A Functional Fibroblast Growth Factor-1 Immunoglobulin Fusion Protein*

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Proteins of the fibroblast growth factor (FGF) family play diverse roles in embryonic development, angiogenesis, and wound healing. The most well studied targets of FGF activity typically are cells of mesodermal and neuroectodermal origin; in addition, expression of FGF-1 (acidic FGF) is increased at several sites of chronic immunologic injury, and recent studies show that FGF-1 also may interact with cells of the immune system. In some human T cells, FGF-1 can induce signals necessary for production of interleukin-2, a key cytokine required for T cell proliferation. To better characterize the interaction of FGF-1 with FGF receptors on T cells, a fusion protein was constructed containing a portion of the constant region of human IgG1 (Fc) at the amino terminus of FGF-1. The Fc-FGF-1 fusion protein retained FGF function as determined by stimulation of tyrosine phosphorylation and DNA synthesis in NIH 3T3 cells. Binding of the intact fusion protein to FGF receptor 1 (FGFR1) on T cells was demonstrated by immunoprecipitation of the receptor bound to Fc-FGF-1 and by flow cytometry showing binding of fusion protein to T cells expressing FGFR1. This functional Fc-FGF-1 protein should prove useful in identifying FGF-expressing cells.

Classical targets for stimulation by heparin binding growth factors FGF-1 and FGF-2 (basic FGF) include cells of mesodermal and neuroectodermal origin such as endothelial cells, smooth muscle cells, fibroblasts, and myoblasts (reviewed in Ref. 1). FGF-1 and FGF-2 potently induce proliferation and migration of these cells as well as angiogenesis, events that are required during normal embryogenesis and tissue repair. Although expression of FGF-1 and FGF-2 at sites of injury is therefore beneficial, some evidence suggests these growth factors may also contribute to vascular pathology by promoting excessive intimal hyperplasia (2–5). FGF-1 expression is increased at several sites of chronic immune injury, including the synovium in rheumatoid arthritis (6–8), the myocardium in human hearts after transplantation (9–11), and in transplanted kidneys undergoing chronic rejection (12, 13). The pathologic lesions in these sites demonstrate cellular responses typical of FGF effects on mesenchymal cells that result in vascular intimal hyperplasia, increased extracellular matrix deposition, and neoangiogenesis. In addition, these sites are characterized by chronic infiltration of T lymphocytes, suggesting there may be interactions between the immune system and fibroblast growth factors as demonstrated by the finding that T cells can produce FGF-2 (14, 15). Evidence that FGFs may have immunoregulatory effects on T cells was first provided in 1985 by Johnson and Torres (16), who showed that FGF, at physiologically relevant concentrations, could replace the requirement for IL-2 or helper cells in production of interferon-γ. Although FGF could activate intracellular signals necessary for interferon-γ production, FGF alone could not stimulate proliferation of T cells. More recent studies show directly that some human T cells express receptors for FGF-1 and that the normally small subpopulation of FGF-responsive T cells is expanded in the peripheral blood of patients with rheumatoid arthritis and in patients after heart transplantation (8, 17). These data suggest that T cells bearing FGF receptors can be stimulated and expanded in the FGF-enriched environment at sites of immune injury and subsequently migrate to the peripheral blood. As found in the earlier studies (16), FGF alone does not stimulate proliferation of T cells but together with engagement of the T cell antigen receptor induces production of IL-2 and proliferation (17). In T cells, FGF thus functions in a manner analogous to other well described “costimulators” (18, 19) to activate a second signal transduction pathway necessary for T cell proliferation and effector function.

