The Cysteine Residue Responsible for the Release of Fibroblast Growth Factor-1 Resides in a Domain Independent of the Domain for Phosphatidylserine Binding*

(Received for publication, August 28, 1995, and in revised form, October 11, 1995)

Francesca Tarantini, Susan Gamble‡, Anthony Jackson and Thomas Maclagл
From the Department of Molecular Biology, Holland Laboratory, American Red Cross, Rockville, Maryland 20855

Fibroblast growth factor (FGF)-1 lacks a classical signal sequence to direct its secretion yet utilizes high affinity cell surface receptors to signal its heparin-dependent angiogenic and neurotrophic activities. We have previously reported that FGF-1 is released in response to temperature stress as a latent homodimer through a pathway that is potentiated by the Golgi inhibitor, brefeldin A (Jackson, A., Tarantini, F., Gamble, S., Friedman, S., and Maciag, T. (1995) J. Biol. Chem. 270, 33-36). In an attempt to further characterize this unconventional secretion mechanism, we sought to define the Cys residue(s) critical for FGF-1 dimer formation and release and to determine whether FGF-1 can associate with known phospholipid components of organelle or plasma membranes, which may be disturbed by brefeldin A. Utilizing FGF-1 Cys mutants, we were able to demonstrate that residue Cys30 is critical for FGF-1 release in response to heat shock. In addition, using solid phase phospholipid binding assays we demonstrate that FGF-1 is able to specifically associate with phosphatidylinerine (PS). Heparin inhibits the association between FGF-1 and PS, and synthetic peptide competition assays suggest that the PS-binding domain of FGF-1 lies between residues 114 and 137. These observations indicate that FGF-1 may be able to associate with the PS component of organelle and/or plasma membranes and that the domains responsible for FGF-1 homodimer formation and PS binding are structurally distinct.

*This work was supported in part by National Institutes of Health Grants HL32348 and HL44336 (to T.M.) and by a fellowship from the University of Florence (to F.T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the Department of Genetics, George Washington University, Washington, D.C.
§To whom correspondence should be addressed: Dept. of Molecular Biology, Holland Laboratory, American Red Cross, 15601 Crabb's Branch Way, Rockville, MD 20855. Tel.: 301-738-0653; Fax: 301-738-0465.

1The abbreviations used are: FGF, fibroblast growth factor; EGF, epidermal growth factor; ER, endoplasmic reticulum; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TBS, Tris-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.
(PE), and L-α-phosphatidyl-DL-glycerol (PG) were purchased from Sigma. Synthetic peptides corresponding to FGF-1 residues 114-137 (KHKAEKNWFVGLKNGSCKGRPT) and 15-29 (FNLPPNGKYPK-LLY) were synthesized using an Applied Biosystems (Foster City, CA) model 431A peptide synthesizer and purified by reversed-phase high pressure liquid chromatography. Amino acid analysis was conducted using an Applied Biosystems Pico-Tag system and amino acid sequencing was done using an Applied Biosystems model 477A protein sequencer.

