Internalization and Degradation of Thrombin and Up Regulation of Thrombin-Binding Sites in Corneal Endothelial Cells*

Naphtali Savion, Jeffrey D. Isaacs‡, Denis Gospodarowicz, and Marc A. Shuman

From the Cancer Research Institute and the Departments of Medicine and Ophthalmology, University of California Medical Center, San Francisco, California 94143

Thrombin binds specifically to bovine corneal endothelial cells. Binding involves the formation of an apparently covalent complex between thrombin and its binding site, M, = 77,000. The cells appear to internalize this complex by adsorptive endocytosis since there is a 10-fold greater amount of thrombin internalized than of prothrombin. Internalization proceeds at a rate of 4 ng of thrombin/1 x 10^6 cells/h and reaches a steady state by 2 h at 37 °C. Approximately 90% of the 125I-thrombin reappears in the extracellular media within 1 h of binding to the cells. Since this released 125I-labeled material cannot be precipitated by an anti-thrombin antibody or trichloroacetic acid, it probably represents degradation of thrombin into small peptides. Chloroquine treatment of the cells completely inhibits degradation of thrombin. This suggests that proteolysis occurs in lysosomes.

Preincubation of corneal cells with physiological concentrations of thrombin for 2 to 4 h results in a concentration-dependent increase in synthesis and subsequent release into the incubation medium of thrombin binding sites. The increase in the rate of release of binding sites is proportional to the duration of preexposure of the cells to thrombin and reaches a maximal increase of approximately 8-fold at 24 h. 125I-thrombin binds to these soluble sites and forms a 77,000-dalton complex similar to that seen with the noninduced binding sites. This complex binds to the cells, is internalized, and then degraded. Binding, internalization, and degradation of thrombin by endothelial cells and the subsequent up regulation of binding sites may represent a mechanism for maintaining low extracellular levels of thrombin.

Previous studies have demonstrated that thrombin binds to platelets (1, 2), endothelial cells (3), and fibroblasts (4). Moreover, apparently spontaneous covalent binding of thrombin to human foreskin fibroblasts, human cervical carcinoma cells, secondary chick embryo cells, and Chinese hamster lung cells concomitant with formation of a complex between 68,000 and 80,000 daltons has been reported (5, 6). Previously, we reported that thrombin binds specifically to bovine corneal endothelial cell binding sites (7). The binding site-thrombin complex resists disruption by heating, sulfhydryl reduction, and SDS-polyacrylamide gel electrophoresis. The linkage behaves as an ester bond, dissociating in the presence of hydroxylamine (7). It has recently been reported that normal human fibroblasts release into culture medium binding sites which form a covalent linkage with thrombin and mediate most of the specific cellular binding of these proteases (8).

In the present communication, we have followed the fate of thrombin-binding site complexes in bovine corneal endothelial cells. Our results indicate that thrombin binding site complexes are internalized and degraded within the lysosome. We have also observed that preincubation of corneal cultures with thrombin results in an increase in the synthesis and number of thrombin binding sites released subsequently into the medium.

EXPERIMENTAL PROCEDURES

Materials—Crystalline bovine serum albumin, Fraction V, lactoperoxidase, and glucose oxidase, type 5, were obtained from Sigma; DMEM and calf serum were purchased from Grand Island Biological Co. (Grand Island, NY); Gentamycin was obtained from Schering (Kenilworth, NJ). Tissue culture dishes were purchased from Falcon Plastics. Carrier-free sodium [125I]iodide was purchased from Amer sham Radiochemicals (Arlington, IL). Human thrombin was a generous gift from J. W. Fenton II, New York State Department of Health, Albany, NY, and was prepared as previously described (9). The specific activities ranged from 2400 to 4000 units/mg. Human prothrombin was purified from fresh-frozen human plasma using the technique of Milettic et al. (10). Fibroblast growth factor (FGF) was purified from bovine brain as previously described (11). Rabbit anti-human thrombin and goat anti-rabbit IgG were prepared by ammonium sulfate precipitation followed by DEAE-cellulose chromatography as previously described (12).

