Covalent Binding of Thrombin to Specific Sites on Corneal Endothelial Cells†

J. D. Isaacs,‡ N. Savion, D. Gospodarowicz, J. W. Fenton II, and M. A. Shuman*

ABSTRACT: Binding of ^{125}I-labeled human α-thrombin to endothelial cells derived from bovine corneas was studied in tissue culture. Specific and saturable binding to the cell surface occurred at 37 °C but to a much smaller extent at 4 °C. Binding of [^{125}I]thrombin to a specific site on these cells with formation of a 77 000-dalton complex was demonstrated by $NaDodSO_4$ (sodium dodecyl sulfate)–polyacrylamide gel electrophoresis. Binding of [^{125}I]thrombin was blocked by a 100-fold excess of unlabeled α-thrombin and by the thrombin inhibitor, hirudin. There are ~100 000 of these thrombin binding sites on the cell surface. Formation of the complex could be detected as early as 15 s, increased rapidly over the next 20–30 min, and then continued at a slower rate for the next 2.5 h. The catalytically active site of the enzyme was required for formation of the $NaDodSO_4$-stable complex as shown by the inability of diisopropyl phosphorofluoride inactivated thrombin to form stable complexes with these cells. The complex was dissociated in $NaDodSO_4$ with 1.0 M hydroxylamine, suggesting an acyl linkage of the enzyme to the cellular binding site. The thrombin–endothelial cell complex was distinct from the thrombin–antithrombin III complex ($M_r \approx 90\,000$) on gel electrophoresis, and its formation was not enhanced by heparin. Additional thrombin–cell complexes ($M_r < 77\,000$) were also identified; however, they represent a small fraction of the total thrombin bound to the cells. These observations demonstrate that α-thrombin is capable of reacting specifically with corneal endothelial cells to form a $NaDodSO_4$-stable complex which requires the catalytically active enzyme.

Thrombin, a serine proteinase, is formed when the zymogen prothrombin is activated during blood clotting. Although it exhibits a limited preferential specificity for arginyl bonds (Elmore, 1973), thrombin has diverse biological effects. After initiation of coagulation, thrombin activates factors V, VIII, and XIII and converts soluble fibrinogen to a fibrin clot (Ware et al., 1947; Therriault et al., 1957; Buluk et al., 1961; Morawitz, 1905). With the exception of factor VIII, activation of these glycoproteins has been shown to be the result of limited proteolysis (Buluk et al., 1961; Nesheim et al., 1979; Esmon, 1979; Bettelheim & Bailey, 1952; Lorand, 1952).

Thrombin also has effects on several different types of cells. These include stimulation of platelet secretion and aggregation (Grette, 1962), mitogenesis of fibroblasts (Chen & Buchanan, 1975) and B lymphocytes (Chen et al., 1976), and stimulation of prostacyclin (PGI_2) formation and release by endothelial cells (Weksler et al., 1978). Thrombin causes a marked reduction in the capacity of endothelial cells to activate plasminogen and hence fibrinolysis (Loskutoff, 1979; Fehrenbacher et al., 1979). The mechanism by which thrombin initiates these cellular effects is unknown.

Reversible and specific binding of thrombin to platelets (Tollefsen et al., 1974; Ganguly, 1974), vascular endothelial cells (Awbrey et al., 1979), and fibroblasts (Carney & Cuningham, 1978) has previously been demonstrated. The binding of thrombin to platelets has been proposed as the first step in the induction of platelet secretion. Although both native thrombin and thrombin inactivated at its serine active site bind identically to platelets (Tollefsen et al., 1974) and endothelial cells (Awbrey et al., 1979), only the active enzyme affects cell function (Davey & Lüscher, 1967). This suggests that there may be at least two cellular reactions in which thrombin is directly involved: the first, binding to the cell surface, and the second, proteolysis, which requires the active site. When platelets are treated with thrombin, a single membrane glycoprotein disappears. This coincides with the appearance of a soluble glycoprotein in the supernatant. (Phillips & Agin, 1977; Mosher et al., 1979). However, it is not apparent at this time whether cleavage of this glycoprotein is necessary for the initiation of platelet secretion and aggregation.

