by immunoblotting utilizing an antibody against the His-tag of the expressed protein (top).

**Fig. 9. Binding of FGF ligand to the FGFRL1 protein.** Samples of the recombinant FGFRL1 protein were bound to nickel-agarose beads and incubated with radioactively labeled FGF2 in the presence of 2 mg/ml serum albumin and 0.2% Triton X-100. After extensive washing of the beads, bound proteins were eluted with hot SDS sample buffer and separated on a 15% polyacrylamide gel. Labeled FGF2 was detected by autoradiography. The preparation of radiiodinated FGF2 is included on the left (Input), competition of the complex formation by 5 μg of unlabeled FGF2 (FGF2* + FGF2) as well as by 10 μl of fetal bovine serum (FGF2* + Serum) is shown on the right.

**Fig. 10. Effect of FGFRL1 on cell proliferation.** MG-63 cells were transfected with FGFRL1 ligated into a eukaryotic expression vector in sense (S) or antisense (AS) orientation. The empty expression vector (−) served as a negative control. The cells were synchronized by starvation and stimulated to proliferate by the addition of insulin, FGF2, and heparin. DNA synthesis of the stimulated cells was visualized by incorporation of bromodeoxyuridine followed by indirect immunofluorescence. The top panels show the immunoreactive cells, and the panels in the middle show the same cells under phase contrast. Quantitative results from three independent experiments are depicted at the bottom in relation to cells transfected with the empty expression vector (100%).
Characterization of FGFR1

noted that the strong signal detected by Kim et al. (20) in samples from human pancreas corresponded to a mRNA species of ~5 kb that was not found in any other tissue. This species must represent an unusual gene product that clearly differs from the human FGFR1 mRNA of ~3 kb characterized in our study (14). Furthermore, no pancreas-specific expression is supported by data from serial analysis of gene expression (SAGE) published on the internet (www.ncbi.nlm.nih.gov/SAGE). At any rate, our in situ hybridization studies with sections from mouse embryos clearly corroborate the preferential expression of FGFR1 in skeletal tissues.

We believe that FGFR1 does not possess any direct signaling function although we cannot rule out the possibility that it might signal in an as yet unknown way. Several lines of evidence may support our notion: (i) FGFR1 does not contain any protein tyrosine kinase domain at its C terminus that would be required for signaling by transphosphorylation. (ii) The cytoplasmic domain of FGFR1 does not appear to interact with any signaling partner. We have employed the yeast two-hybrid system to search for potential interaction partners encoded by cDNA libraries prepared from placenta and chondrocytes. These efforts did not lead to the identification of any meaningful clones. (iii) The intracellular tail of FGFR1 does not appear to interact with any target at a specific subcellular site when expressed in vertebrate cells as a fusion protein with GFP. (iv) The murine FGFR1 sequence exhibits a peculiar frameshift mutation in comparison with the human, chicken, and fish protein, which results in the incorporation of 54 unrelated amino acids at the C terminus of murine FGFR1. Since this portion has not been conserved during evolution, it cannot serve a general, crucial function in all vertebrates.

We believe that FGFR1 might rather have a modulating or inhibitory function in the FGF signaling cascade. It binds FGF ligands and sequesters them from other receptors. Furthermore, it might be able to dimerize, via its heparin binding site, with a true FGF receptor and inhibit the activity at the cytoplasmic domain. Here, the peculiar histidine-rich motif of FGFR1 might play an active role. This motif could either form a complex with tyrosine residues of the true FGF receptors and prevent their phosphorylation or alternatively it could bind the phosphorylated residues after modification and prevent their interaction with downstream effector molecules. In either case, the FGF signal would be attenuated. Our preliminary studies may support this conclusion. When overexpressed in MG-63 cells, the novel receptor significantly reduced cell proliferation. This effect was observed with the sense construct but not with the antisense construct. A similar inhibition of FGF signaling was previously found with an artifically truncated form of human and chicken FGFR1. Ueno et al. (22) prepared an FGFR1 construct that contained the extracellular and the transmembrane domain but lacked the intracellular tyrosine kinase domain. When overexpressed in Xenopus oocytes, this construct inhibited signaling by the wild-type receptor in a dominant negative fashion.

