Human α-thrombin was modified by four different procedures to identify specific active site regions required for receptor binding and stimulation of cell division. Conjugation of α-thrombin with diisopropylphosphofluoridate (DIP-F) or methylsulfonyl fluoride (MS-F) yielded catalytically inactivated preparations. Nitrination or limited proteolysis of α-thrombin led to nitro-α-thrombin or γ-thrombin preparations, respectively. Both possessed very little clotting activity but retained significant esterase activity; they were modified at regions necessary for the binding recognition of fibrinogen.

Measurements of specific binding of these modified thrombins to cultured mouse, hamster, chick, and human fibroblasts revealed no significant binding of nitro-α-thrombin or γ-thrombin. Thus, binding of α-thrombin to each of the cell types examined involved regions of α-thrombin distinct from the catalytic apparatus that are related, if not identical, to regions required for fibrinogen recognition. Binding experiments with catalytic site-conjugated DIP- or MS-α-thrombins revealed significant differences in the α-thrombin receptor among the four cell types; these thrombin forms bound as effectively as α-thrombin to mouse and hamster cells, but did not bind significantly to chick or human cells. Thus, thrombin binding to chick and human cells required the thrombin catalytic apparatus or adjacent active site regions, whereas binding to mouse or hamster cells did not.

The four cell types examined all responded to the mitogenic action of α-thrombin. However, the derivative thrombin forms did not stimulate division of any of the cells significantly, with the exception of nitro-α-thrombin which possessed some residual activity for mouse cells. Since enzymatically inactive DIP- and MS-α-thrombins bound to mouse and hamster cells as effectively as active α-thrombin, the mitogenic activity of α-thrombin on these cells requires the intact catalytic apparatus of the enzyme to interact with and presumably cleave a specific protein component.

Thrombin stimulates cell division in nonproliferating fibroblastic cultures of CE cells (1–7), ME cells (5, 7), HF cells (7–9), and CHL cells (10). The maximal cell number increases range from about 20 to 40% for HF and CHL cells to about 50 to 70% for ME and CE cells. With each cell type, α-thrombin can initiate cell division under chemically defined culture conditions in the absence of added serum or additional growth factors. This has greatly facilitated exploration of the biochemical events in thrombin-initiated cell division.

Previous studies have shown that action of α-thrombin at the cell surface is sufficient to initiate CE cell division (11, 12). As a consequence of these results, we focused our attention on the interaction of thrombin with the cell surface. Binding studies with 125I-α-thrombin revealed a single class of high affinity cell surface receptors on ME cells (13). Evidence was obtained that the thrombin receptors were relatively specific since neither insulin, epidermal growth factor, nor prothrombin significantly competed for the binding of 125I-α-thrombin. It appears that α-thrombin must bind to its receptors to stimulate division of ME cells since low concentrations of serum, which inhibited the mitogenic action of thrombin, markedly reduced its binding to cellular receptors. These low serum concentrations had no effect on either the fibrinogen-clotting activity of α-thrombin or its nonspecific association with cells (13). Binding sites for α-thrombin have also been identified and examined on CE cells (6, 14).

In the present studies, we examined a variety of active site-modified forms of thrombin to determine the extent to which the catalytic apparatus and adjacent active site regions of the enzyme are needed for binding to cellular receptors and for stimulation of cell division. DIP-F or MS-F were used to modify chemically the active site serine because of their large difference in occluding or sterically obstructing regions neighboring this serine. Thrombin forms which retain ester/amidolytic activities but which lack fibrinogen-clotting activity were made by nitrination or limited proteolysis to evaluate binding site regions within the enzyme involved in fibrinogen recognition. Early in these studies, significant differences were observed in the mode of interaction of certain thrombin forms with receptors on cells from different animal species. As a result, the binding and mitogenic potential of α-thrombin and its derivative forms were examined with ME, CHL, CE, and HF cells. Such comparisons enabled us to define features of the active site regions of thrombin required for receptor binding and stimulation of cell division in these cells. This

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Thrombin Binding and Mitogenic Stimulation

comparative approach also allowed us to detect significant differences in the thrombin receptor among cells of different animal species.

