Rånby, M. (1982) *Biochim. Biophys. Acta 704*, 461-469.
Riccio, A., Grimaldi, G., Verde, P., Sebastio, G., Boast, S., & Blasi, F. (1985) *Nucleic Acids Res. 13*, 2759-2771.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Sprengers, E. D., & Kluft, C. (1987) *Blood 69*, 381-387.
Steffens, G. J., Günzler, W. A., Ötting, F., Frankus, F., & Flohé, L. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 1043-1058.
Stump, D. C., Lijnen, H. R., & Collen, D. (1986) *J. Biol. Chem. 261*, 17120-17126.
Thorsen, S., & Philips, M. (1984) *Biochim. Biophys. Acta 802*, 111-118.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655-709.
van Mourik, J. A., Lawrence, D. A., & Loskutoff, D. J. (1984) *J. Biol. Chem. 259*, 14914-14921.
van Zonneveld, A. J., Veerman, H., & Pannekoek, H. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4670-4674.
van Zonneveld, A. J., Veerman, H., MacDonald, M. E., van Mourik, J. A., & Pannekoek, H. (1986b) *J. Cell. Biochem. 32*, 169-178.
van Zonneveld, A. J., Veerman, H., Brakenhoff, J. P. J., Aarden, L. A., Cajot, J. F., & Pannekoek, H. (1987) *Thromb. Haemostasis 57*, 82-86.
Verde, P., Stoppelli, M. P., Galeffi, P., Di Nocera, P., & Blasi, F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4727-4731.
Verheijen, J. H., Mullaart, E., Chang, G. T. G., Kluft, C., & Wijngaards, G. (1982) *Thromb. Haemostasis 48*, 266-269.
Vieira, J., & Messing, J. (1982) *Gene 19*, 259-268.
Wiman, B., & Collen, D. (1978) *Eur. J. Biochem. 84*, 573-578.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103-119.

Thrombin Inactivates Acidic Fibroblast Growth Factor but Not Basic Fibroblast Growth Factor†

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ABSTRACT: Incubation of bovine brain derived acidic fibroblast growth factor (aFGF) with bovine or human thrombin, 0.5 NIH unit/mL, for 24 h at 37 °C results in cleavage of the mitogen, generating a 14-kilodalton fragment which has significantly reduced affinity for immobilized heparin as compared to aFGF, and is at least 50-fold less potent at stimulating mitogenesis. In addition, an 18 amino acid peptide, aFGF(123-140), is generated, identifying one of the thrombin cleavage sites as the Arg-122/Thr-123 bond. The peptide, aFGF(123-140), is neither mitogenic itself nor an inhibitor of the mitogenic activity of aFGF. The cleavage of aFGF by thrombin is inhibited by heparin (50 μg/mL) and is completely blocked by the irreversible thrombin inhibitors D-Phe-Pro-Arg chloromethyl ketone and hirudin. Incubation of aFGF with 50 units/mL thrombin at 37 °C results in rapid cleavage of the mitogen into several fragments. In contrast, incubation of bovine brain derived basic fibroblast growth factor with 1 unit/mL thrombin for 24 h, or 50 units/mL thrombin for 6 h, does not result in significant cleavage of mitogen. The results show that the C-terminal region of aFGF is of functional importance in both mitogenesis and heparin binding. Most importantly, a novel role for anionic heparin-binding growth factors and their fragments is indicated in physiologic and pathologic situations associated with thrombin generation.

Heparin-binding growth factors (HBGF's)[1] are a family of polypeptides with a wide range of mitogenic and nonmitogenic functions in vitro for cells of the vascular, neural, endocrine, and immune systems (Gospodarowicz et al., 1986a; Lobb et al., 1986a). In addition, they induce neovascularization, regeneration, and morphogenesis in vivo (Gospodarowicz, 1976; Gospodarowicz et al., 1986a; Risau, 1986; Slack et al., 1987), suggesting that they are of broad physiologic significance. HBGF's can be grouped into two classes (Lobb et al., 1986a,b): anionic mitogens found in high levels in neural tissue, typified by bovine brain derived acidic fibroblast growth factor (aFGF) (Thomas et al., 1984), and cationic mitogens found in virtually all tissues, typified by bovine pituitary derived basic fibroblast growth factor (bFGF) (Bohlen et al., 1984). Mitogens from both classes have been sequenced (Esch et al., 1985a,b; Gimenez-Gallego et al., 1985, 1986a; Harper et al., 1986; Strydom et al., 1986), and their genes and cDNAs

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[1] Abbreviations: HBGF, heparin-binding growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; FPRC, D-Phe-Pro-Arg chloromethyl ketone; Z-Lys-SBzl, benzyloxycarbonyl-L-lysine thiobenzyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PBS, Dulbecco's calcium- and magnesium-free phosphate-buffered saline; BSA, bovine serum albumin; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Gd-NPF, glia-derived neurite-promoting factor; TCA, trichloroacetic acid; kDa, kilodalton(s); Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; PTH, phenylthiohydantoin.

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have been cloned (Abraham et al., 1986a,b; Jaye et al., 1986). However, despite intensive study, the reasons for the existence of two classes of HBGF, or the physiologic significance, if any, of the strong interaction of these mitogens with heparin, have not been established.

We have examined the relationship between aFGF structure and function by chemical modification of specific amino acid residues and find that the C-terminal region of aFGF is functionally important (Harper & Lobb, 1987). Reductive methylation of lysine residues in aFGF with formaldehyde and sodium cyanoborohydride reduces both its mitogenic potency and its affinity for heparin (Harper & Lobb, 1987). Structural characterization of methylated mitogen of low heparin affinity (La-aFGF) showed extensive modification of only Lys-118, suggesting that this residue plays an important role in heparin binding. Moreover, since LA-aFGF has reduced mitogenic potency, this residue and its local environment may also be involved in the interaction of aFGF with its cell surface receptor (Harper & Lobb, 1987).

