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Identification of a thrombin sequence with growth factor activity on macrophages

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ABSTRACT **In contrast to fibroblasts, the exposure of G_0/G_1-arrested J774 cells, a murine macrophage-like tumor cell line, with either active or esterolytically inactive diisopropyl phosphorofluoridate-conjugated α-thrombin (the enzymatically active form of thrombin, EC 3.4.21.5) results in a mitogenic response as measured by increased [^{3}H]thymidine incorporation. This response to thrombin is optimal at 10 nM and is specifically blocked by hirudin, a high-affinity thrombin inhibitor. When prethrombin 1 [a single-chain prothrombin derivative lacking fragment 1, resulting from the action of thrombin on prothrombin] is cleaved with cyanogen bromide, a fragment (peptide CB67-129) is produced that, like the parent thrombin molecule, is mitogenic for J774 cells but not for fibroblasts. Limited tryptic digests of this fragment retain the ability to stimulate macrophages—a function that can be mimicked by a synthetic tetradecapeptide homologue of CB67-129 (representing residues 367–380 of the human thrombin B chain sequence) but not by any of a series of well-known growth promoters, including platelet-derived growth factor, epidermal growth factor, nerve growth factor, and fibroblast growth factor. The mitogenic effects of this peptide are not limited to J774 cells but can be expressed in other macrophage-like tumor cell lines, including P388D1, RAW, and PU5. In addition to increased [^{3}H]thymidine incorporation, the synthetic B chain peptide stimulates cell proliferation as evidenced by a dose-dependent increase in total protein per culture well and cell number. We conclude that the thrombin molecule contains a macrophage growth factor domain that is separate and distinct from its active center. Thus, thrombin, in addition to its major role in hemostasis and thrombosis, may also have important functions in such basic processes as the inflammatory response and monocytopoiesis.**

It has been recognized for many years that α-thrombin, the enzymatically active form of thrombin (EC 3.4.21.5), like other better-known mitogens [e.g., platelet-derived growth factor (PDGF), epidermal growth factor (EGF), etc.], is capable of initiating proliferation in quiescent cells belonging to the fibroblast family (1–5). As with other growth factors, this stimulation results in enhanced phosphorylation of the S6 ribosomal protein (6) and activation of the Na^+/H^+-antiport system leading to alkalinization of the cytosol (7). However, unlike these other mitogenic peptides, thrombin is a serine esterase whose function as a growth stimulator, at least for fibroblasts, is intimately linked to its esterolytic activity. Thus, while native α-thrombin is capable of evoking DNA synthesis in G_0/G_1-arrested cells, neither enzymatically inactive thrombin [i.e., diisopropyl phosphorofluoridate (iPr_2P-F)-conjugated α-thrombin] nor partially degraded thrombin (i.e., γ-thrombin) share in this capability (3–5).

Recent data from our laboratory have shown that thrombin is chemotactic for peripheral blood monocytes and cells belonging to the monocyte/macrophage family and that this activity is not dependent upon thrombin's enzymatic properties (8–11). We wondered therefore, whether thrombin might also serve as a growth factor for these cells and, if so, whether this mitogenic capability would be independent of esterolytic function. In this communication, we present data that (*i*) establishes enzymatically inactive thrombin as a growth factor for cells of macrophage lineage and (*ii*) localizes this growth-promoting site within or near the "loop B" insertion sequence (12) of the thrombin B chain.

MATERIALS AND METHODS

Reagents. All chemicals were of reagent grade or better. Bovine serum albumin (fraction V) was obtained from Miles; Dulbecco's modified Eagle's medium (DMEM), from KC Biological (Lenexa, KS); fetal calf serum, from HyClone (Logan, UT); hirudin from Sigma; EGF, nerve growth factor (NGF), and fibroblast growth factor (FGF), from Biomedical Technologies (Norwood, MA); and [*methyl*-^{3}H]thymidine (specific activity, 6.7 Ci/mmol; 1 Ci = 37 GBq), from New England Nuclear. PDGF was a gift from T. Deuel (Washington Univ., St. Louis, MO), and L-cell colony-stimulating factor (CSF) designated CSF-1 was provided by E. R. Stanley (Albert Einstein College of Medicine, New York).

