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Receptor-Bound Thrombin Is Not Internalized Through Coated Pits in Mouse Embryo Cells

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The localization of thrombin receptors on mouse embryo (ME) cells was examined using electron microscope (EM) immunocytological techniques. ME cells were fixed with formaldehyde, prior to thrombin binding, and thrombin visualized on cell surfaces using affinity-purified antithrombin rabbit antibody and colloidal gold labeled anti-rabbit IgG. Colloidal gold particles were found in clusters on the surface of cells incubated with thrombin. There were approximately seven particles per cluster observed in thin sections with cluster diameters ranging from 70 to 200 nm. These clusters were not observed on cells incubated without thrombin. The total number of particles present on cells incubated with and without thrombin indicate that the colloidal gold labeling is approximately 98% specific for thrombin. Only four colloidal gold particles out of approximately 1,200 were associated with coated pits. Thus the thrombin receptor clusters do not appear to associate with coated membrane regions. To determine whether receptor-bound thrombin was internalized by receptor-mediated endocytosis, ME cells were incubated with ^{125}I-thrombin and examined using EM autoradiography and the trypsin sensitivity of ^{125}I-thrombin which was associated with the cells. In two types of experiments, where thrombin was incubated with cells at 4°C and the temperature increased to 37°C and where initial incubation was at 37°C, the receptor-directed specific internalization proceeded at approximately the same rate as nonspecific internalization. These studies indicate that thrombin that binds to its receptors on ME cells is not rapidly internalized by receptor-mediated endocytosis.

Key words: thrombin, receptor-mediated endocytosis, coated pits, immunocytochemistry, growth factors

A number of growth factors including insulin and epidermal growth factor (EGF) have been shown to enter cells by receptor-mediated endocytosis [1,2]. In this process, the growth factors bind to receptors, aggregate into clathrin-coated pits, and are rapidly internalized. Shortly after internalization the vesicles appear to lose their clathrin coats and subsequently fuse with lysosomes [2]. Thus, a major function of this endocytic pathway may be to clear receptor-bound molecules from the cell surface and provide a mechanism for their rapid degradation. Indeed, both insulin and EGF

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are rapidly internalized and degraded [3,4]. It is not clear, however, whether this clearance and internalization are involved in the biological actions of these factors.

Previous studies have shown that thrombin action at the cell surface is sufficient to initiate cell division in nonproliferating serum-free cultures of chick embryo cells [5,6]. Thrombin binds to high affinity receptors on a number of cell types [7], but most of the internalization and degradation of thrombin appears to be mediated through "protease nexin" rather than the mitogenic receptor [8]. In chick embryo cells ^{125}I-thrombin accumulates inside the cells and remains intact and active for many hours [9,10]. Thus, neither internalization nor degradation of thrombin appears to be involved in its initiation of proliferative events.

We have recently demonstrated with both immunoflourescent staining [11] and electron microscopy (EM) autoradiography [12] that thrombin receptors are clustered on the surface of cells prior to thrombin binding. From the EM autoradiography studies, it appears that the receptor clusters do not associate with coated regions of the membrane and are not collected in coated pits or internalized into coated vesicles. These studies, however, were somewhat limited by the resolution of EM autoradiographic techniques.

In the present studies, we have utilized colloidal gold labeled IgG to obtain a more direct visualization of thrombin binding and to investigate the relationship between thrombin receptors and coated membrane regions. These studies confirm the clustering of thrombin receptors and that these receptor clusters are not associated with coated membrane regions. We also have used EM autoradiography to follow the binding and internalization of ^{125}I-thrombin to determine whether specific receptor-mediated internalization is occurring by a process other than the coated-pit pathway.

MATERIALS AND METHODS

Materials

Dulbecco-Vogt modified Eagle's medium (DV medium) and medium supplements were purchased from Grand Island Biological Company, New York; calf serum from Irvine Scientific, California; and tissue culture dishes from Lux Scientific, California. Highly purified human thrombin (3,000 NIH units/mg) was generously provided by John W. Fenton II. Sodium iodide (IMS-30, approximately 17 mCi/μg) was purchased from Amersham, Illinois. Chemicals for EM and EM autoradiography were obtained from Polysciences, Pennsylvania, and from Ted Pella, Inc., California.