Secondary structure analysis of aFGF predicts that Lys-118 is contained within a β-turn or loop structure encompassing residues 112–123 (Harper & Lobb, 1987). This part of the aFGF sequence contains not only Lys-118 but also lysines at positions 112 and 113, and arginines at positions 116 and 122. Since such loop structures are often susceptible to proteolysis, this suggested that the C-terminal region of aFGF might be cleaved by trypsin-like enzymes. The effects on aFGF of a number of such proteases are being examined, and in this report, the cleavage and inactivation of aFGF, but not bFGF, by thrombin are demonstrated, suggesting a novel role for anionic HBGF's and their fragments in physiologic and pathologic situations associated with thrombin generation.

Materials and Methods

Materials. Bovine thrombin (680 NIH units/mg) and human thrombin (2000 NIH units/mg) were from Sigma Chemical Co. (St. Louis, MO). Bovine thrombin was used without further purification. Human thrombin was purified to homogeneity by cation-exchange HPLC as described below. Z-Lys-SBzl and the specific thrombin inhibitors hirudin (Seemuller et al., 1986) and FPRC (Kettner & Shaw, 1981) were from Calbiochem-Behring (La Jolla, CA). Heparin was from Hepar Industries (Franklin, OH). Heparin–Sepharose was from Pharmacia (Piscataway, NJ). Hirudin was stored at 4 °C in water as a 50 unit/mL solution, where 1 unit is defined as the amount of hirudin sufficient to inhibit completely the enzymatic activity of 1 NIH unit of thrombin toward Z-Lys-SBzl. FPRC was stored at −20 °C as a 1 mM solution in dry dimethylformamide. Z-Lys-SBzl was stored at 4 °C as a 3 mM solution in dimethyl sulfoxide.

Growth Factor Isolation. Acidic and basic FGF's were isolated from bovine brain (Pel-Freez, Rogers, AR) by heparin–Sepharose affinity chromatography and Mono-S cation-exchange HPLC as described (Lobb et al., 1986a). To generate pure mitogen free of NaCl for iodination, both aFGF and bFGF were eluted from the Mono-S column with a sodium phosphate gradient as described for aFGF (Harper & Lobb, 1987).

HPLC. Peptides were purified by sequential reversed-phase HPLC on a Beckman Ultrapore RPSC (C3) column and an Altex Ultrapore IP (C18) column. For both columns, solvent A was 0.1% TFA in water, and solvent B was 3:2:2 (v/v/v) 2-propanol/acetonitrile/water containing 0.08% TFA. Sample was first applied to the C3 column equilibrated with solvent A at a flow rate of 0.8 mL/min and than eluted with a linear gradient of solvent B (0.75%/min) at the same flow rate. Peptides were rechromatographed on the C18 column using a linear gradient of solvent B of 1%/min, at a flow rate of 0.8 mL/min.

Human thrombin was purified to homogeneity by cation-exchange chromatography according to established techniques (Lundblad et al., 1976). Briefly, thrombin was applied to a Mono-S HR5/5 column (Pharmacia) equilibrated with 0.05 M sodium phosphate, pH 6.5, at a flow rate of 0.8 mL/min and eluted with a linear gradient of sodium chloride (2 mM/min) in the same buffer. Thrombin eluted at about 0.2 M NaCl as a sharp peak consisting of several closely spaced bands of molecular weight ~33K by SDS–PAGE. The protein peak coeluted with hirudin-inhibitable enzymatic activity toward Z-Lys-SBzl.

Iodination. Both aFGF and bFGF were iodinated as described for aFGF (Harper & Lobb, 1987). Briefly, 1–2 μg of either mitogen in ~0.2 M sodium phosphate, pH ~7.0, was iodinated with Enzymobeads (Bio-Rad, Richmond, CA) at room temperature, the beads were pelleted by centrifugation, and the supernatant was fractionated by heparin–Sepharose affinity chromatography. The mitogens had specific activities of ~40 μCi/μg and possessed full mitogenic activity.

Enzymatic Assays. The enzymatic activity of bovine and human thrombin was assessed by the continuous spectrophotometric coupled assay of Green and Shaw (1979) using Z-Lys-SBzl and DTNB. Initial rates of substrate hydrolysis were determined at 25 °C in 0.05 M Hepes, pH 7.5, containing 0.15 M NaCl, by measuring the increase in absorbance at 412 nm. Kinetic constants and enzyme concentrations were evaluated from double-reciprocal plots according to established procedures.

Other Methods. Cleavage of mitogens was examined by SDS–PAGE on 15% gels as described (Lobb & Fett, 1984). Gels were silver stained using a commercially available kit (I.C.N., Irvine, CA), dried, exposed at −70 °C to Kodak XAR5 film with an intensifying screen for 1–4 days, and developed according to established procedures. Amino acid analyses and automated Edman degradation were performed as described (Strydom et al., 1986). DNA synthesis in Balb/C 3T3 cells was measured as described (Lobb & Fett, 1984). Concentrations of pure mitogens were determined by amino acid analysis. In most experiments, a trace quantity of ^{125}I-mitogen was included to allow the determination of mitogen concentration from TCA-precipitable counts.

Results

Thrombin Cleaves aFGF but Not bFGF. To determine the susceptibility of HBGF's to thrombin, aFGF and bFGF (each 4 μg/mL, and containing ^{125}I-mitogen as a tracer) were incubated with various concentrations of bovine or human thrombin for different lengths of time and the mitogens examined by SDS–PAGE, silver staining, and autoradiography. Incubation of aFGF with pure human thrombin (0.5 NIH unit/mL; 250 ng/mL) in 0.05 M Hepes, pH 7.5, containing 0.25 M NaCl/5 mM $CaCl_2$, at 37 °C for 24 h results in the formation of a form of aFGF with an apparent molecular weight of ~14K (Figure 1A,B). No fragments of lower apparent molecular weight could be observed by either silver staining or autoradiography. In contrast, incubation of bFGF with 0.5 unit/mL human thrombin under identical conditions does not result in mitogen cleavage (Figure 1C,D). Identical results were obtained with 1 unit/mL bovine thrombin (not shown).