Endothelial Cell Preparation—BCE cells were established from steer corneas (13). Cells in their 2nd to 7th passage were grown to confluence at 37 °C in 10% CO2 in DMEM containing 10% calf serum and 1% fetal calf serum. One hundred ng/ml of FGF were added every other day to the cultures until the cells were nearly confluent.

Iodination of Thrombin and Prothrombin—Human thrombin (150 units) was radioiodinated at room temperature using 0.05 unit of lactoperoxidase, 0.01 unit of glucose oxidase, 0.2% β-β-glucose, 4 μCi of Na [125I]iodide, 0.01 M benzamidine, and buffer containing 0.2 M NaCl and 0.05 M sodium phosphate (pH 6.7) for 1 min in a total volume of 0.2 ml. 125I-thrombin was separated from free sodium [125I]iodide by gel filtration on Sephadex G-25. Iodination of prothrombin (39 μg) was similar, except that 3 μCi of Na [125I]iodide was used. The buffer contained 0.6 M NaCl and 0.02 M sodium phosphate (pH 7.0). Benzamidine was omitted from the incubation.

Binding of 125I-Thrombin and 125I-Prothrombin to BCE Cells—Confluent monolayers (1.2 x 10^6 cells/35-mm culture dish) were washed 3 times with DMEM supplemented with 0.5% BSA. To
initiate binding, the radiolabeled ligand was added to the cells in 1.0 ml of serum-free medium containing 0.5% BSA. After incubation at 37 °C, the cell monolayers were washed rapidly 10 times with 1 to 2 ml of cold phosphate-buffered saline (PBS), pH 7.4, containing 0.1% BSA. The washed monolayers were lysed in 0.1 ml of buffer containing 15% glycerol, 2% SDS, 75 mm Tris-HCl (pH 6.8), 2 mM PMSF, and 2 mM EDTA (henceforth referred to as SDS-lysis buffer). One mM N-ethylmaleimide and 1.0 mM iodoacetic acid were added to block free sulfhydryl groups. The radioactivity in the lysed samples was counted in a Beckman 300 series γ counter. Specific binding was determined by measuring the difference in cell-bound radioactivity in the presence and absence of a 100-fold excess of unlabeled thrombin. The cell-bound radioactivity in the presence of an excess amount of unlabeled thrombin was considered to be nonspecific and was subtracted.

Polyacrylamide Gel Electrophoresis—Aliquots of 10 or 33 µl of the total solubilized cellular material were applied to 10% polyacrylamide slab gels with a 3% stacking gel, as described by Laemmli (14). After electrophoresis at 20 mA for about 4 h, the slab gel was stained with Coomassie blue (0.1%) in 10% trichloroacetic acid and subsequently destained overnight. The gels were dried and subjected to autoradiography on Kodak NS-2T x-ray film for 1 to 24 h. To quantitate thrombin binding, the areas of the gel corresponding to the bands of radioactivity on the autoradiogram were sliced and counted in a Beckman 300 series γ counter. Calculation of thrombin bound in the complex was corrected for quenching of the γ radioactivity by the polyacrylamide gel using 125I standards.

Immunoprecipitation of Thrombin—The BCE cell incubation medium were collected and centrifuged (10,000 × g, 5 min) to remove cell debris. Protease inhibitors, PMSF and benzamidine, were added to give a final concentration of 1 mM and 10 mM, respectively. Rabbit anti-human thrombin IgG (5 µl) was then added to 0.5 ml of medium for 5 min at 37 °C. At the end of the incubation, 2.5 µl of nonimmune rabbit IgG was added as a carrier, followed by addition of 100 µl of goat anti-rabbit IgG. The suspension was then incubated at 4 °C. After 18 h, the media were centrifuged at 10,000 × g for 20 min, the supernatant was removed, and the pellet was washed twice with PBS. The radioactivity present in both the pellet and supernatant was counted in a Beckman 300 series γ counter.