These findings suggest that FGF and FGF receptors in T cells may function quite differently than in cells in which FGF alone can stimulate proliferation, migration, and secretion of effector molecules such as plasminogen activator (20). Our efforts to investigate T cells that express FGF receptors and the mechanisms by which FGF signals in T cells have been hampered by the lack of reagents that can conveniently identify these cells and allow us to examine the fate of FGF and its receptors in T cells. To address this difficulty, the experiments reported here describe the production and characterization of a fusion protein that includes a portion of the constant region (Fc) of human IgG1 at the amino terminus of full-length FGF-1. In contrast to a previously reported fusion protein of FGF-1 with diphtheria toxin A chain, Fc-FGF-1 retains full FGF function, including the ability to induce DNA synthesis, tyrosine phosphorylation, and FGFR1 binding as well as Fc-mediated binding to protein A and anti-human IgG antibodies.

EXPERIMENTAL PROCEDURES

Production of Fc-FGF-1—Plasmid BS-2 mutFc was kindly provided by Dr. Melanie Spriggs, Immunex Corp. This plasmid includes the
murine IL-7 leader sequence, a nonamer FLAG epitope, CH2 and CH3 domains of human IgG1 (amino acids 242–470 (21)), and a (Gly3Ser)2 repeat to provide a flexible linker between the Fc domain and the amino terminus of the introduced protein (22). The Fc region of this construct includes several amino acid mutations introduced to diminish Fc receptor binding. Centroin tandem anti-rabbit sites were generated as insert containing CH2, CH3 epitope, Fc region, and flexible linker flanked by a 5’ Not site and 3’ SpeI site. The insert and pET23b construct containing FGF downstream of the SpeI site were digested with NcoI and SpeI and ligated together. The insert encodes a protein comprised of an amino-terminal FLAG epitope and human IgG1 Fc fused to the amino terminus of FGF-1. For conditions for iso-1-thio-b-galactopyranoside induction and lysis were modified to improve the yield of fusion protein in the soluble fraction of the bacterial lysate. Iso-1-thio-b-galactopyranoside concentration was decreased to 0.4 mM, and bacteria were grown at 30 °C for 5 h. The bacteria were harvested and resuspended in 80 ml of lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 10 mM glucos, 10 mM NaVO3, and protease inhibitor mixture) on ice for 10 min, sonicated (approximately 5 mg/ml leupeptin, 0.5 mg/ml pepstatin, 1 mg/ml phenylmethylsulfonyl fluoride). After sonication and centrifugation, the supernatant containing Fc-FGF was purified on heparin-Sepharose CI-6B (Amersham Pharmacia Biotech). After initial experiments demonstrated some proteolytic cleavage and release of free FGF-1 from storage at 4 °C, the protein was stored in aliquots, frozen at -70 °C. In some experiments, the protein was further purified by elution from protein A-Sepharose with 0.2 M glycine-HCl (pH 2.5) followed by the addition of 1× Tris (pH 8.5) to pH 7 or dialysis.

**Transfection of Jurkat T cells**—A plasmid containing the human CD2 promoter and locus control region for expression in CD2+ T cells, pH151 (23), was a gift from Dr. Dimitri Kiossis. FGRF1f1a1 cDNA (24) was provided by Dr. Wallace McKeehan. It was released with BamHI, blunt-ended, and ligated into the SpeI site of pH151. A plasmid containing FGRF1f1a1 in the correct orientation was designated pCD2-FGRF1 and used to transfect Jurkat T cells as described below. To produce Jurkat transfectants expressing FGF-1, a cDNA encoding full-length FGF-1 (amino acids 1-154) in pMEXneo was excised, blunt ended, and ligated into the SpeI site of pH151. A plasmid containing FGRF1f1a1 in the correct orientation was designated pCD2-FGFR1 and used to transfect Jurkat T cells as described below. To produce Jurkat transfectants expressing FGF-1, a cDNA encoding full-length FGF-1 (amino acids 1-154) in pMEXneo was excised, blunt ended, and ligated into the SpeI site of pH151. A plasmid containing FGRF1f1a1 in the correct orientation was designated pCD2-FGFR1 and used to transfect Jurkat T cells as described below. To produce Jurkat transfectants expressing FGF-1, a cDNA encoding full-length FGF-1 (amino acids 1-154) in pMEXneo was excised, blunt ended, and ligated into the SpeI site of pH151. A plasmid containing FGRF1f1a1 in the correct orientation was designated pCD2-FGFR1 and used to transfect Jurkat T cells as described below. To produce Jurkat transfectants expressing FGF-1, a cDNA encoding full-length FGF-1 (amino acids 1-154) in pMEXneo was excised, blunt ended, and ligated into the SpeI site of pH151. A plasmid containing FGRF1f1a1 in the correct orientation was designated pCD2-FGFR1 and used to transfect Jurkat T cells as described below. To produce Jurkat transfectants expressing FGF-1, a cDNA encoding full-length FGF-1 (amino acids 1-154) in pMEXneo was excised, blunt ended, and ligated into the SpeI site of pH151. A plasmid containing FGRF1f1a1 in the correct orientation was designated pCD2-FGFR1 and used to transfect Jurkat T cells as described below. To produce Jurkat transfectants expressing FGF-1, a cDNA encoding full-length FGF-1 (amino acids 1-154) in pMEXneo was excised, blunt ended, and ligated into the SpeI site of pH151. A plasmid containing FGRF1f1a1 in the correct orientation was designated pCD2-FGFR1 and used to transfect Jurkat T cells as described below.

