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Visualization of Thrombin Receptors on Mouse Embryo Fibroblasts Using Fluorescein-Amine Conjugated Human α-Thrombin

Darrell H. Carney

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

The localization of thrombin receptors on mouse embryo (ME) cells has been examined by direct fluorescence microscopy using a fluorescein amine-labeled thrombin. Two fluorescein amines, 4-(N-6-aminoethyl thioureal)-fluorescein and 4-(N-6-aminohexyl thioureal)-fluorescein, were synthesized and attached to the carbohydrate moiety of highly purified human α-thrombin by periodate oxidation of the carbohydrate and selective reduction of the Schiff's base using sodium cyanoborohydride. Preparations of fluorescent thrombin with from 1 to 4 fluoresceins per molecule of thrombin retained their ability to proteolytically cleave fibrinogin to form fibrin clots, to bind to thrombin receptors on ME cells, and to initiate cell division. After incubating mitogenic concentrations of the fluorescein amine labeled thrombin with ME cells at 4°C, a diffuse fluorescent pattern was observed over the surface of the ME cells. This diffuse pattern was specific: it was not observed on cells from parallel cultures incubated with fluorescent thrombin plus a 20-fold excess of unlabeled thrombin. Thus, thrombin receptors appear to be distributed randomly over the surface of ME cells prior to interaction with thrombin. Increasing the temperature to 37° C following binding at 4° C resulted in a rapid dissociation of the fluorescent pattern from the cells leaving only the autofluorescent vesicles. This result may reflect the unique ability of thrombin to proteolytically cleave its own receptor.

Key words: thrombin, initiation of cell division, receptor visualization, fluorescent labeling, proteolysis of receptors

Highly purified human α-thrombin initiates division of fibroblast-like cells from several different sources [1–3]. Studies to determine the molecular mechanism of this initiation have been recently reviewed [4]. Briefly, these studies have shown that thrombin action at the cell surface is sufficient to initiate division of chick embryo cells [5, 6] and that specific binding of thrombin at the cell surface is necessary for initiation of cell division [7]. The receptors for thrombin have been identified on mouse embryo fibroblasts by photoaffinity labeling experiments as molecules with a Mr = 50,250 ± 5,700 (n = 4) [8]. An additional thrombin binding protein "protease nexin" has been characterized in cultures of

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human fibroblasts with an apparent molecular weight of 38,000 [9]. The binding of thrombin to this protein, however, does not correlate with initiation of cell division [J. Baker, personal communication].

To determine whether proteolytic cleavage of the thrombin receptor is necessary for thrombin to initiate cell division, we recently prepared several proteolytically inhibited thrombin derivatives and examined their ability to bind to thrombin receptors and to initiate division of chick, mouse, human and Chinese hamster fibroblasts [10]. Two of these derivatives (with active site inhibitors PMSF or DIP) were able to bind to thrombin receptors on mouse and Chinese hamster cells equally as well as unmodified α-thrombin. However, none of the derivatives initiated cell division. These experiments indicate that receptor binding alone is not sufficient for initiation of cell division and suggest that proteolytic cleavage of the thrombin receptor is necessary for initiation.

Further evidence indicating that proteolytic cleavage of the thrombin receptors is necessary for initiation of cell division has come from recent studies with responsive and nonresponsive chick embryo cells. In these cells, thrombin cleavage of an iodinated cell surface protein corresponding in size to the thrombin receptors correlates with initiation of cell division by thrombin [11]. This protein is cleaved and lost from the surface of responsive cells after treatment with mitogenic concentrations of thrombin, but is not lost from cells which are not initiated to divide in response to the same concentration of thrombin [11]. Thus, this evidence as well as that obtained with proteolytically inhibited thrombin indicates that proteolytic cleavage of the thrombin receptor is necessary to initiate cell division.

Proteolytic cleavage of the thrombin receptor could generate a transmembrane signal in at least two ways. First, a fragment of the receptor might be released directly into the cells. A precedent for this type of signal has come from studies with epidermal growth factor (EGF), where a correlation between receptor degradation and initiation of cell division suggests that generation of a receptor fragment could be a part of the mitogenic signal [12]. Second, proteolytic cleavage of the receptor could be necessary for redistribution which might iteself generate a mitogenic transmembrane signal. Indeed, recent studies have indicated that insulin, EGF and many other molecules which bind to receptors aggregate on the cell surface and are then internalized by receptor-mediated endocytosis [13–16]. At present, however, it is unclear whether receptor aggregation and internalization are causally related to generation of a mitogenic signal.