The cleavage of aFGF by thrombin was next examined in the presence of various inhibitors. Thrombin, 0.5 unit/mL, in 0.05 M Hepes, pH 7.5, containing 0.25 M NaCl/5 mM

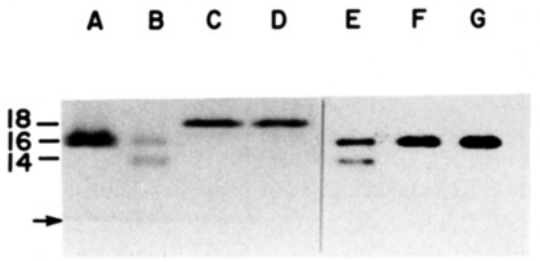

FIGURE 1: Thrombin cleavage of aFGF but not bFGF. Mitogens were incubated for 24 h at 37 °C with human thrombin, examined by SDS–PAGE, and either silver stained (A–D) or silver stained and autoradiographed (E–G). aFGF (4 μg/mL) incubated without (lane A) or with (lane B) human thrombin, 0.5 unit/mL. bFGF (4 μg/mL) incubated without (lane C) or with (lane D) human thrombin, 0.5 unit/mL. aFGF (4 μg/mL, with ^{125}I-aFGF as tracer) incubated with human thrombin, 0.5 unit/mL, after preincubaton of the enzyme either with saline as control (lane E), with 1 unit/mL hirudin (lane F), or with 50 μg/mL heparin (lane G). Molecular weights ($\times 10^{-3}$) are indicated at the left, and the dye front is indicated by the arrow.

$CaCl_2$, was preincubated at 37 °C with either 1 unit/mL hirudin, 10^{-7} M FPRC, 50 μg/mL heparin, or a saline control. After 10 min, aFGF (containing ^{125}I-mitogen as a tracer) was added (final concentration 4 μg/mL) and incubated at 37 °C for 24 h. In the presence of the highly specific thrombin inhibitor hirudin, a 6-kDa protein which forms a 1:1 complex with the enzyme (Seemuller et al., 1986), cleavage of aFGF is totally inhibited (Figure 1E,F). In addition, in the presence of FPRC (10^{-7} M), no cleavage of aFGF occurs (not shown). These results demonstrate that the enzymatic activity of thrombin is required for the generation of the aFGF fragment. Moreover, when heparin (50 μg/mL) is present in the incubation mixture, aFGF is also protected against thrombin cleavage (Figure 1G). Thus, aFGF, but not bFGF, undergoes limited proteolysis upon incubation with 0.5–1.0 unit/mL thrombin.

To examine the effects of higher thrombin concentrations on the mitogens, aFGF and bFGF (each 4 μg/mL, and containing ^{125}I-mitogen as a tracer) were incubated with 50 units/mL human thrombin in 0.05 M Hepes, pH 7.5, containing 0.15 M NaCl/5 mM $CaCl_2$, at 37 °C. Aliquots were removed at hourly intervals for 6 hours, thrombin activity inhibited with 10^{-7} M FPRC, and the extent of mitogen cleavage examined by SDS–PAGE. Figure 2A shows that aFGF is rapidly degraded under these conditions, with ~50% of the mitogen cleaved to the ~14-kDa form after only 1 h. Moreover, further degradation occurs rapidly to yet smaller forms of mitogen, indicating that at high thrombin concentrations multiple cleavage sites exist on aFGF. The rapid degradation of the mitogen is reflected in its rapid loss of mitogenic activity toward Balb/C 3T3 cells[2] (Figure 2B). In contrast, Figure 2C shows that degradation of bFGF is almost negligible over the same time period. After 4 h, some lower molecular weight forms of the basic mitogen are apparent, but they represent a very small percentage of the total mitogen. Consistent with this result, the mitogenic activity of bFGF is not changed even after 6 h of incubation at 37 °C with 50 units/mL thrombin (Figure 2D). Neither aFGF (5 μg) nor bFGF (5 μg) inhibited the hydrolysis of Z-Lys-SBzl by thrombin, indicating that the observed differences in susceptibility are not due to direct thrombin inhibition.

Sites of Thrombin Cleavage in aFGF. To determine the primary thrombin cleavage sites in aFGF, an aliquot of the

[2] Neither active thrombin nor thrombin inactivated with FPRC stimulates mitogenesis in Balb/C 3T3 cells at concentrations ranging from 1 μg/mL to 1 pg/mL.

