Protease Mitogenic Response of Chick Embryo Fibroblasts and Receptor Binding/Processing of Human α-Thrombin*

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Quiescent cultures of chick embryo fibroblasts incubated with human α-thrombin (14-219 pm) incorporated [methyl-3H]thymidine proportional to concentration. Inactivated forms of this protease (e.g. active-site-conjugated α-thrombin or its hirudin complex) had no mitogenic activity and did not compete with [125I]-α-thrombin for binding to specific plasma membrane receptors. The noncoagulant but esterolytic active forms, γ- and nitro-α-thrombins, were weakly mitogenic and correspondingly competed weakly for binding. Trypsin competed equally as well as native thrombin for binding, whereas chymotrypsin, elastase, and human urokinase competed with 80-fold less affinity. Plasma, arginine-specific proteases associated with nerve or epidermal growth factors, insulin, and insulin-like growth factors did not compete for binding. These data demonstrate that (a) functional catalytic residues of the thrombin active site are necessary for mitogenic activity and for specific binding; (b) regions adjacent to the active site, i.e., the high affinity protein recognition site, appear to enhance binding; and (c) the receptor can discriminate between other proteases and binds those which are also mitogens for the avian cells.

The characteristics of [125I]-α-thrombin binding were determined, and it was found to be (i) proportional to cell number; (ii) optimal at pH 8.8; (iii) 70-90% specific; (iv) at equilibrium after 60 min of incubation at 22-24°C or 180 min at 0-4°C (the rate constants for association, i.e. K_a, at 22 and 4°C were 18 and 1.1 x 10^7 M^-1 min^-1, respectively); and (v) essentially nondissociable. Nondissociable thrombin that bound during incubation at 0-4°C was distributed equally between trypsin-sensitive and insensitive compartments. Thrombin associated with the former was released into the media when the cells were incubated at 0-4°C with hirudin or hydroxyamine, or transferred to the insensitive compartment when incubated at 22°C. Finally, confluent cultures of fibroblasts bind 2-3 x 10^12 [125I]-α-thrombin molecules/cell with an apparent binding constant, i.e. K_a, of 0.7 nM (a true K_a could not be determined because of the irreversible nature of thrombin binding). The binding capacity per cell and the apparent K_a value increased proportionally to an increase in culture density.

Replication of cultured avian and mammalian cells can be initiated by numerous factors, e.g. bioregulatory polypeptides, steroids, membrane perturbants, and proteases, which may act alone or in concert. The proteolytic enzyme α-thrombin stimulates cell replication when added to quiescent, serum-deprived cultures of chick embryo fibroblasts (3-11), human embryonic (12) or foreskin fibroblasts (8), mouse embryo cells (8, 13, 14), and human lymphocytes (15). In chick embryo fibroblasts, the mitogenic response was correlated with surface binding and internalization of enzymically active [125I]-α-thrombin. Martin and Quigley (9) observed that specific surface labeling increased linearly up to 1 h, followed by internalization up to 14 h. Zetter et al. (7) observed a surface saturation at 1-4 h and intracellular accumulation of activity up to 12 h. With either trypsin or α-thrombin immobilized on polystyrene beads, Carney and Cunningham (10) showed that mitogenic stimulation can occur without internalization. However, their studies do not exclude the possibility that the receptors are modified and/or internalized during the stimulatory process. Consistent with this view, Glenn and Cunnigham (11) have...
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observed that α-thrombin caused a specific loss of a M, = 43,000 membrane protein(s) from avian fibroblasts. This observation, however, has not provided insight into the mechanism coupling the specific interaction of thrombin with the replicating events. The mitogenic stimulation could result from (i) specific, but reversible interactions between the enzyme and its receptor on the cell membrane; (ii) catalytic events subsequent to binding, such as enzyme acylation and deacylation; or (iii) possibly postbinding compartmentalization of the occupied receptor and other processes.

In an initial evaluation of these possibilities, we examined the saturable interactions of human 125I-α-thrombin with substratum-attached chick embryo fibroblasts with respect to binding affinity, receptor number, and receptor sensitivity. The binding was found to be distinct from that to platelets (18), endothelial cells (19), or mouse embryo fibroblasts (14) in that only catalytically active forms of the enzyme were bound and became reversibly compartmentalized.

EXPERIMENTAL PROCEDURES

Materials
Pathogen-free, COFAL-negative, 11-day embryonated eggs were obtained from Armand Frappier, Laval, Quebec. Temin-modified Eagle's medium was purchased from Schwarz/Mann (Orangeburg, NY); fetal calf serum was from Grand Island Biological Company (Grand Island, NY); hirudin with 1000 thrombin neutralizing units/mg of protein was from Pentapharm Ltd. (Basel, Switzerland); elastase, bovine albumin fraction V, choroic, α-chymotrypsin, lactoperoxidase, plasmin and trypsin were from Sigma (St. Louis, MO); benzamidino hydrochloride, DIP-F1 MS-F, and tetratinemethane were from Aldrich Chemical Co. (Milwaukee, WI); TLCK was from Calbiochem (San Diego, CA); and NPGB was from Nutritional Biochemical Corp. (Cleveland, OH).

