Inactivation of Human Fibroblast Growth Factor-1 (FGF-1) Activity by Interaction with Copper Ions Involves FGF-1 Dimer Formation Induced by Copper-catalyzed Oxidation

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Although the angiogenic proteins acidic fibroblast growth factor (FGF-1) and basic fibroblast growth factor (FGF-2) both interact with the transition metal copper, itself a putative modulator of angiogenesis, a role for copper in FGF function has not been established. Using nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we detect the complete conversion of recombinant forms of human FGF-1 monomer protein to FGF-1 homodimers after exposure to copper ions. In contrast, not all forms of bovine FGF-1 isolated from bovine brain or a recombinant preparation of human FGF-2 completely formed homodimers after exposure to copper ions under similar conditions. Since the copper-induced FGF-1 homodimers reverted to the monomer form in the presence of dithiothreitol, specific alklylation of cysteine residues by pyridylethylation prevented FGF-1 homodimer formation, and preformed FGF-1 homodimers could not be dissociated by the metal chelator EDTA. FGF-1 dimer formation appeared to result from the formation of intermolecular disulfide bonds by copper-induced oxidation of sulfhydryl residues. FGF-1 homodimers bound with similar apparent affinity as FGF-1 monomers to immobilized copper ions, both eluting at 60 mM imidazole. Both human FGF-1 monomer and dimer forms had a 6-fold higher apparent affinity for immobilized copper ions, as compared with human FGF-2, which eluted in the monomer form at 10 mM imidazole. Further, in contrast to FGF-1 monomers, which dissociate from immobilized heparin in 1.0 M NaCl, preformed FGF-1 homodimers had reduced apparent affinity for immobilized heparin and eluted at 0.4 M NaCl. In contrast, the apparent affinity of human FGF-2 for immobilized heparin was unaffected after exposure to copper ions. Heparin appeared to modulate the formation of copper-induced intermolecular disulfide bonds for FGF-1 but not FGF-2, since co-incubation of heparin and copper with FGF-1 monomers resulted in dimers and other oligomeric complexes. FGF-1 copper-induced homodimers failed to induce mitogenesis in [3H]thymidine incorporation assays, an effect which could be reversed by treatment with dithioreitol, whereas FGF-2-induced mitogenic activity was relatively unaffected by pretreatment with copper.

The differences between human FGF-1 and FGF-2 in protein-copper interactions may be due to differing free thiol content and arrangement between the two proteins. A recombinant human FGF-1 mutant containing the two cysteines conserved throughout the FGF family of proteins but lacking a cysteine residue (Cys 131) present in wild-type human FGF-1 but not human FGF-2 readily formed copper-induced dimers. This suggests FGF-1-copper interactions may involve the cysteine residues conserved among all the members of the FGF family. Despite the differences in copper interactions between FGF-1 and FGF-2, copper-induced heterodimers between these two proteins were demonstrated, indicating FGF-1 and FGF-2 can form mixed thiols with each other. The difference in copper interactions between different forms of FGF-1 and FGF-2 are important in further attempts to determine any putative role for copper in FGF action.

Angiogenesis, the formation of new blood vessels, is regulated by a variety of factors influencing the migration, proliferation, and differentiation of the endothelial cell (1). One class of angiogenic factors includes the polypeptides fibroblast growth factor (FGF)1 and -2, commonly known as acidic and basic fibroblast growth factor, respectively. Additional sequences that are related to FGF-1 and FGF-2 form a family of FGFs presently containing seven members (2-4). Structurally, the FGFs share between 30 and 50% sequence similarity at the amino acid level, and each contains 2 conserved cysteine residues within their structures.

It has been demonstrated that native forms of FGF-1 and FGF-2 can interact with the transition metal copper, as each has been purified by heparin-copper biaffinity chromatography (5, 6). In addition, a human mammary tumor-derived growth factor with properties similar to FGFs has also been isolated by its affinity for copper (7). Interestingly, copper is a potent modulator of angiogenesis in vivo (1, 8), and the interaction between FGFs and copper may offer insight into the role of copper as a modulator of angiogenesis (5, 6). Indeed, levels of copper increase in response to an angiogenic stimulus prior to neovascularization in vivo in normocupremic animals (9), and the same angiogenic response is prevented if the copper levels are decreased by pretreatment with the copper chelator desferrioxamine. The differences between human FGF-1 and FGF-2 in protein-copper interactions may be due to differing free thiol content and arrangement between the two proteins. A recombinant human FGF-1 mutant containing the two cysteines conserved throughout the FGF family of proteins but lacking a cysteine residue (Cys 131) present in wild-type human FGF-1 but not human FGF-2 readily formed copper-induced dimers. This suggests FGF-1-copper interactions may involve the cysteine residues conserved among all the members of the FGF family. Despite the differences in copper interactions between FGF-1 and FGF-2, copper-induced heterodimers between these two proteins were demonstrated, indicating FGF-1 and FGF-2 can form mixed thiols with each other. The difference in copper interactions between different forms of FGF-1 and FGF-2 are important in further attempts to determine any putative role for copper in FGF action.

1 The abbreviations used are: FGF, fibroblast growth factor; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid; DTT, dithiothreitol; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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animal is copper-deficient (9). Hypocupreanmic animals also are unable to mount an intense neovascular response to implants of brain tumors (10). Copper may regulate vascular growth by affecting endothelial cell adhesion and migration, since copper ions enhance endothelial cell locomotion in vitro (11), and may alter the composition of the extracellular matrix, since copper treatment increases fibronectin synthesis in vitro (12) and acts as a cofactor for proteins involved in extracellular matrix formation (13).

