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The Release of Fibroblast Growth Factor-1 from NIH 3T3 Cells in Response to Temperature Involves the Function of Cysteine Residues*

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Fibroblast growth factor (FGF)-1 is released from NIH 3T3 cells in response to heat shock as a biologically inactive protein that is unable to bind heparin and requires activation by $(NH_4)_2SO_4$ to generate a biologically active extracellular heparin-binding growth factor (Jackson, A., Friedman, S., Zhan, X., Engleka, K. A., Forough, R., and Maciag, T. (1992) *Proc. Natl. Acad. Sci. USA* 89, 10691–10695). To further study the mechanism of FGF-1 release in response to heat shock (42 °C), we examined the kinetics of FGF-1 release from FGF-1-transfected NIH 3T3 cells and observed that the cells require at least 1 h of exposure to heat shock conditions for the release of FGF-1. Interestingly, agents that interfere with the function of the endoplasmic reticulum-Golgi apparatus, exocytosis, and the multidrug resistance pathway (brefelden A, methylamine, and verapamil, respectively) do not inhibit the release of FGF-1 in response to temperature; rather, they exaggerate the release of FGF-1. Because immunoblot analysis of FGF-1 in the conditioned medium of heat-shocked NIH 3T3 cells revealed the presence of a minor band with an apparent molecular weight of a FGF-1 homodimer and because we have previously shown that FGF-1, but not FGF-2, is able to form a homodimer in response to chemical oxidation by $CuCl_2$ (Engleka, K. A., and Maciag, T. (1992) *J. Biol. Chem.* 267, 11307–11315), we examined whether reducing agents would substitute for $(NH_4)_2SO_4$ and activate extracellular FGF-1. Indeed, dithiothreitol and reduced glutathione are able to individually generate a FGF-1 monomer as a heparin-binding protein from the conditioned medium of heat-shocked NIH 3T3 cell transfectants. To confirm that cysteine residues are involved in the release of FGF-1 in response to temperature, we used mutagenesis to prepare a human FGF-1 Cys-free mutant in which Cys^{30}, Cys^{97}, and Cys^{131} were converted to serine. Analysis of the release of the FGF-1 Cys-free mutant in NIH 3T3 cells transfected with the FGF-1 Cys-free mutant demonstrated that the FGF-1 Cys-free mutant is not released into the conditioned medium in response to temperature. Interestingly, exposure of the NIH 3T3 cell FGF-1 Cys-free transfectants to brefelden A followed by heat shock also demonstrated the absence of the extracellular FGF-1 Cys-free mutant. Finally, ion-exchange and reverse-phase chromatographies of heat-shocked conditioned medium analyzed by FGF-1 immunoblot analysis were able to resolve FGF-1 as a homodimer under nonreducing conditions and as a monomer under reducing conditions. These data demonstrate that FGF-1 utilizes cysteine residues as an important component of its release from NIH 3T3 cells *in vitro* in response to temperature and exits the cell as a biologically inactive homodimer with reduced heparin affinity that requires activation by reducing agents to generate heparin binding and biological activities.

The fibroblast growth factor (FGF)[1] gene family is presently composed of nine family members, and the prototype structures for the FGF family are defined by FGF-1 (acidic) and FGF-2 (basic) (1). A unique structural feature of the FGF prototypes is the absence of classical signal peptide sequence to direct their secretion through a conventional pathway utilizing the endoplasmic reticulum (ER)-Golgi apparatus (1). Because the biology of the FGF prototypes is tightly coupled with developmental, neurotrophic, and angiogenic processes and requires association with high affinity cell-surface tyrosine kinase receptors to modify gene expression and cell division (1), it is important to understand the mechanism by which the FGF prototypes are released from cells.