The following were gifts: crystalline porcine insulin (Lot 1P885) from Dr. R. E. Chance (Ill Lilly and Co., Indianapolis, IN); ILAs from Dr. B. Posner (McGill University, Montreal, Canada); mCPC(PBA)-F from Dr. D. H. Bing (Center for Blood Research, Boston, MA); porbofolic myristic acid from Dr. J. Quigley (Downstate Medical Center, Brooklyn, NY); and the high molecular weight forms of EGF and NGF complexes which have protease activities specific for arginine residues (20, 21) from Dr. T. Maciag (Collaborative Research, Boston, MA) and Dr. M. Young (University of Florida, Gainesville, FL), respectively. Rat pituitary protease which is inhibited by hirudin and generates sulfation factor activity from growth hormone and low molecular weight urokinase were also gifts from Dr. T. Maciag, whereas high and low molecular weight forms of human urokinase with 100 and 84 CTA units/mg of protein, and esterase activity as estimated by titration with NPGB was reduced from 80 to 8%. The modified thrombin was dialyzed against 0.75 m NaCl, frozen at -70°C (I). The preparation used (No. 15) was composed of 0.3% DIP-F, 12% NaCl and 5% DIP-F at pH 7.8. Specific activity was reduced from 2700 to 12 clotting units/mg of protein, and NPGB-titratable active sites decreased from 85.5 to 67%.

The modified thrombin contained 3.4 nitrotyrosines/molecule.

TLCK-α-Thrombin—α-Thrombin was alkylated by repeated treatment at pH 7.4 with a 100-fold molar excess of TLCK over α-thrombin. The specific activity was reduced from 2700 to 12 clotting units/mg and the NPGB-titratable active sites were reduced from 85.5 to 67%.

mCPC(PBA)-α-Thrombin—mCPC(PBA) in 0.75 m NaCl, 5 ml Tris was incubated 2 h at 24°C with mCPC(PBA) (Lot 126) and dialyzed to remove unbound inhibitor. Titration with NPGB indicated complete inhibition. α-Thrombin incubated in the presence of methanol and dialyzed, was similarly prepared and is designated "control α-thrombin."

Hirudin-thrombin—Hirudin-α-thrombin complex was prepared by diluting 125I-α-thrombin in Dulbecco's PBS and incubating it at 24°C with a 20-fold molar excess of hirudin dissolved in buffered saline. 125I-α-Thrombin Binding—Substratum-attached fibroblasts grown on 40-mm culture dishes were rinsed 2 times with 5 ml of PBS (pH 7.4) and incubated for 5 min to remove cell-associated radioactive label. The cells were then incubated at 24°C for 2 h with PBS (pH 6.8) containing 1% (w/v) bovine serum albumin to reduce nonspecific binding and had been exposed to 22°C for 2 h prior to incubation. The cells were then incubated with 2 × 106 cpm of 125I-α-thrombin in 0.1 ml of PBS (pH 7.4) for 5 min to remove cell-associated radioactive label. The cells were then washed two times with chilled PBS and reincubated with PBS (pH 7.4). The samples were incubated for 2 h at 24°C in the presence of unlabeled α-thrombin from that bound in its absence. The protein content of comparably grown cells, attached to plates but rinsed with bovine serum albumin-free PBS, was determined in duplicate by the method of Lowry et al. (27) following digestion in 0.2 M NaOH. The plates were rinsed twice with chilled PBS (pH 7.4) and were incubated for 60 min at 37°C with 2 ml of PBS (pH 7.4) containing 1 μCi/ml of [methyl-3H]thymidine. Following incubation, the plates were rinsed 3 times with a total of 15 ml of ice-chilled PBS + 1.5% bovine serum albumin (pH6.8) and once in saline (pH 7.4). The attached cells were dissolved in 10 ml of 0.5 n NaOH; the sample was transferred to glass tubes; and the plates were rinsed with 1 ml of water, which was combined with the original samples. Radioactivity was determined with an automatic dual channel γ counter (Nuclear Chicago model 4230) at an efficiency of about 40%.

The specific binding of 125I-α-thrombin was obtained by subtracting the cell-bound radioactivity in the presence of excess unlabeled α-thrombin from that bound in its absence. The protein content of comparably grown cells, attached to plates but rinsed with bovine serum albumin-free PBS, was determined in duplicate by the method of Lowry et al. (27) following digestion in 0.2 M NaOH. The plates were rinsed twice with chilled PBS (pH 7.4) and were incubated for 60 min at 37°C with 2 ml of PBS (pH 7.4) containing 1 μCi/ml of [methyl-3H]thymidine. Following incubation, the plates were rinsed 3 times with 5 ml of 10% (w/v) trichloroacetic acid.
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Mitogenic Activity of Human Thrombin Forms—Addition of 0.25–5 pg/ml of α-thrombin to fibroblasts grown for 24 h in serum-free medium stimulated [methyl-3H]thymidine incorporation comparable to 4% fetal calf serum (Fig. 1). Furthermore, 8 pg/ml of 125I-α-thrombin was 80% as active as the unmodified thrombin (data not presented), suggesting that iodination did not appreciably alter this property. The essentially nonclotting forms, γ-thrombin and nitro-α-thrombin, were ~80 and 60% esterase active, respectively, and caused a similar proportional stimulation of [methyl-3H]thymidine incorporation (Fig. 1). On the other hand, catalytically inactivated forms of DIP (Fig. 1), M5-, and mCP(βA)-α-thrombins had <1% esterase activities and were mitogenically inactive (data not presented).

