MOLECULAR AND CELLULAR BIOLOGY, Dec. 1992, p. 5600-5609
0270-7306/92/125600-10$02.00/0
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Vol. 12, No. 12

Identification of a Cysteine-Rich Receptor for Fibroblast Growth Factors

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Received 21 July 1992/Returned for modification 3 September 1992/Accepted 14 September 1992

The fibroblast growth factor (FGF) family consists of seven members whose activities are thought to be mediated by multiple receptors. Here we describe the cDNA cloning, expression, and characterization of a cysteine-rich FGF receptor (CFR) that is distinct from previously identified FGF receptors. The deduced amino acid sequence for CFR suggests that it is an integral membrane protein containing a large extracellular domain comprising 16 cysteine-rich repeated units and an intracellular domain of 13 amino acids. No reported sequences exhibit significant homologies to either the repeated extracellular motif or to the entire CFR amino acid sequence. Several CFR transcripts are present in embryonic chick tissue, suggesting that CFR undergoes alternate mRNA splicing or that related genes are present. Chinese hamster ovary cells transfected with the CFR cDNA express a 150-kDa polypeptide that binds FGF-1, FGF-2, and FGF-4 but does not bind several non-FGF family members. The high degree of evolutionary conservation among vertebrate CFRs and its ability to bind three different FGFs with high affinity suggest that this unique receptor plays an important role in FGF biology.

The fibroblast growth factor (FGF) family comprises seven members that possess significant amino acid homology and often exhibit overlapping biological activities. FGF-1 (acidic FGF), FGF-2 (basic FGF), and FGF-7 (keratinocyte growth factor) were initially identified as potent mitogens for a variety of cell types, while FGF-3 (*int*-2), FGF-4 (K-FGF/*hst*-1), and FGF-5 were first identified as oncogenes (2, 10, 31, 42). FGF-6 was isolated on the basis of its homology to FGF-4 (27, 71). Further characterization of these FGF family members has revealed possible roles as modulators of cellular differentiation, angiogenesis, neurite extension, neuronal cell survival, mesoderm induction, and inner ear induction (2, 10, 31, 58). The majority of the FGF family (FGF-3 through FGF-7) possess signal peptides and are believed to be secreted. In contrast, FGF-1 and FGF-2 possess no secretory signal peptide sequences and are not localized to cellular compartments involved in secretion (15, 38, 39, 52). Despite the absence of signal peptides, both FGF-1 and FGF-2 are present in the extracellular matrix of endothelial cells cultured in vitro (3). Immunohistochemical studies have also localized FGF-2 in the extracellular matrix of a variety of tissues (16, 22). Thus, the FGFs are believed to mediate their biological actions in part via cell surface receptors.

Two distinct types of cell surface receptors for FGF family members have been characterized. These include a family of four tyrosine kinase FGF receptors (FGFR1 to 4) (17, 26, 35, 36, 40, 46-48, 64) and a family of heparan sulfate proteoglycans (HSPGs), of which syndecan is a prototype (30, 55, 61). Syndecan, a heparan/chondroitin sulfate proteoglycan, binds FGF via its covalently attached heparan sulfate chains (30). Recently, we and others have shown that FGF binding and signaling are dependent on the presence of heparan sulfate, suggesting that both FGFRs and HSPGs are essential (7, 45, 56, 70).

Although FGFRs in conjunction with HSPGs clearly participate in mediating some of the biological activities of FGF, there is mounting evidence to suggest that these receptors may not be sufficient for all aspects of FGF signaling. The localization of FGF-2 and FGF-3 in the nucleus (1, 19, 50, 54, 57, 66) suggests that FGFs may also possess an intracellular mode of action. Initiation of translation from CUG codons 5′ of the predicted FGF-2 and FGF-3 AUG start codons yields polypeptides possessing nuclear targeting sequences that direct these products to the nucleus (1, 54). Although the role of nuclear targeting is not yet clear, two experiments correlate nuclear localization with FGF responses. First, the addition of exogenous FGF-2 to endothelial cells promotes G_1-phase nuclear transport of FGF-2 which parallels an increase in ribosomal gene transcription (5, 9). Second, an FGF-1 mutant in which the putative nuclear targeting sequence has been deleted is capable of binding cell surface receptors and stimulating tyrosine phosphorylation but fails to elicit a mitogenic response in endothelial cells (28). Full biological activity of the mutant FGF-1 is restored by the introduction of a heterologous yeast nuclear targeting sequence (28). The existence of two additional FGF mutants that exhibit a restricted spectrum of biological activities also provides evidence for the complexity of FGF-dependent signaling pathways. An FGF-1 mutant (Lys→Glu-132) stimulates tyrosine phosphorylation and *fos* induction but is not capable of stimulating DNA synthesis (11). Similarly, a mutant FGF-2 also exhibits differential stimulation of FGF-mediated activities. This deletion mutant (lacking residues 27 to 32) is capable of stimulating both tyrosine phosphorylation and DNA synthesis at concentrations similar to wild-type FGF-2 but exhibits a greater than 100-fold reduction in its ability to stimulate plasminogen activator activity (51).

We have previously reported the isolation of a 150-kDa FGF receptor which possesses biochemical properties that distinguish it from both HSPGs and FGFRs (12). First, treatment of purified receptor with heparitinase results in no shift in mobility when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), indicating

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that it is not covalently modified with heparan sulfate chains. In contrast to FGFRs, purified cysteine-rich FGF receptor (CFR) exhibits specific binding of FGF-1 and FGF-2 in the absence of heparin or heparan sulfate. Furthermore, CFR is eluted from both FGF-1 and FGF-2 affinity columns by the addition of heparin, indicating that heparin disrupts the interaction between FGF and CFR.

Here, we report the cDNA cloning, expression, and biochemical characterization of a unique FGF receptor. CFR is an integral membrane protein with a novel primary amino acid sequence that binds at least three FGF family members and is highly conserved among vertebrate species. Thus, we speculate that CFR plays an important yet undefined role in FGF biology.

MATERIALS AND METHODS

Growth factors. Human recombinant FGF-2 purified from yeast cells (Zymogenetics, Inc.) was iodinated as previously described (44). Specific activities were determined by MM14 cell cycle exit assay and ranged from 1,000 to 5,000 cpm/fmol (43). Bovine FGF-1 was purified as described previously (43). FGF-4, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF-II) were provided by Claudio Basilico, New York University; Paul Bertics, University of Wisconsin-Madison; Dan Bowen-Pope, University of Washington; and Lilly Research Laboratories, respectively. Insulin was purchased from Sigma. Chicken FGFR1 (cek1) was a gift from Elena Pasquale, University of California, San Diego.

Determination of amino-terminal and internal amino acid sequences. CFR was purified from detergent-solubilized chick membranes by wheat germ agglutinin and FGF-1 affinity chromatography (12). Proteins were electrophoresed through a 7.5% polyacrylamide gel (1.5 mm thick) and electrophoretically transferred onto an Immobilon membrane in buffer containing 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH 10, and 20% high-performance liquid chromatography-grade methanol. Electroblotting was performed at 100 V for 2.5 h at 4°C. Amino-terminal and internal amino acid sequencing was performed at the UW Biotechnology Center (68).

