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Cysteine-Rich FGF Receptor Regulates Intracellular FGF-1 and FGF-2 Levels

MICHAEL E. ZUBER,[1] ZHAOHUI ZHOU,[1] LAURA W. BURRUS,[3] AND BRADLEY B. OLWIN[1,2]*

[1] *Department of Biochemistry, Purdue University, West Lafayette, Indiana 47906*
[2] *Walther Cancer Institute, Indianapolis, Indiana 46208*
[3] *Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138*

The cysteine-rich FGF receptor (CFR) is a 150-kD membrane-associated glycoprotein that specifically binds FGFs. CFR protein is not detectable at the cell surface and immunocytochemistry with anti-CFR antibodies demonstrates that CFR is concentrated in the Golgi apparatus. These data suggest CFR does not function as a plasma membrane FGF receptor. CFR expressed in chinese hamster ovary cells reduces the intracellular accumulation of exogenously applied FGF-1 and FGF-2. A mutant CFR lacking the juxtamembrane, transmembrane and intracellular domains is unable to alter intracellular FGF levels. Mutant CFR is detected throughout the cell, indicating that the domains absent in mutant CFR are required for appropriate subcellular localization and the regulation of intracellular FGF levels. Although the activation of plasma membrane receptors is necessary for cellular responses to FGFs, a requirement for intracellular FGF has also been proposed. The subcellular localization of CFR and its ability to regulate the levels of intracellular FGFs suggests that CFR may be involved in intracellular FGF trafficking and the regulation of cellular responses to FGFs. **J. Cell. Physiol. 170:217–227, 1997.** © 1997 Wiley-Liss, Inc.

FGF-mediated cellular responses require activation of plasma membrane receptors and may involve activation of intracellular targets that require intracellular trafficking of FGFs. Three independent experiments support a requirement of nuclear transport for FGF-1 action. First, FGF-1 targeted to the nucleus of Vero cells induces DNA synthesis, but does not stimulate cellular proliferation (Wiedlocha et al., 1994). Cellular proliferation is restored only when cells are transfected with FGFR-1 (FGF receptor 1 tyrosine kinase), implying that intracellular FGF-1 and the plasma membrane receptor are required for cell division (Wiedlocha et al., 1995). Second, an FGF-1 mutant lacking a nuclear translocation sequence induces intracellular receptor-mediated tyrosine phosphorylation and c-*fos* expression but fails to induce DNA synthesis and cell proliferation (Imamura et al., 1990). The fusion of a heterologous nuclear translocation sequence results in a chimeric molecule with complete biological activity (Imamura et al., 1990). Third, an FGF-1 mutant in which lysine 132 was mutated to glutamic acid elicits tyrosine kinase activity and stimulates protooncogene transcription but does not stimulate DNA synthesis, suggesting the existence of alternate signaling pathways (Burgess et al., 1990).

Similar requirements for intracellular FGF-2 have also been demonstrated. Different biological responses to FGF-2 are observed depending on the isoform of FGF-2 expressed (Bikfalvi et al., 1995). Initiation of translation from CUG codons upstream of the predicted FGF-2 start codon results in the synthesis of larger forms of the growth factor containing nuclear targeting sequences (Acland et al., 1990; Quarto et al., 1991). NIH/3T3 cells expressing only the 18-kD (cytoplasmic) form display enhanced migration but are unable to grow in low serum culture media (Bikfalvi et al., 1995). Cells expressing only the upstream CUG translated (high molecular weight [MW]) forms of FGF-2 grow in low serum but do not display increased mobility (Bikfalvi et al., 1995). These results demonstrate that intracellular FGF-2 is likely to mediate specific cellular responses.

CFR is a 150-kD integral membrane glycoprotein originally purified from embryonic chicken (Burrus and Olwin, 1989). The amino acid sequence of cysteine-rich FGF receptor (CFR) differs from the previously identified FGFRs and heparan sulfate proteoglycans (Burrus et al., 1992). CHO (chinese hamster ovary) cells transfected with a CFR cDNA (Burrus et al., 1992) express a 150-kD protein that specifically binds FGFs. The CHO cells used in these experiments are unresponsive to FGF; hence, the effect of chicken CFR expression on in vitro cellular responses to FGF could not be studied. Attempts to stably express CFR in other FGF-responsive cell types has failed as overexpression of CFR ap-

*Correspondence to: Bradley B. Olwin, now at Molecular Cellular and Developmental Biology, Campus Box 347, University of Colorado, Boulder, CO 80308.

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pears generally cytotoxic. Cell surface labeling of intact cells and FGF binding experiments indicate that the CFR protein is not detectable on the cell surface. Thus, we performed experiments designed to analyze the fate of intracellular FGF as a function of CFR expression to determine if CFR is involved in the regulation of intracellular FGF levels. Here we demonstrate that the levels of intracellular FGF-1 and FGF-2 are regulated by CFR. CFR expressed in CHO cells reduces the intracellular accumulation of FGF-1 and FGF-2. Indirect immunofluorescence indicates that wild-type CFR is concentrated in the Golgi apparatus. A CFR mutant unable to alter intracellular FGF levels lacks the juxtamembrane, transmembrane and intracellular domains of the wild-type CFR. Moreover, this mutant CFR is distributed throughout the cell. The ability of CFR to alter the levels of intracellular FGFs, its detection in the Golgi apparatus, and the inability of mutant CFR to regulate intracellular FGF levels suggest that CFR is involved in intracellular trafficking of FGFs.

MATERIALS AND METHODS

Cell culture

CHO-K1 cells (ATCC, Rockville, MD, #CCL 61) were transfected with a previously described CFR cDNA and a vector control DNA (Burrus et al., 1992). The CHO.*mut*CFR cell line expresses an altered CFR protein due to a spontaneous deletion in the transfected CFR cDNA. The deletion in the CFR cDNA of CHO.*mut*CFR was not engineered, but was a fortuitous transfection artifact. CHO cells were grown on plastic tissue culture plates in DME (Gibco BRL, Gaithersburg, MD) supplemented with 10% bovine calf serum (HyClone Laboratories, Inc., Logan, UT), 25 mM Hepes, 10 mM L-Proline, penicillin and streptomycin (growth medium) in a humidified 5% CO_2 incubator at 37°C. Chick embryo fibroblasts (CEF) were isolated from day 13 chicken embryos. CEF cells were grown on collagen-coated plastic tissue culture plates in DME supplemented with 10% bovine calf serum, 2% chick embryo extract, penicillin and streptomycin in a humidified 5% CO_2 incubator at 37°C.