Properties of 125I-α-Thrombin Binding—The binding of 125I-α-thrombin to chick embryo fibroblasts during 60 min of incubation at 22–24°C was proportional to the cell protein concentration (Fig. 2) and the labeled enzyme concentration (Fig. 7). Confluent, substrate-attached cells bound greater quantities of 125I-α-thrombin with higher specificities than detached cells. Specific binding, i.e. total binding minus binding in the presence of a 100-fold excess of unlabeled α-thrombin ranged between 70 and 90%. The amount specifically bound was between 3 and 40% of the total 125I-α-thrombin applied. This binding was not to protease-binding serum proteins (e.g. α-antitrypsin, antithrombin III or α-macroglobulin, Ref. 28) absorbed to the culture dish, since 125I-α-thrombin bound to culture dishes incubated overnight in medium containing 4% fetal calf serum or to dishes scraped free of fibroblasts with a rubber policeman was <5% of that bound to attached cells and the binding was reduced only slightly by 1 μg/ml of unlabeled α-thrombin (data not presented). In addition, the specific binding to fibroblasts grown in fetal calf serum heated either to 60 or 100°C was not markedly different from that of cells grown in the presence of unheated serum. In two experiments, fibroblasts cultured in unheated serum bound 78.2 ± 1.9 and 82.3 ± 2.4 fmol of 125I-α-thrombin/mg of cell protein, whereas those grown in serum heated to 100 or 60°C bound 64.3 ± 3.3 and 93.0 ± 0.2 fmol/mg of protein, respectively. Although thrombin interactions with antithrombin III are accelerated by heparin (28), the addition of 20 or 50 ng of heparin during thrombin incubation with substrate-attached fibroblasts inhibited binding by 25 and 35%, respectively (data not presented). Finally, the t1/2 for binding was 3.5 h in the presence or absence of fetal calf serum (data not presented), indicating that the binding was not attributable to serum-adsorbed proteins and was indeed associated with the cells.

The optimum pH for maximum specific thrombin binding was between 6.6 and 7.0. As the pH increased, the total 125I-α-thrombin bound decreased markedly (Fig. 3). These results are consistent with what has been observed for thrombin hydrolysis of synthetic esterase substrates where kcat was maximum at pH 8.9–9.3 (24, 29).

Association and Dissociation Kinetics of 125I-α-Thrombin—At 22–24°C, total and specific binding of 0.27 nm 125I-α-thrombin to substrate-attached fibroblasts was linear for 15 min and reached equilibrium by 60 min (Fig. 4). Nonspecific binding accounted for <10% of total binding at 24°C and remained constant after 15 min. The association rate constant, kass, was 1.5 × 10^5 M^-1 min^-1 for a reversible complex. However, such binding is not reversible, because cellular compartment-
Blasts as a function of pH. Fibroblasts were incubated for 60 min at 24°C in phosphate-buffered saline with 1.5% (w/v) bovine serum albumin, containing 0.27 nM 125I-a-thrombin at pH 6.6-8.2. Nonspecific binding was determined in the presence of 1 μg/ml of unlabeled α-thrombin. The data, expressed as the mean ± S.E. of triplicates, are representative of 6 experiments; while those in Figs. 5 and 6A are representative of 3 experiments.