Isolation and sequencing of cDNA clones. A day 13 embryonic chick brain λgt11 library (provided by B. Ranscht, La Jolla Cancer Research Foundation) was screened with a mixture of three distinct monoclonal antibodies that recognize avian CFR (33). Bacteriophage were plated at a density of 5×10^5 per 15-cm plate, using Y1090 as the host bacteria. The plates were incubated for 5 h at 37°C, overlaid with nitrocellulose filters soaked in 10 mM isopropyl-β-D-thiogalactopyranoside, and then incubated for 12 h at 37°C. Nonspecific binding sites were blocked by incubating filters with TBST (TBS [50 mM Tris-Cl {pH 7.4}, 100 mM NaCl] containing 0.1% Triton X-100) with 2% nonfat dry milk (NFDM) added for 1 h. The filters were incubated with three monoclonal anti-CFR antibodies (1/50 dilution of tissue culture supernatants) in TBST containing 0.2% NFDM for 1 h. The filters were washed three times with TBST and incubated with a secondary goat anti-mouse antibody conjugated to alkaline phosphatase (Promega; 1/5,000 dilution) in TBST and 0.2% NFDM for 1 h. After the filters were rinsed three times with TBST, alkaline phosphatase substrate (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) was added and the reaction was stopped by rinsing with water. Purification of positive bacteriophage was accomplished by replating and rescreening positive bacteriophage three times.

cDNA inserts were subcloned into the *Eco*RI site of the pBSKS+ vector (Stratagene) to facilitate DNA sequencing. Double-stranded template DNA was prepared by miniprep or purification on a CsCl gradient. Sequencing was performed by the dideoxynucleoside chain termination method (60) by using Sequenase version 2.0 per manufacturer's instructions (U.S. Biochemicals). Sequencing of the entire clone was accomplished by using a combination of methods that included synthesizing new primers based on cDNA sequence and generating a nested set of deletions with exonuclease III (Erase-a-base; Promega, Inc.).

Data base searches and DNA and amino acid sequence analysis. DNA and amino acid sequences were analyzed by DNA Strider (37) and Genetics Computer Group (20) software packages. TFastA program (49) was used to search the GenBank (8) and EMBL (25) data bases. FastA (49) program was used to search SwissPro (4) and PIR-Prot (21) data bases. Cysteine-rich repeat motifs were aligned by using the Lineup (20) program and the NBRF data base (21) searched with the ProfileMake (24) and ProfileSearch (23) programs.

In vitro transcription and translation of CFR by using the pCITE vector. The CFR cDNA was subcloned into a plasmid containing a cap-independent translation enhancer (pCITE; Novagen, Inc.). Because pCITE must utilize its own start codon, it was necessary to delete CFR's start codon. Hence, the amino-terminal sequence of full-length CFR subcloned into pCITE is <u>MARTS</u>RSRCP (underlined sequence is contributed by the vector). A second construct was generated by removing nucleotide sequence corresponding to amino acids −45 through −9. The deduced amino acid sequence at the amino terminus of the polypeptide encoded by this construct is <u>ME</u>AGPLN. Plasmid DNA was linearized with *Sal*I, extracted with phenol-chloroform, ethanol precipitated, and resuspended in diethyl pyrocarbonate-treated water. A total of 20 μg of template DNA was transcribed with 250 U of T7 RNA polymerase in buffer containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine-HCl, 20 mM dithiothreitol, 1 U of RNasin (Promega) per ml, and 200 μM (each) ATP, CTP, GTP, and UTP at 37°C for 1 h. An additional 250 U of T7 RNA polymerase was added, and the reaction was allowed to proceed for 1 h at 37°C. Template DNA was removed by digestion with 5 U of RNase-free DNase (Promega) at 37°C for 20 min. RNA was translated in vitro by using rabbit reticulocyte lysate in the presence or absence of canine pancreatic microsomes per manufacturer's instructions (Promega). [^{35}S]methionine was included in the reaction so that the translation products could be visualized by autoradiography. When noted, proteinase K was added following translation to a final concentration of 5 μg/ml in the presence or absence of Triton X-100 (0.25%) for 30 min on ice. Samples were separated by SDS–5% PAGE. Gels were treated with En^{3}Hance (Dupont) prior to autoradiography.

Transfection and detection of CHO cells expressing chicken CFR. CHO-K1 cells were cotransfected with the eukaryotic expression vector pDX (6, 13) or pDX containing the CFR cDNA (pDX.CFR) and a plasmid encoding a neomycin resistance gene (pKOneo) (67) by the calcium phosphate method (59). A 20:1 molar ratio of pDX (or pDX.CFR) to pKOneo was utilized for the transfections. Stable transfectants were selected by growing the cells in Dulbecco modified Eagle medium plus 10% calf serum plus 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) plus 10 mM proline containing 1 mg of G418 per ml for 3

days. The G418 concentration was reduced to 400 μg/ml, and the cells were grown for an additional 5 days. Individual colonies were subcloned onto 35-mm plates and expanded for further analysis. To detect CFR expression, proliferating CHO cells were washed two times with ice-cold phosphate-buffered saline and harvested with a rubber policeman. Cells were centrifuged at 18,000 × g for 5 min at 4°C and resuspended in solubilization buffer containing 20 mM Tris (pH 7.4), 100 mM NaCl, 4 mM EDTA, 1 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Membranes were solubilized by incubating samples on a rotating wheel for 30 min at 4°C. Detergent-insoluble material was removed by centrifugation at 18,000 × g for 10 min at 4°C. Protein concentrations of the supernatants were determined by using the bicinchoninic acid assay per manufacturer's instructions (Pierce Chemical Co.). Protein samples, 76 μg of CHO cell extract, and 33 μg of partially purified chick embryo CFR were subjected to electrophoresis in a 7.5% polyacrylamide gel and electrophoretically transferred to an Immobilon membrane for 1 h at 100 V at 4°C in transfer buffer containing 25 mM ethanolamine and 25 mM glycine in 20% methanol. Immobilon membranes were blocked with Tris-buffered saline (TBS) containing 3% nonfat dry milk and 0.05% Tween 20 for 2 h. The blot was incubated with a purified anti-CFR monoclonal antibody diluted 1/500 in blocking buffer for 1 h. Unbound antibody was removed by washing three times for 20 min with blocking buffer. ^{125}I-labeled goat anti-mouse IgG was diluted to 7.5 × 10^5 cpm/ml in blocking buffer and added to the blot for 1 h. The blot was washed with blocking buffer three times for 20 min and subjected to autoradiography.