Recently, Cunningham and co-workers have shown that a photoreactive derivative of α-[^{125}I]thrombin could be cross-linked to a specific site on chick and mouse embryo cells by photolysis (Glenn & Cunningham, 1979; Carney et al., 1979). Additionally, they have presented evidence that thrombin binds spontaneously in an apparent covalent fashion to human foreskin fibroblasts with the formation of a complex similar in molecular weight to the photoaffinity-labeled thrombin–cell complex on embryo cells (Baker et al., 1979). On the premise that thrombin stimulation of cellular events involves a common mechanism among all cell types, we undertook experiments to determine if a similar complex was formed between thrombin and endothelial cells derived from bovine corneas (BCE).[1] In this paper we present evidence for a complex, which resists disruption by sodium dodecyl sulfate ($NaDodSO_4$) and polyacrylamide gel electrophoresis, that is formed between endothelial cells and catalytically active α-[^{125}I]-thrombin.

Materials and Methods

Materials. Crystalline bovine serum albumen, fraction V (BSA), lactoperoxidase, glucose oxidase, type 5, and hirudin

† From the Division of Hematology–Oncology, Department of Medicine and Cancer Research Instijtute, University of California, San Francisco, California 94143 (J.D.I., N.S., D.G., and M.A.S.), and the New York State Department of Health, Albany, New York 12201 (J.F.). *Received March 9, 1980.* This research was supported by Grants HL 21403, EY 02196, and HL 13160 from the National Institutes of Health. A portion of this work was presented at the meeting of the American Society of Hematology, December 1979 (Isaacs et al., 1979).

‡ Fellow, American Heart Association, Northern California Affiliate, Grant 784352-69853.

* Address correspondence to this author at the University of California Medical Center, 1282-M, San Francisco, CA 94143.

[1] Abbreviations used: BCE, bovine corneal endothelial; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumen; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; DIP-thrombin, diisopropyl phosphoryl thrombin; PBS, phosphate-buffered saline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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were obtained from Sigma Chemical Co. (St. Louis, MO); Dulbecco's modified Eagle's medium (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 (Oxnard, CA). Carrier-free sodium [^{125}I]-iodide was purchased from Amersham Radiochemicals (Arlington, IL). A molecular weight standards kit was obtained from Bio-Rad Laboratories (Richmond, CA).

Preparation of Thrombin, Fibroblast Growth Factor (FGF), and Antithrombin III. Human α-thrombin was prepared as previously described (Fenton et al., 1977). The specific clotting activities ranged from 2400–4000 units/mg. Fibroblast growth factor (FGF) was purified from bovine brain as previously described (Gospodarowicz et al., 1978a). Antithrombin III was purified from human plasma by a modification (Shuman & Majerus, 1976) of the method described by Yin et al. (1975).

Corneal Endothelial Cell Preparation. Endothelial cells derived from bovine corneas were isolated as previously described (Gospodarowicz et al., 1977). Cells in their second to fifth passage were grown to confluence at 37 °C in 10% CO_2 in DMEM containing 5% calf serum and 10% fetal calf serum. FGF (50 ng/mL) was added every other day to the cultures.

Iodination of Thrombin. Human α-thrombin (150 units) was radioiodinated at room temperature with 0.05 unit of lactoperoxidase, 0.01 unit of glucose oxidase, 0.2% β-D-glucose, 4 m Ci of sodium [^{125}I]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. [^{125}I]Thrombin was separated from free sodium [^{125}I]iodide by gel filtration.

Diisopropyl phosphoryl (DIP) thrombin and DIP-[^{125}I]-thrombin were prepared as previously described (Shuman et al., 1979). DIP-[^{125}I]thrombin was also prepared by radioiodination of freshly prepared DIP-thrombin using the method described above. Radiolabeled and DIP-thrombin were used within 4 h of preparation.

Binding of [^{125}I]Thrombin. Confluent monolayers containing $\sim 0.85 \times 10^6$ cells were grown on gelatinized 35-mm culture dishes (Gospodarowicz et al., 1977). The cells were washed 3 times with DMEM supplemented with 0.5% BSA. For initiation of binding, the radiolabeled ligand was added to the cells in 1.0 mL of serum-free medium. After incubation at 37 °C, the cell monolayers were washed rapidly 10 times with 1–2 mL of cold phosphate-buffered saline (PBS) with 0.1% BSA. The washed monolayers were lysed in 0.1 mL of buffer containing 15% glycerol, 2% sodium dodecyl sulfate ($NaDodSO_4$), 75 mM Tris-HCl (pH 6.8), 2 mM phenylmethanesulfonyl fluoride, and 2 mM EDTA. *N*-Ethylmaleimide and iodoacetic acid, 1.0 mM, were added to block free sulfhydryl groups (henceforth referred to as $NaDodSO_4$ lysis buffer) (Hynes & Destree, 1977). 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. In experiments to determine the effect of reduction of sulfhydryl bonds, dithiothreitol (DTT) was added to solubilized samples to a final concentration of 0.1 M.