^{125}I-FGF binding and covalent crosslinking to CHO cells

FGF-1 purified from bovine brain and human recombinant FGF-2 were iodinated as previously described (Olwin and Hauschka, 1990). Specific activities were determined by MM14 cell cycle exit assay and ranged from 1,000 to 7,000 cpm/fmol (Olwin and Hauschka, 1986). CHO cells transfected with vector (CHO.*vec*) or CFR cDNA (CHO.*wt*CFR) were plated onto 24-well plates at a density of 1×10^5 cells per well in growth medium. Two days after plating, the cells were counted, washed twice with 4°C serum-free growth medium, and 0.25 ml of 200 pM ^{125}I-FGF-1 or ^{125}I-FGF-2 was added in binding buffer (serum-free growth medium containing 0.15% gelatin, 4°C). A duplicate set of samples was prepared in which a 250-fold molar excess of unlabeled FGF was added in addition to ^{125}I-FGF. Cells were incubated for 1 h at 4°C on a rocker, rinsed rapidly three times with 1 ml ice cold Tris-buffered saline (TBS) containing 2 mM $MgCl_2$ and 0.15% gelatin then incubated 5 min at room temperature in TNT (50 mM Tris Base, pH 7.4; 100 mM NaCl; 1% Triton X-100). Four hundred fifty µl were removed and the ^{125}I-*c*pm determined. Nonspecific binding was defined as the cpm in the presence of excess unlabeled FGF. Specific binding was calculated by subtracting the nonspecific cpm from the total cpm detected in the absence of competitor. Values were normalized for cell number. Error bars represent the S.E.M. of measurements performed in triplicate. For crosslinking experiments, cells were plated in 6-well tissue culture plates at a density of 5×10^4 cells per well and grown for 96 h. After washing cells 2 times with phosphate-buffered saline (PBS), 200 pM ^{125}I-FGF ± 40 nM unlabeled FGF was added to wells in binding buffer. Cells were incubated for 1.5 h at 4°C and washed 2 times with Hepes-buffered saline (HBS: 20 mM Hepes, pH 7.4 containing 100 mM NaCl). Crosslinking reactions were initiated by the addition of 0.15 mM Disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL). Cells were incubated for 15 min at 22°C, washed 2 times with HBS, and harvested with a rubber policeman. Cells were pelleted by centrifugation at $18,000 \times g$ for 5 min at 4°C and resuspended in 20 mM Hepes, pH 7.4 containing 250 mM sucrose, 1 mM EDTA, 20 kallikrein aprotinin units per ml, 1 µg/ml leupeptin, and 1% Triton X-100. Samples were incubated for 1 h at 4°C and detergent-insoluble material was pelleted by centrifugation at $18,000 \times g$ for 10 min at 4°C. The supernatant was electrophoresed in a 7.5% SDS polyacrylamide gel, and subjected to autoradiography with a single intensifying screen.

^{125}I-FGF internalization assay

Proliferating cultures of CHO cells were passaged onto 6-well plates at a density of 5×10^5 cells per well in growth media. The following day, cells were incubated with 500 µl of 200 pM ^{125}I-FGF for various times. Cultures were placed on a Belly Dancer Shaker (Stovall Life Science Inc., Greensboro, NC.) in a humidified 5% CO_2 incubator at 37°C for the length of the incubation. At the end of the incubation period, the cells were counted, and washed rapidly three times with ice-cold TBS + 0.2% bovine serum albumin (BSA) followed by two washes with high salt/low pH buffer (15 mM sodium acetate, 2 M NaCl, 0.2% BSA, pH 4.0). Cells were solubilized by adding 500 µl TNT for 5 min at room temperature and a 450-µl aliquot of the solubilized cell extract was counted in a LKB Clinigamma counter (Wallac, Gaithersburg, MD). The amount of intracellular FGF was calculated using the following equation: fmols intracellular FGF = (average cpm/FGF specific activity). These values were then normalized to cell number. Average cpm and cell number were determined in triplicate. Error bars represent the S.E.M. For multiple clone analysis (Fig. 3), cells expressing

Abbreviations

CEF	chick embryo fibroblasts
CFR	cysteine-rich FGF receptor
CHO	chinese hamster ovary K1 cells
CHO.*mut*CFR	CHO cells expressing mutant CFR
CHO.*vec*	vector transfected CHO cells
CHO.*wt*CFR	CHO cells expressing wild-type CFR
FGFR	FGF receptor

various amounts of CFR were incubated with ^{125}I-FGF for 5 h and processed as above.

Immunoblot analysis

To detect chicken CFR protein, proliferating CHO cells were washed two times with ice-cold PBS and harvested with a rubber policeman. Cells were centrifuged at 300 × g for 5 min at 4°C and resuspended in solubilization buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 4 mM EDTA, 1 μg/ml leupeptin, 1 mM PMSF, 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 using the BCA assay per manufacturer's instructions (Pierce Chemical Co., Rockford, IL). CHO cell extracts were subjected to electrophoresis in a 7.5% SDS polyacrylamide gel and electrophoretically transferred to an Immobilon membrane (Millipore, Bedford, MA). Immobilon membranes were blocked with TBS containing 3% nonfat dry milk and 0.05% Tween-20 (blocking buffer) for 2 h. The blot was incubated for 1 h with a purified anti-CFR mAb (clone 15.E.9 TC10) diluted 1/5,000 in blocking buffer. Unbound antibody was removed by washing three times for 20 min with blocking buffer. ^{125}I-labeled goat-anti-mouse IgG (Promega Corporation, Madison, WI) 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. To determine the relative levels of CFR protein (Fig. 3), individual lanes were cut from the blot and counted in a LKB Clinigamma counter.

FGF degradation assay

Proliferating cultures of CHO cells were passaged using trypsin-EDTA onto 10-cm plastic tissue culture dishes in growth media at a density of 3.5×10^6 cells per dish. The following day the cells were incubated at various time intervals with 3 ml culture media containing 200 pM ^{125}I-FGF. The dishes were continuously rocked to ensure that the cells were covered with media. At the end of the incubation period, the cells were counted and washed rapidly three times with ice-cold TBS containing 0.2% BSA, three times with high salt/low pH buffer then three times with TBS containing 0.2% BSA. Cells were harvested with a rubber policeman, pelleted by centrifuged at 300 × g for 5 min at 4°C and resuspended in solubilization buffer. 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. CHO cell extracts were subjected to electrophoresis through a 12% SDS polyacrylamide gel and autoradiography was used to detect the fate of the internalized ^{125}I-FGF. An equivalent number of cpm were loaded in each lane so a comparison could be made between the different cell types (e.g., FGF-1 internalized by CHO.*vec* and CHO.*wt*CFR cells in Fig. 4a). For Figure 4b, the relative level of the ^{125}I-FGF-2 degradation products were determined using Molecular Imager System GS-363 (BioRad, Richmond, CA). Since the resolution of the individual degradation fragments by SDS-PAGE was not sufficient for analysis, the ^{125}I-cpm of the upper and lower doublets were used. The values reported in Figure 4b are an average of duplicate experiments.