We have previously shown that FGF-1 is released from FGF-1-transfected NIH 3T3 cells in response to heat shock *in vitro* in a form that is biologically inactive and unable to associate with the glycosylaminoglycan heparin (2). However, the heparin binding and growth promoting activities of FGF-1 released in response to temperature can be recovered by $(NH_4)_2SO_4$ precipitation of the conditioned medium (2). While the mechanism of FGF-1 release in response to temperature involves the secretion of a latent protein, this mechanism appears to be specific for FGF-1 since FGF-2 is not released from cells *in vitro* in response to heat shock (3). Thus, to further define the pathway utilized by FGF-1 to gain access to the extracellular compartment in response to temperature stress, we have studied the kinetics of FGF-1 release, the action of pharmacologic agents on the release of FGF-1, and the ability of reducing agents to activate latent extracellular FGF-1 in NIH 3T3 cells transfected with FGF-1. We report that the pathway of FGF-1 release in response to heat shock involves the function of cysteine

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[1] The abbreviations used are: FGF, fibroblast growth factor; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; RCPCR, recombinant circle polymerase chain reaction; PCR, polymerase chain reaction; FBS, fetal bovine serum; DTT, dithiothreitol.

residues that may be important for the secretion of a latent FGF-1 homodimer.

MATERIALS AND METHODS

Cell Culture and Immunoblot Analysis—All chemical reagents were obtained from Sigma unless otherwise noted. NIH 3T3 cells transfected with the pMEXneo expression plasmid (4) containing the coding sequence for FGF-1 (residues 21–154) were obtained and grown as described previously (5), and confluent monolayers were subjected to heat shock (42 °C) for varied periods of time in serum-free Dulbecco's modified Eagle's medium (DMEM). Conditioned media from control and heat-shocked NIH 3T3 cells transfected with wild-type and mutant FGF-1 were centrifuged at 4000 × *g* for 5 min, processed through a 0.22-μm filter, treated with either $(NH_4)_2SO_4$ as described previously (2) or various reducing agents, and passed over a 2-ml heparin-Sepharose 4B column (Pharmacia Biotech Inc.) equilibrated in 50 mM Tris-HCl (pH 7.0) containing 10 mM EDTA (TEB). The column was washed with 10 volumes of TEB, eluted with TEB containing 1.5 M NaCl, dialyzed against TEB, and concentrated (Centricon 10, Amicon, Inc.) to a volume of 40 μl. All samples were resolved by SDS-polyacrylamide electrophoresis (PAGE) (15% (w/v) acrylamide), transferred to nitrocellulose filters as described previously (5), incubated with 10 mM Tris-HCl (pH 8.0) containing 5.0% (w/v) bovine serum albumin, 150 mM NaCl, and 0.1% (v/v) Tween 20 (TCB), and incubated with 1 μg of a rabbit anti-human FGF-1 antibody/ml (5) for 18 h at 4 °C. The filters were washed three times with TCB, and the proteins were detected with ^{125}I-protein A prepared as described (6). Recombinant human FGF-1-(21–154) served as a positive control and was prepared as described previously (7).

The conditioned medium collected from heat-shocked NIH 3T3 cell FGF-1 transfectants was also resolved by Mono-S (Pharmacia Biotech Inc.) high pressure liquid chromatography (HPLC). The medium (120 ml) was dialyzed against 50 mM sodium phosphate (pH 6.0) containing 50 mM NaCl (20 h, 4 °C) and fractionated with a linear gradient as described previously (8). Fractions (2 ml) containing FGF-1 were collected, further resolved by Sep-Pak C_{18} HPLC, lyophilized, resuspended in SDS-PAGE sample buffer, and analyzed by immunoblot analysis using 12% (w/v) SDS-PAGE and FGF-1 antisera as described above.

Plasmid Construction—The pXZ38 plasmid containing FGF-1-(21–154) in the pMEXneo expression vector has been described previously (2). A recombinant human FGF-1-(1–154) construct in which Cys^{131} was replaced by Ser (FGF-1 C131S) was obtained using the recombinant circle polymerase chain reaction (RCPCR) as described previously (9). Briefly, two separate PCRs were utilized for the mutagenesis of Cys^{131} in pUC18. The first PCR used the primers CTCAAGAAGAATGGGAGCTCCAAACGCGGT (sense oligonucleotide; the mutation is underlined here and below) and GCCAACAAACCAATTCTTCTCTGCATG (antisense oligonucleotide), and the second PCR used the primers AGTCCGAGGACCGCGTTTGGAGCTCCCATT (antisense oligonucleotide) and CACTATGGCCAGAAAGCAATC (sense oligonucleotide).