To investigate the possible role of receptor redistribution in the initiation of cell division by thrombin, we have prepared fluorescent thrombin derivatives which retain their biological activity and ability to bind to the thrombin receptor. Using these derivatives we have been able to visualize thrombin receptors on mouse embryo fibroblasts using low mitogenic concentrations of fluorescent thrombin. Incubating the fibroblasts at 37°C following initial binding at 4°C does not lead to aggregation and internalization of the fluorescent thrombin as has been observed for insulin and EGF. Instead, the fluorescence disappears from the cells within minutes. This difference between thrombin and other nonproteolytic growth factors such as insulin and EGF may reflect the unique ability of thrombin to proteolytically cleave its own receptor.

MATERIALS AND METHODS

Cell Cultures

Primary cultures of mouse embryo fibroblasts were prepared from the body walls of 11- to 13-day-old NIH Swiss outbred mouse embryos and cultured in Dulbecco-Vogt-

modified Eagles (DME) medium supplemented with 10% calf serum (Irvine Scientific) as previously described [3]. After 3 to 5 days, the primary cultures were subcultured into 35-mm dishes (6.2×10^4 cells cm^{-2}), or 35-mm dishes with 20 mm^2 coverslips (1.5×10^4 cells cm^{-2}). After 16 hr the cells were rinsed and the medium was changed to serum-free DME medium. Binding, growth and fluorescent labeling experiments were performed 2 days after changing cells to serum-free medium. By this time the cells were nonproliferating as judged by cell number and cell cycle analysis with flow microfluorimetry.

Preparation of Fluorescein Amine-Labeled Thrombin

A complete description of this labeling procedure will be published elsewhere [Carney, Jones and Weigel, manuscript in preparation]. Briefly, two different fluorescein amines were synthesized by reacting fluorescein isothiocynate (FITC) with a 10-fold excess of ethylenediame or hexanediamine in absolute ethyl alcohol to give 4-(N-6-aminoethyl thioureal)-fluorescein or 4-(N-6-aminohexyl thioureal)-fluorescein, respectively. The fluorescein amines were isolated from unreacted FITC and ethylene or hexanediamine by preparative thin-layer chromatography using ethylacetate:acetic acid:water (3:2:1) as a developing solvent.

These fluorescein amines were linked to the carbohydrate moiety of highly purified human α-thrombin (generously provided by Dr. John W. Fenton, II, [17]) by a modification of a procedure described by Yang, Fenton, and Feinman [18] for linking dansyl hydrazine to α-thrombin. Briefly, 1 ml of human α-thrombin (1.83 mg/ml, about 4,000 NIH Units/mg) was dialyzed overnight at 4°C against 0.1 M sodium acetate buffer at pH 5.6 with 0.15 M NaCl and reacted at 0°C in the dark for 30 min with sodium meta periodate (Sigma) at a final concentration of 5 μM. The protein was then dialyzed at 4°C against three changes of 50 mM phosphate buffer at pH 7.6 with 150 mM NaCl. Approximately 1 mg of 4-(N-6-aminoethyl thioureal)-fluorescein or 4-(N-6-aminohexyl thioureal)-fluorescein was dissolved in buffer and added to the oxidized protein. The pH was adjusted to 9.0 by addition of NaOH and the solution was stirred gently at 4°C. After 60 min, 1 mg of sodium cyanoborohydride was added to the mixture and stirring at 4°C was continued. After 18 hr, the fluorescent thrombin preparation was removed from the tube and dialyzed for 2 days against five changes of 50 mM phosphate buffer at pH 7.6 with 750 mM NaCl. Protein concentration and average molar ratio of fluorescein: thrombin were determined as described previously [19] using absorption at 495 and 280 nm. Thrombin samples prepared as described above had maximal molar fluorescein thrombin ratios of 1.1:1 when aminoethyl thioureal fluorescein was used and 4:2:1 when aminohexyl thioureal fluorescein was used. Aliquots of these labeled proteins were frozen and stored at –60°C or stored at 4°C. In all cases experiments were performed within 2 weeks since thrombin labeled in this manner was not stable at 4°C or –60°C for prolonged periods.