**Cell Culture, FGF Stimulation, and Western Blotting**—Hybond. After UV cross-linking, the membrane was prehybridized (5 for 1-2 h) and then hybridized overnight (2-4 h) with 1× hybridization solution with 10% dextran sulfate. After the washes in PBS, 1% Tween 20-20. Western blotting was performed with rabbit anti-phosphotyrosine (Transduction Laboratories) and horseradish peroxidase-conjugated goat anti-rabbit (Santa Cruz Biotechnology). For immunoprecipitation, lysates were incubated with protein A-Sepharose with 0.2 M glycine-HCl (pH 2.5) followed by the addition of 1× Tris (pH 8.5) to pH 7 or dialysis.

**Flow Cytometry with Fc-Jurkat** T cells or transfectants (1-2× 106 cells) were resuspended in Dulbecco's modified Eagle's medium, 0.1% BSA, 2.5 μg/ml heparin. Fc-FGFR was added (100 ng/ml), and the cells were incubated on ice for 1 h. Cells were collected by brief microcentrifuge spin and washed in the same medium and then in PBS with 250 μg/ml heparin unless otherwise indicated and finally with PBS only. The cells were stained with FITC goat-anti-human IgG (Fc) (1.80) or FITC (Fab) goat anti-human IgG (Fc) (0.5 μg) for 1 h on ice. In some experiments, the cells were subsequently stained with phycoerythrin anti-CD3.

**FGF Receptor Cross-linking with Radiolabeled Ligands—** FgF-1α (amino acids 21-154) was produced in E. coli DH5α (25). FGF-1α was labeled with Na125I using chloramine T and purified on heparin-Sepharose as described previously (26). Fc-FGFR was labeled and purified in essentially the same manner except that the incubation time with chloramine T was decreased from 90 to 75 s. For binding of 125I-labeled FGF-1 or Fc-FGFR, 5×105 cells were incubated with 10 ng of FGF-1 or Fc-FGFR, 5×105 cells were incubated with 10 ng of FGF-1 or 20-30 ng of Fc-FGFR in PBS, 0.1% BSA, 2.5 μg/ml heparin in a final volume of 250 μl for 1 h on ice. The cells were collected by brief microcentrifuge spin, washed once in PBS, 0.1% BSA in 250 μl then in PBS containing 250 μg/ml heparin, and finally in PBS. The cells were resuspended in PBS with varying concentrations of bisulfosuccinimidy-suberate (BS3; Pierce) as indicated for 20 min at room temperature. The croslinking reaction was quenched by adding 50 mM Tris-HCl and 10 mM glycine (final concentration) for 10 min at room temperature. For direct analysis, cells were lysed in PBS, 1% Triton X-100, mixed with SDS sample buffer with 1 mM dithiobis, and run on SDS-polyacrylamide gel (10% gel). Other conditions were modified to obtain a clear separation of the bands on the gel. The bands were visualized by autoradiography or by autoradiography and staining with Coomassie Blue or silver staining. Approximatulated as follows.

