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Transmembrane Action of Thrombin Initiates Chick Cell Division

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Thrombin immobilized on polystyrene beads initiates DNA synthesis and cell division in quiescent cultures of chick embryo (CE) cells in serum-free medium. These thrombin beads also produce morphological changes in CE cells similar to those produced by soluble thrombin. The amount of acid-precipitable material released from ^{125}I-thrombin-beads into the culture medium was 60-fold less than the amount of soluble thrombin required to produce an equivalent increase in cell number. Moreover, EM autoradiography of CE cells 10 h after ^{125}I-thrombin-bead addition showed that there was no direct release and accumulation of radioactive material in the cytoplasm of these cells. These results demonstrate that thrombin action at the cell surface is sufficient to initiate proliferation of CE cells.

Key words: **transmembrane action, growth control, initiation of cell division, proteases, cell surface action, thrombin**

Several recent observations have suggested that the response of cells to certain polypeptide hormones and growth factors might require internalization of these polypeptides by the cells. For example, there is evidence that many polypeptides including insulin, nerve growth factor, low-density lipoprotein, and various toxins enter cells by receptor-mediated uptake (for review, see [1]). Also, there is evidence that specific receptors exist in the nucleus for both insulin [2] and nerve growth factor [3].

Trypsin or thrombin initiate cell division when added to quiescent cultures of chick embryo (CE) cells [4–12] or cells of certain mammalian lines and strains [13–21]. In some cell types, this initiation occurs in the absence of serum or other growth factors, indicating that these proteases are themselves mitogens or that they can substitute for other growth factors [7, 20, 21].

Similar to other peptide factors, both trypsin [22] and thrombin [23, 24] are internalized by cells. In some cases the amount of thrombin internalized correlates with

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the amount of cell division that occurs. We have recently demonstrated, however, that internalization of these proteases is not a necessary event in initiation of cell division and that, in fact, action of trypsin or thrombin at the cell surface is sufficient to initiate division of CE cells [12, 25].

Early studies designed to demonstrate the cell surface as the site of action of peptide hormones and growth factors largely utilized peptides linked to cyanogen bromide-activated Sepharose beads. Although most of these preparations were biologically active [26–31], the extensive release of active hormone, sometimes in a "superactive" form, compromised the conclusions of many of these studies [32–36]. In our experiments we have attached ^{125}I-trypsin or ^{125}I-thrombin via peptide bonds to carboxylate-modified polystyrene beads and added these preparations to quiescent cultures of CE cells [12, 25]. Both of these preparations initiate division of CE cells and, in contrast to Sepharose preparations, little intact growth factor is released from the beads into the culture medium or directly into the cells. Therefore, using this approach, we have been able to obtain direct information about the cellular site of trypsin and thrombin action.

In this paper we have characterized the initiation of cell division by thrombin immobilized on polystyrene beads and provide additional evidence that thrombin action at the cell surface is sufficient to initiate cell division. In addition, we propose that the use of similar techniques could provide direct evidence for whether other peptide factors require internalization for their biological responses or whether they also act at the cell surface.

MATERIALS AND METHODS

Highly purified human thrombin (2600 NIH units per mg [37]) was supplied by Dr. John W. Fenton, II. Carboxylate-modified polystyrene beads (0.926 μm diameter) were purchased from Dow Diagnostics. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate was purchased from Sigma, N,N-bis(3-aminopropyl)-1,3-propanediamine from Eastman Organic Chemicals, and cyanogen bromide-activated Sepharose beads from Pharmacia. Dulbecco-Vogt modified Eagle's medium (DV medium) and tryptose phosphate broth were obtained from Flow Laboratories, Inc. Trypsin solution, glutamine, antibiotics, and chicken serum were obtained from Gibco. Chemicals for cell fixation and embedding were obtained from Polysciences and from Ted Pella, Inc. Illford autoradiographic emulsion L4 and K5 were obtained from Polysciences. 4′-6′-diamidino-2-phenylindole (DAPI) was provided by Dr. Eric J. Stanbridge.

Chick embryo (CE) cells were prepared from body walls of 9-day-old chick embryos as described by Rein and Rubin [38]. Primary cells were grown in DV medium supplemented with 2.0% chicken serum and 2.0% tryptose phosphate broth. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to all media used in this study. Nonproliferating secondary cultures of these cells were prepared as described previously [21]. Briefly, primary cultures were subcultured into 35-mm-diameter dishes at a density of 6.2×10^4 cells per cm^2 in DV medium containing 2.0% chicken serum and 2.0% tryptose phosphate broth. After 4 h, the cultures were rinsed with serum-free DV medium and then cultured either in serum-free DV medium or DV medium containing 0.03% chicken serum. After 48 h the indicated additions were made and cell numbers determined 24 h later using a Coulter electronic particle counter. It should be noted that the small polystyrene beads used for these studies were not counted by the Coulter counter.

The percent of nuclei synthesizing DNA was determined as follows. ^{3}H-Thymidine (0.25 μCi/ml) was added to secondary CE cell cultures at the same time that the indicated

additions were made. After 24 h, cells were rinsed two times with PBS, fixed in their dishes with 2% paraformaldehyde for 1 hour at 4°, rinsed again, and then extracted twice with cold 5% trichloracetic acid (TCA) for 5 min. Cells were than rinsed with 95% ethanol three times and allowed to air-dry. A thin film of Illford K5 autoradiographic emulsion was applied to each dish and exposed for 3 weeks at 4°. The emulsion was then developed and nuclei were stained with 4′-6′-diamidino-2-phenylindole (DAPI) by incubating cells at 23° for 20 min in a 1% solution of DAPI in PBS followed by five washes with water to remove free stain. With this procedure, nuclei which incorporated thymidine were covered with autoradiograph grains and all other nuclei were clearly fluorescent. Each culture was examined using a Leitz microscope equipped with phase contrast and ploem fluorescent illumination (250 X). Labeled and unlabeled nuclei were scored in four separate fields.