Radioimmunosorbent assay (RISA) for FGF binding and competition. Anti-CFR monoclonal antibody was purified from ascites fluid by caprylic acid precipitation (65), and 2 μg/ml (100 μl per well) was bound to the wells of a polystyrene microtiter plate in 50 mM $NaHCO_3$, pH 9.5. Nonspecific sites were blocked with TBSTG (TBST containing 0.1% gelatin). Free antibody was removed by rinsing three times with TBSTG and was replaced with sample—either CHO cell extract (100 μg) or CFR partially purified from chick embryos (14 μg). Nonimmunoadsorbed factors were removed by washing three times with TBSTG and ^{125}I-FGF-2 (100 pM) was added in the presence or absence of a 1,000-fold molar excess of unlabeled competitor. Unbound ^{125}I-FGF-2 was removed by washing three times with TBST. The wells were cut from the plate, and the ^{125}I counts per minute remaining in the wells were determined by counting in an LKB Clinigamma counter. Nonspecific binding was defined as counts per minute detected when excess unlabeled FGF-2 was used as a competitor. ^{125}I counts per minute were normalized relative to ^{125}I-FGF-2 binding in the absence of competitors. Total and 100% specific counts per minute represent 5,200 and 4,500 cpm for partially purified CFR and 3,560 and 2,800 cpm for CFR transfected CHO cells, respectively. Nonspecific counts per minute determined by subtraction of counts bound in the presence of excess FGF-2 represented 5% (774) and 20% (767) of the total binding counts per minute for partially purified and CHO expressed CFR, respectively. In the absence of CFR-containing extract, ^{125}I counts per minute were 850. When monoclonal antibody was omitted, the ^{125}I counts per minute were 300 for both partially purified and recombinant CFR. Error bars represent the standard error of the mean of measurements performed in triplicate.

Immunoadsorption of ^{35}S-labeled CHO cell extracts. CHO cells (10^7) were labeled for 10 h with 0.05 mCi of TRAN^{35}S-label (ICN Biochemicals) per ml in methionine- and cysteine-free growth media. Lysates were prepared from ^{35}S-labeled CHO cells, and a RISA was performed as described above, substituting unlabeled FGF-2 for ^{125}I-FGF-2. SDS-PAGE sample buffer was added to the microtiter plate wells and boiled to solubilize remaining proteins. Three wells were pooled, separated by SDS–7.5% PAGE, and treated with En^{3}Hance (DuPont), and the dried gel was subjected to fluorography.

Equilibrium binding and determination of K_d for ^{125}I-FGF-2 binding. For Scatchard analysis, the RISA was performed as described except that 3.5 μg of partially purified CFR and 10 μg of CHO cell extract were used. For ^{125}I-FGF-2 binding to partially purified chick CFR, the specifically bound ^{125}I-FGF-2 was defined as the total binding in the presence of the extract minus the total binding in the absence of the extract. Similarly, for binding to recombinant CFR expressed in CHO cells, the specifically bound ^{125}I-FGF-2 was defined as the ^{125}I-FGF-2 bound to CFR-transfected CHO cell extract minus the amount bound to control-transfected CHO cell extract. The free concentration of ^{125}I-FGF-2 was determined by removing an aliquot of the incubation mixture just prior to termination of the assay.

Northern (RNA) analysis of day 7 embryonic chick RNA. Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (14). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose (Pharmacia) chromatography. For RNase H digestions, samples containing 20 μg of total RNA and 1 mM EDTA were boiled for 1 min and immediately cooled on ice. After addition of 1 μg of antisense oligonucleotide, samples were incubated for 10 min at 25°C. KCl was then added to a final concentration of 50 mM, and samples were incubated for an additional 10 min. After $MgCl_2$ and Tris (pH 8) were added to final concentrations of 56 and 40 mM, respectively, 3 U of RNase H was added and samples were incubated for 30 min at 37°C. RNA samples were phenol-chloroform extracted, ethanol precipitated, and electrophoresed through a 1% agarose gel containing formaldehyde and capillary blotted onto Nytran (Schleicher and Schuell). Blots were hybridized per manufacturer's instructions and washed with 0.1× SSPE (0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) plus 1% SDS at 65°C.

Nucleotide sequence accession number. The nucleotide sequence for CFR has been submitted to GenBank, and the accession number is M95766.

RESULTS

cDNA isolation and primary structure of CFR. To isolate CFR cDNA clones, CFR protein was purified from embryonic chick tissue as previously described (12) and subjected to amino acid sequencing for determination of amino-terminal and internal sequences (68). Purified protein was also used as an antigen for the production of anti-CFR monoclonal antibodies (33). A mixture of three anti-CFR monoclonal antibodies was then used to isolate a cDNA containing the entire open reading frame of CFR (see Materials and Methods).

Twenty positive cDNA clones were identified by screening 4 × 10^5 plaques of a chick embryo cDNA expression library. cDNA inserts isolated from 11 of the positive clones ranged in size from 1.4 to 3.5 kb. All but one of the isolated cDNAs hybridized with the largest cDNA under stringent conditions, suggesting that they possessed identical or highly related sequences. In addition, none of the cDNAs hybridized with a ^{32}P-labeled chicken FGFR1 cDNA (48) under

low-stringency conditions, indicating that the isolated cDNAs were unlikely to be FGFR family members. The two largest cDNAs were identical at their 5′ and 3′ termini. The 5′ sequence encoded a putative signal peptide followed by the experimentally determined amino-terminal amino acid sequence. The 3′ ends of these cDNAs revealed that both possessed short poly(A) tracts immediately preceded by a polyadenylation signal sequence. Two smaller cDNAs also analyzed lacked the first 105 nucleotides present in the longest cDNAs. All four cDNAs were digested with several restriction enzymes to determine if any polymorphisms were detectable. With the exception of the 105 nucleotides that are absent in the smaller clones, the pattern of fragments obtained after electrophoresis of the digested cDNA inserts was indistinguishable. These results suggest that the four cross-hybridizing clones are likely to represent a single transcript species.

The complete nucleotide sequence of one of the two longest clones (3,546 bp) was determined and found to contain an open reading frame of 3,429 nucleotides beginning at an AUG codon five nucleotides from the 5′ end of the cDNA (Fig. 1). This codon lies within a sequence very similar to the consensus sequence determined to be optimal for initiation by eukaryotic ribosomes (32). One hundred eleven nucleotides of untranslated sequence are present at the 3′ end of the isolated cDNA. This region contains a terminal 10-nucleotide poly(A) tract directly preceded by a consensus polyadenylation signal (53) at position 3530 (Fig. 1). The deduced amino acid sequence of CFR reveals a polypeptide of 1,142 residues with a predicted molecular mass of approximately 130 kDa (Fig. 1). The experimentally determined amino-terminal and internal amino acid sequences of the purified CFR protein (68) correspond well with the deduced amino acid sequence for the CFR cDNA, demonstrating that the isolated cDNA encodes CFR. Two hydrophobic sequences of sufficient length to comprise putative signal peptide and membrane-spanning domains were identified by Kyte and Doolittle hydropathy analysis (34). The presence of five consensus sites for asparagine-linked glycosylation is consistent with the observation that purified CFR contains approximately 10 kDa of asparagine-linked carbohydrate (12). The predicted intracellular domain comprises 13 amino acid residues, nearly half of which are basic (Fig. 1). Threonines 1087 and 1091 are consensus sequences for phosphorylation by protein kinase C and cyclic AMP/cyclic GMP-dependent protein kinase, respectively (18, 69).

