Probing Fibroblast Growth Factor Dimerization and Role of Heparin-like Glycosaminoglycans in Modulating Dimerization and Signaling*

Received for publication, November 29, 2000, and in revised form, March 28, 2001
Published, JBC Papers in Press, April 5, 2001, DOI 10.1074/jbc.M010786200

Chi-Pong Kwan‡, Ganesh Venkataraman§, Zachary Shriver‡, Rahul Raman‡, Dongfang Liu‡, Yiwei Qi‡, Lyuba Varticovski‡, and Ram Sasishekaran§**

From the ‡Division of Bioengineering and Environmental Health, the §Harvard-Massachusetts Institute of Technology Division of Health Sciences and Technology, and the ¶Center for Biomedical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and the ¶¶Department of Medicine, St. Elizabeth’s Medical Center, Tufts University School of Medicine, Boston, Massachusetts 02135

For a number of growth factors and cytokines, ligand dimerization is believed to be central to the formation of an active signaling complex. In the case of fibroblast growth factor-2 (FGF2) signaling, heparin/heparan sulfate-like glycosaminoglycans (HLGAGs) are involved through interaction with both FGF2 and its receptors (FGFRs) in assembling a tertiary complex and modulating FGF2 activity. Biochemical data have suggested different modes of HLAG-induced FGF2 dimerization involving specific protein-protein contacts. In addition, several recent x-ray crystallography studies of FGF-FGFR and FGF-FGFR-HLGAG complexes have revealed other modes of molecular assembly, with no FGF-FGF contacts. All these different biochemical and structural findings have clarified less and in fact raised more questions as to which mode of FGF2 dimerization, if any, is essential for signaling. In this study, we address the issue of FGF2 dimerization in signaling using a combination of biochemical, biophysical, and site-directed mutagenesis approaches. Our findings presented here provide direct evidence of FGF2 dimerization in mediating FGF2 signaling.

Fibroblast growth factors (FGFs)1 are involved in a wide range of physiological processes, including morphogenesis, as well as disease processes such as tumor angiogenesis (1–3). The FGF family consists of at least 20 members, including the well characterized acidic FGF (FGF1) and basic FGF (FGF2), both of which are potent mitogens of many cell types (4–5). FGF signaling is mediated primarily through high affinity interactions with cell-surface FGF receptors (FGFRs), transmembrane polypeptides composed of immunoglobulin-like and tyrosine kinase domains (6, 7). FGF binding to different isoforms of FGFR is believed to trigger receptor dimerization, followed by transphosphorylation of specific tyrosine residues (8). In turn, phosphorylated tyrosine residues activate other signaling proteins, leading to cell proliferation, migration, and survival.

For proper presentation to FGFR, FGF2 and other members of the FGF family interact with heparin/heparan sulfate-like glycosaminoglycans (HLGAGs). Consisting of a disaccharide repeat of glucosamine and uronic acid, HLGAGs are heterogeneous in length (10–100 disaccharide units) and chemical composition (including differential sulfation, acetylation, and epitopes such as different repeats and branching of the FGF family interact with HLGAGs, including FGF2 and its receptors (28), a mechanism for dimerization that may or may not extend to other members of the FGF family, viz. FGF2. More recently, several crystallography studies of FGF-FGFR and FGF-FGFR-HLGAG complexes, including FGF2-FGFR1 (29), FGF1-FGFR1 (30), FGF2-FGFR2 (30), and FGF1-FGFR2 (31), suggest the possibility of FGF2 dimerization extending interactions with one another as well as with other proteins in the extracellular matrix and on the cell surface as part of proteoglycans, HLGAGs modulate FGF2 activity by low affinity interactions with specific FGF2- and FGFR-binding sites (13–15), facilitating FGF2 binding to FGFR. HLGAGs promote FGF2-induced activation of FGFR through a number of mechanisms, including regulating the diffusion rate of FGF2 (16, 17) and possibly dictating the specificity of FGF2-FGFR binding through interactions with both FGF2 and FGFR (18–20).

Another hypothesis is that FGF2 binding to HLGAGs induces ligand oligomerization, which in turn induces dimerization and transphosphorylation of FGFR. Biochemical studies have demonstrated that HLGAGs facilitate FGF oligomerization (21–23); however, due to a lack of direct evidence, it is unclear whether this biochemical phenomenon is important for FGF2 signaling. Furthermore, different modes of FGF-FGF interactions have been observed in various studies, drawing into question what modes of FGF oligomerization, if any, are biologically relevant.

Examination of apo-FGF and FGF-HLGAG crystal structures has led to the proposal of preferential FGF2 self-association in a cis-mode, with substantial protein-protein interactions between the adjacent molecules (24); and biochemical studies (including chemical cross-linking, analytical ultracentrifugation, and mass spectrometry) support this model (22, 25, 26). However, NMR studies predict a different mode of FGF oligomerization, viz. a symmetrical FGF2 dimer with possible disulfide bond formation between two surface cysteines (27). Furthermore, the recently solved FGF1-decaysaccharide co-crystal points to an FGF trans-dimer involving no FGF-FGF contacts (28), a mechanism for dimerization that may or may not extend to other members of the FGF family, viz. FGF2. More recently, several crystallography studies of FGF-FGFR and FGF-FGFR-HLGAG complexes, including FGF2-FGFR1 (29), FGF1-FGFR1 (30), FGF2-FGFR2 (30), and FGF1-FGFR2 (31),
have revealed assemblages of two FGFs bound to two FGFRs, with no FGF-FGF contact in the complex. Thus, conflicting biochemical and biophysical evidence makes it unclear whether FGF oligomerization is important for signaling through FGF 

and, if so, which dimerization mode of FGF, involving either protein contact or no protein contact, mediates FGF signaling. This problem is compounded when one considers that the two recent crystal structures of the ternary complex involving FGF, FGF, and HLGAG (32, 33) reveal different stoichiometries for the complex with markedly divergent geometries.