Human thrombin was iodinated by the lactoperoxidase method as described by Martin et al [39]. Thrombin beads were prepared by adding 500 μg of ^{125}I-thrombin or unlabeled thrombin to 25 mg of washed carboxylate-modified polystyrene beads in 50 mM $NaPO_4$, 200 mM NaCl at pH 7.0 (total volume of 0.75 ml) and linked by addition of water-soluble carbodiimide [1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate] to a final concentration of 0.1 M. After 4 h of stirring at 4°, the beads were a) washed five times by centrifugation (10,000g for 15 min) through PBS, b) preincubated for 3 h at 37° in conditioned DV medium (medium taken from 48-h secondary cultures), and c) washed two times and resuspended in 2.5 ml of PBS. Care was taken to minimize microbial contamination. All bead preparations were used within 1 day.

The amount of acid-precipitable material released from ^{125}I-thrombin-beads was assessed using 5% trichloroacetic acid with 0.025% BSA carrier. After 18–24 h at 4°, precipitates were collected by centrifugation, dissolved with 1 N NaOH, and counted in a gamma counter.

For electron microscopy and EM autoradiography cells were: a) fixed in situ for 2 min with 2.5% glutaradehyde in 0.2 M $NaPO_4$, pH 7.4 at 23°;b) fixed in combined glutaraldehyde (1.25%) and osmium tetroxide (1%) in 0.1 M $NaPO_4$ for 30 min at 4° [40]; and c) stained en bloc for 30 min in aqueous uranyl acetate. Cells were then dehydrated in ethanol and embedded in Epon 812 [41]. Plastic dishes were removed and thin sections (600–800 Å) were cut perpendicular to the cell monolayer. For autoradiography, sections were stained and a thin film of Illford L4 emulsion was applied [42]. After a 3-week exposure, the emulsion was developed, sections were restained, and then studied and photographed with a Philips EM 300 electron microscope.

RESULTS

Several different types of thrombin-bead preparations were able to initiate division of CE cells. In preliminary experiments, we attached ^{125}I-thrombin by carbodiimide condensation to carboxylate-modified polystyrene beads and to polystyrene beads substituted with N,N-bis(2-aminopropyl)-1,3-propanediamine (nine carbon spacers with terminal amino groups). We also attached ^{125}I-thrombin to cyanogen bromide-activated Sepharose beads by standard techniques. As shown in Figure 1a, most of the soluble thrombin became attached to these different beads and was not released by successive PBS rinses or by incubation of the beads for 3 h in conditioned medium. When added to quiescent CE cells, each thrombin-bead preparation initiated cell division (Fig. 1b). Examination of the medium for acid-precipitable radioactivity indicated that all of the bead preparations released some thrombin-derived material into the medium (Fig. 1c). However, based on

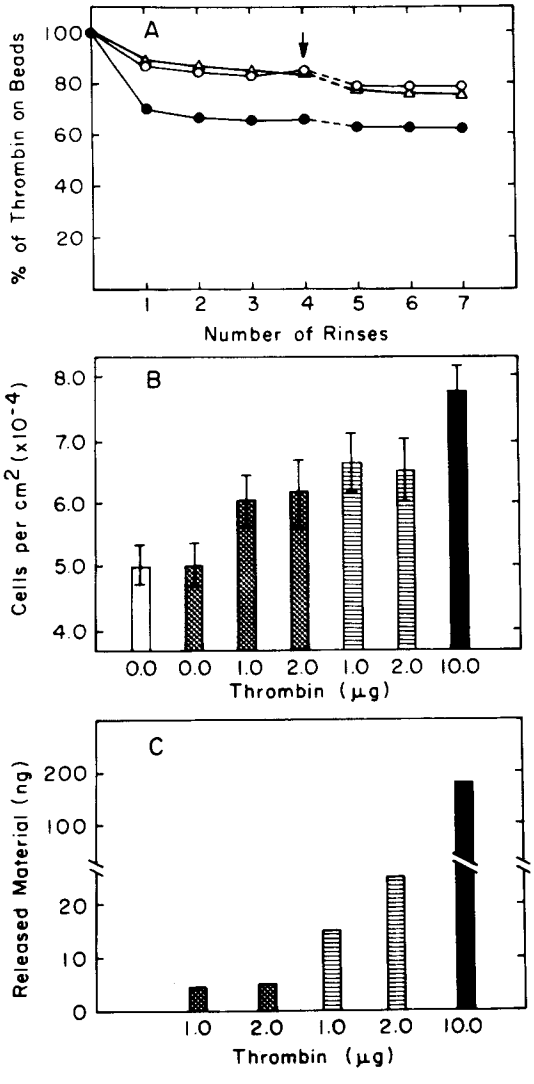

Fig. 1. Effect of thrombin immobilized on different types of beads. Thrombin linked directly to carboxylate-modified polystyrene beads (• and dotted bars) to polystyrene beads via N,N′-bis (3-aminoprophyl)-1, 3-propanediamine (○ and striped bars), and attached to CNBr-activated Sepharose 4B (△ and black bars). A) The percent of the initial ^{125}I-thrombin still present in each bead preparation after the indicated number of rinses through PBS. Initial linkage reactions contained 500 μg of ^{125}I-thrombin per 25 mg of polystyrene beads and 1,500 μg per 250 μl of swollen Sepharose beads. Arrow indicates point at which beads were incubated for 3 h in conditioned medium taken from 48-h secondary CE cell cultures. B) The effect of the bead preparations on cell number determined 24 h after their addition to quiescent CE cells. Open bar represents control cultures with no addition; dotted bar with 0.0 μg of thrombin represents control cultures with unsubstituted carboxylate-modified polystyrene beads (added at the same concentration as those with 2.0 μg of thrombin). C) The amount of acid-precipitable ^{125}I-thrombin-derived material released into the medium from the different bead preparations after 24 h of incubation with CE cells.