A prominent feature of CFR is the presence of 69 cysteines, 6% of the total number of residues. Sixty-seven of these cysteine residues are arranged in 16 conserved repeating units. Alignment of the repeated units revealed that they are approximately 25% identical and arranged in a $CX_{13}DX_{10}CX_7CX_nCL$ motif (Fig. 2). A search of protein data bases with a profile of the repeated sequences failed to identify any polypeptides possessing a similar motif, indicating it is unique. Furthermore, we have searched four data bases with the entire primary amino acid sequence and determined that CFR represents a unique protein.

Biochemical analysis of CFR signal peptide and membrane-spanning domain by in vitro translation. Because of the unique structure of CFR and the proximity of the membrane-spanning domain to the carboxy terminus, we used an in vitro translation system to biochemically characterize the putative signal peptide and membrane-spanning domains identified by hydropathy analysis. Translation of the entire CFR coding region yielded a polypeptide of approximately 143 kDa (Fig. 3A). The apparent molecular mass of CFR was increased to 150 kDa when microsomes were added to the translation mix. As microsomes provide components necessary for processing of secreted and membrane-bound polypeptides, it is likely that the shift in CFR's mobility was the net result of cleavage of the signal peptide and addition of carbohydrate chains.

To further demonstrate that the sequence prior to the experimentally determined amino terminus was functioning as a classical signal peptide, we generated a mutant CFR encoding a polypeptide lacking amino acids −45 through −9 (CFR Δ−45 to −9). CFR Δ−45 to −9 translated in the presence and absence of microsomes exhibited no detectable difference in mobility, suggesting that it did not enter the lumen of the microsomes where glycosylation normally occurs (Fig. 3A). Addition of proteinase K following translation degraded CFR Δ−45 to −9 synthesized in the presence of microsomes (Fig. 3B). In contrast, wild-type CFR translated in the presence of microsomes was resistant to proteinase K and was degraded only upon addition of detergent which disrupts microsome integrity (Fig. 3B). Comparison of the molecular weights of CFR Δ−45 to −9 (CFR without signal peptide or carbohydrate) and of full-length CFR translated in the presence of microsomes (glycosylated CFR with truncated signal peptide) reveals a difference of approximately 10 kDa. This is consistent with the modification of purified CFR with 10 kDa of asparagine-linked oligosaccharide (12). We also determined that mature CFR is anchored in the membrane. Wild-type CFR remains associated with disrupted microsomes while yeast α-factor, which does not contain a membrane-anchoring region, is released (data not shown). This suggests that the second region of hydrophobic residues functions as a membrane anchor.

FGF binding to purified CFR and CFR expressed in CHO cells. In order to examine the FGF-binding characteristics of CFR, the CFR cDNA was expressed in CHO cells. Stable CHO cell transformants were analyzed for CFR expression by immunoblot analysis with anti-CFR monoclonal antibodies (Fig. 4). The mobility of recombinant chicken CFR expressed in CHO cells is indistinguishable from that of native chicken CFR (Fig. 4). The immunopositive-staining proteins migrating more rapidly than 150 kDa are likely to be degradation products, as their relative contribution increases upon storage or treatment with protease (unpublished data). As the antibodies used are specific for avian CFR and do not recognize mammalian CFR, any endogenous CHO cell CFR, if present, is not detected (unpublished data). Immunocytochemical staining revealed that the majority of CFR protein was localized intracellularly, in a region consistent with the Golgi apparatus (33). Because CFR appears to be expressed at low levels on the surface of CHO cells and these cells express significant levels of endogenous cell surface FGF binding proteins (unpublished data), we developed a RISA for analysis of FGF binding (Materials and Methods). For all subsequent analysis, CHO.CFR clone 4 was used.

Specific binding of ^{125}I-FGF-2 to CFR was detected in CFR-transfected CHO cell extracts and in partially purified CFR preparations derived from chick embryos (Fig. 5A). ^{125}I-FGF-2 binding was not detected in cell extracts prepared from CHO cells transfected with the vector alone or from parental CHO cells. Unlabeled FGF-1, FGF-2, and FGF-4 displaced ^{125}I-FGF-2 bound to CFR. Unrelated growth factors, including PDGF-BB, EGF, insulin, and IGF-II, did not compete for binding of ^{125}I-FGF-2 to CFR, demonstrating that CFR has the appropriate ligand specific-

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-45       M A P C G R V R S R C P G P A L L L L L A L A A R P A L A G P P A A A L Q A
1     ccaagatggcgccgtgcggacgtgtccggagccgctgcccgggccccgcgctcctcctgctgctggcgctggcggcgcggccggcgctcgccgggcctcccgctgccgccctgcaggcc

-7    G P G L N A A G Q P A Q G A A P G A A G P R G A R G G G G G S G G G W K L S E E
120   ggacccggcttgaacgcggcggggcagcccgcgcagggagcggcgcccggtgccgcgggcccgcggggagcccgaggcggcggaggcggctccggcggcgggtggaagctgtcggaggag

34    A V C R E D V V R L C S K H S W A N N L A V L E C L Q D V R E P D N E I S S D C
240   gcggtgtgccgcgaggacgtggtgcggctgtgctccaagcacagctggccaacaacctggccgtgctcgagtgcctgcaggacgtccgcgagccagacaacgaaatctcttcagactgc

74    N H L L W N Y K L N L T T D P K F E S V A R E V C K S T I A E I K E C A D E P V
360   aaccacctcctgtggaactacaagctgaacctgactacagatcccaagtttgagtccgtggccagagaagtctgcaagtccaccatcgcagagatcaaggagtgtgcagatgaaccagtt

114   G K G F L V S C L V D H R G N I T E Y Q C H Q Y I T K M T A I I F S D Y R L I C
480   ggcaaaggcttcttggtgtcgtgtttggtggatcacagaggcaacatcacggagtaccagtgccatcagtacatcaccaagatgacagccattatcttcagcgattaccggctgatctgc

154   G F M D D C K A D I N L L K C G S I R P G E K D A H S Q G E V V A C L E K G L V
600   ggcttcatggacgactgcaaggctgacatcaacctcctgaaatgcggcagcatccgacccggggaaaaggacgcccactcccagggagaggtggtggcatgcctggaaaaaggcctggtg

194   K E A E E N D P R V Q V S D Q C K K A I L R V A E L S S D D F H L D R H L Y F A
720   aaagaggcagaggagaacgaccctcgtgttcaggtttctgatcagtgtaagaaggcgatcctccgcgtagcagagctctcttcggatgatttccacttggaccgccatctctactttgcc

234   C R D D R E R F C E N T Q A G E G R V Y K C L F N H K F E E S M S E K C R D A L
840   tgccgagacgacagggagagattctgtgaaaatactcaggctggagaaggccgagtttacaagtgcctctttaatcacaagtttgaagaatcaatgagtgagaagtgccgtgacgcgctg

274   T T R Q K L I A Q D Y K V S Y S L A K S C K S D L K K Y R C N V E N L P R S R E
960   acgacgcgccagaagctgattgcccaggactacaaagtcagctactcactggccaagtcctgtaagagcgacctgaagaagtaccgctgcaacgtggagaacctgccccgctcccgggaa