Cells. J774 murine macrophage-like cells were a gift from Jay Unkeles (Rockefeller University, New York). Human foreskin fibroblast cells (HF cells) were provided by H. Welgus (Washington Univ. School of Medicine). Chinese hamster lung (CHL) fibroblasts and murine macrophage-like cell lines P388D1, RAW, and PU5 were obtained from the American Type Culture Collection.

Human Thrombin Preparations. Preparations of active human α-thrombin and esterolytically inactive human iPr_2P-F-conjugated α-thrombin were provided by John Fenton (New York State Department of Health, Albany, NY). Their preparation and characterization have been described in detail (13).

Thrombin Fragments. The isolation and purification of peptide CB67-129 from human prethrombin 1 [a single-chain prothrombin derivative lacking fragment 1, resulting from the action of thrombin on prothrombin (12)] and the preparation of limited tryptic digests of CB67-129 peptide after citraconylation and subsequent removal of the reversible blocking groups were carried out as described (10, 12, 14). Fractionation and isolation of peptides from this digest were performed by semipreparative reversed-phase HPLC (15) using a 250 × 10 mm Hi-Pore RP-318 column (Bio-Rad).

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Abbreviations: CSF, colony-stimulating factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; HF, human foreskin fibroblast cells; iPr_2P-F, diisopropyl phosphorofluoridate; NGF, nerve growth factor; PDGF, platelet-derived growth factor; CHL, Chinese hamster lung.

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[^{3}H]Thymidine Incorporation. Cells were plated in DMEM containing 10% fetal calf serum in humidified 95% air/5% CO_2 at 37°C at an initial density of 5×10^5 cells per well in 24-well disposable plastic plates (Falcon). Arrest of DNA synthesis (i.e., G_0/G_1) was achieved by incubating cells for 48 hr in serum-free DMEM containing 0.1% bovine serum albumin. The cells were then exposed to various thrombin forms, peptide, or fetal calf serum for 48 hr. Enhanced thymidine incorporation was assessed after a 2-hr pulse of [*methyl*-^{3}H(N)]thymidine (1 μCi/ml). The cells were washed three times in phosphate-buffered saline (140 mM NaCl/3 mM KCl/8 mM Na_2HPO_4/1 mM K_2HPO_4/1 mM $CaCl_2$/5 mM $MgCl_2$, pH 7.4) at 4°C and lysed with 10% CCl_3COOH (30 min at 4°C); the CCl_3COOH-insoluble material was extracted twice with ETOH:ET_2O, 3:1 (vol/vol). The precipitate was solubilized in 1 M NaOH, and aliquots were withdrawn for protein determinations using a dye-binding assay (Bio-Rad) and liquid scintillation spectrometry.

Peptide Synthesis. Tetradecapeptide H-Tyr-Pro-Pro-Trp-Asn-Lys-Asn-Phe-Thr-Glu-Asn-Asp-Leu-Leu-OH, representing residues 367–380 of the human thrombin B chain[‖] and containing the loop B insertion sequence (see Fig. 2), was synthesized by a modification of the method of Merrifield as described (17, 18). The fully protected peptide was simultaneously deprotected and cleaved from its resin support by the two-step HF-catalyzed S_N2 procedure described by Tam *et al.* (19). The crude peptide was definitively purified by ion-exchange chromatography on a DEAE-Sephacel column as described (17). Peptide purity was ascertained by reversed-phase (C_{18}) HPLC (15) during which it migrated as a single peak. The peptide was also found to be homogeneous by TLC, migrating as a single spot in two different solvent systems (17, 18). Amino acid analysis yielded the expected molar ratios.

