A Novel Method for Measuring Cell Surface-bound Thrombin

DETECTION OF IODINATION-INDUCED CHANGES IN THROMBIN-BINDING AFFINITY

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David A. Low‡ and Dennis D. Cunningham

From the Department of Microbiology, College of Medicine, University of California, Irvine, California 92717

A method is presented, which we call the hirudin assay, for measuring picogram amounts of thrombin bound to the cell surface of normal mouse fibroblasts. After incubation of cells with hirudin, virtually all cell-bound thrombin is released into the medium. Importantly, over 90% of cell-associated thrombin is in complex with hirudin as shown by native gel electrophoresis. Therefore, by incubating cells with 125I-hirudin and precipitating 125I-hirudin-thrombin complexes with an antibody to thrombin, we can quantitatively measure the amount of cell-bound thrombin. Thrombin linked to protease-nexin is tightly bound to the cell surface and only a small amount is released into the medium in the presence of hirudin. Thus, the hirudin assay primarily measures free, unlinked thrombin bound to cells.

Comparison of the binding of 125I-thrombin and unlabeled thrombin to mouse cells using the hirudin assay shows that 125I-thrombin detects only about one-half of the binding sites that thrombin does. Additionally, by incubating a constant amount of 125I-thrombin or thrombin with increasing amounts of membrane preparations, most thrombin molecules, but only 30% of 125I-thrombin molecules, bind to cellular receptors. The discrepancies between 125I-thrombin and thrombin binding to mouse cells are caused, at least in part, by the presence of a large fraction of 125I-thrombin containing diiodotyrosine (DIT). We find that 125I-thrombin containing DIT cannot bind to mouse cells. Additionally, 125I-thrombin containing monoiodotyrosine binds to mouse cells with a 2- to 3-fold higher affinity than thrombin. Therefore, a variety of errors in measurement of thrombin binding occurs when using 125I-thrombin as a probe. However, 125I-thrombin containing monoiodotyrosine or DIT links to protease nexit with equal affinity, indicating that 125I-thrombin can be used to accurately measure the amount of thrombin in linkage with protease nexit.

Thrombin, a serine protease which catalyzes the conversion of fibrinogen to fibrin in blood coagulation, has a number of other important biological functions. Thrombin is a potent mitogen for a variety of mammalian fibroblasts in culture (1, 2), stimulates prostaglandin release from endothelial cells (3), initiates release of various platelet factors, and causes platelet aggregation (4). Studies with all of these cell types have shown that each possesses high affinity binding sites for thrombin. By analogy with the action of hormones such as epidermal growth factor (5), these thrombin receptors have been implicated in the action of thrombin on platelets (6, 7), chick embryo fibroblasts (8, 9), and mouse embryo fibroblasts (10).

To study the interaction of thrombin with these binding sites or receptors, thrombin has been iodinated using chloramine-T (11), lactoperoxidase coupled to Sepharose (7), and soluble lactoperoxidase (6). At low levels of iodination, 125I-thrombin appears to retain most of its proteolytic activity as evidenced by its ability to cleave fibrinogen. However, 125I-thrombin loses stability, especially at high specific activities, and must be used soon after labeling (12). Because of this instability, long term cell-binding experiments cannot be done, which precludes determination of receptor occupancy by thrombin over the 2-day course of a mitogenic experiment.

A number of reports have shown that iodination of polypeptide hormones can both alter their affinities for cell surface receptors (13) and their biological activities (13, 14). Although 125I-thrombin retains its ability to cleave fibrinogen (6), it is not known whether the fibrinogen binding site of thrombin is identical with its cellular binding site. Because of these problems and uncertainties when using 125I-thrombin for thrombin-binding studies, we sought an alternative means of detecting cell-bound thrombin. In this paper, we present a cellular binding assay which will quantitatively detect unlabeled thrombin bound to mouse cells. Results obtained using this assay for unlabeled thrombin differ significantly from results obtained using 125I-thrombin. We show that these differences are caused by iodination-induced changes in the affinity of thrombin for its receptor.

EXPERIMENTAL PROCEDURES

Materials—Highly purified human thrombin (about 3100 National Institutes of Health units/mg) and DIP-hT (12.8 unit/mg) were generously supplied by Dr. John W. Fenton, II, Division of Laboratories and Research, New York State Department of Health, Albany, NY 12201 (15). Hirudin (about 1000 units/mg), BSA (radioimmunoassay grade), fibrinogen (bovine type I), aprotinin, and iodotyrosine standards were products of Sigma. PhAsO was obtained from Aldrich, Silica Gel 60 aluminum sheets were from Merck, and lactoperoxidase (320 units/mg) was from Worthington. Ovalbumin (B grade) was obtained from Calbiochem and protein A Sepharose was from Pharmacia. We purchased DV medium from Flow Laboratories; trypsin