In this study, we apply conformational studies and molecular engineering techniques to systematically explore proposed modes of FGF2 oligomerization and to evaluate the importance of FGF-FGF interactions in signaling. Together, our data suggest that an FGF2 dimer involving substantial noncovalent protein-protein contact is readily formed and mediates signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ampicillin, isopropyl-β-D-thiogalactopyranoside, 1,10-phenanthroline, sodium chlorate, and dithiothreitol were from Sigma. Human recombinant wild-type FGF and anti-FGF2 antibody were gifts from Scios Nova (Mountain View, CA). The pET14b expression vector variant and FGF2 were a generous gift from D. M. Ornitz (Washington University). Heparin sodium from porcine intestinal mucosa was from Kabaki Pharmacia (Franklin, OH). Ready-Gel (15% polyacrylamide gel) and Bradford assay, immunoblot assay, and silver staining kits were from Bio-Rad.

Site-directed Mutagenesis, Protein Expression, and Purification of the Cysteine Mutant—Site-directed mutagenesis was carried out through a two-step PCR procedure as described previously (34). PCR products were subcloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Inserts were subcloned into a variant of the pET14b expression vector through the Ndel/SpeI sites. To express recombinant protein, an overnight culture of BL21 cells was transferred to 500 ml of LB medium supplemented with ampicillin (400 mg/liter) and allowed to grow with shaking at 37 °C until cell density reached A600 = 0.5. Isopropyl-β-D-thiogalactopyranoside (1 mm) was added to induce protein expression for 2 h. Protein purification by nickel chromatography was performed as previously described (35, 36). Purity of the protein was assessed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, and concentration was determined by Bradford assay using recombinant wild-type FGF2 as a control.

Oxidative Cross-linking—Purified protein was buffer-exchanged into HEPES with 10-kDa molecular mass cutoff membranes (Millipore Corp., Bedford, MA). Oxidative cross-linking was performed by incubating 50 μg of protein (30 μM final concentration) with 750 μM Cu2+ phenanthroline (made from a 1:1 mixture of 25 mM CuSO4 and 130 mM phenanthroline) in a 100-μl reaction volume at room temperature for 15 min. Longer incubation times (up to 2 h) did not significantly increase the amount of oligomer formed. For heparin treatment, the protein was incubated with 3 μM heparin for 1 h prior to cross-linking. The protein/heparin ratio was 10:1, which was previously shown to be optimal for FGF2 dimer formation (25). Other reaction conditions are indicated in the legend to Fig. 3. The reaction was terminated with 0.1 M EDTA and 10 mM isoacetic acid. Cross-linked products were analyzed by electrophoresis on 15% nonreducing SDS-polyacrylamide gels, followed by silver staining.

Conformational Studies—Conformational studies were performed with the Insight II package (Molecular Simulations, Burlington, MA) on a Silicon Graphics workstation. The coordinates of the FGF2 dimer in the FGF2-FGF1 crystal structure (code 1CVS) and that of free FGF2 (code 4FGF) were obtained from the Protein Data Bank. The sequential dimer was constructed from code 4FGF by translating the coordinates along the 31-A axis. The linker used in the experiment contained a tripeptide with the sequence GAL. However, since the N and C termini of FGF2 in most of the crystal structures are disordered, the modelled linker included the tripeptide sequence and the disordered residues of FGF2. The modelled linker of the form Cterm-GAL-Nterm, where Cterm and Nterm are the disordered C and N termini of FGF2, respectively. By deleting residues from the disordered N terminus, linkers of different lengths could be obtained. The most optimal structure for each of the linkers was obtained as follows. Combinations of structures for the linker were generated from the C terminus of one of the FGF2 monomers to the N terminus of the other monomer in the receptor-bound and sequential dimer using the homology modeling of Insight II. A good starting structure from the randomly generated linker structures in each FGF2 dimer was subjected to energy minimization with the Newton-Raphson method until convergence. Potentials were assigned using the consistent valence force field. Inference force-field parameters among monomers did not lead to significant changes in the model of the cross-linked dimer; and therefore, it did not affect the interpretation of the results.

