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Active Proteinase Inhibitors Associated With Human Breast Epithelial Cells

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The major glycoproteins synthesized by human breast epithelial cells have been characterized [6,8]. The most consistently observed and prominent component in supernatants of organ cultures of breast surgical specimens and of MCF-7 cells was gp 68 which has been immunologically identified as α-1-antichymotrypsin (Achy). In the present study we demonstrate that this glycoprotein can form an irreversible complex with chymotrypsin, which indicates that it is a functional inhibitor. The ^{14}C-glucosamine-labeled gp 68 forms a stable, 88,000-dalton, enzyme-inhibitor complex with chymotrypsin. The molecule is secreted continuously for 9 days into a chemically defined, serum-free medium. In addition to the de novo synthesized inhibitor, another component is adsorbed from fetal bovine serum and subsequently released into serum-free medium. This component also forms an irreversible, 88,000-dalton complex with enzyme. The observations establish that two types of inhibitors are associated with human breast epithelial cells, one actively synthesized and the other derived from serum. Both of these molecules may have significant roles in stabilizing cell surface components and in protecting extracellular matrices from untimely degradation.

Key words: proteinase inhibitors, alpha-1-antichymotrypsin, breast epithelial cells, matrix protection, gp 68

Human breast tissue is composed of epithelial cells arranged in glandular structures which are surrounded by a basal lamina. Components of the basal lamina are organized into large complexes which serve as an extracellular scaffolding to impose structural form on the epithelium. During ontogeny, pregnancy, and lactation, breast tissue undergoes dynamic rearrangement which requires proteolytic remodeling and extensive deposition of basal lamina to support the changes in glandular architecture [4,5,9]. Glycoproteins (gps) also play an important role in establishing and in maintaining three-dimensional tissue structure. We have previously investigated their synthesis by monitoring the incorporation of ^{14}C-glucosamine using organ cultures of human breast surgical specimens to identify the major gps from the breast epithelial tissue and to compare them as function of growth conditions [8,20]. Twenty representative gp families have been identified in the molecular weight range of 20–200 kd. Sixteen to thirty percent of these gps appeared to be serum-related. One of the

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most prominent components, gp 68, has been immunologically identified as alpha-1-antichymotrypsin (Achy). In view of another report on the synthesis of an inactive Achy by melanoma cells [12], the key question that remained was whether the Achy produced by human breast epithelial cells can form complexes with an enzyme. The results of experiments that answer this question affirmatively are provided below.

MATERIALS AND METHODS

Cell Cultures

The human breast epithelial cell line MCF-7 (passages 6–14) was obtained from Dr. David Kingsbury at Naval Biosciences Laboratory (Oakland, CA), and ZR-75-1 (passages 78-88) was from Mason Research Institute (Worcester, MA). Cells were routinely maintained in Dulbecco's Modified Minimum Essential Medium (DMEM) (Flow Laboratories, Rockville, MD), supplemented with insulin (10 μg/ml) (Sigma Chemical Co, St. Louis, MO), gentamycin (50 μg/ml) (GIBCO, Grand Island, NY), and 7% fetal calf serum (FCS) (Biocell, Compton, CA). Cells were maintained in a humidified atmosphere of 90% air–10% CO_2 at 37°C. Crystalline α-chymotrypsin (Worthington Biochemicals, Freehold, NJ) (50 μg/ml) in Dulbecco's phosphate-buffered saline (DPBS) was used for cell passages. Three milliliters containing 150 μg of α-chymotrypsin was added to each T-75 flask (Costar, Cambridge, MA) and incubated at 37°C for 10–45 min. This procedure resulted in single cell suspensions rather than the clumps and sheets of cells that were obtained from tissue culture trypsin dissociation. At each passage the cells were screened for mycoplasma contamination using Hoechst 33258 stain (Calbiochem-Behring Co, La Jolla, CA) [3].

Normal breast epithelial cells were obtained from reduction mammoplasty using a method developed by Dr. Martha Stampfer and were kindly supplied by Dr. Helene Smith and Dr. Adeline Hackett of Peralta Cancer Institute, Berkeley, CA [17,18]. The cells used in these experiments were passaged no more than three times. The identity of the epithelial cells was confirmed by immunofluorescent staining with antibodies to gp 70 purified from milk-fat-globular membranes and to epithelial-specific keratin [11,17].

