

Cysteine-rich Fibroblast Growth Factor Receptor Alters Secretion and Intracellular Routing of Fibroblast Growth Factor 3*

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Expression of the cysteine-rich fibroblast growth factor (FGF) receptor (CFR) in COS-1 cells strongly inhibits the secretion of co-expressed FGF3. By using a column retention assay and affinity chromatography, we demonstrate that at physiological salt concentrations FGF3 binds with strong affinity to CFR *in vivo* and *in vitro*. Furthermore, to show that FGF3 binds to CFR *in vivo*, truncation mutants of CFR with changed subcellular distributions were shown to cause a similar redistribution of FGF3. Although CFR is a 150-kDa integral membrane glycoprotein that is primarily located in the Golgi apparatus, we show here that in COS-1 cells a substantial proportion of CFR is secreted. This is due to a carboxyl-terminal proteolytic cleavage that releases the intraluminal portion of the protein for secretion. However, the apparent size of the integral membrane and secreted CFR appears similar, since the loss of protein mass is balanced by a gain of complex carbohydrates. The released CFR is associated with the extracellular matrix through its affinity for glycosaminoglycans. These findings show that CFR can modulate the secretion of FGF3 and may control its biological activity by regulating its secretion.

Fibroblast growth factors (FGFs)¹ are a family of at least 18 structurally related polypeptides that can modulate the growth, differentiation, migration, and survival of various cell types in culture (reviewed in Refs. 1–4). The properties of FGFs indicate they act primarily as intercellular signaling molecules, by binding to and activating receptors on the surface of the same or adjacent cells. Four high affinity FGF receptor genes (FGFR1–4) have been identified that encode transmembrane tyrosine kinases (5, 6). Moreover, signal transduction requires the presentation of FGFs by a second type of receptor composed of heparan sulfate containing proteoglycan. In addition to the transmembrane signaling receptor complex, a third class of membrane-anchored FGF-binding protein has been identified as a cysteine-rich FGF receptor (CFR) (7, 8).

CFR was purified as an FGF-binding protein from embryonic chick and shown to be a 150-kDa integral membrane glycoprotein (9). It subsequently emerged that the CFR is homologous to a rat protein cloned as a Golgi-specific protein designated MG-160 (10–12). More recently, the human homologue of the same protein was identified as an E-selectin-binding protein (ESL-1) provided it was modified by $\alpha(1,3)$ -fucosylation (13, 14). A comparison of the derived amino acid sequences of the chicken and human homologues shows more than 90% sequence identity, demonstrating a high degree of conservation during evolution (7, 9, 10). Chicken CFR and rat-MG160 were shown to localize primarily to the Golgi apparatus, and ESL-1 was described as a cell surface protein (11, 12, 16).

The sequence of CFR reveals 16 cysteine-rich repeats in the intraluminal domain and a short cytoplasmic tail (7, 12). CFR binds FGF1, FGF2, and FGF4, and its presence has been shown to alter the levels of intracellular FGF1 and FGF2 suggesting a role in the intracellular trafficking of the FGFs (7, 17). However, the CFR shows no recognizable sequence homology to the FGF tyrosine kinase receptors, nor to the proteoglycan co-receptors. The CFR failed to bind several other growth factors tested (platelet-derived growth factor-BB, epidermal growth factor, insulin, and insulin-like growth factor-II), but forms a secreted complex with TGF- β (7, 18).

Previously we showed that mouse FGF3 was retained in the medial Golgi complex of COS-1 cells and only slowly released into the culture medium, raising the possibility that FGF3 export may be controlled (19). Regulation at the level of secretion has been also described for TGF- β , and interestingly, this ligand is also retained in the Golgi complex as an inactive precursor (18, 20). The coincident intracellular localization of FGF3 and the CFR in the Golgi apparatus prompted us to investigate whether there was a functional interaction between these two proteins. We show here that FGF3 binds to the CFR and that the CFR can alter FGF3 secretion and its subcellular localization.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-1 cells were maintained as described previously (16). For transient DNA transfections, plasmid DNA as indicated was introduced into 5×10^5 COS-1 cells by electroporation (450 V/250 microfarads). Between 48 and 72 h after transfection, the cells were harvested for immunoblot analysis or processed for immunofluorescence.

Plasmid Constructions—pCFR1.1 was constructed by inserting anti-RGS(His)₆ epitope downstream of the signal peptide cleavage site of a chicken CFR cDNA (7). The amino-terminal 48 codons of chicken CFR were amplified by PCR using a 3' primer that encodes the RGS-His epitope and included a *Bam*HI site. A second PCR fragment was generated using a 5' primer encoding the same epitope and encompassing the *Bam*HI site to amplify the downstream CFR sequence beginning with codon 49. Both resulting PCR fragments were fused via the newly created *Bam*HI site placing the RGS-His epitope 5 codons downstream

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¹ The abbreviations used are: FGF, fibroblast growth factor; ECM, extracellular matrix; CFR, cysteine-rich FGF receptor; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; TGF- β , transforming growth factor- β .

from the signal peptide cleavage site (17). The modified CFR cDNA was then inserted in the expression vector pKC4 under control of the early SV40 promoter. To obtain the plasmids pCFR2.1 and pCFR3.1, PCR was used to delete the carboxyl-terminal 12 and 34 codons of CFR, respectively. The 3' oligonucleotide primers were used to introduce a stop codon and an *EcoRI* site. The resulting 3' fragments were used to replace the 3' sequences of pCFR1.1 through a naturally occurring *HindIII* site. PGEX-CFR was constructed by subcloning the total CFR3.1 insert as a partial *BamHI-EcoRI* fragment into pGEX2T (Amersham Pharmacia Biotech).