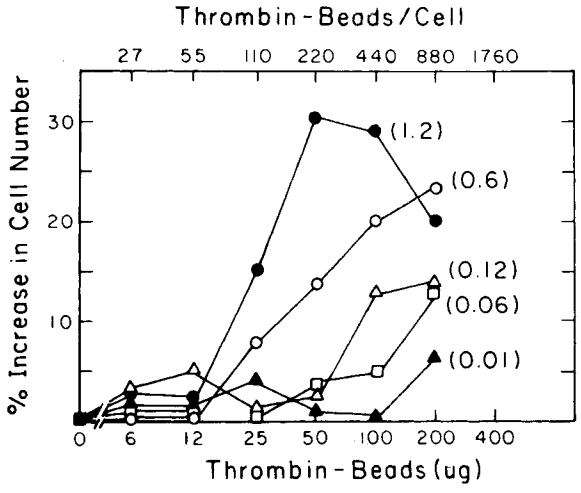

Fig. 2. Effect of polystyrene beads with various amounts of attached thrombin on CE cell number. Indicated amounts of thrombin-beads were added to cultures of CE cells brought to quiescence in serum-free medium and cell number was determined 24 h later. Data are expressed as percent increase in cell number over control populations. In these experiments control plates increased approximately 10% during this period. Microgram quantities along the abscissa represent μg of polystyrene beads added per culture. Numbers in parentheses within the figure represent micrograms of ^{125}I-thrombin attached to 50 μg of polystyrene beads in each preparation.

dose-response curves obtained with soluble thrombin, only the Sepharose-bead preparation released enough material to account for any of the observed initiation. Because the direct linkage of thrombin to carboxylate-modified polystyrene beads resulted in the smallest amount of released material, we utilized this preparation (hereafter referred to simply as thrombin-beads) for all of the following experiments.

To determine if the initiation by these thrombin-beads was dependent upon the concentration of immobilized thrombin, we prepared beads with various amounts of attached ^{125}I-thrombin and added the indicated amounts of each bead preparation to cultures of quiescent CE cells. As shown in Figure 2, addition of 50 μg (220 beads per cell) of the preparation containing 1.2 μg of thrombin produced a 30% increase in cell number over control populations by 24 h. Addition of 50 μg of beads with less thrombin attached resulted in proportionally less initiation of cell division, but by adding more of these beads some initiation was observed even at the lowest thrombin concentration. This indicated that initiation was dependent upon the amount of thrombin that was contacting the cells.

In addition to initiating cell division, the thrombin-beads produced morphological changes in CE cells. These changes can be seen by comparing Figure 3a and Figure 3b. The control CE cells (Fig. 3a) had a flattened appearance with many cell-cell contacts. In contrast, the thrombin-bead treated cells appeared more elongated with fewer cell-cell contacts (Fig. 3b). It also appeared that these cells had migrated into cell islands, leaving exposed areas of clear plastic. Control CE cells treated with ovalbumin-beads did not produce these morphological changes. Interestingly, the morphological changes produced by thrombin-beads were quite similar to those previously reported for soluble thrombin [11]. This suggested that thrombin on the beads was able to interact with the surface of the CE cells in much the same way as soluble thrombin.

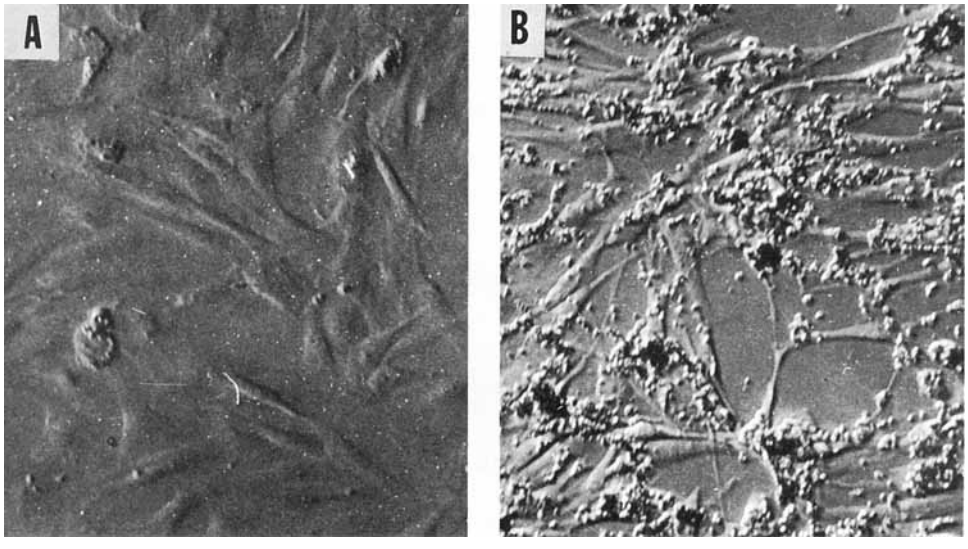

Fig. 3. Effect of thrombin-beads on CE cell morphology. Hoffman optics pictures of CE cells 24 h after the indicated additions (400 ×). A) Control cells with added PBS; B) CE cells with 50 μg of thrombin-beads.