This dissociation, however, was mainly that of 125I-a-thrombin bound in the presence of an excess of unlabeled enzyme, and is, by definition, nonspecifically bound. Subtracting the amount that was nonspecifically bound from the total bound at different time intervals showed that no dissociation of specifically bound 125I-a-thrombin occurred during 60-min of incubation (Figs. 5 and 6A). Of the quantity of enzyme bound...
Substratum-attached fibroblasts were incubated in culture medium for 180 min at 37°C in the presence or absence of 50 μM chloroquine. Following rinsing, cells were incubated for 60 min at 24°C with 0.32 nM \(^{125}\)I-a-thrombin in the absence or presence of unlabeled thrombin and processed as described below or rinsed 2 times with buffer and incubated an additional 60 min at 24°C in 1 ml of phosphate-buffered saline containing 1.5% (w/v) bovine serum albumin. The radioactivity released into the buffer during this latter incubation was removed and measured, and both groups of cells were treated with 25 pg/ml of trypsin at 0-4°C for 30 min. The trypsin-released radioactivity was removed, and the adherent cells were digested with 0.2 M NaOH. The distribution of radioactivity released into the incubation medium and that designated as trypsin-sensitive or trypsin-insensitive was expressed as femtomoles ± S.E. for 3 determinations. The results presented in this table are representative of 3 experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trypsin-insensitive radioactivity (fmol)</th>
<th>Trypsin-sensitive radioactivity (fmol)</th>
<th>Radioactivity released into medium following 60 min of incubation (fmol)</th>
<th>Total (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (−)</td>
<td>87.1 ± 3.4</td>
<td>52.3 ± 1.8</td>
<td>NA*</td>
<td>134.6 ± 5.1</td>
</tr>
<tr>
<td>Untreated (+)</td>
<td>6.7 ± 0.3</td>
<td>14.3 ± 2.1</td>
<td>NA</td>
<td>20.3 ± 0.6</td>
</tr>
<tr>
<td>Chloroquine treated (−)</td>
<td>ND*</td>
<td>ND</td>
<td>NA</td>
<td>174.0 ± 3.4</td>
</tr>
<tr>
<td>Chloroquine treated (+)</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
<td>27.3 ± 0.1</td>
</tr>
<tr>
<td>Untreated (−)</td>
<td>56.0 ± 2.0</td>
<td>26.2 ± 0.5</td>
<td>63.1 ± 2.0</td>
<td>120.1 ± 4.5</td>
</tr>
<tr>
<td>Untreated (+)</td>
<td>10.8 ± 0.3</td>
<td>8.7 ± 0.3</td>
<td>16.5 ± 0.3</td>
<td>25.3 ± 1.1</td>
</tr>
<tr>
<td>Chloroquine treated (−)</td>
<td>132.0 ± 1.9</td>
<td>8.6 ± 0.2</td>
<td>10.2 ± 0.5</td>
<td>143.8 ± 1.9</td>
</tr>
<tr>
<td>Chloroquine treated (+)</td>
<td>15.3 ± 0.5</td>
<td>8.9 ± 0.3</td>
<td>11.6 ± 0.1</td>
<td>23.9 ± 0.8</td>
</tr>
</tbody>
</table>

* NA, not applicable.
* ND, not determined.

Following dissociation for 60 and 140 min in buffer at 0-4°C, ~50% was released during incubation with 25 pg/ml of trypsin (30 min at 0-4°C; Fig. 6C). However, if the 60-min incubation was at 22-24°C, ~40% of the trypsin-sensitive \(^{125}\)I-a-thrombin was transferred to the insensitive form (Fig. 6B). These latter results are similar to those observed using identical methods to evaluate binding and compartmenalization during a 60-min incubation at 22°C. Under this condition, 42% of the initially bound \(^{125}\)I-a-thrombin was released by trypsin (Table II). During a subsequent 60-min incubation at 22°C, the quantity of both trypsin-sensitive and trypsin-insensitive radioactivity fell dramatically and paralleled the appearance of radioactivity in the medium. This was attributed to degraded \(^{125}\)I-a-thrombin, since its appearance was markedly inhibited by 50 μM chloroquine (an inhibitor of lysosomal functions). Chloroquine treatment also resulted in an increase in the quantity of radioactivity associated with trypsin insensitivity, although no mechanism for this is readily apparent. One plausible hypothesis involves the formation of a cell membrane-localized acyl enzyme complex which is sterically protected from proteolysis by trypsin. In this regard, hydroxylamine, which destabilizes covalent acyl bonds, enhanced the dissociation rate of \(^{125}\)I-a-thrombin specifically bound to fibroblasts following an initial 60-min incubation; in buffer at 0-4°C (Fig. 6A). However, hirudin, which inhibits a-thrombin with a Kₜ of 6.3 × 10⁻¹¹ M (30), did not accelerate the dissociation rate when present in the buffer at 70-fold molar excess.

Internalization of thrombin bound during incubation at 22-24°C could not be prevented by treating the fibroblasts with 1% formaldehyde for 30 min at 22-24°C or by incubating them for 60 min at 37°C with 5 mM 2-deoxyglucose, 5 mM KCN, or these chemicals and 10 mM iodoacetic acid (conditions which have been shown to deplete the cells of ATP (31, 32) and to block internalization of bound insulin and epidermal growth factor (33)). Although these treatments reduced specific binding, the quantity of ligand that was trypsin-insensitive was proportionally the same as was found for untreated cells (data not presented).

