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Growth-Promoting Effects of Esterolytically Inactive Thrombin on Macrophages

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It has been recognized for many years that α-thrombin, like other better known mitogens (eg, PDGF, EGF, etc) is capable of initiating proliferation in quiescent cells belonging to the fibroblast family. However, unlike these other peptides, thrombin is a serine protease whose function as a growth stimulator 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 (eg, iPR_2P-α-thrombin) nor partially degraded thrombin (eg, γ-thrombin) shares in this capability. Data from our laboratory have shown that thrombin is chemotactic for peripheral blood monocytes and for cells belonging to the monocyte/macrophage family and that this activity is not dependent upon thrombin's enzymatic properties. Our recent findings demonstrate that thrombin also serves as a growth factor for these cells, and this mitogenic capability is independent of esterolytic function and resides in the same region of the molecule as that responsible for chemotaxis. Additionally, by means of techniques such as computer modeling and peptide synthesis, we have now been able to delineate a distinct mitogenic subsite within this chemotactic thrombin sequence. Thus, the sequence in the thrombin B chain that mediates chemotaxis represents a true cell interactive exosite additionally capable of stimulating growth and possibly other biological functions in cells of macrophage/monocyte lineage.

Key words: growth factor, macrophage, peptide synthesis, thrombin

Abbreviations used: BSA, bovine serum albumin; cpm, counts per minute; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FGF, fibroblast growth factor; HF, human foreskin fibroblast cells; ^{3}[H]-TdR, tritiated thymidine; iPR_2P, diisopropyl phosphorofluoridate; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline (pH 7.4); SEM, standard error of mean; TCA, trichloroacetic acid.

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Thrombin (E.C. 3.4.21.5.) is a serine esterase that plays a central role in initiating procoagulant events. This enzyme, which catalyzes the conversion of fibrinogen to fibrin [3], also stimulates platelet aggregation and release reactions [4,5] and release of arachidonate pathway metabolites from endothelial cells [6]. Interestingly, significant amounts of active thrombin are recoverable intact from the fluid phase of fibrin clots [7–9]. Thus, thrombin is available to the inflammatory cellular components that characterize the early phases of wound healing. In addition, α-thrombin (the physiologically active form of this enzyme) stimulates quiescent fibroblasts arrested at G_0/G_1 to reenter the proliferative phase [10–12]. Although structurally dissimilar, thrombin is similar to a family of polypeptide growth factors (ie, EGF, PDGF, insulin, and insulin-like growth factors) that act to initiate DNA synthesis and cell division [13] in regard to the mitogenic effects. However, unlike these other peptide mitogens, thrombin is a proteolytic enzyme. In studies on the mitogenic effects of thrombin on fibroblasts, only the intact, fully active enzyme (α-thrombin) is capable of stimulating cell division and DNA replication [12,14], whereas esterolytically inactive (ie, iPR_2P-α-thrombin) or partially degraded thrombin forms (ie, γ-thrombin) are essentially inactive [12]. Although the mechanism by which thrombin stimulates fibroblast growth is unknown, its ability to function as an esterase appears to be required.

We have recently demonstrated that thrombin is a potent and selective chemotaxin for monocytes and certain macrophage-like cell lines [15,16] and that its ability to stimulate directed cell movement resides in a specific cell-recognition exosite region on the thrombin B chain that is separate and distinct from its active center [17]. Moreover, the chemotactic region in thrombin appears to reside within a cyanogen bromide fragment (CB67–129) of human prethrombin 1 [18,19] corresponding to residues 338–400* of the thrombin B chain. Structurally, this region in thrombin is unique insofar as it contains a relatively large (9–10 residues) insertion sequence, termed loop "B" by Elion and coworkers [20] (see Table I), which is absent in the closely related enzymes, chymotrypsin and trypsin. By inference, since these latter proteins are devoid of chemotactic activity, this insertion sequence is likely to be an important determinant of thrombin's chemotactic effects [18].

In the present report, we show that in contrast to fibroblasts, the murine macrophage-like cell line J-774 exhibits a mitogenic response to enzymatically inactive thrombin, and that the region in thrombin required for such growth stimulation appears to reside in the B chain cell-recognition exosite. Moreover, we are able to demonstrate that a synthetic tetradecapeptide homologue of this region (residues 367–380) containing the loop "B" insertion sequence [20] is itself capable of stimulating J-774 cell division. These data further indicate that the structural requirements for thrombin mediation of macrophage cell growth and chemotactic effects are clearly dissociable.