RESULTS

Binding and Internalization of 125I-Thrombin to BCE cells—To determine the cellular disposition of bound thrombin, BCE cells were incubated with 125I-thrombin for various time periods, washed, and then treated with trypsin. The trypsinnized cells were centrifuged, and the radioactivity in both the pellet and supernatant measured (Fig. 1). The radioactivity in the supernatant presumably represents thrombin which was present on the cell surface and, therefore, accessible to removal by trypsin. On the other hand, the radioactivity in the pellet represents thrombin which was not removed by trypsin, probably because of internalization by the cell. As shown in Fig. 1, the maximal amount of thrombin trypsinnized from the cells occurred after they were incubated for 1 h with 125I-thrombin. This suggests that saturation of cell surface binding occurs by this time. At saturation, there was 8 ng of surface-bound thrombin/10⁶ cells or 1.38 × 10⁵ surface binding sites/cell. There was a slight increase in the number of surface binding sites between 1 and 6 h of incubation. Only a small amount (10%) of 125I-thrombin which was bound to the cells at 5 min was resistant to removal by trypsinization (Fig. 1B). However, by 1 h, 27% of the 125I-thrombin was resistant to removal. This suggests that the 125I-thrombin is internalized much more slowly than it is bound to the cell surface. The 125I-thrombin was internalized at a constant rate of about 3 ng/h/10⁶ cells. Each cell, therefore, internalized 0.52 × 10⁵ molecules of 125I-thrombin/h. The amount of internalized 125I-thrombin reached a plateau after 2 h. This plateau may represent the termination of the internalization process or a steady state between 2 processes, internalization and degradation of the internalized ligand. The ratio of internalized 125I-thrombin to the total cell-associated 125I-thrombin increased during the first 2 h of incubation with the ligand and was maximal at 38% (Fig. 1B). To distinguish between internalization of thrombin due to fluid phase versus adsorptive pinocytosis, the cells were incubated with either 125I-prothrombin or 125I-thrombin under identical conditions. The uptake of ligand was then measured as described in the above experiments. The ratio of uptake by the cells of enzyme to zymogen was 10:1 at 1 and 3 h. Thus, the small amount of 125I-prothrombin internalized represents either fluid phase pinocytosis or partial conversion of prothrombin to thrombin during the incubation. This is additional evidence that uptake of thrombin by the cells is due to specific binding to the cell surface and adsorptive pinocytosis.

Degradation of 125I-Thrombin by BCE Cells—In order to analyze the fates of 125I-thrombin within the cell, BCE cultures were exposed to chloroquine, an inhibitor of the lysosomal system, and the amount of internalized 125I-thrombin was measured (Fig. 2). Cells were preincubated in the presence or absence of chloroquine for 2 h and then exposed for various periods of time to 125I-thrombin. The cells were then washed, trypsinnized, and spun down, and the radioactivity in the pellet was measured. This radioactivity represented the amount of

![Fig. 1. Binding and internalization of 125I-thrombin by BCE cells.](image-url)
The presence increased by 5-fold the amount of \(^{125}\text{I}\)-thrombin that was inaccessible to trypsinization after activity measured. Aliquots of was a linear accumulation of \(^{125}\text{I}\)-thrombin at an approximate point. Preincubation of cells in the presence of chloroquine for 2 cells not treated with chloroquine after solubilized in 100 then exposed to \(^{125}\text{I}\)-thrombin. Cultures were then washed of \(^{125}\text{I}\)-thrombin removed by trypsin (data not shown). There process, BCE cells were exposed to \(^{125}\text{I}\)-thrombin for 30 min and an additional incubation for 1 h in fresh medium. After the end of this time period, the media were collected and processed for immunoprecipitation of \(^{125}\text{I}\)-thrombin as described under “Experimental Procedures.” The cell layers were washed 10 times with PBS containing 0.1% BSA, lysed with 0.1 N NaOH, and the radioactivity counted in a Beckman y counter.