Polyacrylamide Gel Electrophoresis. Aliquots (0.033 mL) containing one-third of the total solubilized cellular material were applied to $NaDodSO_4$–10% polyacrylamide slab gels with 3% stacking gel, as described by Laemmli (1970). After electrophoresis at 20 mA, the slab gel was stained with 0.1% Coomassie blue in 50% trichloroacetic acid and subsequently destained overnight. The gels were dried and subjected to autoradiography on Kodak NS-2T X-ray film for 48 h. The areas of the gel corresponding to the band of radioactivity on the autoradiogram were sliced and counted in a Beckman γ-300 spectrometer. Calculation of thrombin bound in the 77 000-dalton complex was corrected for quenching of the γ radioactivity by the polyacrylamide gel by using ^{125}I standards.

Hydroxylamine Incubation with [^{125}I]Thrombin–Cell Complex and Antithrombin III–[^{125}I]Thrombin Complex. Antithrombin III was incubated with [^{125}I]thrombin at a molar ratio of 9.5:1 for 15 min at room temperature. Corneal cells were incubated with 0.25 μg/mL [^{125}I]thrombin for 30 min at 37 °C. Both radiolabeled complexes were solubilized in 1.0 mL of $NaDodSO_4$ lysis buffer. The samples were dialyzed against 0.05 M NaCl containing 0.1% $NaDodSO_4$ in a ratio of 1:2000. Following dialysis, aliquots of material were heated at 100 °C for 5 min and then incubated with equal volumes of either 2.0 M hydroxylamine (pH 7.4) or 2.0 M NaCl at 37 °C for 6 h. The incubation mixture was then further dialyzed for 3 h against 1:100 ratio of dialysis buffer (Owen, 1975). Samples of each specimen were electrophoresed and autoradiograms were made.

Isoelectric Focusing of [^{125}I]Thrombin and [^{125}I]-Thrombin–Cell Complex. Nonequilibrium pH gradient electrophoresis with pH 3.5–10.0 ampholines was used to measure the isoelectric points (O'Farell et al., 1977). The isoelectric focusing gels were polymerized in glass tubes (120 × 2.0 mm i.d.), and the samples were run at 450 V for 4 h. The gels were then cut into 3-mm slices, and ^{125}I was counted in a Beckman 310 γ counter. The pH gradient was measured on 3-mm slices of the gel as described (O'Farrel et al., 1977).

Results

Binding of [^{125}I]Thrombin to Corneal Endothelial Cells. Confluent BCE cells were incubated with [^{125}I]thrombin at 4 or 37 °C, and at varying intervals incubation was terminated by washing the cells. The cells were then solubilized with $NaDodSO_4$ lysis buffer (see Materials and Methods), and the amount of [^{125}I]thrombin bound was determined by γ scintillation counting. As shown in Figure 1, binding of [^{125}I]-thrombin could be detected after the shortest time of incubation, 15 s. Thrombin binding at 37 °C was comparatively rapid for the initial 20–30 min; subsequently, binding continued at a slower rate for the next 2.5 h.

The rapidity of thrombin binding suggests that this occured at the cell surface. This does not appear to be the explanation for the additional binding which occured after 30 min of incubation. Further study of the fate of the [^{125}I]thrombin revealed that 95% of thrombin bound to the cells after 5 min of incubation could be removed by trypsinization, 82% after 30 min incubation, and 70% after 1 h of incubation.[2] Thus, thrombin appears to be slowly internalized by the cells. This would account for the slow increase in thrombin associated with the BCE cells after 20–30 min of incubation.

When the cells were incubated with [^{125}I]thrombin at 4 °C, a small amount of thrombin binding occurred within the first few minutes, but no further binding was detected even after 3 h of incubation (Figure 1). All binding experiments were subsequently performed at 37 °C. These studies were also conducted in buffer containing fat-free albumen, since it has been shown that albumen but not fat-free albumen causes a

[2] N. Savion, J. D. Isaacs, D. Gospodarowicz, and M. A. Shuman, unpublished experiments.