**Northern Analysis—** 20 ng of total RNA (from 4-8×105 cells) was electroelrophoresed in a 1% formaldehyde agarose gel and transferred to Hybond. Under UV cross-linking, the membrane was prehybridized (5× Denhardt's, 5× SSPE, 150 μg/ml salmon sperm DNA, 0.1% SDS) and then hybridized overnight (2-4 h) with 1 ng/ml of the FGF-1a probe. After washing with 0.1% SDS 30 min with prehybridization solution with 0.1% SDS first with a 1.4-kilobase FGF18 DNA probe and after stripping with a 400-base pair glyceraldehyde-3-phosphate dehydrogenase probe. Final washes were 0.1× SSPE, 0.1% SDS at 65–65 °C.

**Other Reagents—** Spro-Orange fluorescent protein stain was from Molecular Probes (Eugene, OR), horseradish peroxidase-anti- rabbit Ig was from Western Biotechnology, horseradish peroxidase-anti-mouse Ig was from Boehringer Mannheim, anti-FLAG monoclonal antibody was from CoMab (17 Ci/mg) in 10-5 M sodium orthovanadate. Whole cell lysates were run on 7% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon, blocked with 5% nonfat dry milk in 10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20. Western blotting was performed with rabbit anti-phosphotyrosine (Transduction Laboratories) and horseradish peroxidase-conjugated goat anti-rabbit (Santa Cruz Biotechnology). For immunoprecipitation, lysates were incubated with protein A-Sepharose with 0.2 M glycine-HCl (pH 2.5) followed by the addition of 1× Tris (pH 8.5) to pH 7 or dialysis.

**RESULTS**

**Fc-FGF Binds Heparin and Is Recognized by Anti-FGF-1 and Anti-human IgG—** Purification of the bacterial lysate on heparin-Sepharose yielded a predominant band of approximately 50 kDa corresponding to the predicted molecular mass of the desired Fc-FG-1 fusion protein (Fig. 1A). A portion of the fusion protein was additionally purified using protein A-Sepharose. Western blotting was performed on the eluates with antibodies to FGF-1, human IgG, and the FLAG epitope (Fig. 1B). In each case, the 50-kDa protein stained with antibody, indicating the presence of the correct epitopes. The anti-FLAG antibody was least sensitive for detection of the fusion protein, presumably because of the presence of a single FLAG epitope and hence limited binding by the monoclonal antibody com-
pared with the polyclonal anti-FGF and anti-human IgG antibodies. The additional purification step on protein A-Sepharose appeared to offer no advantage and resulted in substantial loss of protein because of precipitation of the fusion protein after elution with 0.2 M glycine. Therefore, subsequent experiments were performed with Fc-FGF purified on heparin-Sepharose only. Preparations of Fc-FGF stored at 4 °C for more than a week began to show cleavage to lower molecular mass proteins that reacted with anti-FGF by Western blotting (not shown). Fc-FGF stored at −20 °C or −70 °C was stable and showed no release of free FGF detectable by Western blotting when incubated with cells in culture medium overnight at 37 °C (Fig. 1C).

Fc-FGF Stimulates Proliferation of NIH 3T3 Cells and Phosphorylation of p90—To confirm that Fc-FGF was functional, serum-starved NIH 3T3 cells were stimulated with either Fc-FGF or recombinant human FGF-1, and DNA synthesis was measured by [3H]thymidine incorporation. Fc-FGF stimulated DNA synthesis by NIH 3T3 cells in the presence of heparin; the concentration required was approximately 3-fold higher on a molar basis than that required for an equivalent response to recombinant human FGF-1 (Fig. 2). Little or no response was observed in the absence of heparin (data not shown).