1 The abbreviations and trivial names used are: DIP-hT, diisopropyl-phosphate-modified thrombin; BSA, bovine serum albumin; PhAsO, phenylarsine oxide; DV medium, Dulbecco-Vogt modified Eagle’s medium; binding medium, DV medium containing Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (20 mM) and ovalbumin (0.1%); D-PBS, Dulbecco’s phosphate-buffered saline; bis-tris, 2-hydroxyethyl) 1-amino-2-(hydroxymethyl)-1,3-propanedolid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MIT, monoiodotyrosine; DIT, diiodotyrosine; PN, protease nexit.
solution, glutamine, and antibiotics were from Gibco, and calf serum was from Irvine Scientific (Santa Ana, CA). Na-1251 was obtained from Amersham and 1,3,4,6-tetrachloro-3,6-diphenylglycouril (IODO-GEN) was from Pierce.

Iodination and Binding—Hirudin (2 μg) was radioiodinated using IODO-GEN (16) and Na-1251 (1 mCi). The specific activity of 125I-hirudin was determined by spotting dilutions of the postiodination solution onto Whatman SF filter paper discs. After drying, discs were added to a beaker containing Amersham IODO-GEN (21). The amount of hirudin bound to membranes was determined by addition of 0.75 ml of D-PBS containing BSA (1%) and 125I-hirudin (10 ng) to each tube. Membranes were resuspended by agitation and tubes were rocked for 3 h at 37 °C. After centrifugation for 5 min at 12,000 x g to remove membranes, the amount of membrane-associated hirudin in supernatant solutions was determined using the hirudin assay.

Polyacrylamide Gel Electrophoresis and Immobiline Analysis—Medium containing 125I-thrombin and 125I-thrombin-hirudin complexes was analyzed using a bis-tris native gel system, pH 7.5 (System 1505 described by Chrambach et al. (21)) except that the stacking buffer pH was 22 °C. For SDS-PAGE using this bis-tris buffer system, the upper reservoir buffer contained 0.1% SDS. SDS-PAGE using a Tris buffer system was performed as described by Laemmli (22) using a 5% acrylamide stacking gel and a 10% acrylamide separating gel. Quantitation of radioactive gel slices was carried out as described (23).

Gel slices containing 125I-thrombin and 125I-thrombin-PN were electrophoresed through a 5% acrylamide stacking gel (22) into dialysis bags. After dialysis against water, samples were dried using a Speed Vac Concentrator (Savant) and SDS was removed by addition of 200 μl of acetone/triethylamine/acidic acid/water (4:1:1, v/v) and 300 μl of 0.38 mM barium hydroxide were added to the protein precipitates and samples were hydrolyzed in sealed glass ampules for 3–6 h at 110 °C. After neutralization with sulfuric acid, extracts of the barium sulfate precipitate (in acetone/acidic acid, 3:2, v/v) were applied to Silica Gel 60 aluminum sheets using a 1-butanol/acidic acid/water (10:1:1, v/v) developing solvent (25). Standards were identified with ninhydrin and samples were identified by autoradiography.

RESULTS

Assay for the Detection of Picogram Amounts of Thrombin

We developed a highly sensitive assay for detecting thrombin by using the leech salivary protein hirudin. Hirudin (M, ~8000) has a high affinity for thrombin and its interaction with thrombin is highly specific (26, 27). Hirudin was iodinated to a high specific activity (about 2.5 x 10^9 cpm/pg) and incubated with increasing amounts of thrombin. These mixtures, containing 125I-hirudin-thrombin complexes, were incubated with rabbit anti-thrombin antiserum and protein A Sepharose to specifically adsorb 125I-hirudin-thrombin complexes. As shown in Fig. 1, the maximum sensitivity of the assay was about 50 cpm/pg of thrombin. The linear range of these bind to cells containing cell-bound thrombin, only 125I-thrombin-human thrombin preparations do not bind tightly to cellular components and are not measured using the hirudin assay.
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**TABLE I**

| Time (h) | Hirudin | PhAsO | Cells (cpm) | Medium (cpm) | Total (cpm) |
|---------|---------|-------|-------------|--------------|-------------|
| 0       | -       | -     | 0.77        | 0            | 0.77        |
| 2       | -       | -     | 0.19        | 0.43         | 0.62        |
| 2       | -       | +     | 0.22        | 0.61         | 0.83        |
| 2       | +       | -     | 0.05        | 0.78         | 0.83        |
| 2       | +       | +     | 0.05        | 0.78         | 0.83        |

*Time of incubation at 37 °C.