Immunofluorescence—COS-1 cells grown on glass coverslips were transfected with the appropriate plasmids, and 48 h later the cells were fixed and processed as described previously (19). For surface immunostaining, the cells were incubated with antibodies without permeabilizing; alternatively, cells were incubated with the antibodies at 4 °C in the presence of 0.05% sodium azide prior to fixation. After washing in PBS, the stained cells were mounted in 90% glycerol containing *p*-phenylenediamine and viewed with a 63× oil immersion lens on a Zeiss microscope equipped with barrier filters for fluorescein or Texas Red. Rabbit antiserum directed to the carboxyl terminus of mouse FGF3 was diluted 1:200 in PBS. CFR was detected using a mouse monoclonal antibody, anti-LA epitope 15E9 against chicken CFR (8), or a mouse monoclonal antibody against the RGS-His tag (Qiagen). The cis-Golgi compartment was visualized with a rabbit antibody against the cis-Golgi matrix protein GM130 (kindly provided by Dr. Graham Warren, ICRF (21)). For visualizing the endocytotic pathway, cells were incubated with 5 mg/ml lysine-fixable FITC-dextran (10,000 MG, Molecular Probes) in culture medium at 37 °C for 1 h before fixation.

Immunoblot Analysis—The procedures used for preparing cell lysates and ECM have been described in detail elsewhere (19, 22). Samples from equivalent numbers of cells were fractionated by SDS-PAGE in 12.5% gels, transferred to nitrocellulose membranes (Schleicher & Schuell), and then probed with rabbit antiserum to the carboxyl terminus of FGF3 or monoclonal antibodies to detect CFR or the RGS-His epitope (Qiagen). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Digestion of Carbohydrate Side Chains—Medium and cell extracts of CFR-transfected COS-1 cells were concentrated by acetone precipitation, resuspended in denaturation buffer (0.5% SDS and 1% β -mercaptoethanol), and treated with neuraminidase and *N*-glycanase as described by the manufacturer (Biolabs). For neuraminidase treatment, the acetone-precipitated proteins were resuspended in 50 mM sodium citrate, pH 4.5, as recommended by the manufacturer (Biolabs). The digested products were analyzed by SDS-PAGE and immunoblotting.

In Vivo CFR Column Retention Assay—Cell extracts from FGF3 cDNA and pCFR1.1 co-transfected or from FGF3 cDNA alone transfected COS-1 cells lysed by sonification in a 50 mM phosphate buffer, pH 7.8, containing 300 mM sodium chloride were incubated with 50 μ l of Ni²⁺-nitrilotriacetic acid resin (Qiagen) for 2.5 h at 4 °C in sonification buffer at a final volume of 500 μ l. After washing with a 200-fold volume of sonification buffer containing 10 mM imidazole, the bound proteins were eluted with sonification buffer containing increasing concentrations of imidazole, and the eluates were analyzed by immunoblotting using a polyclonal antibody against FGF3 or a monoclonal antibody against CFR. The immune complexes were detected by the enhanced chemiluminescence technique as described under "Experimental Procedures."

GST-CFR Fusion Protein Affinity Chromatography—An overnight 30-ml *Escherichia coli* culture containing PGEX-CFR or a control GST plasmid was diluted 10-fold into LB/ampicillin medium and grown at 37 °C to OD 1.0 before induction with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (BTS). Bacteria were lysed by pulse sonication in lysis buffer (1% Triton X-100, 1.5% *N*-laurylsarcosine, 25 mM triethanolamine, 1 mM EDTA in PBS). 200 μ l of a 50% slurry of GSH-agarose beads (Molecular Probes) was added and incubated at 4 °C overnight. After washing 6 times in an excess volume of PBS, 1% Triton X-100 (PBS-TX), agarose beads containing bound proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining and immunoblot analysis. Interactions between GST-CFR and FGF3 cell-associated proteins were analyzed using 0.5-ml aliquots of FGF3-transfected COS-1 cells (equal to 2×10^5 cells) to which 50 μ l of the prepared GST- or GST-CFR-glutathione-agarose beads were added. The binding reaction was incubated at 4 °C overnight. The beads were washed, resuspended in PBS-TX buffer, and poured into a column. The columns were extensively washed with an excess of PBS-TX, and the retained proteins were analyzed by SDS-PAGE. FGF3-related proteins were detected by immunoblotting.

RESULTS

CFR Is Secreted and Associated with the ECM—As a prelude to investigating the effect of CFR on FGF3 secretion, we have analyzed the subcellular distribution of CFR using two expression plasmids, pCFR1 and pCFR1.1. pCFR1 contains a full-length chicken cDNA encoding CFR, whereas pCFR1.1 additionally incorporates a histidine epitope at the amino terminus of CFR (Fig. 1A). COS-1 cells transfected with pCFR1 or pCFR1.1 contain products of the expected size of 150 kDa that were detected by immunoblotting with a monoclonal antibody (LA epitope) that maps toward the carboxyl terminus of CFR, or in the case of pCFR1.1 protein was detected with an antibody to the histidine tag (Fig. 1B).

The identification of ESL-1 as a cell surface protein and MG-160 as Golgi apparatus-specific, prompted a re-examination of the subcellular location of CFR/ESL-1/MG-160. The ability of CFR to bind heparin also suggested that a secreted product may remain associated with the cell surface and extracellular matrix (ECM). To test these possibilities, pCFR1 and pCFR1.1 were transfected into COS-1 cells and analyzed for cell-associated and secreted products in the presence or absence of heparin (Fig. 1C and data not shown). In the absence of heparin, CFR was associated with the cell, ECM, and medium. However, in the presence of heparin, more CFR was displaced into the medium from the cell surface/ECM, indicating that the CFR is secreted from transfected COS-1 cells.