To determine how the initiation of cell division by thrombin-beads compared with initiation by soluble thrombin, we added similar amounts of soluble thrombin or thrombin-beads to CE cell cultures and looked 24 h later at both cell number and the percent of nuclei labeled by ^{3}H-thymidine (Fig. 4). As shown, thrombin-beads did not initiate division of as many cells as solbule thrombin by 24 h (Fig. 4a). In contrast, the increase in percent of labeled nuclei after 24 h of exposure to ^{3}H-thymidine was about the same with soluble or immobilized thrombin (Fig. 4b). These experiments indicated that the thrombin-beads were capable of initiating DNA synthesis in as many cells as soluble thrombin, but not all of these cells divided by 24 h.

These results also indicate that the initiation of DNA synthesis in the same number of cells required only about twice as much immobilized thrombin as soluble thrombin (Fig. 4b). We also found that the fibrinogen clotting and esterase activities of these thrombin-beads were about 40% as high as soluble thrombin. Thus, in this case, attachment of thrombin to these beads resulted in a similar reduction of both clotting and mitogenic activities.

The above observation that only twice as much immobilized thrombin was required to initiate the same number of cells as soluble thrombin is also very important to our understanding of whether released material could account for the initiation caused by the thrombin beads. Since only twice as much thrombin on beads was required, it follows that if the thrombin-bead initiation were resulting from thrombin released into the medium, then the beads must be releasing at least half of their thrombin. As shown in Figure 1c and in the experiments to follow, this was not the case.

Two different types of experiments were used to evaluate the mitogenic potential of material released from the thrombin-beads. The first of these involved coverslip experiments where populations of cells with and without thrombin-beads shared the same medium. As diagramed in Figure 5, CE cells were plated on small plastic coverslips in 60-mm dishes and brought to quiescence in DV medium containing 0.03% chicken serum.

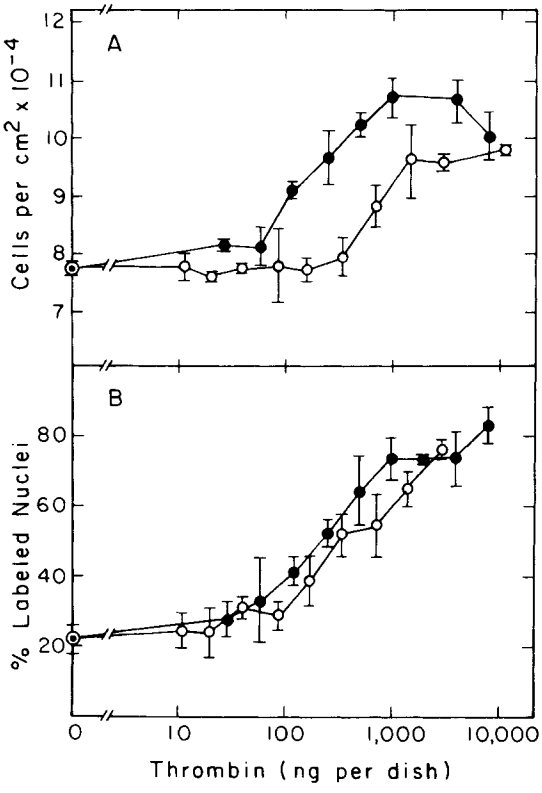

Fig. 4. The effect of soluble and immobilized thrombin on cell number and percent of ^{3}H-thymidine-labeled nuclei. Indicated concentrations of thrombin were added to cultures of CE cells in serum-free medium as described in the Materials and Methods. After 24 h cell number and percent labeled nuclei were determined. A) Effect on cell number; B) percent of nuclei labeled by ^{3}H-thymidine. Soluble thrombin (•), thrombin-beads (○). Error bars on this and Figure 6 represent ± 1 standard deviation from the mean of duplicate determinations.

After 48 h, thrombin-beads were added to some of the dishes. The thrombin-beads became firmly attached to the CE cells by 3 h, making it possible to relocate coverslip cultures with and without thrombin-beads into the same dishes. In these experiments the thrombin-bead-contacted populations increased in cell number from 50–70% over control populations grown in separate dishes, but there was no initiation of cell division on control coverslips sharing medium with the thrombin-bead-contacted cells (Table I).

Another type of coverslip experiment also showed that initiation of cell division by the thrombin-beads was not caused by release of thrombin from the beads. In these experiments a single coverslip culture with thrombin beads was added to a 60-mm dish with fresh serum-free medium. In this situation any released material or soluble factors involved in the thrombin-bead initiation would be diluted from 4- to 25-fold over the previous experiments. As shown in Table I, we found no reduction in the amount of initiation of cell division in these cultures. Together, these coverslip experiments indicated that the observed thrombin-bead initiation of cell division was not the result of soluble released material.