Measurement of Thrombin Biological Activity: Proteolytic Activity, Receptor Binding and Initiation of Cell Division

To determine proteolytic activity we measured the ability of thrombin and thrombin derivatives to convert fibrinogen to fibrin as described by Lundblad et al [20] except that polyethylene glycol was substituted for acacia [21].

Ability of thrombin derivatives to bind to thrombin receptors on mouse embryo fibroblasts was determined by comparing their ability to compete with ^{125}I-labeled α-thrombin for specific binding sites as described previously [7]. Briefly, competition studies were performed in situ on cultures of nonproliferating mouse embryo cells containing approximately

5×10^5 cells per 35-mm dish. The cultures were rinsed and allowed to equilibrate with binding medium (serum-free medium containing 0.5% albumin, buffered with 15 mM Hepes at pH 7.0) for 30 min at 22°C. The medium was then changed to binding medium containing 10 ng per ml of ^{125}I-thrombin with indicated concentrations of unlabeled α-thrombin or fluorescein amine-labeled thrombin and incubated at 22°C for 120 min. Binding was terminated by removing the binding medium and rinsing the cultures through four beakers of phosphate buffered saline (PBS) at 4°C. The cultures were dissolved in 1 ml of 0.5 M NaOH and ^{125}I radioactivity was determined.

Ability of thrombin derivatives to initiate cell division was assessed by adding indicated concentrations of α-thrombin or fluorescein amine-labeled thrombin to nonproliferating cultures of secondary mouse embryo cells in serum-free medium prepared as described above. After 48 hr, cells were detached from their 35-mm culture dishes using 1 ml of PBS containing 0.02% EDTA and 0.05% trypsin. They were then diluted with PBS and counted in a Coulter electronic particle counter. Each experimental point is the mean of duplicate determinations which usually varied less than 5%.

Visualization of Fluorescent Thrombin by Direct Fluorescence Microscopy

Coverslip cultures of nonproliferating mouse embryo fibroblasts (approximately 1.5×10^4 cells per cm^2) were rinsed and equilibrated with binding medium for 30 min at 4°C. The binding medium was then changed to binding medium containing fluorescein amine-labeled thrombin (250 ng/ml) with or without unlabeled thrombin (5 μg/ml) and the cultures were incubated for 5 hr at 4°C. To terminate binding, cultures were rinsed through four beakers of PBS at 4°C. Some of these cultures were rinsed and immediately fixed with freshly prepared 3% formaldehyde for 20 min, others were increased to 37°C prior to rinsing and fixation.

Coverslip cultures were inverted onto microscope slides and examined by fluorescence microscopy using a Leitz Orthoplan microscope equipped with "ploemopack" illuminator and an I_2 fluorescent filter cube. Photographs were all taken through this microscope with 100 × phase objective (numerical apperture of 1.30). All photographs were 30-sec exposures on Kodak Tri-X film pushed to 1600 ASA by developing in Ethol Blue developer, printed on Agfa Rapidoprint contrast 4 single weight paper with identical exposures, and processed with a Kodak Ektamatic rapid processor.

RESULTS

Preparation of Biologically Active Fluorescent Thrombin

Labeling human α-thrombin with fluorescein isothiocyante or other molecules which couple to free-amino groups quantitatively inhibited thrombin activity and ability to bind to thrombin receptors. Therefore, we synthesized two fluorescein amines, 4-(N-6-aminoethyl thioureal)-fluorescein and 4-(N-6-aminohexyl thioureal)-fluorescein. When these fluorescein amines were linked directly to free carboxyl groups on thrombin using carbodiimide condensation there was also a considerable loss of proteolytic activity as judged by the ability of these thrombins to cleave fibrinogen and produce a fibrin clot. As an alternative form of linkage, we attached these same fluorescein amine molecules to the carbohydrate moiety on α-thrombin by periodate oxidation and reduction with sodium cyanoborohydride. As shown below, this procedure yielded thrombin with up to four fluoresceins per thrombin molecule with little, if any, effect on its biological activity.

Proteolytic activity of human α-thrombin did not appear to be affected by linking fluorescein amines to the carbohydrate moiety. As shown in Figure 1, thrombin labeled with 4-(N-6-aminoethyl thioureal)-fluorescein (molar ratio of 1.1 fluoresceins per thrombin) was at least 95% as active as unlabeled thrombin in cleaving fibrinogen to produce a fibrin clot.