Metabolic Labeling With ^{14}C-Glucosamine

Monolayers of cultured breast epithelial cells in T-75 flasks were grown for 7 days in Minimum Essential Medium-α+ (GIBCO) supplemented with insulin (10 μg/ml), gentamycin (50 μg/ml), and 7% fetal calf serum. When cells reached 70% confluency, the flasks were washed and incubated for 72 hr with 7 ml of labeling medium which consisted of MEM-α+, insulin (10 μg/ml), gentamycin (50 μg/ml), 2% fetal calf serum, and 21 μCi of D-[U-^{14}C]glucosamine hydrochloride (> 200 mCi/mmole, Amersham, Arlington Heights, IL). The cell-free supernatants were clarified by centrifugation, extensively dialyzed against phosphate-buffered saline (PBS) at 4°C and stored at −70°C. Specific activity ranged from 250 to 720 dpm per μg of trichloroacetic acid–precipitable protein.

Preparation of Conditioned Serum-Free Medium

Since fetal calf serum contains proteinase inhibitors, conditioned serum-free medium was collected. The cells were grown in the presence of serum until they reached approximately 70% confluency. At this time each T-150 flask was rinsed six times with 15 ml of warm serum-free DMEM. Each rinse remained on the cells for

15 min. The cells were then refed with 15 ml of serum-free DMEM containing insulin (10 μg/ml) and gentamycin (50 μg/ml). After 12 hr, this serum-free medium was removed and replaced with 15 ml of fresh serum-free DMEM and cells were maintained for 24 hr.

The MCF-7 cells do not continue active cell division under these conditions of serum deprivation. They remain attached to the flasks for the first 48 hr following the removal of serum. The conditioned serum-free medium used in the complex formation experiments contained 380 μg of protein that appeared in the 24-hr period starting from hr 12 after the removal of serum-containing medium from one T-75 flask containing 5×10^6 cells. This medium was withdrawn from the flasks, spun for 20 min at 10,000g at 4°C in a Beckman J21B centrifuge, concentrated 18-fold in an Amicon concentrator using a PM-10 membrane (Amicon Corp, Lexington, MA), and dialyzed against phosphate-buffered saline, pH 7.2, at 4°C. It was stored as frozen aliquots at −20°C. The final protein concentration was 460 μg/ml as determined using the BioRad protein assay with gamma globulin as the standard (BioRad Laboratories, Richmond, CA).

Preparation of Conditioned Chemically Defined Serum-Free Medium

In order to obtain conditioned serum-free medium even further removed from serum than 36 hr, MCF-7 was grown in a chemically defined serum-free medium designated MCDB170 and here called defined serum-free (DSF) which was kindly supplied to us by Dr. Martha Stampfer, Lawrence Berkeley Laboratory. (Berkeley, CA). This medium contains 70 μg/ml of protein which is contributed by bovine pituitary extract; the exact content of this medium is reported elsewhere [10].

MCF-7 was plated in the presence of 7% FCS in DMEM. When the cells reached approximately 70% confluency, each T-75 flask was washed six times with 15 ml of serum-free DMEM at 37°C. Each wash remained on the cells for 15 min. The cells were then refed with 10 ml of warm DSF and maintained in a humidified atmosphere of 2% CO_2. Medium was changed at 12 hr, 72 hr, and 132 hr. Both the fresh and conditioned media were stored at 4°C. All DSF media were concentrated using a Amicon PM-10 low protein binding membrane.

Radioiodination of Proteins

Chymotrypsin was iodinated by incubating 250 μg of enzyme and 1 mCi Na ^{125}I (Amersham) with 0.2 ml iodination buffer (0.15 M NaCl, 0.2 M potassium phosphate (pH 7.0)) in a tube coated with 200 μg chloroglycouril (Pierce Chemicals, Rockford, IL) for 10 min at room temperature [7]. The reaction mixture was withdrawn and added to 120 μg of potassium iodide and passed through a column (1 × 20 cm) containing Sephadex G-25 (medium mesh) which was equilibrated with iodination buffer. The fractions containing radiodinated proteins were pooled and stored at 4°C. Labeled preparations were used within 72 hr. The purified Achy (10 μg) was iodinated in a similar way using 50 μg of chloroglycouril and 500 μCi Na ^{125}I. Enzyme inhibitor complexes were formed by incubation with the iodinated enzyme for 15 min at 23°C.