MATERIALS AND METHODS

Thrombin Preparations—Human α-thrombin was prepared from Fraction III paste (obtained through the courtesy of Dr. Fred Feldman as gifts from Armour Pharmaceutical Co., Kankakee, Ill.) and characterized as previously described (15-17). The α-thrombin preparation, used for comparative experiments, was a homogeneous preparation (100% α-thrombin) with high enzyme activity (Table I). α-Thrombin inactivated at its active site serine was made by incubation with DIP-F or MS-F (18, 19). Either DIP-F or MS-F was added in molar excess to the enzyme in 0.75 M NaCl, 10 mM Tris, pH 8.3, at 23°C and repeatedly added at 10- to 15-min intervals until the clotting activity was less than 5% of the parent α-thrombin preparation. The unconjugated reagents were removed by dialyzing against several changes of 0.75 M NaCl at 4°C. Nitro-α-thrombin with low clotting activity but moderate esterase activity was made by treating the enzyme with tetrantromethane at pH 8.0 essentially as described for other proteins by Sokolovsky et al. (20). When about 2% of the original clotting activity remained, nitration of tetrantromethane was removed by centrifugation and the nitro-α-thrombin was dialyzed as above. γ-Thrombin was prepared by either controlled tryptic digestion or autocatalytic digestion of α-thrombin. The tryptic digestion was carried out by passage of α-thrombin at pH 6.2 through a column of trypsin immobilized on agarose with a flow rate that yielded greater than 9% conversion to γ-thrombin (16). To exclude the possibility of any trypsin leached from the resin, γ-thrombin was also prepared by autoxidation. α-Thrombin at about 2 mg of protein/ml was incubated in 3 M NaCl, pH 8, at 23°C for about 3 days to achieve less than 1% of the initial clotting activity (21). The resulting γ-thrombin preparation was dialyzed against 0.75 M NaCl and characterized (see Table I). The percentage compositions of α-, β-, and γ-thrombins were determined by labeling with 125I-DIP-F (New England Nuclear) and quantitating thrombin activity as detected by emulsion grains over the nucleus. The resulting γ-thrombin preparation was dialyzed against 0.75 M NaCl and characterized (see Table I). The percentage compositions of α-, β-, and γ-thrombins were determined by labeling with [125I]-DIP-F (New England Nuclear) and quantitating the relative distribution of radioactivity after electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels (15). Since β- and γ-thrombin have their proteolytic B chain fragments in their enzymatically active states, an Mₜ of 36,500 for α-thrombin was assumed for all thrombin forms (15-17).

Iodination of Thrombin—Thrombin preparations were iodinated using chloroglycouril and Na125I (22). Chloroglycouril (Pierce) was dissolved at 1 mg/ml in CHCl₃; 50 ml of this solution was placed in a glass tube (10 × 75 mm) and the chloroglycouril was coated on the surface by drying under a stream of N₂ (23). Approximately 80 μg of α-thrombin (or other thrombin forms) and 1.0 mCi of Na125I (100 Ci/μm, carrier-free; Amersham) were added to 0.1 M NaCl buffered with 50 mM sodium phosphate at pH 7.0 and containing 160 mM benzamidine (11). The mixture was added to a chloroglycouril-coated tube and incubated for 10 to 20 min at 4°C. Iodination was terminated by removing the reaction mixture from the chloroglycouril-coated tube. Free 125I and benzamidine were removed by gel filtration (Sphedex G-50, medium) and three 15-min dialyses against 0.75 M NaCl buffered with 50 mM sodium phosphate at pH 7.0 at 4°C. The 125I-labeled preparations had a specific activity of 5.0 to 12.6 × 10⁶ cpm/μg 125I-α-thrombin retained 100% of its fibrinogen-clotting activity.

Thrombin Activity Measurements—Specific clotting activities, expressed in U.S. (NIH) units per mg of protein, were determined by measuring the clotting activity at each concentration as previously described (15, 24). Clotting activities relative to that of α-thrombin were measured using chloroglycouril and Hepes buffer (pH 7.4) at 4°C. The buffer solution consisted of 0.2 M Tris and 30 mM CaCl₂ at pH 8.1. An absorbance index of 540 mₜ cm⁻¹ at 247 nm was used for the hydrosulfosalicyclic acid assay.

Cells and Culture Conditions—All cell cultures were grown in plastic tissue culture dishes (Falcon Plastics). CE cells were prepared from the body walls of 9-day-old chick embryos as previously described (26). Primary CE cells were grown for 3 to 4 days in DV medium supplemented with 5.0% calf serum (Irvine Scientific). HF cells were cultured from newborn neonatal foreskin explants as previously described (27). HF cells were grown in DV medium containing 5.0% calf serum and used between passages 7 to 16. CHL cells from the V7 strain (28) were obtained from Dr. John J. Wasmuth (University of California, Irvine). HF and CHL cell cultures were judged to be free of mycoplasma by the Hoechst dye N 3258 as a fluorescent DNA stain for mycoplasma as previously described (29). Penicillin (100 units/ml, Gibco) and streptomycin (100 μg/ml, Gibco) were added to all media used in this study. All cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in air. Nondividing cultures, used in binding and growth studies, were obtained as follows: confluent cultures were rinsed and then removed from stock 100-mm culture dishes with 0.15 M NaCl, 50 mM sodium phosphate (pH 7.4), containing 0.02% EDTA and 0.05% trypsin, resuspended in their appropriate growth medium, and plated as 2-ml aliquots in 35-mm culture dishes. Secondary cultures of HF and CE cells were plated at 5.5 × 10⁴ cells/dish while HF and CHL cells were plated at 2.0 and 3.0 × 10⁵ cells/dish, respectively. After 4 h for the ME and CE cells or 72 h for the HF and CHL cells, the cultures were rinsed with DV medium containing no serum or tryptose phosphate broth and then incubated for 48 h in 2 ml of this serum-free medium prior to use in binding and growth studies.