To determine whether the fluorescent thrombin could bind to thrombin receptors, we incubated nonproliferating cultures of mouse embryo cells with ^{125}I-thrombin (10 ng/ml) for 2 hr at 22°C in the presence of increasing concentrations of unlabeled and aminoethyl fluorescein-labeled thrombin (molar ratio 1.1:1). Under these steady state conditions approximately 90% of the ^{125}I-thrombin specifically bound is associated with receptors on the cell surface [7]. As shown in Figure 2, the competition curves generated with unlabeled and fluorescein-labeled thrombin were virtually identical. This demonstrates that the fluorescein amine-labeled thrombin retained its full ability to bind specific receptors on the mouse embryo cells.

Fluorescein amine-labeled thrombin was also able to initiate division of mouse embryo cells. Figure 3 compares the mitogenic activity of unlabeled and aminohexyl fluorescein-labeled thrombin (molar ratio 4.2 fluoresceins per thrombin). As shown, at thrombin concentrations of 250 ng/ml the increase in cell number over controls with no addition after 56 hr was approximately 58% for aminohexyl fluorescein thrombin and 61% for unlabeled thrombin. At concentrations above 500 ng/ml the ability of fluorescein amine-labeled thrombin to initiate cell division appeared to decrease while stimulation by unlabeled thrombin continued to increase. This may indicate that high concentrations of carbohydrate modified fluorescein thrombin interfere with the normal initiation process. It should also be noted that there was approximately a twofold difference in concentration of labeled and unlabeled thrombin required for half maximal initiation. A similar difference in specific activity of this particular preparation was observed in proteolytic activity and

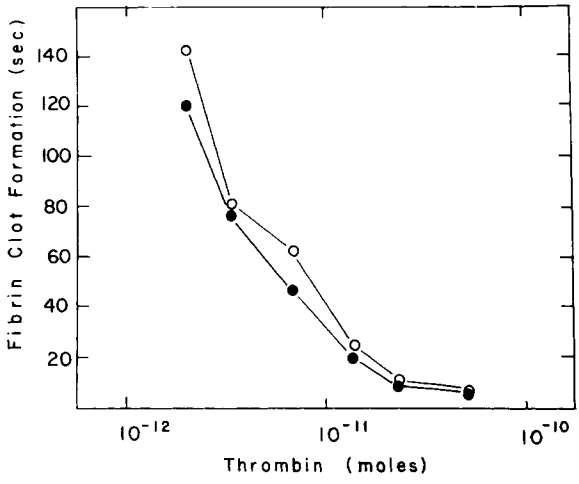

Fig. 1. Fibrinogen clotting activity of fluorescein amine-labeled and unlabeled human α-thrombin. Indicated concentrations of aminoethyl fluorescein-labeled thrombin (○) and unlabeled thrombin (●) were added to a standard fibrinogen solution at 22°C and the time required for fibrin clot formation was determined. Molar ratio of fluorescein to thrombin in this preparation was 1.1.

concentration required to compete for ^{125}I-thrombin binding. The shape of these activity curves, however, were identical to those of unlabeled thrombin (data not presented). This suggests that inactive molecules in this population do not interfere with activity or binding of labeled molecules to the thrombin receptors. Other preparations tested with fluorescein: thrombin ratios of closer to one, retained nearly all of their mitogenic activity consistent with their capacity to bind to thrombin receptors and cleave fibrinogen.

Visualization of Fluorescent Thrombin on ME Cells

In the above results, we have demonstrated that fluorescein amine-labeled thrombin preparations retain their proteolytic activity, can bind to thrombin receptors, and can initiate cell division. These studies, however, relied on average molar ratio of fluorescein to thrombin to determine the extent of labeling, leaving the possibility that the fluorescence was localized on highly fluorescinated, inactive molecules which would only bind nonspecifically to cells or that the fluorescent label might be dissociating from thrombin during incubation. To determine whether the actual molecules which were labeled with fluorescein were binding specifically, we performed competition experiments with fluorescein amine-labeled thrombin with or without a 20-fold excess of unlabeled thrombin and then examined these cultures with fluorescence microscopy. As shown in Figure 4A, when aminohexyl fluorescein-labeled thrombin (250 ng/ml, 4.2 fluoresceins/thrombin) was incubated for 5 hr at 4°C with ME cells, a diffuse pattern of fluorescence could be observed and photographed without the aid of video intensification. In contrast, in parallel cultures incubated with the same fluorescein amine-labeled thrombin plus a 20-

