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Localization of a Chemotactic Domain in Human Thrombin†

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ABSTRACT: The cyanogen bromide fragment CB67–129 of human prethrombin 1, corresponding to residues 54–116 of the thrombin B chain, is a potent chemotaxin for human peripheral blood monocytes and the murine macrophage like cell line, J774. Both of these cell types have been shown to respond chemotactically to α-thrombin and iPr_2P-α-thrombin. Effective concentrations for stimulating directed cell movement with the fragment vary from 10^{-11} to 10^{-7} M. Moreover, CB67–129 and its parent protein compete for the same chemotactic receptor site. Fragment CB67–129, representing residues 54–116 of the human thrombin B chain sequence, contains a nine-residue insertion ("loop B") that is absent in homologous sequences derived from the closely related proteases chymotrypsin and trypsin. Unlike iPr_2P-α-thrombin, iPr_2P derivatives of these latter enzymes possess little or no chemotactic activity, suggesting a relationship between the insertion sequence and thrombin chemotactic activity. The loop B sequence is unique insofar as it contains all of the carbohydrate moieties known to reside in α-thrombin. However, chemotactic activity is only minimally reduced subsequent to hydrolysis by both neuraminidase and β-galactosidase, indicating that receptor recognition and stimulated cell movement are mainly a function of structure of the cyanogen bromide derived fragment rather than of asparagine-linked carbohydrates.

Thrombin is a serine esterase whose generation results from activation of the clotting cascade (Jackson et al., 1975). In addition to its multiple effects on both the plasma (Fenton, 1981; Mann & Lundblad, 1982) and cellular components of the hemostatic system (Davey & Luscher, 1967; Tollefsen et al., 1974; Martin et al., 1975), thrombin has more recently been shown to be a potent mitogen (Buchanan et al., 1976; Cunningham et al., 1979; Perdue et al., 1981), to stimulate prostaglandin synthesis in endothelial cells (Weksler et al., 1978; Awbrey et al., 1979; Fehrenbacker et al., 1979; Loskutoff, 1979) and established cell lines (Becherer et al., 1982), and to promote smooth muscle contraction (White et al., 1980; Ku, 1982; Haver & Namm, 1983). To date, virtually all of these effects have been reported to relate to thrombin's function as an enzyme, requiring an intact active center.

Recently, we reported that α-thrombin as well as a spectrum of active-center and exo-site-modified thrombin forms is a potent chemotaxin for monocytes and certain macrophage-like cell lines. Only when thrombin is complexed with the inhibitors AT3 and hirudin does it lose its ability to stimulate directed cell movement. These data suggest the existence of a unique region in thrombin which is independent of the active center but which is involved with cell membrane recognition and the initiation of chemotaxis (Bar-Shavit et al., 1983a,b,c).

In the present study, we report localization of a chemotactic region within a ~6500-dalton segment of the human thrombin B chain. This peptide (CB67–129) is chemotactic and effectively competes with thrombin as a chemotactic stimulus. The latter finding suggests that both CB67–129 and thrombin alter cell movement through occupancy of the same membrane receptor site.

Materials and Methods

Reagents. Human α-thrombin was prepared and characterized as described previously (Fenton et al., 1977). The specific clotting activity of the α-thrombin preparation was 3975 units/mg. iPr_2P-α-thrombin[1] was prepared by treating α-thrombin repeatedly with 0.2 M iPr_2P-F at pH 8.0 for 30

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[1] Abbreviations: AT3, antithrombin III; FMP, fMet-Leu-Phe-OH; iPr_2P-F, diisopropyl phosphorofluoridate; SEM, standard error of mean; TLCK, N^{α}-tosyl-L-lysine chloromethyl ketone; CNBr, cyanogen bromide; hpf, high power field.

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min until clotting activity was diminished to ~0.5 unit/mg and active sites were titrated an undetectable level. Asialo- and asialoagalacto-α-thrombins were generously provided by Dr. McDonald K. Horne, III, NIH, Bethesda, MD, and were prepared as described (Nilsson et al., 1983). These preparations contained no measurable sialic acid nor galactose as determined by colorimetric and enzymatic methods, respectively. iPr_2P-chymotrypsin and iPr_2P-trypsin were prepared in essentially the same manner as iPr_2P-α-thrombin; bovine enzymes (Worthington, Freehold, NJ) were used as starting materials. Peptide CB67–129 was isolated from a cyanogen bromide digest of human prethrombin 1 by gel filtration on Sephadex G-100 as previously described (Butkowski et al., 1977). The identity of the peptide was verified by quantitative amino acid analysis and NH_2-terminal sequence analysis (Edman & Begg, 1967). Fragments of CB67–129 were prepared by first reversibly blocking the peptide at lysine by citraconylation (Gibbons & Perham, 1970), followed by trypsin digestion and subsequent removal of blocking groups.

