The Role of Surface Proteins in Cell Proliferation as Studied with Thrombin and Other Proteases

(chick embryo fibroblasts/external cell proteins/DNA synthesis/mitogenic activity)

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ABSTRACT This communication explores the capacity of different proteases to stimulate DNA synthesis in resting chick embryo fibroblasts and to cause the removal of cell membrane proteins previously postulated as important in the regulation of growth and division of cells. Thrombin, a highly specific protease and a known mitogen, was incubated with chick embryo fibroblasts, and analysis was made of the cell membrane proteins. Of particular interest were a protein of molecular weight 250,000, which is known to be readily removed by the action of trypsin and is not present in most transformed cells, and two other proteins, which are reduced in amount in transformed as compared to confluent resting cell cultures. None of these three proteins was removed by thrombin when the latter was added to confluent cells in concentrations sufficient to cause significant increase in DNA synthesis twelve hours after stimulation by the protease. The presence or absence of these proteins in the membranes of confluent resting or transformed cells of chick embryo fibroblasts does not seem to be directly related to the process of regulation of DNA synthesis and cellular division.

Changes in cell surfaces are believed to be involved in the regulation of cell growth brought about by viral transformation or by the action of proteases. A mild treatment of confluent resting cells in culture with proteases, in particular the serine proteases, can promote the initiation of cell proliferation and result in release from density inhibition of growth (1, 2). Recent publications have strongly implicated the increased proteolytic activity in cultures of transformed cells with alteration of cell surfaces and a resulting perturbed regulation of cell growth (3, 4). In addition, Hynes (5-7) and Robbins et al. (8) have characterized an extremely trypsin-sensitive component of the external membrane of the cell with an apparent molecular weight of 250,000 (250 k). This protein component is heavily labeled by lactoperoxidase-catalyzed iodination in normal cells and is intriguingly either only weakly labeled or not present in transformed cells (5-13). The putative relationship between cell surface alteration by trypsin and the production of proteolytic enzymes by transformed cells had encouraged the speculation that the removal of the 250-k surface protein may play a direct role in the control of growth of cells in culture.

In this communication we report a study in which confluent resting chick embryo fibroblasts were treated with several proteases. Modification of the composition of the cell surface proteins, in particular the 250-k protein, and cell overgrowth have been measured, and their relationship has been investigated.

Recent studies by Chen and Buchanan (14) have shown that thrombin is a potent mitogen and that potentially pro-thrombin is one of the major sources of mitogenic activity in plasma. They have found that exposure of the arrested cells to purified bovine thrombin alone can bring about a considerable growth stimulation, as measured by thymidine incorporation into DNA or by actual cell count.

Thrombin exhibits a high specificity in the cleavage of peptide bonds. Those so far demonstrated are four arginyl-glycine bonds of fibrinogen (15). A very limited number of arginyl and lysyl bonds of actin are also split by thrombin (16). Thrombin, therefore, seemed to be an excellent probe for the study of cell surface proteins of cells that are undergoing mitogenic stimulation. The results of these studies have indicated that a reassessment of previous speculations on the role of specific membrane proteins in the regulation of cell growth is in order.

MATERIALS AND METHODS

Chick embryo fibroblasts were prepared according to the method of Rein and Rubin (17) from 11-day-old embryos (SPAFAS, Norwich, Conn.). Secondary cultures were obtained from primary cells after 3 days of growth in medium 199 supplemented with 2% tryptose-phosphate broth, 1% calf serum, and 1% heat-inactivated chick serum. Confluent resting cultures were prepared by seeding at 7 × 10⁴ cells per 35-mm dish with Dulbecco modified Eagle's medium supplemented with 0.5% calf serum. The medium was changed on day 2, and the culture (7 × 10⁴ cells per plate) was used on day 4. The protease treatments were carried out in Dulbecco modified Eagle's medium without serum. The rate of DNA synthesis was measured by incubation of the culture for 1 hr with [³H]thymidine 12 hr after protease treatment as described previously (14).