**Fig. 1.** Measurement of thrombin and DIP-thrombin using the hirudin assay. After addition of increasing amounts of thrombin and DIP-thrombin to Microfuge tubes, either low specific activity (1.3 × 10^5 cpm/μg; 35 ng) or high specific activity (2.6 × 10^6 cpm/μg; 15 ng) ^125^I-thrombin was added. Immediately following ^125^I-thrombin addition, rabbit anti-thrombin antiserum was added (5 μl/tube). Each tube contained a total volume of 0.75 ml and a final BSA concentration of 1%. After 30 min at 22 °C, protein A Sepharose (50 μl) was added, the tubes were washed, and the radioactivity in each tube was determined as described under “Experimental Procedures.”

**Assay for the Detection of Thrombin Bound to the Cell Surface of Fibroblasts**

**Release of ^125^I-Thrombin from the Surface of ME Cells—**To measure thrombin bound to the cell surface of fibroblasts, we first determined the conditions which would allow complete dissociation of thrombin from the cell surface. To do this, ^125^I-thrombin was incubated with mouse cells for 15 min. Cells were rinsed thoroughly and incubated in BSA-containing buffer with or without the addition of hirudin or PhAsO. After 2 h, both cell and medium samples were analyzed by SDS-PAGE. Table I shows that after a 2-h incubation in buffer alone, about 75% of the cell-bound ^125^I-thrombin was lost. However, only a fraction of this “dissociated” ^125^I-thrombin was recovered in the medium, indicating that some ^125^I-thrombin was degraded by the cells. To confirm this hypothesis, cells were treated with PhAsO just prior to the 2-h incubation. We have shown previously that PhAsO does not affect binding of ^125^I-thrombin to human fibroblasts but completely blocks its internalization (18). As shown in Table I, pretreatment with PhAsO did not significantly change the amount of ^125^I-thrombin bound to cells after the 2-h incubation. However, all of the ^125^I-thrombin lost from cells during the 2-h incubation was recovered as intact ^125^I-thrombin in the medium. These results indicated that cells internalized and degraded a portion of the ^125^I-thrombin during the 2-h incubation in the absence of PhAsO.

Addition of hirudin during the 2-h incubation resulted in a 95% reduction in cell-bound ^125^I-thrombin. All of this dissociated ^125^I-thrombin was recovered in the medium (Table I). Importantly, this occurred in the absence of PhAsO, indicating that hirudin blocked internalization of ^125^I-thrombin by the mouse cells. Thus, after incubating cells for 2 h in the presence of hirudin, 95% of cell-bound ^125^I-thrombin was lost from the cell surface and could be recovered in the medium.

**Formation of ^125^I-Thrombin-Hirudin Complexes—**Since only thrombin-hirudin complexes were detected by the assay, it was important to show that thrombin which dissociated from cells in the presence of hirudin was also in complex with hirudin. After addition of ^125^I-thrombin to mouse cells cultures, cells were incubated in the presence or absence of hirudin for 2 h. Media samples were then analyzed by native PAGE. In this native gel system, free ^125^I-thrombin barely entered the separating gel, but ^125^I-thrombin-hirudin complexes migrated well into the separating gel. As shown in Fig. 2A, ^125^I-thrombin which dissociated from cells in the absence of hirudin migrated to the stacking gel/separating gel interface. However, at least 90% of ^125^I-thrombin which dissociated from cells in the presence of hirudin was also in complex with hirudin as evidenced by its migration into the separating gel. Importantly, little if any ^125^I-thrombin which bound to cells nonspecifically was found in complex after the 2-h incubation with hirudin (Fig. 2B). Nonspecifically bound ^125^I-thrombin is defined as ^125^I-thrombin which binds to cell cultures in the presence of a large excess of thrombin. A comparison of Fig. 2A and Fig. 2B showed that the amount of ^125^I-thrombin which was not in complex with hirudin was about equal to the amount of nonspecifically bound ^125^I-thrombin. Therefore, in Fig. 2A the fraction of ^125^I-thrombin not in complex with hirudin appeared to be nonspecifically bound ^125^I-thrombin. Taken together, these results showed that after incubation of cells in the presence of hirudin for 2 h, almost all specifically cell-bound ^125^I-thrombin was cell-dissociated and in complex with hirudin.