Secreted CFR Is Truncated at the Carboxyl Terminus—As secretion of a transmembrane protein may involve proteolysis, the size of the non-glycosylated CFR products was determined. Proteins were prepared from pCFR1.1-transfected COS-1 cells and medium and treated with glycosidases as described, and the sizes of the resultant CFR proteins were determined by immunoblotting (Fig. 1D). Digestion of the cell extract with neuraminidase alone did not result in a recognizable decrease in the size of the CFR suggesting that the majority of the intracellular CFR forms have not yet gained sialic acid modifications. However, when the same cell extract was treated with neuraminidase and *N*-glycanase, the 150-kDa form was almost completely reduced to approximately 135 kDa, presumably corresponding to the non-glycosylated translation product after signal peptide cleavage (Fig. 1D). By contrast, the majority of secreted CFR proteins reduced their estimated molecular mass to approximately 112–124 kDa following digestion with neuraminidase alone, consistent with sialic acid modifications, and their mass was further decreased to 100–110 kDa in the presence of additional *N*-glycanase (Fig. 1D). The faint 150-kDa band still visible after digestion with neuraminidase presumably represents residual undigested secreted CFR. Also the weak 135–140-kDa band present in the *N*-glycanase and neuraminidase digestion is probably due to partial digestion. Since the double digestion conditions have been optimized for *N*-glycanase but not for neuraminidase, it is quite likely that some *N*-linked carbohydrates are less accessible and therefore less efficiently cleaved (23). In addition, concentration of the proteins by acetone precipitation prior neuraminidase and *N*-glycanase treatment may have affected the efficiency of the digestions. However, the majority of the secreted protein is sensitive to *N*-glycanase and neuraminidase, and the decrease in mass is consistent with loss of carbohydrate from the five predicted Asn-linked glycosylation sites. This demonstrates that the core protein(s) of secreted CFR are considerably smaller than the intracellular precursor, although they appear to have similar mass because additional sialylation compensates for the reduction of protein content. As the CFR proteins were recognized by a monoclonal antibody against the amino-terminal histidine epitope, differences in mass between the

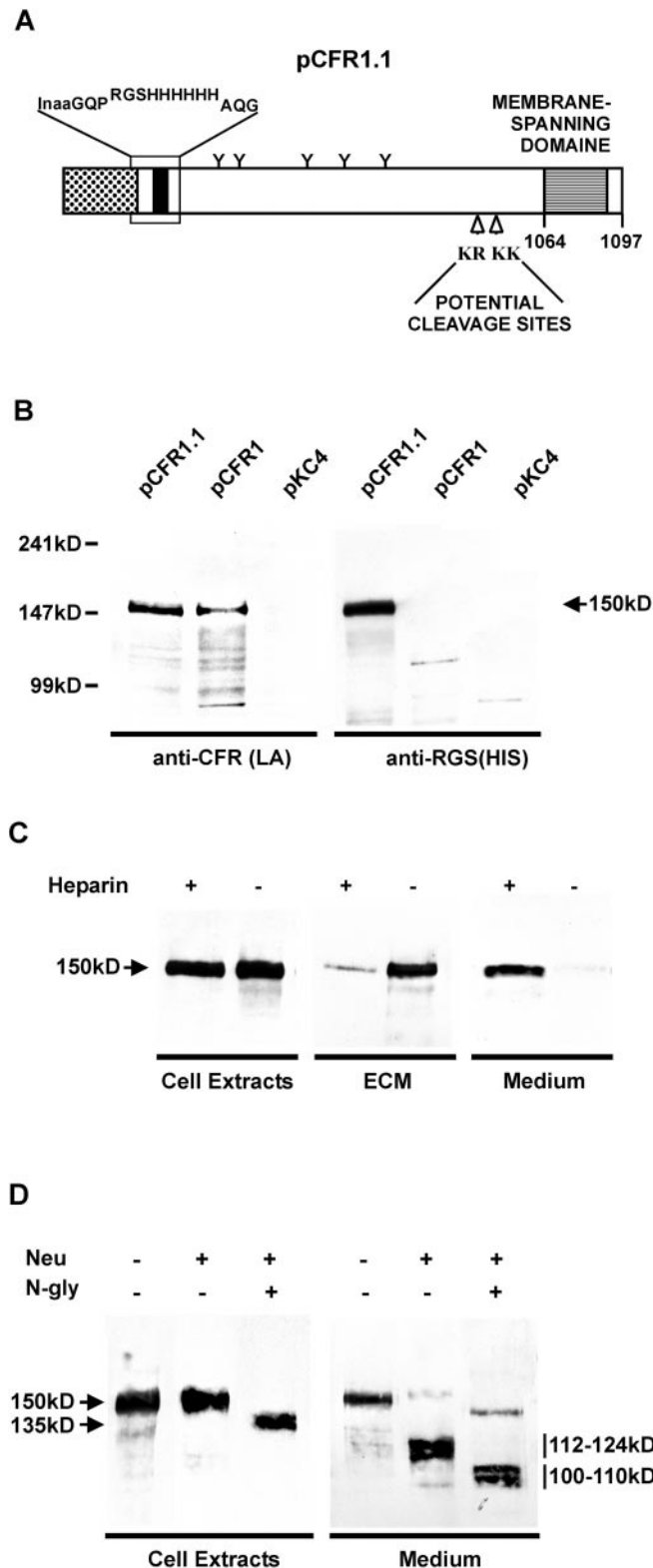


FIG. 1. Presence of chicken CFR in the ECM and culture medium. *A*, schematic depiction of pCFR1.1 showing the main features of the CFR: amino-terminal signal peptide (*heavy stippling*), the transmembrane domain (*horizontal stripes*), and the position of potential *N*-linked glycosylation sites (*Y*). The *shaded box* represents the RGS-His epitope inserted carboxyl-terminal to the signal peptide cleavage site. *B*, extracts from COS-1 cells transfected with pCFR1, pCFR1.1, or the empty vector pKC4 were separated by SDS-PAGE, and the CFR proteins were detected by immunoblotting with an antibody to the CFR or the RGS-His epitope. *C*, COS-1 cells transfected with pCFR1.1 were harvested after 48 h, and the culture fluid was recovered. The cells were washed in PBS and removed from the culture dish with 0.5% Triton X-100 in PBS. The material remaining on the dish was operationally

non-glycosylated extracellular and intracellular forms of the CFRs must occur at their respective carboxyl termini. This would suggest the presence of proteolytic cleavage sites located amino-terminal to the transmembrane domain, which would also provide an explanation for CFR secretion (see Fig. 1*A* for potential position of cleavage sites).