MATERIALS AND METHODS

Reagents

All chemicals were of reagent grade or better. The following were obtained from the sources listed: BSA (fraction V) from Miles (Naperville, IL) DMEM from

*Prothrombin nomenclature [1]. The sequence used for synthesis is according to the protein sequence data of Butkowski et al [2].

TABLE I. Macrophage-Interactive Sequences in the Human Thrombin B Chain

I.[a]

```
          340                                   350
-Leu-Phe-Arg-Lys-Ser-Phe-Gln-Glu-Leu-Leu-Cys-Gly-Ala-Ser-Leu-Ile-Ser-Asn-
                360                                   370          *
Arg-Trp-Val-Leu- Thr-Ala-Ala-[His]-Cyss-Leu-Leu-Tyr-Pro-Pro- Trp-Asn-Lys- Asn-
                    380                               390
Phe-Thr-Glu-Asn-Asp- Leu-Leu-Val-Arg-Ile-Gly-Lys-His-Ser-Arg- Thr-Arg-Tyr-
                                  400
Glu-Arg-Asn-Ile-Glu-Lys-Ile-Ser- Met-
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II.[b]

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          367          370                                   380
       H- Tyr-Pro-Pro- Trp-Asn-Lys-Asn-Phe-Thr-Glu-Asn-Asp-Leu- Leu-OH
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[a]Primary structure of CB67–129, a cyanogen bromide-derived peptide derivative of human prethrombin 1, which represents residues 338–400 of the human thrombin B chain. The structure shown is based on the protein sequence data of Butkowski et al [2]. The nomenclature used is that of the zymogen, prothrombin [1]. The loop "B" insertion sequence (residues 367–375) [20] is underlined. Other important structural features include the active site His 363 (in brackets) and the carbohydrate attachment site at Asn 373, indicated by an asterisk. The amide assignment on Asx 355 and 371 is uncertain [2,1].

[b]Structure of a synthetic loop "B"-containing peptide, which exhibits macrophage-specific mitogenic activity. Since the precise limits of the loop "B" insertion sequence is uncertain, this peptide contains sufficient overlap to accommodate the limits suggested in the literature [20,29,30].

KC Biologicals (Kansas City, MO) FCS from Hyclone (Logan, UT) hirudin from Sigma Chemical Co. (St. Louis, MO) ^{125}I (specific activity = 2.3 Ci/μmol) and methyl^3[H]-TdR (specific activity = 6.7 Ci/mmol) from New England Nuclear (Boston, MA).

Cells

J-774 murine macrophage-like cells were a gift from Dr. Jay Unkeles, Mount Sinai School of Medicine (New York, NY). Chinese hamster lung (CHL) fibroblasts were obtained from the ATCC (Rockville, MD). Human foreskin (HF) fibroblasts were provided by Dr. H. Welgus, Washington University School of Medicine.

Human Thrombin Preparations

Preparations of active human α-thrombin and esterolytically inactive human iPR_2P-α-thrombin were generously provided by Dr. John Fenton, New York State Department of Health (Albany, NY). Their preparation and characterization have been described in detail [21,22].

Thrombin Fragments

The isolation and purification of peptide CB67-129 from human prethrombin 1 and the preparation of limited tryptic digests of this peptide following citraconylation and subsequent removal of the reversible blocking groups were carried out as described [18].

^{125}I-α-Thrombin Preparation

Human α-thrombin was radioiodinated using the iodogen reagent method as previously described [14]. The specific activity of a typical preparation was 120–180 cpm/fmol.

^{3}[H]-TdR Incorporation

Cells were plated in DMEM containing 10% FCS in 95% CO_2 humidified atmosphere at 37°C at an initial density of 5×10^5 per well in 24-well disposable plastic plates (Falcon, Oxnard, CA). Arrest of DNA synthesis (ie, G_0/G_1) was achieved by incubating cells for 48 hr in serum-free DMEM containing 0.1% BSA. The cells were then incubated with various thrombin forms, peptide, or FCS for 48 hr. Enhanced thymidine incorporation was assessed following a 2-hr pulse of N-methyl^{3}[H]-TdR (1 μCi/ml). Cells were then washed three times in PBS at 4°C precipitated with 10% TCA (30 min at 4°C), and the insoluble material was extracted twice with ETOH:ET_2O (3:1 v/v). The precipitate was solubilized in 1 N NaOH, and aliquots was withdrawn for protein determinations using a dye-binding assay (Bio-Rad, Richmond, CA) and liquid scintillation spectrometry.

