The fibroblast growth factor (FGF) prototypes, FGF-1 and FGF-2, lack a signal sequence, but both contain a nuclear localization sequence. We prepared a series of FGF-1 deletion mutants fused to the reporter gene, β-galactosidase (β-gal) and determined that a domain between residues 83 and 154 is responsible for FGF-1 cytosol retention in NIH 3T3 cells. Using a series of FGF-β-gal chimeric proteins prepared by the shuffling of cassette-formatted synthetic FGF prototype genes, we were able to demonstrate that the nuclear localization sequence from the 5′-CUG region of FGF-2 is not able to direct the nuclear association of FGF-1 due to its inability to repress the function of the FGF-1 cytosol retention domain. We also observed that while the FGF-1:β-gal chimera was released in response to heat shock, the FGF-2:β-gal protein was not. Further, replacement of the FGF-1 cytosol retention domain with the corresponding domain from FGF-2 repressed the release of the chimeric protein. These data suggest that the specificity of the stress-induced secretion pathway for FGF-1 involves a carboxyl-terminal domain that is absent in FGF-2 and that the FGF-1 secretion pathway does not restrict the release of high molecular weight forms of FGF-1.

The heparin-binding fibroblast growth factor (FGF) family currently consists of nine structurally related members with broad biological activities (1, 2), and the biological functions of the FGF gene family members are mediated through high affinity FGF receptors, which contain intrinsic tyrosine kinase activity (3). The FGF prototypes, FGF-1 (acidic) and FGF-2 (basic), lack a classical signal sequence for secretion through the conventional endoplasmic reticulum (ER)-Golgi apparatus, and the signal sequences present in other FGF gene family members contribute to their oncogenic potential (1, 2). Because the mitogenic potential of the FGF prototypes are mediated by the function of the FGF prototypes as exogenous proteins, it has been proposed that novel and unconventional secretory pathways may have evolved to regulate their activities as extracellular modifiers of biological responses (1, 2).

We have shown that FGF-1 is secreted from NIH 3T3 cell FGF-1 transfectants by a brefeldin A-insensitive, ER-Golgi-independent pathway in response to heat shock (4, 5). FGF-1 is released as a latent homodimer with reduced affinity for immobilized heparin (4), and both the mitogenic and heparin-binding activities of latent extracellular FGF-1 can be activated by reducing agents (5). Mutagenesis of Cys residues in FGF-1 enabled us to demonstrate that Cys³⁰ is solely responsible for the formation of the FGF-1 homodimer (6). In addition, FGF-1 is a phosphatidylserine-binding protein (6), and FGF-1 at 42 °C is able to attain a molten globule character, which enables it to associate with acidic phospholipid membranes (7). In contrast, FGF-2 is also secreted by a brefeldin A-insensitive pathway (8), and, although the Cys³⁰ residue is conserved between FGF-1 and FGF-2 (1, 2), FGF-2 neither forms a homodimer (9) nor is released in response to temperature stress in vitro (8). Because it is possible that the FGF prototypes are released by different unconventional pathways, we sought to study the release of the FGF prototypes using a strategy of molecular shuffling of four synthetic cassettes, each encoding domains from FGF-1 (10) and FGF-2 (11). This overlapping cassette shuffle strategy has previously enabled us to construct and express a variety of novel recombinant FGF-1:FGF-2 chimeric proteins and determine that residues 65–81 from FGF-2 significantly contribute to the heparin-dependent character of FGF-1 as an exogenous mitogen (11). We have utilized these constructs to obtain stable NIH 3T3 cell FGF-1:FGF-2 cassette shuffle transfectants and studied the intracellular locale and secretory potential of these constructs as β-galactosidase (β-gal) reporter gene fusion proteins. We report that the carboxyl-terminal half of FGF-1 (residues 84–154) is involved in cytosol retention and contributes to the ability of FGF-1 to be released in response to heat shock and that the corresponding domain in FGF-2 is responsible for the inability of FGF-2 to be released in response to temperature stress in vitro. Finally, we also report that there does not appear to be a molecular weight restriction for the size of FGF-1 released in response to temperature stress in vitro, and nuclear-associated FGF-1 does not have access to the FGF-1 secretory pathway.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The construction of plasmids pX245 and pXZ55 was previously described (12). The plasmid pJS2, which contains residues 21–78 of the human FGF-1 gene, was constructed by ligating the 6673-base pair (bp) Scal-EspI fragment of pXZ55 and the 3011-bp Xmal-EspI fragment of pX245 together. The Scal digestion was partial, and both the Scal and Xmal sites were filled in with Klenow fragment (Boehringer Mannheim). The plasmid pJS123, which contains residues 21–117 of the human FGF-1 gene, was constructed in four steps involving three intermediate constructs: pJS23temp, pJS23temp2, and pJS23temp3. The plasmid pJS23temp was constructed by ligating the BamH1 fragment of pXZ55, which contains the