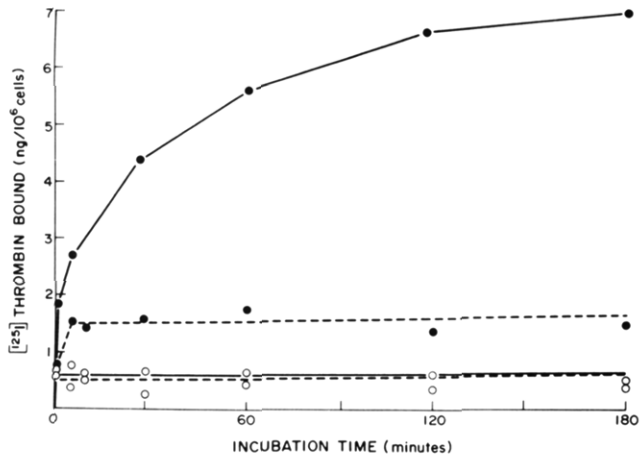

FIGURE 1: Effect of temperature on binding of [^{125}I]thrombin to BCE cells. Confluent BCE cells, 8.5×10^5, were incubated with 0.25 μg/mL [^{125}I]thrombin at 4 (---) and 37 °C (—). At the indicated intervals, the binding assay was terminated by washing and solubilizing the cells in $NaDodSO_4$ lysis buffer. The amount of [^{125}I]thrombin bound to the cells was determined in a γ scintillation counter. Each point is a mean of duplicate experiments. (●) Binding of [^{125}I]thrombin to BCE cells. (○) Binding in the presence of excess unlabeled thrombin (25 μg/mL).

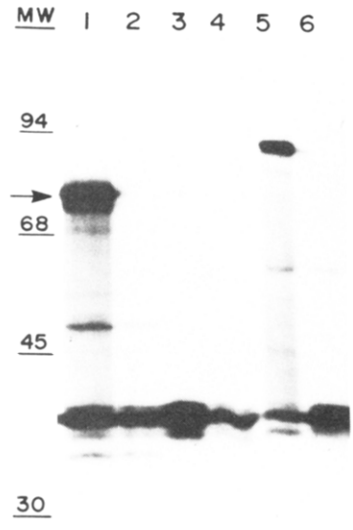

FIGURE 2: Binding of [^{125}I]thrombin to a specific site on confluent BCE cells. [^{125}I]Thrombin was incubated with 8.5×10^5 BCE cells for 30 min at 37 °C. After washing and solubilization, the cells were electrophoresed in a $NaDodSO_4$–10% polyacrylamide gel. An autoradiogram was then made of the gel. (Lane 1) [^{125}I]Thrombin and BCE cells. (Lane 2) [^{125}I]Thrombin, BCE cells, and a 100-fold excess of unlabeled thrombin. (Lane 3) DIP-[^{125}I]thrombin and BCE cells. (Lane 4) DIP-[^{125}I]thrombin, BCE cells, and a 100-fold excess of unlabeled thrombin. (Lane 5) [^{125}I]Thrombin and antithrombin III. (Lane 6) [^{125}I]Thrombin, antithrombin III, and a 100-fold excess of thrombin. The arrow indicates the [^{125}I]thrombin–cell complex, M_r < 77 000.

delay in the rate of thrombin binding to platelets (Martin et al., 1976). No difference in thrombin binding was observed between the two albumen-containing buffers (data not shown).

Specificity and Kinetics of Thrombin Binding to BCE Cells. We next investigated the nature of thrombin binding to these cells. After incubation with [^{125}I]thrombin, the cells were washed, solubilized, and electrophoresed in $NaDodSO_4$–10% polyacrylamide gels. As shown in the autoradiogram from one such gel (Figure 2), a 77 000-dalton complex between [^{125}I]thrombin and BCE cells was formed. Formation of the