Cell Cycle Analysis [27,28]

Adherent J-774 cells were detached by gentle scraping with a rubber policeman, washed once in PBS, and resuspended (10^6 cells/ml) in Krishan's reagent [28] containing propidium iodide (50 μg/ml), ribonuclease A (20 μg/ml, Sigma), Nonidet P-40 (0.3% v/v), and 1 mg/ml sodium citrate, pH 8.3. The resultant nuclei were vortexed and placed on ice for 30 min vortexed again, centrifuged (200g) for 10 min, resuspended in Krishan's reagent, and analyzed immediately in an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL). Fifty thousand nuclei were analyzed for each culture variable. Histogram analysis was performed on a TERAK LSI/1123 microcomputer utilizing software program PARA 1.C written by Dr. C.B. Bagwell (Coulter Electronics, Hialeah, FL).

Peptide Synthesis

Peptide 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 structure II, Table I), was synthesized by a modification of the method of Merrifield, as previously described [23,24]. 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 [25]. The crude peptide was definitively purified by ion exhange chromatography on a DEAE Sephacel column as previously described [24]. Peptide purity was ascertained by reverse phase (C18) high-performance liquid chromatography (HPLC) [26], in which it chromatographed as a single peak. The peptide was also found to be homogeneous by TLC, migrating as a single spot using two different solvent systems [23,24]. Amino acid analysis yielded the expected molar ratios.

^{125}I-α-Thrombin Binding

Human foreskin fibroblasts were grown to confluence on six-well plates (Falcon, Oxnard, CA). Radioiodinated binding studies were carried out essentially as described by Glenn and coworkers [14].

RESULTS

Mitogenic Activity of Nonenzymatically Active Thrombin

Addition of α-thrombin to CHL fibroblasts stimulates DNA replication (Fig. 1). When esterolytically inactive iPR_2P-α-thrombin is substituted, the cells are incapable of entering the replicative phase and remain in G_0/G_1-arrested phase. By contrast, addition of iPR_2P-α-thrombin to the macrophage-like cell line J-774 stimulates DNA replication. As shown in Figure 1, the rate of ^{3}[H]-TdR incorporation varies as a function of α-thrombin and iPR_2P-α-thrombin concentrations over a range of 10^{-11} to 10^{-6} M, with optimal stimulation occurring at 10^{-8} M for both thrombin forms. The active and inactive thrombin forms elicit the same degrees of response in J-774 cells.

The mitogenic effects stimulated by these thrombin preparations is a specific effect of thrombin itself, since hirudin, a leech-derived polypeptide thrombin inhibitor that complexes with both α- and iPR_2-α-thrombin, blocks these effects (data not shown).

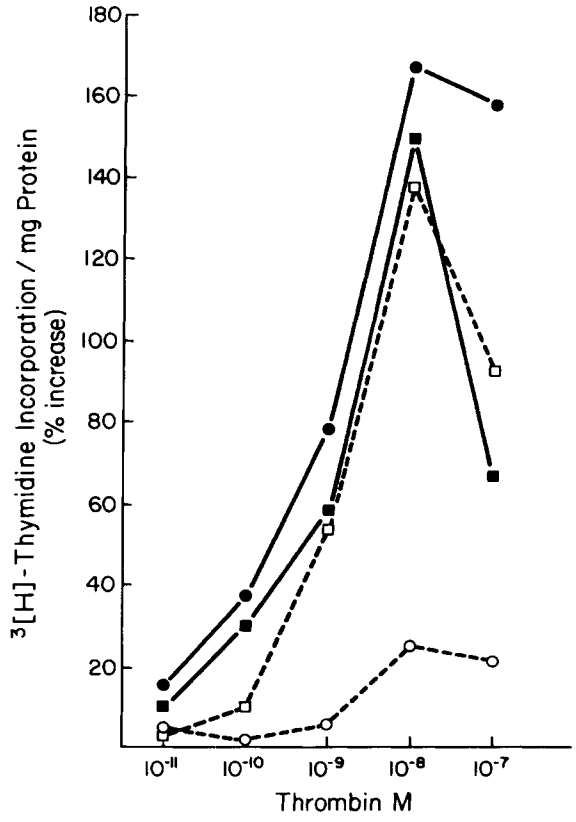

Fig. 1. Effect of α-thrombin and iPR_2P-α-thrombin on ^{3}[H]-TdR incorporation by CHL fibroblasts and J-774 macrophage-like cells. CHL cells were treated with α-thrombin (●) or iPR_2P-α-thrombin (○) with baseline incorporation of ^{3}[H]-TdR (in quiescent state) of 2,000 cpm ± 145 SEM per well. J-774 cells were treated with α-thrombin (■) or iPR_2P-α-thrombin (□) with control levels of 780 cpm ± 42 SEM per well.

