Heparin-Copper Biaffinity Chromatography of Fibroblast Growth Factors*

Yuen Shing
From the Departments of Surgery and Biological Chemistry, The Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115

A novel method is described to separate and identify the various forms of fibroblast growth factor (FGF) based on their differential affinities for both heparin and copper. FGFs were extracted from bovine hypothalamus and purified by batchwise adsorption to heparin-Sepharose. The partially purified FGFs were then applied to an affinity column prepared by mixing equal portions of heparin-Sepharose and copper-Sepharose. The column was rinsed consecutively with the following four reagents: (i) 2 M NaCl, (ii) 0.6 M NaCl, (iii) 0.6 M NaCl plus 10 mM imidazole, and (iv) 0.6 M NaCl. FGFs were then eluted with a linear NaCl/imidazole gradient (from 0.6 M NaCl without imidazole to 2 M NaCl plus 10 mM imidazole). Fractions eluted from the column were analyzed by sodium dodecyl sulfate-gel electrophoresis with silver staining and electrophoretic immunoblot using site-specific antibodies against basic and acidic FGF. The results demonstrate that it is possible to resolve from hypothalamus at least two basic FGF species (with Mr values of 19,000 and 18,000) and three acidic FGF species (with Mr values of 18,000, 16,400, and 16,600). These findings indicate that heparin-copper biaffinity chromatography may have wide applicability in the study of the structure and activity of FGFs.

FGFs that have a broad spectrum of target cells in culture have been reported to induce vascular growth (angiogenesis) in vivo (8, 21). In attempting to further understand the mechanism of these activities and the heparin affinity of FGFs, Folkman and Klagsbrun (3) speculated that the binding of FGFs to heparin might be copper-dependent. This idea was based on several reports in the literature indicating that copper levels in tissue could somehow modulate the intensity of the neovascular response to a given angiogenic stimulus. Such reports included the observations that (i) copper ions augment endothelial cell mitogens in vitro (22), (ii) white of molar copper-deficient diet are unable to mount an angiogenic response to prostaglandin E1 (23) and to implants of a variety of human brain tumors (24), (iii) both ceruloplasmin and heparin become angiogenic when complexed to copper, but not when deprived of copper (25), and (iv) heparin can act as a copper chelator (26, 27). To test this hypothesis, I found instead that while copper was not required for the binding of FGFs to heparin, FGFs appear to have separate binding sites for copper and for heparin. By taking advantage of the unique interactions between heparin, copper, and FGFs, I was able to develop a novel method to resolve the multiple forms of FGFs, based upon biaffinity chromatography, the details of which are presented here.

EXPERIMENTAL PROCEDURES

Isolation of FGFs from Hypothalamus—Bovine hypothalami (100 g) obtained from Pel-Freez were homogenized in 300 ml of 0.15 M (NH4)2SO4 at pH 6 and extracted by stirring at 4°C for 2 h. The crude extract was centrifuged at 15,000 × g for 1 h, and the supernatant solution was loaded directly onto a heparin-Sepharose column (1.5 × 12 cm) pre-equilibrated with 0.6 M NaCl in 10 mM Tris, pH 7. The column was rinsed with 300 ml of 0.6 M NaCl in 10 mM Tris, pH 7. FGFs were subsequently eluted with 40 ml of 2 M NaCl in the same buffer.

Heparin-Copper Biaffinity Chromatography—The heparin-copper biaffinity column was prepared by mixing 3.5 ml each of heparin-Sepharose (Pharmacia LKB Biotechnology Inc.) and chelating Sepharose (Pharmacia LKB Biotechnology Inc.) that had been saturated with copper(II) chloride. The sample (40 ml) of FGF partially purified by batchwise adsorption to heparin-Sepharose was applied directly to this blue color biaffinity column (1 × 9 cm) pre-equilibrated with 2 M NaCl, 10 mM Tris, pH 7. The column was rinsed consecutively with 40 ml each of the following four reagents in 10 mM Tris, pH 7: (i) 2 M NaCl, (ii) 0.6 M NaCl, (iii) 0.6 M NaCl plus 10 mM imidazole, and (iv) 0.6 M NaCl. Finally, FGFs were eluted at a flow rate of 20 ml/h with a linear NaCl/imidazole gradient from 100 ml of 0.6 M NaCl without imidazole to 100 ml of 2 M NaCl plus 10 mM imidazole in 10 mM Tris, pH 7. Fractions (10 ml) were collected and assayed for growth factor activity.