To actually measured how much potentially mitogenic material was released from the

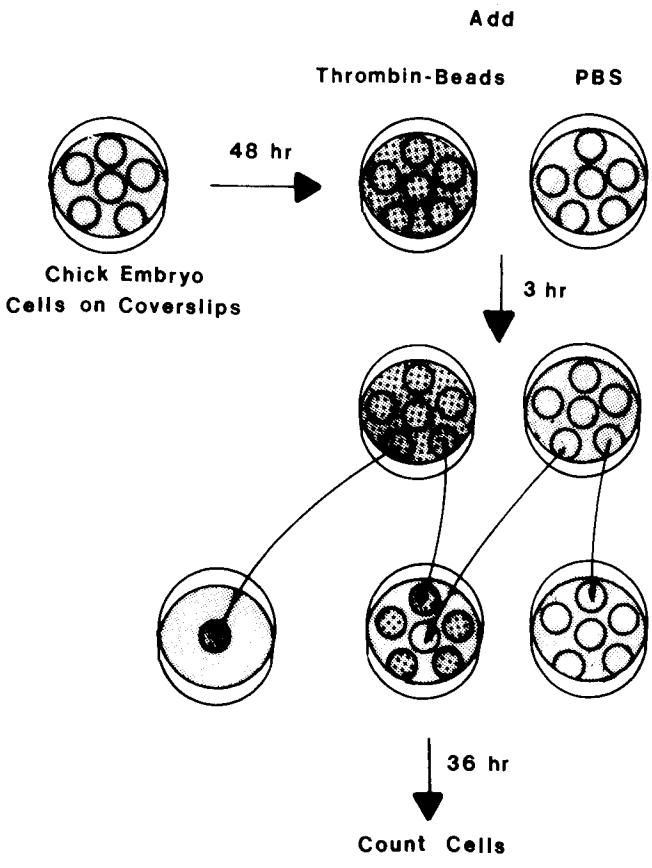

Fig. 5. Diagram of coverslip experiments. CE cells were plated at a density of 5.0×10^4 cells per cm^2 on 13-mm plastic coverslips (Lux) in 60-mm dishes with 6.0 ml of DV medium and brought to quiescence in DV medium containing 0.03% chicken serum. After 48 h, 500 μg of thrombin-beads were added to some of the dishes. Three hours later, coverslips with bead-contacted cells and coverslips with cells only were transferred to new 60-mm dishes with fresh serum-free DV medium. Cell number was determined 36 h later.

beads, we added ^{125}I-thrombin-beads to cultures of quiescent CE cells, measured the amount of acid-precipitable material released into the medium, and compared this amount with the dose-response curve for soluble thrombin added to parallel cultures. As shown in Figure 6a, addition of as little as 25 μg of ^{125}I-thrombin-beads (containing 0.45 μg of ^{125}I-thrombin) resulted in a cell number increase of 20% over controls. It is noteworthy that ovalbumin-beads did not initiate cell division. Thus, the initiation was a result of thrombin. Five nanograms of acid-precipitable material was released into the medium from 25 μg of ^{125}I-thrombin-beads by 24 h (Fig. 6b); however, nearly 300 ng of soluble thrombin was required to achieve the 20% increase in cell number produced by 25μg of ^{125}I-thrombin-beads (Fig. 6c). With 200 μg of thrombin-beads, cell number increased 40% (Fig. 6a) and 32 ng of acid-precipitable material was released by 24 h (Fig. 6b). It should be noted that up to 60 ng of soluble thrombin had little or no effect on cell number, and to achieve a 40% increase required almost 2 μg (Fig. 6c). Thus, in this experiment the amount of acid-precipitable material released from the beads was not sufficient to account for any of the

TABLE I. Effect of Thrombin-Beads on Coverslip Cultures

Additions	Culture conditions	Cells per coverslip ($\times 10^{-4}$)
PBS (control coverslips)	5 per dish	7.0 ± .1
PBS (control coverslips)	1 per dish with 4 thrombin-bead coverslips	5.5 ± .9
Thrombin-beads	4 per dish with 1 control coverslip	10.6 ± 2.4
Thrombin-beads	1 per dish	11.8 ± 1.2

Secondary CE cells were plated on 13-mm plastic coverslips at a density of 5.0×10^4 cells per cm^2 in 60-mm dishes with 6.0 ml of DV medium containing 2.0% chicken serum and 2.0% tryptose phosphate broth. After 4 h the cells were rinsed and the medium was replaced with DV medium containing 0.03% chicken serum. After 48 h, 500 μg of thrombin-beads were added to some of the dishes. Three hours later, coverslips with bead-contacted cells and coverslips with cells only were transferred to new 60-mm dishes with serum-free DV medium. Cell number was determined 36 h later.

observed initiation of cell division and was about 60-fold less than the amount of soluble thrombin necessary for the observed increase in cell number. The total amount of radioactive material released into the medium (acid-soluble and acid-precipitable) from 25 μg and 200 μg of ^{125}I-thrombin-beads by 24 h was only 26 ng and 242 ng, respectively. Therefore, even if one argued that all of the acid-soluble and acid-precipitable material were initially released from the beads as intact thrombin, it would still be about 8-fold less than the amount necessary to produce the increase in cell number caused by the ^{125}I-thrombin-beads.

The above coverslip experiments and experiments measuring the release of radioactive material from ^{125}I-thrombin-beads demonstrated that release of thrombin from the beads into the culture medium did not account for the observed cell division. It was extremely important, however, to consider the possibility that material could be released from the beads directly into cells without appearing in the medium. To examine this possibility we used the technique of ultrastructural (EM) autoradiography.

EM examination of CE cells after incubation with thrombin-beads showed that some of the beads were endocytosed. The amount of this internalization appeared similar to the endocytosis of trypsin-beads where 4 of 225 beads added per cell were endocytosed by 9 h [12]. It should be noted that these endocytosed beads were completely surrounded by an intact membrane. Thus, if the thrombin on these endocytosed beads were involved in initiation, its action was still on the plasma membrane and not the cytoplasm. In addition, we found that ovalbumin-beads were also taken up by these cells. Therefore, the process of bead endocytosis itself did not lead to initiation of cell division.