**Binding as a Function of Concentration**—Specific binding to the attached fibroblasts at 22°C was determined with 0.07-280 nM \(^{125}\)I-a-thrombin, where it was linear to 2 nM and saturated at ~35 fm (Fig. 7). Although we are now aware that this binding is not at equilibrium (i.e. no dissociable component) invalidating Scatchard analysis, the data were so analyzed in order to compare our results with those reported for mouse embryo cells (14), platelets (18) and endothelial cells.
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Such analysis indicated that $^{125}$I-$\alpha$-thrombin binds to a single class of high affinity receptors on the avian fibroblasts with an apparent $K_d$ of $\sim 0.7 \text{nM}$, and that there are $2-3 \times 10^4$ sites/cell, based on a total binding capacity per mg of cell protein of 56 fmol and $2-2.5 \times 10^6$ cells/mg of protein (Fig. 8). In addition, $\sim 10^6$ receptors/cell were found with an apparent $K_d$ of 180 nM when the fibroblasts were incubated with $>1.2$ nM concentrations of $^{125}$I-$\alpha$-thrombin. It is very probable that these are nonspecific and nonfunctional associations.

Specific binding was also determined at 0-4°C following a 3-h incubation with 0.07-8.20 nM $^{125}$I-$\alpha$-thrombin, where it was linear to $0.7 \text{nM}$ and levelled off at $2.7 \text{nM}$ (Fig. 9A). At higher concentrations, large quantities of $^{125}$I-$\alpha$-thrombin were bound at the low affinity sites. Scatchard analysis of these data gave the same number of sites/cell (i.e. $\sim 32,000$ versus the above value at 22-24°C) but with a 3- to 4-fold reduced affinity (Fig. 9B).

Receptor Specificity—Fetal calf, bovine, and chicken serum competed with $^{125}$I-$\alpha$-thrombin for specific binding. This inhibition was proportional to serum concentration and was partially reduced by heating the fetal calf serum to 100°C for 15 min (Fig. 10). Heating for 15 min at 56°C did not reduce the inhibition (data not presented). Insulin and ILAs, which

![Graph](image)

**Fig. 9.** Scatchard analysis of specific $^{125}$I-$\alpha$-thrombin binding to chick embryo fibroblasts. Data from 4 binding experiments, representing 3 determinations each, as described in the legend to Fig. 7, were plotted as bound/free versus bound, corrected for nonspecific binding. Intercepts and slopes were determined by linear regression analysis with a Wang 700 series advanced programming calculator.

Concentrations of 0.07-0.68 nM labeled enzyme were used to calculate values of an apparent $K_d$ and binding sites per cell for the high affinity site, while 1.2-7.1 nM concentrations were used to obtain those for the low affinity or nonspecific site(s).

![Graph](image)

**Fig. 8.** Scatchard analysis of specific $^{125}$I-$\alpha$-thrombin binding to chick embryo fibroblasts. Data from 4 binding experiments, representing 3 determinations each, as described in the legend to Fig. 7, were plotted as bound/free versus bound, corrected for nonspecific binding. Intercepts and slopes were determined by linear regression analysis with a Wang 700 series advanced programming calculator.

Concentrations of 0.07-0.68 nM labeled enzyme were used to calculate values of an apparent $K_d$ and binding sites per cell for the high affinity site, while 1.2-7.1 nM concentrations were used to obtain those for the low affinity or nonspecific site(s).

![Graph](image)

**Fig. 9.** Binding of $^{125}$I-$\alpha$-thrombin to chick embryo fibroblasts as a function of labeled enzyme concentration at 0-4°C. From 0.07 to 8.2 nM $^{125}$I-$\alpha$-thrombin was incubated with substratum-attached fibroblasts for 180 min at 0-4°C in the absence or presence of a 100-fold excess of unlabeled enzyme. The mean specific binding ±S.E. of 5 experiments are plotted in A and analyzed in B by the Scatchard method as described in the legend to Fig. 8.

![Graph](image)

**Fig. 10.** Competition for $^{125}$I-$\alpha$-thrombin binding by various sera, insulin, and insulin-like activity. Chick embryo fibroblasts were incubated for 60 min at 24°C in 1 ml of phosphate-buffered saline containing 0.41 nM $^{125}$I-$\alpha$-thrombin and the indicated concentration of fetal calf serum (•), fetal calf serum heated for 15 min at 100°C (▲) according to the procedure of Cohen and Nissley (56), bovine serum (▼), chicken serum (△), insulin (●), ILAs (○), and 1.5 µg of $\alpha$-thrombin (□). Data are expressed as the mean ±S.E. for 3 determinations. The 100% value for maximum $^{125}$I-$\alpha$-thrombin bound corresponds to 72.9 fmol/mg of protein.

![Graph](image)

**Fig. 11.** Competition for $^{125}$I-$\alpha$-thrombin binding by various thrombins. The percentage of maximum $^{125}$I-$\alpha$-thrombin binding to attached fibroblasts in the presence of increasing concentrations of unlabeled $\alpha$-thrombin (○), $\gamma$-thrombin (●), $\beta$-thrombin (■), DIP-thrombin (△), mesyl-thrombin (▲), TLCK-thrombin (●), and nitro-thrombin (□) was determined following a 60-min incubation at 24°C with 0.28 or 0.42 nM $^{125}$I-$\alpha$-thrombin as described under "Methods." Each point for $\alpha$- and $\gamma$-thrombin is the mean ±S.E. for 3 determinations, and for DIP, TLCK, MS, and nitro-$\alpha$-thrombins, it is that of 9 determinations. The 100% value corresponds to 140 ± 10 fmol/mg of protein.
are also mitogens for the avian cells (34), did not compete with \(1^2S\)-\(a\)-thrombin for binding. Such binding similarly was not affected by phorbol myristic acetate (data not presented). This alkaloid is a tumor promoter and mitogen for mammalian and avian fibroblasts (35) and inhibits specific EGF binding to receptors on HeLa cells (36).