After binding to FGF receptors, FGF-1 and FGF-2 rapidly stimulate tyrosine phosphorylation of several intracellular substrates, including a prominent membrane-associated protein of approximately 90 kDa (27, 28) that may be an important link between FGFR and downstream effector Ras/mitogen-activated protein kinase signaling pathways. Goh et al. (29) identified a 90-kDa protein that is phosphorylated in response to FGF-2 and binds to the SH2 adapter protein GRB-2 (29). This protein is identical to 80K-H, a protein of unknown function. More recently, Kouhara et al. (30) identified a different, novel protein of 92–95 kDa, designated FRAS2 (fibroblast growth factor receptor substrate 2), that is phosphorylated upon FGF-1 stimulation and also binds GRB-2 (30). To determine whether Fc-FGF similarly activates tyrosine phosphorylation via FGFR, Western blotting with anti-phosphotyrosine was performed on NIH 3T3 cells stimulated with FGF-1 or Fc-FGF. Both Fc-FGF and recombinant human FGF-1 induced tyrosine phosphorylation of a prominent 90-kDa protein and proteins between 83–86 kDa (Fig. 3). Taken together, the data indicate that Fc-FGF has functional FGF-1 and Ig properties. The following studies were performed to assure that the functional FGF activity of Fc-FGF resided in the intact fusion protein and did not result from cleavage of free FGF-1.
concentration or source of serum, or duration of passage in vitro. To establish a more predictable system for further understanding of FGF-1 function and stimulation in T cells, Jurkat T cells were stably transfected with a plasmid directing expression of FGFR1β under the control of a T cell-specific promoter and locus control region. In addition, because T cells can produce FGF-2 (14, 15) and isoforms of FGF-2 regulate FGFR encoded receptors capable of binding FGF as evident considering the long Fc domain presumably extending from Fc-FGF bound to FGFR1. Far fewer receptors were expressed by the parental nontransfected Jurkat and fewer yet by Jur/FGFR. When the same amounts of mRNA for FGFR1 (31, 32), Jurkat transfectants expressing FGF-1 (1–154) were produced using the same expression vector. Northern analysis demonstrated that Jurkat transfected with pCD2-FGFR1 (Jur/FGFR) had little or no detectable endogenous mRNA for FGFR1. These cell lines were used first to examine binding of 125I-labeled FGF-1α to demonstrate that the FGFR1 mRNA in Jurkat transfectants produced a functional receptor that binds FGF and second, to confirm that intact Fc-FGF binds to FGFR1.

Nontransfected and transfected Jurkat were incubated with 125I-labeled FGF-1α followed by cross-linking with BS3, a water-soluble, membrane-impermeant cross-linker. The cross-linked cells were solubilized, and FGFR1 binding of labeled FGF-1α was analyzed by SDS-polyacrylamide gel electrophoresis of whole cell lysates and autoradiography (Fig. 5A). In Jur/FGFR, prominent bands were seen at approximately 140 kDa and >200 kDa, corresponding to the approximate molecular masses expected for FGF-1α-coupled to FGF-1α monomers and dimers or higher order oligomers. Fainter bands of the same size were also seen in nontransfected Jurkat, consistent with the low level of FGFR1β that was observed in these cells previously. Virtually no binding of 125I-FGF-1α was seen with Jurkat transfected with FGF-1. When the same procedure was performed with 125I-Fc-FGF, only high molecular mass bands were seen (Fig. 5A). These findings suggested several conclusions. First, the FGFR1 mRNA expressed in Jur/FGFR encoded receptors capable of binding FGF as evident from the strong binding of FGF-1α. Far fewer receptors were expressed by the parental nontransfected Jurkat and fewer yet by Jur/FGFR. Second, the cross-linking conditions used appeared to favor formation of dimers or higher order oligomers with Fc-FGF compared with FGF-1α because no band was observed at the molecular mass expected for cross-linked monomers of Fc-FGF and FGFR1. This may not be surprising considering the long Fc domain presumably extending from Fc-FGF bound to the receptor and the observation that other Fc-fusion proteins spontaneously dimerize (33). Third, cross-linking of Fc-FGF to FGFR in these cells did not demonstrate the band at 140 kDa seen with cross-linking of FGF-1. This suggests that there was not substantial cleavage of labeled FGF-1 from Fc-FGF to produce monomers of labeled FGF-bound FGFR1.