Several physiological variants of FGF receptors that are incapable of signaling because they are lacking the protein tyrosine kinase domain have also been described in the literature (16, 17). These forms are generated by proteolytic cleavage and/or by alternative splicing. Since they can still interact with ligands, they will inhibit FGF signaling. In contrast to these processed forms, FGFR1 is the first receptor of the FGF signaling system with a transmembrane domain but without a kinase domain, which is transcribed from a separate gene.

The concept of a nonfunctional receptor that acts as a molecular trap for ligands is not unprecedented in biology but has been found in several growth factor families. The first example of such a decoy receptor that binds and sequesters the agonist but that is incapable of signaling has been described in the interleukin-1 signaling system (23). IL-1 receptor I possesses a cytoplasmic TIR domain, which plays a key role in the recruitment of an adapter protein. In contrast, IL-1 receptor II contains a short cytoplasmic domain lacking any TIR motif. All the experimental results accumulated so far are consistent with the view that IL-1 receptor II is a pure IL-1 decoy that blocks the IL-1 response. Similar decoy receptors have subsequently been identified in the tumor necrosis factor receptor family (e.g. osteoprotegerin, Fas death receptor) and in the chemokine signaling system (e.g. Duffy, D6). Furthermore, a pseudoreceptor has also been described in the TGF-β signaling system (24). A transmembrane protein designated BAMBI has been identified that is related to TGF-β receptor-I but lacks the intracellular serine/threonine kinase domain. This pseudoreceptor can associate with a normal TGF-β receptor and inhibit TGF-β signaling. Likewise, a receptor without signaling function has been found in the EGF receptor system and termed ErbB3 (25). Similar to the active receptor ErbB4, ErbB3 does interact with neuregulins, but it cannot pass on the signal because it is lacking the kinase activity. All these examples emphasize that it is a common strategy of nature to counteract the effects of growth factors and cytokines by the use of decoy receptors.

It is of interest to note in this context that decoy receptors appear to possess substantially lower affinities for the corresponding ligands than the cognate receptors. IL-1 receptor I has an affinity of 10⁻¹⁰ for its ligands IL-1α and IL-1ra, whereas the negatively acting IL-1 receptor II binds these ligands at least 100 times less efficiently (25). Our FGFR1 shows an affinity for FGF2 which is about two orders of magnitude lower than the affinity of FGFR1. A relatively weak interaction of FGFR1/FGFR5 with FGF2 was also noted by Sleeman et al. (21). In fact these authors reported that the interaction was so low that FGFR5 was not able to compete with FGFR1 for ligand binding. Further experiments will be required to determine the significance of the relatively low affinity of decoy receptors in comparison with their cognate receptors.

The FGF signaling system is used to regulate a variety of functions, including cell proliferation, differentiation, and migration (16, 17). FGFR1 might therefore be involved in the fine-tuning of several different processes. There is indirect evidence that the function might be linked with the formation of the vertebrate skeleton because FGFR1 appears to be expressed in all vertebrates (man, mouse, rat, chicken, fish) but not in invertebrates (Drosophila, Caenorhabditis). Furthermore, FGFR1 is expressed at relatively high level in the cartilage of skeletal tissues, but at much lower level in many other tissues. It might therefore be involved in the control of proliferation and differentiation of chondrocytes. We have investigated this possibility, but so far we could not find any evidence to support this notion. The chicken sternum has widely been used as a model system for chondrocyte differentiation (1, 26). The cranial portion of the sternum, which is mineralized and which contains chondrocytes of a late differentiation stage including many hypertrophic cells, showed an expression level of FGFR1 similar to the caudal portion, which contains proliferative and resting chondrocytes of an early differentiation stage. It is therefore unlikely that FGFR1 will specifically control the differentiation program of chondrocytes.