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β-gal fragment, into the HindIII site of pDS90 (10). The ligation of the 5334-bp BamHI-Smal fragment of pXZ55 and the BamHI-SmalI fragment of pJS23temp, which contains residues 38–117 of the human FGF-1 gene, generated pJS23temp2. The ligation of the 2185-bp BglII-EspI fragment of pXZ55 and the 7591-bp BglII-EspI fragment of pJS23temp, which contains the large HI fragment of pXZ55, was performed by ligating the 7909-bp Scal fragment of pJS23temp3 with the 1905-bp Scal fragment of pXZ55.

The plasmid pJS5, which contains residues 28–154 of the human FGF-1 gene, was constructed by ligating the Scal-Nhel fragment of pXZ55 (12) and the 5702-bp Scal-HindIII fragment of pXZ55 with the 3555-bp Scal-HindIII fragment of pJS23temp. The plasmid pJS20temp was constructed by ligating the 3129-bp Scal fragment of pXZ55 and the 2138-bp Scal fragment of pJS23temp with the 1905-bp Scal fragment of pXZ55.

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RESULTS AND DISCUSSION

The nomenclature for the human FGF-1 and FGF-2 plasmid discuss utilizes the subscription number to describe the FGF cassette number employed in the construction of the individual reporter gene sequence (10, 11). The residues used to describe the FGF-1 and FGF-2 sequences are based upon the translation of the FGF prototypes as AUG-initiated translation products (1). We have previously prepared a synthetic human FGF-1 gene by the ligation of four cassettes encoding the FGF-1 ORF (10) and have reported that while the ligation of the FGF-1 NLS, NYKKPK (residues 21–26), to the reporter gene, β-gal, efficiently translocates the fusion protein, FGF-1:β-gal to the nucleus in transfected NIH 3T3 cells, the FGF-1:β-gal fusion protein remains cytosol-associated (Ref. 12, Fig. 1A–D). Because these data suggest that FGF-1 may contain a domain that is able to repress the intracellular function of the FGF-1 NLS, we (i) prepared a series of FGF-1:β-gal deletion constructs (Fig. 1A), (ii) obtained stable NIH 3T3 cell transfec-
tants, and (iii) assessed the intracellular locale of the reporter gene by immunofluorescence (Fig. 1B, C, D). The intracellular locale of the reporter gene sequence (10, 11). The residues used to describe the FGF-1 and FGF-2 sequences are based upon the translation of the FGF prototypes as AUG-initiated translation products (1). We have previously prepared a synthetic human FGF-1 gene by the ligation of four cassettes encoding the FGF-1 ORF (10) and have reported that while the ligation of the FGF-1 NLS, NYKKPK (residues 21–26), to the reporter gene, β-gal, efficiently translocates the fusion protein, FGF-1:β-gal to the nucleus in transfected NIH 3T3 cells, the FGF-1:β-gal fusion protein remains cytosol-associated (Ref. 12, Fig. 1A–D). Because these data suggest that FGF-1 may contain a domain that is able to repress the intracellular function of the FGF-1 NLS, we (i) prepared a series of FGF-1:β-gal deletion constructs (Fig. 1A), (ii) obtained stable NIH 3T3 cell transfec-
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tants, and (iii) assessed the intracellular locale of the reporter gene by immunofluorescence (Fig. 1B), X-gal staining (Fig. 1C), and subcellular fractionation followed by anti-β-gal immunoblot analysis (Fig. 1D). While the deletion of either cassettes 3 and 4 (residues 84–154) or the deletion of cassette 4 (residues 118–154) does not repress the trafficking of the reporter gene to the nucleus (Fig. 1, A–D), the ligation of cassettes 3 and 4 to