PCR amplification, DNA sequence determination and analysis

Genomic DNA was isolated from CHO cells as previously described (Weeks et al., 1986). The forward and reverse primers used for polymerase chain reaction (PCR) amplification were $^{5'}$CUACUACUACUAUGG-ACATTAAACACC$^{3'}$ and $^{5'}$CAUCAUCAUCAUCATCC-ACACATCAGGC$^{3'}$, respectively and were designed for use with the CloneAmp cloning system (Gibco BRL). The primers correspond to nucleotides 3112 to 3126 (forward) and 3379 to 3392 (reverse) of the chicken the CFR cDNA (GenBank accession number M95766). PCR was performed in PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 2.5 mM $MgCl_2$), 200 μM each of dGTP, dATP, dTTP, dCTP, 0.2 μM each primer, and 0.1 U/μl AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Foster City, CA). Template DNA was either 1 ng plasmid DNA or 500 ng genomic DNA. Temperature cycling was performed in Perkin Elmer Cetus DNA Thermal Cycler 480, 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C for 30 cycles. PCR products were cloned into the pAMP (Gibco BRL) vector using the CloneAmp cloning system. Double-stranded template DNA was prepared by a small scale DNA alkaline extraction procedure. Sequencing was performed by the dideoxynucleoside chain termination method (Sanger et al., 1977) using Sequenase version 2.0 per manufacturer's instructions (US Biochemicals, Cleveland, OH). Both strands of DNA were sequenced to ensure sequence fidelity. DNA and amino acid sequences were analyzed using DNA Strider (Marck, 1988) and Genetics Computer Group (GCG, 1992) software packages.

Indirect immunofluorescence and confocal microscopy of CHO and CEF cells

Proliferating cultures of CHO and CEF cells were plated onto collagen-coated glass coverslips. The following day, the cells were fixed for 8 min with 4% paraformaldehyde in PBS, pH 7.0, and permeablized for 2 min in PBS containing 0.2% Triton X-100. The cells were rinsed, and nonspecific protein binding sites were blocked with 1% BSA in PBS for 1 h prior to antibody incubations. Cells were incubated in 1% BSA in PBS with primary antibodies (1/1,000 dilution of α-Mannosidase II rabbit polyclonal (provided by Dr. Marilyn Farquhar, UC San Diego) and 0.02 μg/ml anti-CFR mAb 15.E.9 TC10) for 1 h, then with 1 μg/ml Texas Red-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG secondary antibodies (Promega Corporation) for 1 h. Stained cells were analyzed by confocal microscopy using a Bio-Rad MRC 1000 Confocal System at Purdue University Cytometry Laboratories.

RESULTS

To determine if CFR affects the binding, intracellular accumulation, or degradation of FGFs, we examined and compared each of these processes in CHO cells expressing the chicken CFR (CHO.*wt*CFR) and vector transfected control (CHO.*vec*) CHO cells. CHO cells were used, as we were unable to isolate stable transformants of Swiss 3T3, NMuMg, NIH 3T3, Raji, MM14, or PC12 cells overexpressing CFR even thought the selection gene and CFR coding sequences were con-

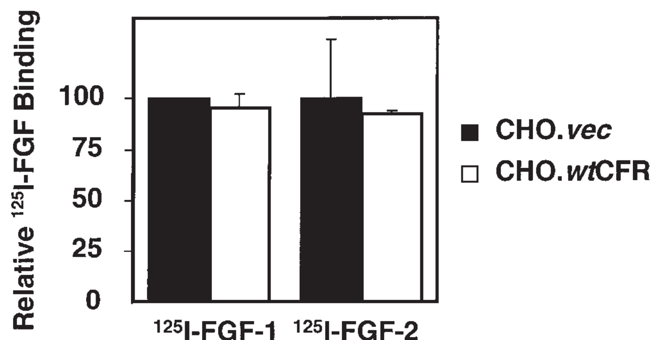


Fig. 1. CFR-dependent FGF binding is not detected on the cell surface of *wt*CFR-expressing CHO cells. ^{125}I-FGF was incubated with intact CHO.*vec* and CHO.*wt*CFR cells under conditions that would minimize internalization of FGF (see Materials and Methods). The cells were washed to remove nonspecifically bound FGF and the relative amount of FGF bound to the cell surface was determined.

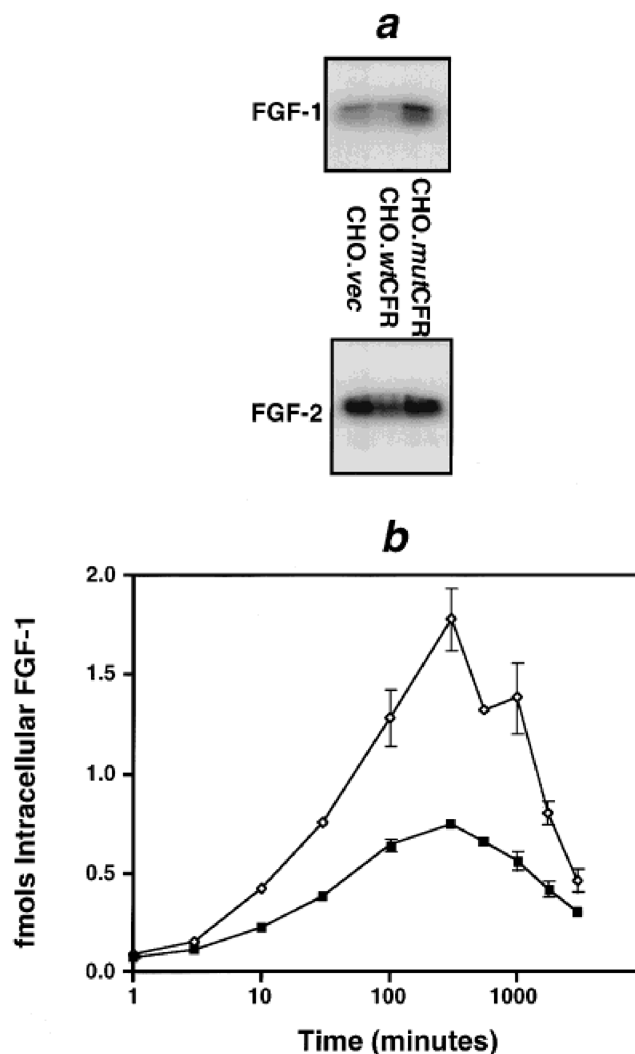


Fig. 2. CFR regulates the level of intracellular FGF-1 and FGF-2 in CHO cells. **a:** *wt*CFR cells, CHO.*vec* cells, and CHO.*mut*CFR cells were cultured in the presence of 200 pM ^{125}I-FGF-1 or 200 pM ^{125}I-FGF-2 for 5 h. At the end of the incubation period, the cells were washed with a low pH, high salt buffer to remove cell surface bound ^{125}I-FGF-1 and ^{125}I-FGF-2. The cells were then solubilized, cytoplasmic extracts prepared, proteins separated by SDS-PAGE and visualized on a phosphoimager. **b:** A time course for ^{125}I-FGF-1 internalization was performed in CHO.*vec* (diamonds) and CHO.*wt*CFR cells (closed squares). Cell extracts were prepared and the total intracellular ^{125}I-FGF was determined. Error bars represent the S.E.M. of measurements done in triplicate.

tained on the same plasmid (data not shown). An analysis involving transfection of different levels of CFR expression vector in Raji cells suggests that overexpression of CFR is cytotoxic (data not shown). Attempts to overexpress CFR in other cell types were abandoned and the effects of CFR overexpression on FGF-1 and FGF-2 trafficking were examined in CHO cells.