Iodination was carried out in confluent cultures with slight modifications of described methods (5, 8, 18, 19). Washed cells in 35-mm Falcon plastic culture plates were iodinated in 1 ml of Dulbecco's phosphate-buffered saline containing 0.9 mM CaCl₂ and 0.9 mM MgCl₂ with addition of 20 μg of lactoperoxidase (Worthington) further purified by chromatography on a Sephadex G-100 column, 25-50 μCl of carrier-free Na¹²¹I (New England Nuclear Corp.) in 0.1 N NaOH, and 2.5 nmol of NaI. The reaction was initiated by addition of 5 μmol of glucose and 0.015 units of glucose oxidase. Reactions were terminated after 10-15 min by five washes of ice-cold Ca²⁺- and Mg²⁺-free phosphate-buffered saline solution. The cells were then dissolved in electrophoresis sample buffer containing 2% sodium dodecyl sulfate, 1% mercaptoethanol, and 2 mM phenylmethane sulfonyl fluoride or 1 mM sodium tetrathionate to inhibit protease activities.
Slab gel electrophoresis was carried out with an apparatus similar to that described by Maizel (20). The separating gel, which was 13 cm in length, consisted of a gradient of 5-15% acrylamide (acrylamide:bisacrylamide = 30:0.8) in a modified Laemmli's buffer (21, 22) containing 0.1% sodium dodecyl sulfate. The gel was made by use of a standard two-chamber linear gradient former. A 4% acrylamide stacking gel (acrylamide:bisacrylamide = 30:1.6) was used. The gel separates proteins in the molecular weight range of 10,000-300,000. The slab gels were dried and subjected to autoradiography on Kodak RP R2 x-ray film. The position and amount of the labeled proteins were determined by scanning the autoradiogram on a Canaio model G 11 densitometer with a digital integrator (Autolab 6300) attachment.

Because of the occasional variability involved in the iodination and cell plating procedures, we would estimate that the error of individual determinations could be as high as 15-20%. The values for the iodinated 250-k protein reported in Table 1 are scored with this variability in mind and should therefore be accepted with this reservation. A partial correction can be made for some of these errors by normalizing the 12SI content of the 250-k band to that of other bands of iodinated proteins on the autoradiogram.

Cells were also labeled with [35S]methionine (185 Ci/mmol,
New England Nuclear Corp.) or L-[14C]amino-acid mixtures (New England Nuclear Corp). Twice crystallized trypsin and L-(1-tosylamido-2-phenyl) ethyl chloromethylketone (TPCK)-treated trypsin were obtained from Worthington Chemical Co. Twice crystallized ficin suspension and bacterial subtilisin, Carlsberg (Type VIII), were from Sigma Chemical Co. Bromelin (1200 U/g) was obtained from Schwarz/Mann and Pronase (grade B) from Calbiochem (98,000 FUK/g).

α-Protease was purchased from Gallard-Schlesinger Chemical Mfg. Corp. Chymotrypsin treated with L-(1-tosylamido-2-lysyl) ethyl chloromethylketone (TLCK) was obtained from Worthington. Topical thrombin was obtained from Parke-Davis Co. and highly purified thrombin (2000 NIH units/mg) was kindly provided by Dr. David F. Waugh of the Massachusetts Institute of Technology. This thrombin migrated as a single band upon electrophoresis with polyacrylamide gel.

RESULTS

Several proteases have been recognized for their properties of stimulating DNA synthesis and cell division in several cell lines. The proteases studied in this investigation have been placed into two categories, depending upon their capacity to hydrolyze a particularly sensitive protein of the cell surface of chick embryo fibroblasts of molecular weight 250,000. An attempt has been made to determine whether removal of this protein in any way correlates with the mitogenic activity of the protease.