314   A R L S Y L L M C L E S A V H R G R Q V S S E C Q G E M L D Y R R M L M E D F S
1080  gccaggctctcctacctgctgatgtgcctggagtctgctgtgcacagaggtcggcaggtgagcagcgagtgccagggagagatgctggactaccgacgcatgctcatggaagacttctcc

354   L S P E I I L S C R G E I E H H C S G L H R K G R T L H C L M K V V R G E K G N
1200  ctgagcccagaaatcatcctgagctgccgtggggagattgagcaccactgctccggctgcaccgcaagggccgcacgctgcactgcctgatgaaagtcgtgcgtggagagaagggcaac

394   V G L N C Q Q A L Q T L I Q E T D P G A D Y R I D R A L N E A C E S V I Q T A C
1320  gtggggctgaactgccagcaggcgctccaaacgctgatccaagaaacggaccctggtgcagattatcgcatcgaccgagccctgaatgaggcctgtgaatctgtcatacagactgcctgc

434   K H I R S G D P M I L S C L M E H L Y T E K M V E D C E H R L L E L Q Y F I S R
1440  aagcacatacggtccggggatcccatgattctgtcctgcctgatggagcacctgtatacggaaaagatggtggaggactgtgagcatcggctcctggaactccagtatttcatatctcgg

474   D W K L D V V L Y R K C Q G D A S R L C H T H G W N E T S E L M P P G A V F S C
1560  gactggaagttggatgtggtcctttaccgaaagtgccagggcgatgcctcccgcctctgccatacccatggctggaatgagaccagcgagctgatgcccccggggctgtgttctcctgc

514   L Y R H A Y R T E E Q G R R L S R E C R A E V Q R I L H Q R A M D V K L D P A L
1680  ttgtacaggcatgcctaccgcacagaggagcagggacggaggctatcacgggagtgcagagcggaggtgcagcggatcctccaccagcgtgccatggatgtgaagctggacccggccctg

554   Q D K C M I D L G K W C S E K T E T G Q E L E C L Q D H L D D L V S D C R D I V
1800  caggacaagtgcatgatcgacctgggcaagtggtgcagtgagaaaacagagacagggcaggaactggaatgtcttcaggaccatctcgatgacttggtgtctgattgcagagacatagtg

594   G N L T E L E S E D I Q I E A L L M R A C E P I I Q T F C H E V A D N Q I D S G
1920  ggaaacctgactgagctggagtctgaggacattcaaatcgaagccttgctgatgagagcatgcgagcccatcatccagaccttctgtcatgaggttgcagataaccagattgattctggg

634   D L M E C L I Q N K H Q K E M N E K C A I G V T H F Q L V Q M K D F R F S Y K F
2040  gacctgatggagtgtttaatacagaacaaacaccagaaggaaatgaatgaaaaatgtgcaatcggagttactcacttccagctggttcaaatgaaagacttcaggttctcctacaagttc

674   K M A C K E D V L K L C P N I K K K V D V V I C L S T T V R N D T L Q D A K E H
2160  aaaatggcttgcaaggaggatgtgctgaaactttgccccaacatcaaaaaaaaggtggatgtagtgatctgcctgagcacaacggtgcgcaatgacactctgcaggacgccaaggagcac

714   R V S L K C R K Q L R V E E L E M T E D I R L E P E L Y E A C K S D I K N Y C Q
2280  agggtatctctgaagtgccgcaagcagctgcgtgtggaggagctagaaatgaccgaggatatcagactggaaccagagctctacgaggcttgtaaaagtgacatcaagaattactgccaa

754   N V P Y G N A Q I I E C L K E I K K Q L S T R C H Q K V F K L Q E T E M M D P E
2400  aacgttccctatggcaatgctcagatcatagaatgcctgaaagaaatcaagaagcagttgagtacccggtgccaccagaaggtgttcaagctgcaggagacagagatgatggatccagag

794   L D Y T L M R V C K Q M I K R F C P E A D S K N M L Q C L K Q N K N S E V M D P
2520  ctggactacacactgatgagagtctgcaagcagatgatcaagcgttttgcccagaagcagactcaaaaaacatgttgcagtgtttgaaacaaaacaagaacagtgaagtgatggatcca

834   K C K Q M I T K R Q I T Q N T D Y R L N P V L R K A C K A D I P K F C Q N I L N
2640  aagtgcaagcaaatgataaccaagcgccagatcacacagaacacagattaccgcctaaatccggtgctgaggaaggcctgcaaagcagacataccaaagttctgccagaatattctgaat

874   R A K D D T E L E G Q V I S C L K L K Y A D Q R L S P D C E D Q I R V I I Q E S
2760  agagccaaggatgatacagagttggagggacaggtcatctcctgcctgaagctgaagtatgcagaccagcgtctctctccagactgcgaggaccaaattcgagtgatcatccaggaatca

914   A L D Y R L D P Q L Q M H C S E E I S S L C A E E A A A Q E Q T G Q V E E C L K
2880  gcccttgattatcgactggatccccagcttcagatgcattgctctgaagagatttccagcttgtgtgctgaagaggcagcagctcaggagcagactgggcaggtggaagaatgcctgaaa

954   V N L L K I K T E M C K K E V L N M L K E S K A D I F V D P V L H T A C A L D I
3000  gtgaacctgctgaaaataaaaaccgaaatgtgcaagaaggaggtgctcaacatgctgaaggagagcaaggcagatatctttgtggacccagtgctgcacacagcctgtgccctggacatt

994   K H H C A A I P P G R G R Q M S C L M E A L E D K R V R L Q P E C K K R L N D R
3120  aaacaccactgtgctgctattcctccagggagaggacgccaaatgtcatgcttaatggaagctttggaggacaagcgtgtgaggctgcagcctgaatgcaagaaacgccttaatgatcgt

1034  I E M W S Y A A K V A P A E G F S D L A M Q V M T S P S K N Y I L S V I T V G I
3240  attgaaatgtggagctatgctgcaaaggttgccccagcggaaggcttctctgaccttgccatgcaagttatgacctctccgtccaagaattacatattgtctgtgatcacggttggcatc

1074  C V L F L I G L M C G R I T K R V T R E L K D R *
3360  tgtgtactcttcctgatcggcctgatgtgtggacgcatcaccaagcgggtgacaagagagctcaaggacaggtagagcctggattgcctgctggagaaatgcctgcccagtgcccagttt

3480  tgtacagccctcctctgtatagtctacccacccccctcttgtgagagaggagaataaaaaaaaaaaaa  3546
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FIG. 1. Nucleotide and predicted amino acid sequence of CFR. Protein sequences corresponding to that obtained from purified receptor preparations are underlined. The amino terminus of the purified protein is indicated by an arrow. Solid black boxes indicate the signal peptide and membrane-spanning domains. Consensus sites for the attachment of asparagine-linked oligosaccharide are demarcated by open boxes. A consensus polyadenylation signal is underlined with a dashed line. The CFR cDNA sequence has been submitted to GenBank under accession number M95766.

ity for an FGF receptor (Fig. 5A). Moreover, a 2 M NaCl wash, which has previously been demonstrated to release ^{125}I-FGF-2 from HSPGs (41), does not release ^{125}I-FGF-2 from CFR (Fig. 5A).