Figure 7 shows an EM autoradiograph of a CE cell 10 h after addition of ^{125}I-thrombin-beads. From autoradiographs such as this, we determined that beads above cells averaged 10.6 grains per section and that beads inside of endocytic vesicles averaged 10.2 grains per section. This indicated that little, if any, material was lost from the beads as they interacted with and were internalized by the CE cells. Nevertheless, to determine if this small difference represented material released into the cytoplasm, we counted grains in the cytoplasm and compared this value with the number of background grains in areas above the cells. In areas above cells where released material would be washed away, we found that 99% of the grains were with 0.3 μm of the edge of the nearest bead. Thus, grains appearing

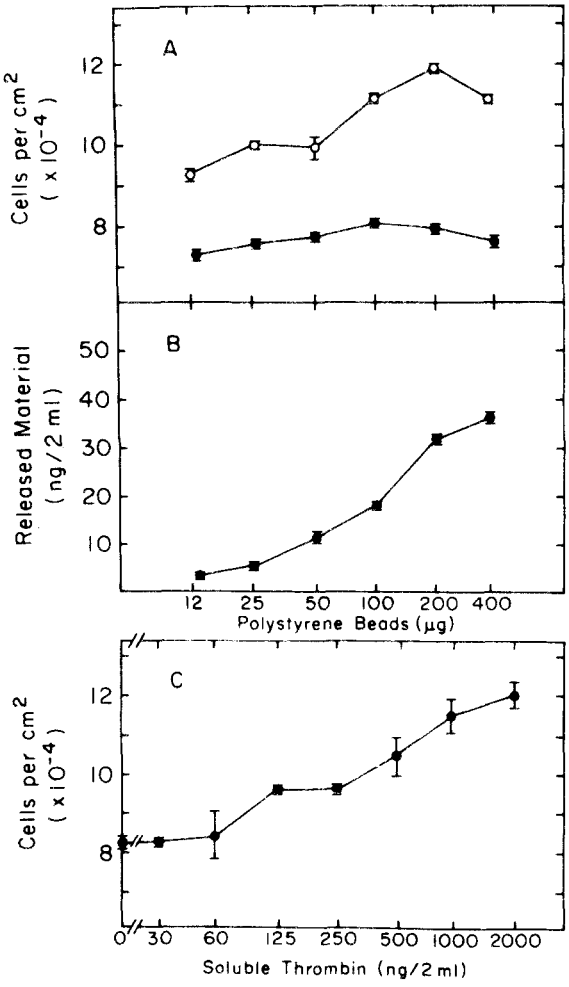

Fig. 6. Initiation of CE cell division by ^{125}I-thrombin-beads and evaluation of the mitogen potential of the released material. A) Effect of ovalbumin-beads (•) and ^{125}I-thrombin-beads (○) on cell number 24 h after their addition to cultures of CE cells quiescent in medium containing 0.03% chicken serum. ^{125}I-Thrombin-beads contained 0.9 μg of ^{125}I-thrombin per 50 μg of beads. Ovalbumin beads were prepared under parallel conditions. B) Amount of acid-precipitable material released from the indicated concentrations of ^{125}I-thrombin beads during the 24 h incubation. C) Effect of various concentrations of soluble thrombin on parallel cultures of quiescent CE cells.

in the cytoplasm farther than 0.3 μm from the edge of beads should represent either background or released material. After scoring grains in cross sections of more than 225 cells and background grains in areas equal to 3 times the cytoplasmic area, we found no difference in grain density. This indicated that all of the grains in the cytoplasm could be explained as background and that by this technique we could not detect the release of any material from the beads into the cytoplasm.

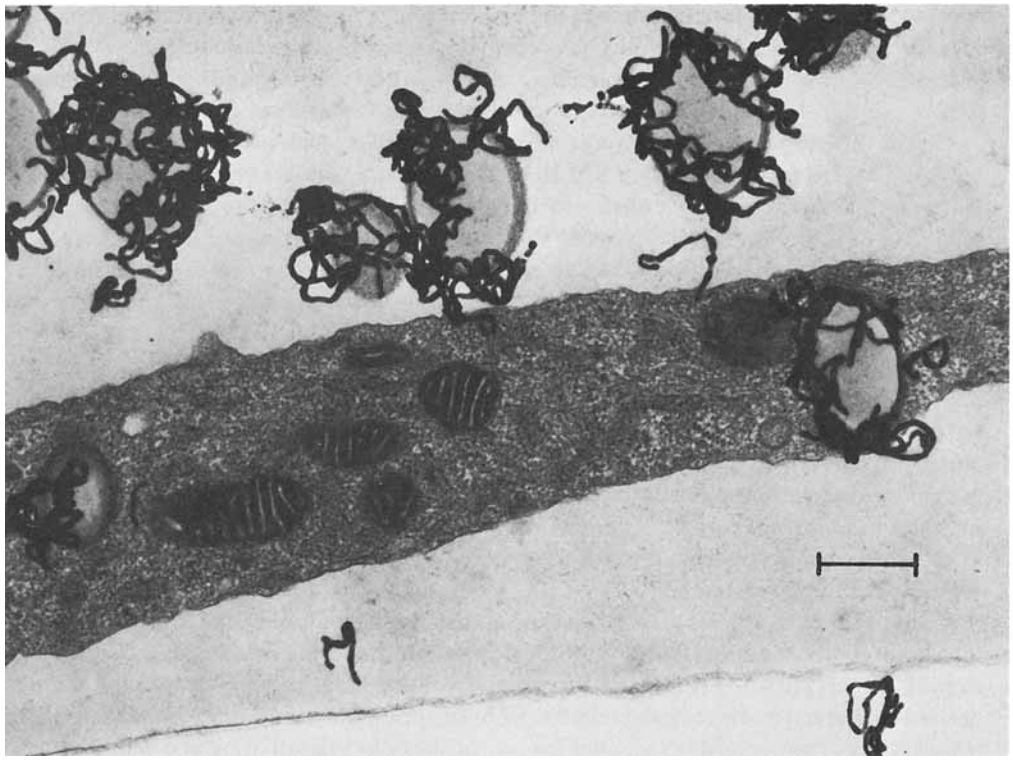

Fig. 7. Ultrastructural autoradiograph of CE cells 10 h after addition of ^{125}I-thrombin-beads. Preparation of samples and EM autoradiography are described in the Materials and Methods. Based on the number of thrombin molecules per bead and the number of grains observed per bead, we calcualte that each grain in these autoradiographs represents from 800 to 1,000 thrombin molecules. In these samples any material released from beads above or below the cells would be washed away. Therefore, the grains in this figure which appear to be floating below the cell near the edge of the epon section represent the random background associated with this technique. Bar represents 0.5 μm.