CFR Co-expression Alters FGF3 Secretion—To examine the effect of CFR on the subcellular distribution and trafficking of FGF3, COS-1 cells were co-transfected with pCFR1.1 and a plasmid (pKC3.2) designed for the efficient expression of secreted FGF3 (19). Four intracellular isoforms of FGF3 have been identified as follows: two major glycosylated species of 31.5 and 30.5 kDa (gp31.5 and gp30.5) and two less abundant non-glycosylated forms, 28.5 and 27.5 kDa (Fig. 2*B*). The higher molecular weight species of each pair of proteins was previously shown to differ from the lower molecular mass members by retention of the signal peptide. These four protein isoforms of FGF3 reside in the secretory pathway, predominantly associated with the medial Golgi compartment (19). A form of FGF3 (gp32.5), which is endoglycosidase H-resistant, is secreted and can associate with the ECM (Fig. 2*A*). As CFR and FGF3 are both retained in the Golgi apparatus, there is a possibility that the presence of an FGF-binding protein in the same compartment is directly influencing the intracellular trafficking of FGF3. To test this possibility, a cDNA encoding FGF3 was co-transfected into COS-1 cells with increasing amounts of pCFR1.1 (Fig. 2*A*). The results show that transfection of even a small amount of CFR cDNA causes a diminished release of FGF3 into the culture medium. This was not due to a reduction of intracellular FGF3 synthesis as the total amount of FGF3 detected in COS-1 cells was not detectably diminished, even at higher concentrations of CFR cDNA (Fig. 2*B*). Under the conditions used, the amount of CFR protein detected was proportional to the amount of input cDNA (data not shown). Thus it would appear that FGF3 is sequestered in the secretory pathway, possibly through an association with CFR. This finding also suggests that the amount of CFR that is secreted would not be sufficient to compensate for the amount retained by the bulk of CFR that resides in the Golgi.

In Vivo and in Vitro Detection of CFR-FGF3 Binding—By using a column retention assay, we tested the capacity of CFR to bind FGF3 *in vivo*. Cell extracts were prepared from COS-1 cells transfected with FGF3 or made from cells that have been co-transfected with the FGF3 cDNA and the histidine tag containing pCFR1.1 plasmid. The extracts were applied to a Ni²⁺-chelate column to test the ability of FGF3 to bind CFR that is attached to the column via its His tag. After washing the columns, the bound proteins were eluted with increasing concentrations of imidazole to compete off the His-tagged CFR protein from the affinity column. The eluates were separated by SDS-PAGE and analyzed by immunoblotting using FGF3 and CFR-specific antibody, respectively. As shown in Fig. 3*A*, FGF3 without CFR is completely eluted from the column with

defined as ECM and recovered in dissociation buffer as described under "Experimental Procedures." Samples of cell extract, ECM, and culture medium were fractionated by SDS-PAGE on a 7.5% gel and immunoblotted with the monoclonal antibody anti-RGS(His) against the His tag of pCFR1.1. The + and - indicate whether the cells were grown in the presence or absence of 10 μ g/ml heparin. The immunocomplexes were visualized by ECL technique using a specific anti-mouse secondary antibody. *D*, cell extracts and culture fluids were recovered from pCFR1.1-transfected COS-1 cells grown in the presence of heparin (10 μ g/ml). Cell proteins were concentrated by acetone precipitation and digested with neuraminidase (*Neu*), or *N*-glycanase (*N-gly*) as indicated. The products were then fractionated by SDS-PAGE, and CFR products were detected by immunoblotting. Product sizes were calculated relative to prestained protein standards.

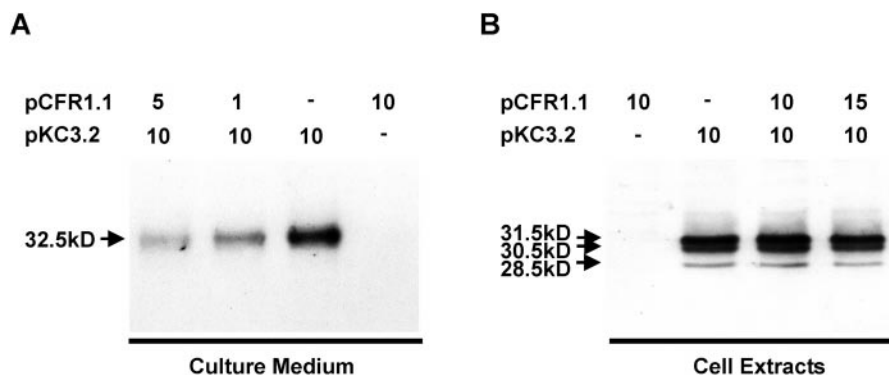


FIG. 2. **CFR reduces the secretion efficiency of FGF3.** COS-1 cells were co-transfected with the indicated amount of pCFR1.1 plasmid DNA, together with 10 μ g of DNA of pKC3.2 or empty vector DNA. 12 h after transfection the cell culture medium was replaced by medium containing 0.1% FCS. After 48 h cell extracts and culture medium were harvested as described under "Experimental Procedures." Cell extracts and the culture medium proteins were analyzed by SDS-PAGE and immunoblotted with a rabbit polyclonal antibody against the carboxyl-terminal peptide of FGF3. The immunocomplexes were visualized by ECL technique. The sizes of the various FGF3 related proteins are indicated in kilodaltons and were calculated relative to prestained protein standards.