CFR regulates the level of intracellular FGF

^{125}I-FGF binding assays were used to determine if cell surface FGF binding was altered in CHO.*wt*CFR versus CHO.*vec* cells. Although CFR was expressed in transfected cells as confirmed by immunoblot analysis, no detectable effect on ^{125}I-FGF binding (Fig. 1) or crosslinking (not shown) was observed. Additional experiments to detect CFR at the cell surface were negative and included cell surface immunolocalization, proteolytic treatment of intact cells, and ^{125}I-labeling of cell surface proteins (not shown).

Immunolocalization experiments indicated that CFR was present intracellularly (see Fig. 7). Therefore, we determined if CFR expression altered the level of intracellular FGF-1 or FGF-2 internalized via cell surface receptors. CHO.*vec* and CHO.*wt*CFR cells were cultured in the presence of ^{125}I-FGF-1 or ^{125}I-FGF-2. Cell surface bound ^{125}I-FGFs were removed and the amount of intracellular FGF was determined. CFR expression reduces the amount of intracellular FGFs in CHO cells (Fig. 2a). A time course for the internalization of ^{125}I-FGF-1 revealed that this CFR-dependent effect is first detected 10 min following the addition of ^{125}I-FGF-1 (Fig. 2b). A maximal reduction in intracellular FGF levels is observed 5 h following the addition of ^{125}I-FGF to the medium. At this time, the levels of intracellular ^{125}I-FGF-1 were reduced 60% (Fig. 2b). Beyond 5 h, the reduction in intracellular ^{125}I-FGF-1 is attenuated until no significant differences are observed (Fig. 2b). Similar results were observed with ^{125}I-FGF-2 (Fig. 2a and data not shown).

To demonstrate that the reduction in intracellular FGF levels is CFR-dependent and not due to clonal phenotypic variation, four additional CFR transfectants were isolated and the effect of CFR expression on the level of intracellular FGF was determined. Chicken CFR was not detected in CHO.*vec* cells, but was detected in five CHO cell lines stably transfected with the chicken CFR cDNA (Fig. 3, inset). CHO transformants expressing higher levels of CFR exhibit reduced intracellular FGF-1 (Fig. 3). Similar results were obtained with FGF-2 (not shown). An inverse linear relationship exists between the level of CFR expression and the level of intracellular FGF-1 (Fig. 3). The inverse correlation is consistent for four of the five cell lines expressing CFR.

CFR could decrease the intracellular level of ^{125}I-FGFs by promoting rapid degradation of the growth factors. However, the fragment patterns of intra-

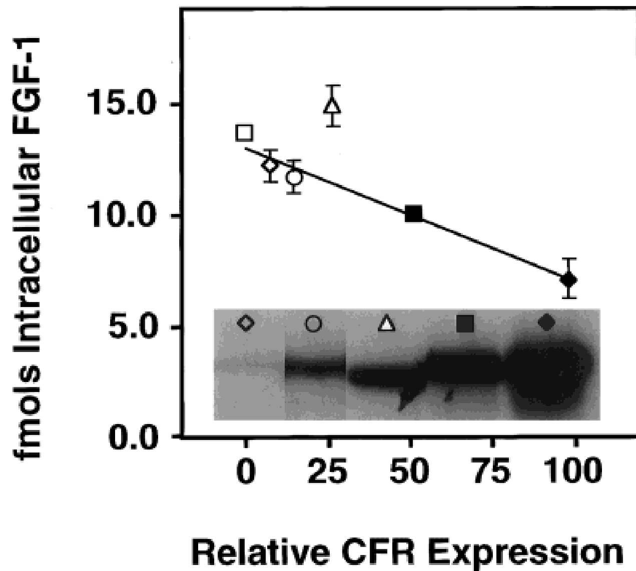


Fig. 3. The level of CFR expression alters the level of intracellular FGF-1. Cellular extracts prepared from stable CHO cell lines expressing CFR were subjected to immunoblot analysis to determine the relative level of CFR present. The fmols of intracellular FGF-1 are plotted as a function of CFR expression. Relative CFR expression levels are as follows: open square (vector transfected) < open diamonds < open circles < open triangles < closed squares < closed diamonds. A linear relationship exists between the level of intracellular FGF and CFR expression (with the exception of CHO.*mut*CFR (open triangles)). **Inset:** Immunoblot analysis for CFR in extracts prepared from CHO.*wt*CFR (open diamonds < open circles < closed squares < closed diamonds) and CHO.*mut*CFR (open triangles) cells.

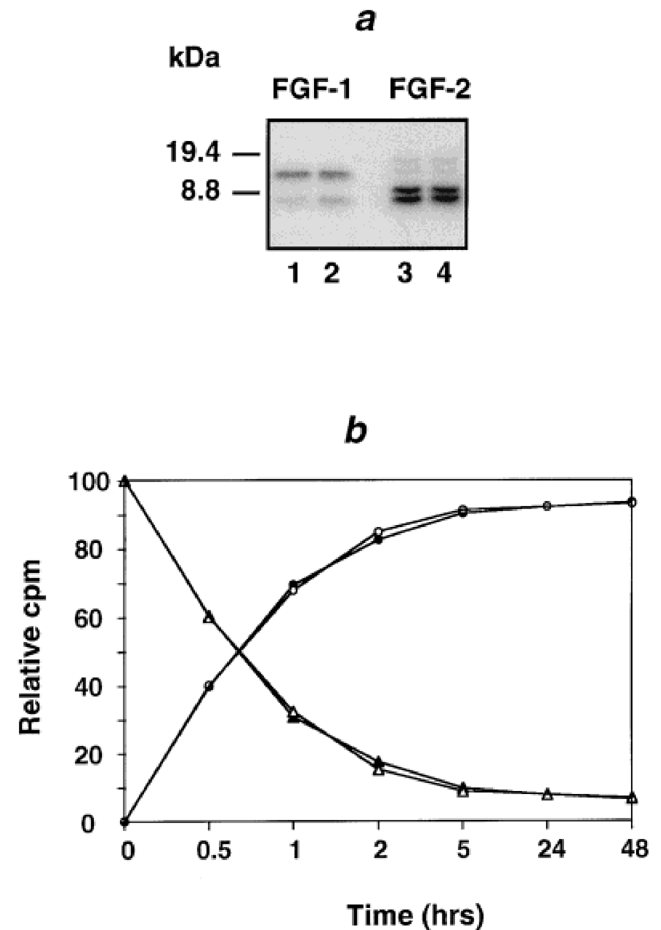


Fig. 4. CFR expression has no detectable effect on the pattern or rate of ^{125}I-FGF degradation. **a:** CHO.*vec* (**lanes 1** and **3**) and CHO.*wt*CFR (**lanes 2** and **4**) cells were cultured in the presence of ^{125}I-FGF-1 (lanes 1 and 2) or ^{125}I-FGF-2 (lanes 3 and 4) for 5 h. After washing to remove cell surface bound ^{125}I-FGF, the cells were solubilized in detergent and SDS-PAGE was used to determine the fate of the intracellular ^{125}I-FGF. Cell extracts were normalized for ^{125}I-cpm prior to electrophoresis to provide a better comparison of degradation patterns. **b:** CHO.*vec* (open circles, open triangles) and CHO.*wt*CFR (closed circles, closed triangles) cells were cultured in the presence of ^{125}I-FGF-2 for various times. Solubilized cell extracts were separated by SDS-PAGE and the relative cpm of the ^{125}I-FGF-2 degradation products (see Fig. 4a) were determined. Upper doublet (triangles), lower doublet (circles). Open and filled symbols represent the results from two independent experiments.