**Measurement of ^125^I-thrombin-PN by the Hirudin Assay—**Over 90% of ^125^I-thrombin specifically bound to mouse cells is bound as free ^125^I-thrombin. The remaining specifically bound ^125^I-thrombin is found in complex with a protein of M_r ~ 38,000 that we have named PN (23, 28). PN probably would not play a significant role in thrombin-binding studies using mouse cells, since it accounts for less than 10% of the total specifically bound ^125^I-thrombin. However, about 70% of ^125^I-thrombin specifically bound to human foreskin fibroblast-like cells is in complex with PN (28). Thus, it was important to determine if, under the conditions described above, thrombin-PN complexes could dissociate from cells and might thus be available for detection by the hirudin assay. ^125^I-Thrombin was incubated with human cells for 2 min to avoid internalization of thrombin which occurs rapidly in these cells (18).
Addition. After rinsing, cultures were incubated with 125I-hirudin for mouse cell cultures. Incubations were for fraction is and hirudin on gels as described under "Experimental Procedures." The first gel incubation to determine levels of intracellular 125I-thrombin-internalization, cells were incubated in the presence of hirudin without the addition of thrombin (10 µg/ml) was added to mouse cell cultures. After 30 min at 37 °C, cultures were washed and incubated with D-PBS containing PhAsO (0.1 mM) for 10 min at 0 °C. After rinsing once in D-PBS, DV medium containing Hepes (20 mM) and either BSA (10 µg/ml) or BSA and hirudin (35 ng) was added and cultures were shifted to 37 °C. After 2 h, culture fluids were analyzed on native polyacrylamide gels as described under "Experimental Procedures." The first gel fraction contained stacking gel and the remaining gel fractions contained separating gel. The arrows indicate the migration positions of 125I-thrombin-hirudin, and 125I-thrombin-hirudin complexes. Radioactivity at the migration position of hirudin in gels is 125I. Each fraction is 2 mm. A, incubations with 125I-thrombin; B, incubations with 125I-thrombin and thrombin. O, no hirudin addition; O, hirudin addition.

After treatment with PhAsO to prevent possible subsequent internalization, cells were incubated in the presence of hirudin for 3 h. Cells were trypsin treated before and after the 3-h incubation to determine levels of intracellular 125I-thrombin-PN. Both cell and media samples were analyzed by SDS-PAGE. Table II shows that cell-bound 125I-thrombin-PN remained at the cell surface throughout the incubation as evidenced by its complete sensitivity to trypsin. After 3 h, only about 16% of total cell bound 125I-thrombin-PN was found in the medium. Thus, 125I-thrombin-PN complexes dissociated very slowly from human cells with the consequence that only a small amount of PN would be available for detection using the hirudin assay.

Comparison of the Cellular Binding of Thrombin and 125I-Thrombin

Scatchard Analysis—To measure thrombin binding to the cell surface, increasing amounts of thrombin were added to mouse cell cultures. Incubations were for 30 min, by which time steady state binding levels had been reached (not shown). After rinsing, cultures were incubated with 125I-hirudin for 3 h. As shown above, under these conditions almost all cell surface-bound thrombin had dissociated and formed complexes with hirudin (Table I, Fig. 2). 125I-Hirudin-thrombin complexes were then measured using the hirudin assay. As shown in Fig. 3A, binding of thrombin to mouse cells was saturable. The results, transformed by the method of Scatchard (29), are shown in Fig. 3B and cumulative results of six separate determinations are presented in Table III. Thrombin appeared to bind to a single class of high affinity (Kd = 2.4 × 10⁻⁸ M) receptors at 37 °C as evidenced by a high linear correlation coefficient. At saturating levels, thrombin detected about 140,000 receptor sites/mouse cell. However, interpretation of binding data obtained at 37 °C can be complicated by ligand internalization (30); therefore, we also performed binding studies at 0 °C to block endocytosis. Our results showed that the apparent affinity of thrombin for its receptor almost doubled, but the apparent number of receptor sites detected remained the same when compared to binding studies performed at 37 °C (not shown).

Because previous studies have used 125I-thrombin for the detection of thrombin-binding (6, 7, 9–12), we compared results obtained using 125I-thrombin to those obtained using the hirudin assay. Thrombin was iodinated by a soluble lactoperoxidase technique (6) and retained 100% of its fibrinogen clotting activity at up to 0.4 mol of I/mol of thrombin. Fig. 3A shows that the total binding of 125I-thrombin to mouse cultures was nonsaturable, in contrast to the total binding of thrombin to mouse cultures determined using the hirudin assay. However, subtraction of the nonspecifically bound 125I-thrombin from total 125I-thrombin at each 125I-thrombin concentration yielded a saturable binding curve (Fig. 3A). As shown in Fig. 3B and Table III, 125I-thrombin detected one class of thrombin-binding sites. As seen for unlabeled thrombin, the binding affinity of 125I-thrombin at 0 °C increased by about 2-fold over the apparent affinity obtained at 37 °C, but an equal number of 125I-thrombin-binding sites was detected at 0 and 37 °C (not shown). However, only about one-half of the thrombin-binding sites detected using the hirudin assay were detected using 125I-thrombin. Additionally, the apparent affinity of 125I-thrombin was over 2-fold higher than the apparent affinity of uniodinated thrombin for its receptor (Table III).