The catalytically active enzyme is required for specific binding, since DIP-, TLCK-, and MS-\(a\)-thrombins, which are chemically modified at the active site, do not compete or compete very poorly with \(1^2S\)-\(a\)-thrombin (Fig. 11). As further support for the conclusion that the catalytic site is required, hirudin-complexed and mCP(PBA)-F-inactivated \(1^2S\)-\(a\)-thrombin failed to bind to substratum-attached cells (Fig. 12). These relatively large inhibitors interact noncovalently and covalently, respectively, with the high affinity or apolar binding sites of \(a\)-thrombin, causing steric occlusion of active site regions in addition to masking the catalytic site (26, 30).

In contrast to the catalytically inactivated forms, nonclotting thrombins, which retain esterolytic activity, were made by nitration of 3-4 out of the 10 tyrosines in \(a\)-thrombin or by proteolytic fragmentation of the \(a\)-thrombin B chain to generate \(\gamma\)-thrombin. Both nonclotting thrombins displayed reduced binding affinities for avian fibroblasts, shown by the displaced competitive binding curves relative to that for \(a\)-thrombin (Fig. 11). These nonclotting thrombins were nevertheless mitogenic but with correspondingly reduced potencies (Fig. 11).

The thrombin receptors further displayed a high degree of specificity for the enzyme. Plasmin, a serine protease, which is also mitogenic for chick embryo fibroblasts (5, 6), did not compete with \(1^2S\)-\(a\)-thrombin for binding (Fig. 13). \(a\)-Chymotrypsin and elastase at high concentrations did compete at 22°C, but receptor and/or thrombin degradation could explain these observations. Urokinase, a plasminogen activator isolated from human urine either as the \(M_r = 55,000-60,000\) or 38,000, biologically active species, competed with \(1^2S\)-\(a\)-thrombin. These preparations were 33 and 66 times less potent on a molar basis than unlabeled \(a\)-thrombin in causing 50% inhibition. Trypsin, which is mitogenic for avian fibroblasts, competed with \(1^2S\)-\(a\)-thrombin for binding at 0–4°C (data not presented) and at 22°C (Fig. 13) with an affinity that was similar to that of \(a\)-thrombin. Although receptor and/or \(1^2S\)-\(a\)-thrombin degradation may explain inhibition at 22°C, trypsin inhibition at 0–4°C, as well as urokinase and nonclotting thrombin inhibition, strongly suggests that the avian fibroblast receptor is not absolutely specific for \(a\)-thrombin.

Large molecular weight, arginine-specific esterases have been isolated complexed with nerve (21, 37) and epidermal growth factors (20, 37). Recently, Maciag (38) has isolated a protease from rat pituitary which cleaves bovine and human growth hormone to generate peptides with sulfation factor activity. Since this activity is inhibited by hirudin, we evaluated the ability of this protein and the other growth factor proteases to inhibit \(1^2S\)-\(a\)-thrombin binding. However, none of these inhibited \(1^2S\)-\(a\)-thrombin binding to either avian (Fig. 13) or mouse embryo fibroblasts (data not presented).

**Fig. 12. Binding of mCP(PBA)-F or hirudin-inactivated \(1^2S\)-\(a\)-thrombin.** In A, \(1^2S\)-\(a\)-thrombin was treated with mCP(PBA)-F or hirudin as described under "Methods," and its binding and that of untreated or control-treated thrombin was determined as a function of concentration. In B, data, expressed as fmol/mg of protein ± S.E. for specifically bound enzyme, were analyzed by the Scatchard method.

**Fig. 13. Competition for \(1^2S\)-\(a\)-thrombin binding by serine proteases and growth factor-processing esterases.** Chick embryo fibroblasts were incubated for 60 min at 24°C in 1 ml of phosphate-buffered saline containing 0.41 nM \(1^2S\)-\(a\)-thrombin and the indicated concentrations of \(\alpha\)-thrombin (C), \(\alpha\)-chymotrypsin (S), elastase (V), high or low molecular weight urokinase (U, and U, respectively), plasmin (P), stabilised nerve growth factor (N), epidermal growth factor binding protein (V), trypsin (X), and rat pituitary growth hormone protease (A). Following incubation, the plates were rinsed and processed as described under "Methods." For \(a\)-thrombin, the mean ± S.E. represents 12 determinations, while it represents 6 determinations for the other proteases. The 100% values were 145–167 fmol/mg of protein.
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![Diagram A: Binding of 125I-α-thrombin as a function of culture density.](image)

**Fig. 14.** Binding of 125I-α-thrombin as a function of culture density. Chick embryo fibroblasts were plated at 4.0 (●) or 9.0 (○) £ 10^5 cells/60-mm dish, then grown for 3–4 days as described under “Methods.” Specific binding as a function of 125I-α-thrombin concentration was determined after a 60-min incubation at 22°C, and data were analyzed by the Scatchard method. Data for a representative experiment based on 3 determinations are presented in A and B. In C, apparent K_d and R_o values for 8 experiments are plotted versus the final cell protein concentration. Regression analysis gave p < 0.05.