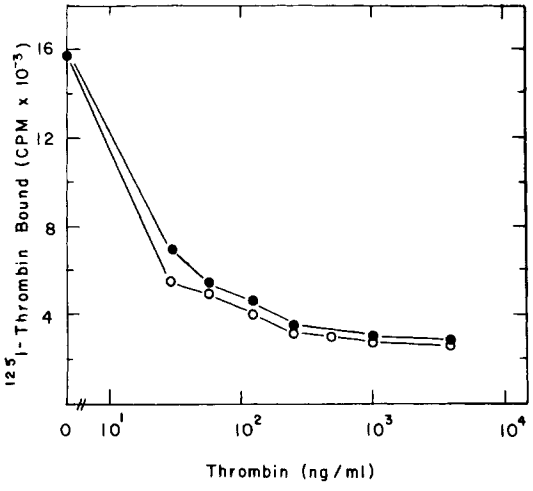

Fig. 2. Ability of fluorescein amine-labeled and unlabeled thrombin to compete for binding of ^{125}I-thrombin to ME cells. Nonproliferating cultures of ME cells (5.5×10^5 cells per 35 -mm dish) prepared as described in Materials and Methods were incubated at 22°C for 2 hr with ^{125}I-thrombin (10 ng/ml, prepared as described previously [7]) with the indicated concentrations of aminoethyl fluorescein-labeled thrombin (○) or unlabeled thrombin (●). Cells were rinsed and the total ^{125}I-thrombin radioactivity bound to the cells was determined. The molar ratio of fluorescein:thrombin in this preparation was 1.1.

fold excess of unlabeled thrombin (Fig. 4B), the only fluorescence observed was that which could be attributed to autofluorescence (compare Fig. 4B and 4C). This demonstrated that the fluorescein amine-labeled thrombin molecules were themselves binding specifically to receptors on ME cells.

It should be noted that in these experiments virtually all of the cells incubated with fluorescein amine-labeled thrombin alone had about the same level of diffuse fluorescence. Thus, the brightly fluorescent cell depicted in Figure 4A does not merely represent a select population of cells which take up fluorescent dye. There was some variation between cells and between cultures in the number of bright spots which could be observed in the cells. These bright spots, however, do not represent aggregated receptors or aggregates of free fluorescein label because they were also observed in cells incubated with excess unlabeled thrombin and in control cells which were not exposed to any fluorescent label (Note Fig. 4C). Thus, these bright spots appear to be due to aggregates or vesiculated autofluorescent molecules within the cells.

The more diffuse brighter fluorescent pattern in all cases was specific for cells incubated with fluorescent thrombin alone and was easily distinguishable from the nonspecific and autofluorescent patterns. In many cases this diffuse fluorescence appeared to have a mottled or fibrous appearance (see also Fig. 5A). This pattern reflects the normal contours and projections of the cell surface. Thus this somewhat irregular diffuse fluorescent pattern suggests that the fluorescein-labeled thrombin is specifically bound under these conditions to receptors randomly distributed over the entire surface of the cells.

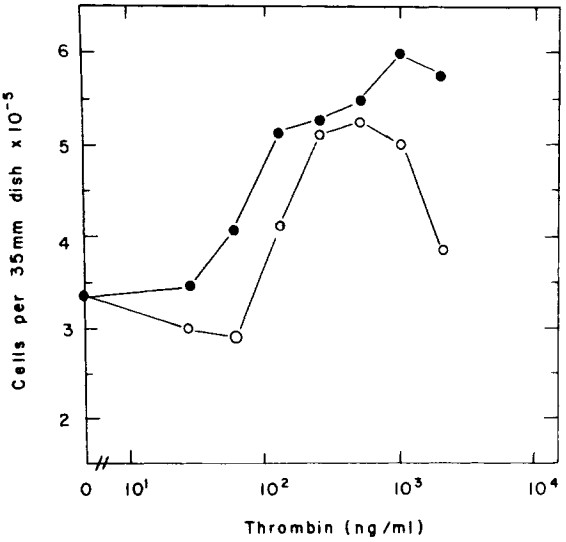

Fig. 3. Effect of various concentrations of fluorescein amine-labeled and unlabeled thrombin on initiation of ME cell division. ME cells were plated in 35-mm dishes at a density of 6.2×10^4 cells per cm^2. After complete cell attachment (16 hr) the cultures were rinsed and the medium changed to serum-free medium. After 2 days the indicated concentrations of aminohexyl fluorescein-labeled thrombin (○) or unlabeled thrombin (●) were added to each culture. Cell number was determined 56 hr later. The molar ratio of fluorescein:thrombin in this preparation was 4.2.