Proteases of Category I: The proteases capable of catalyzing the removal of the 250-k protein from chick embryo fibroblasts after brief exposure at low enzyme concentration and in the absence of serum include trypsin (columns E and F, Fig. 1), α-chymotrypsin (column G), subtilisin (column H), ficin (column I), Pronase (column M), and bromelin (column O) (see also Table 1). In some of the experiments proteases were added just at sufficient concentration to remove the 250-k protein. At these concentrations, only limited mitogenic activities were observed for most of the category I proteases with the possible exception of trypsin. Undoubtedly trypsin and possibly some of the other proteases listed in Expts. 1 and 2 of Table 1 could have elicited greater response had they been administered at higher concentrations and for a longer period of time. However, for scoring of mitogenic activity in our assay system, proteolysis should not occur to the point of removal of the cells from the dish.

In three instances in which we have added proteolytic enzymes (trypsin, subtilisin, and ficin) in increasing but still limiting amounts, we could observe no correlation between the removal of the 250-k protein and DNA synthesis. The results of the experiment with chymotrypsin were particularly significant, since incubation of this protease with chick fibroblasts caused the complete removal of the 250-k protein at both concentrations tested, yet had no significant effect on DNA synthesis. The inactivity of chymotrypsin and the other proteases as mitogens could not be ascribed to the presence of inhibitors or to irreversible damage to the cells during the test period, since addition of thrombin at a level of 1 μg/ml for the remaining part of the 12-hr period, thereafter, resulted in a rate of DNA synthesis equal to that obtained with thrombin alone.

Proteases of Category II: Two proteases, thrombin (columns J and K, Fig. 1) and α-protease (column N), did not catalyze the cleavage of the 250-k protein under the conditions of our experiments. A significant increase in DNA synthesis also did not occur in the cells in the case of α-protease (Table 1, Exp. 2). Hence, from these data alone it was not known whether there was an interaction between α-protease and the cells. However, at the high concentration of α-protease (25 μg/ml) used in this experiment the cells showed the typical morphological changes observed during protease treatment (23).

Effect of Thrombin on DNA Synthesis and Cleavage of the Protease-Sensitive Protein of Chick Fibroblasts. In confirmation of a previous communication (14) we found that thrombin, when added to confluent resting cells, stimulates DNA synthesis and cell division. The level of DNA synthesis after stimulation of chick embryo fibroblasts either with 50 μg/ml of highly purified thrombin for 30 min or with 1 μg/ml for the whole 12 hr at 37°C was considerable and equivalent to that obtained with 2% calf serum (Table 1, Exp. 1).

The retention of the 250-k protein by the fibroblasts after stimulation of the cells with thrombin (50 μg/ml for 30 min) is shown in the gel strip in column J of Fig. 1 and compared to the control strip from untreated cells at the beginning and end of the 12-hr period required for the measurement of DNA synthesis (columns D and L, respectively). In the experiment
reported in Table 1, Exp. 1, a semiquantitative estimation of the intensity of the bands in the region of the 250-k protein was made by measuring the density of the film from the autoradiograph in that area of the gel strip. Within the error of the method, the radioactivity of the 250-k protein band was the same for both thrombin-treated and the untreated control samples. It is clear that even at concentrations of thrombin as high as 50 μg/ml, no reduction of the 250-k band occurs under conditions of mitogenic response to the protease. Measurement was also made of the content of the 250-k protein at various intervals during a 12-hr incubation of fibroblasts with 2 μg/ml of purified thrombin (column K). At all time points tested the content of \(^{125}\)I-labeled 250-k protein was the same as that of the untreated control.

Although purified thrombin does not cause the removal of the 250-k protein, a commercial preparation did exhibit a low but definite proteolytic activity on the cell membrane proteins (Exp. 3, Table 1). It is likely that this activity is a result of the action of contaminating proteases, since chromatography of the commercial sample on Sephadex G-200 showed the presence of three major protein peaks (14).