Gel Electrophoresis

One-dimensional slab gel electrophoresis was carried out according to the procedure of Laemmli using 10% polyacrylamide gels [13]. The gels were stained in 0.025% Coomassie Blue G, destained, and dried on cellulose membranes. Gels

containing ^{125}I-labeled protein samples were autoradiographed on Kodak XAR-5 film with Cronex intensifying screens (Dupont, Wilmington, DE) at −70°C for varying periods of time.

Densitometry

The optical density (OD) of complexes on autoradiographs was determined using a manual densitometer from Brumac Industries (model bmi TRD-85, Huntington Beach, CA), equipped with a 1-mm aperture.

Preparation of Immunoaffinity Columns

Five milliliters of anti-alpha-1-antichymotrypsin (Dako Corp, Santa Barbara, CA) was treated with an equal volume of saturated $(NH_4)_2SO_4$, pH 7.8, at room temperature with constant stirring for 1 hr. After centrifugation at 10,000g the pellet was resuspended in 5 ml of 0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl and dialyzed for 24 hr against the same buffer at 4°C with four changes of 1 liter each. The resulting 33 mg of antibodies was coupled to 4 gm of cyanogen bromide–activated Sepharose CL-4B according to the procedure recommended by Pharmacia. Coupling was greater than 95% efficient on every occasion.

RESULTS

Enzyme-Inhibitor Complex Formation in Serum-Free Conditioned Medium

In order to determine the inhibitory activity of proteins released by human breast epithelial cells, the formation of complexes with iodinated chymotrypsin was studied. When 16 ng of iodinated enzyme was reacted with 25 μg of protein from serum-free medium conditioned by MCF-7 cells, complexes were formed. These complexes are stable to boiling in SDS sample buffer and migrate on SDS-polyacrylamide gels with molecular weights of approximately 88 kd (Fig. 1, lane A). The complexes formed when 25 μg of protein from fetal bovine serum was reacted with chymotrypsin are shown in lane B of the same figure. The 88-kd complexes formed from the medium conditioned by MCF-7 cells and from fetal bovine serum are indistinguishable. Fetal bovine serum contains an additional component which forms a 140-kd complex. The unbound enzyme represented the third band at 25 kd.

Figure 2, lane A, displays a similar autoradiograph of serum-free medium conditioned by ZR-75-1 cells, an established malignant breast epithelial cell line which does not synthesize Achy (manuscript submitted). Lane B contains fetal bovine serum of identical protein concentration reacted with iodinated chymotrypsin. The patterns are almost indistinguishable, which suggest that the breast epithelial cells are releasing into serum-free medium fetal bovine serum components which have previously been adsorbed by cells.

Since we have shown that MCF-7 but not ZR-75-1 cells synthesize and secrete gp 68, a glycoprotein that is immunologically identical to Achy, we wanted to determine if this molecule could also form an 88-kd complex with chymotrypsin. The conditioned serum-free media and fetal bovine serum at the same protein concentrations were incubated with iodinated enzyme, and the incubation mixture was treated with antibodies to human Achy covalently coupled to Sepharose CL-4B. The specifically adsorbed material was eluted by boiling in 2% SDS and subjected to gel electrophoresis. An 88-kd complex was obtained from the medium conditioned by