Growth Factor Assay—FGF activity was assessed by measuring the incorporation of [3H]thymidine into the DNA of quiescent, confluent monolayers of BALB/c mouse 3T3 cells in 96-well plates as previously described (28). One unit of activity was defined as the amount of growth factor required to stimulate half-maximal DNA synthesis in 3T3 cells (about 10,000 cells/0.25 ml of growth medium/well). For

* This work was supported by a grant to Harvard University from Takeda Chemical Industries, Ltd. 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.

1 The abbreviations used are: bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; PFG, fibroblast growth factor; SDS, sodium dodecyl sulfate.
determination of specific activities, protein concentrations of crude extract and the active fraction eluted from heparin-Sepharose column were determined by the method of Lowry et al. (29). Protein concentrations of the pure FGFs eluted from biaffinity column were estimated by comparing the intensities of silver-stained FGF bands on SDS-polyacrylamide gel electrophoresis to those of the protein standards, which were prepared by a serially 2-fold dilution of β-lactoglobulin from 800 ng to 50 ng.

**SDS-Polyacrylamide Gel Electrophoresis—**FGF-containing samples were analyzed by electrophoresis on 15% polyacrylamide gels as described by Laemmli (30). The polypeptide bands were visualized by a silver stain (31).

**Preparation of Anti-FGF Polycolonal Antibodies—**Peptide fragments corresponding to position 1-12 (Pro-Ala-Leu-Pro-Glu-Asp-Gly-Glu-Ser-Gly-Ala-Phe) of basic FGF (8) and position 59-90 (Thr-Glu-Thr-Gly-Thr-Thr-Arg-Leu-Val-Leu-Met-Asp-Thr-Asp-Glu-Leu-Leu-Arg-Pro-Thr-Glu-Thr-Pro-Ser-Glu-Cys-Leu-Val-Arg-Glu-Arg-Glu) of acidic FGF (9) were synthesized by solid phase methods (32,33) using a silver ion as a cross-linking agent (34). Rabbits were injected at multiple dorsal intradermal sites with 500 μg each of keyhole limpet hemocyanin from 800 ng to 50 ng.

**Preparation of Anti-FGF Polycolonal Antibodies—**Peptide fragments were conjugated to keyhole limpet hemocyanin using maleimidohexanoyl-N-hydroxysuccinimide ester as a cross-linking agent (34). Rabbits were injected at multiple dorsal intradermal sites with 500 μg each of keyhole limpet hemocyanin- peptide conjugate emulsified with complete Freund’s adjuvant. Animals were boosted regularly at 3-6-week intervals with 200 μg of KLH-peptide conjugate emulsified in incomplete Freund’s adjuvant. The titer of the antisera after the second booster injection was about 1:15,000 to 1:50,000 as determined in an enzyme-linked immunosorbent assay using unconjugated peptide as the antigen.

**RESULTS**

Hypothalamus is a rich source of mitogens. Approximately $7 \times 10^8$ units of growth factor activity were extracted from 100 g of tissue. More than 95% of this activity bound to heparin-Sepharose in 0.6 M NaCl and were eluted with 2 M NaCl. A single passage of the bovine hypothalamic crude extract over 20 ml of heparin-Sepharose resulted in approximately 100-fold purification. FGFs were further purified by chromatography on a heparin-copper biaffinity column as described under “Experimental Procedures.” The results were summarized in Table I. This purification was possible because of the unique affinities of the multiple forms of FGF for heparin and copper. The principle of this biaffinity chromatography is diagrammatically illustrated in Fig. 1. The success of this method depended on thorough rinsing of the column with alternate solutions of 2 M NaCl and of 10 mM imidazole in the presence of 0.6 M NaCl before starting the NaCl/imidazole gradient. After the rinses, FGFs were eluted with a linear NaCl/imidazole gradient and the growth factor activities in the eluates were measured (Fig. 2, panel A). Growth factor activity was detected starting at fraction 9, which corresponded to about 1.3 M NaCl and 5 mM imidazole.