Recent work with an FGFR-like molecule of planarians may provide some clues to the function of FGFR1 in vertebrates (27). Planarians possess two FGF receptors (FGFR1 and FGFR2) as well as an FGFR-like molecule termed nou-darake.
Although the amino acid sequences of human FGFRL1 and the planarian protein nou-darake are not related, the two proteins exhibit a striking similarity of their domain structures. Nou-darake has two extracellular Ig-loops, a single membrane-spanning domain and a C-terminal domain that lacks the kinase domain characteristic of FGFRs. Nou-darake is specifically expressed in the head region of the animals. Studies with RNA interference showed that the loss of function of nou-darake results in the ectopic induction of brain tissue throughout the body of the animal. The authors concluded that nou-darake may restrict growth of brain tissue to the head region by inhibiting diffusion of FGF to the rest of the body. Although our receptor-like molecule does not appear to be expressed in the brain, the planarian system can still provide some clues to a possible function of FGFRL1 in vertebrates. It is conceivable that FGFRL1 may restrict the diffusion range of FGF in an analogous way in regions where FGF expression and signaling is high. This would be the case in the vertebrate skeleton during development. Thus, FGFRL1 might trap FGF ligands and keep them within the border of this tissue to prevent the uncontrolled proliferation of adjacent cells in neighboring tissues. The tools are now available to investigate this possibility.

Acknowledgments—We thank Drs. Bo Li and Christof Schild for critically reading of our manuscript.

REFERENCES
1. Bianco, P., Descalzi Cancrutta, F., Rimanucci, M., and Cancrutta, R. (1998) Matrix Biol. 17, 185–192
2. Heinegard, D., and Oldberg, A. (1989) FASEB J. 3, 2042–2051
3. Cancrutta, R., Descalzi Cancrutta, F., and Castagnola, P. (1995) Int. Rev. Cytol. 159, 265–358
4. Vorkamp, A. (2001) Osteoarthr. Cartilage 9 Suppl. A, S109–S117
5. de Crombrugghe, B., Lefebvre, V., and Nikashima, K. (2001) Curr. Opin. Cell Biol. 13, 721–727
6. Belluccio, D., and Trueb, B. (1997) FEBS Lett. 415, 212–216
7. Belluccio, D., Schenker, T., Baici, A., and Trueb, B. (1998) Genomics 53, 391–394
8. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
9. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) Current Protocols in Molecular Biology, Greene Publishing Associates, New York
10. Schenker, T., and Trueb, B. (1998) Exp. Cell Res. 239, 161–168
11. Duguid, J. R., and Dinauer, M. C. (1990) Nucleic Acids Res. 18, 2789–2792
12. Walchli, C., Koch, M., Chiquet, M., Odermatt, B. F., and Trueb, B. (1994) J. Cell Sci. 107, 669–681
13. Li, B., and Trueb, B. (2001) J. Biol. Chem. 276, 33333–33335
14. Wiedemann, M., and Trueb, B. (2000) Genomics 69, 275–279
15. Wiedemann, M., and Trueb, B. (2001) Biochim. Biophys. Acta 1520, 247–250
16. Szelenyi, G., and Fallon, J. F. (1999) Int. Rev. Cytol. 185, 45–106
17. Ornitz, D. M. (2001) Bioessays 23, 108–112
18. Pellegrini, L. (2001) Curr. Opin. Struct. Biol. 11, 629–634
19. Labb, R. R., and Fett, J. W. (1984) Biochemistry 23, 6285–6299
20. Kim, I., Moon, S.-O., Yu, K.-H., Kim, U.-H., and Koh, G. Y. (2001) Biochim. Biophys. Acta 1518, 152–156
21. Sleeman, M., Fraser, J., McDonald, M., Yuan, S., White, D., Grandison, P., Kumble, K., Watson, J. D., and Murison, J. G. (2001) Gene (Amst.) 271, 171–182
22. Ueno, H., Gunn, M., Dell, K., Tseng, A., Jr., and Williams, L. (1992) J. Biol. Chem. 267, 1470–1476
23. Mantovani, A., Locati, M., Vecchi, A., Sozzani, S., and Allavena, P. (2001) Trends Immunol. 22, 328–336
24. Onichtchouk, D., Shen, Y. G., Dusch, R., Gawantka, V., Delius, H., Massague, J., and Niehrs, C. (1999) Nature 401, 480–485
25. Eggerl, S., and Klambt, C. (2001) Curr. Biol. 11, R292–R295
26. Romanoff, A. L. (1960) The Avian Embryo, The Macmillan Company, New York
27. Cebria, F., Kobayashi, C., Umesono, Y., Nakazawa, M., Mineta, K., Ikeo, K., Gujnikori, T., Itoh, M., Taira, M., Sanchez Alvarado, A., and Agata, K. (2002) Nature 419, 620–624