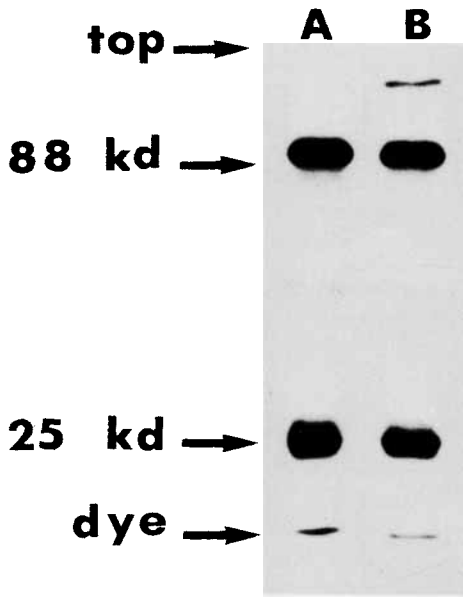

Fig. 1. Autoradiography of enzyme-inhibitor complexes formed by incubation of ^{125}I-chymotrypsin with MCF-7-conditioned serum-free medium or with fetal bovine serum. Sixteen nanograms of iodinated chymotrypsin (specific activity 1.26×10^6 cpm/μg) was incubated for 15 min at 23°C with 25 μg of protein from serum-free medium conditioned by MCF-7 cells (lane A) or with an equal amount of protein from fetal calf serum (lane B). The reaction was stopped by the addition of 2X SDS sample buffer, and a 10% acrylamide gel was run as described in Materials and Methods. The labeled inhibitor-enzyme complex of 88 kd is identified on the autoradiograph. The 25-kd band represents uncomplexed enzyme.

MCF-7 cells (Fig. 3, lane A), whereas no such complex resulted from fetal bovine serum (lane B). None of the fetal bovine serum components cross-react with antibodies to human Achy, as determined from immunodiffusion experiments and Western blots of fetal bovine serum treated first with antibody to Achy followed by iodinated protein A (data not shown).

Enzyme-Inhibitor Complex Formation in Chemically Defined Serum-Free Medium

In order to eliminate the problem of adsorption of fetal bovine serum inhibitors by MCF-7 cells, an 8-day incubation period was carried out in defined serum-free (DSF) medium. Medium conditioned by MCF-7 cells was collected from 12–72 hr and 132–192 hr cultures and concentrated. Time 0 is the point at which the cells were washed six times and placed in the chemically defined medium which contained no fetal bovine serum. Stable complexes were formed when 400 ng of iodinated chymotrypsin was reacted with 21 μg protein from DSF medium conditioned by MCF-7 cells for 12–72 hr (Fig. 4, lane A), 132–192 hrs (lane B), or with an equal amount of protein from unconditioned medium which has seen no cells (lane C). These complexes were analyzed using SDS slab gels. The DSF media conditioned by MCF-7 cells (lanes A and B) contained an 88-kd complex which was not present in the unconditioned medium (lane C). In addition to the 88-kdcomplex, radioactive bands

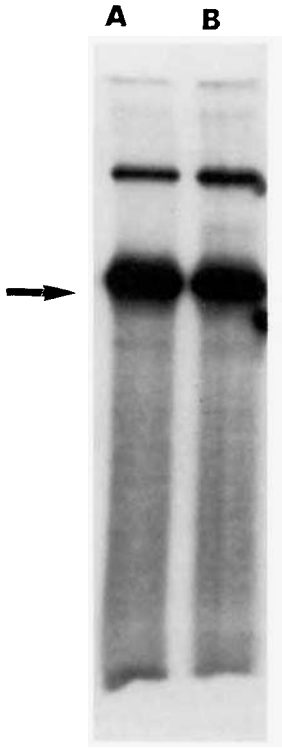

Fig. 2. Autoradiography of enzyme-inhibitor complexes formed by reaction of ^{125}I-chymotrypsin with serum-free medium conditioned by ZR-75-1 cells and with fetal bovine serum. Forty nanograms of ^{125}I-chymotrypsin (specific activity 4.5×10^4 cpm/μg) was incubated for 15 min at 23°C with 21 μg of protein from serum-free medium conditioned by ZR-75-1 cells (lane A) or with an equal amount of protein from fetal bovine serum (lane B). The reaction was stopped by addition of 2X SDS sample buffer. A 10% polyacrylamide gel was run as described in Materials and Methods and an autoradiograph was prepared. The arrow indicates the labeled 88-kd enzyme-inhibitor complex.

could be observed at 62, 54, and 45 kd in both conditioned and unconditioned medium. These lower-molecular-weight bands must originate from the bovine pituitary extract which is present in the medium. The appearance of the 88-kd band in conditioned medium suggests that this molecule which binds iodinated chymotrypsin is synthesized by the breast epithelial cells.

There is no decrease in the amount of enzyme-binding protein released by MCF-7 cells over an 8-day period. When the serum-free medium is replaced at 12 hr and every 60 hr thereafter, the cells released as much Achy between the 132–192 hr period as they did during the 12–72 hr periods as can be ascertained from densitometry scans of the complexes (Fig. 5). Under identical conditions of protein and enzyme concentrations, the intensity of the iodinated enzyme inhibitor bands was approximately the same. This rules out that the 88-kd complex detected under this experimental condition is formed with a protein from fetal bovine serum which the cells adsorb and slowly release during serum-free conditions, as one would expect a diminishment of the bands over an 8-day period.

