Journal of Supramolecular Structure
and Cellular Biochemistry 15:49–61 (1981)
Control of Cellular Division and Development: Part A: 373–385

Cleavage of Cell Surface Proteins by Thrombin

Martin Moss and Dennis D. Cunningham

Department of Microbiology, College of Medicine, University of California, Irvine, California 92717

This study was based on our previous findings that the mitogenic action of thrombin on cultured fibroblasts can result from interaction of thrombin with the cell surface in the absence of internalization, and that the proteolytic activity of thrombin is required for stimulation of cell division. This prompted us to look for thrombin-mediated cleavages using 2-dimensional gel electrophoresis of labeled cell surface proteins. Surface membrane components were labeled by 3 procedures: 1) proteins were labeled by lactoperoxidase-catalyzed iodination using $^{125}I^-$; 2) galactose and galactosamine residues of glycoproteins were oxidized with galactose oxidase and reduced with ^3H-$NaBH_4$; and 3) glycoproteins were metabolically labeled by incubating cells with ^3H-fucose. Labeling with the first 2 procedures was carried out after thrombin treatment; in contrast, cells metabolically labeled with ^3H-fucose were subsequently treated with thrombin to look for proteolytic cleavages. Collectively, these studies indicated that only about 5 cell surface proteins were thrombin-sensitive, consistent with the high specificity of this protease. Each of the labeling procedures revealed a thrombin-sensitive cell surface glycoprotein which was identified as fibronectin by immunoprecipitation experiments. In addition, cell surface proteins of about 140K and 55K daltons were thrombin-sensitive. However, cell surface proteins of about 45K daltons and 130K to 150K daltons were increased after thrombin treatment. These experiments were conducted on an established line of Chinese hamster lung cells with the eventual goal of studying thrombin-mediated cleavages of cell surface proteins in a large number of cloned populations derived from this line that are either responsive or unresponsive to the mitogenic action of thrombin. This approach should permit identification of proteolytic cleavages that are necessary for thrombin-stimulated cell division.

Key words: labeling of cell surface proteins, two-dimensional gel electrophoresis, fibronectin

Addition of thrombin to cultured nonproliferating fibroblastic cells leads to about one round of DNA synthesis and cell division. This stimulation has been observed with several kinds of early passage cells in culture, including strains derived from chick embryo, mouse embryo, and human neonatal foreskins [1–4]. In contrast, many established cell lines appear to be refractory to the stimulation by thrombin [4], although 3 different

Received August 18, 1980; accepted October 21, 1980.

0275-3723/81/1501-0049$04.00

Chinese hamster cell lines derived from ovary, lung, and whole embryo readily divide after thrombin treatment [5, 6]. With the above fibroblastic cells, stimulation of cell division can be brought about by adding highly purified thrombin to serum-free cultures in the absence of other mitogens. In addition, thrombin can potentiate the action of certain growth factors on fibroblastic cells [7, 8]. With human vascular endothelial cells, thrombin is not mitogenic by itself but will potentiate the action of epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor [9, 10]. Although the biological role of thrombin-stimulated cell division has not yet been directly examined, it is reasonable to suggest that it might be involved in tissue repair following injury [1]. Large quantities of thrombin are produced at very localized regions of tissue damage and could stimulate cell division either by itself or by augmenting the action of other growth-promoting agents.

Studies on the mechanism by which thrombin stimulates fibroblast proliferation have been greatly aided by its ability to produce cell division in chemically defined serum-free medium. Early experiments were directed at identifying the cellular site of thrombin action. By covalently linking thrombin to carboxylate-modified polystyrene beads, it was possible to show that cell surface action of thrombin was sufficient to bring about cell division [11, 12]. Although these studies did not rule out an additional intracellular action of thrombin, they demonstrated that internalization was not *necessary* for stimulation. Subsequent experiments on the binding of ^{125}I-thrombin revealed cell surface binding sites or receptors that were highly specific for thrombin [13, 14]. Studies on the mechanism by which cells bind thrombin have shown that several cellular components are involved. One of these has some novel properties that distinguish it from most cellular receptors. This component (protease-nexin) is released from the cells and forms a covalent linkage with thrombin; these complexes then bind back to cells via the protease-nexin portion of the complex [15]. Thus, the binding of thrombin to fibroblastic cells is complex; studies are in progress to evaluate the role of each of the thrombin-binding components in thrombin-stimulated cell division. A fundamental question regarding the mechanism by which thrombin initiates cell division is whether the stimulation requires its proteolytic activity. This issue was examined with 2 preparations of thrombin that had been derivatized at the catalytic-site serine. These enzymatically inactive thrombins bound to mouse embryo and Chinese hamster lung cells as effectively as active thrombin but did not cause the cells to divide, thus demonstrating a requirement for proteolysis [16].

The above results prompted a search for thrombin-mediated cell surface cleavages that are required for stimulation of cell division. The first experiments were conducted with secondary chick embryo cells, which are responsive to the mitogenic action of thrombin, and chick embryo cells at their 25th population doubling, which are responsive to serum stimulation but which do not divide after thrombin treatment [17]. These studies revealed a 43K dalton cell surface component that was thrombin-sensitive on the responsive cells. An apparently identical component was present on the cells that were unresponsive to thrombin, but on these cells this component was not cleaved by thrombin. Thus, cleavage of the 43K dalton component on responsive chick cells appears necessary for thrombin-stimulated cell division [17].