Construction of Dimeric FGF2 (dFGF2)—Based on the results from the conformational studies, the two DNA sequences of FGF2 were ligated and subcloned into an expression vector as outlined in Fig. 4A. Ndelsac1 sites were introduced by PCR 5‘ and 3‘ to the first sequence, whereas Sacs1/SpeI sites were introduced to the second. Both the first and second sequences encode FGF2 with the first nine N-terminal residues removed. To facilitate purification of dFGF2, a His, tag and a thrombin cleavage site were introduced by PCR 5‘ to the first sequence, and a T7 tag and another thrombin cleavage site were introduced similarly 3‘ to the second sequence. Upon subcloning of the PCR product of the first sequence into pCR2.1-TOPO (which carries an internal SpeI site), a SacI/SpeI double digest was performed to linearize the vector. The PCR product from the second sequence was subcloned similarly, and the insert was excised by a SacI/SpeI double digest. Ligation between the linearized vector and the insert from the second sequence yielded in a tandem of two tandem of FGF2 DNA sequences. DNA sequencing was performed to confirm the identities of the fused DNA sequences. Protein expression and purification were performed as described above, except that a T7 affinity column (Novagen, Madison, WI) was used as described by the manufacturer after nickel chromatography. Biochemical studies were performed to ensure that dFGF2 was folded properly. Immunoblot analysis using a monoclonal antibody raised against the native form of wild-type FGF2 showed that the eluants from the nickel and T7 affinity chromatographies were recognized by the antibody in a concentration-dependent fashion (data not shown).

**CD Spectroscopy**—dFGF2 was concentrated to 1 μM and buffer-exchanged into 10 mM sodium phosphate (pH 7.2). CD spectroscopy of dFGF2 was performed in a quartz cell with a 1-mm path length (Starrz, Atascadero, CA) at room temperature. Data were recorded in an average of 20 scans between 195 and 260 nm on an Aviv 62SD spectropolarimeter.

**Protein Mass Spectrometry**—MALDI mass spectrometry was completed by diluting a solution of FGF2, FGF1, and a heparinase-derived HLGAG decasaccharide (consisting of a trisulfated disaccharide repeat unit) in 10 mM sodium phosphate (pH 7.0). To 1 μl of this sample was added an equimolar amount of the purified dFGF2 construct. The sample was allowed to come to equilibrium for 30 min at 4 °C. 1 μl of the sample was then immediately spotted on the MALDI target with 1 μl of a saturated sinapinic acid solution in 50% acetonitrile. After drying, the sample was washed with water, dried under a stream of nitrogen, and subjected to mass spectral analysis. MALDI mass spectrum of dFGF2 were acquired in the linear mode with a PerSeptive Biosystems, Framingham, MA) fitted with a 337-nm nitrogen laser. Delayed extraction was used to increase resolution (25 kV, grid at 91%, guide wire at 0.25%, pulse delay of 350 ns, and low mass gate at 2000).

**SMC Proliferation Assay**—SMCs isolated from bovine aorta were maintained in propagation medium supplemented with 10% bovine calf serum, 2 mM L-glutamine, and antibiotics. The proliferation assay of SMCs, as measured by tritium incorporation, was performed as follows. Cells were split at 95% confluence and seeded onto 24-well plates at 1 ml/well. After 24 h, cells were serum-starved in medium supplemented with 0.1% bovine calf serum for another 24 h. An appropriate amount of growth factor was added to eight wells for each protein concentration tested. 75 mM sodium chloride was added to half of the wells for each condition. After 21 h, [3H]thymidine (1 μCi/ml) was applied to each well and incubated for 3 h. Cells were washed with phosphate-buffered saline, and 0.5 ml of 1 N NaOH was subsequently added. The contents of each well were transferred to scintillation vials filled with 5 ml of ScintiSafe Plus 50% scintillation fluid (Fisher, Fair Lawn, NJ). Total [3H]thymidine incorporation was measured by liquid scintillation counting.
Analysis of various binding sites on FGF2. The surface of an FGF2 molecule can be approximated as the faces of a parallelepiped. Of the six faces, two opposite faces represent the receptor-binding sites (pointing into and out of the plane of the paper), whereas the other four (denoted as oligomerizing and heparin binding) represent directions about which FGF can associate. Note that two of the three oligomerizing directions are aligned along the same plane. Translation of FGF2 molecules along these two directions forms the basis of FGF2 oligomerization.

Cells were seeded at a density of ~1–2 × 10^5/well onto 96-well plates coated with fibronectin-like polymer (Sigma). Appropriate amounts of growth factor were added to the wells using a multichannel pipette. Each experimental condition was tested in six different cells. Cell viability was assessed after 18 h using a cell titer 96 proliferation assay (Promega) by measuring absorbance at 490 nm.

An angiogenic assay in the rat cornea—Pellets containing superlattice with FGF2 or superlattice alone were prepared as described by Kenyon et al. (37). Briefly, suspensions of sterile FGF2 solution containing appropriate amounts of FGF2 (5 and 20 μg) and dFGF2 (5 μg) were prepared and speed-vacuumed for 5 min. 10 μl of 12% Hydron in ethanol was added, and the suspension was deposited onto an autoclave-sterilized nylon mesh. The mesh was stacked between two layers of fiber covered with a thin film of Hydron. After drying on a sterile Petri dish for 30 min, the fibers of the mesh were pulled apart. With the aid of a dissecting microscope, uniformly sized pellets were selected from ~200 pellets produced. Each pellet contained ~1.5 and 6 pmol of FGF2 or 0.7 pmol of dFGF2. Control pellets containing no FGF2 were also prepared.

For pellet implantation, male Harlan Sprague-Dawley rats (400–450 g; n = 5) were anesthetized with ketamine (80 mg/kg) or xylazine (10 mg/kg). Using an operating microscope, an intrastromal linear keratotomy was performed with a surgical blade (Bard-Parker No. 15, Becton Dickinson Labware, Franklin Lakes, NJ) parallel to and 2 mm away from the limbus. A lamellar micropocket was dissected toward the cornea. A lamellar micropocket was dissected toward the cornea. A lamellar micropocket was dissected toward the cornea.