Plasmid Construction—FGF-1C30S/C97S and FGF-1 Cys-free in the eukaryotic expression vector pMEXneo and FGF-1, FGF-1 Cys-free, and FGF-1 C131S in the prokaryotic expression vector PETc were obtained as described previously (4). There are three different constructs in which two of the three Cys residues present in the human FGF-1 sequence (Cys30, Cys97, and Cys131) were alternatively replaced by Ser. These FGF-1 mutants, each containing a single Cys residue, were inserted into the pMEXneo expression vector (11) and named FGF-1 Cys30 (C97S/C131S), FGF-1 Cys97 (C30S/C131S), and FGF-1 Cys131 (C30S/C97S), respectively. The FGF-1 Cys30 (C97S/C131S) construct, in which only the Cys30 of the protein was conserved, was obtained as follows. The FGF-1 Cys-free/pMEXneo plasmid was purified using a Qiagen column (Qiagen, Inc.) and digested with BamHI/EcoRI restriction enzymes (Boehringer Mannheim); the 867-base pair band was purified by agarose gel electrophoresis and subcloned into BamHI/ EcoRI-digested FGF-1C121-154/pMEXneo, gel-purified prior to ligation. The ligation was conducted with T4 ligase (Life Technologies, Inc.), and the product was analyzed by the dideoxy sequencing method (Sequenase, U.S. Biochemical Corp.). The FGF-1 Cys97 (C30S/C131S) construct, in which only Cys97 was conserved, was obtained by ligation of the 867-base pair fragment from the FGF-1 C131S/pET3c plasmid by digestion with BamHI/EcoRI into the BamHI/EcoRI compatible ends of the FGF-1 Cys-free/pMEXneo plasmid. Purification of plasmids, digestion, phosphorylation of bands, ligation, and analysis of the product were conducted as described previously (4). For the FGF-1 Cys131 (C30S/C97S) construct, in which only Cys131 was conserved, the mutated sequence was cloned first into the pET3c vector (4). To obtain the FGF-1 C30S/C97S/pET3c construct, FGF-1/pET3c and FGF-1 Cys-free/pET3c were digested with the restriction enzymes, SphI. The SphI-digested fragment, gel-purified from FGF-1 Cys-free/pET3c, was ligated into the SphI-compatible ends of FGF-1/pET3c. Purification, ligation, and analysis of the product were conducted as described previously (4). The FGF-1 C30S/C97S/pET3c construct was digested with BamHI/EcoRI, and the 867-base pair fragment was gel-purified and subcloned into the BamHI/EcoRI-compatible ends of FGF-1 Cys-free/pMEXneo.

Transfection, Heat Shock, and Immunoblot Analysis—NIH 3T3 cells were maintained in DMEM, 10% (v/v) fetal bovine serum (FBS). When the cells were 60-70% confluent, fresh medium was added, and the transfection was conducted using the calcium phosphate precipitation method with either 5 μg of Qiagen-purified FGF-1 Cys30, FGF-1 Cys97, or FGF-1 Cys131 DNA as described (4). The medium was changed with fresh DMEM containing 10% (v/v) FBS after 24 h, and 48 h, and at the time of transfection the selection was started with DMEM, 10% (v/v) FBS containing 800 μg/ml genetically. The medium containing the antibiotic was changed every 2nd day, and geneticin-resistant colonies were isolated and grown separately in DMEM containing 10% (v/v) FBS and 400 μg/ml geneticin. The individual NIH 3T3 cell transfectants were grown to confluence and subjected to heat shock (42 °C, 2 h) as described previously (4). Conditioned media were collected, and latent FGF-1 was activated with 0.1% (w/v) dithiothreitol and fractionated by heparin-Sepharose chromatography, and the 1.5 mM NaCl elution fractions were resolved by 12.5% (w/v) SDS-polyacrylamide gel electrophoresis and subjected to FGF-1 immunoblot analysis as described (4).

To determine the FGF-1 lipid-binding ability, FGF-1 was added to the liposome assay. Phospholipid binding assays were performed using polystyrene flat bottom wells (Dynatech Labs) as described (12). Briefly, PS, PC, PI, PE, and PG were dissolved in ice-cold methanol to the desired concentrations (ranging from 1 to 10 μg/ml) and sonicated twice at 4°C for 5-10 s, and 100 μl of individual phospholipid was used to coat each well. Wells coated with maleimide as a control were used as a control, and the wells were kept at 25°C for 18 h to evaporate the methanol. Nonspecific binding was minimized by incubation of the phospholipid-coated wells with TBS, containing 0.5% (v/v) gelatin, for 1 h at 37°C. The wells were washed three times with TBS containing 0.05% (v/v) Tween 20, and the binding assay was performed in TBS. For the heparin competition experiments, TBS containing 4 units/ml heparin (The Upjohn Co.), pH 7.4, was used as a binding buffer. The labeled protein, 125I-FGF-1 or 125I-EGF, was added to the binding buffer, and the wells were incubated for 1 h at 37°C, aspirated, and washed three times with TBS containing 0.05% (v/v) Tween 20. The amount of radiolabeled protein bound to the phospholipid was quantitated by an automatic γ counter. When the phospholipid binding assay was performed under conditions of increased ionic strength, 25-150 mM NaCl was added to the binding buffer prior to the addition of 125I-FGF-1. Similarly, the synthetic peptides were also added to the well prior to the addition of 125I-FGF-1 during the synthetic peptide competition experiments. RESULTS AND DISCUSSION