To further insure that the diffuse pattern observed did not reflect fluorescent molecules which were released from thrombin during incubation, cells were incubated with free fluorescein amines under conditions identical to those used in the experiments depicted in Figure 4. In all cases, with free fluorescein concentrations up to 50 ng/ml (about 10 X the amount of fluorescein in 250 ng of fluorescent thrombin), there was no fluorescence observed above that which was due to autofluorescence alone (data not presented). Cells incubated with thrombin plus free fluorescein amines also did not appear to bind or internalize the free fluorescent molecules. Thus from these experiments it has been possible to show that the fluorescein amine-labeled thrombin binds specifically to receptors on the surface of mouse embryo cells at 4°C, that free fluorescein amines cannot duplicate this pattern, and by the diffuse but somewhat complex appearance of the fluorescent pattern, that at 4°C the thrombin receptors to which these molecules bind are randomly distributed over the entire cell surface.

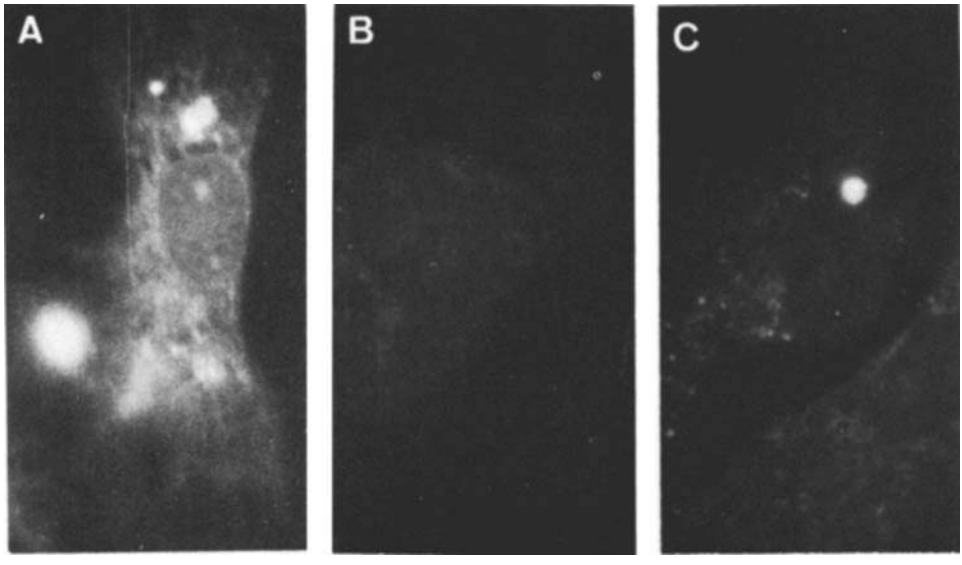

Fig. 4. Specificity and pattern of aminohexyl fluorescein thrombin binding to ME cells at 4°C. Aminohexyl fluorescein-labeled thrombin (250 ng/ml) was incubated with nonproliferating cultures of ME cells plated on glass coverslips at 4°C for 5 hr in the presence or absence of unlabeled thrombin (5 μg/ml). These coverslip cultures and ones incubated without fluorescent thrombin were then rinsed four times with PBS at 4°C, fixed with 3% formaldehyde and photographed using identical exposures through a fluorescent microscope as described in Materials and Methods. (× 4,250) A) Aminohexyl fluorescein thrombin alone. B) Aminohexyl fluorescein plus 5 μg/ml unlabeled thrombin. C) Autofluorescence of cells incubated without fluorescent thrombin.
Note: The molar ratio of fluorescein to thrombin in this preparation was 4.2. A similar diffuse pattern was also observed with aminoethyl fluorescein thrombin preparations (molar ratios of ~ 1.0); however, these patterns were much dimmer and only clearly discernable as specific patterns on cultures with low autofluorescence. It should also be noted that after a 5-hr incubation at 4°C, thrombin binding is approaching steady state with almost all of the cell-associated thrombin molecules on the cell surface [7].