RESULTS

Framework for This Study—The three-dimensional structure of FGF2 has been thoroughly elucidated by a variety of biophysical techniques, including solution NMR and crystallography (13, 27–33, 38–40). All have pointed to roughly the same basic structure for FGF2, whether free, bound to its HLGAG ligand, or complexed with the receptor. An analysis of all of these structures suggests that three orthogonal surfaces exist on FGF2 (Fig. 1). As indicated in Fig. 1, the first surface has been implicated in binding of FGF2 to its high affinity protein receptor. Through rigorous biochemical and site-directed mutagenesis studies, a second orthogonal surface has been implicated in HLGAG binding. The third surface, orthogonal to both of the first two, has been implicated in FGF2 oligomerization.

Biochemical and structural studies have suggested different modes of FGF2 oligomerization within the third surface both in the presence and absence of HLGAGs (24, 27, 28). As schematically represented in Fig. 2, three modes of HLGAG-induced FGF2 dimerization have been proposed. Specific protein-protein contacts are involved in both the sequential and symmetrical FGF2 dimers (Fig. 2A and C, respectively), but not in the HLGAG-bridged or sandwich dimer (Fig. 2B). It was earlier demonstrated that FGF2 was capable of dimerization and oligomerization in the absence of heparin using an amine-specific chemical cross-linker with an 11-Å spacer (25). This observation is not consistent with the proposed HLGAG-bridged dimer in Fig. 2B since, in this FGF sandwich model, there are no residues on neighboring FGF2 molecules proximal to one another and thus available for covalent cross-linking with an 11-Å spacer (additional experiments described below are not consistent with this dimer mode). Therefore, we focused our initial experiments on determining whether either of the dimer models involving protein contacts (represented in Fig. 2, A and C) is an accurate representation of FGF2 dimerization mediated by HLGAGs.

Strategy to Investigate FGF Dimerization: Oxidative Cross-linking through Surface-exposed Cysteine Residues on FGF2—To establish the presence of proximal contacts between FGF2 molecules and to distinguish between different modes of FGF2 dimerization, we performed oxidative cross-linking experiments targeting the cysteine residues of FGF2 using copper phenanthroline, an oxidative agent used widely for disulfide bond formation (41). This approach is anticipated to probe for atomic distance interactions between the FGF molecules through the introduction of a disulfide bond between two FGF2 molecules. As discussed below, by taking advantage of the surface-exposed cysteine residues in FGF2 and through rationally engineering cysteine residues on the surface of FGF2, we systematically explored possible modes of FGF2 dimerization.

Oxidative Cross-linking of Wild-type FGF2—There are four cysteines in FGF2, two of which are surface-exposed (Cys69 and Cys87), and two of which are buried in the protein core (Cys35 and Cys87). The surface positions of the two exposed cysteines (Cys69 and Cys87) in wild-type FGF2 are related to each other by 90°. Taking advantage of the surface-exposed cysteine residues in the wild-type structure of FGF2, we performed oxidative cross-linking studies to test the proposed symmetrical mode of FGF2 dimerization in Fig. 2C, as this model predicts facile cross-linking between two FGF2 molecules (27). Under mild oxidative conditions, wild-type FGF2 showed very little oligomer formation in the presence and absence of heparin (Fig. 3A, lanes 1 and 2). (Several control experiments were performed to ensure authenticity of the data and are described below.) The absence of significant dimers or oligomers suggests either that the FGF-FGF interface does not involve molecular contacts or that the contacts are such that the two surface-
FGF2 Dimerization in FGF2-mediated Signaling

exposed cysteines are not at the dimer interface. Our observation is not consistent with the proposed symmetrical mode of FGF2 dimerization wherein dimerization is mediated by disulfide bond formation between Cys69 of each monomer (27).

Rational Design of the Cysteine Mutant—In a previous study (24), we had performed an extensive analysis of all FGF2 crystal structures available at that time and identified protein-protein interfaces (p-p' and q-q') that were conserved along the two unit-cell axes. Based on our analysis, we had proposed an FGF2 dimerization model in which FGF2 molecules were preferentially self-associated in a sequential fashion and HLGAG binding stabilized FGF2 dimers and oligomers that were subsequently presented to FGFR for signaling. In this model, noncovalent FGF-FGF interactions translated along the oligomerization direction (Fig. 2B) are expected to lead to FGF2 oligomerization. If this model indeed describes a mode of FGF oligomerization, then we would predict that, by substituting cysteine residues near the protein-protein interface between adjacent FGF2 molecules, intermolecular disulfide bonds could be created under mild oxidative conditions. The sequential dimer formed in this fashion would be stabilized by significant protein-protein contacts. As a first step toward testing this hypothesis, we searched for candidate pairs of residues in the p-p' interface that, when mutated to cysteine residues, could generate a disulfide linkage in a facile manner upon oxidative cross-linking. Through conformational studies, we found that optimal disulfide bond formation would be achieved when Arg81 and Ser100 were mutated into cysteines, as schematically represented in Fig. 3B. The two introduced cysteines are located on the opposite sides of FGF2 such that intramolecular disulfide bond formation would be disfavored. The two original cysteines, Cys69 and Cys87, were mutated to serines such that the total number of surface cysteines within the primary amino acid sequence of FGF2 remained the same. This protein, with four mutations (R81C/S100C/C69S/C87S), is hereafter referred to as the cysteine mutant. The cysteine mutant was constructed by site-directed mutagenesis as described under “Experimental Procedures.” The protein retained biological activity to stimulate cell proliferation compared with the wild-type protein (data not shown), suggesting that the introduced mutations did not grossly alter protein folding.