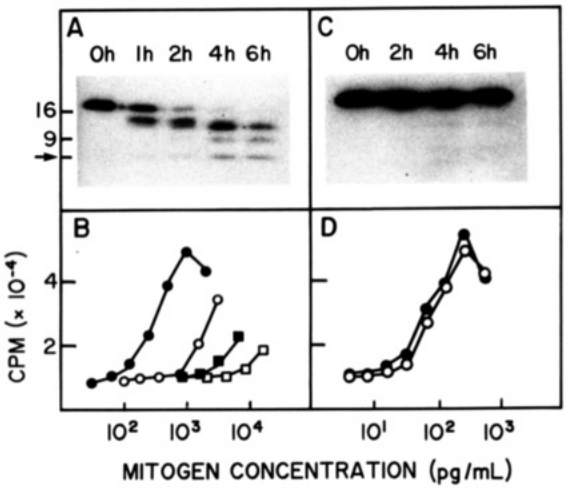

FIGURE 2: Effects of high thrombin concentrations on aFGF and bFGF. Mitogens (4 μg/mL) were incubated with 50 units/mL human thrombin at 37 °C; aliquots were removed at hourly intervals, quenched with FPRC, and examined for degradation and for mitogenic potency toward 3T3 cells. (A) Progressive degradation of aFGF by thrombin, as judged by SDS–PAGE and autoradiography. Times of incubation with thrombin are indicated above each lane. (B) Mitogenic potency of aFGF after incubation with thrombin for 0 (●), 2 (○), 4 (■), and 6 h (□). (C) Lack of degradation of bFGF by thrombin. Incubation times are indicated above each lane. (D) Mitogenic potency of bFGF after incubation with thrombin for 0 (●) and 6 h (○). For panels A and C, molecular weights ($\times 10^{-3}$) are indicated at the left. The arrow indicates the dye front.

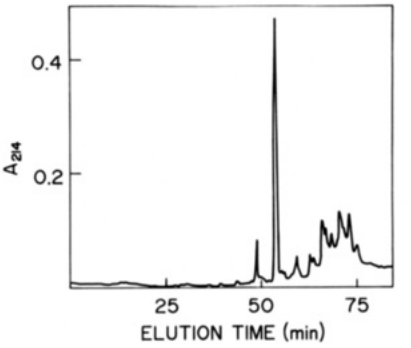

FIGURE 3: Isolation of thrombin-generated aFGF peptide by reversed-phase HPLC. aFGF was incubated with 1 unit/mL bovine thrombin at 37 °C for 24 h, and the reaction mixture was fractionated directly on a reversed-phase C3 column (see text). A single major peak was obtained, eluting at 54 min. SDS–PAGE of all fractions revealed that the peaks eluting between about 60 and 75 min represent multiple forms of uncleaved mitogen, of the 14-kDa fragment, and of various forms of thrombin.

mitogen (97 μg in 0.5 mL of 0.05 M Hepes, pH 7.5, containing 0.5 M NaCl/5 mM $CaCl_2$) was incubated with bovine thrombin (1 unit/mL) at 37 °C for 24 h, and the reaction mixture was fractionated directly on a reversed-phase C3 column (Figure 3). A single major peak was obtained, eluting at a 54 min. SDS–PAGE of all fractions revealed that the peaks eluting between about 60 and 75 min consisted of multiple forms of uncleaved mitogen (Lobb et al., 1986a), of the 14-kDa fragment, and of various forms of thrombin. In contrast, the peak eluting at 54 min did not silver stain after SDS–PAGE. This peak was rechromatographed (not shown) and subjected to amino acid analysis. Table I shows that this peptide has the exact composition expected for the 18-residue C-terminal fragment of aFGF. To confirm this assignment, the peptide was subjected to automated Edman degradation (Table I). This resulted in the sequence Thr-His-Phe-Gly-Gln-Lys-Ala, which corresponds to the amino-terminal sequence of aFGF(123–140) (Strydom et al., 1986), demon-

Table I: Amino Acid Composition and Partial Sequence of Peptide Generated by Thrombin Cleavage of aFGF

composition		sequence	
amino acid	amount[a]	cycle	identification[b]
Asp	1.4 (1)	1	Thr (86)
Glu	1.2 (1)	2	His (107)
Ser	1.8 (2)	3	Phe (278)
Gly	1.4 (1)	4	Gly (116)
His	0.9 (1)	5	Gln (60)
Arg	0.1 (0)	6	Lys (78)
Thr	1.0 (1)	7	Ala (45)
Ala	1.1 (1)		
Pro	2.0 (2)		
Tyr	0.1 (0)		
Val	1.0 (1)		
Met	0.1 (0)		
Ile	1.0 (1)		
Leu	2.9 (3)		
Phe	1.9 (2)		
Lys	0.9 (1)		
Cys	N.D.[c] (0)		
Trp	N.D. (0)		

[a] Relative molar amounts of amino acids are given. The number of residues expected for the peptide corresponding to the 18 C-terminal amino acids of aFGF (Gimenez-Gallego et al., 1985; Strydom et al., 1986) is given in parentheses. 110 pmol of peptide was analyzed in duplicate. [b] 700 pmol of peptide was subjected to automated Edman degradation, and PTH-amino acids were identified, as described elsewhere (Strydom et al., 1986). The yield of PTH-amino acid in picomoles is given in parentheses. No identification could be made beyond cycle 7. [c] N.D., not determined.

strating that one of the primary thrombin cleavage sites in aFGF is the Arg-122/Thr-123 bond. This is consistent with the results of SDS–PAGE (Figure 1), since aFGF(1–122) and aFGF(123–140) have molecular weights of about 14K and 2K, respectively. However, we have no evidence at present to show that the 14-kDa fragment is aFGF(1–122). In addition to cleavage at the Arg-122/Thr-123 bond, cleavage may also occur at other basic amino acids in this region. Studies are in progress to resolve this point.

The reversed-phase HPLC-purified peptide, aFGF(123–140), was also examined in mitogenesis assays. Concentrations of peptide ranging from 10^{-11} to 10^{-7} M neither stimulated mitogenesis in quiescent Balb/C 3T3 cells nor inhibited the mitogenesis induced by 2×10^{-11} M (320 pg/mL) aFGF. In addition, the peptide does not bind to heparin–Sepharose equilibrated with PBS.