The RCPCR method was also used to obtain a FGF-1 C30S mutant in pUC18. As described above, four primers were used in two separate PCRs. One PCR was performed with the primers AAGCCCAAACTCCTCTACTCTAGCAACGGG (sense oligonucleotide) and CTTGTAATTCCCTGGAGGCAGATTAAA (antisense oligonucleotide), and the other PCR was performed with the primers GAAGTGGCCCCCGTTGCTAGAGTAGAGGAG (antisense oligonucleotide) and CTGAGGATCCTTCCGGATGGCACA (sense oligonucleotide). The same four primers were also used for the mutagenesis of Cys^{30} in the FGF-1 C131S mutant in pUC18. In this case, the FGF-1 C30S/C131S mutant was obtained and eventually used in the last set of RCPCRs for the mutagenesis of nucleotides encoding Cys^{97} in order to obtain the FGF-1 Cys-free mutant. In this case, the primers CAGACACCAAATGAGGAATCTTTGTTCCTG (sense oligonucleotide) and TGAGCCGTATAAAAGCCCGTCGGTGTC (antisense oligonucleotide) were used in the first PCR, and the primers CAGCCTTTCCAGGAACAAAGATTC (antisense oligonucleotide) and GAGGAGAACCATTACAACACC (sense oligonucleotide) were used in the second PCR. Sequence analysis demonstrated that two extra point mutations were inadvertently introduced (TTC to TCC, F36S; GAA to GAT, E63D). To obtain the FGF-1 Cys-free clone without the extra point mutations, the FGF-1 C30S and FGF-1 C30S/F36S/E63D/C97S/C131S (five-point) mutants were transferred into the prokaryotic expression vector pET3c. As described previously (9), PCR was used to add unique sites for the restriction enzymes *Nde*I and *Bgl*II to the ends of the DNA sequence encoding the two FGF-1 mutants. The FGF-1 C30S and FGF-1 five-point mutants were amplified with 30 cycles using the primers TACGGCATATGGCTGAAGGGGAAATCACC (sense oligonucleotide; *Nde*I site) and TACGAACAGATCTCTTTAATCAGAAGA (antisense oligonucleotide; *Bgl*II site). The two *Nde*I- and *Bgl*II-digested inserts were subcloned into compatible *Nde*I/*Bam*HI sites in the pET3c plasmid. The ligation was performed with T4 ligase (Life Technologies, Inc.), and the reaction products were confirmed by sequencing using the dideoxy method with Sequenase (U. S. Biochemical Corp.) following the manufacturer's instructions. The FGF-1 five-point pET3c plasmid was then digested with *Nco*I and *Eco*RI; the band (741 base pairs) was purified and ligated into FGF-1 C30S pET3c previously digested with *Nco*I and *Eco*RI, treated with calf intestinal phosphatase, and gel band-purified prior to ligation. DNA sequencing of the insert in pET3c confirmed the FGF-1 Cys-free sequence, without the two extraneous mutations. The FGF-1 Cys-free insert was transferred from pET3c into the eukaryotic expression vector pMEXneo (4). The FGF-1 Cys-free pET3c plasmid was digested with *Bam*HI and *Eco*RI; the band (867 base pairs) was purified and subcloned into *Bam*HI/*Eco*RI-digested pSF23[2] that had been treated with calf intestinal phosphatase and purified by agarose electrophoresis prior to ligation. DNA sequencing of the insert in pMEXneo confirmed the presence of the FGF-1 Cys-free sequence.