cellular ^{125}I-FGF-1 and ^{125}I-FGF-2 in CHO.*vec* and CHO.*wt*CFR cells (Fig. 4a) are indistinguishable at 5 h. This corresponds to the time at which a maximal difference in intracellular FGF levels is observed (see Fig. 2). In addition, the rate of ^{125}I-FGF-2 degradation (Fig. 4b) is not significantly different in CHO.*vec* and CHO.*wt*CFR cells. Taken together, these results indicate it is unlikely that CFR increases the rate of FGF degradation in CHO.*wt*CFR cells.

Characterization of a spontaneous CFR mutant that does not affect the intracellular level of FGF

The CFR-expressing CHO subclone (CHO.*mut*CFR) that is unable to reduce intracellular FGF-1 levels synthesizes a CFR protein product that migrates with an apparent molecular weight 5 to 10 kD smaller than wild-type CFR (Fig. 3, inset). Therefore, we hypothesized that characterization of this spontaneously generated CFR mutant could provide information regarding the mechanism involved in CFR-dependent reduction of intracellular FGF levels.

Mutant CFR binds FGF

One possibility is that *mut*CFR may be altered such that it no longer binds FGFs, thereby inhibiting its ability to regulate intracellular FGF levels. CFR prepared from extracts of CHO.*wt*CFR and CHO.*mut*CFR cells were assayed for ^{125}I-FGF-2 binding as previously described (Burrus et al., 1992). Both CFR preparations specifically bind ^{125}I-FGF-2 (Fig. 5). The FGF binding kinetics for wild-type and mutant CFR are indistinguishable (not shown). A series of CFR deletion mutants have been constructed and the FGF binding domain is intact in *mut*CFR (Zhou et al., in press). Taken together, these results indicate that *mut*CFR binds FGF with affinities similar to *wt*CFR.

Mutant CFR sequence

To characterize *mut*CFR, we synthesized CFR-specific PCR primer pairs and amplified CFR cDNA from genomic DNA isolated from CHO.*vec,* CHO.*wt*CFR and CHO.*mut*CFR cells. A product of 281 bp was amplified from genomic DNA isolated from CHO.*vec* and

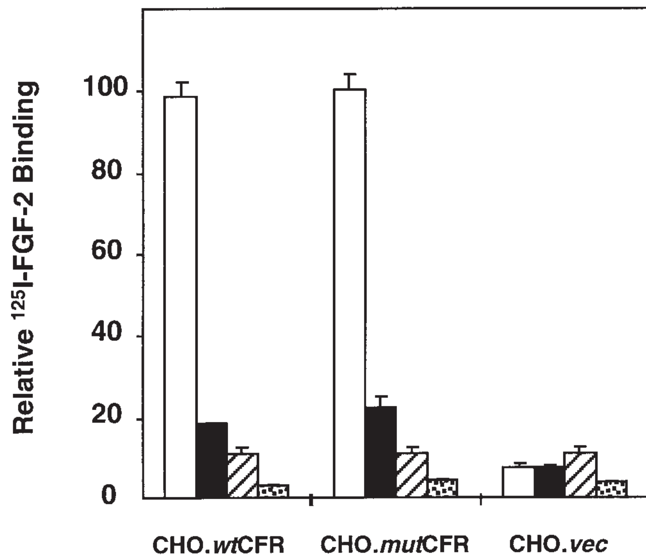


Fig. 5. Mutant CFR binds ^{125}I-FGF-2. A radio-immunosorbant assay was used to quantify ^{125}I-FGF-2 binding (open bars) to extracts prepared from CHO.*wt*CFR, CHO.*mut*CFR and CHO.*vec* cells. Controls include the addition of a 250-fold molar excess of FGF-2 (closed bars), no cell extract (hatched bars) and no anti-CFR mAb (stippled bars).

CHO.*wt*CFR cells (Fig. 6a, lanes 3 and 5, respectively). A second PCR product of approximately 200 bp was amplified from genomic DNA isolated from CHO.-*mut*CFR cells (Fig. 6a, lane 4).

The PCR product amplified from CHO.*vec* cells codes for the hamster CFR. Chicken and hamster CFR were amplified from genomic DNA isolated from CHO.*wt*CFR cells. The 281-bp fragment from CHO.-*mut*CFR cells contained only hamster CFR cDNA. The smaller product amplified from CHO.*mut*CFR cells contains chicken CFR cDNA containing a 106-bp deletion (Fig. 6b).

The deletion in *mut*CFR shifts the open reading frame and introduces a premature in frame stop codon (Fig. 6b). Thus, the predicted amino acid sequence of *mut*CFR lacks the juxtamembrane, transmembrane and intracellular domains present in *wt*CFR. The predicted molecular weight of *mut*CFR is 7 kD smaller than that of *wt*CFR, consistent with the migration of the mutant protein observed by SDS-PAGE (Fig. 3, inset).

Subcellular localization of wild-type and mutant CFR

Mutant CFR lacks the juxtamembrane, transmembrane and intracellular domains present in *wt*CFR. Therefore, we speculated that the cellular localization of wild-type and *mut*CFR may differ and correlate with the inability of *mut*CFR to alter intracellular FGF levels.

To test this hypothesis, we examined the cellular localization of both *wt*CFR and *mut*CFR by indirect immunofluorescence. Preliminary experiments indicated that CFR was concentrated in a region of the cell consistent with the Golgi apparatus. Therefore, cells were stained for chicken CFR using an anti-CFR mouse mAb and for α-Mannosidase II (a resident Golgi protein) using a rabbit polyclonal antibody (Velasco et al., 1993). As the anti-CFR mAb is specific for avian CFR (Kudla, submitted for publication), no detectable staining is observed in CHO.*vec* cells (Fig. 7a). The α-Mannosidase II antibody exhibits a staining pattern consistent with that of the Golgi apparatus (Fig. 7b). In CHO.*wt*CFR cells, the localization of the Golgi marker α-Mannosidase II overlaps that of CFR (Fig. 7d vs. c). However, note that some CFR staining is also observed in regions adjacent to α-Mannosidase II-stained areas (Figs. 7 and 8).