We have previously reported that FGF-1 is released from FGF-1-transfected NIH 3T3 cells in response to heat shock as a latent homodimer, and we were able to utilize a FGF-1 Cys-free mutant to demonstrate the functional importance of Cys residues (4). Because two of the three Cys residues (Cys30 and Cys97) present in the human FGF-1 sequence are conserved among all known species of FGF-1 (1), we sought to establish whether a critical Cys residue exists for FGF-1 homodimer formation during the stress-induced FGF-1 secretion. Using the recombinant circle polymerase chain reaction, we created three FGF-1 mutants in which two of three Cys residues were converted to Ser (Fig. 1A). Stable NIH 3T3 transfectants for each of these mutants were obtained, and their expression levels were examined by immunoblot analysis. As shown in Fig. 1B, the level of FGF-1 mutant expression was comparable with the expression of FGF-1 in the wild-type NIH 3T3 transfectants. These transfected cells were individually examined for their ability to release FGF-1 in response to heat shock. As shown in Fig. 1B, extracellular FGF-1 was observed in the conditioned medium from FGF-1 wild-type and FGF-1 Cys30 NIH 3T3 transfectants but not in the media conditioned by heat shock from either the FGF-1 Cys-free, FGF-1 Cys97, or FGF-1 Cys131 transfectants. These data suggest that Cys30, positioned near the NH2-terminal nuclear translocation signal in FGF-1 (8), is critical for the entry of the protein into the heat shock-induced FGF-1 secretion pathway.
were analyzed for their ability to interact with FGF-1. The acidic phospholipids, PS, PI, and PG, and the neutral phospholipids, PC and PE, were evaluated in solid phase phospholipid binding assays to assess the ability of the protein to bind phospholipid-coated polystyrene wells. In these assays, 125\textsuperscript{I}-

FGF-1 was able to associate with PS but not with either PE, PG, PC, or PE (Fig. 2A). In contrast, 125\textsuperscript{I}-EGF, also an acidic protein of similar size (13), was unable to interact with any of the phospholipids examined (data not shown). The association between 125\textsuperscript{I}-FGF-1 and PS was saturable and dependent on the concentration of PS with half-maximal binding occurring at approximately 4 \mu g/ml (Fig. 2B).

An analysis of the FGF-1 protein sequence for the presence of a consensus sequence for phospholipid binding (14) revealed a putative structural motif in the carboxyl-terminal region of FGF-1 between residues 112 and 132. Because of the presence of several basic amino acids in this sequence, we anticipated that the interaction between PS and FGF-1 may involve ionic bonds. Indeed, the interaction between 125\textsuperscript{I}-FGF-1 and PS was reduced by increasing the ionic strength of the binding buffer (Fig. 3A). We also sought to determine whether this domain was indeed responsible for PS binding. Using a synthetic peptide containing residues 114–137 from the FGF-1 sequence, it was possible to compete for the PS binding of 125\textsuperscript{I}-FGF-1 in the solid phase binding assay (Fig. 3B). In contrast, a second peptide corresponding to FGF-1 residues 15–29, which includes the basic residues involved in nuclear translocation (8), was not able to compete with 125\textsuperscript{I}-FGF-1 for PS binding (Fig. 3B).