Cells. Murine macrophage like J774A.1 cells were obtained from the American Tissue Type Collection, Rockville, MD. Cells were grown in RPMI-1640 medium containing 10% fetal calf serum, 1% glutamine, 1% nonessential amino acids, and antibiotics at 37 °C in a humidified CO_2 incubator. Human peripheral blood monocytes were harvested from blood samples obtained from normal volunteers as previously described.

Chemotaxis Assays. Chemotaxis was determined in modified Boyden chambers (Ahlco Manufacturing Co., Inc., Southington, CT) by a double filter technique, modified as previously described (Bar-Shavet et al., 1983a,c). The chemotactic peptide fMet-Leu-Phe-OH (FMP) was used as control (Schiffman et al., 1975).

Results and Discussion

Cleavage of the single-chain precursor of human thrombin, prethrombin 1, with cyanogen bromide results in four major fractions following gel filtration on Sephadex G-100 columns (Butkowski et al., 1977). Of these fractions, only CB67–129, representing residues 54–116 (M_r ~6500) of the human thrombin B chain, possesses significant ability to stimulate cell mobility in both the murine macrophage like cell line J774 and human peripheral blood monocytes. As shown in Figure 1, CB67–129 promotes cell migration in J774 cells and monocytes at concentrations ranging from 10^{-11} to 10^{-7} M with the optimum dose for J774 mobilization at 10^{-9} M (Figure 1A) and for monocytes at ~10^{-7} M (Figure 1B). The degree of response at these concentrations is approximately equal to that of thrombin. Attempts to further fractionate CB67–129 into smaller biologically active peptides resulted in a complete loss of chemotactic potency (Figure 1B).

The nature of chemotaxis is such that motile cells move only in response to an increase in concentration gradient of chemotaxin (Zigmond & Hirsch, 1973; Zigmond, 1977). To determine whether the migratory response to CB67–129 represents movement along a gradient (i.e., chemotaxis) or simply increased random movement (i.e., chemokinesis), assays were run in which the concentration of peptide was systematically varied in the upper and lower compartments of the Boyden chambers ("checkerboard analysis"). As evident in Table I, significant migration occurs only when the peptide concentration in the lower compartment of the Boyden chamber exceeded that of the upper compartment.

It is well established that the chemotactic response to formylated peptide is a cell membrane receptor mediated event (Becker, 1979; Pike et al., 1980; Weinberg et al., 1981), and this is likely true for the thrombin-induced chemotaxis as well

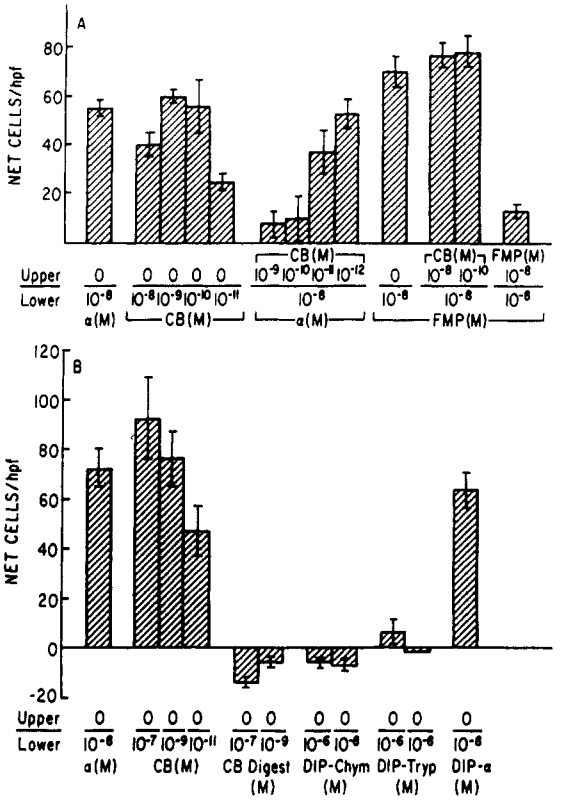

FIGURE 1: Stimulated migration of murine macrophage like J774A.1 cells (panel A) or human peripheral blood monocytes (panel B) as a function of peptide CB67–129 (CB) concentration. Comparison is made with the chemotactic effects of other test substances, including the tryptic digest of CB67–129 (CB Digest), α-thrombin (α), iPr_2P-α-thrombin (DIP-α), related esterases including iPr_2P-chymotrypsin A (DIP-Chym) and iPr_2P-trypsin (DIP-Tryp), and the chemotactic peptide FMP. The concentrations of test substances in the upper compartment (Upper) vs. the lower compartment (Lower) of the Boyden chambers are indicated. A representative experiment is shown. Each bar represents the mean of triplicates ± SEM.