If the subcellular localization of CFR to the Golgi apparatus is due to overexpression in CHO cells, then this pattern should not be observed in chicken cells expressing endogenous CFR. Chick embryo fibroblasts were costained for chicken CFR and α-Mannosidase II (Fig. 7e and f). CFR was localized to the Golgi apparatus of chicken embryo fibroblast cells, demonstrating that native CFR has a staining pattern indistinguishable from that of CFR expressed in CHO cells. Similar staining patterns were also observed with three epitope-distinct anti-CFR mAbs (not shown). When the anti-CFR mAb was preincubated with CFR purified from chicken embryos, no staining was detected (not shown).

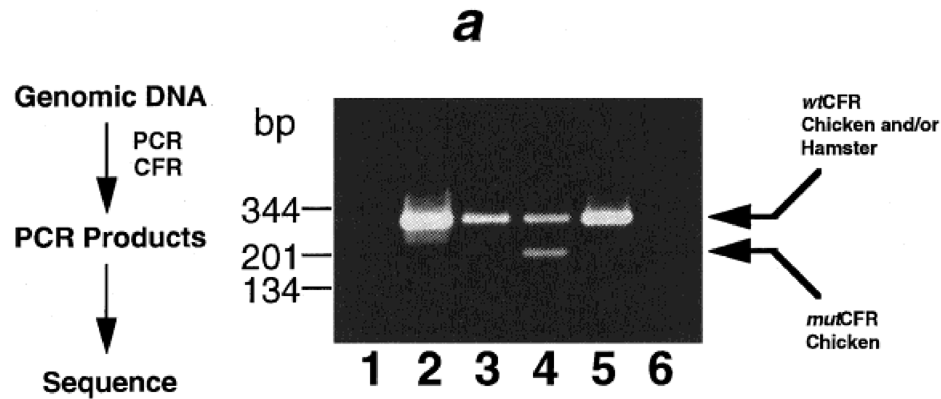
In contrast to *wt*CFR, *mut*CFR is detected in the Golgi apparatus and additional subcellular compartments (Fig. 8d–f). The staining pattern for α-Mannosidase II in CHO.*mut*CFR cells is similar to nontransfected and *wt*CFR transfected CHO cells, indicating the Golgi apparatus is intact and has not been compromised by *mut*CFR expression. Similar to *wt*CFR, *mut*CFR staining was not detected in nonpermeabilized cells (not shown).

DISCUSSION

Our results demonstrate that CFR overexpression reduces the levels of intracellular FGF-1 and FGF-2 in CHO cells. Reduction of intracellular FGF levels may alter biological responses to these factors, as transport of exogenously applied FGFs to the nucleus appears to be required for their function (Amalric et al., 1991; Bouche et al., 1987; Cao et al., 1993; Wiedlocha et al., 1994; Zhan et al., 1993). In addition, FGF-2 and FGF-3 isoforms derived from alternative translational initiation sites are found in the nuclei of cells in which they are expressed (Acland et al., 1990; Florkiewicz and Sommer, 1989; Powell and Klagsburn, 1991; Prats et al., 1989; Quarto et al., 1991; Zhan et al., 1993).

The reduction of intracellular FGF-1 and FGF-2 levels is CFR-dependent, as CHO transfectants expressing higher levels of CFR have correspondingly reduced levels of intracellular FGF (Fig. 3). At least four independent mechanisms could explain this effect. CFR may: (1) increase FGF degradation, (2) decrease FGF internalization, (3) sequester FGF in a subcellular compartment, or (4) transport internalized FGF out of the cell. The first mechanism is unlikely since the fragment pattern and rate of ^{125}I-FGF degradation are not affected by the expression of CFR in CHO cells (Fig. 4).

CFR could reduce the internalization of FGF, thereby inhibiting the intracellular accumulation of the growth factor. CFR may bind FGF at the cell surface and inhibit the interaction of the ligand with FGF-internaliz-



b

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3114  GACATTAAACACCACTGTGCTGCTATTCCTCCAGGGAGAGGACGCCAAATGTCATGCTTA
      GACATTAAACACCAC---------------------------------------------
1037  D  I  K  H  H  C  A  A  I  P  P  G  R  G  R  Q  M  S  C  L
      D  I  K  H  H  •  •  •  •  •  •  •  •  •  •  •  •  •  •  •

3174  ATGGAAGCTTTGGAGGACAAGCGTGTGAGGCTGCAGCCTGAATGCAAGAAACGCCTTAAT
      ------------------------------------------------------------
1057  M  E  A  L  E  D  K  R  V  R  L  Q  P  E  C  K  K  R  L  N
      •  •  •  •  •  •  •  •  •  •  •  •  •  •  •  •  •  •  •  •

3234  GATCGTATTGAAATGTGGAGCTATGCTGCAAAGGTTGCCCCAGCGGAAGGCTTCTCTGAC
      -ATCGTATTGAAATGTGGAGCTATGCTGCAAAGGTTGCCCCAGCGGAAGGCTTCTCTGAC
1077  D  R  I  E  M  W  S  Y  A  A  K  V  A  P  A  E  G  F  S  D
       I  V  L  K  C  G  A  M  L  Q  R  L  P  Q  R  K  A  S  L  T

3294  CTTGCCATGCAAGTTATGACCTCTCCGTCCAAGAATTACATATTGTCTGTGATCACGGTT
      CTTGCCATGCAAGTTATGACCTCTCCGTCCAAGAATTACATATTGTCTGTGATCACGGTT
1097  L  A  M  Q  V  M  T  S  P  S  K  N  Y  I  L  S  V  I  T  V
       L  P  C  K  L  R  P  L  R  P  R  I  T  Y  C  L  *

3354  GGCATCTGTGTACTCTTCCTGATCGGCCTGATGTGTGGACGCATCACCAAGCGGGTGACA
      GGCATCTGTGTACTCTTCCTGATCGGCCTGATGTGTGGACGCATCACCAAGCGGGTGACA
1117  G  I  C  V  L  F  L  I  G  L  M  C  G  R  I  T  K  R  V  T

3414  AGAGAGCTCAAGGACAGGTAG  3434
      AGAGAGCTCAAGGACAGGTAG
1137  R  E  L  K  D  R  *    1142
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Fig. 6. Characterization of *mut*CFR. **a:** Genomic DNA was isolated from CHO cells transfected with CFR and subjected to PCR analysis using primers designed to amplify the transmembrane and juxtamembrane domains of CFR. Template DNA used in the amplification was vector plasmid (**lane 1**), vector plasmid containing chicken CFR cDNA (**lane 2**), genomic DNA isolated from CHO.*vec* cells (**lane 3**), CHO.*mut*CFR cells (**lane 4**), and CHO.*wt*CFR cells (**lane 5**). **Lane 6** is amplification in the absence of added template DNA. Hamster CFR is amplified from CHO genomic DNA since the primers used for amplification of the chicken CFR perfectly match the hamster CFR DNA sequence (not shown). **b:** The nucleotide and predicted amino acid sequence of chicken *wt*CFR (top sequence) and *mut*CFR (bottom sequence) are compared. A 106-bp deletion (dashed line) in the chicken *mut*CFR cDNA results in a shift of the open reading frame (dots) and the introduction of a premature stop codon (asterisk). Numbers left of the nucleotide and amino acid sequences represent the base numbers and residue numbers of chicken *wt*CFR. These sequence data are available from GenBank under accession numbers M95766 and U48395.