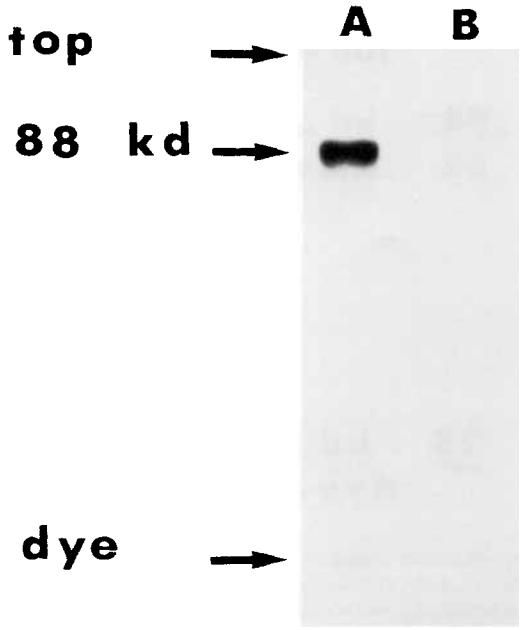

Fig. 3. Immunoaffinity purification of ^{125}I-chymotrypsin complexed with Achy from MCF-7-conditioned serum-free medium or from bovine fetal serum. Three hundred nanograms of ^{125}I-chymotrypsin (specific activity 1.26×10^6 cpm/μg) was reacted with 100 μg of protein from serum-free medium conditioned by MCF-7 cells (lane A) or with an equivalent amount of protein from fetal bovine serum (lane B) for 15 min at 23°C. Antibodies to human Achy covalently coupled to Sepharose CL-4B were added to each reaction mixture and incubated for 1 hr at 4°C. The specifically adsorbed material was eluted in boiling 2% SDS. Electrophoresis was carried out on a 10% polyacrylamide slab gel and an autoradiograph prepared. An arrow indicates the 88-kd complex from MCF-7-conditioned medium which was specifically adsorbed to antibodies to human Achy.

Enzyme-Inhibitor Complex Formation in Culture Supernatants Containing ^{14}C-Glucosamine-Labeled Glycoproteins

Experiments were designed to test whether the biosynthetically labeled Achy in the culture supernatants is accessible for complexing with chymotrypsin. MCF-7 culture supernatant containing ^{14}C-labeled glycoproteins was incubated with chymotrypsin. The incubation mixture was treated with Sepharose CL-4B covalently coupled with antibodies to Achy. This matrix is capable of binding to both free and enzyme-complexed inhibitors. The specifically adsorbed material was eluted by boiling in 2% SDS and subjected to gel electrophoresis followed by fluorography. In addition to the uncomplexed inhibitor of 66 kd, an 88-kd complex was detected. A densitometer scan of these bands from the fluorograph is shown in Figure 6. Since ^{14}C-labeled 88-kd complex was seen only after the addition of chymotrypsin, its presence suggests the formation of stable enzyme-inhibitor complexes by the de novo synthesized Achy.

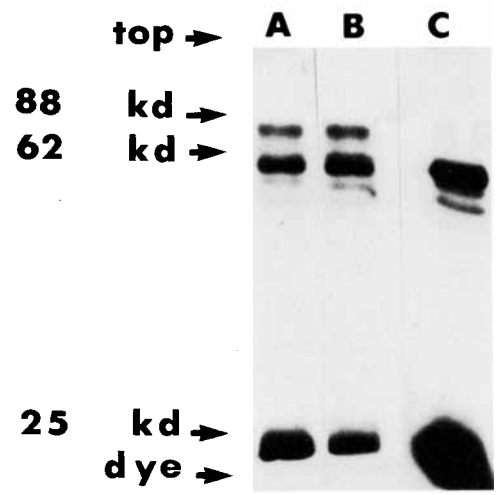

Fig. 4. Autoradiography of enzyme-inhibitor complexes formed by incubation of iodinated chymotrypsin with defined serum-free medium, conditioned or unconditioned, by MCF-7 cells. One hundred sixty nanograms of ^{125}I-chymotrypsin (specific activity 4.5 × 10^4 cpm/μg) was incubated for 15 min at 23°C with 21 μg of protein from defined serum-free medium conditioned by MCF-7 cells for 12–72 hr (lane A), 132–192 hr (lane B), or with an equivalent amount of protein from unconditioned medium (lane C). The reaction was stopped by the addition of an equal volume of 2X SDS sample buffer. A 10% polyacrylamide slab gel was run and an autoradiograph was prepared. An arrow indicates the 88-kd band which appears only in medium conditioned by MCF-7 cells. The 62-kd complex which is present in both conditioned and unconditioned medium is also indicated.