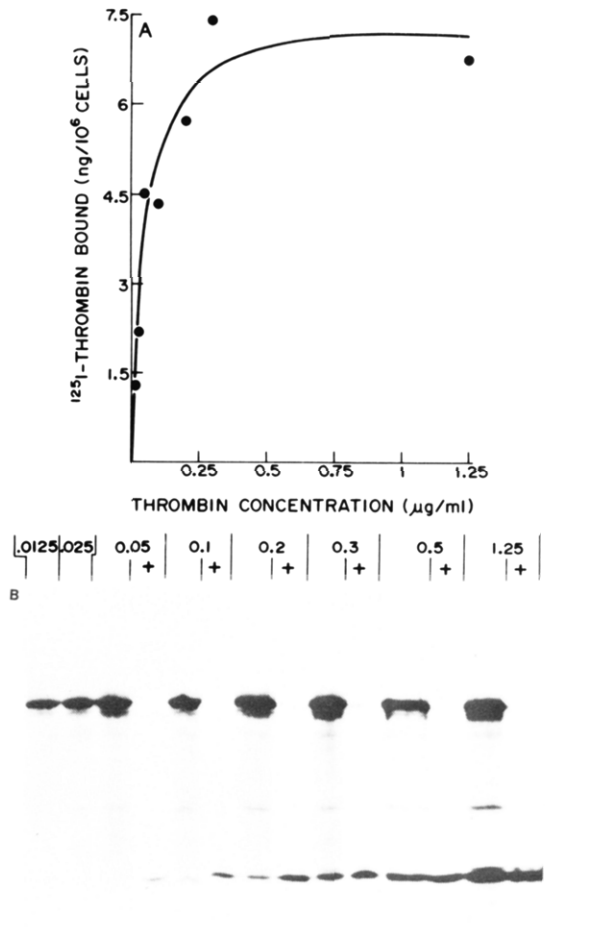

FIGURE 3: [^{125}I]Thrombin binding to BCE cells as a function of thrombin concentration. BCE cells were incubated with [^{125}I]thrombin for 30 min at 37 °C, and the cultures were washed, then solubilized in $NaDodSO_4$, and subjected to gel electrophoresis; the amount of thrombin bound in the 77 000-dalton complex was determined as described under Materials and Methods. (A) (●) [^{125}I]Thrombin and BCE cells. (B) Autoradiogram of gel from which data in (A) were derived. (+) indicates binding in the presence of a 100-fold excess of unlabeled thrombin.

radiolabeled complex was completely blocked by the addition of a 100-fold excess of unlabeled thrombin (Figure 2). This suggests specific binding of thrombin to this endothelial cell site. With the assumption of an M_r of 36 500 for α-thrombin (Fenton et al., 1977), the M_r of the BCE binding site is ~40 500.

The effects of increasing the concentration of [^{125}I]thrombin on formation of the 77 000-dalton complex is shown in Figure 3. Thrombin binding was terminated after 30 min of incubation with the cells, when surface binding is near maximal and when there is little internalization of thrombin (~18%). The cells were solubilized, electrophoresed, and the amount of radioactivity in the 77 000-dalton complex was determined. Binding of [^{125}I]thrombin was maximal at a concentration 0.5 μg/mL. There are ~100 000 molecules of thrombin bound per cell. As shown in the autoradiogram in Figure 3B, virtually all of the specific binding is covalent at 30 min. The free [^{125}I]thrombin appears to be bound nonspecifically since it is not blocked by excess unlabeled thrombin.

Characterization of Thrombin–Cell Complex. Since the thrombin–BCE cell complex resists disruption by heating at 100 °C in $NaDodSO_4$, reduction with diothiothreitol, and

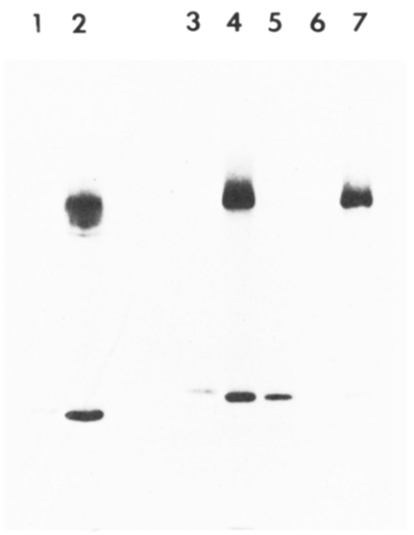

FIGURE 4: Characterization of the [^{125}I]thrombin–cell complex. Autoradiogram of polyacrylamide gel. The [^{125}I]thrombin–BCE cell complex or [^{125}I]thrombin was treated as follows: (lane 1) [^{125}I]-thrombin and 0.1 M DTT; (lane 2) 0.25 μg/mL [^{125}I]thrombin, cells, and 0.1 M DTT; (lane 3) [^{125}I]thrombin; (lane 4) 0.25 μg/mL [^{125}I]thrombin and cells; (lane 5) 0.25 μg/mL [^{125}I]thrombin, cells, and 25 μg/mL thrombin; (lane 6) 0.25 μg/mL [^{125}I]thrombin, cells, and hirudin (10 units/mL); (lane 7) 0.25 μg/mL [^{125}I]thrombin, cells, and 25 μg/mL DIP-thrombin.

$NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4), an apparent covalent, nondisulfide bond is formed between thrombin and the binding site. This complex did not form with [^{125}I]thrombin alone, which was treated identically with the [^{125}I]thrombin–cell incubation mixture (Figure 4), demonstrating that the 77 000-dalton complex was not a dimer of thrombin.

Additional [^{125}I]thrombin–cell complexes, M_r < 77 000, were also observed (Figure 2). They represented a small fraction of the total amount of [^{125}I]thrombin bound to these cells and may represent degradation products of the 77 000-dalton complex or thrombin bound to different sites on these cells. Formation of these complexes was also blocked by an excess of unlabeled thrombin.

When DIP-thrombin was added in a 100-fold excess prior to incubation of [^{125}I]thrombin with endothelial cells, 40% inhibition of binding was detected. This could be explained by a 0.4% contamination of the DIP-thrombin with native thrombin. Addition of a 5–50-fold excess of DIP-thrombin resulted in proportionately less inhibition (Figure 4). Although specific binding of DIP-[^{125}I]thrombin to BCE cells could be demonstrated (data not shown), formation of the 77 000-dalton complex was not detected by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2), indicating that the active-site serine of thrombin is necessary for apparent covalent binding to the cell.

Thrombin but not DIP-thrombin binds covalently to the protease inhibitor antithrombin III (Rosenberg & Damus, 1973). One possibility, therefore, would be that the BCE cell binding site is cellular-associated antithrombin III. As shown in Figure 2, the antithrombin III–[^{125}I]thrombin complex migrates more slowly (M_r < 90 000) when electrophoresed on the same slab gel as the thrombin–cell complex.

The glycosaminoglycuronan, heparin, is known to greatly accelerate binding of thrombin to antithrombin III (Rosenberg & Damus, 1973). Addition of heparin to BCE cells immediately prior to the addition of [^{125}I]thrombin resulted in no increase in the amount of [^{125}I]thrombin bound to the cells after a 5-min incubation (a time at which there is less than half-maximal thrombin binding) compared to an equivalent incubation without heparin (data not shown). This is additional indirect evidence that the 77 000-dalton complex is not the result of thrombin binding to antithrombin III associated with BCE cells.

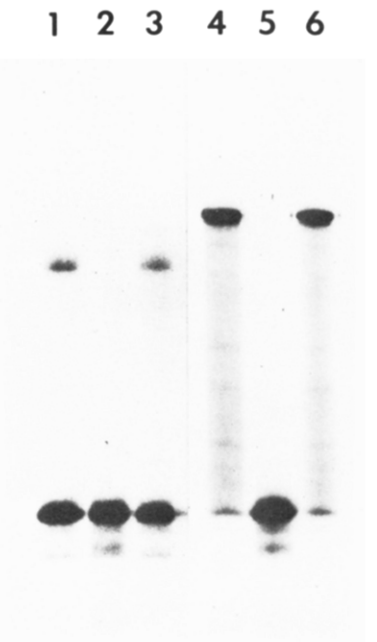

FIGURE 5: Effect of hydroxylamine on [^{125}I]thrombin–antithrombin III and [^{125}I]thrombin–BCE cell complexes. [^{125}I]Thrombin was incubated with either antithrombin III or BCE cells as described in Figure 2. The samples were solubilized (see Materials and Methods) and dialyzed against 0.1% $NaDodSO_4$ and then hydroxylamine or sodium chloride, final concentration 1.0 M, or isotonic buffer was added for 6 h at 37 °C. The samples were again dialyzed against 0.1% $NaDodSO_4$ and then electrophoresed as described in Figure 2. (Lanes 1–3) [^{125}I]Thrombin and BCE cells treated with (1) isotonic buffer, (2) hydroxylamine, and (3) hypertonic sodium chloride. (Lanes 4–6) [^{125}I]Thrombin and antithrombin III treated with (4) isotonic buffer, (5) hydroxylamine, and (6) hypertonic sodium chloride.

Further evidence for the specific binding of thrombin in this reaction was provided by experiments using the antithrombin leach protein, hirudin. Hirudin blocks platelet secretion induced by thrombin (Detwiler & Feinman, 1973). Hirudin has been used to measure specific binding of [^{125}I]thrombin to platelet membranes (Ganguly & Sonnichsen, 1976; Tam & Detwiler, 1978) as well as intact platelets (Tam et al., 1979). When hirudin was incubated with BCE cells prior to the addition of [^{125}I]thrombin, binding was completely blocked (Figure 4, lane 6). This suggests that [^{125}I]thrombin, rather than a trace radiolabeled contaminant, binds to the endothelial cell.