Copper also confers an angiogenic capability to other macromolecules. The copper-binding protein molecule ceruloplasmin is an acute phase protein whose concentration increases during inflammation, infection, and tissue injury (14). Ceruloplasmin is angiogenic in vivo but only when complexed to copper (15, 16). Likewise, heparin, itself a potentiator of angiogenesis in vivo (1), is a copper chelator (17, 18) and can initiate an angiogenic response in the presence of copper (15, 16). Further, the plasma tripeptide, Gly-His-Lys, which may be liberated from collagen (19), is also angiogenic in vivo when complexed to copper (16, 20, 21). Finally, the association of angiogenin, an angiogenic polypeptide with ribonuclease activity (22), with plasma membrane components may be modulated by copper (23).

The mechanism utilized by copper to mediate angiogenic phenomena remains unclear but may involve interactions with angiogenic molecules such as FGFs. Oxidative damage to polypeptides catalyzed by transition metals such as copper has been postulated as an underlying process in several biological systems (24, 25). Functions of protein-bound metal ions also include the regulation of catalysis, the stabilization of folded conformations and macromolecular complexes, and the modulation of gene expression by acting through regulatory proteins (26). In this report, we describe the formation of stable but biologically inactive FGF-1 homodimers induced by copper through the formation of apparent intermolecular disulfide bonds and characterize differences in copper interactions between different forms of FGF-1 and FGF-2.

**EXPERIMENTAL PROCEDURES**

Construction, Production, and Purification of FGF-1 Proteins—The constructs for FGF-1 and FGF-2 (1-154) were prepared, cloned into expression vectors pET3c (FGF-1) or pKK233-2 (FGF-1; 1-154) and used to transform BL21 (DE3) Lys-S (FGF-1) or DH5α (FGF-1-(21-154)) cells as described (27). The amino acid sequence of the full-length FGF-1 construct corresponds to amino acids 1-164 of the human FGF-1 sequence (28). The amino acid sequence encoded in the FGF-1 (21-154) construct corresponds to FGF-1 residues 21-154, representing a human analogue of bovine FGF-1 (21-154) (29) with the addition of a methionine and an alanine for glycine substitution (corresponding to sense nucleotides). The second PCR used primers AGTCCGAG- CAGAAGA (corresponding to antisense nucleotides; NdeI site) and TACGAACAGATCTCTTTAAT (corresponding to sense nucleotides) to add unique sites for the restriction enzymes NdeI and EglII to the ends of the DNA sequence encoding the mutagenized FGF-1. This material was ligated into a HindIII/EaI fragment of pET3c containing 1 ng of each primer, 1 ng of the pUC18 vector containing AGCAATC (corresponding to sense nucleotides). Both reactions were performed, cloned into expression vector pET3c (FGF-1) or pKK233-2 (FGF-1-(21-154)), and used to transform BL21 (DE3) Lys-S cells and lysates from four selected colonies were analyzed by immunoblotting with anti-FGF-1 antibodies. Amino acid sequencing of the purified protein confirmed the human FGF-1 sequence through the first 15 amino-terminal residues.

E. coli transformed with the appropriate expression plasmids were grown in Luria Broth containing 100 μg/ml ampicillin and chloramphenicol in 0.5-liter cultures, as detected by immunoblotting with anti-FGF-1 antibodies. The purified PCR products were combined, and the reaction products were transformed into DH5α cells. Digestion of the FGF-1/pET3c DNA with EcoRI and HindII revealed the presence of insert in 10 of 10 selected colonies. DNA sequencing of the insert of the pET3c construct was confirmed by the dideoxy method with Sequenase (U. S. Biochemical Corp.) following the manufacturer’s instructions and confirmed the presence of the desired mutation. The FGF-1 C131S/pET3c construct was transformed into BL21 (DE3) Lys-S cells and lysates from four selected colonies were analyzed by immunoblotting with anti-FGF-1 antibodies. Amino acid sequencing of the purified protein confirmed the human FGF-1 sequence through the first 15 amino-terminal residues.