Cells and Cell Culture

Primary cultures of mouse embryo (ME) cells were prepared from the body walls of 11- to 13-day-old mouse embryos and cultured in DV medium supplemented with 10% calf serum. After 3–5 days, the primary cultures were subcultured into 60-mm Permalux dishes (5.2×10^6 cell cm^{-2}). After 16 hr the cells were rinsed and the medium changed to serum-free DV medium. EM autoradiography and colloidal gold receptor localization experiments were performed 2 days later. At this time the cells are quiescent (90% G_0/G_1) and are mitogenically responsive to human thrombin at a concentration of 125 ng/ml [7].

Thrombin Antibodies

Antithrombin antibodies were raised in rabbits as described [13] using multiple injections of highly purified human α-thrombin that had been proteolytically inacti-

vated by incubation with diisopropylphosphofluoridate [14]. Specific thrombin antibodies were purified from immune rabbit serum by affinity chromatography using highly purified thrombin coupled (by cyanogen bromide) to CL Sepharose 4B as previously described for purification of antitubulin antibodies [15]. These antithrombin antibodies form single precipitin bands with human α-thrombin in Ouchterlony diffusion tests and can precipitate ^{125}I-thrombin following incubation with whole formalin-fixed Staph A (Bethesda Research Laboratories, Maryland). Antibodies aliquots were stored at 4°C or at −20°C to avoid repetitive freezing and thawing.

Colloidal Gold Thrombin Label

Experiments were performed on monolayer cultures of nonproliferating ME cells. Cultures were rinsed and allowed to equilibrate with binding medium (serum-free DV medium containing 0.5% albumin buffered with 15 mM HEPES at pH 7.0) for 10 min at 23°C. Cells were fixed 10 min in a freshly made solution of 3% paraformaldehyde in PBS, rinsed twice in binding medium, and incubated with 125 ng/ml thrombin for 2 hr at 23°C. Following binding, cells were rinsed in PBS and incubated with monospecific rabbit antithrombin for 60 min at 23°C. The cells were rinsed in PBS and labeled with goat-antirabbit IgG-gold colloidal particles (EY Laboratories, California) for 60 min at 23°C. The cultures were rinsed with PBS and fixed in their culture dishes with 3% glutaraldehyde in 0.1 M PIPES buffer for 60 min at 23°C. The cells were post fixed in 1% O_sO_4 in 0.1 M PIPES buffer, rinsed twice in PBS and distilled water, dehydrated in graded ethanol, and embedded in Epon 812. Culture dishes were removed and the Epon/cell surface reembedded in Epon 812. Thin sections (approximately 70 nm thick) were cut perpendicular to the cell monolayer with a diamond knife (Diatome) on an LKB-2 ultramicrotome and mounted on 200-mesh grids. Sections were examined and photographed on a Philips 201 electron microscope at 80 Kv.

Iodination of Thrombin

Thrombin was iodinated using lactoperoxidase as described previously [7]. Using a 1:1 ratio of radioactive to nonradioactive iodine, thrombin was prepared with specific activities up to 4.3×10^4 CPM/ng, corresponding to approximately 1.1 iodine/thrombin molecule. The proteolytic activity of each iodinated thrombin preparation was determined by its ability to convert fibrinogen to fibrin [16]. All iodinated thrombin used for these experiments retained greater than 80% of its proteolytic activity.

^{125}I-Thrombin Binding

Experiments were performed on monolayer cultures of nonproliferating ME cells. Cultures were rinsed and allowed to equilibrate with binding medium for 30 min at 4°C or 37°C. Medium was then changed to binding medium containing 125 ng per ml of ^{125}I-thrombin ± 2.5 μg per ml (20-fold excess) of unlabeled thrombin to determine both total and nonspecific thrombin binding. Cells were incubated at 37°C for 10, 35, and 60 min. Cells chilled to 4°C were incubated with ^{125}I-thrombin for 3.5 hr at 4°C, at which time the cells were rinsed. To examine redistribution of ^{125}I-thrombin the temperature was increased to 37°C for 1.5, 5, and 10 min. After appropriate incubations, all cells were prepared for quantitation of specific and nonspecific internalized and surface-bound ^{125}I-thrombin by counting ^{125}I radioactivity and by EM autoradiography. In all cases, specific thrombin binding was estimated

by subtracting the amount of radioactivity or number of grains observed per cell in the presence of unlabeled thrombin (nonspecific binding) from the total amount observed.