In the present studies we extended the search for thrombin-mediated cell surface cleavages by employing 2-dimensional gel electrophoresis to better resolve individual cell surface proteins. In addition, 3 different approaches were used to label cell surface proteins to maximize the likelihood of detecting cleavages by thrombin. The first involved labeling tyrosine residues of cell surface proteins using $^{125}I^-$ and lactoperoxidase [18]. The second involved labeling galactose and galactosamine residues of cell surface glyco-

proteins with ^{3}H-$NaBH_4$ following oxidation with galactose oxidase [19]. In the third approach, cell surface glycoproteins were metabolically labeled with ^{3}H-fucose [20]. These experiments were conducted with a line of Chinese hamster lung (CHL) cells, which are very sensitive to the mitogenic action of thrombin. Our eventual goal is to determine which cleavages are necessary for the stimulation of cell division by examining thrombin-mediated cell surface alterations in a series of cloned responsive and unresponsive cells derived from this line.

MATERIALS AND METHODS

Cells and Cell Culture

CHL fibroblasts from the V79 strain were obtained from Dr. John J. Wasmuth, University of California, Irvine. Cell stocks were grown in Dulbecco-Vogt-modified Eagle's medium (DV medium) supplemented with 5% calf serum (Irvine Scientific), designated DV-5.

Radioactive Labeling of Cell Surface Proteins

Lactoperoxidase-catalyzed iodination. Replicate cultures of CHL cells (1.8×10^5 to 3.6×10^5 cells/cm^2) were used for thrombin treatment and subsequent radioactive labeling. Serum-containing growth medium was aspirated from culture dishes and replaced with serum-free medium (DV-0) after one rinse with DV-0. Thrombin-treated cultures received highly purified human thrombin [21] (4,300 NIH units/mg, kindly provided by Dr. John W. Fenton, II) at a final concentration of 20 μg/ml in phosphate-buffered saline (PBS), while control cultures received PBS alone. The cultures were then incubated at 37°C for 30 min. After the incubation, each culture dish was rinsed 4 times with Dulbecco's PBS (0.137 M NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.87 mM $CaCl_2 \cdot 2H_2O$, 0.49 mM $MgCl_2 \cdot 6H_2O$, pH 7.2). Na ^{125}I (Amersham Radiochemical Centre, 15–17 mCi/μg iodine) in Dulbecco's PBS (400 μCi/ml) was then added to the cultures, followed by 85 μl/ml Enzymobeads (Biorad) as a source of lactoperoxidase and glucose oxidase. Cell surface labeling was initiated by addition of glucose to a final concentration of 10 mM. After 20 min at 23°C, radioactive labeling was terminated by aspirating the reaction mixture and rinsing the cultures 4 times with phosphate-buffered iodide (10 mM Na_2HPO_4–NaH_2PO_4, 150 mM NaI pH 7.2). Preliminary experiments showed that labeling with Enzymobeads led to specific activities that were 4- to 8-fold greater than those obtained with soluble lactoperoxidase plus soluble glucose oxidase, immobilized lactoperoxidase plus soluble glucose oxidase, or immobilized lactoperoxidase plus H_2O_2. ^{125}I-labeled cell lysates were prepared by scraping cells from the culture dishes in cell lysis buffer (0.5% NP40, 0.1 mM $MgCl_2$, 50 mM Tris HCl, pH 7.0). The lysates were then treated with nucleases for 10 min at 0°C (Ribonuclease A, Miles Laboratories, 0.1 mg/ml; Deoxyribonuclease I, Worthington Biochemicals 0.1, mg/ml). Following nuclease digestion, sodium dodecyl sulfate (SDS) and 2-mercaptoethanol were each added to a final concentration of 2% and the ^{125}I-labeled lysates were heated for 5 min at 100°C. The lysates were stored at −20°C.

Labeling of cell surface glycoproteins with galactose oxidase and ^{3}H-$NaBH_4$. This procedure was performed essentially as described by Baumann and Doyle [19]. As for iodination, replicate cultures of CHL cells were treated with thrombin or PBS for 30 min at 37°C. The cultures were then rinsed 4 times with Dulbecco's PBS containing 2 mM phenylmethyl sulfonylfluoride (PMSF) and then incubated for 5 min at 23°C with non-

labeled 1 mM $NaBH_4$ in Dulbecco's PBS. Terminal sialic acid residues were removed by treatment with neuraminidase (Calbiochem) at a final concentration of 5 units/ml in Dulbecco's PBS containing 2 mM PMSF for 15 min at 37°C. After rinsing the neuraminidase-treated cultures 3 times with Dulbecco's PBS containing 2 mM PMSF, galactose oxidase (Sigma) was added to a final concentration of 6 units/ml in Dulbecco's PBS containing 2 mM PMSF. The cultures were then incubated at 37°C for 15 min. Tritiation of the oxidized sugar residues was accomplished by incubating the cultures in Dulbecco's PBS containing 2 mCi/ml ^{3}H-$NaBH_4$ (Amersham, 7.26 Ci/mM) for 5 min at 23°C. ^{3}H-labeled cells were then lysed as described for iodination.

Metabolic labeling with ^{3}H-fucose. Cultures of log-phase growing CHL cells in DV-5 were incubated with 10 μCi/ml L-[6^{3}H] fucose (Amersham, 26 Ci/mmole) for 36 h. The cultures were then rinsed with DV-0 and treated with thrombin or PBS as described above for iodination. Cell lysates were prepared as described above.