Receptors for other peptide growth factors and hormones have also been reported to be evenly distributed over the surface of cells at 4°C [14–16]. In these cases, warming the cells to 22° or 37°C following 4°C binding resulted in aggregation of the fluorescent hormone-receptor complexes and their internalization into vesicles by receptor mediated endocytosis.

To determine whether a similar process occurred with the thrombin receptor complexes, we incubated cells for 5 hr at 4°C with fluorescein amine-labeled thrombin as described above and then increased the temperature to 37°C. As shown in Figure 5, rather than aggregating or being internalized into vesicles, as the temperature was increased the fluorescent thrombin seemed to disappear from the ME cells. This phenomena was observed in separate experiments with both ethylamine and hexylamine fluorescein-labeled thrombin preparations. In all cases as early as 1 min after increasing the temperature to 37°C there was considerably less diffuse fluorescence and by 5 min the diffuse pattern was gone leaving just the autofluorescent vesicles. These unexpected results suggest that the interaction between thrombin and its receptor might be quite different from that of other nonproteolytic growth factors and hormones.

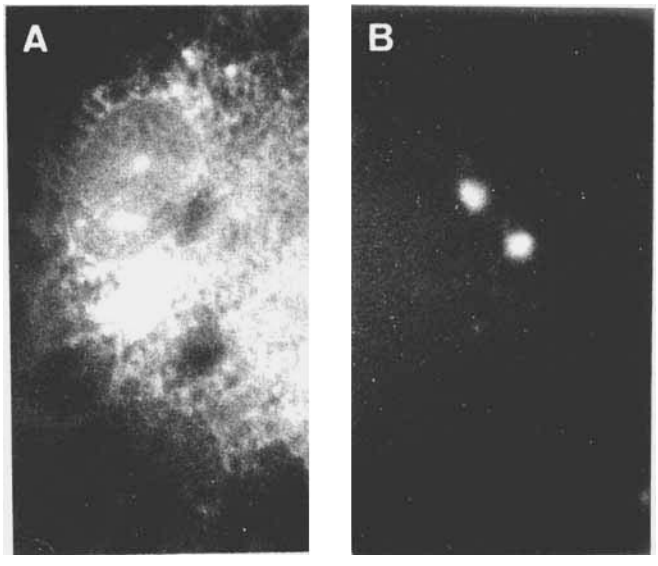

Fig. 5. Effect of 37°C incubation on the cellular pattern of fluorescent thrombin. Coverslip cultures of ME cells were prepared and incubated with aminohexyl fluorescein thrombin (250 ng/ml) as described in Figure 4. After 5 hr of incubation at 4°C, the cultures were either rinsed and fixed for examination (A) or incubated for 5 min at 37°C and then rinsed and fixed for examination (B). The cultures were then photographed as in Figure 4 using identical exposures, processing and printing (see Materials and Methods).

DISCUSSION

As an initial step in determining whether thrombin receptor redistribution on the surface of fibroblasts plays a role in thrombin initiation of cell division, we have synthesized a new fluorescent derivative and coupled this molecule to the carbohydrate moiety on human α-thrombin. Fluorescent thrombin prepared in this manner retains its proteolytic activity, its ability to bind to thrombin receptors on ME cells, and its ability to initiate ME cell division. Most importantly, with this derivative, thrombin receptors on the surface of ME cells can be visualized by direct fluorescence microscopy.

A number of recent studies have utilized fluorescent derivatives of insulin, EGF, and α-2-macroglobulin to visualize the interaction of these molecules with receptors on the surface of cells [13,14]. These molecules appear to bind to receptors which are diffusely distributed and then to rapidly aggregate and be internalized into vesicles [16]. From ultrastructural studies with ferritin derivatives it appears that such molecules are internalized into coated vesicles [15, 16]. These coated vesicles, however, are much smaller than the large fluorescent agggregates observed with video intensified fluorescence microscopy. Therefore, it is not clear exactly what these large patches represent. A further problem in some of these studies has been a lack of specificity of the fluorescently labeled hormone. For example, highly fluorescent insulin has been prepared by conjugating FITC-labeled lactalbumin to insulin. This preparation retained less than 9% of its ability to bind to insulin receptors and less than 2% of its biological activity [22]. In such cases, it is difficult to be convinced that visualized redistributions are truly receptor mediated.