It should be noted that up to 8% of ^{125}I-thrombin and essentially all urokinase binding to ME cells can be attributable to formation of protease-nexin complexes in conditioned medium and the binding of these complexes back to the cells [8,17]. In the present experiments, where the cells are rinsed, equilibrated with binding medium, and then introduced to fresh binding medium containing thrombin, we do not observe any protease-nexin-^{125}I-thrombin complexes on $NaDodSO_4$ gels and little if any specific urokinase binding (Crossin and Carney, unpublished). Thus, under these conditions ^{125}I-thrombin binding, as well as unlabeled thrombin binding for immunocytological localization, should not be complicated by protease-nexin binding.

Measurement of Trypsin-Sensitive and Trypsin-Insensitive Thrombin

The amount of soluble ^{125}I-thrombin internalized by ME cells was measured by determining the amount of trypsin-insensitive radioactivity as described by Zetter et al [9]. Briefly, following ^{125}I-thrombin exposure, cells were incubated at 37°C for 10 min in PBS containing 10 μg/ml trypsin. Cells were removed from the plates and placed into 1.5-ml microfuge tubes and allowed to incubate an additional 15 min. The cells were sedimented at 8,700g for 2 min, the supernatant removed, and the ^{125}I radioactivity associated with the supernatant (surface-bound, trypsin-sensitive) and the cell pellet (internalized, trypsin-insensitive) was measured in a gamma counter.

EM Autoradiography

Following incubation with ^{125}I-thrombin, cells were fixed, embedded, and sectioned as previously described. To increase autoradiographic resolution, sections were stained with uranyl acetate and lead citrate prior to overlaying a 0.1 μm film of Ilford L4 emulsion [5]. Exposure was carried out for 2 months at 4°C. The emulsion was developed in Kodak Microdol-X for 5 min, and the sections were restained with uranyl acetate and lead citrate before examination.

RESULTS

Visualization of Thrombin on Cells Using Colloidal Gold IgG

Previous studies from our laboratory using immunoflourescence and EM autoradiography have indicated that thrombin receptors are clustered on the surface of cells prior to thrombin binding. To confirm these observations more directly, we have utilized colloidal gold IgG immunocytological labeling of thrombin bound to specific receptors on mouse embryo (ME) cells. For these studies, ME cells were fixed with formaldehyde prior to incubation with thrombin to restrict receptor mobility. Previous studies with ^{125}I-thrombin binding have shown that this fixation restricts receptor internalization, but that it does not affect the amount of ^{125}I-thrombin bound or the proportion of ^{125}I-thrombin specifically bound to thrombin receptors [12]. Furthermore, immunofluorescent studies of thrombin binding to ME cells have indicated a similar pattern of thrombin binding to fixed cells and to nonfixed cells incubated with thrombin at 4°C [11]. As shown in Figure 1, EM micrographs of prefixed cells incubated with a mitogenic concentration of thrombin (125 ng/ml) for 2 hr at 23°C show colloidal gold associated with the plasma membrane in discrete clusters. These

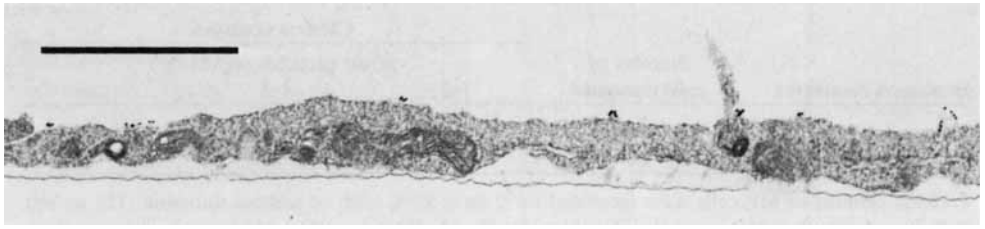

Fig. 1. Electron micrograph of prefixed ME cell incubated with thrombin, antithrombin, and colloidal gold IgG. Cells were fixed in formaldehyde, incubated for 2 hr at 23°C with thrombin (125 ng/ml), and labeled with colloidal gold IgG as described in Methods. As shown, colloidal gold particles (small electron-dense dots) are associated with the plasma membrane in discrete clusters. As described in Table I, these colloidal gold clusters were not observed on cells incubated without thrombin. Scale bar=0.5 μm.

clusters were periodically spaced on both upper and lower surfaces of some cells, much the same as the localization and spacing of autoradiographic grains observed with EM autoradiography following binding of ^{125}I-thrombin to both fixed and nonfixed cells [12]. It should be noted, however, that the even spacing was not observed on all cells, perhaps reflecting the heterogeneity of these ME cell populations [11,12].