Measurement of Cellular Proliferation—Broad concentration ranges of thrombin preparations were added to nondividing cultures of CE, ME, or CHL cells; respective cell numbers were measured in duplicate 24, 48, or 72 h later using a Coulter electronic particle counter (7). For HF cells, broad concentration ranges of thrombin preparations were added to nondividing cultures, and 12 h later, 0.4 μCi of [3H]thymidine (6 Ci/mM; Schwarz/Mann) was added to each culture dish. Cultures were incubated an additional 24 h and were then rinsed two times with 0.15 M NaCl, 50 mM sodium phosphate (pH 7.4), fixed with 3% formaldehyde at 23°C overnight, rinsed again with 0.15 M NaCl, 50 mM sodium phosphate (pH 7.4), and extracted twice with 10% trichloroacetic acid. The nuclear monolayer was then rinsed with 0.15 M NaCl, 50 mM sodium phosphate (pH 7.4), and 95% ethanol, and allowed to dry. It was then overlaid with Ilford K5 emulsion. The emulsion was developed after 4 days of exposure at 4°C. For each dish, greater than 1,000 nuclei were scored for incorporation of radioactivity as detected by emulsion grains over the nucleus.

Binding Assays—For all cell types, the medium on nondividing cultures was changed to binding medium (serum-free DV medium containing 0.05% bovine albumin buffered with 0.015 M Hepes (pH 7.4)) and treated with 25 mM Heps buffer (pH 6.7) containing 0.5% NP40, 1 mM dithiothreitol, 1 mM CaCl₂, for 3 min on ice to produce a nuclear monolayer (30). The monolayer was rinsed with 0.15 M NaCl, 50 mM sodium phosphate (pH 7.4), fixed with 3% formaldehyde at 23°C overnight, rinsed again with 0.15 M NaCl, 50 mM sodium phosphate (pH 7.4), and extracted twice with 10% trichloroacetic acid. The nuclear monolayer was then rinsed with 0.15 M NaCl, 50 mM sodium phosphate (pH 7.4), and 95% ethanol, and allowed to dry. It was then overlaid with Ilford K5 emulsion. The emulsion was developed after 4 days of exposure at 4°C. For each dish, greater than 1,000 nuclei were scored for incorporation of radioactivity as detected by emulsion grains over the nucleus.

Binding Assays—For all cell types, the medium on nondividing cultures was changed to binding medium (serum-free DV medium containing 0.1% bovine serum albumin buffered with 0.015 M Heps at pH 7.4), and the cells were equilibrated at 37°C for 30 min. The medium was then changed to binding medium containing the indicated concentration of 125I-labeled α-thrombin or 125I-labeled thrombin derivative forms. Cells were incubated for 60 min at 37°C to achieve steady state binding. The binding was terminated by rapidly rinsing the cells five times with 0.15 M NaCl, 50 mM sodium phosphate (pH 7.4) at 4°C. To dissolve cellular materials, cultures were treated with 0.5 ml of 1.0 N NaOH for 1 h at 37°C. An additional 0.5-ml rinse of each dish was pooled with each sample and radioactivity was measured with a γ counter. In each experiment aliquots from several samples were removed for protein quantitation by the method of Lowry et al. (31).

Binding competition experiments, 1000-fold concentration ranges of unlabeled thrombin derivative forms were used to compete for the binding of 0.28 nM 125I-α-thrombin. In all other experiments the binding of indicated concentration ranges of 125I-α-thrombin or 125I-labeled thrombin forms was examined, and the protocol could nonspecific binding was measured as the amount of radioactivity bound to cultures after incubation in binding medium containing excess unlabeled α-thrombin (110 nM) in addition to the 125I-labeled thrombin forms. Specific binding was obtained by subtracting nonspecific binding from total radioactivity bound to cultures incubated only with the 125I-labeled form. In all cases, were each measured on duplicate cultures.

