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The Synthesis of a Proteinase Inhibitor, α-1-Antichymotrypsin, by Human Breast Epithelial Cells

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The synthesis and release of glycoproteins were studied in organ cultures of human breast surgical specimens and in established breast epithelial cell lines, MCF-7 and MDA-MB-231. Biosynthesis was monitored by the incorporation of ^{14}C-glucosamine. Labeled macromolecules in the culture supernatants were analyzed by biochemical and immunological techniques. One to 8% of the labeled glycoproteins from benign breast and infiltrating ductal carcinoma specimens was precipitated by antibodies produced against human serum α-1-antichymotrypsin. Twelve percent of the total glycoproteins from the culture supernatants of the MCF-7 cell line was identified as α-1-antichymotrypsin. Both the normal serum and the human breast epithelia-derived proteinase inhibitor can be resolved into similar subclasses by two-dimensional gel electrophoresis. MDA-MB-231 and MCF-7 cells which were extensively washed with EDTA, serum-free medium, and phosphate-buffered saline retain this proteinase inhibitor on their cell surfaces. Three to 4% of the total cell-surface iodinated components was immunoprecipitated by these specific antibodies. Since α-1-antichymotrypsin is a potent inhibitor of neutral proteinases such as cathepsin G, the demonstration of its synthesis by benign and malignant breast epithelial cells is of considerable interest. This glycoprotein may represent the epithelia's own protective shield of cell surface components and the cell's attempt to moderate the effects of invading leukocytes. In addition, it may play a regulatory role in the maintenance of three-dimensional glandular structures.

Key words: human breast epithelia, glycoproteins, proteinase inhibitors, organ culture, α-1-antichymotrypsin, breast adenocarcinoma, glandular structure

Human breast epithelial cells form well-defined, three-dimensional glandular structures. These complex structures are maintained by lateral-lateral cell interactions, basal lamina, and connective tissue support. The development of glandular tissue is a dynamic process involving active deposition and resorption of the macromolecules involved in these interactions. Under various pathological conditions such as neoplasia, the three-dimensional integrity of these structures is characteristically altered. Earlier work suggested that this gradual breakdown of tissue integrity and the dynamic development of breast tissue may be mediated by proteinases [1–3]. The object of our study was to monitor the biosynthesis and the

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release of glycoproteins as a function of breast epithelial cell arrangement. Benign and malignant human surgical specimens were maintained in organ culture under conditions where the three-dimensional tissue integrity was least altered. We now report that one of the actively-synthesized glycoproteins is α-1-antichymotrypsin, Achy, a known inhibitor of the serine-type neutral proteinases such as cathepsin G and chymotrypsin. This glycoprotein is also one of the major products from the established breast epithelial cell line, MCF-7. A portion of the released inhibitor is retained at the cell surface as well. The demonstration that this molecule is synthesized by the breast epithelia suggests that Achy may play an important role in maintaining three-dimensional glandular structures and in protecting the tissue from the neutral proteinases of invading leukocytes.

MATERIALS AND METHODS

Organ Cultures

The general procedure for organ cultures has been described earlier [4]. Breast surgical specimens from two benign breast tissues and three infiltrating ductal carcinomas were cut into 1.0 mm cubes and placed on stainless steel-screens within 35 × 10 mm petri dishes (Falcon Plastics, Oxnard, CA) containing 3.0 ml of culture medium. Two screens were used per dish and each screen contained from 20 to 30 cubes of explant. Cultures were carried out in Medium 199 (Flow Laboratories, Rockville, MD) supplemented with 2 mM glutamine, 100 units/ml of penicillin, 100μg/ml streptomycin, 10μg/ml insulin, and 1 to 5% of the patient's own serum. To each dish, 4 μCi of [^{14}C]-glucosamine was added (250–350 mCi/mmole, New England Nuclear Corp. Boston, MA). After 72 hr of incubation the cell-free supernatants were extensively dialyzed against phosphate-buffered saline (PBS), pH 7.2, at 4°C and concentrated by negative pressure dialysis.

Cell Cultures

Cultures of MCF-7 (passage no. 23) and MDA-MB-231 (passage no. 103) were obtained from Dr. W. A. Nelson-Rees at Naval Biosciences Laboratory, Oakland, California. Monolayers of 5 × 10^6 cells per 175 cm^2 culture flasks (Falcon Plastics) were incubated for 72 hr in RPMI-1640 medium (Flow Labs.) containing 0.5% fetal calf serum and 25 μCi of [^{14}C]-glucosamine. The cell-free supernatants were extensively dialyzed against PBS at 4°C and concentrated by negative pressure dialysis.

Two-Dimensional Gel Electrophoresis

Labeled glycoproteins from culture supernatants and immunoprecipitates were analyzed by two-dimensional gel electrophoresis using the O'Farrell technique as described for human plasma proteins [5]. The first dimension was isoelectric focusing in the presence of urea, nonionic detergent, and reducing agents. The presence of urea interferes with the accurate determination of isoelectric points; therefore, only apparent pIs are indicated on figure legends. The pH 4.0 to 6.0 gradient was made by using a 4:1 mixture of pH 4.0–6.0:pH 3.5–10.0 LKB ampholytes (LKB Western Instr., Pleasant Hill, CA). This particular gradient was chosen since it provides excellent resolution for many of the serum glycoproteins. The second dimension was electrophoresis in 10% polyacrylamide slab gels in the

presence of sodium dodecyl sulfate. The gels were stained with either 0.1% Coomassie Brilliant Blue G or with periodic acid-Schiff, PAS [6]. Molecular weight markers were phosphorylase B, 93,000 daltons; bovine serum albumin, 68,000 daltons; ovalbumin, 43,000 daltons and soybean trypsin inhibitor, 21,000 daltons. Labeled glycoproteins were identified on the gels by fluorography. Gels were infused with Enhance (New England Nuclear), dried, and exposed to preflashed Kodak XR-5 film (Eastman Kodak Company, Rochester, NY) at 70°C for varying periods of time.