Effects of Hydroxylamine on [^{125}I]Thrombin–Cell Complex. Previously it was shown that hydroxylamine dissociates the thrombin–antithrombin III complex, presumably by disruption of a carboxylic ester bond (Owen, 1975). To determine whether the 77 000-dalton complex is the result of a similar bond, we solubilized the cells after incubation with [^{125}I]-thrombin and treated them with hydroxylamine at a final concentration of 1.0 M for 6 h at 37 °C. As shown in Figure 5, both the [^{125}I]thrombin–endothelial cell complex and the antithrombin III–[^{125}I]thrombin complex were completely dissociated when each was incubated with hydroxylamine. This was not simply a result of an increase in ionic strength since 1.0 M NaCl had no effect on either complex. These results suggest that thrombin binds to a specific site on the cell by an ester bond similar to its binding to antithrombin III. It may be that the 77 000-dalton complex represents an

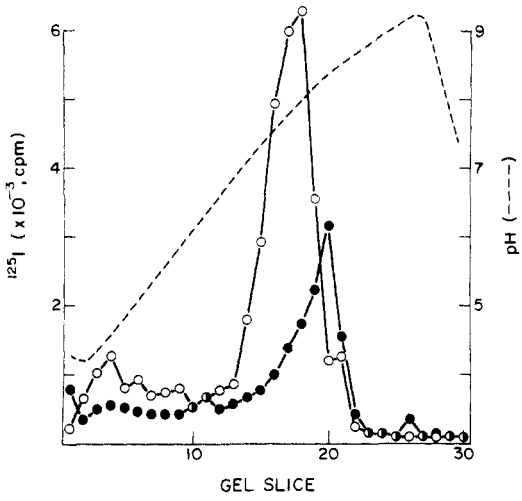

FIGURE 6: Isoelectric focusing of [^{125}I]thrombin and [^{125}I]-thrombin–BCE cell complex. BCE cells were incubated with [^{125}I]thrombin as described in Figure 2. The cells were washed, solubilized in buffer containing 9.5 M urea, 2% (w/v) Nonidet NP40, 2.0% Ampholyne (pH range 3.5–10), and 5% β-mercaptoethanol, and applied to gels, 60 000 cpm/gel (●). For comparison, 100 000 cpm/gel [^{125}I]thrombin was also electrophoresed (○). The pH gradient was measured in individual gel slices (---).

enzyme–substrate intermediate that is seen on electrophoresis because of disruption of the charge relay system by $NaDodSO_4$ denaturation.

Isoelectric Focusing of [^{125}I]Thrombin–BCE Cell Complex. For further characterization of the thrombin–cell complex, the cells were washed after incubation with [^{125}I]thrombin, solubilized, and then subjected to isoelectric focusing in a pH 3.5–10 ampholine gradient. One major peak, p*I* 8.4, was observed (Figure 6). Electrophoresis of [^{125}I]thrombin alone revealed a p*I* of 8.0, indicating that the cellular binding site has a more basic p*I* than that of [^{125}I]thrombin. The p*I* we obtained for thrombin is more basic than previously reported (Fenton et al., 1977). This may be due to iodination.

Discussion

We have demonstrated that thrombin binds specifically to distinct BCE cellular sites. The rapidity with which it takes place suggests that binding occurs at the cell surface. While cellular internalization is markedly inhibited at 4 °C, this does not account for the much greater thrombin binding during the first 30–60 min at 37 °C since most of the bound enzyme could be removed from intact cells by trypsinization. Furthermore, we have discovered that thrombin binding to BCE cellular sites which are present in the incubation media is also markedly reduced at 4 °C, indicating that binding itself is temperature dependent.[2] Also, internalization of thrombin by chicken embryo fibroblasts takes several hours (Zetter et al., 1977).