Analysis of the eluates from fraction 9 to fraction 20 by SDS-polyacrylamide gel electrophoresis followed by silver staining revealed the existence of multiple protein bands (Fig. 2, panel B). Further analysis of these protein bands by electrophoretic immunoblot (Western blot) using site-specific polyclonal antibodies raised against either bFGF (Fig. 2, panel C), or aFGF (Fig. 2, panel D) demonstrated that it was possible to resolve from hypothalamus at least two bFGF species with $M_r$ values of 19,000 and 18,000 and three aFGF species with $M_r$ values of 18,000, 16,400, and 15,600. Basic FGF (fractions 10 to 13) and acidic FGF (fractions 14 to 20) triggered a half-maximal response at concentrations of 0.24 and 1 ng/ml, respectively. Interestingly, they appear to elute from the heparin-copper biaffinity column in an order opposite of that from the heparin affinity column alone (28). This result indicates that aFGF species probably have stronger affinities for copper than those of the bFGF species.

**DISCUSSION**

Basic FGF was first sequenced and initially reported as a 146-amino acid polypeptide (8). Subsequently, both amino-terminally extended (13, 14) and truncated (15-17) forms of bFGF were demonstrated in a variety of cells and tissues. Recently, the gene for bovine bFGF has been cloned (37), and the nucleotide sequence predicts a 155-amino acid bFGF translation product. This is consistent with the finding of the existence of a 154-amino acid form of bFGF which is extended by 8 residues on the amino-terminal side (13). Based on these facts and the apparent molecular weights of FGFs shown in Fig. 2, it is likely that the $M_r = 18,000$ band of hypothalamic bFGF species corresponds to the 154-amino acid form of bFGF. This raises an interesting possibility that the $M_r = 19,000$ band of hypothalamic bFGF species might correspond to a larger precursor of bFGF.

A primary structure of 140 amino acids has been determined for acidic FGF (9-12). Similar to bFGF, both amino-terminally extended and truncated forms of aFGF have been demonstrated (19, 20). Amino acid sequence analysis of a 154-amino acid extended form and a 134-amino acid truncated form of aFGF (19) and the original 140-amino acid of aFGF itself are all in agreement with the gene sequence data (38). These are consistent with the possibility that the $M_r = 18,000$, 16,400, and 15,600 bands of the hypothalamic aFGF species shown in Fig. 2 actually correspond to a 154-amino acid precursor of aFGF, the 140-amino acid aFGF and a 134-amino acid truncated form of aFGF, respectively.

We have previously reported the separation of hypothalamic bFGF and aFGF by heparin-Sepharose affinity chromatography (28). With heparin-copper biaffinity chromatography, the multiple forms of both basic and acidic FGFs can further be resolved and identified. An additional advantage of using the biaffinity column includes its capability of purifying FGFs from the heparin-contaminated sample. Furthermore, the principle of this biaffinity chromatography appar-

### Table I

| Purification step | Total protein | Total activity | Specific activity | Activity recovery | Purification |
|------------------|---------------|----------------|-------------------|------------------|-------------|
| Crude extract*   | 1,302,000     | 680,000        | 0.52              | 100              | 1           |
| Heparin adsorption | 4,100         | 200,000        | 48.08             | 29.4             | 92.5        |
| Heparin-copper biaffinity chromatography | 2.4 | 40,000 | 16,667 | 5.9 | 32,052 |
| Basic FGF        | 12.3          | 52,000         | 4,228             | 7.6              | 8,131       |

*From 100 g of starting tissue.
Elution Reagents

2 M NaCl
→

0.6 M NaCl
→

0.6 M NaCl + 10 mM Imidazole
→

0.6 M NaCl
→

(Gradient)
→

2 M NaCl + 10 mM Imidazole
→

FGF

Bi-affinity Column

Heparin
Copper

FGF

A.