We have previously reported that two polypeptides of 45 and 70 kDa copurify with CFR (12) and we now have evidence demonstrating that these polypeptides associate with CFR to form a complex (11a). To demonstrate that the

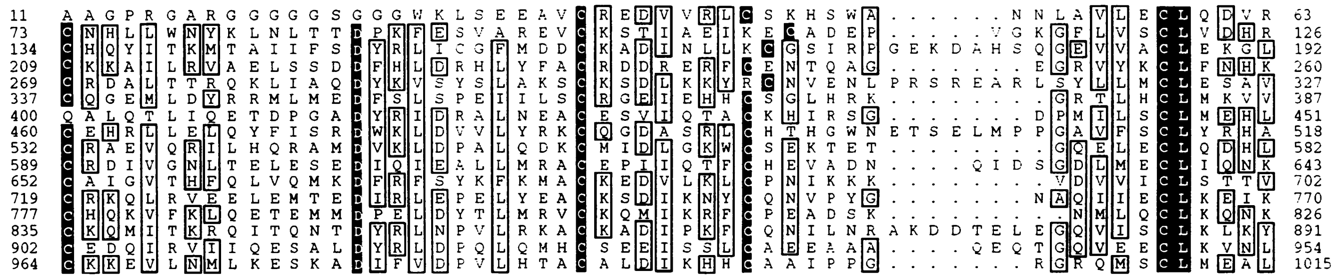

FIG. 2. Alignment of the 16 cysteine-rich repeats. Black boxes indicate amino acids residues that are nearly identical in each of the 16 repeats. Residues in columns containing at least eight highly similar residues are in open boxes. Amino acids scoring at least 0.4 in a Dayhoff comparison table (63) were considered highly similar. Although lysine and histidine score less favorably, both residues score at 0.4 compared with arginine and glutamine. Hence, lysine and histidine were grouped together in columns containing arginine and/or glutamine residues. Numbers to the left and right of the repeats correspond to the first and last amino acid of each repeated unit, respectively.

binding of FGF-2 to CFR in CHO cell extracts does not require the presence of additional proteins, a RISA was performed with extracts from [^{35}S]Met- and [^{35}S]Cys-labeled CHO cells and unlabeled FGF-2 (Fig. 5B). A single ^{35}S-labeled protein of 150 kDa (chicken CFR) was immunoadsorbed from CFR-transfected CHO cell extracts, demonstrating that additional proteins are not likely to be required for specific binding of ^{125}I-FGF-2 to CFR.

The affinity of ^{125}I-FGF-2 chick embryo CFR and recombinant CFR expressed in CHO cells was determined by Scatchard analysis of equilibrium binding data (62). The binding of ^{125}I-FGF-2 to partially purified CFR (Fig. 6A and C) and recombinant CFR (Fig. 6B and D) was saturable and specific, yielding dissociation constants (K_d) of 1.0 ± 0.3 and 1.1 ± 0.5 nM, respectively. These K_d values represent the mean from three independent experiments and are likely to be greater than those determined for CFR in a native membrane environment, as these assays are performed on detergent-solubilized CFR. Purified and recombinant CFR exhibit virtually identical affinities for ^{125}I-FGF-2.

Characterization of CFR transcripts. Northern analysis of day 7 embryonic chick poly(A)$^+$-selected RNA revealed a major transcript of 8.6 kb and several smaller, less abundant transcripts including a 3.8-kb mRNA (Fig. 7, upper panel). The transcript from which the CFR cDNA clone was produced has not yet been identified. In order to determine whether the 8.6-kb transcript represented an unprocessed CFR message, four antisense oligonucleotides were hybridized with CFR transcripts present in total RNA preparations, and the resultant DNA:RNA hybrids were digested with RNase H. The 8.6-kb transcript was sensitive to digestion with all four oligonucleotides (Fig. 7, upper panel). The observed fragment sizes suggest that sequence corresponding to the contiguous CFR cDNA lies approximately 100 bp from the 5′ end of the mRNA. Thus, the majority of the

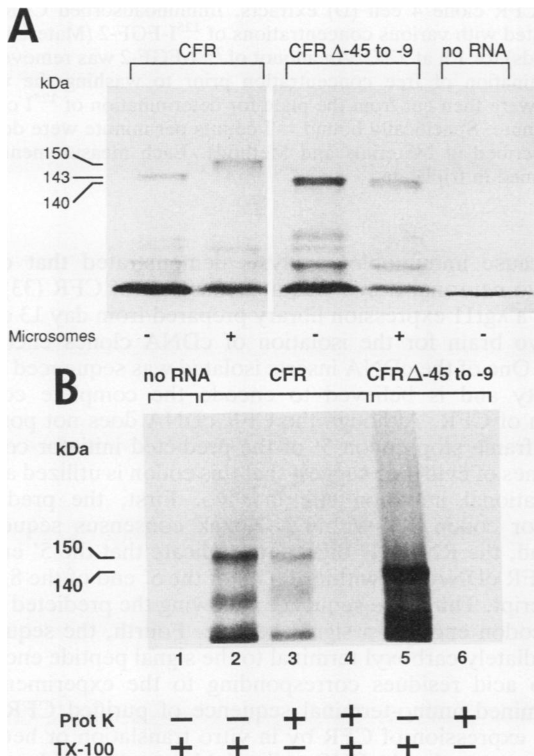

FIG. 3. Expression of CFR by in vitro translation. (A) CFR and CFR Δ−45 to −9 cRNA translated in vitro by utilizing rabbit reticulocyte lysate in the presence (+) or absence (−) of canine pancreatic microsomes. A control translation with no added RNA is included. (B) CFR and CFR Δ−45 to −9 cRNA were translated by using reticulocyte lysate in the presence of canine pancreatic microsomes. Proteinase K (Prot K) with or without Triton X-100 (TX-100) detergent was added following translation where noted.

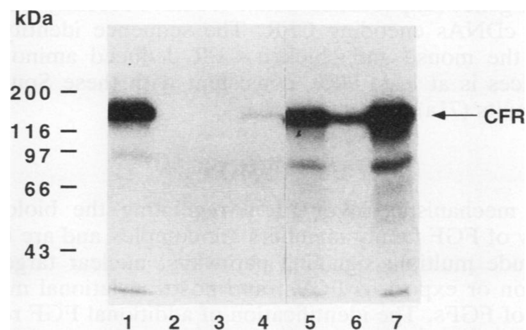

FIG. 4. Expression of CFR in CHO cells. Several G418-resistant clones were analyzed for CFR expression by immunoblotting cell extracts as described in Materials and Methods. Partially purified chick embryo CFR (lane 1), parental CHO-K1 cells (lane 2), vector-transfected CHO-K1 cells (CHO.pDX) (lane 3), and CFR-transfected clones 1 (lane 4), 2 (lane 5), 3 (lane 6), and 4 (lane 7) were analyzed.