FGF-1 Cys-free Mutant Transfection—Murine NIH 3T3 cells were grown in DMEM and supplemented with 10% (v/v) fetal bovine serum (FBS). One hour prior to transfection using the calcium phosphate precipitation method (10), fresh medium was added, followed by the addition of 5 μg of plasmid DNA. After 24 h, the medium was changed with DMEM containing FBS, and 48 h after transfection, the cells were split at 1:10 and 1:30 ratios in DMEM containing 10% (v/v) FBS and 800 μg/ml Geneticin. The medium was changed every second day, and Geneticin-resistant colonies were recovered after 2 weeks. The transfected cells were maintained in DMEM containing 10% (v/v) FBS and 400 μg/ml Geneticin.

RESULTS AND DISCUSSION

The kinetics of FGF-1 release in response to temperature was studied in an attempt to determine the rapidity of the release process *in vitro*. As shown in Fig. 1, we did not observe significant levels of extracellular FGF-1 using $(NH_4)_2SO_4$ activation of the latent heparin binding property of FGF-1 until after 1 h of exposure of the FGF-1-transfected NIH 3T3 cell population to heat shock conditions. While small levels of extracellular FGF-1 were present 30 min after heat shock, we failed to detect extracellular FGF-1 at earlier time points (Fig. 1*A*). In addition, we were unable to detect ^{35}S-labeled FGF-1 by pulse-chase analysis of [^{35}S]Met/Cys-labeled NIH 3T3 cell transfectants 60 min after heat shock using similar methods (data not shown). Because the expression of FGF-1 in the NIH 3T3 cell transfectants is constitutive and these cells contain cytosolic FGF-1 levels of ~30 ng/10^6 cells, it was surprising that FGF-1 was released with significantly delayed kinetics following heat shock. Thus, it is possible that the cytosolic store of intracellular FGF-1 may not be accessible to the temperature-induced FGF-1 release pathway, and this is consistent with the identification of a cytosolic retention domain in the structure of FGF-1.[2]

During the course of these (Fig. 1*A*, *lanes 7*, *9*, and *11*) and prior (2) immunoblot experiments with conditioned medium activated with $(NH_4)_2SO_4$, we noted the appearance of a minor band migrating with an apparent M_r of ~38,000, a position that corresponds with the apparent M_r of a FGF-1 homodimer previously generated by chemical oxidation of the recombinant human protein using $CuCl_2$ (9). Because the FGF-1 homodimer prepared by chemical oxidation is biologically inactive and weakly associates with heparin (9) and the form of FGF-1 released into the conditioned medium in response to heat shock is also biologically inactive and unable to associate with heparin at 0.7 M NaCl (2), we questioned whether we could recover the heparin binding properties of FGF-1 from the heat-shocked conditioned medium using reducing agents rather than by ac-

[2] J. Shi, S. Friedman, X. Hu, X. Zhan, and T. Maciag, manuscript in preparation.