14-kDa Fragment Has Reduced Heparin Affinity and Mitogenic Activity. Acidic FGF (25 μg/mL, containing ^{125}I-mitogen as a tracer) was incubated with bovine thrombin (0.5 unit/mL) in 0.05 M Hepes, pH 7.5, containing 0.2 M NaCl/5 mM $CaCl_2$, at 37 °C. After 24 h, the reaction was quenched with 10^{-7} M FPRC, and the sample was applied to a 1-mL heparin–Sepharose column equilibrated with PBS at a flow rate of 30 mL/h. The column was washed with PBS and then eluted with 10 mM Tris·HCl, pH 7.0, containing first 0.6 M NaCl and then 2.0 M NaCl. TCA-precipitable radioactivity was found in the unbound fraction and in the 2.0 M NaCl eluate, but not in the 0.6 M NaCl eluate (not shown). The unbound fraction and the 2.0 M NaCl eluate were then examined for their mitogenic potency toward Balb/C 3T3 cells. Figure 4A shows that the 2.0 M NaCl eluate exhibits a dose response curve typical of the native mitogen, with maximal activity at about 1 ng/mL. In contrast, the fraction not retained by the heparin–Sepharose column exhibits a dose response curve with maximal activity at about 100 ng/mL, indicating a difference in mitogenic potency of approximately 2 orders of magnitude. When these two fractions were examined by SDS–PAGE and autoradiography (Figure 4A, insert), the unbound fraction contained the 14-kDa fragment, while the fraction retained by heparin–Sepharose in the presence of 0.6 M NaCl, and eluted with 2.0 M NaCl, contained uncleaved mitogen. In four separate experiments, two with bovine thrombin and two with human thrombin, the 14-kDa fragment was not retained by heparin–Sepharose equilibrated with PBS and was 40–100-fold less potent a mitogen than aFGF itself.[3]

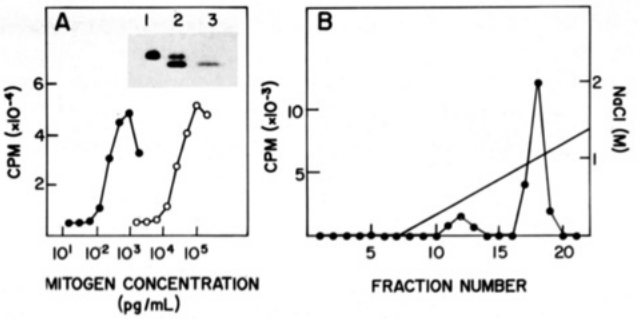

FIGURE 4: The 14-kDa fragment has reduced mitogenic potency and heparin affinity. (A) aFGF containing ^{125}I-mitogen as a tracer was partially cleaved by thrombin, the mixture was applied to a 1-mL heparin–Sepharose column equilibrated with PBS, and the mitogenic potency of the fractions either retained by the column and eluted with 2.0 M NaCl (●) or not retained by the column (○) was assessed. The mitogen concentrations were based on the specific radioactivity of ^{125}I-aFGF. Since the C-terminal region of aFGF contains no tyrosine (Strydom et al., 1986), the specific radioactivity of the mitogen does not change upon thrombin cleavage. Insert: SDS–PAGE and autoradiography of the fraction retained by the heparin–Sepharose column (lane 1), the reaction mixture prior to fractionation on the column (lane 2), and the fraction not retained by the column (lane 3). (B) aFGF containing ^{125}I-mitogen as a tracer was partially cleaved by thrombin, the reaction mixture was applied at a slow flow rate to a 1-mL heparin–Sepharose column equilibrated with 10 mM Tris·HCl, pH 7.0, and the column was eluted with a linear gradient of NaCl in the same buffer. TCA-precipitable radioactivity (●) was eluted from the column with about 0.4 and 1.0 M NaCl and corresponded to the 14-kDa fragment and uncleaved aFGF, respectively. The NaCl gradient is indicated by the solid line.

In a second series of experiments, the 14-kDa fragment was applied to heparin-Sepharose under less stringent conditions. Acidic FGF (6 μg/mL, containing ^{125}I-mitogen as a tracer) was incubated with human thrombin (30 units/mL) in 0.05 M Hepes, pH 7.5, containing 0.2 M NaCl/5 mM $CaCl_2$, at 37 °C for 90 min. The reaction mixture was quenched by addition of 10^{-7} M FPRC and then applied to a 1.0-mL column of heparin-Sepharose equilibrated with 10 mM Tris·HCl, pH 7.0, at a flow rate of 6 mL/h. The column was washed with the same buffer and then eluted with a linear gradient of NaCl in 10 mM Tris·HCl, pH 7.0. Figure 4B shows that TCA-precipitable radioactivity was found in two fractions, one eluting with about 0.4 M NaCl and the second with about 1 M NaCl. When examined by SDS–PAGE and autoradiography, the first peak contained the 14-kDa fragment while the second contained the uncleaved mitogen (not shown). Taken jointly, these results show that the 14-kDa fragment has a markedly reduced affinity for heparin as compared to the native mitogen.

Discussion

Thrombin enzymatically inactivates acidic fibroblast growth factor by cleavage at 1 or more bonds in the C-terminal region, including the Arg-122/Thr-123 bond, releasing an 18 amino acid peptide from the C-terminus and generating a 14-kDa

[3] Preliminary experiments indicate that the 14-kDa fragment also has reduced mitogenic potency toward bovine capillary endothelial cells (P. A. D'Amore and R. R. Lobb, unpublished observations).

fragment which is least 50-fold less potent at stimulating mitogenesis as compared to the uncleaved mitogen. Moreover, upon incubation of aFGF with high concentrations of thrombin, the rapid generation of yet smaller fragments occurs. In contrast, incubation of bovine brain derived basic fibroblast growth factor with 1 unit/mL thrombin for 24 h, or 50 units/mL thrombin for 6 h, does not result in significant cleavage of mitogen. Since the cleavage of aFGF by thrombin is inhibited by heparin, the results suggest a novel role for both thrombin and heparin-like molecules in the regulation of anionic, but not cationic, HBGF function in vivo.