Table I: Monocyte Chemotaxis to CB67-129[a]

		Upper Compartment, CB67-129 (M)			
		0	10^{-11}	10^{-9}	10^{-7}
Lower Compartment, CB67-129 (M)	10^{-11}	47 ± 10	-3 ± 3	0 ± 3	9 ± 4
	10^{-9}	76 ± 11	49 ± 8	-11 ± 5	-1 ± 6
	10^{-7}	92 ± 17	28 ± 6	10 ± 6	-11 ± 3

[a] Results are net movement/hpf. Data are the mean of triplicates ± SEM. A representative experiment is shown.

(Bar-Shavit, 1983c). Presumptive evidence that migratory response to peptide CB67–129 is similarly receptor mediated and, specifically, the result of occupancy of thrombin receptor is shown in Figure 1A. Here, assays were performed in which varying concentrations of CB67–129 were placed in the upper compartment of the Boyden chamber and the optimal chemotactic dose of α-thrombin, CB67–129, or FMP (Bar-Shavit, 1983b,c) in the lower compartment. Under these circumstances, a ligand (e.g., CB67–129), which competes for the same binding site as the chemoattractant (e.g., thrombin), should block migration. Indeed, the data show that cell movement toward α-thrombin (Figure 1A) was inhibited when the upper compartment contained the thrombin fragment and, moreover, that the degree of inhibition varied as a function

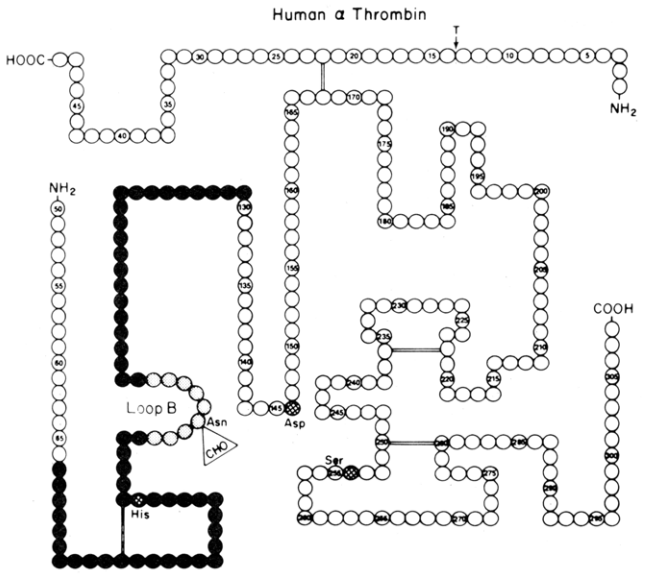

FIGURE 2: Bead model of human α-thrombin. The sequence nomenclature is that of prethrombin 2 (Downing et al., 1975). Prethrombin 2 is subsequently converted to a two-chain structure by factor Xa, following cleavage at residue 49, and to α-thrombin by autocatalytic removal of the NH_2-terminal 13 residues of the shorter A chain at the site marked T (Elion et al., 1977). Residues involved in the charge relay system are crosshatched. Peptide CB67–129 with its associated internal disulfide bond (histidine loop) is indicated by the darkened beads. The carbohydrate-containing loop B insertion (residues 99–107) is shown.

of concentration of the competing ligand. By contrast, cell movement toward FMP was unaffected by the presence of the thrombin fragment in the upper compartment. These observations suggest that CB67–129 and α-thrombin compete for the same binding site or receptor on the macrophage cell membrane and that this receptor is distinct from that mediating FMP chemotaxis. In this regard, it is of interest that computer-generated three-dimensional models of thrombin by Bing and co-workers (Bind et al., 1981) predict that most of the B-chain region included in CB67–129 is surface exposed and therefore capable of interacting with membrane receptors.

CB67–129 contains several unique structural features including the active site histidine (His-92), an internal disulfide bond ("histidine loop"), and a carbohydrate-containing sequence insertion termed "loop B" (Figure 2) (Elion et al., 1977). We have previously shown, through the use of TLCK-modified thrombin, that the active site histidine is unlikely to contribute to chemotactic activity (Bar-Shavit, 1983b). To establish whether the carbohydrate groups are required for chemotaxis, the terminal sialic acid and penultimate galactose groups of α-thrombin were quantitatively removed by sequential neuraminidase and β-galactosidase digestion, and the altered thrombins were assessed for chemoattractant activity. As indicated in Figure 3, the asialo-α-thrombin was unimpaired in its ability to elicit cell movement, while asialogalacto-α-thrombin was somewhat less potent. Considering the marked effects carbohydrate removal would have on charge and conformation of this region in thrombin, it is likely that the chemotactic activity of both thrombin and peptide CB67–129 relates mainly to their primary structure rather than to the asparagine-linked carbohydrate moiety.