Two-Dimensional Gel Electrophoresis

Cell lysates were analyzed by 2-dimensional gel electrophoresis with modifications of the techniques of O'Farrell [22] and Ames and Nikaido [23]. Equal amounts of TCA-precipitable radioactivity from lysates of control and thrombin-treated CHL cells were applied to isoelectric focusing (IEF) tube gels (3 mm × 10 cm) prepared as described by O'Farrell. Before IEF each SDS-denatured sample was adjusted to 8.0 M urea and 17% NP40 using O'Farrell solution A, 50% NP40, and ultrapure urea. After IEF for a total of 7,200 volt-hours [22], each IEF gel for 2-dimensional analysis was equilibrated for 2 h in O'Farrell solution 0 [22] containing 9 M urea. The urea was necessary to electrophorese some iodinated cell surface proteins out of the IEF gel. Duplicate IEF gels which were not equilibrated were cut into 5 mm slices; each slice was incubated in 10 mM KCl for 2 h and then analyzed for pH. The SDS-equilibrated IEF gels were loaded on top of a 7½–15% polyacrylamide gradient slab gel (1 mm × 10 cm × 15 cm), which had a 1 cm stacking gel made of 5% acrylamide without any sample wells. The use of gradient polyacrylamide slab gels instead of constant concentration gels for the second dimensions greatly increased the resolution. The IEF gel was then overlaid with O'Farrell solution P [22] (without 2-mercaptoethanol). Molecular weight markers were applied to a single sample well formed on one side of the IEF gel-agarose overlay. The molecular weight markers were as follows: human plasma fibronectin (FN) (240K), β-galactosidase (120K), phosphorylase A (90K), bovine serum albumin (68K), pyruvate kinase subunit (57K), muscle actin (43K), carboxypeptidase B (35K), and soybean trypsin inhibitor (21K). SDS gel electrophoresis was carried out at 23 mA/gel for 4 h. The gels were then fixed and stained overnight in 0.25% Coomassie brilliant blue, 50% ethanol, 7% acetic acid, and subsequently destained in 5% ethanol 7% acetic acid. Two-dimensional gels of tritium-labeled samples were infiltrated with 2,5-diphenyloxazole as described by Bonner and Laskey [24] and then fluorographed at −80°C with Kodak X-Omat R film. Gels containing ^{125}I-labeled samples were autoradiographed at −80°C using Dupont Cronex "Lighting-Plus" intensifying screens. Average exposure times were 10–20 days for ^{3}H-fucose gels, 2–4 days for ^{3}H-$NaBH_4$ gels, and 24 h for ^{125}I gels.

Immunoprecipitation of ^{125}I-Cell Surface Proteins With Anti-Fibronectin IgG

Confluent cultures of CHL cells were labeled with $^{125}I^-$ as described above. They were then lysed in PBS, pH 7.2 containing 1.0% NP40 and 2 mM PMSF. The lysates were nuclease-treated as above and then incubated with rabbit anti-human-FN IgG (kindly

supplied by Mr. Channing Der, University of California, Irvine), for 30 min at 23°C and 6 h at 4°C. ^{125}I-labeled-immunoprecipitates were isolated with protein-A Sepharose beads (Pharmacia). Briefly, 50 μl of a 50% slurry of protein-A Sepharose beads in PBS were combined with 150 μl of the lysate-anti-FN IgG mixture. The samples were occasionally swirled during a 30 min incubation at 23°C, after which the beads were removed by centrifugation. The beads were then washed extensively in 0.5% NP40, 0.05 M NaCl, 0.01 M Na_2HPO_4-NaH_2PO_4, pH 7.5. Immunoprecipitated ^{125}I-proteins were then released from the beads by treatment with 2% SDS, 2% 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.0 for 3 min at 75°C.

RESULTS

The required thrombin treatment for producing a maximal cell number increase (about 60%) with nonproliferating CHL cells in serum-free medium is 500 ng/ml (14 nM) for 48 h (data not presented). However, during this extended incubation in the absence of serum, a small fraction of the cells round up from the culture dish and eventually lose viability as judged by the loss of their ability to exclude trypan blue. Because these cells might be permeable to thrombin as well as the enzymes for radioactively labeling cell surface proteins, we chose a thrombin treatment of 20 μg/ml for 30 min to examine thrombin-mediated cleavages of cell surface components. This short incubation in the absence of serum did not cause the cells to round up or increase their permeability to trypan blue. Although this thrombin concentration was much higher than those used in the long-term treatments to stimulate cell division, it was chosen to maximize the opportunity for detecting thrombin-sensitive cell surface proteins. If a given protein must be cleaved by thrombin for cell division to occur, cleavage of only a fraction of the total cell surface population of that protein might be required, just as occupancy of only a fraction of the total cell surface receptors for a given hormone is frequently sufficient for production of the hormone response [25, 26]. Thus, thrombin-mediated cleavages that are necessary for cell division might be difficult to detect using mitogenic concentrations of thrombin. With this issue in mind, the present studies were initiated to identify thrombin-sensitive cell surface proteins with the realization that it would next be important to analyze whether a given cleavage was necessary for the biological response.

An early step in these experiments involved optimizing the 2-dimensional gel electrophoresis procedures of O'Farrell [22] and Ames and Nikaido [23] as described in Materials and Methods to obtain maximal resolution of individual membrane proteins from Chinese hamster lung cells. Autoradiograms of 2-dimensional gels containing membrane proteins, which had been radioactively labeled by several different procedures (Figs. 2–5), revealed that there was some streaking or "stuttering." In contrast, Figure 1 shows the Coomassie-stained total cellular proteins of one such gel. As can be seen, the procedures employed yielded a high degree of resolution of total cellular proteins. Thus, the apparent streaking of the radioactively labeled membrane proteins was attributable to the properties of these polypeptides and was probably a result of carbohydrate modifications, which produced microheterogeneity in membrane-abundant glycoproteins.

It should be emphasized that the thrombin-mediated changes in cell surface components described below are ones that were *consistently* observed in a large number of experiments. Although close inspection of the autoradiograms in Figures 2, 4, and 5 suggest some additional changes brought about by thrombin, some of these were not reproducible.