The discrepancies between binding results obtained using 125I-thrombin and thrombin could have been caused by radiiodination-induced alterations in thrombin-binding. Alternatively, the different methods of measurement of cellular binding of 125I-thrombin and thrombin could have caused
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**Fig. 3. Binding of $^{125}$I-thrombin, I-thrombin, and thrombin to mouse cells.** Increasing amounts of $^{125}$I-thrombin (0.3 mol of I/mol of thrombin), I-thrombin (0.3 mol of I/mol of thrombin, or 1.5 mol of I/mol of thrombin) and thrombin in binding medium (2 ml) were added to mouse cell cultures (10$^5$ cells/35-mm dish) for 30 min at 37°C. After rinsing cultures in D-PBS, the amount of cell-bound $^{125}$I-thrombin was determined by measuring the radioactivity remaining on dishes as described under “Experimental Procedures.” The amounts of cell-bound I-thrombin and thrombin were measured using the hirudin assay as described under “Experimental Procedures.” A shows the relationship between the amount of iodinated or uniodinated thrombin added to cells and the amount of thrombin bound to cells. $\triangle$, total cell-bound $^{125}$I-thrombin; $\bigcirc$, cell-bound $^{125}$I-thrombin in the presence of thrombin (10 $\mu$g/ml), defined as nonspecifically-bound $^{125}$I-thrombin; $\bullet$, specifically-bound $^{125}$I-thrombin (total cell-bound $^{125}$I-thrombin minus nonspecifically-bound $^{125}$I-thrombin); $\bigtriangleup$, cell-bound 1-thrombin (0.3 mol of I/mol of thrombin). B shows a Scatchard transformation (29) of both the results presented in A and additional results. $\bullet$, specifically-bound $^{125}$I-thrombin; $\bigtriangleup$, cell-bound 1-thrombin (0.3 mol of I/mol of thrombin); $\bigcirc$, cell-bound thrombin. C shows a Scatchard transformation of additional binding results. $\bigcirc$, cell-bound thrombin; $\bigtriangleup$, cell-bound 1-thrombin (1.5 mol of I/mol of thrombin).

A number of differences in the results obtained. To determine if either or both of these possibilities was occurring, we iodinated thrombin with unlabeled iodide. Cellular binding of two I-thrombin preparations was measured using the hirudin assay. One thrombin preparation contained a low molar ratio of iodide to thrombin (0.3 mol of I/mol of thrombin) and retained all of its fibrinogen clotting activity. The other thrombin preparation contained a high molar ratio of iodide to thrombin (1.5 mol of I/mol of thrombin) and retained only 30% of its fibrinogen clotting activity. Our results showed that low levels of iodination increased the apparent affinity of thrombin for mouse cells (Fig. 3B) whereas high iodination levels decreased thrombin binding affinity (Fig. 3C). The results presented in Figs. 3B and 3C also showed that iodination of thrombin did not alter determination of the total number of thrombin-binding sites on mouse cells. These results suggested that the reduced number of thrombin binding sites determined using $^{125}$I-thrombin (Fig. 3B) was not solely the result of iodination-induced changes in thrombin binding.

**Specific Activity Effects—**Additional data supporting the conclusion that low levels of iodination of thrombin increased its apparent binding affinity are shown in Fig. 4. $^{125}$I-Thrombin was diluted with thrombin to one-fifth the original specific activity. Both diluted and undiluted $^{125}$I-thrombin preparations were incubated with mouse cells at 37°C and the specific activity of each preparation was used to calculate the amount of thrombin bound to cells. The results in Fig. 4 showed that cellular binding of these $^{125}$I-thrombin preparations was similar at low thrombin concentrations. However, at higher thrombin concentrations, it appeared that more $^{125}$I-thrombin was cell-bound using the lower specific activity preparation. These results, in agreement with results presented in Fig. 3, indicated that iodination increased the apparent affinity of thrombin for cellular binding sites.

**Membrane Binding Assay—**To determine what fraction of the molecules in $^{125}$I-thrombin and thrombin preparations could bind to cell surface-binding sites, we performed a membrane binding assay (31). Increasing amounts of mouse cell membranes were incubated with a constant amount of $^{125}$I-thrombin or unlabeled thrombin. The results presented in Fig. 4 show that the specific activity of a preparation of $^{125}$I-thrombin and thrombin bound to a single class of binding sites on mouse cells.

**TABLE III**

Comparison of the binding of $^{125}$I-thrombin and thrombin to mouse cells

| Ligand       | $K_d$ | $B$   |
|--------------|-------|-------|
| Thrombin     | $2.4 \times 10^{-9}$ | $140,000 \pm 28,000$ |
| $^{125}$I-thrombin | $9.8 \times 10^{-10}$ | $66,000 \pm 11,000$ |

* The values shown are the means ± 1 S.D. from 6 separate experiments, each using a different mouse cell preparation.
showed that at least 85% of thrombin in unlabeled preparations could bind to mouse cell receptors when measured using the hirudin assay. This is a minimal estimate since it appeared that some thrombin was sequestered by the membranes as evidenced by the reduction in detected thrombin, and thus membrane-released thrombin, at the largest membrane addition (Fig. 5). In contrast, results obtained using 125I-thrombin showed that only about 30% of the thrombin in these preparations appeared to bind to mouse cell receptors. Thus, iodination of thrombin at levels which did not appear to affect the ability of thrombin to cleave fibrinogen prevented the binding of a large portion of thrombin molecules to the mouse cell surface.