In previous EM autoradiographic studies, the distribution and spacing of autoradiographic grains indicated that a number of receptors were clustered in each location. As shown in Table I, most of the colloidal gold particles were observed in thin sections as aggregates of five or more on the surface of ME cells. An average of seven colloidal gold particles were observed in each cluster in the thin sections examined. The observed diameter of clusters with five or more particles varied from 70 to about 200 nm. Since the estimated maximal length for a single IgG molecule is approximately 23 nm [18], it would be impossible for these colloidal gold particles all to cluster around a single thrombin molecule bound to the cell. It would thus appear that these clusters represent true clusters of multiple thrombin molecules bound to the cell surface. Considering the differences between observed thin-section diameter and actual spherical diameter [19], these observations suggest that the average thrombin receptor cluster might occupy a space on the order of 200 nm in diameter.

Cells incubated in parallel without thrombin had very few colloidal gold particles on their surface. As shown in Table I, most of these particles were observed as single particles. In examining an equivalent number of cell sections, only 20 particles were found on cells incubated without thrombin, compared to approximately 1,200 where thrombin was incubated with cells. Thus, the antibody-directed colloidal gold labeling was more than 98% specific for thrombin. In addition, this shows that the aggregates observed represent receptor-bound thrombin and not antibody aggregation on the cell surface.

As shown in Figure 2, coated-pit regions of the membrane could be easily identified in these micrographs. In all, 85 coated pits were observed. Large aggregates of colloidal gold particles were not observed in any of these pits, and only three of the 85 coated pits contained any colloidal gold. Of these three coated pits, one had two particles and the other two had one particle each. Of the total number of colloidal gold particles observed (approximately 1,200), the four associated with coated pits

TABLE I. Clustering of Colloidal Gold–IgG Complexes on ME Cell Surfaces

Incubation conditions	Number of cells examined	Clusters observed (Gold particles per cluster) 1–2	3–5	>5
Thrombin (125 ng/ml)	69	46 (20%)	63 (27%)	122 (53%)
No thrombin	60	15 (94%)	1 (6%)	0

Prefixed cultures of ME cells were incubated for 2 hr at 23°C with or without thrombin (125 ng/ml). Following incubation, cells were rinsed and incubated with affinity-purified rabbit antithrombin antibody and colloidal gold antirabbit IgG. Thin sections were prepared and EM analysis performed as described in Methods. All gold particles lying closer than 100 nm to an adjacent particle were scored as being within a cluster. Numbers presented represent the number of times clusters of given size were observed. The percentages in parentheses represent the frequency of observing clusters of these sizes. Total number of colloidal gold particles observed following incubation with thrombin was 1,231 and without thrombin 20. The specific binding of colloidal gold IgG to thrombin was thus greater than 98%.

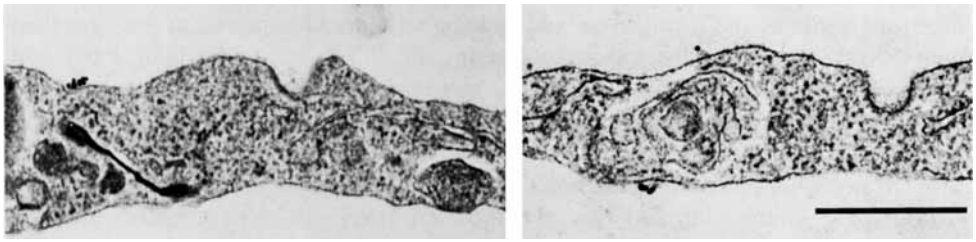

Fig. 2. Electron micrographs of coated pits on ME cells incubated with thrombin (125 ng/ml). Cells were prepared and labeled with colloidal gold as described in the legend to Figure 1. Note that the colloidal gold clusters are not associated with the coated pits. Scale bar=0.5 μm.

represent less than 0.4%. Therefore, thrombin receptor clusters do not appear to associate with coated membrane regions in these cells.

Is Thrombin Internalized by Receptor-Directed Endocytosis?