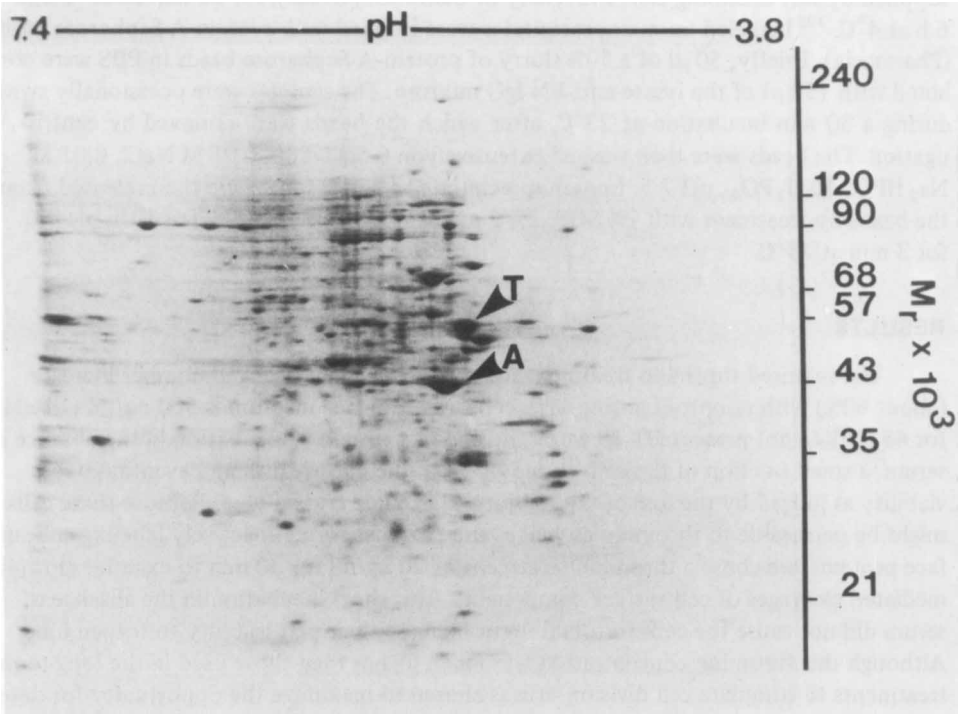

Fig. 1. Coomassie-stained total CHL cell proteins separated by 2-dimensional gel electrophoresis. CHL cells were lysed as described in Materials and Methods. Approximately 80 μg of total cellular protein was separated by IEF in the first dimension and SDS-polyacrylamide gel electrophoresis (7½–15% gradient) in the second dimension. The gels were fixed and stained with Coomassie brilliant blue as described in Materials and Methods. The arrows indicate migration of actin (A) and tubulin (T). Migrations of molecular weight standards are shown at right.

Figure 2 shows autoradiograms of ^{125}I-labeled cell surface proteins of control (left panel) and thrombin-treated (right panel) CHL cells. Control experiments indicated that the proteins labeled by this procedure that were thrombin-sensitive were cell surface rather than intracellular components: 1) there was no detectable cell lysis judged by trypan blue exclusion; 2) these proteins were not labeled when lactoperoxidase was omitted; and 3) *these* proteins were not similarly labeled after iodination of lysed cells. Also, the thrombin-sensitive proteins were cellular rather than serum proteins since they were not detected after iodination of serum. In Figure 2, the lactoperoxidase-catalyzed iodinations were carried out *after* the thrombin treatment; thus, the "absence" of a component from the thrombin-treated autoradiogram could be a result of thrombin-mediated internalization of the intact component in addition to cleavage or release of it from the cells.

The arrows in Figure 2 denote 4 changes that were consistently observed in the autoradiograms prepared from thrombin-treated cells. The component that becomes visible at about 35K daltons is apparently thrombin that was bound to the cell surface and subsequently iodinated. The arrow at about 240K daltons shows a heterogeneous component

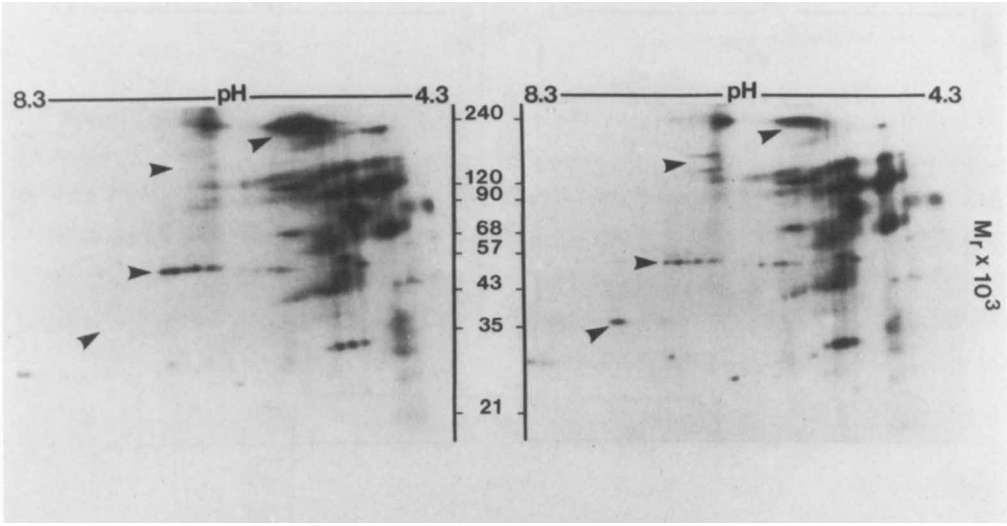

Fig. 2. Two-dimensional gel electrophoresis of ^{125}I-labeled cell surface proteins from control (left panel) and thrombin-treated (right panel) cells. The procedures are described in Materials and Methods. The arrows denote thrombin (35K daltons) and proteins affected by thrombin treatment, which migrated with apparent molecular weights of 240K, 130K to 150K, and 55K daltons.

that migrated to a pH of about 6.0 and was decreased by thrombin. The arrow at about 130K to 150K daltons shows two components that migrated to a pH of about 6.5 that were increased after thrombin treatment. Whether these are fragments resulting from thrombin-mediated cleavages or whether they are components whose appearance in the cell surface labeling pattern is stimulated by thrombin has not yet been evaluated. The arrow at about 55K daltons denotes a "series" of components migrating to a pH of about 6.5 that were deminished by thrombin treatment. In several but not all experiments we also observed a thrombin-sensitive component of approximately 45K daltons that migrated to a pH of 6.1. It should be emphasized that the apparent "stutter" patters of some iodinated components was not due to multiple iodinations; similar patterns were observed when cell surface glycoproteins were labeled with ^{3}H-$NaBH_4$ after oxidation with galactose oxidase (Fig. 4) or metabolically labeled with ^{3}H-fucose (Fig. 5).