Effects of Tyrosine Iodination on Cellular Binding of 125I-Thrombin—As previously noted, the reduction in the number of thrombin-binding sites measured when using 125I-thrombin versus thrombin was not due solely to iodination-caused changes in thrombin-binding affinity. In addition, if 125I-thrombin preparations were not uniformly labeled, and if only certain labeled thrombin molecules could bind to cells, this would result in an incorrect determination of the specific activity of bindable 125I-thrombin and an erroneous estimation of the number of thrombin-binding sites. We tested the possibility that cell-bound 125I-thrombin contained different 125I-Tyr residues from unbound 125I-thrombin by subjecting them to partial enzymatic digestion with Staphylococcus V8 protease and chymotrypsin (32). We found that these proteases yielded the same 125I-Tyr-containing polypeptide fragments for both 125I-thrombin and cell-bound 125I-thrombin, indicating that the 125I-thrombin preparations contained similar 125I-Tyr residues. However, our interpretation of these results would be compromised if neither V8 protease nor chymotrypsin could cleave between the different iodinated tyrosines postulated.

Since it appeared that similar tyrosines were labeled in cell-bound and unbound 125I-thrombin preparations, we determined whether there were differences in labeling at a single Tyr. 125I-Thrombin was incubated with mouse cells in the presence or absence of unlabeled thrombin. After 30 min, solubilized cellular proteins were analyzed by SDS-PAGE and gel slices containing 125I-thrombin were pooled and eluted by electrophoresis, and iodotyrosine standards were added. After hydrolysis for 6 h, samples were analyzed by thin layer chromatography. As shown in Fig. 6a, about 70% of the radioactivity was in MIT and about 30% was in DIT. However, mouse cell-bound 125I-thrombin contained 94% of the radioactivity in MIT and only 6% in DIT (Fig. 6b). This small amount of 125I-thrombin containing DIT could have been derived from nonspecifically bound thrombin since about 20% of the 125I-thrombin in these experiments was nonspecifically bound. As shown in Fig. 6d, about 22% of the radioactivity in nonspecifically bound 125I-thrombin was DIT. Therefore, the small amount of 125I-thrombin containing DIT which bound to mouse cells appeared to be bound nonspecifically. These results showed that 125I-thrombin preparations contained a significant amount of DIT and that this diiodinated thrombin could not specifically bind to mouse cells.
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We showed previously that PN mediates most of the specific cellular binding of thrombin to human cells (28). Therefore, we determined if $^{125}$I-thrombin containing DIT could bind to PN. $^{125}$I-Thrombin was incubated with human cells and solubilized cellular proteins were analyzed by SDS-PAGE. Gel fractions containing $^{125}$I-thrombin-PN complexes were pooled and analyzed as described above. Results in Fig. 6c show that $^{125}$I-thrombin containing DIT linked to PN. Since the fraction of DIT found in complex with PN was similar to that found in the $^{125}$I-thrombin preparation, we conclude that $^{125}$I-thrombin containing either MIT or DIT is equally able to form a linkage with PN.

The results presented in Fig. 6a indicated that about one-third of the total radioactivity in $^{125}$I-thrombin was in DIT. However, this appeared to be a low estimate since experiments in which $^{125}$I-thrombin was directly hydrolyzed for only 3 h and analyzed by thin layer chromatography showed that about one-half of the iodotyrosine radioactivity was in DIT (Fig. 7). Again, we found that cell-bound $^{125}$I-thrombin contained little, if any, DIT. Importantly, a significant amount of $^{125}$I was present. Since about 90% of this $^{125}$I-thrombin preparation was precipitated with trichloroacetic acid, it appears that free $^{125}$I was released from iodotyrosine. Release of $^{125}$I also occurred in the experiment presented in Fig. 6, although a comparison of levels of released $^{125}$I could not be made due to the different assay conditions employed in the experiments presented in Figs. 6a and 7. Detection of iodotyrosine standards added before hydrolysis of samples indicated that DIT was less stable than MIT. This would be expected if the rate of loss of I from MIT and DIT was equal since DIT $\rightarrow$ MIT $\rightarrow$ Tyr. Therefore, it appears that at least 50% of the radioactivity in $^{125}$I-thrombin was in DIT.

Together, these results showed that a large fraction of radioactivity in $^{125}$I-thrombin preparations was present in DIT (Figs. 6a and 7). $^{125}$I-Thrombin containing DIT was apparently unimpaired in its ability to cleave fibrinogen and form a linkage with PN (Fig. 6c), but was unable to bind to mouse cells (Figs. 6a and 7). Additionally, thrombin containing MIT appeared to have a higher affinity for mouse cells then unlabeled thrombin (Table III, Figs. 3 and 4). These iodination-induced alterations in the interaction of thrombin with mouse cells caused errors in the determination of both the binding affinity and the total number of binding sites for thrombin on mouse cells.