RESULTS

The addition of active but not esterolytically inactive thrombin (iPr_2P-F-conjugated α-thrombin) stimulated DNA synthesis in CHL fibroblasts (Fig. 1*A*). This finding is in contrast to that obtained with the murine macrophage-like cell line J774 as well as with other macrophage cell lines (i.e., P388D1, RAW, and PU5; data not shown), wherein both forms of thrombin were seen to enhance markedly (>100%) the incorporation of [^{3}H]thymidine into quiescent cells (Fig. 1*A*) as well as to stimulate cell proliferation (data not shown). The mitogenic effect of thrombin was observed over a wide range of concentrations (10 pM to 1 μM) but was optimal at 10 μM. Both forms of thrombin elicited the same degree of response in J774 cells and were specifically blocked in their ability to stimulate [^{3}H]thymidine incorporation by prior complexing with hirudin, a leech-derived thrombin inhibitor (Fig. 1*B*). This apparent specificity of thrombin as a macrophage mitogen was further emphasized by the failure of other known growth-promoting agents, including PDGF, EGF, NGF, and FGF at concentration ranges of up to 500 ng/ml, to elicit proliferation in these cells (Table 1).

The observation that thrombin promoted cell division in J774 cells independently of its esterolytic function and that such activity was inhibitable by hirudin suggests that the region in thrombin responsible for these effects may either coincide with or overlap the thrombin chemotactic exosite (9, 10). To test this hypothesis, the thrombin-derived chemotactic fragment (10) peptide CB67-129 (Fig. 2) was assessed for its ability to act as a mitogen. Peptide CB67-129 stimulated [^{3}H]thymidine incorporation in J774 cells over a concentration range of 10 pM to 1 M, with optimal stimulation occurring at 10 nM (Fig. 3*A*). The degree of response elicited with this cyanogen bromide-derived fragment was, at a minimum, equal to that elicited with either active or enzymatically inactive thrombins when both were tested at their optimal concentrations. CB67-129 had no stimulatory effect on CHL fibroblasts.

To determine whether the mitogenic effects of the CNBr peptide might be expressed by smaller peptide fragments, the lysine groups of CB67-129 were reversibly blocked by citraconylation (14); then the peptide was digested with trypsin. Under these conditions, cleavage of peptide CB67-129 was limited exclusively to sites containing arginyl bonds. After removal of the lysine side-chain-blocking groups, the resultant digest was tested for J774 mitogenic