Because of its size and relative abundance in the gel profile of ^{125}I-labeled cell surface proteins, the 240K thrombin-sensitive cell surface protein appeared similar to fibronectin (FN). To determine its relatedness to FN, immunoprecipitation of cell lysates containing ^{125}I-labeled cell surface proteins was carried out using rabbit anti-FN IgG and protein-A Sepharose beads. Figure 3 shows the 2-dimensional gel profiles of ^{125}I-labeled cell surface proteins (upper left gel), ^{125}I-labeled cell surface proteins that were not immunoprecipitated (upper right gel), and ^{125}I-labeled cell surface proteins that were immunoprecipitated with anti-FN IgG and protein-A Sepharose beads (lower gel). Three major ^{125}I-labeled cell surface proteins were apparent in the immunoprecipitated samples: one at 240K, labeled F (pH migration of 6.0 to 6.5), one at 85–90K, labeled X (pH migration of 5.0), and a series at 55K labeled Y (pH migration centering around 6.5). Comparison of the migration in 2-dimensional gels of purified human plasma FN and the immunoprecipitated ^{125}I-labeled

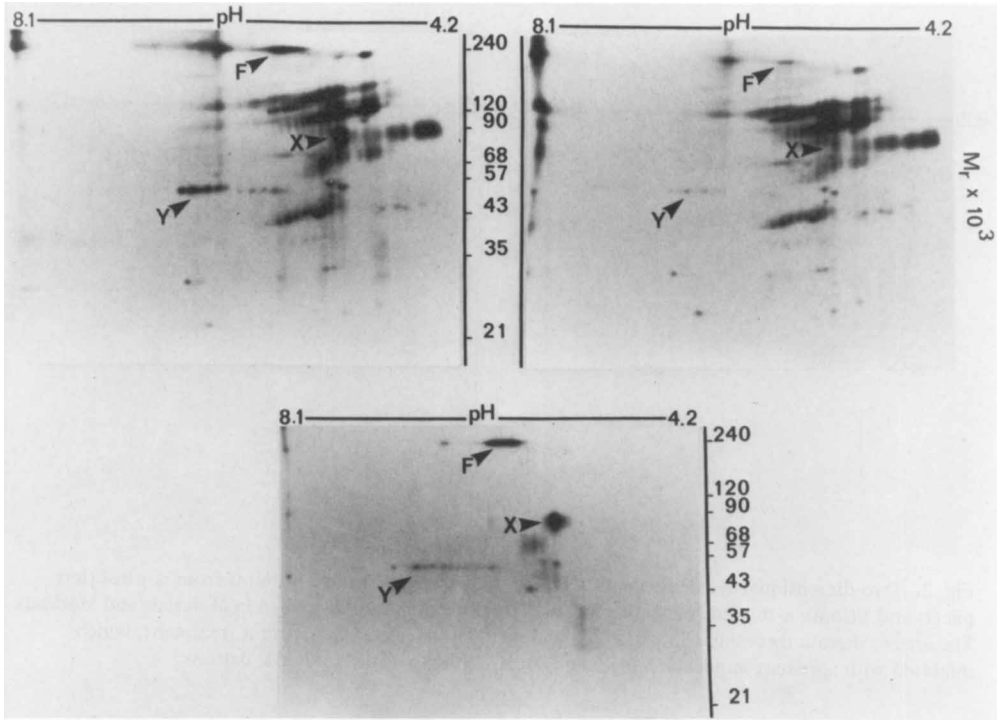

Fig 3 Immunoprecipitation of ^{125}I-labeled cell surface proteins with rabbit anti-human fibronectin IgG Cell surface proteins of CHL cells were labeled with ^{125}I, solubilized in NP40, immunoprecipitated with rabbit anti-FN IgG, and separated by 2-dimensional gel electrophoresis as described in Materials and Methods The upper left panel shows the profile of labeled cell surface proteins The upper right panel shows ^{125}I-labeled cell surface proteins that were not immunoprecipitated The lower panel shows ^{125}I-labeled cell surface proteins that were immunoprecipitated The arrows denote 3 major immunoprecipitated polypeptides with apparent molecular weights of 240K (F), 90K (X), and 55K (Y)

240K component showed them to have the same apparent molecular weight and very similar pH migrations However, the ^{125}I-labeled cell surface component migrated over a broader pH range than the human plasma FN, indicating a greater charge variance among individual molecules of the cell-associated 240K component This was possibly due to additional carbohydrate heterogeneity In fact, the data presented in Figures 4 and 5 indicate that the cell-associated 240K component is a heterogeneous glycoprotein since it was labeled by ^{3}H-NaBH$_4$ after galactose oxidase treatment and after incubating cells with ^{3}H-fucose Further relatedness between the ^{125}I-labeled 240K cell surface protein and authentic FN was demonstrated by displacement of immunoprecipitated ^{125}I-labeled 240K with excess nonlabeled human plasma FN (data not shown) The immunoprecipitated 55K dalton ^{125}I-labeled component (Fig 3, component Y) appears to be the same series of 55K dalton polypeptides that were found to be thrombin-sensitive (Fig 2) The coprecipitation of the 55K dalton component and FN initially suggested a possible interaction between it and FN This possibly appears unlikely, however, since we observed that immunoprecipitation