The recombinant human FGF-2 was supplied by Synergen and corresponds to human placental FGF-2 (30, 31). A recombinant human FGF-1 construct in which the cystine residue at position 131 (FGF-1; 1-154) was replaced by serine was made using recombinant circle PCR methods (32, 33). With oligonucleotides synthesized on an Applied Biosystem 380B DNA synthesizer by the phosphoramidite method and purified by ethanol precipitation, two separate reactions were set up for the mutagenesis of nucleotides encoding Cys-131 in the human FGF-1 insert in pUC18 (27). One PCR used the primers CTCAAGGAAGATTCGCACACCGTG (corresponding to sense nucleotides; mutation underlined) and GCCCAAAAACATTTCTCTGACT (corresponding to antisense nucleotides). The second PCR used primers AGTCCGAG-GACCGCTTGGATGCCTGAAATCC (corresponding to sense nucleotides; mutation underlined) and CACTATGGCCGAAAGCAATC (corresponding to sense nucleotides). Both reactions contained 1 ng of each primer, 1 ng of the pUC18 vector containing the synthesized cDNA encoding FGF-1 (27), and 4.2 units of Taq polymerase (Promega Biotechnology). Reverse transcriptase (termed reverse transcriptase) was amplified as described (33) in 100 μl for 30 cycles using a Perkin-Elmer thermal cycler. Each PCR product was phenol/chloroform-extracted, ethanol-precipitated, run on a 0.8% (w/v) agarose gel, and stained with ethidium bromide. The appropriate sized bands were cut out, electroblotted from the gel piece, and purified using an Elu-Tip column (Schleicher & Schuell). The purified PCR products were combined, denatured, and reannealed as described (33) before transformation into E. coli DH5α. Each of the PCR products was digested with EcoRI and HindIII, and the resulting DNA was phenol/chloroform-extracted, concentrated, ethylenediamine in 1% (w/v) agarose, and purified from the gel. The insert was subcloned into compatible Ndel/BamHI sites of the plasmid pET3c, which had been purified by a Qiagen column (Qiagen, Inc.), gel band-purified, digested with Ndel and BamHI, phosphorylated with calfintestinal phosphatase, and again gel-purified before ligation. Subcloning was performed with T7 ligase (GIBCO-BRL Life Technologies, Inc.), and the reaction products were transformed into DH5α cells. Digestion of the FGF-1/pET3c DNA with BamHI and EcoRI revealed the presence of insert in 10 of 10 selected colonies. DNA sequencing of the insert of the pET3c construct was confirmed by the dideoxy method with Sequenase (U. S. Biochemical Corp.) following the manufacturer’s instructions and confirmed the presence of the desired mutation. The FGF-1 C131S/pET3c construct was transformed into BL21 (DE3) Lys-S cells and lysates from four selected colonies were analyzed by immunoblotting with anti-FGF-1 antibodies. Amino acid sequencing of the purified protein confirmed the human FGF-1 sequence through the first 15 amino-terminal residues.

Copper-induced FGF Homodimers—Tissue homogenates were prepared from males of the inbred Sprague-Dawley strain as described (33) before transformation into E. coli BL21 (DE3) Lys-S cells and lysates from four selected colonies were analyzed by immunoblotting with anti-FGF-1 antibodies. Amino acid sequencing of the purified protein confirmed the human FGF-1 sequence through the first 15 amino-terminal residues.

**Treatment of FGFs with Copper**—Five micrograms of HPLC-purified FGF-1 or FGF-2 in a final volume of 50 μl (6 μM) were incubated for 30 min at 25°C in 20 mM Tris-HCl, pH 7.4 containing 0.5 μM copper(II) or 10 mM sodium phosphate, pH 7.4, containing 0.5 mM EDTA. The reaction was stopped by the addition of 10 μl of 1 M Tris-HCl, pH 7.4, containing 0.5 mM EDTA. In some experiments, heparin (Sigma) was added at the indicated concentrations and incubated for 30 min at room temperature prior to CuCl2 addition. In experiments involving [3H]FGF-1, recombinant
human FGF-1-(21-154) was radiolabeled as described (36), except 100 μg/ml of heparin was used in place of bovine serum albumin as a carrier and added to the dimer formation reactions as indicated. SDS-PAGE (15% (w/v) polyacrylamide) was performed with the buffer system of Laemmli (37). Acrylamide and SDS were purchased from Bio-Rad Laboratories, and all other reagents were purchased from Sigma Chemical Company. EDTA was added to the indicated concentrations and the samples were incubated on ice for 2 h with occasional agitation. The mixture was spun at 10,000 g for 10 s to pellet bound protein. The supernatant containing free protein was removed and mixed with nonreducing SDS-PAGE sample buffer containing 10 mM EDTA for electrophoretic analysis.

Preformed FGF-1 homodimers were purified by reverse-phase HPLC for use in mitogenic assays. HPLC-purified recombinant human FGF-1 was exposed to 10 mM CuCl2 and then dialyzed against 0.1% trifluoroacetic acid (v/v) in water before being loaded onto a Vydac 5C18 column. The column was developed with an acetonitrile gradient as described above for FGF-1 monomer protein. FGF-1 dimer forms eluted at 37% (v/v) acetonitrile and were fully resolved from FGF-1 monomer protein, which eluted at 35% (v/v) acetonitrile. The protein concentration of the HPLC-purified FGF-1 homodimers was determined by amino acid analysis.

Batch Adsorption to Heparin Sepharose and Chelating Sepharose—Chelating Sepharose (Pharmacia LKB Biotechnology, Inc.) was saturated with CuSO4 as described in the manufacturer’s instructions and washed extensively. FGF-1 or FGF-2 monomers or preformed FGF-1 dimers produced in reactions as described above were added to 50 μl of a 50% (v/v) slurry of charged copper-Sepharose in 20 mM HEPES, 2 M NaCl, pH 7.4, in a final volume of 100 μl. Imidazole or EDTA was added to the indicated concentrations and the samples were incubated on ice for 2 h with occasional agitation. The mixture was spun at 10,000 x g for 10 s, and the supernatant with unbound protein was removed and added to nonreducing SDS-PAGE sample buffer containing 10 mM EDTA for electrophoretic analysis.

Modification of Cysteine Residues by Pyridylethylamine—HPLC-purified FGF-1 (6 mM) was incubated under nitrogen in 6 mM guanidine HCl, 0.1 M Tris-HCl, pH 8.5, with or without 5.5 mM DTT. after 10 s to pellet bound protein. The supernatant containing free protein was removed and mixed with nonreducing SDS-PAGE sample buffer containing 10 mM EDTA for electrophoresis.