Oxidative Cross-linking of the Cysteine Mutant—Under exactly the same oxidative conditions as applied to the wild-type protein, the cysteine mutant yielded a markedly higher amount of oligomers. Notably, the extent of oligomerization was elevated by preincubating the protein with heparin (Fig. 3A, lanes 3 and 4). In addition, cross-linking of a FGF mutant that lacked one of these cysteines at the interface (i.e. either the R81C or S100C mutation) resulted in significantly less oligomer formation, further suggesting that the covalent dimer was formed through disulfide bond formation between the designed Cys81 and Cys100. Together, these observations strongly support the sequential mode of FGF2 dimerization and also suggest that the extent and stability of FGF2 oligomers are increased by binding to HLGAGs (24). Several controls were performed to ensure the authenticity of specific cysteine-mediated FGF2 oligomerization. Addition of a reducing agent such as dithiothreitol converted the observed dimers and oligomers into monomers (Fig. 3C, lane 4), indicating that the original cross-linking pattern was the result of disulfide-linked oligomers. Also, oligomerization was abolished when the cysteine mutant was denatured prior to cross-linking (Fig. 3C, lane 3), suggesting that oligomerization was mediated through the native structure of the protein and that the observed oligomers were not formed due to nonspecific protein aggregation. In addition, since two cysteines (Cys25 and Cys92) were buried in the protein core, they could potentially contribute to the observed oligomerization if the protein was unfolded during cross-linking. To exclude this possibility, the primary amino acid sequence of the cysteine mutant was further altered by substituting the two internal cysteines with serines i.e. additional Cys69/C92S mutations were introduced. The introduction of these two additional mutations did not change the cross-linking pattern (data not shown), further indicating that only the surface-exposed Cys81 and Cys100 contributed to disulfide-induced oligomerization. Taken together, these oxidative cross-linking studies support a model wherein FGF2 monomers form sequential dimers via a substantial protein-protein interface, and this interaction is further promoted by binding to HLGAGs. These results are consistent with other experimental
studies, including analytical ultracentrifugation of FGF2 with an octasaccharide, chemical cross-linking, and mass spectrometry of FGF2 with or without addition of exogenous HLGAGs (21, 22, 25, 26).

Attempts to purify the cross-linked dimers for further biochemical and biological characterizations were unsuccessful. Therefore, we adopted an alternative strategy of constructing an FGF2 dimer using a combination of conformational studies and genetic engineering tools, enabling us to investigate the biological importance of FGF2 dimers. This latter point is of special importance since the above biochemical studies indicate that, although a cis-FGF dimer does preferentially form in solution, it might form only under non-physiological conditions (i.e. high protein concentrations, heparin/protein ratios of 1:10, etc.). However, by constructing a defined FGF2 dimer and testing its biological activity, we can determine whether the oligomer mode indicated by the biochemical studies, viz. a cis-dimer involving substantial protein contact, is able to form an active signaling complex at the cell surface.

Engineering of a Tandemly Linked FGF2 Dimer through a Liner to Probe Contact and Non-contact FGF-FGFR Interactions: Design of dFGF2—Conformational studies of FGF-FGFR interactions led to the proposal that receptor clustering is facilitated by receptor binding to an FGF dimer (42). However, the recently solved structures of 2:2 FGF-FGFR complexes, which are proposed to be active signaling complexes, revealed no contact between the two FGF molecules (29–33).

To determine whether FGF-FGF interaction is important for FGFR binding and concomitant signaling, we “forced” FGF2 molecules into a cis-dimerization mode by engineering a dimeric FGF2 protein containing a tripeptide linker. By deleting residues from the N terminus of the protein, we could control the size of the linker between the two FGFs. Since there are at least 15 N-terminal residues that are disordered in all the FGF2 crystal structures, including the proposed active FGF2-FGFR crystal structures, we expect that these deletions would not significantly affect the folding of the protein. To find the optimal linker sequence length that would facilitate the distinction between the two modes of FGF-FGF interaction, we explored combinations of linker sequences with different lengths that could link the FGF2 monomers in both FGF-FGF interaction modes as described under “Experimental Procedures.” Our conformational studies showed that a linker with nine residues deleted from the N terminus would optimally link two FGF2 molecules in the sequential dimer, but would form a highly constrained structure when linking the two FGF2 molecules observed in the FGF2-FGFR1 crystal structure (data not shown). A dimeric protein (dFGF2) containing a tripeptide linker and two FGF2 molecules, linked C- to N-terminal, each with the nine N-terminal residues removed, was constructed (Fig. 4). This engineered FGF2 dimer is an ideal candidate to discriminate between a contacting FGF2 dimer and a non-contacting FGF2 dimer as observed in the FGF2-FGFR1 structure. The protein was expressed in Escherichia coli and purified as described under “Experimental Procedures.”