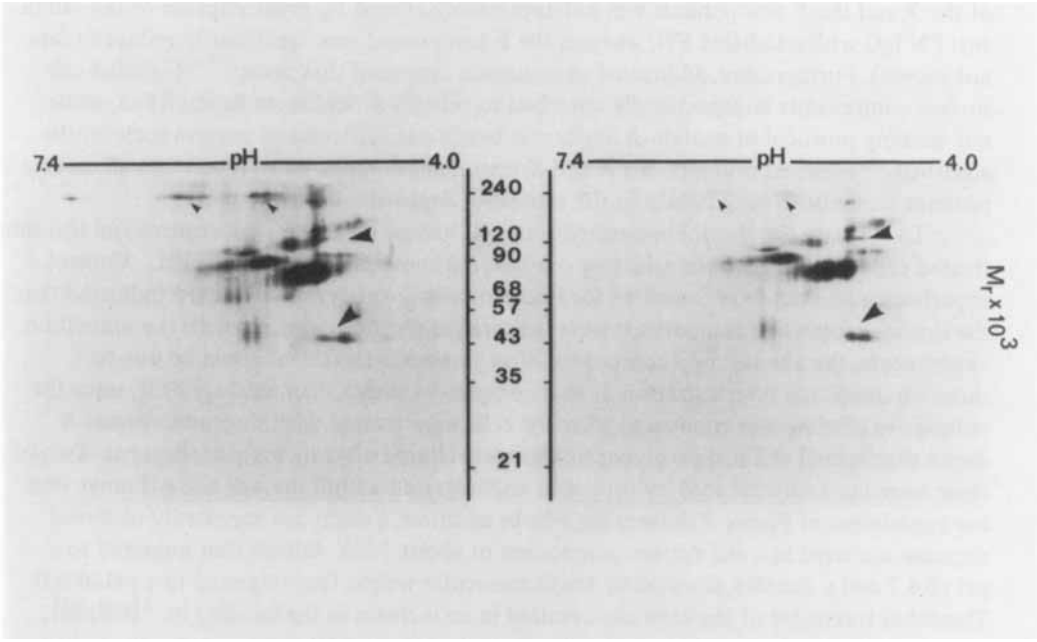

Fig 4 Two-dimensional gel electrophoresis of surface glycoproteins of control (left panel) and thrombin-treated (right panel) cells labeled by ^{3}H-$NaBH_4$ after galactose oxidase treatment The procedures are described in Materials and Methods The arrows denote glycoproteins migrating with apparent molecular weights of 240K, 140K, and 45K daltons that were affected by thrombin treatment

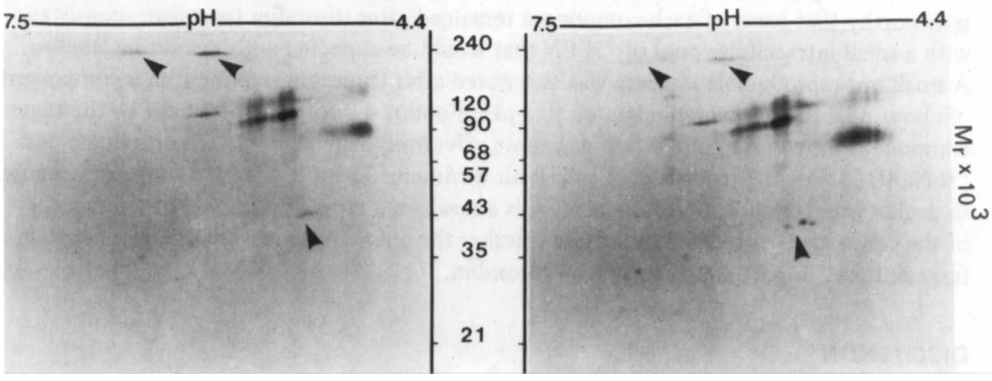

Fig 5 Two-dimensional gel electrophoresis of surface glycoproteins of control (left panel) and thrombin treated (right panel) cells metabolically labeled with ^{3}H fucose The procedures are described in Materials and Methods The arrows denote glycoproteins of 240K and 45K that were affected by thrombin treatment

of the X and the Y components was not appreciably altered by preadsorption of the rabbit anti-FN IgG with unlabeled FN, whereas the F component was significantly reduced (data not shown). Furthermore, additional experiments suggested that several ^{125}I-labeled cell surface components nonspecifically adsorbed to protein-A Sepharose beads. Thus, while our washing protocol of protein-A Sepharose beads was sufficient to remove some of the adsorbed ^{125}I-labeled proteins, the X and Y components appeared to represent cell surface proteins that adsorb very tightly to the protein-A Sepharose beads.

To evaluate the thrombin-sensitivity of cell surface glycoproteins, control and thrombin-treated cells were exposed to galactose oxidase and subsequently to ^{3}H-$NaBH_4$. Control experiments like the ones described for lactoperoxidase-catalyzed iodination indicated that the thrombin-sensitive components were cell surface glycoproteins. As with the iodination experiments, the absence of a component after thrombin treatment could be due to thrombin-mediated internalization of that component rather than cleavage of it, since the radioactive labeling was conducted after the cells were treated with thrombin. Figure 4 shows that several cell surface glycoproteins were altered after thrombin treatment. Two of these were markedly reduced by thrombin and migrated within the size and pH range that the experiment in Figure 3 showed for FN. In addition, a slight but repeatedly observed decrease occurred in a cell surface component of about 140K daltons that migrated to a pH of 4.7 and a doublet at a slightly lower molecular weight that migrated to a pH of 5.0. Thrombin treatment of the cells also resulted in an increase in the labeling by ^{3}H-$NaBH_4$ after galactose oxidase treatment of a component of about 45K daltons that migrated to a pH of 4.7.