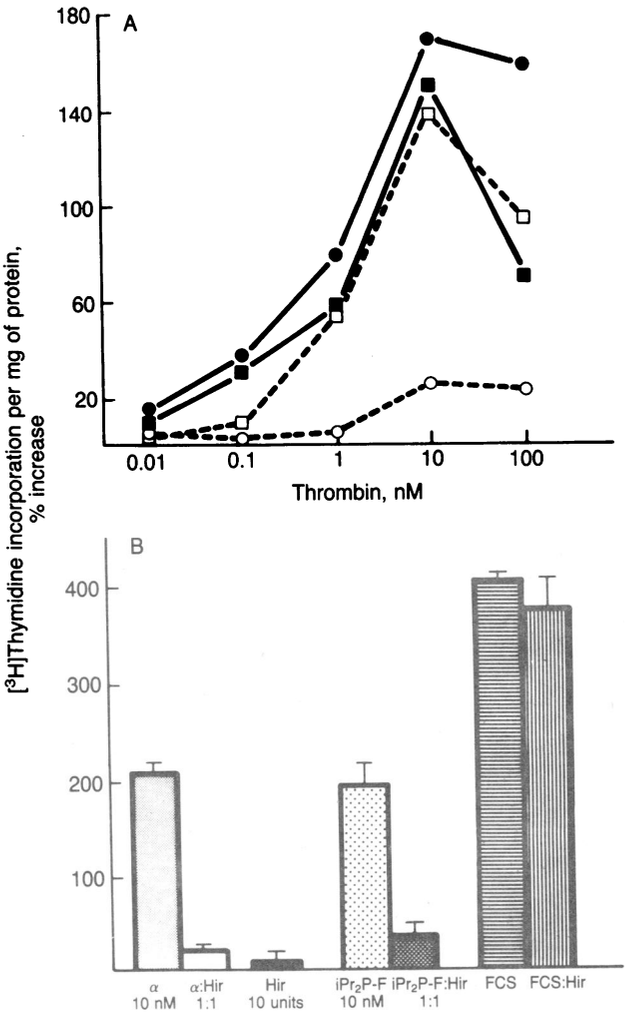

FIG. 1. Specific [^{3}H]thymidine incorporation by CHL fibroblasts and J774 murine macrophage-like cells after stimulation with either α-thrombin, iPr_2P-F-conjugated α-thrombin, or fetal calf serum. (*A*) CHL cells were treated with α-thrombin (●) or iPr_2P-F-α-thrombin (○); J774 cells were treated with α-thrombin (■) or iPr_2P-F-α-thrombin (□). Baseline [^{3}H]thymidine incorporation in quiescent CHL was 2000 cpm ± 145 SEM per well and in quiescent J774 was 780 cpm ± 42 SEM per well. (*B*) J774 cells were treated with 10 nM α-thrombin (α), iPr_2P-F-α-thrombin (iPr_2P-F), or 10% fetal calf serum (FCS) either in the presence or absence of hirudin (Hir). Baseline [^{3}H]thymidine incorporation in quiescent J774 cells for this experiment was 5000 cpm ± 420 SEM per well. A representative experiment is shown with data expressed as the mean of triplicate determinations. Similar data were obtained in at least three separate experiments. Comparable results were obtained substituting other murine macrophage-like cell lines (i.e., P388D1 and RAW) for J774 cells under essentially the same conditions.

[‖] Prothrombin nomenclature (16). The sequence used for synthesis is according to the protein sequence data of Butkowski *et al.* (12).

Table 1. Mitogenic stimulation of growth-arrested macrophage-like tumor cell lines

Cells	Mitogen	Amount per well*	[^{3}H]Thymidine incorp. per mg of protein,† (% increase)
J774	Loop B‡	10 μM	140 ± 8.5
J774	PDGF§	≥500 ng	9.4 ± 0.3
J774	EGF§	≥500 ng	5.2 ± 0.2
J774	FGF§	≥500 mg	0 ± 0.4
J774	NGF§	≥500 mg	0 ± 0.5
J774	Loop B‡	10 nM	190 ± 12
P388D1	Loop B‡	100 nM	226 ± 22
RAW	Loop B‡	10 nM	276 ± 18
PU5	Loop B‡	1 nM	112 ± 4.4

*Concentration yielding optimal specific [^{3}H]thymidine incorporation is shown. Where stimulation was not significantly greater than control (i.e., ≥1.5-fold), the highest concentration tested is indicated.

†[^{3}H]Thymidine incorporation (incorp.) was determined as described. Each value represents the mean percentage increase ± SEM from triplicate cultures in a representative experiment. Comparable data was obtained in at least three independent experiments. Underlined values indicate significant stimulation.

‡Loop B peptide.

§These preparations stimulated a ≥4-fold increase in [^{3}H]thymidine incorporation in quiescent HF cells at the concentration shown. Baseline incorporation in unstimulated cells was 280 cpm ± 21 SEM per well.

activity. The CB67-129 digest stimulated mitogenic activity (as evidenced by [^{3}H]thymidine incorporation) at levels comparable to that of the intact CB67-129 fragment (Fig. 3*A*). Fractionation of the digest by reversed-phase HPLC provided evidence suggesting that activity was present in fractions containing residues 356–382 (Fig. 2) of the thrombin B chain (data not shown). This region of thrombin chain differs from homologous regions in two closely related (but chemotactically inactive) serine esterases, chymotrypsin and trypsin,