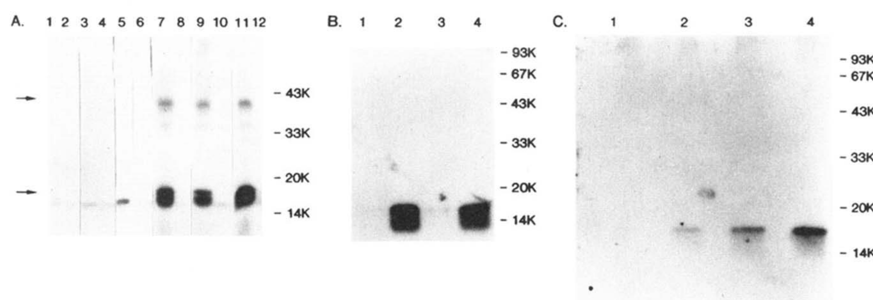

FIG. 1. *A*, kinetics of FGF-1 release in response to temperature. Conditioned media were derived from NIH 3T3 cell pMEXneo/FGF-1 transfectants maintained either at 37 or 42 °C for varied time periods as described previously (2). The conditioned media were collected, treated with 90% (w/v) $(NH_4)_2SO_4$, centrifuged (9000 × *g*, 40 min), resuspended in TEB, dialyzed against TEB (18 h, 4 °C), and adsorbed to heparin-Sepharose, and the 1.5 M NaCl eluates were analyzed by immunoblot analysis for FGF-1. *Lanes 1* and *2* represent conditioned medium after 5 min at 42 and 37 °C, respectively, and *lanes 3* and *4* (15 min), *lanes 5* and *6* (30 min), *lanes 7* and *8* (1 h), *lanes 9* and *10* (90 min), and *lanes 11* and *12* (2 h) represent conditioned medium harvested at the times indicated in parentheses from control (37 °C; *even-numbered lanes*) and heat-shocked (42 °C; *odd-numbered lanes*) NIH 3T3 cells. *B*, activation of FGF-1 from heat-shocked conditioned media using reducing agents rather than $(NH_4)_2SO_4$. Conditioned media were derived from NIH 3T3 cell pMEXneo/FGF-1 transfectants maintained at 42 °C for 2 h. The conditioned media were collected and adsorbed to heparin-Sepharose, and the 1.5 M NaCl eluates were analyzed by immunoblot analysis for FGF-1. *Lane 1*, conditioned medium from heat-shocked (42 °C) cells; *lane 2*, conditioned medium from heat-shocked (42 °C) cells treated with 0.1% (w/v) DTT; *lane 3*, conditioned medium from heat-shocked (42 °C) cells treated with 1 mM glutathione (oxidized form); *lane 4*, conditioned medium from heat-shocked (42 °C) cells treated with 1 mM glutathione (reduced form). *C*, immunoblot analysis of FGF-1 from media conditioned by heat shock and brefelden A. NIH 3T3 cell pMEXneo/FGF-1 transfectants were grown to confluence and pretreated with brefelden A (0.5 μg/ml) for 30 min. Serum-free DMEM containing brefelden A (0.5 μg/ml) was added at time 0, and the monolayer was maintained at 42 °C for 2 h. Control NIH 3T3 cell pMEXneo/FGF-1 transfectants were maintained under identical conditions at 37 and 42 °C. The conditioned media were collected, 1 mM phenylmethysulfonyl fluoride was added, the media were adsorbed with heparin-Sepharose, and the 1.5 M NaCl eluates were analyzed by immunoblot analysis for FGF-1 as described previously (2). *Lane 1*, conditioned medium from 37 °C control cells; *lane 2*, conditioned medium from cells pretreated with brefelden A at 37 °C; *lane 3*, conditioned medium from heat-shocked (42 °C) cells; *lane 4*, conditioned medium from heat-shocked (42 °C) cells pretreated with brefelden A.

tivation with $(NH_4)_2SO_4$. As shown in Fig. 1*B*, 0.1% (w/v) dithiothreitol (DTT) and 1 mM reduced glutathione, but not 1 mM oxidized glutathione, were able to activate the heparin binding property of FGF-1 in the conditioned medium of heat-shocked NIH 3T3 cell FGF-1 transfectants. These data suggest that FGF-1 may be released into the extracellular compartment as a latent FGF-1 complex that may be associated with itself or another protein by a disulfide bridge since reducing agents such as DTT and glutathione are able to substitute for $(NH_4)_2SO_4$ and activate the heparin binding activity of FGF-1 released into the extracellular compartment in response to temperature stress.

We also utilized pharmacologic agents known to impair the secretory function of the ER-Golgi apparatus (11), exocytosis (12), and the multidrug resistance pathway (13) in order to determine whether these pathways are utilized during FGF-1 release in response to temperature stress. As shown in Fig. 1*C*, NIH 3T3 cells transfected with FGF-1 released FGF-1 into the conditioned medium as a result of heat shock for 2 h at 42 °C. However, rather than inhibit the release of FGF-1 in response to temperature, pretreatment of the NIH 3T3 cell transfectants with brefelden A (0.5 μg/ml) resulted in an exaggerated level of FGF-1 in the conditioned medium (Fig. 1*C*). Similar results were also obtained by pretreatment of the FGF-1-transfected NIH 3T3 cell monolayer for 30 min with 10 mM methylamine and 10 μg/ml verapamil (data not shown). These results suggest that the release of FGF-1 in response to temperature utilizes a pathway that is independent of the conventional secretory pathway mediated by the ER-Golgi apparatus.