RESULTS

Properties of Thrombin Preparations—Characterization data for the thrombin preparations used in these studies were obtained at both Albany and Irvine (Table I). The reference
Thrombin Binding and Mitogenic Stimulation

Characterization of thrombin preparations

<table>
<thead>
<tr>
<th>Property</th>
<th>Unit</th>
<th>α-</th>
<th>DIP-α-</th>
<th>MS-α-</th>
<th>NO₂-α-</th>
<th>γ (Trypsin)</th>
<th>π (Autodigestion)</th>
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<tr>
<td>Modification&quot;</td>
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<td></td>
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<td>Thrombin forms detected by</td>
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<tr>
<td>[³⁵Cl]DIP-F labeling and electrophoresis&quot;</td>
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<tr>
<td>Specific fibrinogen clotting activity (Fibrometer)*</td>
<td>Units/mg</td>
<td>&gt;3000</td>
<td>0.53</td>
<td>7.6</td>
<td>4.2</td>
<td>0.79</td>
<td>16.9</td>
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<td>Active site titration with NPGB&quot;</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative fibrinogen clotting activity (Spectrophotometer)*</td>
<td>% of α</td>
<td>100</td>
<td>0.86</td>
<td>3.83</td>
<td>1.00</td>
<td>0.29</td>
<td>8.8</td>
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<tr>
<td>Relative TosArgMe activity&quot;</td>
<td>% of α</td>
<td>100</td>
<td>0.76</td>
<td>3.38</td>
<td>52.1</td>
<td>83.3</td>
<td></td>
</tr>
</tbody>
</table>

"Performed at Albany, NY.
"a Determined for the initial unmodified α-thrombin preparation.
"a-amine derivatives of α-thrombin were used as an electrophoretically homogeneous [³⁵Cl]DIP-F incorporating enzyme; it had high specific fibrinogen-clotting activity of about 3,000 units/mg and was about 85% active as judged by active site titration with NPGB. The three chemically modified forms of α-thrombin were prepared from thrombin preparations that were greater than 95% homogeneous (compositions of starting preparations are given in parenthesis in Table I). Both DIP- and MS-α-thrombins are derivatized at the catalytic serine 205 in the thrombin B chain; the former is conjugated with the catalytic serine residue. Both and MS preparations exhibited very low clotting activities while remaining >95% active (95.2)*, 97.1*, and 96.4* of the esterase activity of γ-thrombin and very low clotting versus esterase activity of α-thrombin and retained about 50% of the clotting activity but retained about 50% of the clotting activity of α-thrombin (Table I). This differential loss of activity results from nitration of about 4 of the 10 tyrosines in the enzyme (20).

Preparations of virtually noncoagulant thrombin were made by both trypsin and autolytic conversion of α- to γ- and subsequently γ- thrombin forms. The former method produced essentially complete conversion to γ-thrombin with no detectable radioactive remaining α-thrombin (Table I), whereas the latter method permitted the preparation of γ-thrombin without possible contamination with trypsin. Both methods produced homologous non clotting thrombin forms, which arise from proteolytic cleavage of the thrombin B chain at about one-third and about two-thirds from the end of the chain yielding β- and γ-thrombin, respectively (15–17). These γ-thrombin preparations exhibited very low clotting activities while retaining esterase activities similar to α-thrombin (Table I).

Because of the sensitivity of turbidimetric clotting detection versus the traditional clotting end point, and the sensitivity of the turnover substrate TosArgMe versus the titrant substrate NPGB measurements, the relative activities of the modified thrombin forms were compared with those of the reference α-thrombin preparation using these more sensitive measures (Figs. 1 and 2). These data illustrate the very low clotting versus esterase activity of γ-thrombin and very low enzymic activities of the DIP- and MS-α-thrombin preparations. Such marked differences among the thrombin preparations revealed by these detailed characterizations permitted meaningful studies on the nature of the interactions required between thrombin and the cells during binding to receptors and stimulation of cell division.

Mitogenic Activity—The mitogenic potentials of the various thrombin forms were compared using four cultured fibroblastic cell types which respond to the mitogenic action of α-thrombin. With ME, CHL, and CE cells, the mitogenic activities of the thrombin preparations were determined by measuring cell number (Figs. 3, ME, CHL, and CE); with HF cells, mitogenic potential was evaluated by [³H]thymidine autoradiography (Fig. 3, HF). These data confirm that α-thrombin is mitogenic for these four different cell types. The greatest responses were about 70 and 50% increases in cell number with ME and CE cells, respectively. The CHL and HF cells responded to a lesser extent. In contrast, with one exception noted below, none of the inactivated thrombins produced a significant mitogenic response in any of the cell types.