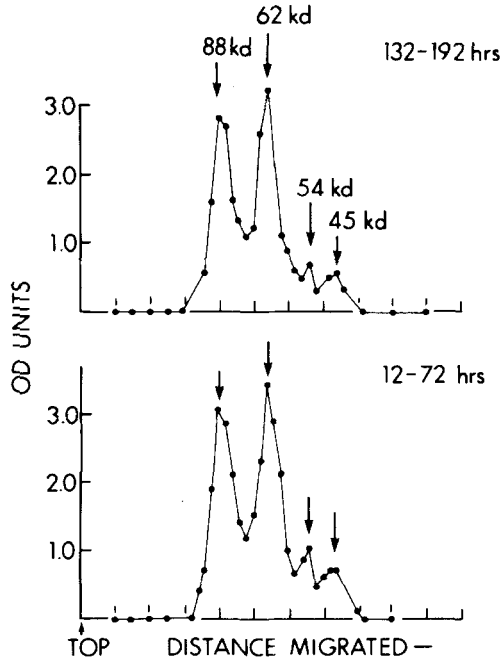

Fig. 5.

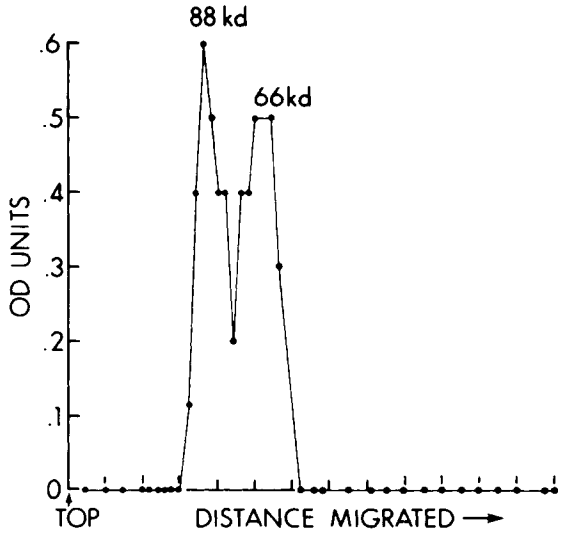

Fig. 6. Densitometer scan of fluorograph of immunoaffinity purified chymotrypsin complexed with ^{14}C-glucosamine-labeled Achy from MCF-7 culture supernatant. Cells were maintained in MEM-α+ medium supplemented with 1% fetal bovine serum and ^{14}C-glucosamine for 48 hr. Samples of culture supernatant containing 415 μg of protein (specific activity 721 cpm/μg) were incubated for 15 min at 23°C with 1 μg of chymotrypsin. Achy antibody coupled to cyanogen-bromide-activated Sepharose CL-4B was added to the mixture and incubated for 1 hr at 4°C. The specifically adsorbed material eluted in boiling 2% SDS. Electrophoresis was carried out on a 10% polyacrylamide slab gel and a fluorograph was prepared. The bands present on the fluorograph were scanned with a densitometer. The 88 kd peak represents the labeled Achy-chymotrypsin complex. The uncomplexed inhibitor comprises the 66 kd peak.

DISCUSSION

The intent of this study was to determine what type of proteins are associated with human breast epithelial cells that can form irreversible complexes with serine proteinases which cleave hydrophobic bands. The present paper serves as the first report on the identification of a passively adsorbed fetal bovine serum component and an actively synthesized glycoprotein which form complexes with chymotrypsin. The study used established breast adenocarcinoma cell lines.