Prior to investigating the biological activity of dFGF2, we performed biochemical studies to ensure that dFGF2 was folded properly. First, as mentioned under “Experimental Procedures,” we assessed the overall folding of the protein by immunoblotting. The dFGF2 construct stained at approximately twice the level of wild-type FGF2. In addition, when the purified protein was heat-denatured in the presence of 1% SDS, the intensity upon immunoblotting was drastically reduced to the background levels (data not shown). The above results suggested that dFGF2 was properly folded with respect to the epitope recognized by this antibody. To assess the overall secondary structure, the banding position from near-UV CD spectroscopy of dFGF2 was analyzed. The CD spectrum showed a negative minimum near 200 nm (Fig. 5), which is characteristic of native FGF2 (25). In addition, dFGF2 bound to a heparin-POROS column and was eluted only at 1.8 mM NaCl (compared with 1.2 mM NaCl for FGF2; data not shown). Not only did this latter result suggest that dFGF2 was properly folded, it also suggested that dFGF2 had a higher affinity for HLGAGs than did FGF2, perhaps through a cooperative binding interaction between the two linked FGF units and the heparin column. If this is the case, then dFGF2 might have a reduced dependence on exogenous HLGAGs for activity. We therefore explored the functional attributes, including the effect of HLGAGs on dFGF2 activity, as described below.

**Stoichiometry of FGF2-FGFR-HLGAG Interactions—**Mass spectrometry was used to determine whether dFGF2 could compete with wild-type FGF2 for binding to FGFR2. MALDI mass spectrometric analysis of dFGF2 yielded a species consistent with the expected mass for dFGF2 of 37,066 Da (data not shown). As a next step, we investigated the nature of wild-type FGF2-FGFR interactions both in the presence and absence of HLGAGs. These experiments indicated that, in the absence of HLGAGs, wild-type FGF2 bound FGFR with a stoichiometry of 1:1 (Fig. 6A), consistent with FGF-FGFR crystal structures (29, 30). However, addition of an HLGAG decasaccharide (consisting of a trisulfated disaccharide repeat unit that is known to bind with high affinity to FGF2 and to support FGF2-mediated signaling) resulted in the formation of a detectable 2:2:1 FGF-FGFR-HLGAG complex (Fig. 6B). Addition of dFGF2 to this complex resulted in the formation of a new 1:2 complex of dFGF2-FGFR (Fig. 6B, inset). Notably, we could detect no dFGF2-FGFR species with decasaccharide bound. This species could be absent because the complex either does not form in solution or is not ionized and detected under the conditions of this experiment. In addition, since the ionization efficacies of the various species undoubtedly differ from one another, with the larger species (especially those containing the decasaccharide) being less amenable to ionization than the smaller species, quantitative estimates of the amount of complex formed in this case are not warranted. However, detection of a 1:2 dFGF2-FGFR complex indicates that this species does form at protein levels that approximate those present at the cell surface.

Together, these results indicate that 1) one molecule of dFGF2 having protein contact is able to support receptor dimerization; 2) one of the roles for HLGAGs in FGF binding to FGFR is to support FGF and/or FGFR oligomerization; and 3) biochemically, one mode of FGF oligomerization and receptor binding involves a dimer with substantial protein-protein contact. To determine whether the complexes observed via mass spectrometry have a biological role, we tested the ability of dFGF2 to signal both in vitro and in vivo.

**Biological Activity of dFGF2—**To test if FGF-FGF contacts are involved in signaling, dFGF2 was first assayed for its biological activity in vitro. Mitogenicity of dFGF2 was tested on SMCs treated with or without chlorate. Because chlorate treatment inhibits the biosynthesis of HLGAGs and thereby depletes cell-surface HLGAGs, the dependence of HLGAG binding on the activity of dFGF2 for signaling can be evaluated. With intact cell-surface HLGAGs (no chlorate treatment), both wild-type FGF2 and dFGF2 were active in mediating a proliferative response on SMCs (Fig. 7A). Importantly, the molar concentrations required to achieve half-maximal proliferation by wild-type FGF2 and dFGF2 were 270 and 60 pm, respectively. Hence, dFGF2 exhibited 4.5-fold more activity compared with wild-type FGF2 in promoting cell proliferation under
these in vitro conditions. In chlorate-treated SMCs, whereas wild-type FGF2 produced only a moderate response in proliferation, a marked increase in the proliferative response was exhibited by dFGF2, achieving ~80% of full proliferation in HLGAG-depleted cells (Fig. 7B). The results from the SMC proliferation assay suggest a higher potency in stimulating proliferation and a lower dependence on HLGAG for signaling by dFGF2.

In addition to SMCs, FGF2 is a potent angiogenic factor well known for its ability to induce cell survival in endothelial cells. Therefore, we determined the ability of dFGF2 to promote cell viability in HUVECs. Using the colorimetric dye, which reflects the mitochondrial integrity of viable cells, the HUVEC survival assay provides a sensitive way to measure endothelial cell viability mediated by the growth factors added. In serum-deprived HUVECs, cell viability was ~50% of that of HUVECs grown in 10% serum (Fig. 8). Addition of various concentrations of wild-type FGF2 and dFGF2 partially recovered cell viability in a dose-dependent manner. Again, dFGF2 was more active than wild-type FGF2 in stimulating survival in HUVECs on a molar basis, consistent with its elevated potency observed in SMCs. Taken together, the biological activity of dFGF2 from two independent cell types demonstrates that the dimeric construct binds to and activates FGFR to elicit various downstream signals as measured by the biological assays.