At present, the physiologic significance of the existence of two classes of HBGF is unknown, since both classes stimulate the same in vitro and in vivo activities on the same spectrum of cell types (Gospodarowicz et al., 1986a). Moreover, while the two classes of HBGF differ in mitogenic potency, tissue distribution, and sensitivity to added heparin (Gospodarowicz et al., 1986a; Lobb et al., 1986a), the presence of bFGF in the same tissues as aFGF, and the complexity of the interactions of heparin with cells in vitro [see, e.g., Gospodarowicz and Cheng (1986) and Gospodarowicz et al. (1986a,b)], has rendered the significance of these differences unclear. The susceptibility of aFGF but not bFGF to cleavage by thrombin provides both a structural basis for and a functional difference between the two classes of HBGF which may provide important clues as to the in vivo role of anionic HBGF's. Because of the close anatomical association of nerves with blood vessels (Bloom & Fawcett, 1968), tissue injury results in concurrent damage to both the neural system and the vascular tree, and thus damaged neural tissue is generally embedded in a fibrin clot. Because much of the thrombin generated during coagulation associates with fibrin (Fenton et al., 1985), high concentrations of thrombin ($\sim$100 units/mL) are maintained in situ. As Figure 2 demonstrates, under such conditions released aFGF would be rapidly converted to smaller fragments and inactivated, while released bFGF would not be degraded, and would maintain its biological activity. This raises the possibility that the generation of aFGF fragments, and particularly aFGF(123–140), may be of far greater physiologic significance under these conditions than the release of the intact mitogen itself. If this is the case, then one might predict that aFGF would be more potent than bFGF in vivo in certain physiologic and pathologic situations involving thrombin generation. It will be interesting to examine the bioactivity of anionic HBGF fragments in assays other than in vitro mitogenesis, including chemotaxis, neurite outgrowth, morphogenesis, and induction of neovascularization.

The results also point to a defined regulatory role for heparin-like molecules in vivo in the protection of anionic HBGF's against thrombin cleavage, either directly via the formation of a heparin/HBGF complex or indirectly via enhancement of the inactivation of thrombin by antithrombin III or protease nexin (Scott & Baker, 1983; Marcum et al., 1986). In this regard, it is intriguing that both thrombin and heparin have important functional interactions with many of the cell types which respond to HBGF's. For example, HBGF's are potent endothelial cell mitogens, and the central role of the endothelial cell in hemostasis (Gimbrone, 1986) depends upon the critical interactions of both thrombin and heparin with the endothelial cell surface (Marcum et al., 1986; Esmon, 1987). In addition, HBGF's are also potent inducers of neurite outgrowth and neuronal cell survival (Togari et al., 1983; Morrison et al., 1986; Wagner & D'Amore, 1986; Walicke et al., 1986; Schubert et al., 1987). HBGF's thus belong to a larger class of heparin-binding proteins which promote neurite outgrowth, including laminin (Manthorpe et al., 1983), purpurin (Schubert & LaCorbierre, 1985), and glia-derived neurite-promoting factor (Gloor et al., 1986). Thrombin, on the other hand, is a potent inhibitor of neurite outgrowth (Monard et al., 1983) and binds with high affinity to neuronal cells, but not astrocytes (Means & Anderson, 1985). Of the neurite-promoting factors, Gd-NPF is a serine protease inhibitor closely related to protease nexin. Gd-NPF inactivates thrombin (Guenther et al., 1985), and the protease inhibitors used in this study, hirudin and FPRC, are as efficient as Gd-NPF at promoting neurite outgrowth (Monrad et al., 1983). Clearly, the proteolytic regulation of anionic HBGF function in the neural system by thrombin, thrombin inhibitors, and heparin-like molecules deserves further study.

The present results not only point to an important role for thrombin in the regulation of the function of anionic HBGF's but also suggest a general regulatory role for proteases and their inhibitors in anionic HBGF function. For example, other proteases generated during coagulation may also inactivate aFGF, as may proteases generated during fibrinolysis. Plasminogen activator secretion is increased by endothelial cells during neovascularization (Kwaan & Astrup, 1964; Gross et al., 1983), and neuronal cells during neurite extension (Monard, 1985; Patterson, 1985), resulting in high local concentrations of plasmin. In preliminary experiments, we have also found that plasmin, but not urokinase, cleaves aFGF in the C–terminal region. Proteolytic processing by trypsin-like proteases has also been postulated to occur at basic dipeptides in aFGF, generating neuropeptide-like fragments (Gimenez-Gallego et al., 1985). It is likely that a complex system involving heparin-binding proteins, heparin-like molecules, proteases, and protease inhibitors, and possibly heparinases and heparinase inhibitors, regulates anionic HBGF function in vivo.

At present, the structural basis for the differential sensitivity of aFGF and bFGF to thrombin is not known. Detailed quantitative studies on 30 polypeptides and proteins cleaved by thrombin (Chang, 1985) show that cleavage at Arg is strongly favored over cleavage at Lys and is most favorable at X-Pro-Arg-Y, where X and Y are any amino acid, or at X-Arg-Y, where either X or Y is glycine. Consistent with these observations, one of the primary thrombin cleavage sites in aFGF is at Arg-122, which is preceded by Pro-121. This same sequence is present in human brain derived aFGF, despite several sequence differences in this region of the two mitogens (Gimenez-Gallego et al., 1986a; Harper et al., 1986). In contrast, the residue in bovine and human basic FGF homologous to Arg-122 is a lysine (Esch et al., 1985a; Abraham et al., 1986b). Moreover, while bovine and human bFGF's show 98% sequence identity, one of the only two sequence differences occurs at the residue that precedes this lysine, where the Pro in bovine bFGF is replaced by Ser in the human mitogen (Abraham et al., 1986b). Thus, on the basis of the studies of Chang (1985), thrombin cleavage of bFGF should be strongly disfavored as compared to aFGF, particularly with the human mitogen. However, at high thrombin concentrations, a FGF is cleaved at several bonds, while bFGF remains resistant (Figure 2). It is possible that differences in three-dimensional structure, as well as primary sequence, may also contribute to the differential sensitivity of the two classes of mitogen.