D. Western Blot

(39) Anti-acidic FGF

C. Western Blot

(Anti-basic FGF)

B. Silver Stain

FIG. 1. Diagrammatic illustration of the principle of heparin-copper biaffinity chromatography. When the column as shown in the right panel is initially rinsed with 2 M NaCl after the FGF-containing sample has been loaded, most of the heparin-binding proteins including FGFs are detached from the heparin moiety, but FGFs remain bound to the column because of its affinity for copper. This step eliminates most of the heparin-binding proteins except those which are being bound to copper such as FGFs. On the other hand, when the column is subsequently rinsed with 10 mM imidazole in 0.6 M NaCl, most of the copper-binding proteins including FGFs are detached from the copper moiety, but FGFs remain bound to the column because of its affinity for heparin. This step eliminates most of the copper-binding proteins except those which are being bound to heparin such as FGFs. At this point, virtually most of the non-FGF proteins should have been eluted from the column. The remaining FGFs can then be eluted with a linear NaCl/imidazole gradient and the various forms of FGFs are eluted according to their respective affinity for both heparin and copper.

ently can be applied to purify many other proteins which have affinities for more than one ligand. For example, a lysine-zinc biaffinity column would probably be very useful in identifying and purifying plasminogen activators which have been reported to have affinities for both lysine (39) and zinc (40).

Acidic FGF species appear to have a stronger affinity for copper than those of the bFGF species. This unique characteristic of aFGF allows them to bind more avidly to the copper moiety of the biaffinity column and therefore greatly facilitates their separation from bFGF. However, copper-Sepharose column alone following heparin-Sepharose column is not sufficient for the complete purification of either bFGF or aFGF (data not shown). Recently, copper-Sepharose affinity chromatography has been reported in the purification of a human mammary tumor-derived growth factor, which binds to heparin and has a molecular weight of 16,000 (41). The relationship of this growth factor to the previously reported $M_r = 18,000$ heparin-binding mitogen purified from rat chondrosarcoma (2, 42) and the hypothalamus-derived FGFs demonstrated here is presently unknown. However, in view of their reported characteristics, it is conceivable that they are structurally related although their relationship can only be ascertained by direct comparison of their amino acid sequences.

Despite the apparent importance of FGF, its exact structure has been the subject of some controversy since its identification. Due to the differences in tissue and species sources as well as purification procedures, it has been difficult to make direct comparisons of the various forms of FGFs isolated in separate laboratories. Furthermore, although it has been well established that both basic and acidic FGFs exist in multiple forms, it has been difficult to isolate them in their native forms in a reproducible manner. The observation that various FGF species can be separated and eluted from the biaffinity column in an order of descending molecular weight as shown in Fig. 2 may eventually lead to the development of a chromatographic condition that allows the further identification and complete separation of the native forms of the varied FGF molecules in various tissue and species sources.

Acknowledgments—I am grateful to Dr. Judah Folkman for constant support and encouragement. I also thank Dr. Michael Klagesbrun for helpful discussion, Drs. Bruce Zetter and Patricia D’Amore for critically reviewing this manuscript, and Dr. Joachim Sasse for providing the anti-FGF antibodies.

REFERENCES
1. Shing, Y., Folkman, J., Murray, J., and Klagesbrun, M. (1983) J. Cell Biol. 97, 395a
Heparin, Copper, and FGF

2. Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., and Klagsbrun, M. (1984) *Science* 223, 1296-1298
3. Folkman, J., and Klagsbrun, M. (1987) *Science* 235, 442-447
4. Baird, A., Esch, F., Mormede, P., Ueno, N., Ling, N., Bohlen, P., Ying, S. Y., Wehenpen, W. B., and Gilmour, R. (1986) *Recent Prog. Horm. Res.* 42, 143-205
5. Gospodarowicz, D., Neufeld, G., and Schweiger, L. (1986) *Mol. Cell. Endocrinol.* 46, 187-204
6. Schreiber, A. B., Kenney, J., Kowalski, J., Thomas, K. A., Gimenez-Gallego, G., Rios-Candelore, M., DiSalvo, J., Barrettait, D., Courty, J., Courrois, Y., Moenner, M., Loret, C., Burgess, W. H., Mehlman, T., Friel, T., Johnson, W., and Maceig, T. (1985) *J. Cell Biol.* 101, 1623-1659
7. Lobb, R., Sasse, J., Sullivan, R., Shing, Y., D'Amore, P., Jacobs, J., and Klagsbrun, M. (1986) *J. Biol. Chem.* 261, 1924-1928
8. Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., Kiepper, R., Gospodarowicz, D., Bohlen, P., and Gilmour, R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 6507-6511
9. Gimenez-Gallego, G., Rokdek, J., Bennett, C., Rios-Candelore, M., DiSalvo, J., and Thomas, K. (1985) *Science* 230, 1385-1388
10. Esch, F., Ueno, N., Baird, A., Hill, F., Denoroy, L., Ling, N., Gospodarowicz, D., and Gilmour, R. (1985) *Biochemistry* 24, 945-951
11. Burgess, W. H., Mehlman, T., Marshak, D. R., Fraser, B. A., and Maceig, T. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 7216-7220
12. Strydom, D. J., Harper, J. W., and Lobb, R. R. (1986) *Biochemistry* 25, 945-951
13. Ueno, N., Baird, A., Esch, F., Ling, N., and Gilmour, R. (1986) *Biochemistry* 25, 951-959
14. Klagsbrun, M., Smith, S., Sullivan, R., Shing, Y., Davidson, S., Smith, J. A., and Sasse, J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 1838-1843
15. Gospodarowicz, D., Cheng, J., Lui, G. M., Baird, A., Esch, F., and Bohlen, P. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 287-291
16. Baird, A., Esch, F., Bohlen, P., Ling, N., and Gospodarowicz, D. (1995) *Biochemistry* 24, 945-951
17. Gospodarowicz, D., Baird, A., Cheng, J., Lui, G. M., Esch, F., and Bohlen, P. (1986) *Endocrinology* 118, 82-90
18. Thomas, K. A., Rios-Candelore, M., and Fitzpatrick, S. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 387-391
19. Burgess, W. H., Mehlman, T., Marshak, D. R., Fraser, B. A., and Maceig, T. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 7216-7220
20. Gauthier, P., Frater-Schoen, M., Muller, T., and Bohlen, P. (1986) *Eur. J. Biochem.* 160, 357-361
21. Thomas, K. A., Rios-Candelore, M., Gimenez-Gallego, G., DiSalvo, J., Bennett, C., Rokdek, J., and Fitzpatrick, S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 6409-6413
22. McMahen, B. R., and Reilly, W. (1980) *Exp. Cell Res.* 130, 147-157
23. Ziche, M., Jones, J., and Gullino, P. M. (1982) *J. Natl. Cancer Inst.* 69, 475-482
24. Alpern-Eitan, H., and Brem, S. (1965) *Surg. Forum* 36, 498-500
25. Raju, K. S., Alessandri, G., and Gullino, P. M. (1984) *Cancer Res.* 44, 1579-1584
26. Grushka, E., and Cohen, A. S. (1982) *Anal. Lett.* 15, 1277-1288
27. Stivale, S. S. (1977) *Fed. Proc.* 36, 88-88
28. Klagsbrun, M., and Shing, Y. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 805-809
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265-275
30. Leemomi, U. K. (1970) *Nature* 227, 680-685
31. Oakley, B. R., Kirsch, D. R., and Morris, N. R. (1980) *Anal. Biochem.* 105, 361-363
32. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* 85, 2148-2154
33. Sekikibara, S. (1971) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins* (Weinstein, B., ed) Vol. 1, pp. 51-85, Marcel Dekker, New York
34. Ishikawa, E., Imagawa, M., Hashida, S., Yoshitake, S., Hamaguchi, Y., and Ueno, T. (1983) *J. Immunossed.* 4, 292-327
35. Tombini, H., Stafelini, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350-4354
36. Nakane, F. K. (1968) *J. Histochem. Cytocem.* 16, 557-560
37. Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, A., Friedman, J., Herrud, K. A., Gospodarowicz, D., and Fiddes, J. C. (1986) *Science* 233, 545-548
38. Jaya, M., How, M., Berges, W., Ricca, C. A., Chiu, I. M., Ravera, M. W., O'Brien, S. J., Modii, W. S., Masiag, T., and Dohran, K. N. (1986) *Science* 233, 541-548
39. Radcliffe, R., and Heinze, T. (1975) *Arch. Biochem. Biophys.* 189, 185-194
40. Rijken, D. C., and Collen, D. (1981) *J. Biol. Chem.* 256, 7035-7041
41. Rowe, 1. M., Kasper, S., Shiu, R. P. C., and Friesen, H. G. (1986) *Cancer Res.* 46, 1408-1412
42. Shing, Y., Folkman, J., Hausdenschl, C., Lund, D., Crum, R., and Klagsbrun, M. (1985) *J. Cell. Biochem.* 29, 275-287