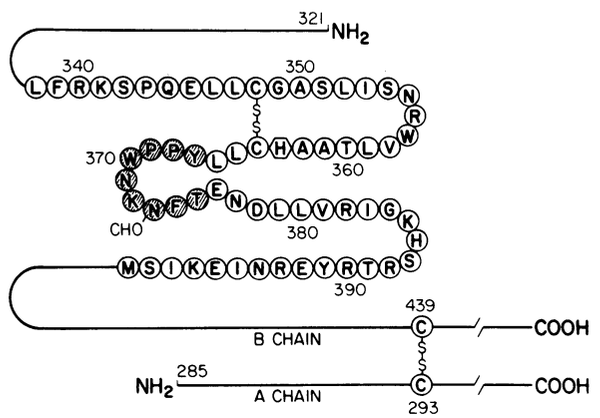

FIG. 2. Model of human α-thrombin, based on the protein sequence data of Butkowski *et al.* (12). The sequence nomenclature used is that of prothrombin (16), a single chain zymogen, which is subsequently activated and cleaved into a two-chain structure at residues 272 and 321 by factor X_a (20). The active enzyme so produced deletes the first 13 residues of the A chain at residue 285 by an autocatalytic event (20), yielding α-thrombin. The structure of peptide CB67-129 is shown, with the loop B insertion sequence (i.e., residues 367–375) indicated by crosshatching. The locations of active site His-363 (hexagon) and carbohydrate attachment site (at Asn-373), important structural features of this fragment, are also indicated (21, 22). The amide assignment on Asx-355 and 371 is uncertain (16), as is the precise limits of the loop B thrombin B chain insertion sequence. The synthetic loop B-containing peptide we prepared contains sufficient overlap to accommodate the limits suggested in the literature (20, 21).

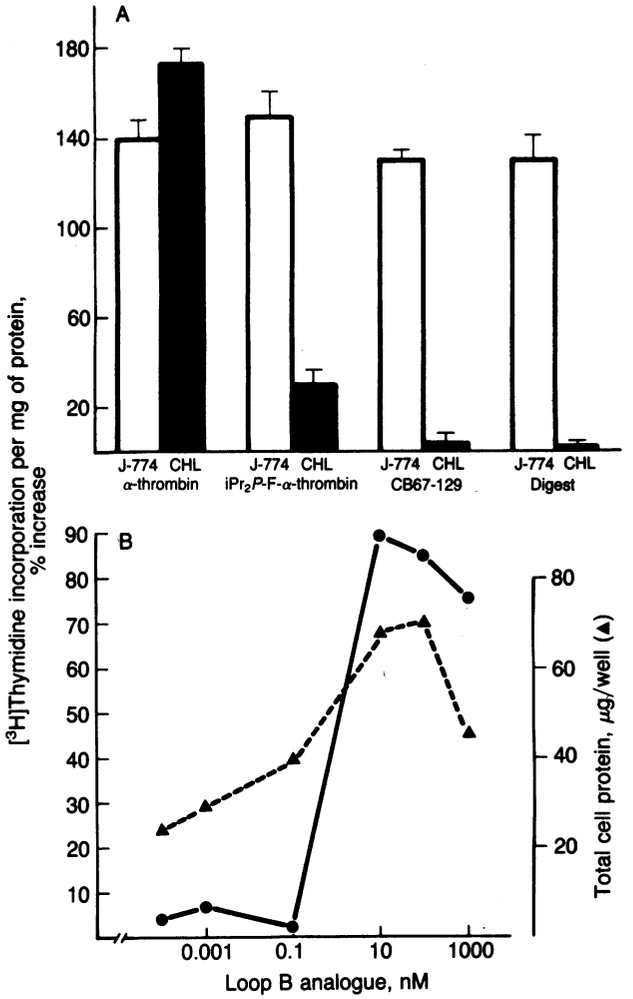

FIG. 3. (*A*) Specific [^{3}H]thymidine incorporation by J774 cells and CHL fibroblasts after stimulation with either the indicated thrombin derivatives at 10 nM or total tryptic digest of citraconylated CNBr-derived peptide CB67-129 at 10 nM. (*B*) For comparison, the effect on specific [^{3}H]thymidine incorporation and total cell protein levels after exposure of J774 cells to a synthetic homologue of the thrombin loop B insertion sequence is shown. Assay and experimental conditions are similar to those in Fig. 1.

principally by the presence of a unique 9–10 residue insertion sequence termed "loop B" (21, 22) (Fig. 2).

To establish whether the loop B sequence itself might account for the biological activity of the parent CB67-129 peptide, a loop B-containing homologue of the CNBr fragment [representing residues 367–380 of the human thrombin B chain (Fig. 2)] was synthesized, and its ability to stimulate mitogenic activity and cell proliferation in J774 cells was determined. This synthetic peptide was capable of stimulating both [^{3}H]thymidine incorporation and protein synthesis in G_0/G_1-arrested J774 cells over a concentration range paralleling that of both thrombin and CB67-129, with optimal stimulation occurring at 10 nM (Fig. 3*B*). Concomitant with these changes was a dose-dependent increase in cell proliferation. As an example, in a representative experiment, 24 hr after exposure of the growth-arrested cells to optimal concentrations of this peptide (i.e., 10 nM), cell numbers increased approximately 2-fold from a control value of 1.2 to 2 $\times$ 10^5 cells per culture well. The ability of this peptide to stimulate cell proliferation is shown in Fig. 4. Moreover, this growth-promoting effect was not limited to J774 cells; other murine macrophage-like cell lines including P388D1, RAW, and PU5, were likewise stimulated by this synthetic peptide (Table 1). Thus, the loop B region in the thrombin molecule