Mitogenic Effects of the Thrombin Chemotactic Peptide

The fact that thrombin promotes cell division in these cell types independent of its esterolytic function, and that such activity is inhibitable by hirudin, suggests that the region in thrombin responsible for these effects may either coincide or overlap with the thrombin chemotactic exosite. Our earlier studies have shown that the site on thrombin responsible for initiating directed cell movement is localized within peptide CB67–129, a cyanogen bromide-derived cleavage fragment from the midportion of the thrombin B chain, which includes the active site histidine [18]. The primary structure of this fragment is depicted in Table I (structure I). To test this hypothesis, the thrombin-derived chemotactic fragment, peptide CB67–129, was assessed for its ability to act as a mitogen. As indicated in Figures 2 and 3 and in Table II, peptide CB67–129 stimulates ^{3}[H]-TdR incorporation in J-774 (but not on fibroblasts, data not shown) over a concentration range of 10^{-11} to 10^{-6} M, with optimal stimulation occurring at 10^{-8} M. Flow cytometric analysis (as shown in Fig. 3) indicates that the peptide (CB67–129) is capable of increasing the proportion of cells in S-phase from 5.6% to 18.75%. The degree of response elicited with this cyanogen bromide-derived fragment is approximately equal to or greater than that elicited with either active or enzymatically inactive thrombins when both are tested at their optimal concentrations.

Our previous studies on the chemotactic effects of peptide CB67–129 indicated a requirement for the intact peptide [18] (Table II). To determine whether the same would be true for its mitogenic effects, the lysl ϵ-amino groups in peptide CB67–129 were reversibly blocked by citraconylation, and the modified fragment was subjected to tryptic digestion [18]. Under these conditions, cleavage of the peptide was limited exclusively to arginyl bonds. Following removal of the lysine side-chain blocking groups, the resultant digest was tested for J-774 mitogenic activity. In contrast to previously reported loss of chemotactic effects, the digest stimulated mitogenic activity (as evidenced by ^{3}[H]-TdR incorporation) at levels equal to that of the intact CB67–129 fragment (Table II; Fig. 3) and by flow cytometric analysis (Fig. 3). The digest is capable of inducing growth-arrested J-774 cells to increase entry to S-phase from 5.6% to 17.1%. Thus, the structural requirements for chemotactic and mitogenic

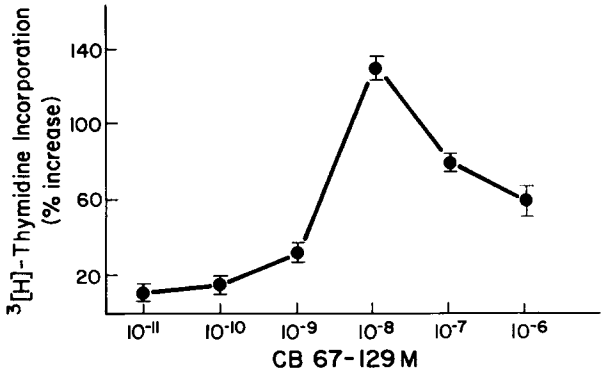

Fig. 2. Effect of the CNBr-derived peptide (CB67–129) on ^{3}[H]-TdR incorporation by J-774 cells. Cells were arrested at G_0/G_1 phase by incubation in serum-free medium containing 1 mg/ml BSA, then incubated with the peptide. Maximal stimulation is observed at 10 nM.