Because thrombin receptor clusters do not appear to associate with coated pits, one must question whether or not there is specific internalization of thrombin that is related to thrombin binding to these clustered receptors. To answer this question, we examined specific and nonspecific internalization of ^{125}I-thrombin utilizing both EM autoradiography and the amount of trypsin-sensitive and -insensitive radioactivity associated with the ME cells.

Figure 3 is a representative EM autoradiograph of ^{125}I-thrombin binding to receptors on ME cells. As shown, most of the autoradiographic grains are still found on the cell surface after a 1-hr incubation at 37°C. By comparing the average number of autoradiographic grains observed per cell, with the amount of ^{125}I-thrombin bound per culture, we estimate that each grain in these autoradiographs represents approximately 40 thrombin molecules.

One method of examining receptor-mediated endocytosis has been to load surface receptors at 4°C, where there is little if any endocytosis, and then to increase the temperature to 37°C and follow redistribution of labeled ligand. As shown in Figure 4, if ^{125}I-thrombin (125 ng/ml)is incubated with ME cells for 3.5 hr at 4°C,

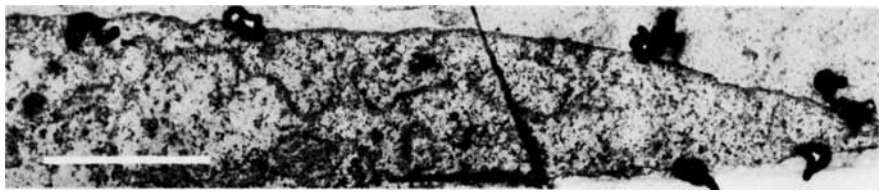

Fig. 3. EM autoradiograph of ^{125}I-thrombin bound to receptors on ME cells. The cells were incubated with ^{125}I-thrombin (125 ng/ml) for 1 hr at 37°C and prepared for EM autoradiography as described in Methods. In this micrograph the dark squiggles represent autoradiographic grains or tracks through emulsion. These grains appear to be evenly spaced approximately 1.5 μm apart over the surface of the cell. Since each grain represents about 40 molecules of ^{125}I-thrombin, this even spacing indicates clusters of ^{125}I-thrombin molecules [12]. It should be noted that one of these grains, the third from the left, is approximately 0.35 μm from the surface; thus, this grain would be scored as an internalized grain in Figure 6. In these experiments, examining thin sections through 38 cells showed 18.8 grains per cell section total binding, of which 5.0 grains per cell section were nonspecific as determined in sections through 56 cells incubated with ^{125}I-thrombin in the presence of a 20-fold excess of unlabeled thrombin. Thus, approximately 73% of the grains represent specific binding. This corresponds to the percentage of specific binding determined by counting radioactivity on parallel plates. Scale bar = 2.0 μm.

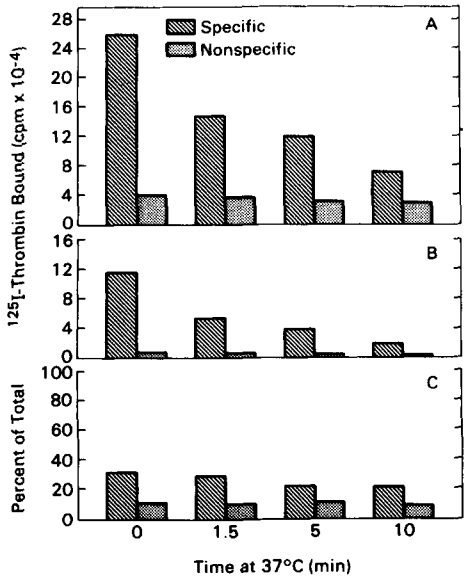

Fig. 4. The effect of 37°C incubation on specific and nonspecific ^{125}I-thrombin internalization. ME cells were incubated with ^{125}I-thrombin (125 ng/ml) for 3.5 hr at 4°C, and the temperature was increased to 37°C for 1.5, 5, and 10 min. The quantities of ^{125}I-thrombin internalized were measured by determining trypsin-sensitive and trypsin-insensitive radioactivity, as described in Methods. A) Trypsin-sensitive radioactivity (surface-releasable); B) trypsin-insensitive radioactivity (internalized); C) trypsin-insensitive radioactivity expressed as a percent of total (trypsin-sensitive plus -insensitive) radioactivity for both specific and nonspecific thrombin binding.