DISCUSSION

Initiation of cell division by proteases such as trypsin and thrombin has been used as a model system for identifying mechanisms involved in initiation of cell division. This system offers several advantages. First, initiation occurs in many different cell types including chick embryo cells [4–12] and certain mammalian cell strains and lines [13–21]. Second, initiation can be studied under serum-free conditions without additional growth factors [7, 20, 21]. Third, initiation is specific for certain proteases. For example, proteases such as chymotrypsin and α-protease do not initiate cell division, whereas, trypsin and thrombin do [11]. In addition, the well characterized proteolytic actions of these enzymes can be monitored to determine how they might interact with cellular proteins to cause initiation.

Several studies have examined the effects of proteases on ^{125}I or ^{131}I-labeled cell surface proteins [6, 9, 11, 17, 19, 43, 44]. Although several proteins have been identified which are cleaved by various proteases, none of these cleavages appear to be causally related to initiation of cell division. For example, the LETS protein, originally implicated

because it was removed by trypsin and not found in transformed cells, is removed from the cell surface by chymotrypsin (which is nonmitogenic) but is not removed by mitogenic levels of thrombin [9]. Another implicated surface protein, the 205K protein, also appears to be removed by proteases which do not initiate cell division as well as by those that do [11].

The difficulty in demonstrating a cell surface protein which is causally involved in initiation has led to the suggestion that these proteases may initiate cell division by acting on intracellular sites. Trypsin and thrombin have both been reported to be internalized by cells [22–24]. Studies with normal and Rous sarcoma virus-transformed CE cells have even shown that internalization of thrombin correlates with the amount of cell division initiated by thrombin in these two cell types [23]. Also, studies comparing chymotrypsin and thrombin have shown that chymotrypsin, which does not initiate cell division, is internalized by CE cells much more slowly than thrombin [24].

In contrast to the above studies, we have found that trypsin and thrombin action at the cell surface is sufficient to initiate CE cell division when these proteases are immobilized on polystyrene beads [12, 25]. From these data alone, however, we cannot rule out the possibility that during incubation with soluble thrombin the thrombin that is internalized may also be able to initiate cell division. To examine this possibility, we are currently introducing soluble thrombin into the cytoplasm of CE cells by fusing thrombin-containing vesicles with the cell membrane.

In the present studies we further characterized the initiation of cell division by immobilized thrombin and provided additional evidence that thrombin acts at the cell surface to produce this initiation. In these experiments, we found that the amount of thrombin-derived acid-precipitable material released from the thrombin-beads into the medium during incubation was not enough to account for any of the observed initiation and was, in fact, 60-fold less than the amount of soluble thrombin required to initiate the same amount of cell division as the thrombin-beads. In addition, in coverslip experiments where populations of cells with and without thrombin-beads shared the same medium, only bead-contacted cells were initiated to divide. Thus, initiation did not result from "superactive" soluble peptide fragments or from thrombin-derived material which had lost its radioactive label. Therefore, the initiation of cell division by thrombin-beads could not be explained by soluble material released into the medium.

Another possibility which must be considered in this type of study is that thrombin could be released from the beads directly into the CE cells. We examined this possibility using ultrastructural autoradiography of CE cells 10 h after addition of ^{125}I-thrombin-beads. In these studies random background accounted for all of the grains observed in the cytoplasm. Therefore, there was no detectable direct uptake of material from the beads nor any uptake of material that had been released into the medium.

In our previous studies with both trypsin-beads and thrombin-beads the amount of initiation of cell division observed by 24 h was somewhat less than that observed with the soluble form of these proteases. This left the possibility that within the cell population only part of the cells were responsive to proteases acting at the cell surface. In the present studies we compared the effects of soluble thrombin and thrombin-beads on both cell number and percent of nuclei that became labeled with ^{3}H-thymidine. We found that, indeed, soluble thrombin produced a greater increase in cell number, but the increase in percent labeled nuclei was almost the same for soluble thrombin and thrombin-beads. Thus, all of the cells that were responsive to soluble thrombin were responsive to the thrombin-beads.

Another issue that is important to consider is whether cell division caused by pro-

tease action at the cell surface might result from a facilitation of the action of other growth factors present in the medium. This is an important consideration in the case of trypsin and thrombin because both of these enzymes have been reported to enhance the mitogenic response of certain cells to serum or other mitogenic factors [15, 18, 45]. However, the following results show that initiation of cell division by thrombin-beads does not require the presence of serum or other growth factors. First, cells in serum-free medium which have been rinsed and incubated for 48 h to remove any remaining serum factors are still initiated by soluble thrombin [21] and by thrombin-beads [25]. Second, increasing the serum concentration present during incubation of CE cells with thrombin-beads had no effect on the amount of initiation observed [25]. Third, in coverslip experiments where a single coverslip culture with bead-contacted cells was added to a 60-mm dish with 6 ml of fresh serum-free medium there was no decrease in the amount of thrombin-bead-initiated cell division. In these coverslip experiments any factors present in the medium or secreted by the cells into the medium would be diluted by at least 25-fold.