To further define the form of FGF-1 released by NIH 3T3 cell FGF-1 transfectants in response to temperature stress, FGF-1 present in the conditioned medium was fractionated by ion-exchange HPLC (Fig. 2*A*). FGF-1 immunoblot analysis of this sample performed in the absence of reducing agents resolved a series of bands with apparent M_r values between 33,000 and 36,000, while identical analysis in the presence of a reducing agent revealed a single band with an apparent M_r of 17,000 (Fig. 2*B*). The triplet band observed in the nonreduced sample was similar to that previously observed with the FGF-1 ho-

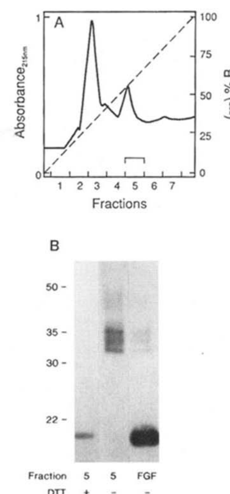

FIG. 2. *A*, ion-exchange HPLC of FGF-1 released in response to heat shock. NIH 3T3 cell FGF-1 transfectants were exposed to temperature stress, and conditioned media were collected and processed as described under "Methods and Materials." The post-Mono-S fraction defined by the boundary was pooled, adsorbed to a Sep-Pak resin (Waters), and eluted with 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The fraction was lyophilized and processed for FGF-1 immunoblot analysis. *B*, immunoblot analysis of the fractions collected by ion-exchange HPLC. The fractions were resuspended in SDS-PAGE sample buffer with or without DTT and resolved by SDS-PAGE, and FGF-1 was identified as described under "Methods and Materials." *Lane 3* contains recombinant human FGF-1 without DTT.

modimer prepared by chemical oxidation with $CuCl_2$ (9). Indeed, the recombinant FGF-1 standard also contained a small level of the triplet FGF-1 homodimer when the protein was resolved in the absence of reductant (Fig. 2*B*, *third lane*). These data suggest that FGF-1 is released into the conditioned medium in response to temperature as a homodimer.

To confirm the importance of cysteine residues for the release of latent FGF-1 in response to heat shock, we utilized the recombinant circle polymerase chain reaction strategy to prepare a human FGF-1 Cys-free mutant. Expression of the FGF-1