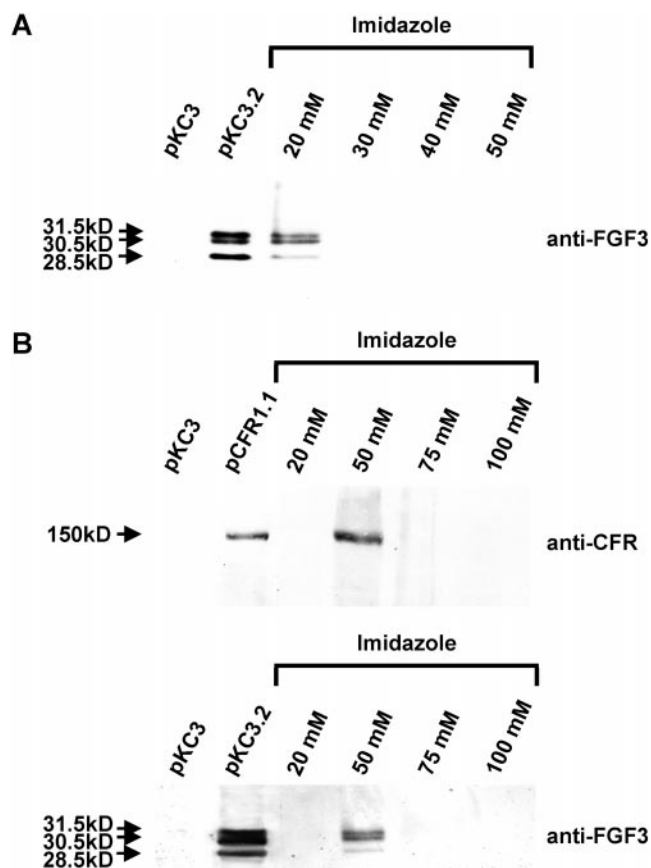


FIG. 3. **Binding of FGF3 to CFR *in vivo*.** COS-1 cells were transfected with FGF3 cDNA (A) or co-transfected with FGF3 cDNA and pCFR1.1 containing the His₆ cassette (B). The cell extracts were incubated for 2.5 h at 4 °C with the Ni²⁺-nitrilotriacetic acid resin. The bound proteins were eluted with increasing concentrations of imidazole as indicated and analyzed by immunoblotting using a polyclonal antibody against FGF3 and a monoclonal antibody against the His tag.

20 mM imidazole, whereas FGF3 co-expressed with CFR elutes at much higher concentrations of imidazole and at the same concentration as the CFR (Fig. 3B). To determine whether cell-associated FGF3 interacts with recombinant CFR *in vitro*, COS-1 cells expressing FGF3 were used to prepare a total cell extract, and the ability of immobilized CFR to bind FGF3 in the extract was assessed. *E. coli* containing an expression plasmid

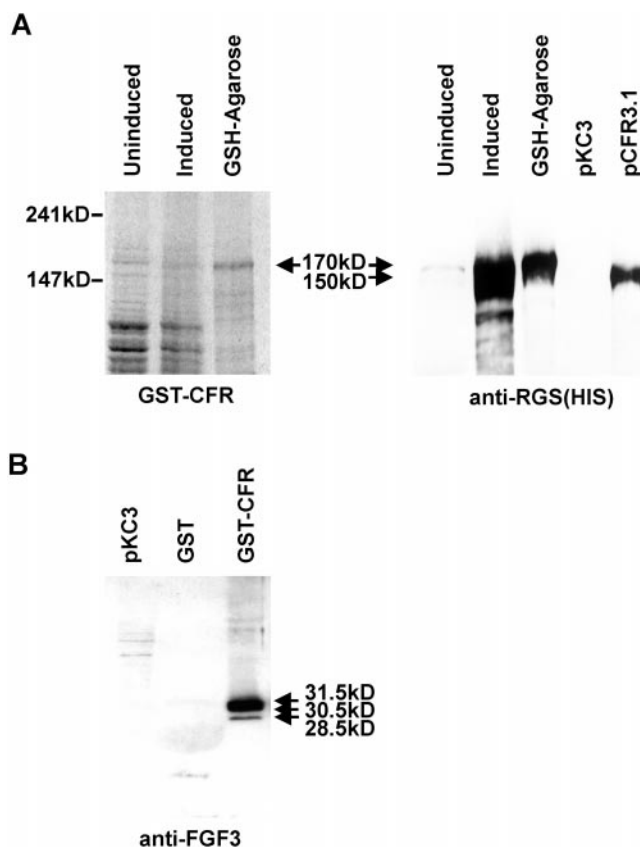
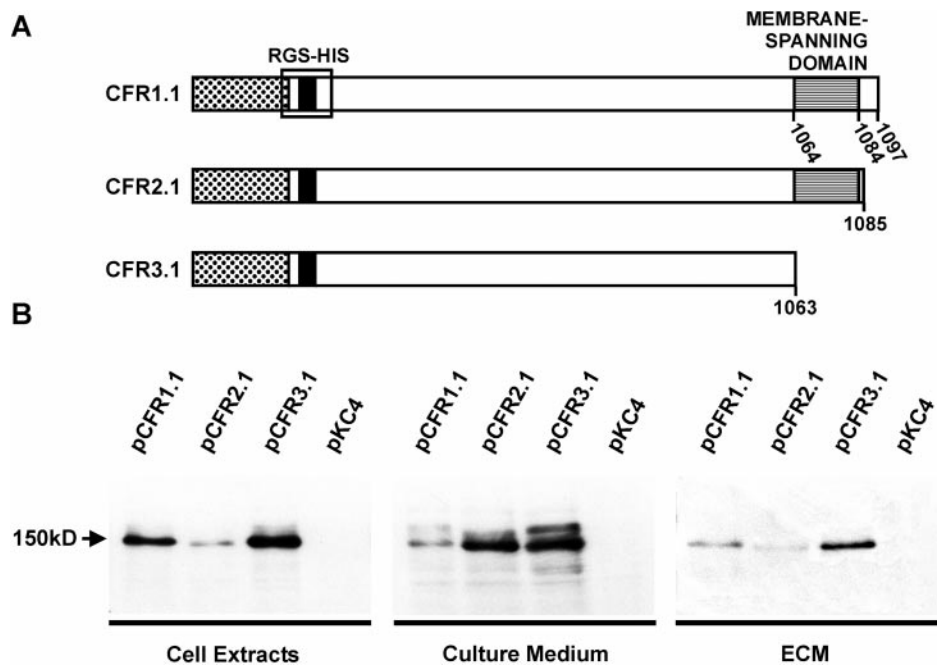


FIG. 4. **CFR binds FGF3 *in vitro*.** A, left panel, a Coomassie Blue-stained SDS-PAGE analysis of bacterial cultures uninduced or induced (3 h) to express a GST-CFR fusion proteins as indicated and described under "Experimental Procedures." The right-hand tracks show the proteins recovered from an aliquot (5 μ l) of the washed GSH-agarose resin after binding of the bacterial extracts. Right panel, an immunoblot analysis of the bacterially expressed GST-CFR fusion proteins and the CFR proteins recovered from an aliquot (5 μ l) of the washed GSH-agarose beads after binding GST-CFR. B, an immunoblot of the FGF3 proteins retained by the loaded resins after incubation with an extract of FGF3-expressing COS-1 cells (right two lanes) or incubation of the GST-CFR resin with a cell extract from COS-1 cells containing the empty expression vector pKC3.