In Vivo Potency of dFGF2—To extend the above in vitro conditions...
findings, the ability of dFGF2 to induce angiogenesis in an experimental in vivo model was investigated. The activities of FGF2 and dFGF2 were compared, side by side, using the rat corneal pocket assay, the results of which are shown in Fig. 9. As anticipated, control pellets containing no FGF2 (i.e. no angiogenic stimuli) failed to induce appreciable angiogenesis (Fig. 9A). FGF2 induced an angiogenic response in a dose-dependent manner, with little angiogenesis induced at a protein level of 1.5 pmol (Fig. 9B) and more extensive angiogenesis induced at...
6 pmol (Fig. 9C). Thus, the extent of angiogenesis induced by FGF2 is accurately reflected both by the length of induced vessels and the circumference of those vessels. Compared with FGF2, dFGF2 induced more extensive angiogenesis in the corneas of rats at a lower concentration of 0.7 pmol (Fig. 9D). With dFGF2, induced blood vessels were longer, of larger circumference, and more plentiful, as measured by “clock hours” or the extent of angiogenesis around the limbus. In fact, 0.7 pmol of dFGF2 was a better angiogenic stimulus than FGF2 at an extent of angiogenesis around the limbus. In fact, 0.7 pmol of dFGF2, as measured in in vitro cell culture experiments, was retained in an in vivo animal model, suggesting that the dFGF2 construct is a potent biological mediator.

**DISCUSSION**

**FGF-FGF Dimer Involves Protein Contact**—Several signaling pathways mediated by growth factors and cytokines involve binding of dimeric or oligomeric ligands to their cell-surface receptors to facilitate receptor dimerization (43), a key step leading toward activation of intracellular signaling cascade. For the FGF family of growth factors, many biochemical studies have pointed to different modes of dimerization and oligomerization in the presence and absence of HLGAGs that are essential for signaling. In this study, we examined three possible modes of HLGAG-mediated FGF2 association (Fig. 2, A–C) by evaluating earlier and current biochemical findings. The observation that FGF2 can oligomerize in the absence of HLGAG (25) is inconsistent with the non-contacting HLGAG-bridged dimerization mode (Fig. 1B). Our results from the oxidative cross-linking studies of wild-type FGF2 do not support the proposed symmetrical dimerization, which is potentially mediated by intermolecular disulfide bonds (27). Through rational design of a disulfide-mediated sequential dimer (cysteine mutant) based on earlier extensive analysis of FGF2 crystal structures (24), we demonstrated (a) a marked increase in the amount of oligomers formed compared with wild-type FGF2, which has the same number of surface cysteines, but at different positions; (b) a higher extent of oligomerization by preincubating this cysteine mutant with heparin; and (c) that the observed oligomers involve specific protein contacts and are disulfide-mediated. The above findings strongly support a model in which FGF2 molecules self-associate through specific FGF-FGF interactions in a sequential fashion and that HLGAG serves to provide a “platform” to stabilize the intermolecular interactions between FGF2 molecules (24).

**dFGF Is Biologically Active**—To test our hypothesis of an active FGF2 dimer involving protein-protein contact and to distinguish it from the FGF2 dimer observed in the FGF-FGFR co-crystal structures that lack protein-protein contact, we constructed a tandemly linked dFGF2 molecule using conformational studies and genetic engineering tools. dFGF2 was designed such that the short distance between the two FGF2 molecules within the dimeric protein would allow for substantial FGF-FGF interactions while making the non-contacting dimer mode less favorable and therefore enable us to determine whether a contacting FGF2 dimer can elicit biological activity. Though mass spectrometry, we show that dFGF2 interacts with FGF2 at a ratio of 1:2, suggesting that dFGF2 can bind to a dimer of FGF2. Furthermore, these results indicate that one mode of dimerizing is a subunit protein contact. However, additional FGF2 and its receptor can interact is through the binding of FGR to an FGF2 dimer. These biochemical findings are supported by the biological activity of the dFGF2 molecule.

To test whether a contacting FGF2 dimer can elicit biological activity, dFGF2 was subjected to two independent in vitro assays. As determined by both the SMC and HUVEC survival assays, dFGF2 exhibited elevated biological activity compared with wild-type FGF2. This effect was especially pronounced in the SMC assays, in which dFGF2 was severalfold more active than wild-type FGF2 and only 30% less active in the absence of HLGAGs as in their presence (as opposed to wild-type FGF2, whose activity was significantly reduced in the absence of cell-surface HLGAGs). These findings suggest that dFGF2, in which FGF-FGF interactions are predicted to be substantial, forms an active signaling complex with the receptor. In addition, proliferation of chlorate-treated SMCs demonstrated that dFGF2 was less HLGAG-dependent for signaling. This observation can be rationalized if one considers that one primary mechanism by which HLGAGs mediate FGF2 activity is by stabilizing two FGF2 molecules in a dimeric mode to facilitate receptor dimerization. Because dFGF2 is already dimeric, its dependence on HLGAGs for proper presentation to the receptor is lower compared with wild-type FGF2. We have also extended these studies to show that the dFGF2 construct is a potent pro-angiogenic agent in vivo, much more so than wild-type FGF, thus providing compelling evidence that the dFGF2 construct, involving substantial protein-protein contact, forms an active signaling complex at the cell surface.