The mitogenic potentials of the inactivated thrombin forms were expressed as a per cent of the mitogenic activity of α-thrombin on each of the four cell types (Table II). The mitogenic responses of all the cell types to DIP-α-thrombin and γ-thrombin produced by trypsin treatment were less than 4% of the response to α-thrombin. Also, MS-α-thrombin had little mitogenic activity; the largest response was with nitro-α-thrombin where its activity was more variable among the

Fig. 1. Time required by α-thrombin and derivative thrombin forms to convert fibrinogen into a fibrin clot. Clotting times were measured for several concentrations of each thrombin preparation as described under "Materials and Methods." The standard deviation of duplicate determinations was typically ±0.75 s.
cell types. With CHL cells it was only 5% as active as α-thrombin; however, with ME cells it exhibited 31% of the mitogenic potential of α-thrombin. CE and HF cells were intermediate in their response to nitro-α-thrombin.

**Competition of α-Thrombin Binding by Inactivated Thrombin Forms**—The binding capacity of the various thrombin forms was initially measured by their ability to compete for the specific binding of 125I-α-thrombin to ME cells. These cells were incubated with 0.28 nM 125I-α-thrombin alone or with a broad range of concentrations of unlabeled α-thrombin or derivative thrombin forms (Fig. 4). Both DIP- and MS-α-thrombins competed for binding of 125I-α-thrombin nearly as well as α-thrombin; however, neither nitro-α-thrombin nor γ-thrombin produced by autolysis effectively competed for the binding of 125I-α-thrombin.

**Binding of 125I-α-Thrombin and 125I-Labeled Thrombin Forms to Various Cultured Cells**—When the above competition experiments were extended to other cells, significant differences were found among the four cell types. Therefore, quantitative evaluation of the binding of the derivative thrombin forms was carried out by labeling them and performing direct binding measurements. Nonproliferating cultures of ME, CHL, CE, or HF cells in serum-free medium were incubated for 1 h at 37°C with concentrations ranging from 0.14 to 8.8 nM of either 125I-α-thrombin or one of the 125I-labeled thrombin forms. Excess unlabeled α-thrombin was used to determine the amount of nonspecific binding of the thrombin forms to the α-thrombin receptor on the different cell types, since use of the homologous unlabeled thrombin form to determine nonspecific binding might detect binding of the modified thrombin to cellular components other than the α-thrombin receptor. These data (Figs. 5 and 6) were assessed using traditional Scatchard plots (32). The dissociation constants (Kd values) were determined by estimating the concentration of each 125I-labeled thrombin form required for half-maximal binding to each of the four cell types (Table III). As

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**Fig. 2.** Ability of α-thrombin and derivative thrombin forms to hydrolyze TosArgMe to a UV-absorbing product. Esterase activity for several concentrations of each thrombin preparation was measured as described under "Materials and Methods" and is shown as the rate of formation of product (nm min⁻¹).

**Fig. 3.** Ability of α-thrombin and derivative thrombin forms to stimulate mitogenesis in four different cell types. Thrombin-stimulated growth of nondividing cultures of ME, CHL, and CE cells was measured by measuring the cell number of thrombin-treated cultures compared to the cell number of parallel untreated cultures. In HF cells, the mitogenic potential of the thrombin preparations was measured as per cent of total nuclei which incorporated [3H]thymidine into DNA as described under "Materials and Methods." Standard deviations were typically <2.5% of the mean (extremes, 0.01% to 9.8%).
TABLE I
Mitogenic potential of α-thrombin and thrombin derivative forms

The per cent values are ratios of the concentration of α-thrombin to concentration of thrombin derivative forms required to produce a given mitogenic response. Each per cent value is an average of several comparisons made over a range of concentrations centering around the ones that yielded a half-maximal response.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>α-Thrombin</th>
<th>DIP-α-thrombin</th>
<th>Mesyl-α-thrombin</th>
<th>Nitro-α-thrombin</th>
<th>γ-Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME</td>
<td>100</td>
<td>1.86</td>
<td>7.16</td>
<td>31.40</td>
<td>3.94</td>
</tr>
<tr>
<td>CHL</td>
<td>100</td>
<td>0.74</td>
<td>1.73</td>
<td>5.22</td>
<td>0.40</td>
</tr>
<tr>
<td>CE</td>
<td>100</td>
<td>3.48</td>
<td>4.30</td>
<td>12.11</td>
<td>2.60</td>
</tr>
<tr>
<td>HF</td>
<td>100</td>
<td>&lt;0.05</td>
<td>2.28</td>
<td>8.05</td>
<td>1.80</td>
</tr>
</tbody>
</table>

Fig. 4. Competition for binding of 125I-α-thrombin to ME cells by unlabeled α-thrombin and derivative thrombin forms. The percentage of maximum binding of 0.28 nM 125I-α-thrombin to ME cells (0.04 fm/μg of cell protein) in the presence of the indicated concentrations of each thrombin preparation was measured as described under "Materials and Methods." Nonspecific binding of 125I-α-thrombin was subtracted from each determination prior to calculation of per cent of maximal binding.