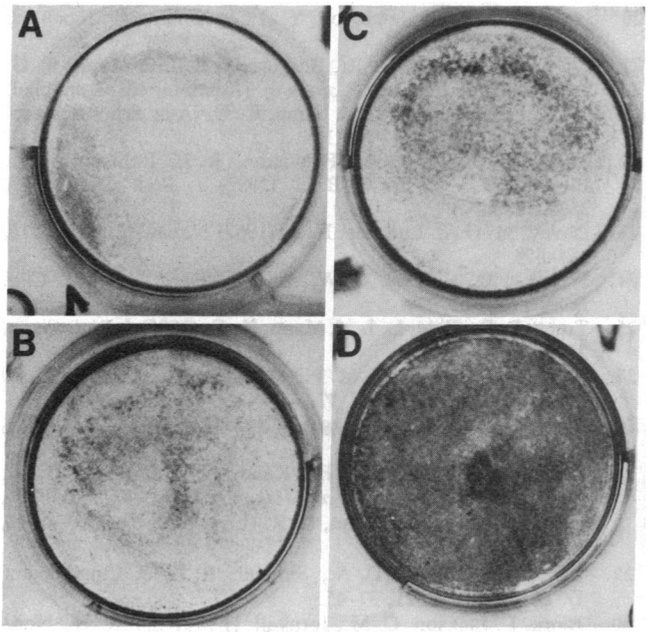

FIG. 4. Proliferative response of J774 cells to synthetic loop B analogue in serum-free culture. Cells were plated in DMEM containing 10% fetal calf serum in six-well (3.5-cm diameter) dishes (Costar, Cambridge, MA) at an initial density of 3×10^4 cells per cm^2. Cells arrested at G_0/G_1 phase were obtained by transferring the cells to serum-free medium, and incubating for 48 hr (conditions were the same as for the [^{3}H]thymidine incorporation assay). At this time, either synthetic loop B analogue or 10% fetal calf serum was added, and the cells were incubated an additional 48 hr. The medium then was aspirated carefully so as not to disturb the cell monolayer, and 0.6 ml of a 0.2% solution of crystal violet in 10% formalin was added to each well. After 20 min of fixation and staining, the wells were rinsed with water and air-dried. (*A*) Serum-starved control. (*B*) Loop B analogue (100 nM). (*C*) Loop B analogue (1 μM). (*D*) Fetal calf serum (10%).

appears responsible, at least in part, for the nonesterolytic macrophage-growth factor activity exhibited by enzymatically inactive thrombin.

DISCUSSION

Thrombin is a serine protease that plays a central role in initiating procoagulant events. This role includes the conversion of fibrinogen to fibrin (20), the stimulation of platelet aggregation and release reactions (23), and the mobilization of arachidonate pathway metabolites from endothelial cells (24). Since assembly of the prothrombin activation complex may occur on the cell membranes of mononuclear inflammatory cells (25), it is not surprising that significant amounts of active thrombin accumulate in the fluid phase of the fibrin gel (26, 27), in part, because some of the latter cells are trapped or migrate into the gel during the early phases of wound healing. Thus, thrombin is both a product of and available to the broad variety of cells, both inflammatory and reparative, that are involved in the wound-healing process.