RESULTS

Copper-induced Formation of Stable FGF Dimers—The purified recombinant form of human FGF-1 migrated at its corresponding molecular weight (M, 17,000) on SDS-PAGE under nonreducing conditions (Fig. 1A, lane 1). However, pre-exposure of FGF-1 to increasing concentrations of CuCl2 resulted in an increase in apparent M, with the recovery of a series of bands migrating between 34 and 38 kDa (Fig. 1A, lanes 2–7). Copper(II) exposure resulted in virtually complete recovery of the proteins as dimers. Maximal dimer formation occurred in the presence of 10 mM Cu(II). Additional complexes of higher Mr in the separating or stacking gel were not observed. In some experiments, a small amount of dimer formation occurred in the absence of copper ions (e.g. Fig. 4A, lane 1). However, such dimers exhibited a slightly different mobility on SDS-PAGE than the copper-induced dimers (e.g. Fig. 4A, lane 2). No difference in dimer formation was observed for FGF-1 protein purified from reversed-phase or Mono-S HPLC columns (data not shown). Copper(II) chloride and CuSO4 were both effective in causing FGF-1 dimer formation. However, copper(I) chloride was less effective on a molar basis, and a shift in apparent Mr, was not observed for FGF-1 with other metals tested under similar conditions, including CaCl2, FeCl3, FeSO4, CoCl2, NiCl2, CdCl2, ZnCl2, HAuCl4, and HgCl2 (data not shown). Preformed FGF-1 dimers were stable in 0.1% (w/v) SDS containing 10 mM EDTA and were resistant to dissociation by heat (100 °C) and acidic conditions (pH 4.0) (data not shown). However, the dimers reverted to the monomer form in the presence of reducing agent (Fig. 1A, lane 8). FGF-1 dimer formation was also observed after the addition of the sulfhydryl oxidizing reagent diamine dicarboxylic acid bis(N,N-dimethylamide) (diamide) (data not shown). Dimerization was prevented by the co-incubation of EDTA with CuCl2 in the reaction, and dimers were not formed in the presence of the oxidant H2O2 alone or together with CuCl2 (data not shown). Some of the less intense bands that appeared between 34 and 38 kDa might be due to oxidation by ammonium persulfate, since the heterogeneity of the dimer bands was reduced when samples were analyzed on pre-run gels or in gels where ammonium persulfate was replaced with riboflavin monophosphate as the cross-linking agent (data not shown).

Although a preparation of purified recombinant human FGF-2 (M, 18,000) migrated at its corresponding molecular weight on nonreducing SDS-PAGE (Fig. 1C, lane 1), pre-exposure to 10 mM CuCl2 did not fully induce homodimer formation, although some dimerization did occur (Fig. 1C, lane 2). Increased FGF-2 homodimer formation was not observed after pretreatment with an extended concentration range of 0.001–100 mM CuCl2 (data not shown).

Prevention of Dimer Formation by S-Pyridylethylamine—Since FGF-1 dimers were stable in SDS and to boiling and acidification, but not to reducing agent, and since both copper and diamide are sulfhydryl oxidizing reagents, dimer formation appeared to involve sulfhydryl residues. The involvement of cysteine residues in FGF-1 dimer formation was investigated by specific pyridylethylation of cysteine residues. Denaturation of the protein in guanidine HCl in the presence of reducing agent did not prevent subsequent dimer formation when these agents were removed, although complete recovery of FGF-1 in the dimer form was not observed (Fig. 1D, lane 1). However, derivatization of reactive cysteines without reduction prevented copper-induced dimer formation for FGF-
**Formation and characterization of copper-induced FGF homodimers.** A, purified recombinant human FGF-1 (6 μM) was incubated in the presence of 0 (lane 1), 0.001 (lane 2), 0.01 (lane 3), 0.1 (lane 4), 1 (lane 5), 10 (lane 6 and lane 8), or 100 (lane 7) mM CuCl₂ as described under "Experimental Procedures." The dimer formation reaction was stopped by addition of Laemmli SDS sample buffer (37) containing 10 mM EDTA without (lanes 1–7) or with (lane 8) 100 mM dithiothreitol and boiling and subjected to SDS-PAGE. The destained gel is shown with molecular weight markers as indicated. B, purified recombinant human FGF-1-(21–154) (6 μM) was incubated in the presence of 0 (lane 1) or 10 (lane 2) mM CuCl₂. The samples were analyzed by SDS-PAGE under nonreducing conditions, and the resulting destained gel is shown. C, purified recombinant human FGF-2 (6 μM) was incubated in the presence of 0 (lane 1) or 10 (lane 2) mM CuCl₂ as described and analyzed by nonreducing SDS-PAGE. D, purified FGF-1 (6 μM) was either subjected to denaturation in 6 M guanidine HCl with DTT (lane 1) or further pyridylethylated without (lane 2) or with (lane 3) prior reduction as described under "Experimental Procedures." The samples were desalted by reversed-phase HPLC and subjected to dimer formation reactions with 10 mM CuCl₂. The samples were analyzed by SDS-PAGE under nonreducing conditions. E, purified forms of bovine FGF-1 (6 μM) (lanes 1 and 3) or FGF-1-(21–154) (6 μM) (lanes 2 and 4) from earlier eluting peaks (35% (v/v) acetonitrile) from reversed-phase HPLC were incubated with 0 (lanes 1 and 2) or 10 mM (lanes 3 and 4) CuCl₂ as described under "Experimental Procedures." The samples were analyzed by nonreducing SDS-PAGE (15% (v/v) acrylamide), and the resulting destained gel is shown. F, purified forms of bovine FGF-1 (6 μM) (lanes 1 and 3) or FGF-1-(21–154) (6 μM) (lanes 2 and 4) distinct from those in E as evidenced by later elution (37% (v/v) acetonitrile) in reversed-phase HPLC were incubated with 0 (lanes 1 and 2) or 10 mM (lanes 3 and 4) CuCl₂. The samples were analyzed by nonreducing SDS-PAGE, and the resulting destained gel is shown. G, 250 ng of purified FGF-1 C131S mutant protein was incubated in the absence (lane 1) or presence (lane 2) of 10 mM CuCl₂ as described and analyzed by nonreducing SDS-PAGE and immunoblotting with anti-FGF antibodies. The resulting autoradiograph detecting immunoreactive FGF-1 is shown.