The results also have a number of implications for the relationship between aFGF structure and function. For example, the significantly reduced affinity of the 14-kDa fragment for heparin is unexpected, since the fragment must contain the

majority of the basic amino acids present in aFGF. This suggests that the strong interaction between aFGF and heparin is not simply the result of additive interactons between clusters of basic amino acids in different regions of the molecule. The results suggest a more complex interaction in which the C-terminal region of aFGF plays a central role, perhaps to stabilize a critical structural conformation or to trigger a major conformational change in the mitogen upon heparin binding. In addition, the results support the conclusions of our previous structure/function study of aFGF (Harper & Lobb, 1987). On the basis of chemical modification of lysine residues, we suggested that Lys-118 and its local environment are of functional importance. Mitogen modified almost exclusively at this residue has reduced affinity for both heparin and its cell surface receptor, as well as reduced mitogenic potency. The results suggested that Lys-118 and its local environment may be directly involved in heparin binding and that the binding of aFGF to heparin may be oligatory for optimal aFGF function. Thrombin cleavage of aFGF at the Arg-122/Thr-123 bond is inhibited by heparin, and the 14-kDa fragment has significantly reduced heparin affinity, confirming an important role for the C-terminal region of the mitogen in the heparin interaction. Moreover, the fragment also has significantly reduced mitogenic potency, again indicating a correlation between heparin affinity and mitogenic potency.

These results and others (Schreiber et al., 1985; Halperin & Lobb, 1987) are consistent with obligatory heparin binding of aFGF for optimal function, with a heparin-dependent increase in mitogenic potency of about 50-fold. This has significant implications for the development of antagonists of anionic HBGF's since either the enzymatic destruction of heparin-like molecules or their blockage with heparin-binding proteins would lead to inhibition of function. In addition, such a heparin-dependent increase would provide a simple explanation for the potentiation of anionic HBGF function in vitro which has been observed in many laboratories (Gimenez-Gallego et al., 1986b; Uhlrich et al., 1986; Wagner & D'Amore, 1986). However, heparin also stabilizes HBGF's toward inactivation (Schreiber et al., 1985; Gospodarowicz & Cheng, 1986), and it has been suggested that the potentiation of aFGF function in vitro may be due to such stabilization. The preferential inactivation of anionic HBGF's by thrombin, and their protection by heparin, is consistent with the latter explanation. Indeed, recent studies also show that heparin protects the anionic HBGF endothelial cell growth factor against inactivation by trypsin, thermolysin, and serum proteases (Rozengart et al., 1987). In preliminary experiments, we have found that incubation of ^{125}I-aFGF for 24 h at 37 °C with some, but not all, lots of bovine serum results in the appearance of some of the 14-kDa fragment. Mitogen cleavage is inhibited by heparin and eliminated by FPRC or hirudin, suggesting that low levels of thrombin are responsible. However, the mitogen is never more than ~50% cleaved, even after 48-h incubation, indicating that inactivation by serum proteases cannot account for the 30–100-fold potentiation of anionic HBGF activity often seen. It is much more likely that proteases associated with the cell surface regulate anionic HBGF function. These two explanations for the heparin potentiation of anionic HBGF function, namely, obligatory heparin binding for optimal activity and heparin-dependent protection against protease inactivation, are not, of course, mutually exclusive.

Most importantly, both this report and our chemical modification study (Harper & Lobb, 1987) indicate the critical importance of the C-terminal regon of aFGF to its function. Whether the C-terminal region of bFGF serves a related function is unknown. However, it is interesting to note that although aFGF and bFGF share overall 50% sequence identity (Esch et al., 1985b; Strydom et al., 1986), the C-terminal region of the two mitogens is the most highly conserved, with 14 of 17 identical residues, including a continuous sequence of 9 identical amino acids (Strydom et al., 1986). With the availability of the genes for HBGF's (Abraham et al., 1986a,b; Jaye et al., 1986), site-directed mutagenic studies on basic amino acids in the C-terminal region, as well as the generation of forms of HBGF serially truncated at the C-terminus, should prove particularly fruitful in understanding the relationship between HBGF structure and function.

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REFERENCES

Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, A., Friedman, J., Hjerrild, K. A., Gospodarowicz, D., & Fiddes, J. C. (1986a) *Science (Washington, D.C.) 233*, 545–548.

Abraham, J. A., Whang, J. L., Tumulo, A., Mergia, A., Friedman, J., Gospodarowicz, D., & Fiddes, J. C. (1986b) *EMBO J. 5*, 2523–2528.

Bloom, W., & Fawcett, D. W. (1968) in *A Textbook of Histology*, Ninth ed., pp 304–357, W. D. Saunders, Philadelphia, PA.

Bohlen, P., Baird, A., Esch, F., Ling, N., & Gospodarowicz, D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5364–5368.

Chang, J.-Y. (1985) *Eur. J. Biochem. 151*, 217–224.

Esch, F., Ueno, N., Baird, A., Hill, F., Denoroy, L., Ling, N., Gospodarowicz, D., & Guillemin, R. (1985a) *Biochem. Biophys. Res. Commun. 133*, 554–562.

Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Bohlen, P., & Guillemin, R. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6507–6511.

Esmon, C. T. (1987) *Science (Washington, D.C.) 235*, 1348–1352.

Fenton, J. W., II, Schuman, M., & Walz, D. A. (1985) *Ann. N.Y. Acad. Sci. 485*, 2–4.