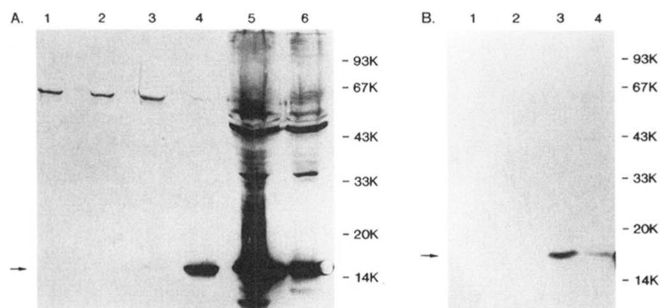

FIG. 3. **Release of FGF-1 Cys-free mutant in response to brefelden A and heat shock.** NIH 3T3 cells were transfected with the FGF-1 Cys-free mutant as described under "Materials and Methods." *A*, immunoblot analysis of FGF-1 and FGF-1 Cys-free release in response to heat shock. FGF-1-transfected (~30 ng of intracellular FGF-1/10^6 cells) and FGF-1 Cys-free mutant-transfected (~90 ng of intracellular FGF-1/10^6 cells) NIH 3T3 cells were subjected to heat shock (2 h, 42 °C); conditioned media were collected, treated with 90% (w/v) $(NH_4)_2SO_4$, processed as described in the legend to Fig. 1, and adsorbed to heparin-Sepharose; and the 1.5 M NaCl eluates were analyzed by immunoblot analysis for FGF-1. *Lane 1*, conditioned medium from control (37 °C) FGF-1 Cys-free transfectants; *lane 2*, conditioned medium from heat-shocked (42 °C) FGF-1 Cys-free transfectants; *lane 3*, conditioned medium from control (37 °C) FGF-1 transfectants; *lane 4*, conditioned medium from heat-shocked (42 °C) FGF-1 transfectants; *lane 5*, cell lysate from FGF-1 Cys-free transfectants; *lane 6*, cell lysate from control FGF-1 transfectants. *B*, immunoblot analysis of FGF-1 and FGF-1 Cys-free release in response to brefelden A pretreatment and heat shock. FGF-1-transfected and FGF-1 Cys-free mutant-transfected NIH 3T3 cells were treated with brefelden A as described under "Materials and Methods" and subjected to heat shock (2 h, 42 °C); conditioned media were collected, treated with $(NH_4)_2SO_4$ as described above, and adsorbed to heparin-Sepharose; and the 1.5 M NaCl eluates were analyzed by immunoblot analysis for FGF-1. *Lane 1*, conditioned medium from FGF-1 Cys-free transfectants pretreated with brefelden A; *lane 2*, conditioned medium from control FGF-1 Cys-free transfectants; *lane 3*, control FGF-1 transfectants pretreated with brefelden A; *lane 4*, control FGF-1 transfectants.

Cys-free mutant in the prokaryotic pET3c expression system yields a recombinant protein with heparin binding properties and mitogenic activity similar to those previously reported (8) for wild-type recombinant human FGF-1 (data not shown). This is consistent with the report that cysteine residues are not required for biological activity (14). NIH 3T3 cells were transfected with the FGF-1 Cys-free mutant using the eukaryotic expression vector pMEXneo (4), and the release of the FGF-1 Cys-free mutant in response to heat shock was examined using the NIH 3T3 cell transfectants. As shown in Fig. 3*A*, we were readily able to detect the FGF-1 Cys-free mutant in the cytosol of heat-shocked NIH 3T3 cell transfectants. However, we were unable to detect the FGF-1 Cys-free mutant in the extracellular compartment following $(NH_4)_2SO_4$ activation of the conditioned medium (Fig. 3*A*). Because brefelden A pretreatment of NIH 3T3 cell FGF-1 transfectants resulted in an enhanced level of FGF-1 in the conditioned medium after heat shock (Fig. 1*C*), we examined whether the FGF-1 Cys-free mutant could be released into the conditioned medium following heat shock of the NIH 3T3 cell FGF-1 Cys-free mutant transfectants pretreated with brefelden A. As shown in Fig. 3*B*, we were unable to detect the appearance of the FGF-1 Cys-free mutant in the conditioned medium of the brefelden A-pretreated NIH 3T3 cell FGF-1 Cys-free mutant transfectants following $(NH_4)_2SO_4$ activation of the conditioned medium. Additional experiments using 0.1% (w/v) DTT activation yielded results similar to those shown in Fig. 3 (*A* and *B*). These results suggest that the release of FGF-1 in response to temperature involves the intracellular function of FGF-1 cysteine residues that may play a role in the formation of a latent homodimer.

The secretory pathway utilized by FGF-1 is unique since FGF-2 is not released from NIH 3T3 cells transfected with FGF-2 in response to heat shock (3) and, unlike FGF-1, FGF-2 does not readily associate to form FGF-2 homodimers in response to Cu^{2+} oxidation (9) even though 2 of the 3 cysteine residues are conserved between FGF-1 and FGF-2 (1). However, we have not eliminated the possibility that FGF-1·FGF-2 heterodimers may be released from NIH 3T3 cells in response to temperature. Thus, our data suggest that in response to temperature stress, FGF-1 is released into the extracellular compartment as a functionally inactive homodimer that is able to associate poorly with immobilized heparin, and the latent FGF-1 homodimer may be activated by reducing agents such as glutathione to generate heparin binding and mitogenic activities. Furthermore, this mechanism argues that the redox state within tissue microenvironments may play a major role in the pathway of hypoxia-mediated angiogenesis and may also resolve the functional importance of adding reducing agents to cell culture environments for the maximal growth stimulation of a variety of diploid mammalian cell types.

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