To get around problems of possible loss of specificity in labeled thrombin molecules, we have utilized a new procedure for direct fluorescent labeling of human α-thrombin using 4-(N-6-aminoethyl thioureal)-fluorescein or 4-(N-6-aminohexyl thioureal)-fluorescein [Carney, Jones and Weigel, manuscript in preparation]. This fluorescein amine was linked to the carbohydrate moiety of thrombin using a modification of a procedure described by Yang, Fenton and Feinman [18] for coupling dansyl hydrazine to human α-thrombin. This gentle procedure for fluorescent labeling should have wide applicability for a number of glycoprotein molecules. In the case of thrombin, molar ratios of 1.1 and 4.2 fluoresceins per thrombin with ethyl amine and hexyl amine derivatives were achieved, respectively, suggesting that the length of the spacer arm separating the fluorescein and carbohydrate molecules is important in determining the extent of labeling. These fluorescent thrombin preparations retained virtually all of their ability to cleave fibrinogen and form a fibrin clot and their ability to bind to the thrombin receptors. In addition, they were able to initiate division of nonproliferating ME cells 50–90% as effectively as unlabeled human α-thrombin. Thus, we have prepared fluorescent thrombin with up to four fluoresceins per molecule with little effect upon interactions between thrombin and the cell surface or its substrate molecules.

When fluorescein amine-labeled thrombin (250 ng/ml) was incubated for 5 hr at 4°C with ME cells, a diffuse pattern of fluorescence was observed over the surface of the cells. In parallel cultures, incubated with both fluorescent thrombin and a 20-fold excess of unlabeled α-thrombin, the diffuse pattern was not detectable indicating that this pattern was specific for fluorescent thrombin bound to thrombin receptors. This concentration of fluorescent thrombin produced a 58% increase in number of ME cells relative to controls over a 56-hr incubation. Thus, with this fluorescent thrombin preparation the interactions between thrombin and its receptors were visualized at normal mitogenic concentrations.

In the present study, when fluorescein amine-labeled thrombin was incubated with ME cells at 4°C for 5 hr and then the temperature increased to 37°C, there was no observable receptor redistribution on cell surfaces or internalization into vesicles. Instead, the diffuse pattern disappeared. This was not accompanied by an apparent increase in the number or intensity of fluorescent vesicles, which in these cells appear to be due to autofluorescence.

There are several possible explanations for the disappearance of the fluorescent pattern as the temperature was increased on these cells. For example, it is possible that the carbohydrate label was cleaved from thrombin by cell surface enzymes which were inactive at 4°C. In kinetic experiments with ^{125}I labeled thrombin at the same concentration, we have observed that at 37°C there is a dissociation of labeled thrombin from ME cells at about 1 hr [Crossin and Carney, unpublished observations]. This suggests that a dissociation of thrombin from cells following receptor binding might be a normal step in the sequence of events leading to a mitogenic signal. It is also possible that in the present experiments incubating the ME cells at 4°C altered a cytoskeletal anchorage of the receptors and accelerated the release of thrombin or the thrombin receptor complex as the temperature was increased. Along these lines, we have recently discovered that drugs which interfere with microtubule polymerization affect thrombin binding and its mitogenic acticity on ME cells [Crossin and Carney, manuscript in preparation].

Finally, the rapid dissociation of thrombin at 37°C could represent proteolytic cleavage of thrombin receptors dramatized in these experiments in which binding occurred at 4°C where thrombin is proteolytically inactive. Indeed, several lines of evidence indicate that proteolytic cleavage of the thrombin receptor is necessary for thrombin to initiate cell division [10, 11]. Thus, the dissociation of fluorescent thrombin observed in the present experiments at 37°C may reflect cleavage of the thrombin receptor. This would also explain why this type of dissociaton has not been observed with nonproteolytic mitogens. Further studies utilizing other types of labeled thrombin, indirect immunofluorescence, and a more sensitive video intensified fluorescence microscope are underway to sort out these possibilities.

Establishing that thrombin receptors on ME cells can be visualized using mitogenic concentrations of fluorescent thrombin provides the basis for determining whether or not changes in distribution or release of thrombin from the cell surface correlate with initiation of cell division. The unexpected finding that the fluorescent thrombin pattern was lost after increasing the temperature to 37°C, if substantiated by other techniques, may point to the unique ability of thrombin as a proteolytic mitogen to cleave its own receptor and thus initiate cell division.

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