The last procedure that was used to examine the thrombin sensitivity of cell surface components involved metabolic labeling with ^{3}H-fucose, a glycoprotein precursor that is incorporated mainly into cell surface glycoproteins [20]. In these experiments, the cells were labeled with ^{3}H-fucose and subsequently treated with thrombin. Since thrombin treatment followed the labeling of cellular proteins, the absence of a component in thrombin-treated cells would indicate cleavage of the component of removal of it from the cells, and not simply internalization without cleavage. Figure 5 shows that this procedure revealed a thrombin-mediated decrease of 2 components that migrated to an apparent molecular weight of about 240K daltons and a pH range of 6.0 to 6.5, as does FN. It is noteworthy that some of each component remained after thrombin treatment, consistent with a small intracellular pool of ^{3}H-FN that would be expected with metabolic labeling. A small but reproducible increase was also noted after thrombin treatment in a component of about 45K daltons which migrated to a pH of about 4.7 to 5.0. This might be the same component shown in Figure 4 that was more effectively labeled by galactose oxidase and ^{3}H-$NaBH_4$ following treatment of cells with thrombin. There is not yet enough information to decide whether such increased labeling is a result of a rapid stimulation of appearance of the component at the cell surface or whether the component represents a glycoprotein fragment resulting from proteolysis by thrombin.

DISCUSSION

The present studies were based on our previous findings that cell surface action of thrombin is sufficient to stimulate cell division [11, 12] and that the proteolytic activity of thrombin is required for the stimulation [16]. Thus, cleavage of one or more cell surface proteins is a critical step for thrombin-stimulated cell division; this limited proteolysis may trigger the series of events that finally leads to DNA synthesis and cell division. At this point in our limited understanding of molecular events that can lead to cell proliferation, it is important to identify cell surface proteins that are susceptible to cleavage by thrombin.

To maximize our ability to detect proteolytic cleavages, the present experiments were conducted on labeled surface membrane components that were separated by 2-dimensional gel electrophoresis [22, 23]. The methods to label the membrane components included one that labeled tyrosines of surface proteins ($^{125}I^-$ and lactoperoxidase), a procedure that labeled galactose and galactosamine residues of surface glycoproteins ^3H-$NaBH_4$ and galactose oxidase) and a procedure that metabolically labeled glycoproteins (^3H-fucose). With the first 2 procedures we employed the standard controls to show that cell surface rather than intracellular proteins were labeled and that the thrombin-sensitive components were not serum proteins [27]. It has previously been shown that glycoproteins metabolically labeled with ^3H-fucose occur mostly at the cell surface [20]. In the experiments employing ^3H-fucose, cells were treated with thrombin *after* metabolic labeling. Thus, a component that was judged thrombin-sensitive on the autoradiograms was either removed from the cell (perhaps as a result of thrombin cleaving an adjacent membrane protein) or directly cleaved by thrombin. However, with the 2 other labeling procedures, cells were labeled *after* thrombin treatment. In these cases, thrombin sensitivity of a cell surface component could be a result of thrombin-mediated internalization without cleavage, as well as removal from cells or direct cleavage by thrombin. In addition, thrombin could produce cell surface alterations that might change the accessibility of a given protein to the labeling reagents. Collectively these procedures indicated that only about 5 cell surface proteins were thrombin-sensitive, consistent with the high specificity of this serine protease [28].

With each of the 3 labeling procedures, a glycoprotein that was identified as FN was found to be thrombin-sensitive. Previous studies have shown that thrombin stimulates the release of apparently intact FN from cultured human fibroblasts [29]. However, experiments with chick embryo fibroblasts did not reveal a reduction of cell surface FN after thrombin treatment [17, 30, 31]. Thermolysin, papain, and elastase, however, readily removed cell surface FN from chick embryo fibroblasts [31]. It is noteworthy that thrombin is very mitogenic for chick embryo fibroblasts but that these cells do not divide after treatment with thermolysin or elastase [31]. Thus, it appears that, with chick cells, removal of cell surface FN is neither necessary nor sufficient for protease-stimulated cell division [31]. However, since cell surface FN is apparently involved in the attachment of many cells to their extracellular matrix [32], thrombin-mediated alterations in cell surface FN could be involved in perturbations of this attachment and perhaps changes in cell migration or other events that might be a part of the total wound healing process in vivo.

A thrombin-sensitive cell surface component of 205K daltons was revealed in earlier studies on chick embryo fibroblasts [33]. However, this protein was also removed by nonmitogenic proteases including α-protease, thermolysin, and papain [31]. Thus, the 205K polypeptide is not simply a negative effector molecule whose removal from the cell surface is sufficient to stimulate cell division. These results are consistent, however, with the possibility that proteolysis of 205K by thrombin produces a specific peptide that is a positive signal in or on the cell. In the present experiments we did not detect on CHL cells a 205K dalton component that was thrombin-sensitive. This could be a result of a difference in the cells that were used or the procedures to analyze cell surface proteins.

Previous studies have shown that a cell surface component of about 43K daltons that is labeled by $^{125}I^-$ and lactoperoxidase on chick embryo fibroblasts appears to be involved in thrombin-stimulated cell division [17]. This component was thrombin-sensitive on chick embryo cells that were responsive to the mitogenic action of thrombin. On 4 separately isolated populations of chick embryo cells that divided after serum treatment but not after thrombin treatment, there was an apparently identical component, but it was not thrombin-sensitive.