To determine whether monomeric cross-linking of Fc-FGF to FGFR1 could be identified, a titration of the cross-linker was performed (Fig. 5B) as described in the original characterization of BS3 (34). In the absence of cross-linker, monomers and spontaneous dimers of labeled Fc-FGF were present. With increasing concentration of BS3, monomers and dimers of free Fc-FGF decreased and new higher molecular weight bands appeared at molecular weights appropriate for monomers of Fc-FGF bound to FGFR1 (approximately 170–190) as well as higher order oligomers of Fc-FGF. Based on this titration, subsequent immunoprecipitation experiments were performed in the absence and presence of BS3 at 0.5 mM final concentration. Jurkat and transfectants were incubated with 125I-labeled Fc-FGF and immunoprecipitated with anti-FGFR1 without prior cross-linking. As shown in Fig. 5C, immunoprecipitation of FGFR1 from nontransfected Jurkat in the absence of cross-linking (1st lane) revealed association of Fc-FGF monomer. A faint band corresponding to Fc-FGF dimers was also seen. In Jur/FGFR (2nd lane), immunoprecipitation of FGFR1 in the absence of cross-linking revealed association of both Fc-FGF monomer and dimer, with substantially increased amounts of...
The fusion protein compared with nontransfected Jurkat, consistent with the larger number of receptors expressed by the transfectant. As seen before with the whole cell lysates, Jur/FGR (3rd lane) showed the least binding of Fc-FGF to receptor. The results show that Fc-FGF is co-precipitated with FGR1. These experiments were also performed with cross-linking before lysis and immunoprecipitation (Fig. 5C, 4th–6th lanes). In Jurkat (4th lane), bands corresponding to monomers of FGR1 bound to labeled Fc-FGF monomers, and dimers are seen. In Jur/FGR (6th lane), free Fc-FGF monomers and dimers are also present along with fusion protein cross-linked to FGR1, whereas little binding to Jur/FGR is seen (6th lane). These results confirm binding of Fc-FGF to FGR1.

As an alternative approach to demonstrate binding of intact fusion protein to FGR1, the fusion protein was immunoprecipitated via its Fc domain with protein A-Sepharose followed by blotting with antibody to FGR1 to identify FGR1 that co-precipitated with Fc-FGF (Fig. 6). Unlabeled Fc-FGF was allowed to bind at 4 °C, the lysates were immunoprecipitated with protein A alone, and the eluted proteins were immunoblotted with anti-FGR1 (Fig. 6A). In Jur/FGR that had not been cross-linked (5th lane), prominent bands were seen at approximately 120 and 100 kDa. The 120-kDa band is at the molecular mass expected for FGR1β, demonstrating the co-precipitation of FGR1β with Fc-FGF. The identity of the 100-kDa band is not known. Lower amounts of FGR1 are seen for nontransfected Jurkat (4th lane) and Jur/FGR (6th lane), consistent with the results found in Fig. 5. Similar results were seen with binding of Fc-FGF at 37 °C (Fig. 6B, 1st lane), and the specificity of the immunoprecipitation is demonstrated by the absence of a FGR1 band in the transfec tant with no added fusion protein (Fig. 6B, 2nd lane). When the cells were cross-linked before lysis (Fig. 6A, 1st–3rd lanes), the Jur/FGR transfec tants showed additional bands at 170–190 and >250 kDa, consistent with the molecular masses of cross-linked Fc-FGF/FGR1β monomers and dimers (Fig. 6A, 2nd lane).