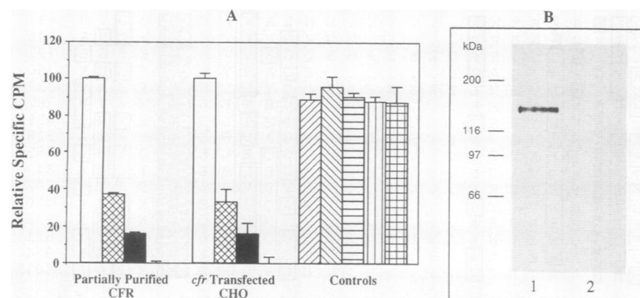

FIG. 5. Demonstration of CFR ligand binding specificity. (A) FGF binding and competition assay with the RISA. Binding of ^{125}I-FGF-2 (□, 100 pM) to partially purified chick CFR and recombinant CFR expressed in CHO.CFR 4 cells. Binding was performed in the presence of a 1,000-fold molar excess of the following unlabeled growth factors: FGF-1 (▨), FGF-4 (■), FGF-2 (only error bars visible), PDGF (▨), EGF (▨), insulin (▤), IGF-II (▥), and without competitor but following a 2 M NaCl wash (▦). Nonspecific binding was defined as the amount of ^{125}I-FGF-2 bound in the presence of excess unlabeled FGF-2. The values shown (specific binding) were derived by subtracting the nonspecific binding from the total binding. (B) Autoradiogram of SDS-PAGE gel showing ^{35}S-labeled proteins present in wells following a RISA. CHO were labeled with [^{35}S]Met and [^{35}S]Cys, and a RISA was performed as described except that unlabeled FGF-2 was used in place of ^{125}I-FGF-2. Labeled extracts were from CHO.CFR clone 4 cells (lane 1) and CHO.pDX cells (lane 2).

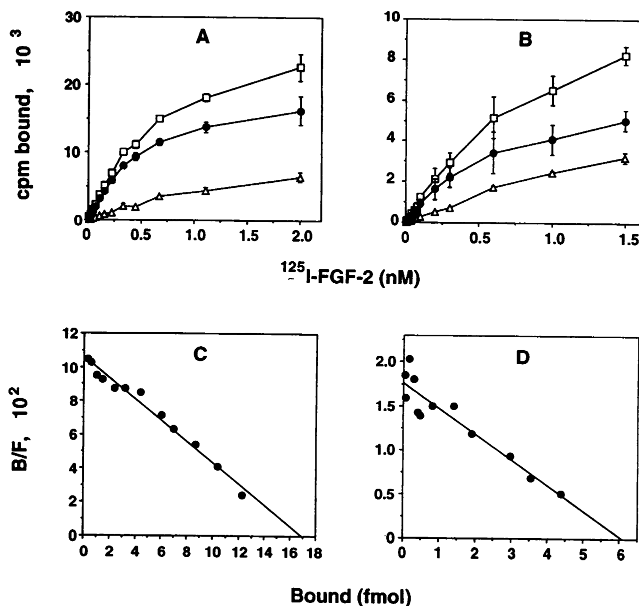

FIG. 6. Quantitative analysis of ^{125}I-FGF-2 binding to CFR. Equilibrium binding of ^{125}I-FGF-2 to CFR partially purified from embryonic chick (A) and CHO.CFR 4 cell extracts (B) was performed by using the RISA as described. The symbols □, △, and ● refer to total, nonspecifically and specifically bound ^{125}I counts per minute, respectively. The equilibrium binding data were plotted by the method of Scatchard (62) for embryonic chick (C) and CHO.CFR clone 4 cell (D) extracts. Immunoadsorbed CFR was incubated with various concentrations of ^{125}I-FGF-2 (Materials and Methods) for 1 h at 22°C. An aliquot of ^{125}I-FGF-2 was removed for determination of free concentration prior to washing the wells. Wells were then cut from the plate for determination of ^{125}I counts per minute. Specifically bound ^{125}I counts per minute were defined as described in Materials and Methods. Each measurement was performed in triplicate.

8.6-kb transcript is likely to be composed of 3′ untranslated sequence (Fig. 7, lower panel). The presence of additional CFR transcripts is suggestive of alternate mRNA splice variants and/or mRNAs encoding related proteins. Two of the transcripts are not sufficiently long to encode the entire CFR open reading frame and may encode truncated versions of CFR. This result is not unexpected, as a number of truncated cell surface receptors, including FGFRs, are encoded by transcripts that yield only the extracellular portion of these receptors (29). The biological role of these truncated, secreted receptor forms is presently unknown.

Comparison of CFR homology among vertebrate species. To assess the relatedness of chicken CFR with that of other species, the chicken CFR cDNA was hybridized to genomic DNA prepared from several vertebrate species. Chicken CFR cDNA hybridized at high stringency to all vertebrate species examined, indicating the presence of a highly related gene or genes (unpublished data). We have recently isolated mouse cDNAs encoding CFR. The sequence identity between the mouse and chicken CFR deduced amino acid sequences is at least 90%, consistent with these Southern blot results (71a).

DISCUSSION

The mechanisms involved in regulating the biological activity of FGF family members are complex and are likely to include multiple signaling pathways, nuclear targeting, secretion or export of FGFs, and posttranslational modification of FGFs. The identification of additional FGF receptors will aid in understanding the mechanisms involved in mediating FGF's diverse biological effects. Two distinct types of FGF receptors have been previously characterized and include the FGFR family and HSPGs. In this paper, we report the cDNA cloning and biochemical characterization of a third type of FGF receptor that we have designated CFR for cysteine-rich FGF receptor.

Because immunoblot analysis demonstrated that chick embryo neuronal tissue was a rich source of CFR (33), we chose a λgt11 expression library prepared from day 13 chick embryo brain for the isolation of cDNA clones encoding CFR. One of the cDNA inserts isolated was sequenced in its entirety and is believed to encode the complete coding region of CFR. Although the CFR cDNA does not possess an in-frame stop codon 5′ of the predicted initiator codon, five lines of evidence suggest that this codon is utilized as the translational initiation site in vivo. First, the predicted initiator codon lies within a Kozak consensus sequence. Second, the RNase H digestions indicate that the 5′ end of the CFR cDNA lies within 100 bp of the 5′ end of the 8.6-kb transcript. Third, the sequence following the predicted initiator codon encodes a signal peptide. Fourth, the sequence immediately carboxyl terminal to the signal peptide encodes amino acid residues corresponding to the experimentally determined amino-terminal sequence of purified CFR. Finally, expression of CFR by in vitro translation or heterologous expression in CHO cells yields a polypeptide of the expected molecular weight. The 3′ end of the cDNA possesses a stop codon followed by 111 nucleotides terminating in a short poly(A) stretch. Although a polyadenylation recognition sequence is present, we have not yet determined whether the poly(A) stretch is encoded by the CFR gene or is the result of polyadenylation.