Fig. 5. Scatchard analysis of the specific binding of 125I-α-thrombin and 125I-labeled derivative thrombins to ME and CHL cells. Concentrations ranging from 0.14 nM to 8.8 nM of each 125I-labeled thrombin preparation were incubated with cells for 60 min at 37°C. Nonspecific binding was determined at each concentration by including 110 nM unlabeled α-thrombin. Free ligand was determined by measuring the radioactivity in aliquots of binding medium taken at each concentration for each thrombin preparation. Each point is the average of duplicate determinations of both specific and nonspecific binding measurements.

Fig. 6. Scatchard analysis of the specific binding of 125I-α-thrombin and 125I-labeled derivative thrombins to ME and HF cells. These experiments were conducted as described in the legend to Fig. 4.

TABLE II
Dissociation constants (Kd values) for 125I-α-thrombin and 125I-labeled thrombin derivative forms with the various cells

Kd values were calculated by determining the concentration of each 125I-labeled thrombin preparation required for half-maximal binding to each cell type.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>α-Thrombin</th>
<th>DIP-α-thrombin</th>
<th>Mesyl-α-thrombin</th>
<th>Nitro-α-thrombin</th>
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</thead>
<tbody>
<tr>
<td>ME</td>
<td>1.37</td>
<td>1.28</td>
<td>0.91</td>
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<tr>
<td>CHL</td>
<td>1.92</td>
<td>1.51</td>
<td>1.92</td>
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<tr>
<td>CE</td>
<td>1.20</td>
<td>1.20</td>
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<td></td>
</tr>
<tr>
<td>HF</td>
<td>1.86</td>
<td></td>
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</tr>
</tbody>
</table>

* Binding was nonsaturable up to a ligand concentration of 8.8 nM.

Shown, 125I-α-thrombin exhibited high affinity, saturable binding to all cells, suggesting that each cell type possessed receptors for α-thrombin.

The ME and CHL cells, furthermore, bound 125I-DIP- and 125I-MS-α-thrombin in a fashion similar to that of α-thrombin (Fig. 5). The Kd values determined support the conclusion that the binding of these derivatives to ME and CHL cells was quite similar to the binding of 125I-α-thrombin (Table III). These results show that the inability of DIP- or MS-α-thrombins to stimulate the division of ME and CHL cells was therefore not due to an inability of the serine-inactivated α-thrombin forms to bind to the thrombin receptors on these cells. Instead, inhibition of the mitogenic response, resulting from inactivation of the enzyme by conjugation at its catalytic site, indicates that catalysis by the enzyme is necessary for initiation of cell division.

Unlike the ME and CHL cells, neither CE nor HF cells displayed any significant binding of either 125I-DIP- or 125I-MS-α-thrombins (Fig. 6). Most of the binding of these derivatives to CE and HF cells was nonspecific, since it was not saturable and not competed by unlabeled α-thrombin. Therefore, the intact catalytic apparatus is necessary for α-thrombin to bind to its receptors on CE and HF cells. These results show that the α-thrombin receptors of CE and HF cells differed significantly from those of ME and CHL cells.

Neither 125I-nitro-α- nor 125I-γ-thrombin showed a significant level of binding to any of the cell types (Figs. 5 and 6). These results agree well with the competition binding data obtained with the ME cells (Fig. 4). The low level of binding of both 125I-nitro-α- and 125I-γ-thrombin can be accounted for by small amounts of contaminating active α-thrombin indicated by the low level clotting activities of both preparations.
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(Table I). Since these thrombin forms were modified at regions of the thrombin active site other than the catalytic apparatus, these active site regions appear to be critical for thrombin binding to cellular receptors.

**DISCUSSION**

Selectively modified thrombin forms enabled evaluation of the requirements for the catalytic apparatus and adjacent active site regions of α-thrombin in its binding to cellular receptors and stimulation of cell division. Both DIP-α-thrombin and MS-α-thrombin were inactive as a result of covalent conjugation at the active site serine (Ser 205 of the thrombin B chain). Thus, both fibrinogen clotting and esterase activities were markedly inhibited. These two forms, however, differ in that the DIP conjugate is a sterically obstructive double branched chain phospho-linked group, whereas the MS conjugate is a relatively small single methylsulfonyl-linked group.

In contrast, nitro-α-thrombin and γ-thrombin are modified at active site regions distinct from the catalytic apparatus. Nitration of tyrosines causes α-thrombin to preferentially lose clotting activity relative to esterase activity; critical tyrosines are implicated for the binding recognition of fibrinogen (16, 17). γ-Thrombin arises from proteolytic cleavage of the α-thrombin B chain into three noncovalently associated fragments (16, 17). Although γ-thrombin retains most of the parent estero/amidolytic activity, it possesses less than 1% of the clotting activity. This differential loss of activity which accompanies the proteolytic fragmentation of the thrombin B chain is believed to be the consequence of protein conformational changes which prevent fibrinogen recognition (16, 17).