4 £ 10^5 cells/dish (Fig. 14A). This increase was accounted for by an increase in receptor number per cell, but the apparent K_d increased (Fig. 14B). The results of 8 similar experiments for fibroblasts plated between 4 and 9 £ 10^5 cells/60-mm dish and grown for 3–4 days prior to assay were plotted as a function of the final protein content of the culture dish (Fig. 14C). As the culture density doubled, there was a corresponding doubling of the R_o value and a 40% increase in the apparent K_d (p < 0.05).

**DISCUSSION**

Induction of the mitogenic signal by α-thrombin with its accompanying pleiotropic changes, e.g. glucose transport stimulation, cell shape changes, and decreased adhesion (5, 6), required catalytically active enzyme. For example, treating α-thrombin with DIP-F or MS-F, which form covalent complexes with α-thrombin (1, 2), is a potent inhibitor of 125I-α-thrombin binding to platelets (30), mouse embryo cells, and chick embryo fibroblasts (Fig. 12). However, since α-thrombin complexed with hirudin is catalytically inactive (e.g. no clotting or esterase activities), the failure to bind to the cells may be explained accordingly. The essentially nonscotting forms, γ and nitro-α-thrombin, which were prepared by limited trypsinic fragmentation and nitration of α-thrombin (2), respectively, retained ~80% of the esterase activity of the parent form. These forms competed with 125I-α-thrombin for binding to the avian cells, but were 8- and 3-fold less effective than α-thrombin, respectively (Fig. 11). They were also correspondingly less mitogenic (Fig. 1), suggesting that binding is a prerequisite, while catalytic activity is essential for mitogenic stimulation. Their reduced ability to compete may imply that the protein binding regions in the active site of α-thrombin assist in the binding to avian receptors but that such binding alone is insufficient for establishing stabilized interactions. In support of this idea, the methysulfonfyl group of MS-α-thrombin is relatively small compared with that of DIP- or TLCK-α-thrombin, and the degree of overlap with the protein-binding region is minimal, yet MS-α-thrombin competes much more poorly than γ- or nitro-α-thrombin (Fig. 11).

Elastase, α-chymotrypsin, and the high and low molecular weight forms of human urokinase all competed with α-thrombin for binding but with reduced affinities. However, plasmin, which is mitogenic for chick fibroblasts (5), did not compete. Neither did the arginine esterases implicated in progrowth factor processing (e.g. EGF-binding protein; 20, 37), the γ subunit of 75 NGF (21, 37), nor rat pituitary protease which activates the pituitary growth hormone (38). Of the serine proteases examined, trypsin displays the highest affinity for the receptors on the avian fibroblasts: it was also species specific. The competitive binding observed at 0–4°C (data not presented) and 22–24°C (Fig. 13) was similar to that of α-thrombin, and it was not the consequence of 125I-α-thrombin or receptor degradation. The presence of trichloroacetic acid-soluble 125I was not detected following 60 min of incubation at 22°C in the presence of 1 µg/ml of trypsin, and trypsin did not prevent 125I-α-thrombin binding to human foreskin fibroblasts,

1 Other evidence implicating enzymatically active thrombin for binding to cultured avian fibroblasts also was applicable for binding to mouse C3H 10T1/2 embryonic fibroblasts, mouse MMC-E epithelial cells (39), human genital, and deltoid skin fibroblasts, but not to mouse secondary embryo cells. These results confirm and are consistent with the observations of Glenn et al. (40).

2 mCIP(PBA)- and PMS-α-thrombins, but not the hirudin-complexed enzyme, bind to and are compartmentalized by mouse embryo cells indistinguishably from the binding and compartmentalization of untreated α-thrombins, but are mitogenically inactive (J. F. Perdue, W. Lubensky, E. Kivity, and J. W. Fenton II, unpublished data).
MMC-E epithelial cells, C3H 10T½ cells or mouse embryo cells. These results strongly support the proposition that both enzymes are acting at a common site and that the induction of the mitogenic signal is the consequence of this interaction.