encoding GST or a GST-CFR fusion protein was used to prepare extracts as described (Fig. 4A). After incubating the bacterial extracts with GSH-agarose beads, they were washed and incubated with cell extracts containing FGF3 as described. After extensive washing the presence of FGF3 retained by the

FIG. 5. Effect of carboxyl-terminal truncations on the secretion of CFR. A, schematic depiction of the parental CFR cDNA present in plasmid vector pCFR1.1, pCFR2.1, and pCFR3.1 containing carboxyl-terminal truncations. Most of the marked features are described in the legend to Fig. 1. B, immunoblot analysis of cell extracts, ECM, and culture medium from COS-1 cells transfected with pCFR1.1, pCFR2.1, pCFR3.1, or the control vector pKC4 (see "Experimental Procedures" and Fig. 1 for procedures). The CFR proteins were detected with a monoclonal antibody (LA) against CFR.



beads was assessed by immunoblotting (Fig. 4B). The GST-CFR fusion protein was clearly able to retain FGF3, whereas even an excess (about 5 times over GST-CFR fusion protein) of the control GST protein did not, demonstrating a strong affinity of FGF3 for CFR.

FGF3 Secretion Is Modulated by CFR—Targeting signals that affect the trafficking and secretion of integral membrane proteins often reside on the intra-luminal domain or close to the transmembrane domain. Assuming that deletion of the carboxyl-terminal domains may alter the localization or secretion of CFR, a concomitant change of the FGF3 secretion efficiency and subcellular localization would provide evidence that CFR binds FGF3 *in vivo*. Two truncation mutants of pCFR1.1 were generated; one contained a deletion encompassing the intra-luminal region (pCFR2.1), and for the other the deletion extended to a position amino-terminal to the transmembrane domain (pCFR3.1) (Fig. 5A). The expression and secretion of the truncated CFRs were analyzed by immunoblotting the culture medium, cell extracts, and ECM in comparison with the full-length protein (Fig. 5B). The ratio of the relative amount of cell extract/medium/ECM analyzed was 1:4:8. As expected, the truncated protein encoded by pCFR3.1 and lacking a transmembrane domain was efficiently secreted as a soluble protein. Cells expressing pCFR2.1 also produced a predominantly secreted CFR protein, despite retaining the sequences encoding the transmembrane domain. Although 80–90% of the wild-type CFR protein remains cell-associated, 30–50% of the truncated CFR proteins (CFR2.1 and CFR3.1, respectively) were found in the extracellular space. However, all extracellular CFR-related products had the same apparent size, indicating that the secreted proteins derived from full-length and truncated forms are similar, presumably through proteolytic cleavage at sites amino-terminally located to the transmembrane domain as described above.

To determine the effect truncated CFR proteins might have on FGF3 secretion, they were co-expressed in COS-1 cells, and the distribution of FGF3 between cell and extracellular compartments was assessed (Fig. 6A). Although co-expression of the truncated CFRs substantially reduced the amount of FGF3 secreted into the culture medium, more FGF3 was found under

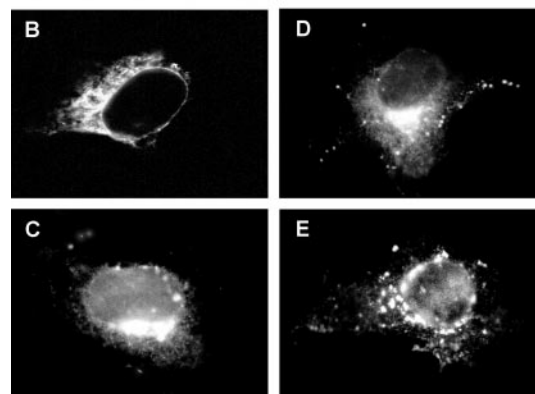
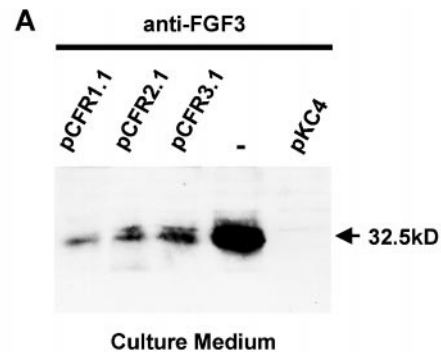


FIG. 6. Effect of carboxyl-terminal truncated CFRs on the secretion and subcellular localization of FGF3. A, an immunoblot analysis of culture medium from COS-1 cells co-transfected with 10 μg of pKC3.2, together with 5 μg of pCFR1.1, pCFR2.1, pCFR3.1, or the empty vector pKC4. FGF3 proteins were detected with a rabbit antiserum directed to FGF3. The immunocomplexes were visualized by ECL. The subcellular localization of FGF3 in COS-1 cells transiently co-transfected with 10 μg of pKC3.2 together with 5 μg of DNA of the empty vector pKC4 (B) or 5 μg of each of the CFR cDNAs pCFR1.1 (C), pCFR2.1 (D), and pCFR3.1 (E). The FGF3-related proteins were detected by immunostaining with the rabbit antiserum and visualized with Texas Red-conjugated secondary antibodies. When co-transfected with CFR, FGF3 proteins are localized in additional vesicular compartments. Representative examples are shown.

conditions where more CFR was exported. To investigate further the influence of CFR and the CFR mutants on the intracellular distribution of FGF3, the subcellular location of FGF3 and CFR was examined by immunofluorescence staining. Expression of FGF3 alone showed the typical dense juxtannuclear staining expected from a protein mainly localized in the medial Golgi as previously reported (Fig. 6*B*). However, co-expression of FGF3 with normal or truncated CFRs revealed additional staining of FGF3 in reticular structures, as well as peripheral vesicular structures (Fig. 6, *C–E*). This latter staining was more pronounced when FGF3 was co-expressed with truncated CFRs, indicating a change in FGF3 trafficking.