**Effect of Thrombin on Other Proteins of Chick Embryo Fibroblasts.** Since only a few of the membrane proteins of chick embryo fibroblasts can be labeled with \(^{125}\)I, cells were allowed to grow in the presence of either a mixture of \(^{14}\)C-labeled amino acids or with \(^{35}\)S]methionine. The proteins labeled with \(^{14}\)C or \(^{35}\)S were then subjected to electrophoresis on polyacrylamide slab gels by the same procedure used for \(^{125}\)I-labeled proteins. Columns A and B of Fig. 1 show, respectively, the \(^{14}\)C and \(^{35}\)S-labeled whole cell proteins in control samples that had not been treated with protease. The pattern of bands of whole cell proteins could not be distinguished from that of cells grown in \(^{35}\)S]methionine but treated with 10 μg/ml of thrombin for 12 hr (column C, Fig. 1). There is no readily detectable alteration in the protein profiles of these three samples. Specifically, the trypsin-sensitive external membrane protein and the transformation-specific proteins of molecular weights 206,000 and 47,000 (8, 24) do not show obvious differences in the thrombin-treated and the control cells.

**DISCUSSION**

In a search for cell surface components that undergo changes during transformation or during mitogenic stimulation brought about by proteases, Hydes (5–7) and Robbins et al. (8) have demonstrated in fibroblasts that a cell surface protein of molecular weight 250,000 is especially sensitive to trypsin action and is not present in most transformed cells. However, some transformed cells are known, in fact, to contain this protein (9, 25). This protein apparently is not fibroblast-specific, since a trypsin-sensitive external cell surface protein of similar molecular weight is found in a rat myogenic line (N. N. H. Teng, unpublished result). Wickus and Robbins (24) and Robbins et al. (8) have also shown that two other proteins are reduced in amount in transformed as compared to untransformed cell cultures. It has, therefore, been tempting to postulate that these specific cell surface components might play some role in the regulation of DNA synthesis and cell proliferation. We have tested this hypothesis by two approaches. In the first case we have shown that at very low concentrations of several proteases, namely, trypsin, α-chymotrypsin, subtilisin, and ficin, there can be nearly complete removal of the 250-k protein without a corresponding increase in DNA synthesis. Of particular importance was the comparison of the effects of thrombin and chymotrypsin. Thrombin is a potent mitogen that does not remove the protease-sensitive protein, whereas chymotrypsin removes this protein from the cell surface, yet has negligible mitogenic activity. Therefore, the protease-mediated loss of the 250-k protein is not an event sufficient by itself to bring about cell division nor is its removal necessary for cell proliferation.

In further experiments where we have examined the profile of other bands of the cell membrane proteins of chick embryo fibroblasts that had been labeled with \(^{35}\)S]methionine, we could detect no changes in the protein pattern of cells that had not been treated as compared to those that had been stimulated to synthesize DNA with thrombin. Of particular interest in this respect were two bands, one of molecular weight 206,000, the other, 47,000, which have been shown to be reduced in amount in transformed cells (8, 24). Therefore, we cannot with assurance detect alterations of surface components when thrombin was used as the mitogenic agent. However, these experiments do not rule out the possibility that subtle changes of the composition of surface proteins other than those specifically mentioned do occur through the action of thrombin and that the alterations might be required in cell growth regulation.

Of some interest was the comparison of results obtained with purified thrombin and a commercial preparation. The latter preparation in the concentrations used caused a slow but significant removal of the 250-k protein from chick embryo fibroblasts. Undoubtedly, this activity results from other contaminating proteases. The experiments with commercial thrombin illustrate the hazard of interpreting data obtained with reagents other than those of highest purity. Thrombin is a protease of great specificity known to hydrolyze four arginyl-glycine peptide bonds of fibrinogen (15). Yet, it is capable of causing the conversion of zymogens present in plasma or serum, for example, plasminogen, to active forms, which themselves might be the primary agents responsible for producing alterations of cell membrane components (26). Conversely, some serine proteases are also capable of activating zymogens which could be mitogens themselves (15). The conversion of prothrombin to thrombin by trypsin is an example. Thus, the interpretation of data obtained from experiments in which impure thrombin and other protease preparations are used, or in which serum or plasma has been fortified by a purified protease, should be suspect.

Because of the narrow range of peptide bonds that thrombin can cleave (15), it should be an important and useful probe for investigations of the role of changes in cell surfaces during mitogenic stimulation or during transformation. As an example, several investigators (27–30) have reported the effect of purified thrombin on the proteins of the intact plasma membrane of human platelets.

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