**DISCUSSION**

**Hirudin Assay**—The present method for detecting picogram amounts of unlabeled thrombin bound to the surface of mouse cells depends upon quantitative release of thrombin from the cell surface and formation of thrombin-hirudin complexes after incubation with hirudin. Cell surface-bound but not internalized thrombin is measured by the assay since only thrombin released from cells and in complex with $^{125}$I-hirudin is detected. We showed that by incubating mouse cells in the presence of hirudin for 2 h, at least 95% of cell-bound $^{125}$I-thrombin was released into the medium (Table I). However, it appears from results with human cells (Table II) that $^{125}$I-thrombin-PN complexes are tightly bound to the cell surface and only a small fraction of $^{125}$I-thrombin-PN is released into the medium in the presence of hirudin. This confirms results we have previously obtained showing that $^{125}$I-thrombin-PN binds to human cells with an extremely high affinity (18). Because only about 10% of specifically-bound $^{125}$I-thrombin of mouse cells is in complex with PN, we did not determine whether $^{125}$I-thrombin-PN complexes were also tightly bound to mouse cells. Such a determination would be difficult since to avoid rapid internalization of $^{125}$I-thrombin-PN, very short times of incubation with $^{125}$I-thrombin must be used (18). This severely limits the amount of $^{125}$I-thrombin-PN that can be formed at the surface of cells. However, since human cell-derived PN can bind to mouse cells, it seems likely that $^{125}$I-thrombin-PN complexes also dissociate very slowly from mouse cells.

In addition to the requirement that thrombin must be dissociated from the cell surface, thrombin must also be in complex with hirudin to be measured by the hirudin assay. We showed in Fig. 2A that almost all of the cell-dissociated $^{125}$I-thrombin migrated as $^{125}$I-thrombin-hirudin complexes. Only a small amount of nonspecifically bound $^{125}$I-thrombin appeared to form a complex with hirudin (Fig. 2B). One explanation for this is that most of the nonspecifically bound $^{125}$I-thrombin might be damaged thrombin, either present in the unlabeled thrombin preparation or induced by iodination. Alternatively, interaction of thrombin with nonspecific sites on the cells or dishes could alter thrombin. In either case, our finding that nonspecifically bound $^{125}$I-thrombin was measured poorly by the hirudin assay was corroborated in cell binding studies. When using $^{125}$I-thrombin, the fraction of $^{125}$I-thrombin bound nonspecifically increased as a linear function of the concentration of $^{125}$I-thrombin. Thus, at high $^{125}$I-thrombin concentrations, a large fraction of total cell-bound $^{125}$I-thrombin was nonspecifically bound (Fig. 3A). However, when measuring binding of unlabeled thrombin using the hirudin assay, high thrombin concentrations were reached, the amount of cell-bound thrombin approached saturating levels (Fig. 3A). Together, these results showed that almost all specifically bound thrombin released from mouse cells in the presence of hirudin was also in complex with hirudin.

Although we have used the hirudin assay primarily to

Fig. 7. Determination of the relative amounts of MIT and DIT in $^{125}$I-thrombin and a comparison of their ability to bind to mouse cells. Binding buffer containing $^{125}$I-thrombin (TH) (0.5 pg/ml) was added to mouse cells for 30 min at 37°C. Cells were then rinsed in D-PBS and treated with PhAsO as described in Fig. 2. Cell-bound $^{125}$I-thrombin was dissociated by addition of D medium (0.75 ml) containing Hepes (20 mM), ovalbumin (2 mg/ml), and hirudin (35 ng) for 3 h at 37°C. Both this culture fluid, which contained cell-dissociated $^{125}$I-thrombin, and the initial $^{125}$I-thrombin preparation (which was adjusted to contain the same amount of ovalbumin and hirudin as cell-dissociated $^{125}$I-thrombin) were hydrolyzed in barium hydroxide as described under "Experimental Procedures." $^{3}^{3}$Tyr was extracted (in 2 N ammonium hydroxide/ethanol, 1:1, v/v) and analyzed by thin layer chromatography as described under "Experimental Procedures." The development time was 3.5 h. *Arrows* indicate the position of standards.

*It should be noted that $^{125}$I-thrombin was used to determine the fate of cell-bound thrombin. Since the apparent binding affinity of $^{125}$I-thrombin appeared to be higher than that of thrombin (Figs. 3 and 4), our quantitations provide a minimal estimate of the amount of cell-dissociated thrombin.