Two experimental approaches were used to evaluate the binding of these derivatives to α-thrombin receptors on cells. In one, the ability of modified thrombin forms to compete for the binding of $\text{^{125}I}-\text{a-thrombin}$ to ME cells and that nitro-α-thrombin and γ-thrombin did not. Direct binding of $\text{^{125}I}$-labeled thrombin forms to directly bind to cellular receptors was measured. Both experimental procedures showed that DIP- and MS-α-thrombins bound to the α-thrombin receptor on ME cells and that nitro-α-thrombin and γ-thrombin did not. Direct binding of $\text{^{125}I}$-labeled forms allowed us to compare quantitatively the binding capacity of DIP- and MS-α-thrombins with that of α-thrombin. The $K_D$ values for these derivatives were essentially identical with that of α-thrombin and agreed closely with our previously published data on $\text{^{125}I}$-α-thrombin binding to ME cells (13).

In the present studies, chloroglycouril was used for labeling thrombin preparations with $\text{^{125}I}$- rather than lactoperoxidase as in our previously reported binding studies with $\text{^{125}I}$-α-thrombin (13). Since chloroglycouril-coated glass efficiently iodinates proteins under nonoxidizing conditions at 4°C (22), we chose this method and found that the $\text{^{125}I}$-α-thrombin had an unaltered clotting activity. Furthermore, the labeled enzyme gave about 75% specific binding to CE cells compared to about 40% with preparations labeled by the lactoperoxidase method.

The binding of α-thrombin to each cell type examined appeared to involve regions of the α-thrombin active site distinct from the catalytic apparatus that are related, if not identical, to regions required for its binding to fibrinogen. This conclusion is based on the finding that $\text{^{125}I}$-nitro-α-thrombin and $\text{^{125}I}$-γ-thrombin did not bind to any of the cell types to a significant extent. The low levels of binding that were observed in a few cases could totally be accounted for by residual α-thrombin in these preparations. Similarly, we found that neither nitro-α-thrombin nor γ-thrombin significantly competed for the binding of $\text{^{125}I}$-α-thrombin to ME cells. In contrast to our results, Perdue found that γ-thrombin competed less efficiently than α-thrombin but to a significant extent for $\text{^{125}I}$-α-thrombin binding to CE cells. However, experimental conditions under which the present binding studies were conducted differed in several respects from the conditions employed by Perdue and may account for this difference.

In addition to this region of α-thrombin that is distinct from the catalytic site, the binding of α-thrombin to CE and HF cells requires the active catalytic site. This conclusion is based on the finding that neither $\text{^{125}I}$-DIP- nor $\text{^{125}I}$-MS-α-thrombins bound to CE or HF cells, a result in close agreement with competition binding studies on CE cells conducted by Perdue. In contrast to CE and HF cells, both ME and CHL cells bound $\text{^{125}I}$-DIP- and $\text{^{125}I}$-MS-α-thrombins as well as $\text{^{125}I}$-α-thrombin, demonstrating that the catalytic site of α-thrombin is not required for binding to receptors on the latter cells. Thus, the properties of the α-thrombin receptor are different in cells from different animal species. We previously reported that the apparent molecular weights of the thrombin receptors from CE (M, about 43,000) and ME cells (M, about 50,000) were different (33). Another difference in the α-thrombin receptor on cells from different animal species is its ability to form a direct covalent linkage with thrombin (34). This linkage occurs very readily with HF cells, and to a lesser extent with CE, ME, or CHL cells (10).

With the exception of some residual mitogenic activity of nitro-thrombin on ME cells and some of the other modified thrombin forms possessed mitogenic activity on any of the cells that could not attributed to residual α-thrombin. With CE and HF cells none of these forms bound to the α-thrombin receptor; therefore, it was not possible to demonstrate directly that specific regions of α-thrombin are necessary for stimulation of cell division. Our results with CE cells agree with results on stimulation of DNA synthesis by Perdue who found that all of the derivatives stimulated less $\text{^{3H}}$-thymidine incorporation into DNA than did α-thrombin, although his measurements do show a greater mitogenic response to nitro-α-thrombin and γ-thrombin than we found by counting increases in cell number. This difference may reflect the cellular heterogeneity in secondary CE cell populations in which some cells may be capable of initiating DNA synthesis in response to the modified thrombins and yet be incapable of completing cell division. With ME and CHL cells, it is possible to conclude that the active catalytic site of α-thrombin is necessary for stimulation of cell division. DIP- and MS-α-thrombins effectively bind to these cells; however, the ability of these forms to stimulate ME and CHL cell division corresponded to the amount of their residual proteolytic activities.