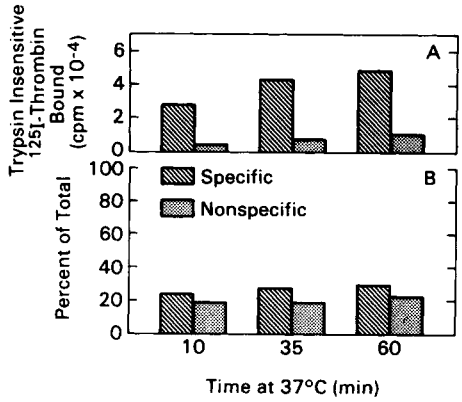

Fig. 5. The rate of specific and nonspecific internalization of ^{125}I-thrombin during 37°C incubation. ME cells were incubated with ^{125}I-thrombin (125 ng/ml) for 10, 35, and 60 min at 37°C. Trypsin-sensitive and -insensitive radioactivities were determined as described in Methods. A) Trypsin-insensitive (internalized) radioactivity; B) trypsin-insensitive radioactivity expressed as percentage of total (trypsin-sensitive plus -insensitive) radioactivity for both specific and nonspecific thrombin binding.

approximately 75% of the ^{125}I radioactivity is removed from the surface of cells by incubation with trypsin. In parallel experiments with EM autoradiography, approximately 95% of the grains are located within 0.2 μm of the cell surface. Thus, at least part of the trypsin-insensitive material may be trapped at the cell surface or may be conformationally altered so that it can no longer be cleaved by trypsin. If there were specific receptor-mediated endocytosis of the ^{125}I-thrombin, we would expect the amount of trypsin-insensitive material to increase rapidly as the temperature was increased to 37°C. As shown, however, there is a decrease in both trypsin-sensitive and trypsin-insensitive radioactivity. This rapid loss suggests that under these conditions there is a loss of bound thrombin from the surface of ME cells. It should be noted that the percentage of total specific and nonspecific trypsin-insensitive material remains at approximately the same level as temperature is increased to 37°C, suggesting that any internalization that is occurring is not specifically associated with the thrombin receptors.

It seemed possible that the 4°C incubation might be altering the affinity of thrombin or the location of binding such that, as the temperature was increased, molecules of thrombin which might normally be internalized were being lost. To avoid any possible 4°C artifacts, ^{125}I-thrombin was incubated with ME cells for various lengths of time at 37°C. As shown in Figure 5A, with increasing length of incubation there is a slight increase in the amount of trypsin-insensitive radioactivity under both specific and nonspecific binding conditions. However, the percentage of total radioactive thrombin (trypsin-sensitive and -insensitive) remained fairly constant during this period (Fig. 5B). This suggests that under these conditions, as well, there is little if any specific receptor-mediated endocytosis.

These observations were confirmed by counting the number of EM autoradiographic grains on the surface of cells and those that were judged to be internalized ($\geqslant$0.2 μm from the surface). As shown in Figure 6A, the number of grains observed on the surface increased during a 60-min incubation. During this period the number of grains localized inside the cell showed only a slight increase (Fig. 6B). Most of the internalized grains, however, represent nonspecific internalization rather than spe-

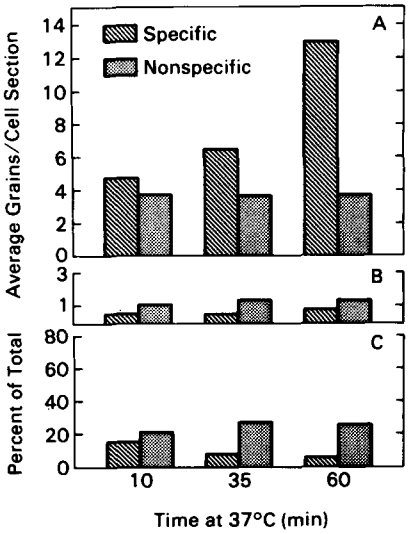

Fig. 6. Rate of binding and internalization of ^{125}I-thrombin during 37°C incubation as determined by location of EM autoradiographic grains. ME cells were incubated with ^{125}I-thrombin (125 ng/ml) for 10, 35, and 60 min at 37°C and prepared for EM autoradiography as described in Methods. Sections through approximately 50 cells were examined for each determination of total and nonspecific thrombin localization at each time point. In all, 286 cells were examined, and the location of 2,261 grains was determined. A) Surface bound ^{125}I-thrombin expressed as average number of grains per cell thin section (grains less than 0.2 μm from the cell surface (see Fig. 3). B) Internalized ^{125}I-thrombin (grains farther than 0.2 μm from the cell surface). C) Internalized ^{125}I-thrombin autoradiographic grains expressed as percentage of total (surface-bound and internalized) for both specific and nonspecific thrombin binding.