Subcellular Localization of Normal and Truncated CFR Proteins and Co-localization with FGF3—To determine whether this routing of FGF3 was due to its association with CFR, the subcellular localization of both wild-type and truncated CFR was examined by immunofluorescence staining. In agreement with previous reports, wild-type CFR was primarily concentrated in areas adjacent to the nucleus in a position characteristic of the Golgi complex (Fig. 7*A*) (10, 11, 15, 16). To establish the juxtannuclear staining as Golgi, the COS-1 cells were stained with two antibodies, one against the His tag and a second against another cis-medial Golgi matrix protein GM130 (21). A comparison of the two staining patterns showed a substantial overlap, confirming the Golgi apparatus as the main intracellular location for CFR (Fig. 7, *C* and *D*). The cell surface/ECM location of the CFR predicted from the cell fraction study was not apparent in the staining pattern with fixed and permeabilized cells, probably due to the strong intracellular signal. Therefore, to investigate the predicted cell surface staining, transfected COS-1 cells were fixed in 4% paraformaldehyde but not permeabilized prior to antibody staining. As can be seen in Fig. 7*B*, cell surface staining, particularly of the microvilli, was now clearly revealed by this procedure. In presence of heparin, cell surface staining for CFR was significantly diminished but not abolished (data not shown). In addition to the juxtannuclear and plasma membrane staining, CFR was also present in numerous cytoplasmic vesicles seen before in the FGF3 co-transfection experiment (Fig. 5, *B–E*). These structures are reminiscent of the endosomal/lysosomal system suggesting a dynamic pattern of intracellular trafficking (Fig. 7*A*).

A similar analysis using the deletion mutants pCFR2.1 and pCFR3.1 showed the intracellular distribution of the truncated CFR mutants was changed. Surprisingly, both deletion mutants still showed a juxtannuclear staining as observed for wild-type CFR, despite the loss of the transmembrane domain and cytoplasmic tail for the product encoded by pCFR3.1 (Fig. 7, *E* and *F*). Moreover, staining of peripheral vesicular structures was considerably more pronounced with the truncated CFRs. The identity of these structures as endocytotic vesicles was suggested by co-staining pCFR3.1-transfected cells for CFR and showing partial coincidence with the uptake of lysine-fixable FITC-labeled dextran added 1 h prior to fixation (Fig. 7, *G* and *H*). The staining pattern of FGF3 in the co-transfected experiments was very similar to the pattern observed for normal or truncated CFR proteins, respectively. Co-staining of pCFR3.1- and pKC3.2-transfected COS-1 cells with a mouse monoclonal antibody against the His tag and a rabbit antiserum against FGF3, respectively, and detection of the immunocomplexes with species-specific secondary antibodies obtained an almost coincident staining pattern in agreement with a new co-localization of CFR and FGF3 in the peripheral vesicles (Fig. 7, *I* and *J*). Thus the reduced secretion of FGF3 probably reflects its sequestration by CFRs in the medial Golgi

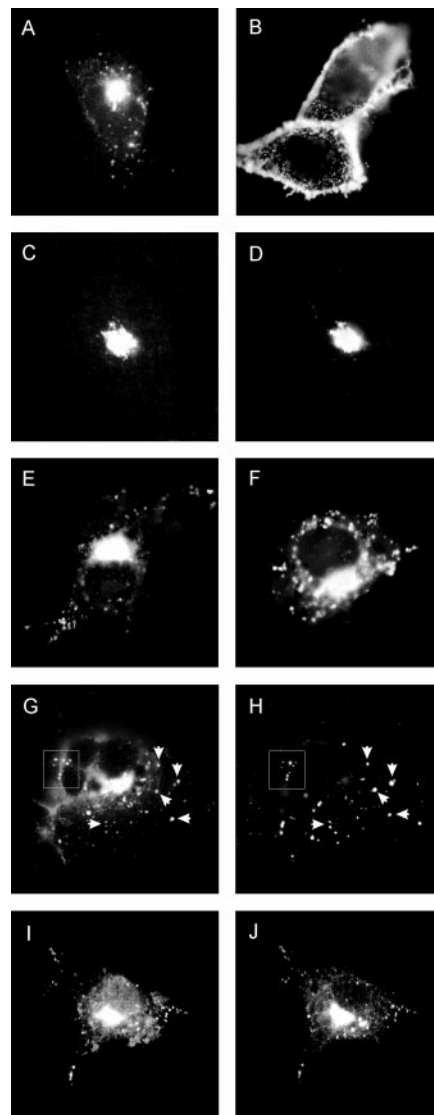


FIG. 7. Intracellular localization of wild-type and mutant CFR by immunofluorescence microscopy. COS-1 cells were transfected with pCFR1.1, fixed, and permeabilized for intracellular staining (*A*) or left unpermeabilized for selective cell surface staining (*B*). CFR proteins were detected with the monoclonal antibody anti-RGS(His) against the His tag. *C* and *D*, partial co-localization of CFR and the cis-Golgi matrix protein GM130 by immunofluorescence microscopy. COS-1 cells were transfected with pCFR1.1, fixed, and permeabilized. The cells were stained with the monoclonal antibody anti-RGS(His) against the His tag and a rabbit antiserum against GM130. *C* shows detection of anti-RGS(His) antibody with a FITC-coupled secondary antibody, and *D* shows staining with anti-GM130 with a Texas Red-coupled secondary antibody. *E* and *F*, intracellular localization of mutant CFR by immunofluorescence microscopy. COS-1 cells expressing pCFR2.1 (*E*) or pCFR3.1 (*F*) were stained with anti-RGS(His) and visualized with a Texas Red-tagged secondary antibody. CFR-related proteins are present in the endosomal compartment (*G* and *H*). To localize the endosomal/lysosomal compartment, COS-1 cells transfected with pCFR3.1 were also incubated with FITC-dextran prior to fixation. The CFR protein was detected with a Texas Red-labeled antibody (*G*), and the fluorescence from the FITC-dextran is shown in *H*. To facilitate the comparison of the staining pattern, some of the vesicular structures were highlighted by arrows or boxed. *I* and *J* show co-staining of COS-1 cells transfected with pKC3.2 and pCFR3.1 demonstrating co-localization of FGF3 and CFR proteins in the endosomal compartment using the mouse monoclonal anti-RGS(His) to detect CFR and a rabbit polyclonal antiserum against FGF3.

complex, and then it would be either secreted or routed to the endocytic pathway in association with CFR. The staining pattern and the co-expression experiments imply that FGF3 is

captured and can be redistributed by CFR proteins to intracellular compartments normally not entered by FGF3.