Studies from Buchanan's laboratory were the first to demonstrate that thrombin is mitogenic for avian fibroblasts (1). Subsequently, this finding was extended to fibroblasts from other species and was found to depend upon thrombin's activity as a protease (2, 3, 5). In the present study, we provide conclusive evidence that thrombin also functions as a mitogen for several macrophage-like tumor cell lines and that, in contrast to the situation in fibroblasts, thrombin's ability to stimulate growth-arrested macrophages is entirely independent of its enzymic activity. In addition, we have localized the macrophage mitogenic site to the same region of the thrombin B chain [i.e., residues 338–400 (Fig. 2)] known to stimulate chemotactic movement in monocytes and macrophage-like cells (10). Since this chemotactic region contains all of the carbohydrate moieties associated with thrombin [attached to Asn-373 (Fig. 2)], the majority of this sequence is probably surface-expressed [corroborated by computer modeling studies (28)] and therefore available for interacting with cell-surface receptors. In addition, this region of the thrombin B chain contains the loop B insertion sequence (Fig. 2), a unique structural feature of the thrombin molecule that is absent from two closely related (but chemotactically inactive) serine esterases, chymotrypsin and trypsin, with otherwise high degrees of sequence homology to thrombin (22). On the basis of these observations, we theorized that the loop B insertion sequence is critical to the expression of thrombin's chemotactic activity and mitogenic potential. In fact, the latter assumption is supported by the following findings: (*i*) native α-thrombin, esterolytically inactive iPr_2P-F-α-thrombin, and fragment CB67-129 are approximately equivalent in their abilities to stimulate proliferation in growth-arrested macrophages; (*ii*) limited tryptic digests of fragment CB67-129, which retain intact the loop B insertion sequence, are also mitogenic for these cells; and (*iii*) most convincingly, these effects can be reproduced in a synthetic tetradecapeptide homologue of this thrombin B chain sequence.

At the present time, we have no certain understanding of the possible involvement of thrombin, and more specifically the loop B region, in modulating macrophage/monocyte proliferation *in vivo*. It is clear, from the experiments described above, that such activity does not depend upon an intact, functional thrombin molecule; therefore, noncoagulant degraded thrombins [e.g., β- and γ-thrombins (13)], substrate-bound thrombin, or even thrombin fragments might serve as authentic mitogens. Moreover, especially because thrombin fragments possess biological activity, such activity would not necessarily depend upon or be modulated by ubiquitous inhibitors of thrombin's enzymic function such as protease nexin (29), antithrombin III (30), or heparin cofactor II (31). From these considerations, it is certainly likely that thrombin can function effectively as a stimulator/regulator of macrophages and monocytes within the milieu of sites of tissue injury and, perhaps, normal hematopoietic bone marrow.

Finally, among the more intriguing possibilities for explaining the unique properties of the loop B sequence is that this region in thrombin is structurally homologous to CSFs, a family of growth factors that are capable of stimulating hematopoietic progenitor cells. Two members of the family, CSF-1 and granulocyte/macrophage (GM)-CSF, appear to directly stimulate proliferation of mononuclear phagocytes (32, 33). Although sequence data for CSF-1 is unavailable, no significant homology is apparent between GM-CSF, whose sequence is known, and the growth-promoting region in thrombin (33). Moreover, preliminary unpublished data from this laboratory (R.B.-S.) indicate that purified murine CSF-1 fails to stimulate proliferation of growth-arrested J774 cells. These findings are consistent with the relatively low number of CSF-1 receptors present on this cell [in comparison to CSF-1-responsive cells (34)] and further suggest that CSF-1 and thrombin interact with different receptor sites on the J774 cell membrane.