In summary, these data show that immunoprecipitation of Fc-FGF co-precipitates intact Fc-FGF and conversely, immunoprecipitation of the fusion protein via its Fc domain demonstrates that it is bound to FGR1. Scatchard analysis demonstrated that Jur/FGR expressed approximately 30,000 high affinity FGF receptors/cell compared with approximately 4,000 receptors on nontransfected Jurkat. The dissociation constant for FGF-1 was 0.18 nM compared with 2.5 nM for Fe-FGF (data not shown). These results are very similar to findings reported for a fusion protein of FGF-7 with Ig CH (33).

Flow Cytometry with Fc-FGF—A potential application of the fusion protein is identification of FGR1-expressing cells in mixed populations; therefore Fc-FGF was tested for its utility in flow cytometry (Fig. 7). After incubation with Fe-FGF, Jurkat and Jur/FGR were stained with FITC-anti-human IgG directly or first washed with 250 µg/ml heparin to remove Fe-FGF from low affinity sites on the cell surface. Nontransfected Jurkat (Fig. 7A) showed significant binding of Fe-FGF detected by FITC-anti-human IgG followed by phycoerythrin-anti-CD3. Jurkat, FITC mean channel 8.43; D, Jur/FGR, FITC mean channel 184.36; E, Jur/FGR, FITC mean channel 4.13.

DISCUSSION

The experiments presented here show that a fusion protein comprised of human IgG1 Fc at the amino terminus of FGF-1 retains both FGF function and IgG constant region function, shown by stimulation of DNA synthesis and tyrosine phosphorylation in NIH 3T3 cells, binding to heparin and FGR1, and binding to protein A and antibodies to human IgG. These
results differ in a potentially important way from a previously reported FGF-1 fusion protein, aFGF-dtA, a construct with diphtheria toxin A chain at the carboxyl terminus of FGF-1 (35, 36). aFGF-dtA failed to induce cell proliferation or DNA synthesis in NIH 3T3 cells, although it did induce tyrosine phosphorylation, suggesting it bound to FGFR on NIH 3T3 cells (35). In contrast to native FGF-1, aFGF-dtA was found only in the cytoplasmic fraction and failed to translocate to the nucleus. Whether the fusion protein in the cytoplasmic fraction was internalized in the cytosol or remained bound to surface FGFR was not determined. In U2OS Dr1 cells that express diphtheria toxin receptors but are resistant to toxin action, combination of aFGF-dtA with dtB resulted in translocation to the nucleus via toxin receptors and induced DNA synthesis, but no tyrosine phosphorylation was induced because these cells lack FGF receptors (35). Further experiments in cells transfected with FGFR4 showed that this FGF receptor could translocate native FGF-1 to the nucleus, but not aFGF-dtA, despite high affinity binding of both native FGF-1 and the fusion protein. These data were interpreted to indicate that both FGFR-dependent tyrosine phosphorylation and nuclear translocation of FGF-1 must occur to induce cell proliferation (36). If this is the case, the experiments presented here suggest that Fc-FGF-1 does translocate to the nucleus as well as inducing tyrosine phosphorylation via FGF receptors and retains the functions of native FGF-1. The structural features that might be responsible for the distinct functional properties of the two fusion proteins are unclear at this time.

A concern with fusion proteins or epitope tags engineered at the amino terminus of FGF-1 is that proteolytic cleavage at the amino terminus is common during purification of native FGF-1, leading to the isolation of functional molecules lacking up to 20 amino-terminal amino acids (37–39). The addition of protease inhibitors during purification of FGF-1 from tissue markedly increased the yield of full-length FGF-1 (39). If similar cleavage events occur with an amino-terminal fusion protein, then functional FGF (e.g. 21–154) may be released from the fusion protein as a result of this proteolysis. We cannot exclude the possibility that some of the functional response of NIH 3T3 cells to Fc-FGF-1 results from such proteolysis, but it is unlikely that most of the activity of the fusion protein results from release of free FGF. The amount of fusion protein required to elicit DNA synthesis in NIH 3T3 cells was only moderately higher on a molar basis than for recombinant human FGF, and Western blotting of the fusion protein after incubation with cells did not reveal a substantial amount of degradation that would result in liberation of substantial free FGF (Fig. 1C).