![Diagram](image)

**Fig. 2.** Time course of \(^{125}\text{I}\)-thrombin internalization in the presence or absence of chloroquine. BCE cells were preincubated for 2 h in the presence (○) or absence (□) of chloroquine (50 μM) and then exposed to \(^{125}\text{I}\)-thrombin (0.25 μg/ml) for various periods of time. Cultures were then washed 10 times with cold (4 °C) PBS containing 0.1% BSA and trypsinized as described in Fig. 1. The pellets were solubilized in 100 μl of SDS sample buffer and the remaining radioactivity measured. Aliquots of 10 μl were subjected to gel electrophoresis, and the gel pattern is inserted in the figure. A, B, C, pellets of cells not treated with chloroquine after 1, 2, and 6 h, respectively; D, E, F, pellets of chloroquine-treated cells after 1, 2, and 6 h respectively. The arrow indicates the 77,000-dalton thrombin-binding site complex.

\(^{125}\text{I}\)-thrombin internalized and accumulated at each time point. Preincubation of cells in the presence of chloroquine increased by 5-fold the amount of \(^{125}\text{I}\)-thrombin that was inaccessible to trypsinization after 6 h of incubation with \(^{125}\text{I}\)-thrombin (Fig. 2). Chloroquine had no effect on the amount of \(^{125}\text{I}\)-thrombin removed by trypsin (data not shown). There was a linear accumulation of \(^{125}\text{I}\)-thrombin at an approximate rate of 4 ng/h/10^6 cells.

To show further that chloroquine inhibits the degradation process, BCE cells were exposed to \(^{125}\text{I}\)-thrombin for 30 min in the absence or presence of chloroquine followed by washing and an additional incubation for 1 h in fresh medium. After incubation, the radioactivity present both in the medium and cell layer was measured. The amount of radioactivity in the medium precipitable by an anti-thrombin IgG, followed by anti-IgG, was also measured. The results shown in Table I demonstrate that the untreated cells released 88% of the cell-associated radioactivity into the media after 1 h of incubation and that 94% of this represents material which was not precipitated by anti-thrombin IgG (approximately 98% of \(^{125}\text{I}\)-thrombin is immunoprecipitated under these conditions). In contrast, the chloroquine-treated cells released into the medium only 7% of the total cell-associated \(^{125}\text{I}\)-thrombin, of which 67% was immunoprecipitable. This may represent intact \(^{125}\text{I}\)-thrombin which dissociated from the cell surface. The nature of the \(^{125}\text{I}\)-thrombin accumulated within the BCE cells was studied by SDS-polyacrylamide gel electrophoresis. The autoradiograph in Fig. 2 shows the accumulation in the cells treated with chloroquine of a 77,000-dalton component that appears to be identical with the covalent \(^{125}\text{I}\)-thrombin-binding site complex observed previously (7). The data provided in Fig. 2 and Table I indicate that internalized \(^{125}\text{I}\)-thrombin is degraded by lysosomal enzymes. The rate of degradation of

| Incubation Time (Hours) | \[^{125}\text{I}\]-Thrombin Bound to the Cells after 30-min Incubation |
|------------------------|---------------------------------------------------------------|
| 0                      | [Graph showing binding data]                                  |

**Fig. 3.** Release of cell-associated \(^{125}\text{I}\)-thrombin from BCE cells and the nature of the released material. Confluent cultures were exposed to \(^{125}\text{I}\)-thrombin (0.25 μg/ml) for 30 min at 37 °C. Unbound radioactivity was then removed by washing the cells 10 times with cold (4 °C) PBS containing 0.1% BSA. Fresh medium (1 ml) was added, and the dishes were further incubated at 37 °C. At the time points indicated in the figure, the incubation media were collected and subjected to immunoprecipitation with rabbit anti-thrombin IgG followed by goat anti-rabbit IgG. The cell monolayers were washed with PBS containing 0.1% BSA, solubilized, and the radioactivity counted. ○—○, \(^{125}\text{I}\) associated with the BCE cells; □—□, immunoprecipitable \(^{125}\text{I}\) in media; □—□, nonimmunoprecipitable \(^{125}\text{I}\) in media.