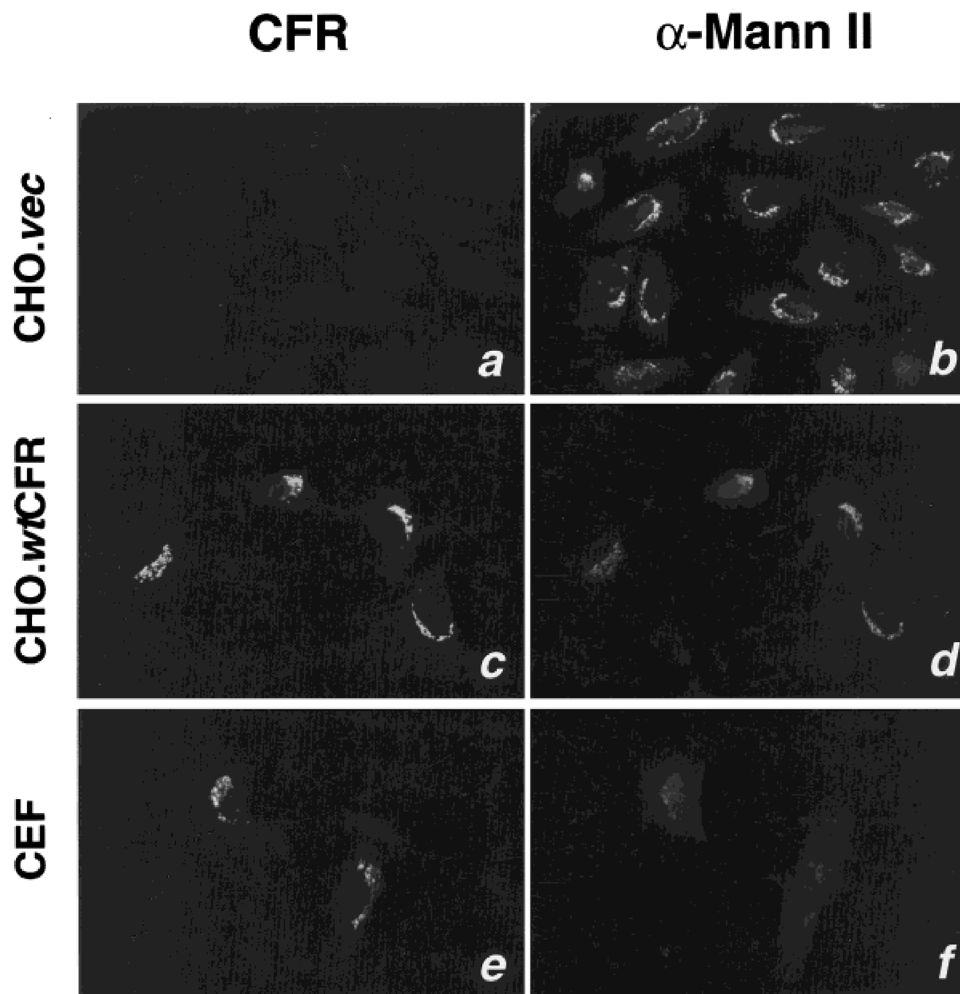

Fig. 7. Subcellular localization of *wt*CFR is consistent with localization to the Golgi apparatus. Indirect immunofluorescence was used to determine the intracellular localization of CFR. CHO cells (**a–d**) and chick embryo fibroblasts (**e, f**) were doubly stained with antibodies to CFR and the Golgi marker α-Mannosidase II. CFR (**c, e**) is detected in a perinuclear region consistent with Golgi apparatus but is not detected in vector-transfected cells (**a**). α-Mannosidase II (**b, d, f**) localization overlaps that of CFR (**c** vs. **d** and **e** vs. **f**). The polyclonal antibody prepared against rat α-Mannosidase II weakly recognizes chicken α-Mannosidase II protein (**f**).

ing receptors. However, we have been unable to detect CFR on the surface of CHO.*wt*CFR cells. Moreover, the binding or crosslinking of FGF-1 and FGF-2 to cell surface receptors is not affected by CFR expression (Fig. 1). Together, these results are inconsistent with CFR binding exogenous FGF at the cell surface of intact CHO.*wt*CFR cells.

It is possible that a secreted form of CFR could bind extracellular FGF and inhibit FGF internalization. Medium conditioned by CHO.*wt*CFR cells contains CFR protein that is approximately 3–5 kD smaller than cell-associated CFR (not shown). This observation is consistent with a posttranslational modification (possibly proteolytic cleavage) resulting in the release of CFR from the cell. As mutant CFR lacks the transmembrane domain necessary for anchoring the protein in the lipid bilayer, *mut*CFR is also present in conditioned medium. If secreted CFR were responsible for reducing intracellular FGF accumulation, then wild-type and mutant CFR should have similar effects. However, secreted *mut*CFR does not affect the intracellular level of FGF-1 (Fig. 3).

CFR could target FGF to a specific subcellular compartment such as the nucleus. Our data represent total intracellular CFR and a preliminary examination of cytoplasmic and nuclear FGF levels suggests CFR may alter the subcellular localization of FGFs (unpublished data). However, changes in intracellular distribution of FGF-1 or FGF-2 does not account for the reduction in intracellular FGFs observed in cells overexpressing *wt*CFR.

We propose that CFR may inhibit the intracellular accumulation of FGFs in CHO cells by rapidly exporting FGF internalized by cell surface receptors. The subcellular localization of CFR appears to be a critical factor in controlling the levels of intracellular FGFs. CFR localization coincides with that of the resident Golgi protein α-Mannosidase II. Although α-Mannosi-