**Formation of Copper-induced Bovine FGF-1 Dimers**—Since bovine forms of FGF-1 differ in the position of 1 cysteine, as compared with the human form, and copper-induced homodimer formation appeared to involve cysteine residues, bovine FGF-1 proteins were tested for homodimer formation in the presence of copper ions. FGF-1 and FGF-1-(21–154) purified from bovine brain eluted from reversed-phase HPLC in a succession of heterogenous peaks, each containing mitogenically active FGF-1 forms possibly with different disulfide arrangements (38). Heterogeneous forms of biologically active FGF-1 and FGF-1-(21–154) purified from bovine brain also migrated at their corresponding Mr on SDS-PAGE under nonreducing conditions (Fig. 1E, lanes 1 and 2; Fig. 1F, lanes 1 and 2). However, only one set of FGF-1 proteins was recovered in the dimer form upon exposure to copper (Fig. 1E, lanes 3 and 4; Fig. 1F, lanes 3 and 4). The earliest eluting primary peaks (35% (v/v) acetonitrile) consisting of FGF-1 and FGF-1-(21–154) did not exhibit copper-induced dimer formation under the conditions used in this study (Fig. 1E, lanes 3 and 4). However, the next successive eluting peaks (37% (v/v) acetonitrile) containing FGF-1 and FGF-1-(21–154) were capable of copper-induced dimer formation (Fig. 1F, lanes 3 and 4).

**Formation of Copper-induced FGF-1 C131S Dimers**—Since chemical modification of cysteine residues prevented copper-induced FGF-1 dimer formation and different FGF forms displaying differential dimer-forming properties also contain variant cysteine contents and arrangement, the role of cysteine residues in FGF-1-copper interactions was investigated by mutational analysis. A recombinant form of human FGF-1 was produced containing a serine for cysteine substitution at position 131 so that this protein only contained the 2 cysteine residues conserved throughout the FGF family (2-4). FGF-1 C131S bound to immobilized heparin and was biologically active in [³H]thymidine incorporation assays (data not shown). The human FGF-1 C131S recombinant protein migrated at the expected monomer position in the absence of copper (Fig. 1G, lane 1) and formed a dimer after pre-exposure to copper ions (Fig. 1G, lane 2).

**Affinity of Copper-treated Human FGF-1 and FGF-2 for Heparin**—The interactions between copper-treated human FGF-1 and FGF-2 with immobilized heparin was analyzed using batch adsorption to heparin-Sepharose. FGF-1 and FGF-2 monomers bind to heparin-Sepharose with high apparent affinity and elute near 1.0 M NaCl (35) and 1.6 M NaCl.
Interactions of Human FGF-1 and FGF-2 with Immobilized Copper—The association of human FGF-1 and FGF-2 monomers with copper was also examined by batch adsorption to copper-charged chelating Sepharose. FGF-1 monomers bound to copper-Sepharose in 1 M NaCl (Fig. 3A, lane 1). The protein eluted in the dimer form with increasing concentrations of the competitive antagonist imidazole (Fig. 3A, lanes 2–6). Complete elution occurred at 60 mM imidazole for human FGF-1, as was evidenced by the amount of respective protein recovered when the resin was stripped of copper ions by EDTA (Fig. 3B, lane 1). Similarly, preformed copper-induced FGF-1 dimers bound to copper-Sepharose (Fig. 3B, lane 1) and eluted with increasing concentrations of imidazole (Fig. 3B, lanes 2–6). The amount of protein recovered by the addition of 60 mM imidazole was similar to that recovered by the addition of EDTA (Fig. 3B, lane 7). In contrast, FGF-2 protein bound to copper-Sepharose in 1 M NaCl (Fig. 3C, lane 1), but the protein eluted in the monomer form with increasing concentrations of imidazole (Fig. 3C, lanes 2–6). Complete elution occurred at 10 mM imidazole, as indicated by the amount of protein recovered by the addition of EDTA (Fig. 3C, lane 7).

**Effect of Heparin on FGF Dimer Formation**—Heparin potentiates the mitogenic activity of FGF-1, possibly by inducing a conformational change in the polypeptide (40, 41). In contrast, heparin does not effect FGF-2 action, as compared with FGF-1 (42). The effect of heparin on dimer formation was examined by including heparin along with CuCl₂ in the dimer formation reaction. When increasing concentrations of heparin were incubated with human FGF-1 prior to the addition of CuCl₂, recovery of protein bands of apparent Mr, 68,000 and higher were observed (Fig. 4A, lanes 2–7). The appearance of the higher M, species was accompanied by the loss of one of the dimer species (Fig. 4A, lanes 2–7). The addition of heparin to human FGF-2 prior to copper treatment did not alter the amount of homodimers formed or produce additional FGF-2.
complexes of molecular weight different from the monomer (Fig. 4B).