J. Baker and D. Low, unpublished observations.
determine levels of cell-bound thrombin, it is possible that other applications may be found. The hirudin assay is more sensitive than the radioimmunoassay developed for thrombin (19). As shown in Fig. 1, DIP-thrombin is poorly detected using the hirudin assay. This is surprising because DIP-thrombin was used as an immunogen to raise antibodies to thrombin (19) and DIP-thrombin binds hirudin (26). DIP-thrombin-hirudin complexes thus appear to have a different conformation than thrombin-hirudin complexes, preventing anti-thrombin antibody from interacting with thrombin antigenic determinants. Binding of hirudin near the active site of thrombin (33) may play a role in inducing this postulated conformational change.

Comparison of 125I-Thrombin and Thrombin Binding to ME Cells—125I-Thrombin has been used in previous studies to measure thrombin binding to a variety of cell types (6, 9–12). Our results showed that the binding properties of 125I-thrombin differ from thrombin when assayed using mouse cells (Figs. 3–6, Table III). We found two reasons for these binding differences: the binding affinity of 125I-thrombin containing MIT appeared to be higher than the binding affinity of thrombin (Figs. 3, 4, and 6), and the binding affinity of 125I-thrombin containing DI T was greatly reduced or negligible (Figs. 6 and 7).

In six separate determinations, we found that the apparent binding affinity of 125I-thrombin was 2- to 3-fold higher than that of thrombin (Table III). Our determination of the binding affinity of 125I-thrombin for mouse cells is in close agreement with the binding affinity that we reported previously (34). Additional data which suggested that 125I-thrombin had a higher affinity for cellular binding sites than thrombin was shown in Fig. 4. In this experiment, if the binding affinities of 125I-thrombin and thrombin were equal, then reduction of the specific activity of 125I-thrombin preparations with thrombin would not alter measurement of the amount of cell-bound thrombin. We found that more 125I-thrombin was apparently bound to cells using the lower specific activity preparation. Thus, it appeared that thrombin could not compete equally with 125I-thrombin for cellular binding sites.

125I-Thrombin preparations contained 50% or more of their radioactivity in DI T (Fig. 7). Thus, at least one-third of the 125I-thrombin molecules in these preparations were unable to bind to mouse cells, with the effect of lowering the apparent affinity of 125I-thrombin. It seems likely, therefore, that the actual binding affinity of 125I-thrombin containing MIT is at least 1.5-fold higher than the apparent binding affinity we estimated from Scatchard analysis (Fig. 3, Table III). We also showed that the binding affinity of thrombin that contained high levels of iodide was reduced by about 2-fold compared to thrombin (Fig. 3C). This may have resulted either from an increase in levels of I-thrombin containing DI T or iodination of another Tyr residue critical for cellular binding, which would have the effect of lowering the apparent binding affinity.

Although the binding affinity of thrombin containing high levels of iodide was about one-half the value determined for thrombin, the same total number of thrombin binding sites was detected (Fig. 3C). In contrast, 125I-thrombin detected only about one-half of the binding sites detected by thrombin (Fig. 3B, Table III). The reason for this difference in the detection of binding sites by 125I-thrombin and I-thrombin appears to be that the specific activity determined by 125I-thrombin was erroneously high. This occurred because 125I-thrombin preparations contained a mixture of MIT and DI T, and only the MIT derivative bound to mouse cells (Figs. 6 and 7).

We showed previously that DIP-thrombin bound to mouse cells (34) but did not form a linkage with PN (28). These results indicated that the active site serine of thrombin was involved in the linkage of thrombin to PN but was not required for thrombin-binding to mouse cells. The results presented in this paper also indicated that the thrombin site which binds to mouse cells and the site which links PN are not identical. We found that although 125I-thrombin containing DI T did not bind to mouse cells, its ability to link to PN was unimpaired (Fig. 6c). These results corroborate those which showed that under optimal conditions with human cells almost all 125I-thrombin was found in complex with PN and that iodination of thrombin did not affect its ability to link to anti-thrombin III (35).

Through use of techniques presented here and elsewhere (18, 28, 36), it is now possible to accurately determine the amount of thrombin bound to the mouse cell surface, both free and in complex with PN. We showed in Table II that thrombin-PN complexes dissociated very slowly from the cell surface. Therefore, only a small amount of thrombin-PN would be available for interaction with 125I-hirudin in the hirudin assay. It also seems unlikely that the hirudin could bind to thrombin-PN since hirudin blocks formation of thrombin-PN (18). By using the hirudin assay for detection of non-PN bound thrombin, the errors inherent in using 125I-thrombin, discussed above, can be avoided. Since iodination of thrombin does not impair its ability to link to PN (Fig. 6c), 125I-thrombin can be used to measure thrombin in complex with PN.

Finally, through use of the hirudin assay, it will now be possible to accurately measure thrombin-binding during the course of an experiment in which cells are mitogenically stimulated by thrombin. These studies, now in progress, should allow us to evaluate whether binding of thrombin to its receptor is a necessary event in thrombin-induced stimulation of cell division.

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