Interestingly, the crystallographic structure of FGF-1\textsuperscript{2} demonstrates that Lys\textsuperscript{127} and Lys\textsuperscript{132} of the putative phosphatidyserine binding domain are positioned around a sulfate molecule from the freezing medium (15). Since this sulfate may be interchangeable with phosphate, it is possible that Lys\textsuperscript{127} and Lys\textsuperscript{132} may be responsible for the binding of FGF-1 to the phosphatidylserine present in phosphatidylserine. Moreover, because the heparin-binding domain has been localized to a domain within the carboxyl-terminal region of FGF-1 (16), we asked whether heparin could compete with PS for 125\textsuperscript{I}-FGF-1 binding. Indeed, heparin was able to inhibit the interaction between 125\textsuperscript{I}-FGF-1 and PS (Fig. 3C). Several proteins have been described that have Ca\textsuperscript{2+}-dependent and -independent phospholipid-binding domains, and those that bind specifically to acidic phospholipids include members of the annexin family (17). Annexins have been implicated in membrane trafficking, signal transduction, cell-cell and cell-matrix interactions, inhibition of coagulation, and other activities, including Ca\textsuperscript{2+}-regulated exocytosis and membrane fusion (17). Neuromodulin and neuroregulin (18, 19) are among the proteins sharing the putative phospholipid-binding consensus sequence with FGF-1 and are brain-specific protein kinase C substrates. Interestingly, several functions have been proposed for these proteins, including an involvement in neurotransmitter release (19). Synaptotagmin, a component of exocytic vesicles whose functional domains as a trafficking macromolecule are cytosol-oriented, has been described as a Ca\textsuperscript{2+}-dependent PS-binding protein (20) and is also involved in the recycling of clathrin-coated endocytic vesicles (21). While the ability of FGF-1 to interact with PS appears to be Ca\textsuperscript{2+}-independent and FGF-1 does not contain a Ca\textsuperscript{2+}-binding structural motif, it is likely that FGF-1 is not able to bind Ca\textsuperscript{2+}. However, FGF-1 is well described as a Cu\textsuperscript{2+}-binding protein (10, 22).

\textsuperscript{2} Amino acid numbers refer to FGF-1 residues 1–154 as defined by the open-reading frame cDNA sequence (1).

---

**FIG. 2.** Solid phase 125\textsuperscript{I}-FGF-1 phospholipid binding assay. A, phosphatidylycerine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PEA), phosphatidylserine (PS), and phosphatidylglycerol (PG) diluted in methanol at a final concentration of 8 \mu g/ml were used to coat the wells. Wells coated with methanol alone served as a negative control. Nonspecific binding was blocked with 0.5% (w/v) gelatin, and the wells were washed with 0.05% (v/v) Tween 20. The binding of 125\textsuperscript{I}-FGF-1 was performed as described under “Experimental Procedures,” and the data are reported as counts/min bound per well. B, PS diluted in methanol at a final concentration of 1, 3, 6, and 9 \mu g/ml was used to coat the wells. Wells coated with methanol alone served as a negative control. Nonspecific binding was blocked with 0.5% (w/v) gelatin, and the wells were washed with 0.05% (v/v) Tween 20. The binding of 125\textsuperscript{I}-FGF-1 was performed as described under “Experimental Procedures,” and the data are reported as counts/min bound per well.