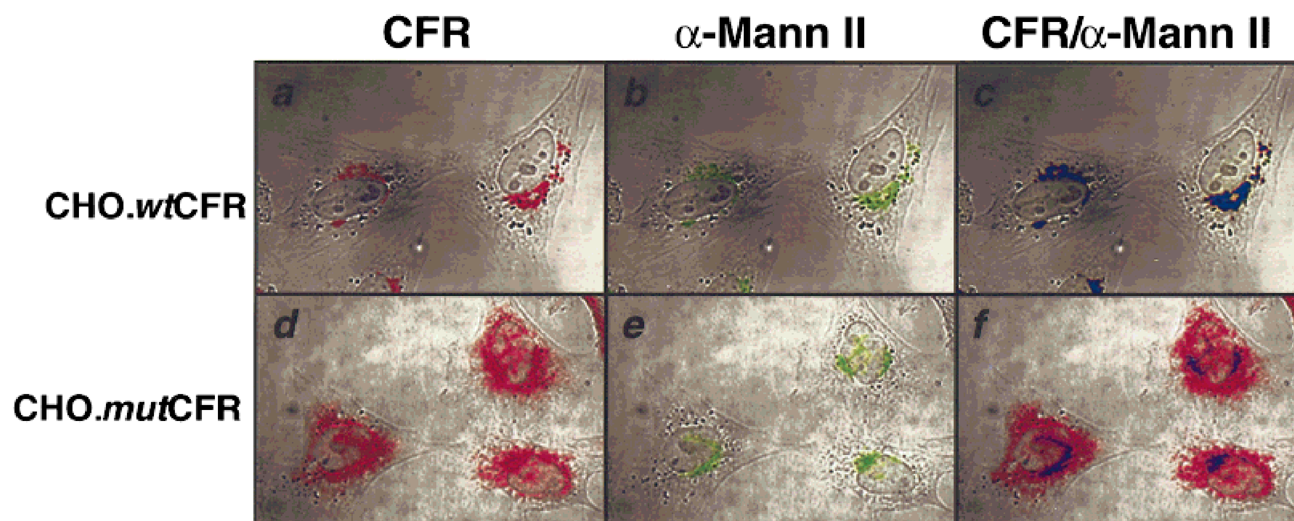

Fig. 8. *mut*CFR is distributed throughout the cell. CHO.*wt*CFR (**a–c**) and CHO.*mut*CFR (**d–f**) cells were stained as in Figure 7. *wt*CFR (a) is localized to the Golgi apparatus while *mut*CFR (d) is not limited to the Golgi. Staining for the Golgi marker α-Mannosidase II protein indicates that the Golgi apparatus is intact in both cell types (b, e). In c and f, CFR and α-Mannosidase II staining images have been overlaid to illustrate the relative localization of the proteins. Red, CFR localization; green, α-Mannosidase II localization; violet, regions of the cell in which CFR and α-Mannosidase II are detected simultaneously.

dase II localization within the Golgi is cell type-specific, in CHO cells it is localized to the medial Golgi (Velasco et al., 1993). Localization of chicken CFR to the medial Golgi is consistent with reports describing the subcellular localization of the rat CFR homologue in the medial cisternae of rat brain neurons, astrocytes, adenohypophysis and cultured rat pheochromocytoma (PC-12) cells (Croul et al., 1990; Gonatas et al., 1989). Interestingly, chicken CFR is modified with sialic acid (unpublished data). Since the enzymes required for sialylation are located in the trans-Golgi and trans-Golgi network (TGN) of most cell types (Berger et al., 1987; Roth et al., 1985, 1986), chicken CFR is likely to return to the medial Golgi after being sialylated in the trans-Golgi or TGN. Consistent with this interpretation, rat CFR was recently shown to cycle from the TGN where it is sialylated, to the medial Golgi in PC12 cells (Johnston et al., 1994). Rapid cycling of CFR is also consistent with the effects of CFR on FGF internalization, in which CFR decreases the level of intracellular ^{125}I-FGF-1 in as little as 10 min following the addition of FGF to the culture media (Fig. 2).

Several reasons could explain the relatively modest reduction in intracellular FGFs in CHO.*wt*CFR-expressing cells as compared to control cells. First, the amount of FGF internalized is a small percentage of the total FGF added to the cells (1–2%). Second, the CHO cells express an endogenous hamster CFR mRNA. The levels of chicken CFR protein present may not greatly exceed the levels of endogenous hamster CFR. As all of our monoclonal antibodies are avian-specific, we were not able to assess the relative levels of hamster CFR protein present in CHO cells. Third, only a fraction of the overexpressed CFR may be active, as CFR associates with two additional proteins of 70 and 45 kDa (Burrus and Olwin, 1989) that may be limiting in CHO cells. Thus, a 2 to 3-fold reduction in the levels of intracellular FGFs is consistent with a role for CFR in this process. Finally, if CHO cells synthesize FGFs, these may interact with CFR and alter the capacity of CFR to affect the intracellular levels of exogenously applied FGFs.

It was not possible to directly test the effects of CFR on FGF function in FGF-responsive cells, as all attempts to overexpress CFR in cell types other than CHO cells were unsuccessful. It is likely that overexpression of CFR is cytotoxic for most cells we tested, as few transformants were obtained. CHO cells may be somewhat resistant to the effects of CFR overexpression, as these cells are not responsive to FGFs despite the presence of FGF receptor tyrosine kinases (Olwin and Hauschka, 1989).

The primary site of CFR localization in the Golgi apparatus suggests that an active mechanism is involved in its retention. A number of membrane proteins have been localized to the Golgi apparatus. In the TGN, retention appears to be a dynamic process dependent on a cytoplasmic retrieval motif (Bos et al., 1993; Bosshart et al., 1994; Humphrey et al., 1993). Much less is known of how proteins are retained in the stacks of the Golgi complex, although transmembrane sequences appear to be involved (Hobman et al., 1995; Machamer, 1991, 1993; Machamer and Rose, 1987, 1990). Although *wt*CFR appears to be localized to the Golgi, *mut*CFR is also detected throughout the cell (Fig. 8). Residues 1042 to 1142, which are absent in *mut*CFR, therefore appear necessary for the appropriate subcellular localization of CFR. As *mut*CFR does not alter intracellular FGF levels, the subcellular localization of CFR may play a critical role in this process. Analysis of CFR deletion mutants will be useful in further characterizing the domain(s) required for the subcellular localization of *wt*CFR.

We do not understand the mechanism used by CFR

that results in the reduction of intracellular FGFs. The decrease in intracellular FGFs does not result from changes in FGF binding, FGF degradation, or major changes in subcellular localization of FGFs. It is therefore possible that CFR is involved in the targeting of internalized FGFs to the Golgi with subsequent release from the cell. Internalized proteins can be targeted to lysosomes for degradation; however, some molecules are transported to the Golgi apparatus (Trowbridge et al., 1993). CFR-containing vesicles recycling from the TGN could fuse with endocytosed FGF-containing vesicles and transport the FGF to the medial Golgi. CFR bound FGF would then be transported from the cell. FGF could be released from CFR in the Golgi apparatus or, alternatively, a posttranslational modification could result in the release of the cell-associated FGF/CFR complex such as proteolytic processing of CFR. This hypothesis predicts that CHO cells overexpressing CFR should contain higher levels of ^{125}I-FGF-1 and ^{125}I-FGF-2 in the medium. However, we were unable to consistently detect an increase in either factor in the medium of CHO.*wt*CFR vs. CHO.*vec* transfected cells. This is due to the low level of ^{125}I-FGFs recycled and released (1–2 fmol) compared to the levels added (100 fmol) and the amount bound to the cell surface (~2 fmol).

Recycling and release of FGFs by CFR is the hypothesis consistent with our observations. First, the secreted form of CFR is likely to be a proteolytic fragment that lacks the transmembrane domain and intracellular region. Second, the inability of *mut*CFR to alter the intracellular level of FGFs correlates with the aberrant subcellular localization of *mut*CFR. The distribution of *mut*CFR throughout the cell may indicate an inability of the mutant protein to cycle back to the medial Golgi once *mut*CFR has left the TGN. Therefore, if *mut*CFR-containing vesicles could fuse with endocytosed FGF vesicles, *mut*CFR could not transport FGF out of the cell. In summary, the ability of CFR to regulate the level of intracellular FGF suggests that CFR may be capable of altering the biological responses to these growth factors.

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