DISCUSSION

In this study we show that FGF3 can associate with the CFR, and this could account for its poor secretion and retention in the Golgi apparatus (19). CFR protein (MG160) is primarily located in the Golgi apparatus, consistent with previous reports, but we also detect a soluble form of the protein that associates with the cell surface and ECM (11, 12, 16, 17). From the size of the core protein after carbohydrate removal, the secreted CFR is cleaved at the carboxyl terminus, which removes the intra-luminal and transmembrane domain allowing the protein to be secreted. Since we could never detect extracellular or cell-associated CFR forms with a significantly higher molecular mass than 170 kDa, which might correspond to a CFR protein with a sialylated intact protein backbone, the residual bands present after treatment of CFR medium proteins with glycosidases derive most likely from partial digestion. The extracellular and soluble CFR appears to be associated with the ECM and/or cell surface through an affinity with glycosaminoglycans, since soluble heparin is efficient at competing CFR from the cell surface and ECM. CFR/MG160 appears to be homologous to ESL-1, a high affinity binding protein for E-selectin. ESL-1 was identified as a cell surface glycoprotein expressed on myeloid and some lymphoid cells (11, 12, 15, 16). However, in the light of the results presented here, it would be interesting to know whether cell surface ESL-1 is present as an integral membrane protein or associated with the cell surface by its affinity to glycosaminoglycans. Although the bulk of the intracellular CFR is Golgi complex-associated, it was also found in endosomal vesicles that were identified by co-staining with FITC-labeled dextran. Interestingly, the CFR carboxyl-terminal deletion mutants that lack either the cytoplasmic domain or both cytoplasmic and transmembrane domain showed a more pronounced staining of endosomal compartment. This suggests that the deletion mutations have weakened but not destroyed the Golgi retention and have allowed a greater amount of CFR to enter endosomes. Moreover, a spontaneous carboxyl-terminal truncation mutant of CFR which lacks the cytoplasmic, transmembrane, and juxtamembrane regions appeared to be widely distributed in the cell suggesting that the intraluminal juxtamembrane domain is important for targeting and retention of CFR to the medial Golgi (17).

The effect of CFR on the biological properties of FGFs remains puzzling. A recent study looking at the effect on exogenously applied FGF1 or FGF2 to Chinese hamster ovary cells expressing elevated levels of CFR demonstrated a significant reduction in the intracellular levels of these FGFs (17). As CFR did not appear to block entry of these FGFs, or reduce their intracellular stability, the effect was ascribed to an enhanced trafficking of the FGFs from endocytic compartments to the cell exterior, possibly via the Golgi apparatus (17). The fate of FGF3 represents a very different situation; in this case FGF3 is synthesized as a naturally secreted ligand that would be expected to encounter CFR in the Golgi apparatus. Furthermore, we show here that FGF3 binds CFR *in vivo* and *in vitro* (Figs. 3 and 4). In cell culture, increased intracellular levels of CFR result in a progressive reduction in the secretion of FGF3 without a noticeable effect on FGF3 synthesis (Fig. 2B). In COS-1 cells, the gp30.5 and gp31.5 products associated with the medial Golgi have a half-life time in excess of 4 h and are continuously sensitive to endoglycosidase H digestion and represent the majority of the steady state level of FGF3-related protein (19). The secreted 32.5-kDa form of FGF3 is derived from the smaller products by secondary modification in a Golgi

or post-Golgi compartment (19). In the presence of heparin to displace the already secreted form from its binding to cell surface heparan-sulfate proteoglycans, no cell-associated gp32.5-kDa product is detectable, suggesting that as soon as FGF3 leaves the medial Golgi the further steps of secretion and modification appear to happen very fast (19, 22). Since we have been unable to detect a cell-associated gp32.5-kDa product as a consequence of co-expression of CFR (Fig. 2B), we concluded that CFR must interact with the FGF3 forms when associated with the medial Golgi before they had undergone further modification. However, since the overall amount of intracellular FGF3 protein is not changed within the limits of our assay, the half-life of the FGF3 products routed via CFR in the endosomal-lysosomal compartment is presumably considerably lower than those in the medial Golgi.

The sequestration of FGF3 by CFR was further supported by the behavior of the carboxyl-terminal deletion mutants of CFR. The degree to which the CFR proteins change the amount of extracellular FGF3 positively correlates to their own Golgi retention, with the CFR3.1 mutant having the smallest effect on the FGF3 release.

In essence, the redistribution of truncated CFR to intracellular vesicles was reflected by an analogous distribution of FGF3 when present in co-transfected COS-1 cells. The CFR potentially contains a Golgi retention and endosomal targeting signal that might shuttle internalized CFR-FGF complex between the endosomal compartment and Golgi via the trans-Golgi network and thereby also provide an interface with the cell surface via endosomes. A possible retrieval of FGF3 by the CFR from the endosomal compartment back to the trans-Golgi network could also account for the significant retardation of FGF3 secretion. As the deleted CFRs may be less efficiently retrieved from the plasma membrane, this would explain their accumulation in the endosomal/lysosomal compartment and also their diminished effect on the FGF secretion.

Recently, CFR was also identified as part of a secreted but latent TGF- β complex and was termed latent TGF- β -complexed protein-1 (LTCP-1) (18). It is interesting that TGF- β as well as FGF3 are retained in the Golgi apparatus as a pre-secreted store. Thus, the presence of CFR/MG-160 in the latent transforming growth factor- β complex and its localization to the medial cisternae of the Golgi complex suggest a possible function in the processing and secretion of a member of another growth factor family (19, 20). The CFR/MG160/ESL-1/LTCP-1 appears to be a multifunctional protein, although its role within the organism is not clear. It is interesting that ESL-1, a ligand for E-selectin, is a cytokine modulated by cell adhesion molecule that causes the binding of neutrophils to the endothelium. Both FGFs and TGF- β have been implicated as having an involvement in tissue repair and inflammatory responses, albeit with opposing effects. Therefore, we would speculate that maybe this multi-functional protein may help to balance or buffer the effects of these powerful cell signaling molecules in a developmental or tissue repair context.

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