Gimbrone, M. A. (1986) in *Vascular Endothelium in Hemostasis and Thrombosis* (Gimbrone, M. A., Ed.) pp 1–14, Churchill, Livingstone, New York.

Gimenez-Gallego, G., Rodkey, J., Bennett, C., Rios-Candelore, M., DiSalvo, J., & Thomas, K. (1985) *Science (Washington, D.C.) 230*, 1385–1388.

Gimenez-Gallego, G., Conn, G., Hatcher, V. B., & Thomas, K. A. (1986a) *Biochem. Biophys. Res. Commun. 138*, 611–617.

Gimenez-Gallego, G., Conn, G., Hatcher, V. B., & Thomas, K. A. (1986b) *Biochem. Biophys. Res. Commun. 135*, 541–548.

Gloor, S., Odink, C., Guenther, J., Nick, H., & Monard, D. (1986) *Cell (Cambridge, Mass.) 47*, 687–693.

Gospodarowicz, D. (1976) *Prog. Clin. Biol. Res. 9*, 1–19.

Gospodarowicz, D., & Cheng, J. (1986) *J. Cell. Physiol. 128*, 475–484.

Gospodarowicz, D., Neufeld, G., & Schweigerer, L. (1986a) *Cell Differ. 19*, 1–17.

Gospodarowicz, D., Massoglia, S., Cheng, J., & Fujii, D. K. (1986b) *J. Cell. Physiol. 127*, 121–136.

Green, G. D. J., & Shaw, E. (1979) *Anal. Biochem. 93*, 223–226.
Gross, J. L., Moscatelli, D., & Rifkin, D. B. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2623–2627.
Guenther, J., Nick, H., & Monard, *D.* (1985) *EMBO J. 4*, 1963–1966.
Halperin, J. A., & Lobb, R. R. (1987) *Biochem. Biophys. Res. Commun. 144*, 115–122.
Harper, J. W., & Lobb, R. R. (1988) *Biochemistry 27*, 671–678.
Harper, J. W., Strydom, D. J., & Lobb, R. R. (1986) *Biochemistry 25*, 4097–4103.
Jaye, M., Howk, R., Burgess, W., Ricca, G. A., Chiu, I.-M., Ravera, N. W., O'Brien, S. J., Modi, W. S., Maciag, T., & Drohan, W. N. (1986) *Science (Washington, D.C.) 233*, 541–544.
Kettner, C., & Shaw, E. (1981) *Methods Enzymol. 80*, 826–842.
Kwaan, H.-C., & Astrup, T. (1964) *J. Pathol. Bacteriol. 87*, 409–414.
Lobb, R. R., & Fett, J. W. (1984) *Biochemistry 23*, 6925–6929.
Lobb, R. R., Harper, J. W., & Fett, J. W. (1986a) *Anal. Biochem. 154*, 1–14.
Lobb, R. R., Sasse, J., Sullivan, R., Shing, Y., D'Amore, P., Jacobs, J., & Klagsbrun, M. (1986b) *J. Biol. Chem. 261*, 1924–1928.
Lundblad, R. L., Kingdon, H. S., & Mann, K. G. (1976) *Methods Enzymol. 45*, 156–176.
Manthorpe, M., Engvall, E., Ruoslahti, E., Longe, F. M., Davis, G. E., & Varon, S. (1983) *J. Cell Biol. 97*, 1882–1890.
Marcum, J. A., Rosenberg, J. S., Bauer, K. A., & Rosenberg, R. D. (1986) in *Vascular Endothelium in Hemostasis and Thrombosis* (Gimbrone, M. A., Ed.) pp 70–98, Churchill Livingstone, New York.
Means, E. D., & Anderson, D. K. (1985) *Ann. N.Y. Acad. Sci. 485*, 314–322.
Monard, D. (1985) *Sel. Top. Neurobiochem. 36*, 7–12.
Monard, D., Niday, E., Limat, A., & Solomon, F. (1983) *Prog. Brain Res. 58*, 359–364.
Morrison, R. S., Sharma, A., De Vellis, J., & Bradshaw, R. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7537–7541.
Patterson, P. H. (1985) *J. Physiol. (Paris) 80*, 207–211.
Risau, W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3855–3859.
Rozengart, T., Friesel, R., Clark, R., & Maciag, T. (1987) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 46*, 2116.
Schreiber, A. B., Kenney, J., Kowalski, W. J., Friesel, R., Mehlman, T., & Maciag, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6138–6142.
Schubert, D., & LaCorbierre, M. (1985) *J. Cell Biol. 101*, 1071–1077.
Schubert, D., Ling, N., & Baird, A. (1987) *J. Cell Biol. 104*, 635–643.
Scott, R. W., & Baker, J. B. (1983) *J. Biol. Chem. 258*, 10439–10444.
Seemuller, U., Dodt, J., Fink, E., & Fritz, H. (1986) in *Proteinase Inhibitors* (Barrett, A. J., & Salvesen, G., Eds.) pp 337–359, Elsevier, New York.
Slack, J. M. W., Darlington, B. G., Heath, J. K., & Godsave, S. F. (1987) *Nature (London) 326*, 197–200.
Strydom, D. J., Harper, J. W., & Lobb, R. R. (1986) *Biochemistry 25*, 945–951.
Thomas, K. A., Rios-Candelore, M., & Fitzpatrick, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 357–361.
Togari, A., Baker, D., Dickens, G., & Guroff, G. (1983) *Biochem. Biophys. Res. Commun. 114*, 1189–1193.
Ulrich, S., Lagente, O., Lenfant, M., & Courtois, Y. (1986) *Biochem. Biophys. Res. Commun. 137*, 1205–1213.
Wagner, J. A., & D'Amore, P. (1986) *J. Cell Biol. 103*, 1363–1367.
Walicke, P., Gowan, W. M., Ueno, N., Baird, A., & Guillemin, R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3012–3016.