Mitogenic Activity of Copper-treated FGFs—The biological activity of FGF-1 monomer and purified FGF-1 dimer was measured in a 3T3 cell DNA synthesis assay (40) in the presence of heparin. As shown in Fig. 5A, FGF-1 monomer was maximally active at 500 PM, whereas the copper-induced FGF-1 dimer was not active as a polypeptide mitogen between concentrations of CuCl₂, the activity of FGF-1 was abolished, whereas FGF-2 activity remained unaltered (Fig. 5B).

Copper (mM)

| Copper (mM) | FGF-1 Monomer | FGF-1 Dimer | FGF-1 Dimer + DTT |
|------------|---------------|-------------|------------------|
| 0.0001     | 0.001         | 0.01        | 0.1              |
| 0.01        | 0.1           | 1           | 10               |
| 1           | 10            | 100         | 1000             |
| 10          | 100           | 1000        | 1000             |
| 100         | 1000          | 10000       | 10000            |

| FGF (mM)   | DNA Synthesis (%) of Control |
|------------|-----------------------------|
| 0.0001     | 100                         |
| 0.001      | 92                          |
| 0.01       | 67                          |
| 0.1        | 0.76                        |
| 1          | 0                          |
| 10         | 0.08                        |
| 100        | 0.001                       |
| 1000       | 0.0001                      |

Formation of Human FGF-1/FGF-2 Heterodimers—Since bovine forms of FGF-1 and FGF-2 can be purified simultaneously by heparin-copper bioaffinity chromatography (5, 6), we investigated whether heterodimers between FGF-1 and FGF-2 formed by copper-induced oxidation may be potential reaction products when these proteins are present together with copper ions. As a preliminary experiment to further characterize FGF dimer formation in more complex reaction mixtures, [³²P]FGF-1-(21-154) (15 ng, 100,000 cpm/ng) was included in the dimerization reactions so that incorporation of labeled protein into dimers could be characterized. Unlike unlabeled FGF-1 monomers, which could be fully converted to the dimer form in the presence of copper, no radiolabeled protein was detected in the dimer position in the presence of copper in the absence of unlabeled FGF-1 (Fig. 6A, lane 1).

As increasing amounts of unlabeled FGF-1 were included in the dimer formation reactions, increasing amounts of radiolabeled dimers were observed (Fig. 6A, lanes 2–7). Although the majority of the labeled protein did not form dimers even in reactions in which unlabeled FGF-1 completely dimerized, a 10-fold excess or greater amount of unlabeled protein could be used to detect radiolabeled homodimers. To determine whether the electrophoretic mobility of the radiolabeled FGF-1-(21-154) was dependent on the presence of the unlabeled protein, excess unlabeled FGF-1-(21-154) (15 kDa) was added to the dimer formation reaction. The presence of unlabeled FGF-1-(21-154) (15 kDa) as opposed to unlabeled FGF-1 (17 kDa) produced radiolabeled dimers of differing mobilities (Fig. 6A, lane 8).

To examine whether human FGF-1 and FGF-2 form heterodimers in the presence of copper, radiolabeled FGF-1-(21-154) was added to reactions that also included FGF-2. In the absence of copper, radiolabeled FGF-1-(21-154) ran as a monomer in the presence of excess FGF-1 (Fig. 6B, lane 1) or...
whether excess FGF-1 (Fig. 6B, lane 2) was added to dimer formation reactions in the presence of 0 (lane 1), 0.012 (lane 2), 0.06 (lane 3), 0.12 (lane 4), 0.6 (lane 5), 1.2 (lane 6), or 6 (lane 7) μM unlabeled FGF-1 or 6 μM unlabeled FGF-1-(21-154) (lane 8) in the presence of 10 mM CuCl₂ as described under “Experimental Procedures.” The product of the reaction was analyzed by nonreducing SDS-PAGE, and the resulting autoradiograph detecting ¹²⁵I-FGF-1-(21-154) is shown. B, radiolabeled FGF-1-(21-154) was added to dimer formation reactions in the absence (lanes 1 and 2) or presence (lanes 3-5) of 10 mM CuCl₂ as described. In addition, the reactions contained 6 μM unlabeled FGF-1-(21-154) (lanes 1 and 3), 6 μM unlabeled FGF-2 (lanes 2 and 4), or 6 μM of both unlabeled FGF-1 and FGF-2 (lane 5). The reactions were analyzed by nonreducing SDS-PAGE followed by autoradiography to detect ¹²⁵I-FGF-1-(21-154).

FGF-2 (Fig. 6B, lane 2). In the presence of copper, a portion of the radiolabel appeared in the dimer position but with slightly differing electrophoretic mobility, depending on whether excess FGF-1 (Fig. 6B, lane 3) or FGF-2 (Fig. 6B, lane 4) was included. When equivalent amounts of FGF-1 and FGF-2 were added together with ¹²⁵I-FGF-1-(21-154) in the presence of copper, the labeled dimer had an electrophoretic mobility similar to that of when ¹²⁵I-FGF-1-(21-154) was incubated with FGF-1 alone (Fig. 6B, lane 5).