cific. As shown in Figure 6C, the percentage of specifically bound thrombin that is internalized actually decreases during this period. These numbers are quite different from the rapid increase in specific internalization expected if receptor-mediated endocytosis of thrombin were occurring. It thus appears that in normal interaction between thrombin and its receptors on ME cells, there is little if any specific receptor-mediated endocytosis.

DISCUSSION

A great deal of emphasis has been directed recently to receptor-mediated endocytosis and to the possible role of this process in the biological effects of various types of hormones and growth factors. The present studies have shown that thrombin binds to clustered receptors on the surface of mouse embryo cells, and that these clusters are not associated with coated pits.

Thrombin binding to ME cells was visualized by immunocytological staining with colloidal gold IgG. Cells were prefixed with formaldehyde to restrict receptor redistribution and incubated first with a mitogenic concentration of thrombin (125 ng/ml), then with affinity-purified antithrombin antibody and colloidal gold IgG. Under these conditions, EM micrographs showed discrete clusters of gold particles on the cell surfaces. These clusters (approximately seven particles per location) were specific for thrombin and were spread over an area with a diameter of up to 200 nm, indicating antibody binding to a number of clustered thrombin molecules. Interestingly, out of

approximately 1,200 gold particles counted, only four were associated with coated pits. This lack of association confirms our previous EM autoradiography indications that thrombin receptors are not found in coated pits or coated vesicles [12]. Thus, it is unlikely that this lack of association of colloidal gold IgG with coated pits is the result of selective interference of antibody binding to thrombin in these structures. Other investigators have recently used colloidal gold IgG to visualize the binding of asialoglycoprotein to its receptors in frozen sections [20]. In these studies, colloidal gold particles were observed in vesicles and membrane invaginations, many of which may have represented coated pits. It would thus appear that the present techniques would have detected thrombin in coated pits if it were there.

Other molecules that appear to be rapidly internalized and degraded also bind to receptors which are clustered at 4°C or on prefixed cells. For example, up to 58% of asialoorosomucoid appears to bind clustered receptors [21], and approximately the same amount of low density lipoprotein binds clustered receptors [22]. These clustered receptors, however, all seem to associate with coated membrane regions. Epidermal growth factor also appears to associate with coated pits, in that 34% of the EM autoradiographic grains observed following ^{125}I-EGF binding to human fibroblasts were associated with coated pits [23]. These molecules that associated with coated pits appear to be rapidly internalized and degraded by lysosomal enzymes. This does not appear to be true for thrombin.

^{125}I-thrombin is internalized by fibroblast-like cells. In chick embryo cells, however, ^{125}I-thrombin has been shown to accumulate inside of the cells and remain intact and active for many hours [9,10]. Thus, one might not expect thrombin to be internalized by the same process as molecules marked for intracellular degradation. In human cells, ^{125}I-thrombin has been reported to be internalized and degraded, but this internalization and degradation appears to be mediated through complex formation with protease-nexin in the medium and association with the protease-nexin receptor [8]. In the present studies, we incubated ^{125}I-thrombin with ME cells in fresh medium without protease-nexin to minimize any protease-nexin-thrombin complex formation. In this case, virtually all of the specific binding of thrombin to ME cells is to the high-affinity thrombin receptors. Under these conditions, we were unable to demonstrate rapid specific receptor-mediated internalization with either accumulation of trypin-insensitive ^{125}I-thrombin or internal localization of EM autoradiographic grains. Thus, these studies also suggest that the internalization and/or internalization and degradation of thrombin reported previously [9,10] is likely to be mediated through protease-nexin rather than through the thrombin receptor.