There are at least two differences between the binding of [^{125}I]thrombin to BCE cells which we have observed and binding of thrombin to umbilical vein endothelial cells reported by Awbrey et al. (1979). The former is not dissociated by $NaDodSO_4$–polyacrylamide gel electrophoresis and does not occur with DIP-thrombin. There are several possible explanations for these differences. One possibility is that there are two distinct cellular binding sites, one to which both thrombin and DIP-thrombin bind reversibly and a second site to which only catalytically active thrombin binds. A second possibility is that both thrombin and DIP-thrombin bind to the same molecule, but an intact catalytic apparatus is necessary to form $NaDodSO_4$-stable complexes with the binding sites. Our observation that DIP-thrombin did not form $NaDodSO_4$ stable complexes nor block the apparent covalent binding of native thrombin is consistent with the hypothesis that there are BCE cellular sites to which only native thrombin binds. The number of thrombin molecules bound covalently and the relative amounts of covalent and noncovalent binding appears to vary considerably depending on the type of tissue from which the cells are derived. Thus there are many more apparent covalent binding sites associated with BCE cells than with bovine aortic endothelial cells,[3] and there are fewer binding sites associated with platelets than either type of endothelial cell.[4] Only a small amount (10–15%) of thrombin bound to fibroblasts forms an $NaDodSO_4$-stable complex (Carney et al., 1979). However, by cross-linking a photoactivated [^{125}I]thrombin conjugate to fibroblasts, these investigators also showed that thrombin probably binds to a larger number of these same sites noncovalently, since radiolabeled complexes with the same molecular weight as that of the spontaneously formed thrombin–cell complex (M_r = 80 500) were formed. If thrombin binds both covalently and noncovalently to the same site, it will be important to determine whether either type of binding results in cellular stimulation.

In studies of the interaction between purified thrombin and antithrombin III, proteolysis of the heavy chain of thrombin in several places resulting in smaller thrombin–inhibitor complexes was observed (Jesty, 1979). Also, thrombin cleaves the inhibitor but the cleavage product is retained by a disulfide bridge (Jörnvall et al., 1979). It is possible that the thrombin–cell complexes of M_r < 77 000 represent partial degradation of the thrombin portion of the complex. Alternatively, such complexes may result from partial degradation of the binding site or from thrombin bound to additional sites of M_r < 40 500. The observation that an intact catalytic apparatus is necessary for cellular stimulation by thrombin suggests that a proteolytic event is part of this reaction. Proteolysis could release a biologically active peptide or result in activation of a second enzyme. Proteolytic removal of a membrane protein could also result in cellular stimulation (Carney et al., 1979). Alternatively, binding to the receptor without proteolysis may be sufficient in and of itself for thrombin to exert its effect on cells. Further characterization of this complicated interaction will be necessary in order to resolve this issue.

Although thrombin binding to the proposed 40 500-dalton site and to antithrombin III is similar (each represents an apparent covalent complex and both are dissociable with hydroxylamine), we have obtained indirect evidence that the BCE cell binding site is not antithrombin III. They differ in molecular weight, and heparin does not accelerate thrombin binding to the sites on BCE cells. Immunological and structural studies of the cellular binding site are under way to resolve this issue.

It has been proposed that binding of thrombin to a 35 000–43 000-dalton receptor on chicken and mouse embryo cells is essential for stimulating mitogenesis of these cells (Glenn & Cunningham, 1979; Baker et al., 1979). Because thrombin is mitogenic neither for bovine endothelial cells (Gospodarowicz et al., 1978b) nor for platelets, it would appear that binding to the 40 500-dalton site on BCE cells serves another purpose in the interaction between thrombin and these cells. Binding may be the initial step by which thrombin stimulates prostacyclin synthesis or inhibits plasminogen ac-

[3] J. D. Isaacs, N. Savion, D. Gospodarowicz, and M. A. Shuman, unpublished results.

[4] T. Maerowitz, M. Botney, L. K. Johnson, M. A. Shuman, J. W. Fenton II, K. C. Glenn, W. F. Bennett, and D. D. Cunningham, unpublished results.

tivation by BCE cells, or both. The concentration of thrombin that saturates the surface covalent binding sites and the concentration necessary for maximal inhibition of activation of plasminogen (Loskutoff, 1979; Fehrenbacher et al., 1979) and prostacyclin production (Weksler et al., 1978) by endothelial cells is similar, ~0.5 μg/mL (2.0–2.5 units/mL). The observation that thrombin forms a similar complex with a specific site on fibroblasts, endothelial cells, and platelets suggests that there may be a common mechanism by which thrombin interacts with these cells.

In addition to modulating cell function, binding of thrombin to endothelium at its serine active site would result in inactivation of its proteolytic activity. Thus, thrombin binding to endothelial cells may also serve as a mechanism for inhibiting coagulation once it has been activated. The significance of thrombin binding to the 40 500-dalton site on endothelial cells will ultimately depend on demonstration of a direct relationship between binding and a physiological effect.

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