Since we have demonstrated that trypsin and thrombin both initiate cell division by action at the cell surface, we are presently examining the interaction between these proteases and surface macromolecules. In addition to the proteolytic nature of this interaction, we have recently found that soluble ^{125}I-thrombin binds specifically with high affinity to surface receptors on mouse embryo cells and that factors which inhibit this specific binding also inhibit initiation of cell division by thrombin (Carney and Cunningham, manuscript in preparation).

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REFERENCES

1. Neville DM, Chang T-M: Receptor-mediated protein transport into cells. Entry mechanisms for toxins, hormones, antibodies, viruses, lysosomal hydrolases, asialo-glycoproteins, and carrier proteins. In Kleinzeller A, Bronner F (eds): "Current Topics in Membranes and Transport." New York: Academic, 1978, vol 10, p 65.
2. Goldfine ID, Smith GJ, Wong KY, Jones AJ: Proc Natl Acad Sci USA 74:1368, 1977.
3. Andres RY, Jeng I, Bradshaw RA: Proc Natl Acad Sci USA 74:2785, 1977.
4. Sefton BM, Rubin H: Nature (London) 227:843, 1970.
5. Vaheri A, Ruoslahti E, Hovi T: Control of Proliferation in Animal Cells. Clarkson B, Baserga R (eds): Cold Spring Harbor Laboratory, 1974, p 305.
6. Blumberg PM, Robbins PW: Cell 6:137, 1975.
7. Chen LB, Buchanan JM: Proc Natl Acad Sci USA 72:131, 1975.
8. Cunningham DD, Ho T-S: Proteases and Biological Control. Reich E, Rifkin DB, Shaw E (eds): Cold Spring Harbor Laboratory, 1975, p 795.
9. Teng NNH, Chen LB: Proc Natl Acad Sci USA 72:413, 1975.
10. Hovi T, Vaheri A: J Cell Physiol 87:245, 1976.
11. Zetter BR, Chen LB, Buchanan JM: Cell 7:407, 1976.
12. Carney DH, Cunningham DD: Nature (London) 268:602, 1977.
13. Burger MM: Nature (Lonson) 227:170, 1970.

14. Noonan KD, Burger MM: Exp Cell Res 80:405, 1973.
15. Noonan KD: Nature (London) 259:573, 1976.
16. Kaplan JG, Bona C: Exp Cell Res 88:388, 1974.
17. Buchanan JM, Chen LB, Zetter BR: Cancer Enzymology. Schultz J, Ahmad F (eds): New York: Academic, 1976, p 1.
18. Brown M, Kiehn D: Proc Natl Acad Sci USA 74:2874, 1977.
19. Chen LB, Teng NNH, Buchanan JM: Exp Cell Res 101:41, 1976.
20. Pohjanpelto P: J Cell Physiol 91:387, 1977.
21. Carney DH, Glenn KC, Cunningham DD: J Cell Physiol 95:13, 1978.
22. Hodges GM, Livingston DC, Franks LM: J Cell Sci 12:887, 1973.
23. Zetter BR, Chen LB, Buchanan JM: Proc Natl Acad USA 74:596, 1977.
24. Martin BM, Quigley JP: Chemistry and Biology of Thrombin. Lundbald RL, Fenton JW, Mann KG (eds): Ann Arbor Science, 1977, p 531.
25. Carney DH, Cunningham DD: Cell: 14:811, 1978.
26. Cuatrecasas P: Proc Natl Acad Sci USA 63:450, 1969.
27. Blatt LM, Kimm KH: J Biol Chem 246:4895, 1971.
28. Oka T, Topper YJ: Proc Natl Acad Sci USA 68:2066, 1971.
29. Anderson J, Melchers F: Proc Natl Acad Sci USA 70:416, 1972.
30. Greaves MF, Bauminger S: Nature (London) New Biol 235:67, 1972.
31. Frazier WA, Boyd LF, Bradshaw RA: Proc Natl Acad Sci USA 70:2931, 1973.
32. Davidson MB, Van Herle AJ, Gerschenson LE: Endocrinology 92:1442, 1973.
33. Garwin JL, Gelehrter RD: Arch Biochem Biophys 164:52, 1974.
34. Bolander FF II, Fellows RE: Biochemistry 14:2938, 1975.
35. Kolb JH, Renner R, Hepp KD, Weiss L, Wieland OH: Proc Natl Acad Sci USA 74:248, 1975.
36. Topper YJ, Oka T, Vonderhaar BK, Wilchek M: J Cell Physiol 89:647, 1976.
37. Fenton JW II, Fasco MJ, Stackrow AB, Aronson DL, Young AM, Finlayson JS: J Biol Chem 252:3587, 1977.
38. Rein A, Rubin H: Exp Cell Res 49:666, 1968.
39. Martin BM, Wasiewski WW, Fenton JW II, Detwiler TC: Biochemistry 15:4886, 1976.
40. Trump BF, Bulger RE: Lab Invest 15:368, 1966.
41. Luft JH: J Biophys Biochem Cytol 9:409, 1961.
42. Salpeter MM, Fertuck HC, Salpeter EE: J Cell Biol 72:161, 1977.
43. Hynes RO: Proc Natl Acad Sci USA 70:3170, 1973.
44. Teng NNH, Chen LB: Nature (London) 259:578, 1976.
45. Zetter BR, Sun T-T, Chen LB, Buchanan JM: J Cell Physiol 92:233, 1977.