**Table I**

Effect of chloroquine on degradation of \(^{125}\text{I}\)-thrombin by BCE cells

| None | Chloroquine-treated cells |
|------|---------------------------|
| 119,200 | 10,200 |
| 13,900 | 10,200 |
| 12.0 | 67 |
| 10,200 | 10,200 |
| 15,300 | 10,200 |
| 6 | 6 |

Confluent BCE cultures were washed and incubated in DMEM supplemented with 0.5% BSA and preincubated for 2 h in the presence or absence of chloroquine (50 μM). \(^{125}\text{I}\)-Thrombin (0.5 μg/ml) was then added to the dishes for 30 min at 37 °C, followed by 10 washes with fresh medium; the cells were then incubated with a fresh medium for an additional hour. At the end of this time period, the media were collected and processed for immunoprecipitation of \(^{125}\text{I}\)-thrombin as described under “Experimental Procedures.” The cell layers were washed 10 times with PBS containing 0.1% BSA, lysed with 0.1 N NaOH, and the radioactivity counted in a Beckman y counter.

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The binding site complex from the cells is shown in Fig. 4. Incubation was continued in fresh media for the indicated times and cell layers then lysed in SDS sample buffer. Aliquots of 50 μl were subjected to slab gel electrophoresis and autoradiography. The area on the gel corresponding to the 77,000 molecular weight complex was sliced, the radioactivity measured, and the amount of 125I-thrombin in the complex determined.

Fig. 5 (center). Up regulation of thrombin binding sites by preincubation of BCE cells in the presence of thrombin. Confluent BCE cultures were washed 3 times with DMEM containing 0.5% BSA and then preincubated for 24 h in the same media containing thrombin at the concentrations indicated in the figure. At the end of the preincubation period, the cultures were washed 3 times with DMEM containing 0.5% BSA and incubated again in the same medium for 1 h with 125I-thrombin (0.25 μg/ml). The cultures were washed and the radioactivity associated with the cells was measured.

FIG. 6 (right). Time course of the up regulation of thrombin binding sites induced by thrombin. Confluent BCE cultures were washed as described in Fig. 5 and preincubated with thrombin (2 μg/ml) for the time periods indicated in the figure at 37°C (●) or 4°C (○). At the end of the preincubation period, the cultures were washed and then incubated with 125I-thrombin and the amount bound determined as described in Fig. 5.

Table II

|                | 125I-Thrombin bound to the cells after 1-h incubation | 125I-Thrombin associated with the cells after subsequent 2-h incubation | % of total bound |
|----------------|------------------------------------------------------|---------------------------------------------------------------------|-----------------|
| Control        | 259,800                                              | 20,800                                                             | 8               |
| Preincubation with thrombin | 434,200                                      | 25,700                                                             | 6               |

125I-thrombin by the BCE cells is shown in Fig. 3. One hour after binding to the cells, most of the 125I-thrombin reappears in the media as nonimmunoprecipitable radioactivity. Degradation of the enzyme appears to account for the inability to immunoprecipitate it, since the radioactive material in the media was also nonprecipitable by trichloroacetic acid (data not shown). Disappearance of the 77,000-dalton 125I-thrombin-binding site complex from the cells is shown in Fig. 4. The data shown in Figs. 3 and 4 demonstrate that the rate of disappearance of the complex from within the cells parallels the appearance of degradation products in the incubation medium.

Effect of Preincubation of BCE Cells with Thrombin on the Appearance of Thrombin Binding Sites—To determine whether prolonged exposure of BCE cells to thrombin regulates the number of binding sites, cells were preincubated with unlabeled thrombin for 24 h at 37°C. The cells were then washed, and the amount of 125I-thrombin bound after a 1-h incubation determined. As shown in Fig. 5, there was a marked increase in 125I-thrombin binding to those cells pre-exposed to unlabeled thrombin. The extent of the increase in binding of 125I-thrombin was dependent on the concentration of thrombin to which the cells were exposed during the preincubation period. Increased 125I-thrombin binding was seen when the cells were preincubated with concentrations of thrombin as low as 0.05 μg/ml. Maximal increase in thrombin binding was seen when the cells were preincubated with 1.0 μg/ml of thrombin.