β-mercaptoethanol, 0.75 mM bromphenol blue, pH 6.8) at 95 °C for 3 min. Samples were resolved by 7.5% (w/v) SDS-polyacrylamide gel electrophoresis and electropherotically transferred to a 0.45-μm Hybond C nitrocellulose membrane (Amersham Corp.). Immunoblot analysis was performed by incubating the membranes with TBS buffer (50 mM Tris, pH 7.4, containing 150 mM NaCl, 0.1% (v/v) Tween-20 containing 5% (w/v) BSA for 1 h at room temperature followed by incubation with anti-β-gal antibody (1:500 dilution in TBS buffer) for 1 h at room temperature. The membranes were washed three times with TBS, incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (Bio-Rad) (1:30,000 dilution) for 30 min, and washed three times with TBS, and the proteins were detected using the ECL detection system (Amersham).

Heat Shock Analysis—Heat shock experiments were performed as described previously (4). Briefly, NIH 3T3 cell transfec-
tants were grown on fibronectin-coated plates. The medium was changed to Dulbecco’s modified Eagle’s medium (serum-free) prior to heat shock, and the cells were incubated at 42 °C for 2 h. The conditioned medium was collected, filtered through a 0.22-μm filter (Corning), activated with 0.1% (v/v) dithiothreitol for 2 h at 37 °C, and processed over a 1-ml heparin-Sepharose-4B (Pharmacia) column equilibrated with 50 mM Tris, pH 7,4, containing 10 mM EDTA. The column was washed with 50 ml of TEB and eluted with 2.5 ml of 1.5 M NaCl in TEB. The cells were lysed in phosphate-buffered saline containing 1% (w/v) Triton X-100, sonicated for 15 s, clarified by centrifugation at 4 °C, and processed over a heparin-Sepharose-4B column as described previously (5). Both cell lysate and conditioned medium eluates were concentrated to a Centricon 10 (Amicon), resolved by 7.5% (v/v) SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblot analysis using anti-β-gal antibodies. NIH 3T3 cell transfec-
tants incubated for 2 h at 37 °C were used as controls.
the FGF-1 NLS in cassette 1 does repress the trafficking of the reporter gene to the nucleus (Fig. 1, A–D). These data suggest that the carboxyl-terminal half of FGF-1 is involved in the cytosol retention of FGF-1, and residues 84–154 may be important for this function.

In order to determine whether the temperature stress-induced FGF secretion pathway is utilized by FGF-1 and FGF-2, NIH 3T3 cell FGF-1(1-2-3-4):β-gal and FGF-2(1-2-3-4):β-gal transfectants (Fig. 2A) were examined for their ability to release the growth factor/reporter gene chimera in response to heat shock. As shown in Fig. 3, A and B, the FGF-1(1-2-3-4):β-gal chimeric protein, was released in response to heat shock as a structurally intact polypeptide with an apparent molecular mass of approximately 118 kDa. These data suggest that the temperature stress-induced pathway is specific for FGF-1 and that this pathway can release FGF-1 as a large molecular weight reporter gene chimera.

Because the temperature stress-induced secretory pathway appears to be specific for FGF-1, we used a cassette shuffle strategy to assess whether specific domains are involved in the release of FGF-1. As shown in Fig. 3, A and B, the substitution of either cassette 3 or 4 from FGF-2 for the corresponding cassette in FGF-1 (Fig. 2A) inhibited the release of the FGF-1(1-2-3):FGF-2(4):β-gal and FGF-1(1-2-4):FGF-2(3):β-gal chimeric proteins in response to temperature stress from their respective NIH 3T3 cell transfectants. In addition, the introduction of cassettes 3 and 4 from FGF-2 as replacement for their corresponding cassettes in FGF-1 (Fig. 2A) also inhibited the release of FGF-1(1-2):FGF-2(3-4):β-gal (Fig. 3, A and B). In addition, the majority of stable NIH 3T3 cell transfectants exhibited similar staining as shown in Fig. 2B by X-gal staining and by β-gal immunofluorescence (data not shown). These data suggest that the domain comprising residues 84–154 from FGF-1 may be involved in the regulation of FGF-1 secretion and that the corresponding domain in FGF-2 is able to repress the release of the FGF reporter gene chimera in response to heat shock.