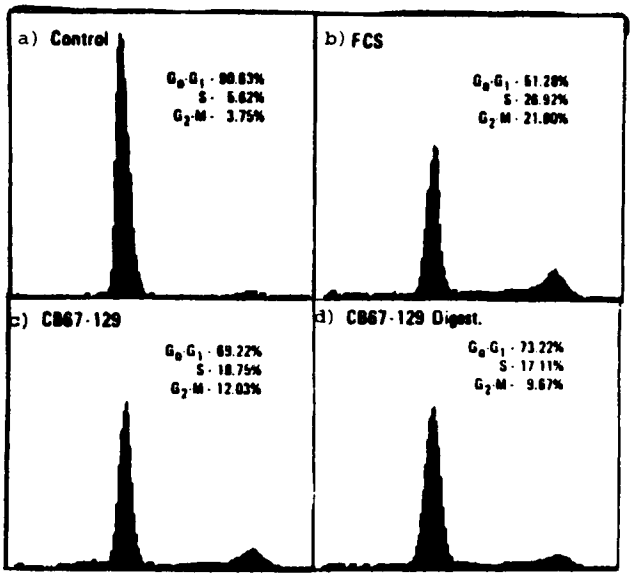

Fig. 3. Flow cytometric analysis of cell cycle distribution in J-774 cells. Analyses were carried out 18 hr following stimulation with 10% FCS (b), the peptide CB67–129 (c), or CB67–129 digest (d), as compared to control levels (a). The abscissa represents relative fluorescence (ie, DNA per nucleus) and the ordinate cell number. Note the increased proliferative activity (percentage of cells entering S-phase) observed in panels b–d, as compared to baseline control (panel a).

TABLE II. Thrombin-Directed Cell Migration and Mitogenesis on Monocytes/Macrophages*

	Migration (cells/hpf)	^{3}[H]-TdR Incorporation by J-774 Cells (% increase)
α-thrombin	*120 ± 10*	*142 ± 9*
iPR_2P-α-thrombin	*135 ± 9*	*170 ± 8*
Hirudin-ga-thrombin complex	9 ± 3	12 ± 2
Prothrombin	10 ± 2	12 ± 3
AT3-α-thrombin complex	4 ± 0.5	10 ± 3
CB67–129	*110 ± 6*	*138 ± 7*
CB67–129 digest	2 ± 0.5	*139 ± 11*
Synthetic B-loop peptide	3 ± 0.4	*140 ± 9*

*Chemotaxis was determined as described [15] using modified Boyden chambers and human peripheral blood monocytes. Mitogenesis was established by the degree of ^{3}[H]-TdR incorporation into TCA-insoluble fraction of stimulated J-774 cells. Significant values are indicated in italics.

stimulation of monocytes and macrophages by this unique region in thrombin are clearly dissociable.

A Synthetic Peptide Representing the Loop "B" Region in Thrombin Has Growth Factor Activity for J-774 Cells

Fragment CB67–129, which is derived from the thrombin B chain, contains a unique nine- to ten-residue insertion sequence termed loop "B" located immediately carboxy-terminal to the active site histidine group [20]. Computer model analysis of CB67–129 shows that this insertion sequence is surface expressed and is therefore accessible to cell interactions [19]. Our previous studies on the ability of the thrombin chemotactic fragment to stimulate directed cell movement suggested that while the

loop "B" insertion sequence contributes significantly to thrombin's chemotactic activity, it alone is insufficient to stimulate monocyte/macrophage chemotaxis [18,19]. To determine whether the loop "B" sequence itself might possess biological function independent from its parent CB67–129 peptide, loop "B"-containing homologue of the cyanogen bromide fragment (representing residues 367–380 of the human prothrombin sequence) was synthesized (Table I, structure II), and its ability to stimulate mitogenic activity in J-774 cells was determined. As indicated in Figure 4 and Table II, this synthetic homologue is capable of stimulating both ^{3}[H]-TdR incorporation and protein synthesis in G_0/G_1-arrested J-774 cells over a concentration range paralleling that of both thrombin and CB67–129, with optimal stimulation for both effects occurring at 10^{-8} M. Two lines of evidence further indicate that this increased ^{3}[H]-TdR incorporation following stimulation with the peptide represents stimulation of cell growth rather than simply acceleration of DNA repair. First, this synthetic peptide stimulates increased J-774 proliferation as compared with unstimulated control (data not shown). Second, the ability of this peptide to stimulate the progression of J-774 cells arrested at G_0/G_1 into S-phase was assessed by means of flow cytometric analysis using the fluorescent DNA-binding