Immunoprecipitation of FGFR1 after binding to fusion protein, either with protein A, anti-human IgG, or anti-FGFR1, also demonstrates that the bound fusion protein is intact. Taken together, the results suggest that the functional activities identified are because of binding of intact fusion protein to cell surface FGFR1.

Other fusion proteins with members of the FGF family have been reported, including keratinocyte growth factor (FGF-7, KGF) with a mouse IgG1 (hinge, CH2, CH3) at the carboxyl terminus (33), recombinant FGF-2-saporin toxin (40), and fusions of FGF-1 with Pseudomonas exotoxin (41). The KGF-HFc fusion protein maintained both KGF and IgG Fc functions as evident from 1) induction of DNA synthesis in KGF-responsive cells, 2) specific binding to NIH 3T3 cells transfected with KGF receptor and not FGFR1 or FGFR2 shown by flow cytometry, 3) immunoprecipitation with anti-mouse IgG, and 4) immunohistochemical staining of KGF receptor in tissues. As seen with Fc-FG-1, the KGF-HFc protein spontaneously formed dimers, functional assays required somewhat higher concentrations of the fusion protein, and there was a very similar 10-fold difference in receptor affinity for KGF versus KGF-HFc (Kd 0.13 nM versus 1.4 nM). Based on these limited data with the FGF-7 fusion protein and Fc-FG-1 described here, the addition of IgG Fc domains to FGF family members appears able to preserve function and receptor binding despite the relatively large size of the added Fc region compared with the FGF. The reported toxin conjugates of both FGF-1 (41) and FGF-2 (40) are at the carboxyl termini of the respective FGFs and bind to high affinity FGFR. In the FGF-2-saporin fusion protein, proteolytic processing at the carboxyl terminus of FGF-2 appears to be required for liberating the toxin in the cytoplasm after receptor-mediated internalization to obtain maximal toxicity. Thus, determining whether amino-terminal or carboxyl-terminal fusions with FGF family members are most suitable for the desired goal may be largely empirical.

Fc-FGF may have a number of useful applications for studies of FGF function and cells expressing FGFR. Flow cytometry demonstrated binding with nontransfected Jurkat, expressing as few as 4,000 high affinity FGFR/cell; however, the shift from control is very modest and at the limit of detection. Jur/FGFR transfectants expressing approximately 25–35,000 high affinity FGFR1 are easily identified. Taken together, the results suggest that cells expressing 8,000-10,000 high affinity receptors should be readily identifiable by flow cytometry with Fc-FGF. Experiments in which varying percentages of Jur/FGFR were mixed with human peripheral blood mononuclear cells revealed that as few as 3% Jur/FGFR could be easily identified, whereas addition of Jur/FGFR as 1% of the total cell population was essentially indistinguishable from background (data not shown). This reagent could thus be useful for sorting mixed populations to obtain cells expressing either high or low levels of receptor. Because the frequency of FGFR\(^+\) T cells in human peripheral blood ranges from 1/2000 to 1/40,000 CD3\(^+\) T cells by functional assay (17), it is unlikely that flow cytometry with Fc-FGF will be useful in demonstrating these cells in unselected peripheral blood lymphocytes. Studies are in progress to determine whether the fusion protein can be used for immunohistology to identify FGFR\(^+\) cells in tissue sections that may be useful in studies of developmental biology, oncology, and angiogenesis.

A second potential application of Fc-FGF is in tracking the intracellular fate of FGF and its receptors. Preliminary studies using immunofluorescent localization show that the appearance of membrane-bound Fc-FGF differs when cells are exposed to the fusion protein at 37 °C versus 4 °C. Diffuse membrane staining, present after 4 °C incubation, becomes highly focal with incubation at 37 °C, suggesting that oligomerization of the receptors can be directly observed by immunofluorescence (data not shown). If intact fusion protein remains bound by receptors after internalization, this approach may allow visualization of the nuclear localization of FGF and its receptors, and investigation of the nuclear factors with which they associate by immunoprecipitation via the Fc domain.

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