**DISCUSSION**

The transition metal copper acts as a modulator of angiogenesis in vivo and confers angiogenic capability to several macromolecules it complexes with, including heparin (1, 8). The potent angiogenic polypeptide FGF-1 interacts with immobilized copper ions, as well as heparin, as demonstrated by heparin-copper biaffinity chromatography (5). Although it is known heparin potentiates the biological activity of FGF-1 in vitro (40), possibly by inducing a favorable FGF-1 structural conformation optimal for receptor interactions (40, 41), the role of copper in FGF-1 structure and function has not been established. Since heavy metals play a role in the formation of various structural motifs in proteins, in the stabilization of macromolecular complexes or metal-catalyzed modification of proteins (24-26), the role of copper in FGF-1 function was first investigated by examining whether copper had any effects on FGF-1 structure as an oxidant. We have identified a shift in the apparent M₀ of FGF-1 when micromolar concentrations of purified recombinant forms of human FGF-1 (full-length and des(1-20) forms) were exposed to copper and analyzed by SDS-PAGE under nonreducing conditions. This alteration in electrophoretic mobility to moieties approximately twice the size of the monomer is consistent with the formation of FGF-1 homodimers. The observation that virtually all the monomer could be converted to the dimer form in the absence of complexes of higher M₀ by exposure to copper ions but not other metals is consistent with the known structural interaction between copper and FGF-1 (5). Denaturation of FGF-1 in guanidine HCl and DTT prior to exposure to copper resulted in incomplete recovery of FGF-1 in the dimer form. This may indicate a native structural conformation is required for complete dimer formation. Specific forms of native bovine FGF-1 also dimerized in a similar manner after copper exposure indicating copper-induced homodimer formation is not an artifact of the recombinant human protein. Finally, copper-induced FGF-1 dimers were distinguished from dimers that form spontaneously as evidenced by slightly altered mobilities during nonreducing electrophoresis. Copper-induced dimers may represent fully oxidized FGF-1 forms as opposed to partially oxidized, spontaneously formed disulfide-linked dimers.

This is in contrast to a preparation of recombinant human FGF-2 that did not fully form homodimers after exposure to copper in conditions under which human FGF-1 could be fully converted from the monomer to the dimer form. In addition, not all FGF-1 forms purified from bovine brain were capable of dimer formation in their native state since only certain FGF-1 fractions discriminated by reversed-phase HPLC showed copper-induced dimer formation. The existence of bovine FGF-1 proteins that are unable to form copper-induced dimers may explain why heparin-copper biaffinity chromatography can be used to purify biologically active bovine FGF-1 despite the potential for damaging side reactions involving copper oxidation (5).

The copper-induced FGF-1 dimers appear to form via covalent intermolecular disulfide bonds because (i) the dimers were stable in SDS and EDTA and remained intact after thermal or chemical treatment, (ii) preformed dimers reverted to the monomer form in the presence of reducing agent, and (iii) pyridylethylthlation of cysteine residues with or without prior reduction prevented dimer formation. The heterogeneity of the protein bands observed for the FGF-1 dimers may reflect differences in intermolecular and/or intramolecular disulfide bonds. Dimer formation caused by the production of free radicals and hydrogen peroxide from the oxidation of thiol groups (Fenton or Haber-Weiss reactions) seems unlikely since the addition of H₂O₂ alone or together with cupric ions did not induce dimer formation. Therefore, FGF-1 dimer formation appears to result from copper-induced oxidation of available sulfhydryl groups. Attempts to further characterize the cysteine status in copper-induced FGF-1 dimers by chemical modification were hampered by the inability to completely purify heterogeneous dimer forms from each other.

The involvement of cysteine residues in copper-induced FGF dimer formation may also explain the different abilities of human FGF-1, bovine FGF-1, and human FGF-2 proteins to form copper-induced homodimers, since each differs in cysteine content and/or arrangement (Fig. 7). Alternatively,
FGF-2 may not form copper-induced dimers due to its lower affinity for copper than FGF-1 (5, 6). The inability of FGF-2 to form copper-induced dimers also does not appear to result from an intrinsic inability to form dimers, since spontaneously-formed disulfide-linked FGF-2 dimers have been reported (43, 44). However, we cannot rule out the possibility that the inability to form copper-induced FGF-2 dimers is due to the method of its purification.

Since copper-induced FGF-1 dimer formation appears to involve cysteine residues, a biologically active recombinant human FGF-1 mutant protein containing the 2 cysteine residues conserved throughout the FGF family but lacking cysteine residue 131 present in wild-type human FGF-1 but not in bovine FGF-1 or human FGF-2 (Fig. 7) was produced to further examine the role of cysteines in FGF-1-copper interactions. Like native FGF-1, the FGF-1 C131S mutant was converted to the dimer form after pretreatment with copper, suggesting that copper-induced dimer formation is not due to the presence of nonconserved cysteines in human FGF-1 or to FGF forms with an odd number of cysteines, one of which could form disulfide-linked dimers with thiol groups in other molecules. Copper-induced FGF-1 dimer formation by the FGF-1 C131S protein suggests FGF-1-copper interactions occur via available cysteine residues present in each identified member of the FGF family. This may explain the formation of copper-induced 125I-FGF-1-(21-154)/FGF-2 heterodimer complexes.

The interaction between human FGF-1 and FGF-2 with immobilized copper ions was also examined. It has been demonstrated that both bovine FGF-1 and FGF-2 bind to heparin-copper biaffinity columns and elute with 2.0 mM NaCl and 10 mM imidazole (5, 6). In the present study, human FGF-1 was completely eluted from copper-Sepharose at 60 mM imidazole but in the form of a homodimer. Dimer formation by copper-Sepharose is reasonable since copper is an oxidant while immobilized. Preformed FGF-1 dimer had similar copper-binding properties. This suggests that the FGF-1 copper-binding site remains intact after dimer formation, possibly indicating that copper-binding residues such as cysteine, as well as histidine and tryptophan, may be available for interactions with copper. Human FGF-2 had a 6-fold less apparent affinity than human FGF-1 for immobilized copper ions eluting from copper-Sepharose at 10 mM imidazole. This affinity is similar to that of bovine FGF-2 as reported previously (5, 6). However, unlike human FGF-1, which eluted from immobilized copper as a homodimer, human FGF-2 was dissociated in the monomer form.