probe, propidium iodide [27,28]. By these means, the increase in relative fluorescence (indicating an increase in cell DNA content) can be assessed as a function of cell number, and responsive subpopulations of cells can be defined accordingly. In a representative experiment, we found that while the quiescent population of J-774 cells contains 7.47% cells in S-phase, this fraction increases more than threefold to 23.5% following stimulation of these cells with the synthetic peptide at 10^{-8} M, its optimal mitogenic concentration based on our ^{3}[H]-

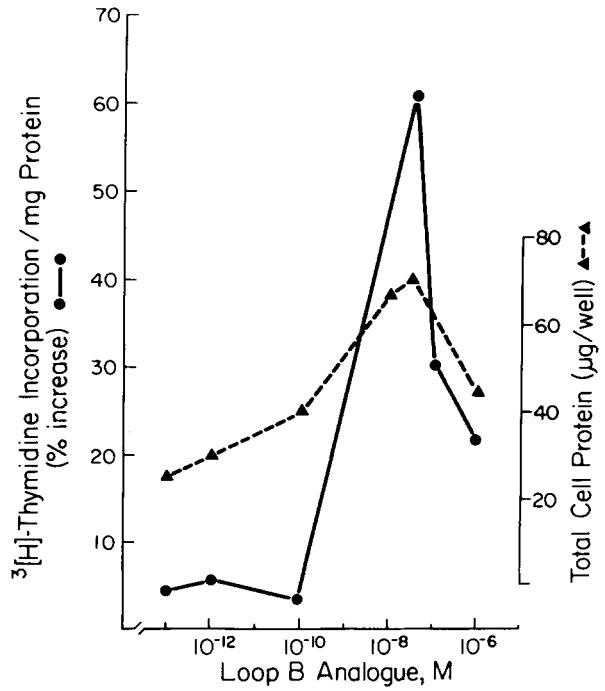

Fig. 4. Effect of the synthetic homologue of thrombin loop "B" insertion sequence on ^{3}[H]-TdR incorporation and protein levels in J-774 cells. Maximal stimulation is observed at 10 nM under experimental conditions, similar to those in Figure 1.

TdR incorporation data. Thus, this loop "B" sequence in the thrombin B chain appears wholly responsible for the nonenzymatic growth factor activity exhibited by enzymatically inactive thrombin. It is likely that the mitogenic effects of this peptide, as is true for polypeptide growth factors [13], is receptor mediated. Studies on the elucidation of loop "B" peptide-binding sites on the macrophage cell membrane are presently in progress.

Relationship of Domain Requirements for Thrombin Mitogenic Stimulation of Macrophages Versus Fibroblasts

Since, with the exception of the requirement for esterolytic function, thrombin appears to be a potent mitogen for both fibroblasts and macrophages, the question arises as to whether the loop "B" sequence, which is mitogenic for macrophages, plays any significant role in stimulating fibroblast proliferation. In contrast to its effects on growth-arrested J-774 cells, the loop "B"-containing synthetic peptide failed to elicit any significant ^{3}[H]-TdR incorporation in quiescent CHL or HF fibroblasts (data not shown). However, it is still possible that the loop "B" sequence alone might be insufficient to stimulate fibroblasts but that it may be required for binding of thrombin to the fibroblast cell membrane. To test this, the ability of the synthetic loop "B" peptide to inhibit ^{125}I-α-thrombin binding to HF fibroblasts was determined. As illustrated in Figure 5, whereas unlabeled human α-thrombin completely inhibited binding of the radioligand to these cells, no inhibition was evident using the synthetic peptide over the same concentration range. Thus, at least over the concentration range of peptide tested, it is apparent that the site requirements for thrombin mediation of macrophage and fibroblast mitogenicity are not related, since the loop "B" sequence is neither itself mitogenic nor is it required for thrombin binding to the fibroblast surface.

DISCUSSION

Studies from Buchanan's laboratory were the first to demonstrate that thrombin is mitogenic for avian fibroblasts [10]. Subsequently, this finding was extended to

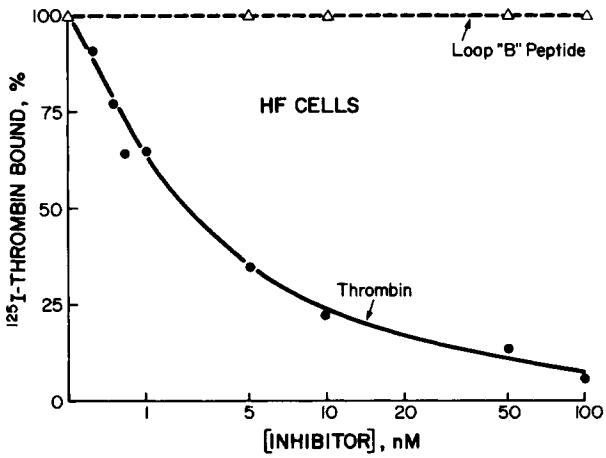

Fig. 5. Competition of ^{125}I-α-thrombin binding to HF cells by unlabeled α-thrombin and the synthetic loop "B" peptide. The percentage of maximal binding of 2.3 nM ^{125}I-α-thrombin to HF cells (per 5×10^5 cells) in the presence of the indicated concentrations of each thrombin preparation or loop "B" peptide was measured as described in "Materials and Methods."