As shown in Fig. 6, preincubation of the cells with thrombin for at least 2 h was necessary before an increase in subsequent 125I-thrombin binding was seen. By 8 h of preincubation, there was a marked increase in binding of 125I-thrombin to the cells. Maximal stimulation of an increase in binding sites was seen after 24 h of preincubation of thrombin with the cells. Induction of additional binding sites was temperature-dependent. Preincubation of the cells with thrombin at 4°C resulted in no increase in subsequent binding of 125I-thrombin.

As shown in Table II, the 125I-thrombin which bound to BCE cells pretreated or untreated with thrombin was degraded to a similar extent. This suggests that the additional 125I-thrombin bound to the pretreated cells was also internalized.

It has been previously observed that normal human fibroblasts release binding sites for thrombin into the incubation medium. The released binding sites react with thrombin to form an apparently covalent complex which then binds rapidly to the cell surface. The same phenomenon has been observed with BCE cells (data not shown). To determine the effect of pretreating BCE cells with thrombin on the number of cellular binding sites and those released into the culture media, the cells were preincubated with thrombin for 24 h, washed, and the incubation continued in fresh medium. At the times indicated in Fig. 7, the medium was either changed...
Internalization, Degradation, and Up Regulation of Thrombin Binding Sites

Fig. 7. Increase in the release of thrombin binding sites into the incubation medium induced by preincubation of BCE cells with thrombin. BCE cultures were incubated in the presence (○) (B, C, and D) or absence (□) (E, F, and G) of thrombin (2 µg/ml) for 24 h, as described in Fig. 5. The cultures were washed 5 times with DMEM containing 0.5% BSA and exposed to fresh media. At the time points indicated in the figure, the incubation medium was either changed (---) or retained (-----) and 125I-thrombin added. Incubation was terminated after 30 min, and the cultures were washed, lysed in SDS sample buffer, and 125I-thrombin binding measured. In addition, aliquots (10 µl) of the solubilized samples from incubations without a prior change in media were further analyzed by slab gel electrophoresis and autoradiography. A, 125I-thrombin; B and E 1-h incubation; C and F, 3-h incubation; D and G, 8-h incubation. The arrow indicates the 77,000-dalton thrombin-binding site complex and the arrowhead indicates the free thrombin.

DISCUSSION

In this study, we followed the fate of the cell surface-bound 77,000-dalton thrombin-binding site complex. The complexes were found to be extensively internalized; within 1 h, as much as 3 to 4 ng of thrombin was internalized/10⁶ cells. The internalization of 125I-thrombin-binding site complexes observed in the present study was much faster than the observed rate of thrombin internalization by chick embryo fibroblasts (15). In the latter case, the rate of internalization was so slow and the amount internalized so small that it was not clear whether thrombin was transported entirely by fluid phase pinocytosis or to some extent also by site-specific uptake. In the case of the thrombin-binding site complexes internalized by BCE cells, the uptake was clearly site-specific, and addition of unlabeled thrombin successfully abolished the uptake of the iodinated complexes. Furthermore, BCE cells internalized 10-fold more 125I-thrombin than 125I-prothrombin, an observation which indicates the specificity of the thrombin internalization process and also excludes the possibility that thrombin internalization proceeds by fluid phase pinocytosis. Rather, it is a specific binding site-mediated process.

In contrast to a previous report (15) that the internalized 125I-thrombin accumulated within fibroblasts as intact thrombin and 2 large fragments of 19,500 and 22,000 daltons, the 125I-thrombin-binding site complex internalized by BCE cells apparently was rapidly degraded to a very low molecular weight material. This was reflected by the fact that the radioactive material released into the media could not be precipitated by antithrombin IgG or trichloroacetic acid. Internalization and degradation of thrombin are similar to the processes observed with the mitogen, epidermal growth factor (16-18), low density lipoprotein (19-21), and α, macro-