Binding of $^{125}\text{I}$-a-thrombin to the avian fibroblasts was optimum at a pH of 6.8 (Fig. 3). It was proportional to cell number (Fig. 2) and labeled enzyme concentration (Figs. 7 and 9A). Scatchard analysis of the specific binding indicated each cell has 20,000-40,000 homogenous receptors which bind the protease with an apparent $K_d$ of 0.7 nM (Figs. 8 and 9B). This $K_d$ value compares favorably with those of 1–0.12–2, and 0.1 nM calculated for $^{125}\text{I}$-a-thrombin binding to mouse embryo cells (14), human platelets (18, 23), and endothelial cells (19), respectively. However, such $K_d$ assignments are most likely invalid, since we have observed that mouse cells irreversibly bind and compartmentalize PMS- or mCP(PBA)-$^{125}\text{I}$-a-thrombin, as well as $^{125}\text{I}$-a-thrombin, similar to avian cells. Compartmentalization was operationally defined as the inability of such bound $^{125}\text{I}$-a-thrombin to be released upon subsequent incubation with 25 µg/ml of trypsin at 0–4°C. Compartmentalization could result from proteolytically induced structural changes in the receptor, rendering the bound $^{125}\text{I}$-a-thrombin inaccessible to trypsin, or the sequestering of the $^{125}\text{I}$-a-thrombin and/or its receptor complex within coated pits or microvesicles as is thought to occur with low density lipoprotein and EGF (41, 42). Compartmentalized $^{125}\text{I}$-a-thrombin is degraded presumably by lysosomes, since radioactivity released into the culture medium was dramatically reduced with 50 µM chloroquine (Table 1) concomitant with an increase in intracellular radioactivity.

$^{125}\text{I}$-a-Thrombin bound to chick cells further appears to have a $t_{1/2}$ of 3.5 h in both serum-free and serum-containing medium. In the presence of 5 or 10 ng/ml of unlabelled a-thrombin, the $t_{1/2}$ was greatly shortened, such that by 4 h, specific binding was not detectable. Such short $t_{1/2}$ values compare with the $t_{1/2}$ of 5–6 h for the glucose transport protein in these same cells (43) and 14–18 h for the receptor which binds an insulin-like growth factor. The rapid, almost complete loss of specific binding suggests that the compartmentalized binding sites are not recycled back to the plasma membrane, as has been suggested for the receptor which binds asialoglycoprotein (44, 45), but rather steady state binding requires the continual synthesis and insertion of new receptors. This may convey information to the cell which is translated into a mitogenic response, or it may be completely unrelated to this signaling event.

How irreversible binding occurs and the chemical nature of the avian fibrobast receptor are presently unknown. Answers to both questions may be found in the analogous relationships of protease inactivation by antithrombin III (46–49), α-protease inhibitor (50), and α-plasma inhibitor (51, 52). These inhibitory plasma proteins initially form a dissociable Michaelis complex with the enzyme. This complexing involves enzyme active site regions adjacent to the catalytic site, since the sterically occluded DIPI-proteases do not form complexes (46, 52). However, catalytically inactive anhydrotrypsin or carboxamidomethyl trypsin forms a dissociable complex with α-plasmin inhibitor, but not with α-protease inhibitor (52), illustrating that enzymic activity may or may not be necessary for complex formation. Acyl bond formation between the active site serine in the enzyme and the cleaved peptide bond in the inhibitors occurs with a markedly reduced rate at 0°C compared with that at 37°C (51) and is believed to be preceded by the formation of tetrahedral intermediates (53) as detected with synthetic substrates (54). Such tetrahedral formation and/or subsequent peptide bond cleavage in the inhibitor (48–50, 53) cause(s) protein conformational changes in the complex (17) which may prevent enzyme turnover but accelerate its rate of degradation.

Many of our observations of thrombin binding to the avian fibroblasts are consistent with the formation of a stable or slow turnover enzyme complex (e.g. acyl-enzyme complex). (i) Only the catalytically active thrombin forms bond. (ii) Binding occurs more slowly at 0–4°C than at 22–24°C. (iii) The specifically bound enzyme at 0–4°C dissociates very slowly from the surfaces of the cells. (iv) Treating the cells with 100 µM hydroxylamine increased the quantity of thrombin which dissociated from the cell surface by 30% (Fig. 5A). (v) The $t_{1/2}$ was accelerated in the presence of unlabelled thrombin.

If a-thrombin complexing with its receptor is analogous to that with antithrombin III (46–49), then it may cleave its receptor, assuming that the COOH-terminal protein is not disulfide-bonded. In this regard, Baker et al. (55) observed that α-thrombin-induced mitogenesis in chick embryo fibroblasts was correlated with the cleavage of a lactoperoxidase-$^{125}$I-labeled $M_r = 43,000$ membrane component. A similar molecular weight component was also found for these cells by cross-linking with photoactivatable $^{125}$I-a-thrombin (11). However, an SDS- and heat-stable, but hydroxylamine-sensitive complex of $^{125}$I-a-thrombin and a putative receptor protein has not been identified for avian cells, although such complexes have been observed with α-thrombin or EGF for human foreskin fibroblasts and with insulin for H-4 hepatoma cells. The future isolation and chemical characterization of a solubilized receptor from the avian cells will hopefully provide insight into the nature of this cell membrane component and the mechanisms whereby the specific interaction with α-thrombin induces the mitogenic events.

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REFERENCES
Thrombin Receptors of Chick Embryo Fibroblasts