The deduced amino acid sequence of CFR revealed char-

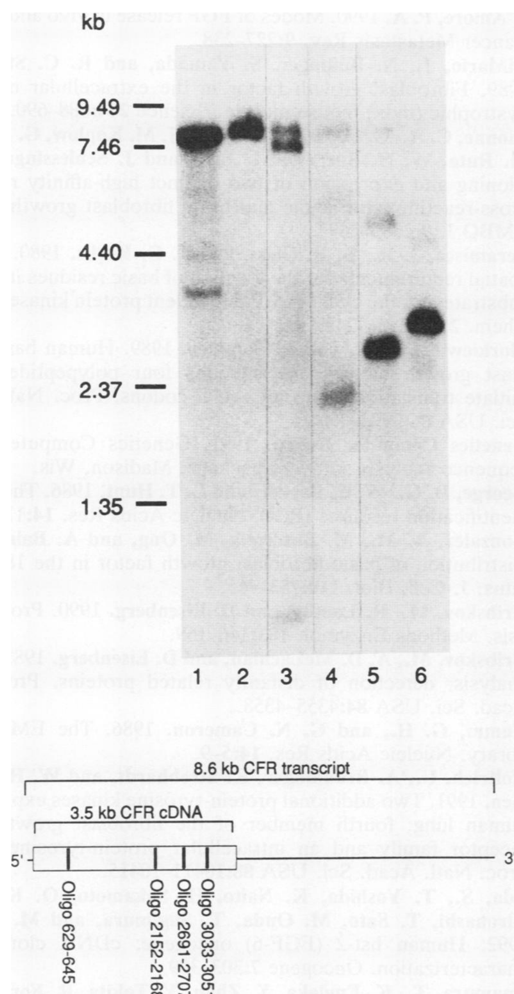

FIG. 7. Characterization of CFR transcripts. (Upper panel) Northern analysis of day 7 embryonic chick RNA. Blots were hybridized with ^{32}P-labeled CFR cDNA and washed under stringent conditions. A total of 3 μg of poly(A)$^+$-selected RNA and 15 μg of total RNA were electrophoresed in lanes 1 and 2, respectively. Lanes 3 to 6 contain total RNA digested with RNase H in the presence of antisense oligonucleotides corresponding to the following regions of the CFR cDNA sequence: nucleotides 629 to 645 (lane 3), nucleotides 2152 to 2168 (lane 4), nucleotides 2691 to 2707 (lane 5), and nucleotides 3033 to 3051 (lane 6). The presence of the 8.6-kb transcript in samples is due to incomplete digestion by RNase H. (Lower panel) Schematic of the CFR 8.6-kb transcript and relative positions of the four antisense oligonucleotides.

acteristics typical of cell surface transmembrane receptors including (i) a signal peptide, (ii) a membrane spanning domain that anchors CFR in the lipid bilayer, and (iii) consensus sites for N-linked glycosylation. Because CFR is glycosylated, the presence of five consensus sites for asparagine-linked glycosylation on the amino-terminal side of the membrane-spanning domain suggests that CFR is oriented in the membrane such that these sites and the majority of the sequence are present on the outside of the cell. This receptor is unusual, as it possesses no known homologies to other proteins, exhibits a 50- to 60-amino-acid cysteine-rich repeated motif, and has an extremely short (13-residue) cytoplasmic domain. The sixteen cysteine-rich repeats compose virtually the entire extracellular domain. The 30-kDa change in mobility of purified CFR analyzed by SDS-PAGE under reducing and nonreducing conditions suggests that some of these cysteine residues are involved in disulfide linkages (12). Thus, it is possible that the function of these repeated units is to maintain the structural integrity of CFR. Finally, if CFR functions as a signal transducer, association with additional intracellular proteins would be expected, as the cytoplasmic domain is too short to possess an inherent catalytic function. CFR forms a complex with two additional proteins of 70 and 45 kDa that do not possess N-linked carbohydrate, suggesting that they may associate with the intracellular domain of CFR.

Because the parental CHO-K1 cells used in these experiments express a large number of high-affinity ^{125}I-FGF-2 binding sites and the levels of cell surface CFR are low, differences in ^{125}I-FGF-2 binding or cross-linking between intact control and CFR expressing cells were minimal. To measure binding of ^{125}I-FGF-2 to CFR, we developed a RISA to quantitatively assess the affinity and number of CFR binding sites present in solubilized membrane extracts. Binding of ^{125}I-FGF-2 to CFR was displaced by the addition of excess unlabeled FGF-1, FGF-2 or FGF-4 but not by unrelated growth factors including EGF, insulin, IGF-II, and PDGF. Because FGF-1, FGF-2, and FGF-4 have very different isoelectric points, it is unlikely that the binding of FGF family members to CFR is due to nonspecific ionic interactions. Scatchard analysis of ^{125}I-FGF-2 binding to either purified or recombinant CFR revealed a single class of sites with a dissociation constant of ~1 nM. As these binding studies were performed on detergent-solubilized CFR, we believe that this value is likely to underestimate the affinity of the endogenous protein in its native membrane environment. We have not yet determined the relative affinities of additional FGF family members for CFR, and thus, it is impossible to predict if CFR preferentially binds certain FGFs.

The binding of ^{125}I-FGF-2 to CFR further distinguishes CFR from the HSPGs and FGFRs. ^{125}I-FGF-2 bound to HSPGs is removed by a 2 M NaCl wash, whereas ^{125}I-FGF-2 bound to CFR is resistant to this wash. We and others have previously shown that binding of FGFs to FGFRs requires the presence of an HSPG or heparan sulfate (7, 45, 56, 70). In contrast to FGFRs, CFR is capable of interacting with FGFs in the absence of heparan sulfate (unpublished data). Thus, the binding sites for FGFs on the FGFRs and CFR may be fundamentally different, perhaps explaining why no sequence conservation is observed between CFR and the FGFRs even though they have similar ligand specificity. It is interesting to speculate that these fundamental differences with respect to heparan sulfate may help resolve why the addition of exogenous heparin has disparate effects on the biological activity of FGF in different cell lines.

If CFR is of fundamental importance in FGF action, the primary sequence would be expected to be conserved across evolutionarily distinct species. We initially examined CFR homology among several vertebrate species by Southern analysis. All vertebrate species examined appear to possess DNA sequences with significant homology to CFR, as hybridization of these genomic DNAs to chicken CFR cDNA occurred at high stringency. Additional evidence for a high degree of homology among the predicted amino acid sequences of chicken and mouse CFR has been obtained from the sequencing of a partial mouse CFR cDNA. The overall sequence identity between chicken and mouse over a region spanning approximately 3/4 of the protein is at least 90% (71a). This sequence identity is comparable to the predicted

amino acid identity observed for the chicken and mouse FGFR1 and FGFR2 cDNAs (35, 48). Thus, these data suggest that the function of CFR is likely to be conserved and that CFR plays an important role in FGF biology.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AR-39467 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases. B.B.O. is a PEW Scholar in the Biomedical Sciences and a Scholar of the Shaw Foundation. L.W.B. and B.A.L. were supported by Cell and Molecular Biology Training Grant GM07215. M.E.Z. is supported by Merck, Sharpe & Dohme.

L.W.B. and M.E.Z. contributed equally to this work. We thank Mike Sheets for suggesting the RNase H experiment.

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