**FIG. 3.** The heparin, ionic strength, and structural dependence of the association between 125\textsuperscript{I}-FGF-1 and phosphatidylycerine. A, PS diluted in methanol at a final concentration of 8 \mu g/ml was used to coat the wells. Wells coated with methanol alone served as a negative control. Nonspecific binding was blocked with 0.5% (w/v) gelatin, and the wells were washed with 0.05% (v/v) Tween 20. The binding was performed as described under “Experimental Procedures” except that increasing concentrations of NaCl, ranging between 25 and 150 mM, were added to the binding buffer before incubation with 125\textsuperscript{I}-FGF-1. Data are reported as counts/min bound per well. B, PS diluted in methanol at a final concentration of 8 \mu g/ml was used to coat the wells. Wells coated with methanol alone served as a negative control. Nonspecific binding was blocked with 0.5% (w/v) gelatin, and the wells were washed with 0.05% (v/v) Tween 20. The binding was performed as described under “Experimental Procedures” except that peptid e 114–137 and peptide 15–29 at 5, 10, and 50 ng/well were added to the binding buffer prior to the addition of 125\textsuperscript{I}-FGF-1. Data are reported as counts/min bound per well. C, PS diluted in methanol at a final concentration of 8 \mu g/ml was used to coat the wells. Wells coated with methanol alone served as a negative control. Nonspecific binding was blocked with 0.5% (w/v) gelatin, and the wells were washed with 0.05% (v/v) Tween 20. The binding was performed with 125\textsuperscript{I}-FGF-1 in TBS with and without 4 units/ml heparin, and the data are reported as counts/min bound per well. 1, control, PS-free with 125\textsuperscript{I}-FGF-1; 2, PS with 125\textsuperscript{I}-FGF-1 without heparin in TBS; 3, heparin and 125\textsuperscript{I}-FGF-1 without PS; and 4, PS, heparin, and 125\textsuperscript{I}-FGF-1 in TBS.
also interesting that Cu^{2+} is able to oxidize the formation of FGF-1 homodimer (10), and since the FGF-1 homodimer is released in response to heat shock, Cu^{2+} may be involved in the regulation of the FGF-1 secretion pathway. This may be particularly appropriate for the FGF-1 release mechanism since the domain responsible for FGF-1 homodimer formation resides near the NH_{2} terminus while the domain involved in PS binding resides near the COOH terminus of FGF-1.

Recently, Mach and Middaugh (9) reported that a thermally modified state of the FGF-1 protein is able to interact with negatively charged lipid vesicles at a neutral pH. In this system, a change in protein structure from its native, globular state to a partially unfolded "molten globular" state is required to obtain the binding of FGF-1 to unilamellar vesicles (9). Interestingly, the temperature range that is required to obtain the unfolded state (9) is consistent with the temperature that is able to stimulate the release of FGF-1 (3), and our data are also consistent with the observation that FGF-1 is able to bind to acidic phospholipids (9), with a specific affinity for PS. Further, the crystal structure for FGF-1 also predicts that residue Cys^{30} may not be accessible to solvent and may also require a significant structural rearrangement for it to participate in intermolecular disulfide bond formation. Indeed, our data argue that heat shock may enable FGF-1 to unfold and thus not only provide access of residue Cys^{30} for intermolecular disulfide bond formation but may enable FGF-1 to disrupt membrane structural integrity attaining characteristics of "molten globules" as described by Bychkova et al. (23) and van der Goot et al. (24) for protein-membrane insertion and/or translocation. Since "molten globule" character has been assigned to FGF-1 as an acidic phospholipid-dependent state (9, 25), it is likely that the "molten globule" character of FGF-1 may be specific for PS.

It is intriguing that the PS-binding domain in FGF-1 resides near the carboxyl-terminal region of the protein. It is possible that under conditions of temperature stress, the FGF-1 secretion pathway is activated and thermally modified FGF-1 undergoes dimerization through the Cys residue at position 30. Under these conditions, FGF-1 may still be able to maintain its ability to interact with the PS component of membranes through the carboxyl-terminal region. The interaction of FGF-1 with membranes may stabilize the partially unfolded state of the protein exposing domains that may be involved in the association of FGF-1 with other phospholipid-related molecules and/or other phospholipid-binding proteins. Alternatively, the interaction of the FGF-1 homodimer with PS may be dependent upon a structural rearrangement, which may provide solvent accessibility to PS. Due to the prevalent representation of PS in the inner face of plasma membranes it is likely that this cytosolic surface represents the target of FGF-1-PS binding activity. However, the significance of this protein-phospholipid interaction in relation to the transport of FGF-1 through the plasma membrane during temperature-induced secretion remains to be determined.