Active chymotrypsin labeled with ^{125}I was used to probe for the existence of inhibitors in conditioned serum-free culture media. The presence of functional inhibitors can be ascertained by the formation of irreversible enzyme-inhibitor complexes. Such complexes eliminate all detectable chymotryptic activity [21]. It is known that cultured cells grown in the presence of fetal bovine serum adsorb proteinase inhibitors, such as fetuin, and gradually release them back into the serum-free medium

Fig. 5. Densitometer scan of an autoradiograph of enzyme-inhibitor complexes formed from conditioned defined serum-free medium. Lanes A and B from Figure 4 were scanned with a densitometer. Enzyme-inhibitor complexes are indicated with arrows. The 88-kd complex is formed only in defined serum-free medium conditioned by MCF-7 cells. There is no decrease in the intensity of this band over a 8-day period.

[14,15,22]. The expected molecular weight of the fetuin-chymotrypsin complex is 88 kd, which makes it feasible to postulate that at least a portion of the 88-kd complex is contributed by fetuin, which has an apparent molecular weight of 68 kd. The complexes formed by these serum inhibitors are essentially irreversible, stable to reduction by 2-mercaptoethanol and to boiling in sodium dodecyl sulfate. The appearance of the 88-kd complex obtained from fetal bovine serum, MCF-7, and ZR-75-1 cells was identical (Figs. 1,2). This finding is consistent with an adsorbed serum component since the ZR-75-1 cells which do not synthesize α-1-antichymotrypsin still release a component capable of complex formation. No biosynthetically incorporated label could be detected in the 88-kd complex from the serum-free medium of ZR-75-1, nor could this complex be immunologically identified with antibodies to human Achy (data not shown). This observation suggests that the ZR-75-1 cells serve as an indicator for the substantial amount of the passively adsorbed fetal bovine serum components capable of forming the 88-kd complexes.

The adsorption of serum proteins made it difficult to identify the inhibitor synthesized by the cells. However, the cell-synthesized product could be distinguished immunologically using antiserum to human Achy. Complexes of iodinated chymotrypsin and components of conditioned serum-free medium or fetal bovine serum were allowed to form and subsequently treated with antibodies to Achy covalently coupled to Sepharose CL-4B. The MCF-7-synthesized Achy which complexed with the enzyme can be seen in Figure 3, lane A. None of the serum-derived inhibitors and their complexes were recognized by specific antibodies to Achy (Fig. 3, lane B). This demonstrates that a portion of the 88-kd complex visualized in Figure 1 comes from human Achy synthesized by the MCF-7 cells.

To further ascertain the cellular origin of the active inhibitor, the cells were maintained in a defined serum-free medium containing bovine pituitary extract. This extract contains three components which are capable of forming stable complexes with chymotrypsin, all of which have molecular weights below the expected weight of the Achy-chymotrypsin complex (Fig. 4, lane C). We detected the appearance of the 88-kd complex after MCF-7 cells had been grown in the medium for 60 hr (lane A), which suggests that this complex is formed from a cell product. This suggestion is confirmed by two sets of observations. The amount of 88-kd complex did not decrease in the period from 6 to 8 days with three changes of medium (Fig. 4, lane B, and Fig. 5) which would be expected if it was a component adsorbed from serum. The second observation is the immunoadsorption of a ^{14}C-labeled 88-kd enzyme-glycoprotein complex. MCF-7-derived glycoproteins were metabolically labeled with ^{14}C-glucosamine and allowed to accumulate in complete culture medium. Active enzyme was added to this medium. Using specific antibodies to human Achy, it was possible to purify the ^{14}C-labeled Achy and its enzyme complex. The immunoaffinity-purified components were resolved by gel electrophoresis and visualized by fluorography. A scan of this fluorograph establishes the presence of a labeled band at 88 kd and an additional uncomplexed inhibitor which is seen at 68 kd (Fig. 6). This establishes unequivocally that the synthesized glycoprotein is indeed able to bind to a serine proteinase and form a complex.

The name alpha-1-antichymotrypsin may be a misnomer. The inhibitor purified from serum has been extensively characterized as being a more potent inhibitor of cathepsin G than of chymotrypsin [1]. Cathepsin G is a matrix-degrading enzyme that is released by neutrophil leukocytes and mast cells [2,16,19]. Thus, the demon-

stration that an active Achy is synthesized by human breast epithelial cells allows one to infer that its function may be to protect the integrity of glandular tissue structures from destructive enzymes released by invading leukocytes. The regulation of the synthesis of this inhibitor may also correlate with developmental events which require the adsorption or the deposition of basal lamina structures during ontogeny.

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