When thrombin was bound at 4°C for 3½ hours, the cells rinsed, and the temperature increased to 37°C, there appeared to be a loss of ^{125}I-thrombin from the cell surface, but no increase in internalized thrombin. These results might suggest that under these conditions receptor conformation or altered receptor interactions result in either a shedding of the thrombin-receptor complexes or a rapid release of thrombin from its receptors. This type of rapid loss of thrombin binding was previously observed on ME cells following the 4°C binding of fluorescein-amine-labeled thrombin [24]. Binding thrombin at 4°C does not appear to alter the affinity of thrombin for its receptors [7]. It is thus possible that the 4°C incubation alters the subsequent interactions of the thrombin-receptor complex or cytoskeletal interactions that may be involved in receptor anchorage. In any event, under these conditions little if any specific internalization of thrombin occurs.

To assure ourselves that the 4°C binding was not causing an artifactual loss of molecules, which might be specifically internalized, we also examined the amount of surface-bound and internalized ^{125}I-thrombin during incubation at 37°C. Under these conditions the amount of surface-bound ^{125}I-thrombin increased about 3-fold from 10 to 60 min. Most of this increase was due to specific receptor binding. In contrast, there was only a slight increase in internalized thrombin, most of which was nonspecific. It should be noted that previous reports of internalization of ^{125}I-thrombin in chick embryo cells have shown that little internalization occurs during the first 30–60 min of incubation [10]. In these studies, cells were also rinsed and introduced to fresh medium containing radioactive thrombin at the beginning of the experiment. As mentioned above, it now appears, at least in mouse and human cells, that most internalization of thrombin is mediated by protease-nexin present in conditioned medium [8]. Thus, lack of internalization in the absence of protease-nexin supports our findings that ^{125}I-thrombin is not internalized by thrombin receptor-mediated endocytosis. Furthermore, it is likely that the specific internalization observed in chick cells after several hours of incubation could be accounted for by cell-secreted protease-nexin-thrombin complex formation. Recent studies have shown that protease-nexin-thrombin complex formation acts to prevent, rather than to stimulate, cell proliferation [25]. It would thus appear that the mitogenic effects of thrombin are generated through surface interactions of thrombin with its receptors rather than any long-range internalization of thrombin complexed to protease-nexin.

The small amount of specific receptor-mediated internalization that was observed in the present experiments proceeded at approximately the same rate as the nonspecific internalization. This might suggest that the thrombin-receptor clusters are internalized at approximately the same rate as the rest of the cell-surface membrane. This type of internalization is clearly different from receptor-mediated endocytosis through coated pits. Thus, these data are consistent with our colloidal gold IgG demonstration that thrombin does not associate with coated pits on prefixed cells, and our previous EM autoradiographic evidence that ^{125}I-thrombin does not associate with coated pits or coated vesicles during 37°C incubation [12]. It is possible that thrombin receptors and/or coated pits in ME cells are unique in their apparent lack of association. However, it should be noted that these cells do internalize and degrade thrombin presumably through the protease-nexin receptor system. Thus, it is likely that the nexin receptor does associate with coated pits. Epidermal growth factor binds and is rapidly internalized by these cells [26], which is similar to the binding patterns observed in other cells. The coated pits are thus likely to be active in these cells. These results suggest that ME, and perhaps other cells as well, have two classes of receptor—one which is involved in endocytic degradation (receptor mediated endocytosis), and one which is not. It seems quite possible that other molecules that exhibit slower internalization will also be shown not to associate with coated pits.

If thrombin binding to its receptors does not stimulate receptor-mediated endocytosis, it would appear that this process is not associated with the mitogenic effects of thrombin. This finding is consistent with previous studies that have shown that thrombin action at the cell surface is sufficient to initiate cell division [5,6]. These results do not explain the requirement for continued exposure to growth factors for up to 8 hr to commit quiescent cells to enter a proliferative phase [27,28]. We have recently shown that microtubule stabilization by taxol can inhibit initiation of DNA synthesis by thrombin and EGF when added up to 8 hr following growth-factor

addition [26]. Thus, it is possible that some form of continuous transmembrane signal which elicits cytoskeletal rearrangements is involved in the commitment phase of initiation. Recent studies have also suggested that prolonged surface receptor occupancy [28] and perhaps microaggregation of EGF on cell surfaces [29] are involved in eliciting a mitogenic effect. Thrombin may therefore not be the only growth factor that appears to act at the cell surface. Since thrombin is a proteolytic enzyme, however, its biological activity and mode of action may still be distinct from growth factors such as EGF, which might still require internalization and partial degradation of their receptors to initiate cell division [27]. To address these questions it will be extremely important to apply sensitive visualization techniques to examine the interaction of molecules such as EGF with mitogenically responsive cells.

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