In view of these results, we looked for a similar component in the present studies on CHL cells. In several experiments we found a cell surface component of about 45K daltons that migrated to a pH of 6.1, which was iodinated with $^{125}I^-$ and lactoperoxidase in control but not in thrombin-treated cells. However, in subsequent experiments we could not detect this component; the reason for this apparent discrepancy is not yet clear. It is noteworthy that the present experiments, in which CHL cell surface glycoproteins were labeled with ^{3}H-$NaBH_4$ after galactose oxidase treatment or with ^{3}H-fucose, did not reveal a 45K dalton component that was diminished or absent after thrombin treatment.

A cell surface component with an apparent molecular weight of about 45K daltons, which migrated to a pH of about 4.7 to 5.0 upon isoelectric focusing, was found in the present studies to be increased after thrombin treatment. The increase was not large but was reproducible and detected both by ^{3}H-$NaBH_4$ and galactose oxidase labeling after thrombin treatment and by thrombin treatment after metabolic labeling with ^{3}H-fucose. It was not detected by $^{125}I^-$ and lactoperoxidase labeling. Thrombin treatment could bring about an increased amount of a given cell surface protein either by stimulating its appearance in the membrane via a synthetic route or by direct proteloysis of a cell surface protein, which would yield the given protein fragment. At this time we do not have the data that would permit a choice between these possibilities.

Cell surface proteins of about 55K and 140K daltons were found to be diminished by thrombin in the present experiments. Although it is not possible to describe the relationship, if any, of these changes to thrombin-stimulated cell division, we are in the process of developing approaches to examine the biological significance of cell surface changes produced by thrombin. The key element of this approach is the isolation of cloned CHL cell lines that are either responsive or unresponsive to the mitogenic action of thrombin. By examining thrombin-mediated cell surface changes in a large number of responsive and unresponsive clones, it should be possible to identify which of the surface changes are *necessary* for thrombin-stimulated cell division. A cell surface cleavage brought about by thrombin that occurs in all of the responsive clones but that does not take place or is altered in some of the unresponsive clones is likely to be necessary for the biological response.

ACKNOWLEDGMENTS

This work was supported by a research grant (CA-12306) from the National Institutes of Health. D.D.C. is a recipient of a Research Career Development Award (CA-00171) from the National Institutes of Health. M.M. received support from a National Institutes of Health Postdoctoral Fellowship (GM-07465). We thank Cindy Rofer for technical assistance and Dr. John W. Fenton II for generous gifts of human thrombin [21].

REFERENCES

1. Chen LB, Buchanan JM: Proc Natl Acad Sci USA 72:131, 1975.
2. Pohjanpelto P: J Cell Physiol 91:387, 1977.
3. Carney DH, Glenn KC, Cunningham DD: J Cell Physiol 95:13, 1978.
4. Buchanan JM, Chen LB, Zetter BR: In Schultz J, Ahmad F (eds): "Cancer Enzymology." New York: Academic Press, 1976, p 1.
5. Simmer RL, Baker JB, Cunningham DD: J Supramol Struct 12:245, 1979.
6. Moss M, Wiley HS, Low DA, Cunningham DD: unpublished results.
7. Zetter BR, Sun TT, Chen LB, Buchanan JM: J Cell Physiol 92:233, 1977.
8. Cherington PV, Pardee AB: J Cell Physiol 105:25, 1980.

9. Gospodarowicz D, Brown KD, Birdwell CR, Zetter BR: J Cell Biol 77:774, 1978.
10. Zetter BR, Antoniades HN: J Supramol Struct 11:361, 1979.
11. Carney DH, Cunningham DD: Cell 14:811, 1978.
12. Carney DH, Cunningham DD: J Supramol Struct 9:337, 1978.
13. Carney DH, Cunningham DD: Cell 15:1341, 1978.
14. Perdue J, Kivitz E, Lubenskyi W: J Cell Biol 83:255a, 1979.
15. Baker JB, Low DA, Simmer RL, Cunningham DD: Cell 21:37, 1980.
16. Glenn KC, Carney DH, Fenton JW II, Cunningham DD: J Biol Chem 255:6609, 1980.
17. Glenn KC, Cunningham DD: Nature 278:711, 1979.
18. Hubbard AL, Cohn ZA: J Cell Biol 64:438, 1975.
19. Baumann H, Doyle D: J Biol Chem 253:4408, 1978.
20. Atkinson PH, Summers DF: J Biol Chem 246:5162, 1971.
21. Fenton JW II, Fasco MJ, Stackrow AB, Aronson DL, Young AM, Finlayson JS: J Biol Chem 252:3587, 1977.
22. O'Farrell PH: J Biol Chem 250:4007, 1975.
23. Ames GF-L, Nikaido K: Biochemistry 15:616, 1976.
24. Bonner WM, Laskey RA: Eur J Biochem 46:83, 1974.
25. Hollenberg MD, Cuatrecasas P: Proc Natl Acad Sci USA 70:2964, 1973.
26. Kahn CR: J Cell Biol 70:261, 1976.
27. Hynes RO: In Pain RH, Smith BJ (eds): "New Techniques in Biophysics and Cell Biology." London: John Wiley and Sons, 1976, vol 3, p 147.
28. Blomback B, Hessel B, Hogg D, Cloesson G: In "Chemistry and Biology of Thrombin." Ann Arbor: Ann Arbor Science, 1977, p 275.
29. Mosher DF, Vaheri A: Exp Cell Res 112:323, 1978.
30. Blumberg PM, Robbins PW: Cell 6:137, 1975.
31. Zetter BR, Chen LB, Buchanan JM: Cell 7:407, 1976.
32. Vaheri A, Mosher DF: Biochim Biophys Acta 516:1, 1978.
33. Teng NNH, Chen LB: Nature 259:578, 1976.