again or retained, and 125I-thrombin was added. The amount bound after a 30-min incubation was then determined. Although there was an increase in 125I-thrombin bound to cells preincubated with thrombin whether or not the medium was changed prior to addition of the 125I-thrombin, much more thrombin bound to those cells maintained in the same media (Fig. 7). This indicates that thrombin stimulates an increase in the number of cell-associated binding sites (approximately 2-fold) and, to a much greater extent, an increase in the rate of release of binding sites into the media (approximately 6-fold). The effect of thrombin pretreatment persisted for several hours after removal of the thrombin, as indicated by the progressive increase in the number of binding sites released into the media for up to 8 h after removal of thrombin. That the increase in thrombin binding was due to an increase in the formation of the 77,000-dalton thrombin-binding site complex can be seen in the autoradiogram in Fig. 7.

In order to study whether thrombin induces synthesis and subsequent release of thrombin binding sites or only stimulates the release of pre-existing intracellular binding sites, cells were preincubated in the presence or absence of 2 µg/ml of thrombin for 24 h. Cells were scraped with a rubber policeman in 1 ml of DMEM, homogenized vigorously with a tight glass-glass homogenizer, and incubated for 1 h in the presence of 125I-thrombin (0.25 µg/ml). Samples were subjected to slab gel electrophoresis and the amount of 125I-thrombin-binding site complex formed was determined. Cells preincubated in the presence of thrombin showed an 87% increase in the amount of 125I-thrombin-binding site complexes in comparison to the amount observed in cells not preincubated with thrombin. Thus, the 6-fold increase in binding sites in the medium must have resulted from an increase in the synthesis of binding sites.
globulin (22, 23). This may indicate that 125I-thrombin is internalized by a similar mechanism, although the internalization seems to be faster with the other ligands. Others have shown that epidermal growth factor (24), low density lipoprotein (25), and α₂-macroglobulin (26) receptor complexes cluster in coated pits where they are internalized. The internalization of 125I-thrombin may, therefore, proceed through receptor clustering into coated pit regions which seem to represent a general pathway for receptor-mediated endocytosis (27).

The phenomenon of down regulation of receptor sites after prolonged exposure to a ligand has been previously observed with epidermal growth factor (16) and low density lipoprotein (28). In surprising contrast, instead of down regulation of receptor sites, we observed an up regulation phenomenon after thrombin preincubation. Preincubation of BCE cells with thrombin for 24 h caused a 6-fold increase in the amount of thrombin binding sites released into the incubation medium and thereby further increased the ability of the cells to bind, internalize, and degrade thrombin. The up regulation of thrombin binding sites was dependent on the thrombin concentration during the preincubation period, and maximal effect was observed at the same concentration as observed for saturation of 125I-thrombin binding to the cell surface (7). The process was time-dependent, and maximal release of binding sites was observed after a 24-h preincubation of cells with thrombin. Failure of the cultures to increase the number of thrombin binding sites when the preincubation with thrombin was done at 4 °C could be explained by one or both of the following possibilities: 1) The up regulation process is dependent on the apparent covalent binding of thrombin, which is inhibited at 4 °C (7); 2) The up regulation is dependent on a metabolic process which is inhibited at 4 °C. The observation that the preincubation with thrombin did not deplete the amount of cell-associated thrombin binding sites, in spite of the fact that it induced a marked release into the incubation medium, suggests that thrombin, rather than stimulating the release of pre-existing intracellular binding sites, induces the synthesis of thrombin binding sites.

It is possible that binding to these sites may mediate the previously observed cellular response to thrombin (29-33). However, the up regulation of binding sites by thrombin described in this manuscript may reflect a new role for the apparent covalent binding of thrombin to BCE cells. Both the binding site on the cell surface and those released into the incubation medium may serve as a mechanism by which thrombin is bound, inactivated, and eventually degraded. A continuous exposure of cells under pathological conditions, such as trauma to the circulatory system, to free active thrombin may induce an increase in thrombin binding sites which are released by the cells, bind, and inactivate the thrombin. Internalization and degradation of thrombin within the lysosomal system would then follow. Therefore, the processes of internalization, degradation, and up regulation may represent a mechanism for maintaining a low extracellular level of thrombin.

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