fibroblasts from other species and was found to depend upon thrombin's activity as a protease [14,12]. Unlike fibroblasts, quiescent macrophage-like cells can be induced by inactivated thrombin to enter S-phase, as monitored by ^{3}[H]-TdR incorporation and flow cytometry analysis. The degree of stimulation appears to be dose dependent, with the optimal concentration required for these effects being the same for inactive iPR_2P-α-thrombin as for the fully active enzyme. The DNA-synthetic-stimulating effects observed here are specific for thrombin, since complexes formed with the leech-derived polypeptide thrombin inhibitor, hirudin, are devoid of this effect. Hirudin itself has neither stimulatory nor inhibitory effects on these cells; quiescent cells; quiescent cells remain arrested, while serum-stimulated cells in the presence of hirudin show the same level of induction as with serum alone. Our finding that alteration of thrombin's catalytic site does not affect its mitogenic activity suggests the existence of a specific region within the molecule that is involved with cell recognition/interaction. Thus, the interaction of this site with the cell leads to cell division and eventually to replicative DNA synthesis. Since our previous studies have demonstrated the existence of a cell-recognition domain within the thrombin B chain that appears to be responsible for directed cell movement of monocytes/macrophages by thrombin [18,19], our present studies indicate that this same region may be responsible for thrombin's mitogenic effects as well. This region is contained within the thrombin B chain-derived cyanogen bromide fragment CB67–129, representing residues 338–400 of the thrombin B chain sequence. As indicated in Figure 2, exposure of quiescent cells to fragment CB67–129 produces a dose-dependent enhancement of ^{3}[H]-TdR incorporation. Furthermore, tryptic digests of citraconylated CB67–129 fragment, although failing to elicit stimulation of mononuclear cell movement, show a similar dose-dependent stimulation of ^{3}[H]-TdR incorporation as does the intact fragment. Moreover, since computer-modeling studies of this sequence indicate that only residues carboxy-terminal to the active site histidine (His 363) are surface expressed [19], it is likely that only sequences within this portion of the cyanogen bromide fragment are physiologically relevant. Of interest is that within this surface expressed region is a unique nine- to ten-residue insertion sequence termed loop "B" by Elion et al [20]. This insertion sequence, which is absent in the closely related serine esterases chymotrypsin and trypsin, as well as in other vitamin K-dependent clotting factors, is a unique structural feature of thrombin [29,30]. As shown in Figure 3, a synthetic 14-residue homologue of the loop "B" sequence stimulates significant DNA synthesis in quiescent J-774 cells, as indicated by ^{3}[H]-TdR incorporation. This increased DNA synthesis represents a mitogenic response and not DNA repair, since the peptide also stimulates an increase in amounts of total protein per culture well and a significant shift in resting cells entering S-phase, as indicated by flow cytometric analysis (R. Bar-Shavit, unpublished data).

Interestingly, despite their efficacy in stimulating fibroblast growth, neither epidermal growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, nor L-cell-derived CSF-1 are capable of stimulating a mitogenic response in these macrophages (R. Bar-Shavit, unpublished data). It is not surprising, therefore, that the loop "B" peptide is ineffective as a mitogen for fibroblast cells, and it does not appear to be involved in thrombin binding sites in these cells (Fig. 5). Based on these observations, the loop "B" sequence in thrombin should be considered a mitogenic and growth factor subsite that is effective in *selectively* stimulating effects in cells of monocyte/macrophage lineage. A corollary

to these findings is that this region has no role in initiating the mitogenic effects of α-thrombin on fibroblasts and that these latter effects require contributions from other sites in the thrombin molecule.

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, and therefore noncoagulant degraded thrombins (eg, β- and γ-thrombins [22], 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 [31], antithrombin III [32], or heparin cofactor II [33]. From these considerations, it is 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.

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