Our previous studies showed that inhibition of binding of α-thrombin to its cell surface receptors on ME cells results in inhibition of thrombin-stimulated cell division (13). Thus, mitogenic stimulation in ME cells requires both binding of α-thrombin to its receptors and a catalytic function of the enzyme.

We have previously reported a similar conclusion for thrombin-stimulated cell division of CE cells (38). By very different approaches, we showed that thrombin must cleave a cell surface component to stimulate division of these cells. In these studies, CE cell surface proteins were labeled with $\text{^{125}I}$, and it was found that an $M_s = 43,000$ cell surface protein must be cleaved by α-thrombin for stimulation of cell division. This protein was present on responsive cells and four separately isolated populations of CE cells that did not divide after α-thrombin treatment. However, it was cleaved by α-thrombin only on the responsive cells, and it was insensitive to α-thrombin proteolysis on the four unresponsive populations.

Platelets also effectively bind inactivated thrombins that have been derivatized at their catalytic site; however, these derivatives do not stimulate physiologic responses in platelets (35–37).
Other approaches using a photoreactive conjugate of $^{125}$I-$\alpha$-thrombin indicated that the $\alpha$-thrombin receptor on CE cells was also an $M_r = 43,000$ component. Thus, on the basis of the similarity in molecular weights, it appears that $\alpha$-thrombin must cleave its receptor to stimulate division of CE cells.

These data on the binding and mitogenic potential of the various thrombin forms have enabled us to develop a conceptual model for the interactions between $\alpha$-thrombin and its receptors that lead to binding and stimulation of cell division (Fig. 7). For significant binding interactions, all cell types examined (CE, ME, HF, and CHL cells) required exosite regions removed from the catalytic apparatus of the enzyme, since neither nitro-$\alpha$-thrombin nor $\gamma$-thrombin bound to appreciable extents to any of the cells. Such an exosite region in the enzyme (Site A) is presumably complementary to a binding site region in the cellular receptor (Site B). The interactions between these regions (Site A of the enzyme and Site B of the receptor) is envisioned to be noncovalent and to depend on the extent of compatibility between the two regions. Since ME and CHL cells bound $^{125}$I-DIP- or $^{125}$I-MS-$\alpha$-thrombins similar to $^{125}$I-$\alpha$-thrombin, these cells appear to have receptor binding site regions (Site B) with high affinities for the enzyme exosite regions (Site A). These enzyme exozites are thus independent of active site regions adjacent to the enzyme catalytic apparatus (Site C) which reacts with a second cellular receptor site (Site D) to generate the mitogenic signal. In contrast, CE and HF cells did not bind either $^{125}$I-DIP- or $^{125}$I-MS-$\alpha$-thrombins. This indicates that the intact catalytic apparatus or adjacent active site region (Site C) is required for binding to these cells and that receptor Site B of CE and HF cells interacts relatively weakly with Site A of thrombin. The high affinity binding of $\alpha$-thrombin to CE and HF cells may be the consequence of an enzyme intermediate complex involving the catalytic apparatus (e.g. an acyl enzyme complex formed between Sites C and D).

Since stimulation of cell division requires the catalytically active enzyme, the mitogenic signal is apparently produced by interaction of Site C on $\alpha$-thrombin with Site D of the receptor. This would require the formation of an enzyme-receptor intermediate and probably the formation of a receptor cleavage product. This cleavage product would correspond to the CO$_2$H-terminal portion of a protein substrate and could be the signal for cell division as a newly formed enzyme or biologically active peptide hormone. Alternatively, the signal could be the consequence of the enzyme-receptor complex where a proteolytic cleavage of the receptor causes a conformational change. Because thrombin-stimulated cell division is dose related to the initial enzyme concentration, the enzyme-receptor complex probably turns over slowly. The latter possibility is supported by the finding that stable covalent complexes have been demonstrated to form with $^{125}$I-$\alpha$-thrombin and binding components on a variety of cell types (10, 34).

The present results will provide meaningful direction to future experiments on the mechanism of thrombin-stimulated cell division. They have revealed that it will be critical to examine the specific role played by the $\alpha$-thrombin receptor in a variety of cell types, since the properties of the receptors are different in cells from different animal species. At the same time, they have indicated that stimulation of cell division by $\alpha$-thrombin requires its intact catalytic apparatus (and presumably its proteolytic activity) in ME and CHL cells. Thus, it will be important in future studies to identify in a variety of cell types proteolytic cleavages by thrombin that are required for stimulation of cell division. It appears that this can be done most effectively by looking for cell surface components that are cleaved by $\alpha$-thrombin on cells that are responsive to its mitogenic action but which are not cleaved or are absent on cells that do not divide after thrombin treatment.

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REFERENCES
Thrombin Binding and Mitogenic Stimulation