FGF-2 (17, 18) and FGF-3 (19, 20) are able to utilize alternative 5'-CUG translation initiation start sites, which are able to direct the intracellular traffic of these translation products to the nucleus. Because FGF-1 does not contain this feature
(21), we sought to determine whether the FGF-1 cytosol retention domain would repress the ability of the multiple NLS domain encoded by the FGF-2 alternative 5'-CUG translational initiation sequence to traffic FGF-1 to the nucleus. As shown in Fig. 2, B and C, the addition of this multiple NLS domain to the synthetic FGF-2 gene inhibited the ability of the reporter gene to remain cytosol-associated and directed the FGF-2:β-gal chimeric protein to the nucleus in the NIH 3T3 cell transfectants. Likewise, the replacement of cassettes 1 and 2 in FGF-2 with the corresponding cassettes from FGF-1 also enabled the reporter gene to translocate to the nucleus (Fig. 2, B and C).

However, the replacement cassettes 3 and 4 in FGF-2 with the corresponding cassettes from FGF-1 not only inhibited the nuclear traffic of the reporter gene (Fig. 2, B and C) but enabled the chimera to be released from the NIH 3T3 cell transfectants in response to heat shock (Fig. 2D). These data suggest that the domain in FGF-1 responsible for cytosol retention is able to repress the nuclear trafficking properties of the multiple NLS domain present in the alternative 5'-CUG-initiated high molecular weight FGF-2 translation product.

In order to determine whether nuclear-associated FGF-1 is able to access the FGF-1 secretion pathway, an additional construct was prepared in which the entire SV40T ORF was ligated in-frame at the 5'-end of the FGF-1:β-gal ORF, and the intracellular traffic and stress-induced release of the translation product was assessed. As shown in Figs. 2C and 3, A and B, the SV40T:FGF-1:β-gal chimera was nucleus-associated and was not released in response to heat shock following expression in NIH 3T3 cells. These data suggest that the domain responsible for FGF-1 cytosol retention may have evolved to ensure participation of FGF-1 in the temperature stress-induced secretion pathway.

The FGF prototype cassette shuffle strategy has enabled us not only to confirm the specificity of the FGF-1 secretion pathway induced by temperature stress (4–6), but it has also enabled us to determine the importance between the FGF prototype carboxyl-terminal domains as a structural correlate for this specificity. Indeed, the similarities between the FGF prototypes within this domain are accentuated by the ability of these sequences to act as potential cytosol retention domains that are able to repress the function of the NLS in their respective AUG-initiated translation products. However, differences within this domain between the FGF prototypes are revealed by the ability of the FGF-1-derived domain to repress the function of the NLS in the 5'-CUG-initiated high molecular weight FGF-2 translation product.

Previous FGF-1 mutagenesis studies have identified the importance of Cys30 in the formation of the stress-induced FGF-1 homodimer (6), and we have proposed that temperature stress
may enable Cys30 to gain access to solvent for homodimer formation. Indeed, Cys30 is conserved in FGF-2 (1); yet, interestingly, FGF chimeric proteins containing the carboxyl-terminal domain from FGF-2 also contain Cys30 but are not secreted in response to heat shock. Thus, we suggest that the carboxyl-terminal domain in FGF-2 may prevent solvent accessibility of Cys30 for FGF-2 homodimer formation under temperature stress conditions, and this feature may be involved in determining the specificity between the FGF prototypes for secretion in response to heat shock. This is consistent with the ability of FGF-1 but not FGF-2 to undergo Cu2+-induced homodimer formation (9) and heparin-induced conformational changes (11), although FGF-1 and FGF-2 are both Cu2+-and heparin-binding proteins (9).

The forced nuclear traffic of intracellular FGF-1 by SV40 T is also noteworthy, since it was difficult to obtain stable NIH 3T3 cell transfectants using the NLS from either the SV40 large T antigen or histone 2B genes to force the nuclear traffic of intracellular FGF-1 as a result of their diminished proliferative capacity in vitro (data not shown). Indeed, this is consistent with the reports that the nuclear traffic of intracellular FGF-2 (22) and FGF-3 (23) as a result of alternative 5'-CUG translation initiation also results in a reduction of proliferative capacity in vitro and may help explain why the AUG-initiated FGF prototype translation products are localized in the cytosol. Since FGF-1 gains access to the nuclear compartment as a result of its receptor-dependent traffic from the cell surface (12), we suggest that the nuclear traffic of FGF-1 during the entire G1 period of the cell cycle (24) may function, in part, to restrict endocytotic FGF-1 from participation in the FGF-1 secretion pathway.