Acknowledgments—We thank K. Wawzinski for expert secretarial support and W. H. Burgess for the synthetic peptides, S. Garfinkel for critical review of the manuscript, and the editorial staff for the astute comment concerning the solvent-inaccessible position of FGF-1 Cys^{30}.

REFERENCES
1. Burgess, W. H., and Maciag, T. (1989) Annu. Rev. Biochem. 58, 575–606
2. Miyamoto, M., Naruo, K.-I., Seko, C., Matsumoto, S., Kondo, T., and Kurokawa, T. (1993) Mol. Cell. Biol. 13, 4251–4259
3. Jackson, A., Friedman, S., Zhan, X., Engleka, K. A., Forough, R., and Maciag, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10691–10695
4. Jackson, A., Tarantini, F., Gamble, S., Friedman, S., and Maciag, T. (1995) J. Biol. Chem. 270, 33–36
5. Lippincott-Schwartz, J., Yuan, L.-C., Bonifacio, J. S., and Klausner, R. D. (1989) Cell 56, 801–813
6. Sato, S., Burdett, J., and Hughes, R. C. (1993) Exp. Cell Res. 207, 8–18
7. Endicott, J. A., and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171
8. Zhan, X., Hu, X. G., Friedman, S., and Maciag, T. (1992) Biochem. Biophys. Res. Commun. 188, 982–991
9. Mach, H., and Middaugh, C. R. (1995) Biochemistry 34, 9913–9920
10. Engleka, K. A., and Maciag, T. (1992) J. Biol. Chem. 267, 11307–11315
11. Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T., and Barbadic, M. (1989) Mol. Cell. Biol. 9, 24–33
12. Ortel, T. L., Devore-Carter, D., Quinn-Allen, M. A., and Kane, W. H. (1992) J. Biol. Chem. 267, 4189–4198
13. Carpenter, G., and Cohen, S. (1990) J. Biol. Chem. 265, 7709–7712
14. Vondrnikov, A. V., Bogatcheva, N. V., and Gusev, N. B. (1992) Biochem. J. 284, 911–916
15. Zhu, X., Komiyama, C., Chirino, A., Fain, G. S., Fox, G. M., Arakawa, T., Hsu, B. T., and Red, D. C. (1991) Science 251, 90–93
16. Burgess, W. H., Shaheen, A. M., Hampton, B., Donohue, P. J., and Winkles, J. A. (1991) J. Cell. Biol. 113, 131–138
17. Raynal, P., and Pollard, H. B. (1994) Biochim. Biophys. Acta 1197, 63–93
18. Houbre, D., Devore-Carter, D., Guo, K. J., and Baudin, J. (1991) J. Biol. Chem. 266, 7121–7131
19. Baudin, J., Devore-Carter, D., Van Dorselaer, A., Black, D., and Matthes, H. W. D. (1991) J. Biol. Chem. 266, 229–237
20. Perin, M. S., Fried, V. A., Mignery, G. A., Jahn, R., and Sudhof, T. C. (1990) Nature 345, 260–263
21. Zhang, J. Z., Davletov, B. A., Sudhof, T. C., and Anderson, R. G. W. (1994) Cell 78, 751–760
22. Shing, Y., Folkman, J., Weisz, P. B., Joullie, M. M., and Ewing, W. R. (1990) J. Biol. Chem. 265, 108–111
23. Bychkova, V. E., Pain, R. H., and Pitsyn, O. B. (1988) FEBS Lett. 238, 231–234
The Cysteine Residue Responsible for the Release of Fibroblast Growth Factor-1
Resides in a Domain Independent of the Domain for Phosphatidylserine Binding
Francesca Tarantini, Susan Gamble, Anthony Jackson and Thomas Maciag

J. Biol. Chem. 1995, 270:29039-29042.
doi: 10.1074/jbc.270.49.29039

Access the most updated version of this article at http://www.jbc.org/content/270/49/29039

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 25 references, 11 of which can be accessed free at http://www.jbc.org/content/270/49/29039.full.html#ref-list-1