Thus, the biochemical, cell culture, and in vivo assays are consistent with the proposal that a contacting FGF2 dimer is involved in the active signaling complex. These findings appear to be inconsistent with the different FGF2-FGFR crystal structures, which show no FGF-FGF interactions. Such an inconsistency may reflect the inherent complexity and multifaceted nature of the FGF system. One possible explanation is that the different structural configurations of FGF-FGFR may reflect the different states, viz. “on” or “off” states, of the signaling complex. A similar observation has been made in other systems (44, 45). For instance, in the case of erythropoietin, it has been noted that certain mimetic peptides can dimerize the receptor, but fail to induce signaling (46) due to the formation of an inactive complex at the cell surface. Furthermore, the ultimate biological end point of erythropoietin signaling, i.e., whether erythropoietin signaling results in proliferation or differentiation, is sensitive to how the receptors are brought together (44).

Another system in which the mode of dimerization plays a critical role in determining activity is tumor necrosis factor binding to tumor necrosis factor receptor-1. Unliganded tumor necrosis factor receptor-1 exists as an inactive dimer, with its catalytic domains over 100 Å apart (47). Binding of tumor necrosis factor to its receptor brings the catalytic domains of the receptors proximal to one another, initiating intracellular signaling cascades (48). Thus, similar to these cases, it is conceivable that a mode of FGF2 dimerization involving protein-protein interactions could lead to a cooperative FGF2-FGFR interaction by promoting subsequent oligomerization and signaling, whereas non-contacting FGF2 dimerization may lead to an inactive complex.

In addition, it must be noted that other studies have suggested that monomeric forms of FGF2 may form active signaling complexes (49, 50). For instance, in a recent study, it was found that covalently linked complexes of monomeric FGF with a pool of heparin dodecasaccharides were able to promote cell proliferation in vitro (50). However, as observed in that study, the covalent FGF-HLGAG complex was less active than uncomplexed FGF in promoting [3H]thymidine incorporation. In contrast, the dFGF2 construct presented in this study is several times more potent in biological assays than wild-type FGF, with reduced dependence on exogenous HLGAGs for activity. Nevertheless, it is possible that monomeric complexes of FGF do signal, albeit with less apparent activity compared with oligomeric forms of FGF2.
In summary, we report here that FGF2 does have a preference to oligomerize, and the studies contained herein point to the fact that this oligomerization interface involves protein-protein contact. In addition, a DFG2 construct based on these biochemical findings has potent biological activity, consistent with the hypothesis that FGF oligomers are potent mediators of FGFR dimerization and concomitant signaling.

Acknowledgments—We thank David Berry and Cindy Ku for technical assistance and Isabel de Aos Scherpenseel for advice. We would also like to thank Scios Nova and Dr. David Omitz for making available reagents used in this study.

REFERENCES
1. Ornitz, D. M. (2000) Bioessays 22, 108–112
2. Taipale, J., and Keski-Oja, J. (1997) FASEB J. 11, 51–59
3. Hanahan, D., and Folkman, J. (1996) Cell 86, 353–364
4. Gospodarowicz, D., Ferrara, N., Schweigerer, L., and Neufeld, G. (1987) En- do. Rev. 8, 95–114
5. Nugent, M. A., and Iozzo, R. V. (2000) Int. J. Biochem. Cell Biol. 32, 115–120
6. Jaye, M., Schlessinger, J., and Dionne, C. A. (1992) Biochim. Biophys. Acta 1135, 185–199
7. Wilkie, A. O., Morriss-Kay, G. M., Jones, E. Y., and Heath, J. K. (1995) Curr. Biol. 5, 500–507
8. Schlessinger, J., Lax, I., and Lemmon, M. (1996) Cell 83, 357–360
9. Gallagher, J. T. (1997) Biochem. Soc. Trans. 25, 1206–1209
10. Salmivirta, M., Lidholt, K., and Lindahl, U. (1996) FASEB J. 10, 1270–1279
11. Turnbull, J., Powell, A., and Guimond, S. (2001) Trends Cell Biol. 11, 75–82
12. Sasisekharan, R., and Venkataraman, G. (2000) Curr. Opin. Chem. Biol. 4, 626–631
13. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1999) J. Biol. Chem. 274, 1116–1120
14. Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Svhahn, C. M., and Waksman, G. (1995) Science 268, 432–436
15. Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeeman, W. L. (1993) Science 259, 16382–16389
16. Dowd, C. J., Cooney, C. L., and Nugent, M. A. (1999) J. Biol. Chem. 274, 5236–5244
17. Flavemetha, R., Moscatelli, D., and Rifkin, D. B. (1990) J. Cell Biol. 111, 1651–1659
18. Guimond, S. E., and Turner, J. E. (1999) Curr. Biol. 9, 1343–1346
19. Kan, M., Wu, X., Wang, F., and McKeeman, W. L. (1999) J. Biol. Chem. 274, 15947–15954
20. Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P., and Gallagher, J. T. (1998) J. Biol. Chem. 273, 22936–22942
21. Ornitz, D. M., Yaoan, A., Flanagan, J. G., Svhahn, C. M., Levi, E., and Leder, P. (1992) Mol. Cell. Biol. 12, 240–247
22. Herr, A. B., Ornitz, D. M., Sasisekharan, R., Venkataraman, G., and Waksman, G. (1997) J. Biol. Chem. 272, 16382–16389
23. Spivak-Krutman, T., Lennmon, M. A., Dikir, I., Ladbury, J. E., Pincasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., and Lax, I. (1994) Cell 79, 1015–1024