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1. Chen, L. B. & Buchanan, J. M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 131–135.
2. Pohjanpelto, P. (1977) *J. Cell. Physiol.* **91,** 387–392.
3. Carney, D. H. & Cunningham, D. D. (1978) *Cell* **14,** 811–823.
4. Carney, D. H., Glenn, K. C. & Cunningham, D. D. (1978) *J. Cell. Physiol.* **95,** 13–22.
5. Perdue, J. F., Lubensky, W., Kivity, E., Sonder, S. A. & Fenton, J. W., II (1981) *J. Biol. Chem.* **256,** 2767–2776.
6. Chambard, J.-C., Franchi, A., Le Cam, A. & Pouyssegur, J. (1983) *J. Biol. Chem.* **258,** 1706–1713.
7. Paris, S. & Pouyssegur, J. (1984) *J. Biol. Chem.* **259,** 10989–10994.
8. Bar-Shavit, R., Kahn, A., Fenton, J. W., II, & Wilner, G. D. (1983) *J. Cell Biol.* **96,** 282–285.
9. Bar-Shavit, R., Kahn, A., Wilner, G. D. & Fenton, J. W., II (1983) *Science* **220,** 728–731.
10. Bar-Shavit, R., Kahn, A., Wilner, G. D., Mann, K. G. & Fenton, J. W., II (1984) *Biochemistry* **23,** 397–400.
11. Bar-Shavit, R., Kahn, A., Fenton, J. W., II, & Wilner, G. D. (1983) *Lab. Invest.* **49,** 702–707.
12. Butkowski, R. J., Elion, J., Downing, M. R. & Mann, K. G. (1977) *J. Biol. Chem.* **252,** 4942–4957.
13. Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M. & Finlayson, J. S. (1977) *J. Biol. Chem.* **252,** 3587–3598.
14. Gibbons, I. & Perham, R. N. (1970) *Biochem. J.* **116,** 843–849.
15. Fulmer, C. S. & Wasserman, R. H. (1979) *J. Biol. Chem.* **254,** 7208–7212.
16. Degen, S. J. F., Macgillivray, R. T. A. & Davie, E. W. (1983) *Biochemistry* **22,** 2087–2097.
17. Wilner, G. D., Nossel, H. L., Canfield, R. E. & Butler, V. P., Jr. (1976) *Biochemistry* **15,** 1209–1213.
18. Wilner, G. D., Thomas, D. W., Nossel, H. L., Robbins, P. F. & Mudd, M. S. (1979) *Biochemistry* **18,** 5078–5082.
19. Tam, J. P., Heath, W. F. & Merrifield, R. B. (1983) *J. Am. Chem. Soc.* **105,** 6442–6454.
20. Jackson, C. M. & Nemerson, Y. (1980) *Annu. Rev. Biochem.* **49,** 765–811.
21. Elion, J., Downing, M. R., Butkowski, R. J. & Mann, K. G. (1977) in *Chemistry and Biology of Thrombin*, eds. Lundblad, R. L., Fenton, J. W., II, & Mann, K. G. (Ann Arbor Science, Ann Arbor, MI), pp. 97–111.
22. Furie, B., Bing, D. H., Feldmann, R. J., Robison, D. J., Burnier, J. P. & Furie, B. C. (1982) *J. Biol. Chem.* **257,** 3875–3882.
23. Davey, M. G. & Luscher, E. F. (1967) *Nature (London)* **216,** 857–858.
24. Weksler, B. B., Ley, C. W. & Jaffe, E. A. (1978) *J. Clin. Invest.* **62,** 923–930.
25. Tracy, P. B., Eide, L. L. & Mann, K. G. (1985) *J. Biol. Chem.* **260,** 2119–2124.
26. Wilner, G. D., Danitz, M. P., Mudd, M. S. & Hsieh, K.-h. (1980) *J. Lab. Clin. Med.* **97,** 403–411.
27. Kaminski, M. & McDonagh, J. (1983) *J. Biol. Chem.* **258,** 10530–10535.
28. Bar-Shavit, R., Bing, D. H., Kahn, A. J. & Wilner, G. D. (1985) in *UCLA Symposia on Molecular and Cellular Biology*, eds. Czech, M. P. & Kahn, C. R. (Liss, New York), Vol. 23, pp. 329–338.
29. Baker, J. B., Low, D. A., Simmer, R. L. & Cunningham, D. D. (1980) *Cell* **21,** 37–45.
30. Bjork, I., Jackson, C. M., Jornvall, H., Lavine, K. K., Nordling, K. & Salsgiver, W. J. (1982) *J. Biol. Chem.* **257,** 2406–2411.
31. Tollefsen, D. M., Majerus, D. W. & Blank, M. K. (1982) *J. Biol. Chem.* **257,** 2162–2169.
32. Stanley, E. R., Guilbert, L. J., Tushinski, R. J. & Bartelmez, S. H. (1983) *J. Cell. Biochem.* **21,** 151–159.
33. Wong, G. G., Witek, J.-A. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., Brown, E. L., Kay, R. M., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R. J., Hewick, R. M., Wang, E. A. & Clark, S.C. (1985) *Science* **228,** 810–815.
34. Morgan, C. J. & Stanley, E. R. (1984) *Biochem. Biophys. Res. Commun.* **119,** 35–41.