As noted above, one of the unique structural features of CB67–129 is that it contains the loop B insertion, and consequently, it is tempting to speculate about the possible involvement of the latter structure in chemotaxis. That the loop B insertion does play an important role in eliciting cell

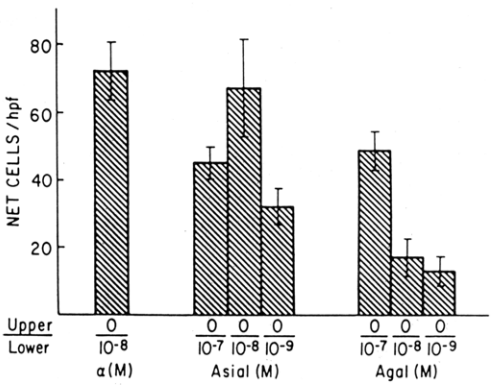

FIGURE 3: Effect of carbohydrate modification of α-thrombin on monocyte chemotaxis. The chemotactic activities of asialo-α-thrombin (Asial) and asialogalacto-α-thrombin (Agal) are shown in comparison with intact α-thrombin. A representative experiment is shown. Each bar represents the mean of triplicates ± SEM.

movement is suggested by the observation that neither iPr_2P-chymotrypsin nor iPr_2P-trypsin is chemotactic for monocytes (Figure 1B). Both these serine esterases bear significant homology to α-thrombin (especially chymotrypsin) but lack the loop B insertion. On the other hand, tryptic digests of CB67–129, which contain intact loop B fragments, failed to elicit cell movement (Figure 1B), indicating that the insertion sequence, per se, lacks some element essential for initiating biological activity. In this regard, we have observed that tryptophan-modified thrombins[2] are devoid of chemotactic activity, suggesting that these groups contribute significantly to the stimulation of cell movement. The role of these groups and other sites on CB67–129 in promoting chemotaxis is presently under investigation.

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Registry No. CB67–129, 88391-92-8; thrombin, 9002-04-4.

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3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine, a Hydrophobic, Photoreactive Probe, Labels Calmodulin and Calmodulin Fragments in a Ca^{2+}-Dependent Way†

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ABSTRACT: 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID), a highly hydrophobic, carbene-generating photoreactive probe, labels calmodulin and some of its proteolytic fragments in the Ca^{2+}-bound conformation only. It is assumed that [^{125}I]TID labels hydrophobic sites exposed by the binding of Ca^{2+}. The finding offers a new and powerful means to characterize calmodulin sites that play a role in the interaction with targets.

Calmodulin belongs to a family of highly homologous proteins that contain one to four Ca^{2+}-binding domains of similar properties (Klee & Vanaman, 1982; Teo & Wang, 1973). Kretsinger (1975) developed the general "EF-hand" model for this class of proteins on the basis of the crystal structure of parvalbumin. He predicted that the Ca^{2+}-binding domains of homologous proteins will always be composed of two α-helical regions flanking a calcium-binding loop, as is the case for parvalbumin. This view has gained general acceptance and has recently been corroborated by the determination of the crystal structure of the bovine intestinal calcium-binding protein (Szebenyi et al., 1981).

Ca^{2+} binding to calmodulin induces large conformational changes in the protein [for reviews see Klee & Vanaman (1982) and Krebs (1981)], exposing hydrophobic sites (La Porte et al., 1980) that are probably responsible for the binding of phenothiazines and other drugs as originally reported by Levin & Weiss (1977). These sites are often thought to be involved in the interaction of calmodulin with its target proteins (Klee & Vanaman, 1982). In this report a new method to identify these sites will be described. The method is based on the use of a radioactively labeled photoreactive probe, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID),[1] which so far has been used exclusively to label the intramembrane segments of transmembrane proteins (Brunner & Semenza, 1981). It has also been possible to label with this probe various proteolytic fragments of calmodulin in a Ca^{2+}-dependent way. The method is useful in identifying hydrophobic sites of the calmodulin molecule (or of other Ca^{2+}-binding proteins) and also, due to its high sensitivity, in

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[1] Abbreviations: [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; TFP, trifluoperazine.

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