Preformed FGF-1 dimers had decreased apparent affinity for the glycosaminoglycan heparin as demonstrated by adsorption to heparin-Sepharose. While FGF-1 monomer binds to heparin with high affinity eluting near 1.0 mM NaCl (35), preformed copper-induced FGF-1 dimers bound to heparin but eluted near 0.4 mM NaCl. This suggests that, unlike the copper-binding site that remains intact for FGF-1 after copper oxidation, dimer formation may mask the heparin-binding site(s) in FGF-1 or otherwise induce a conformation that interferes with the structural interactions between FGF-1 and heparin. In contrast, similar pretreatment of human FGF-2 with copper ions did not affect the apparent affinity of FGF-2 for heparin consistent with the inability of FGF-2 to form copper-induced dimers.

Although the copper-induced FGF-1 homodimer has reduced heparin affinity, heparin is able to modulate the interaction between copper and FGF-1. Addition of heparin prior to copper addition resulted in the loss of at least one FGF-1 dimer species and the formation of oligomeric complexes of higher apparent $M_r$. Heparin may induce a structural conformation or mask cysteine residues in FGF-1 that prevent the formation of intermolecular disulfide bonds favorable for dimer formation and allow the formation of intermolecular disulfide bonds that result in FGF-1 complexes of higher order than dimers. Heparin didn't influence copper-induced FGF-2 dimer formation under identical conditions. This correlates with the ability of heparin to modulate the activity and structural conformation of FGF-1 (40) but not FGF-2 (42). Although FGF-1 is unstable in the absence of heparin, the formation of copper-induced FGF-1 oligomers of specific $M_r$ that occurs in the presence of heparin suggests that differences in stability between human FGF-1 and FGF-2 do not totally explain differences in copper interactions between these molecules.

The FGF-1 homodimer was purified by reversed-phase HPLC and found to be inactive as a mitogen in a DNA synthesis assay. However, copper-induced FGF-1 inactivation was fully reversible by reduction of the protein, a property requisite for any functional role for copper-induced FGF-1 inactivation that may occur in vivo (45). In addition, FGF-1 but not FGF-2 mitogenic activity could be abolished by pretreatment with copper. The amount of copper needed to inactivate FGF-1 corresponded to concentrations that were shown to cause complete dimer formation. FGF-1 inactivation by copper-induced dimer formation appears to occur through the induction of a structural conformation that reduced its affinity for heparin-like molecules and/or FGF receptors. This may be due to dimer formation alone or additional uncharacterized copper-catalyzed damage to FGF-1.

The copper-binding site in FGF-1 or FGF-2 has not been identified but seems to involve available cysteine residues. The availability of the cysteine residues in the FGFs as they occur in vivo is unknown. The cysteine arrangement is heterogeneous throughout the seven members of the FGF family, as well as among different animal species (Fig. 7) (2-4). Among FGF proteins, only cysteines at positions 30 and 97 are conserved across species and in all members of the FGF family presently identified. Chemical modification of cysteines in biologically active purified forms of FGF-1 and FGF-2 provides indirect evidence for the presence of a disulfide bond, whereas the remaining cysteines exist as free thiols (43, 46-49). However, recombinant forms of purified human FGF-1 and FGF-2 containing combinations of cysteines or none at all were biologically active to varying degrees, suggesting a disulfide bond in FGF-1 or FGF-2 was not required for activity (50, 51). This is consistent with the state of cysteine residues in FGF-1 and FGF-2 as inferred from their recently characterized three-dimensional crystallographic structures (52-54). The spatial location of the conserved cysteines in recombinant mutant forms of bovine FGF-1 and human analogues of FGF-2 in the absence of heparin are not compatible for disulfide bond formation and are not accessible to solvent. This corresponds to work suggesting native bovine FGF-2 exists with all 4 cysteines in reduced form with only the 2 non-conserved cysteines accessible to chemical modification (55). The ability of the FGF-1 C131S mutant containing the 2 conserved cysteines to form copper-induced dimers suggests at least one of these cysteines is available for thiol/disulfide interchange. Preliminary studies show that exposure to 10 $\mu$M CuCl$_2$ induces changes in fluorescence spectra of recombinant human FGF-1. Such changes in the conformational structure of FGF-1 may alter thiol availability. In addition, as with other heparin-binding proteins (56), heparin induces conformational changes in FGF-1 (40, 41) that also may alter the

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2 K. A. Engleka and T. Maciag, unpublished results.
accessibility of cysteine residues for potential interactions with copper.

The formation of FGF-1 dimers is an important component of the interaction between copper and human FGF-1. Although we have not been able to show modulation of FGF-1 activity by copper oxidation in vivo, we suggest the copper-FFG interactions described here are important in purification procedures utilizing copper-affinity chromatography. In addition, differences in FGF interactions with copper may be reflective of important structural differences such as thiol availability. Copper exposure may be useful as a method to discriminate between heterogeneous forms of biologically active FGFs. Finally, the reversible copper-induced inactivation of human FGF-1 may be indicative of a redox mechanism that potentially could be used to regulate FGF-1 function.

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