Cell Surface Iodination

Monolayers of established cell lines were dissociated with EDTA (20 mg/100 ml in PBS). The dispersed cells were washed three times with MEM media (Flow Laboratories) and three times with PBS. Viability was greater than 95% as determined by trypan blue exclusion. Iodination was carried out as described earlier [7,8]. Cells were resuspended in PBS, 10^6 cells per 0.5 ml, and added to a scintillation vial which was precoated with 25 μg of Iodogen (Pierce Chemical Corp., Rockford, IL). The reaction was started with the addition of 0.5 mCi of [^{125}I]-iodide (Amersham, Arlington Heights, Illinois) and allowed to proceed for 10 min at room temperature with gentle shaking. The reaction was terminated by withdrawing the cells from the vial and washing them three times with phosphate-buffered sodium iodide (NaI is 0.8%) containing 1 mM phenylmethylsulfonylfluoride, (PMSF). The cells were lysed by incubating at room temperature for 10 min in PBS containing 0.5% NP-40 and 1 mM PMSF [9]. Subcellular particles were removed by centrifugation at 1000*g* for 10 min and the supernatant was repeatedly dialyzed against PBS containing PMSF.

Immunoprecipitation

Extensively dialyzed and concentrated culture supernatants or cell lysates were used for quantitative immunoprecipitation. Rabbit antisera against normal human serum Achy were obtained from Dako Corporation (Santa Barbara, CA). For controls, preimmune rabbit antisera were used after adsorption with glutarldehyde-fixed human red blood cells and fixed human serum proteins and following dilution to the same concentration as the immune sera. Electrophoretically pure human Achy was kindly provided by Dr. James Travis, University of Georgia, Athens, Georgia, who purified it from human plasma using selective precipitation and Cibacron Blue–Sephadex affinity chromatography [10]. Achy was ^{125}I-labeled by Iodogen (Pierce Chemicals) and the labeled protein (40,000 cpm/ng) was used as a tracer to establish conditions whereby 95% of this glycoprotein is specifically immunoprecipitated. All culture samples, lysates, and sera were centrifuged at 100,000*g* for 30 min in a Beckman Airfuge to sediment nonspecific aggregates prior to incubation with specific antibodies. Typical immunoprecipitations required 0.5 ml of culture supernatants in duplicate containing 3000–7000 dpm of ^{14}C-labeled trichloroacetic acid-precipitable glycoproteins. The samples were incubated with 10–50 μl of rabbit antisera or preimmune sera at 4°C for 12 hr. Goat anti-rabbit IgG was added (100 μl per 1 μl undiluted antisera) and further incubated for 5 hr at room temperature. The antigen–antibody complexes were sedimented by centrifugation and washed three times with PBS. The amount of radioactivity was determined in a Beckman LS 9000 liquid scintillation counter.

TABLE I. Immunoprecipitation of Biosynthetically-Labeled α-1-Antichymotrypsin From Culture Supernatants

Culture specimens	Total dpm per 3.0 ml supernatant	dpm precipitated with preimmune sera	dpm precipitated with anti-Achy	Percentage specifically precipitated with anti-Achy
MCF-7 cell line	12,360	756	2,230	11.9
Benign breast tissue	16,500	768	1,340	3.5
Benign breast tissue	45,900	1650	5,240	7.8
Infiltrating duct CA	16,900	726	1,130	2.4
Infiltrating duct CA	4,860	306	570	5.4
Infiltrating duct CA	18,240	690	858	0.9
Control: ^{125}I-Achy + MCF-7 supernatant	95,400	3130	94,500	95.8

Immunoprecipitation of cell lysates was carried out in the presence of 0.1% NP-40, and the ^{125}I-labeled proteins were measured in a Packard auto-gamma counter. A portion of the precipitates was solubilized in 2% SDS–sample buffer [5] and analyzed by two-dimensional gel electrophoresis as described earlier.

RESULTS

Organ cultures of breast surgical specimens release a portion of their newly synthesized and [^{14}C]-glucosamine-labeled glycoproteins into culture supernatants. The amount of nondialyzable and trichloroacetic acid-precipitable glycoproteins varies significantly in different surgical specimens. Glycoproteins with 4800 dpm to 45,900 dpm of incorporated label appeared in 3.0 ml of the supernatants of five different organ cultures after 72 hr of incubation (Table I). The variations in the amounts of released proteins are functions of epithelial cell abundance and viability in the tissue specimens. Since these parameters are not readily measured, a quantitative comparison of glycoprotein synthesis by benign and malignant breast surgical specimens is not feasible.

Our earlier observations revealed that one of the labeled glycoproteins from organ cultures was serologically related to the serum proteinase inhibitor, Achy. In order to confirm the synthesis of Achy and to estimate the proportion of labeled glycoproteins present as this proteinase inhibitor, quantitative immunoprecipitation was performed on the culture supernatants (Table I). We demonstrate that 3.5 and 7.8% of the total labeled glycoproteins from two benign breast tissues were recognized as Achy by specific antibodies. Similarly, 0.9 to 5.4% of the total labeled components was precipitated from the organ culture supernatants of three infiltrating ductal carcinomas. The immunoprecipitated Achy from the supernatant of an infiltrating ductal carcinoma produced patterns similar to its normal serum-derived equivalent on two-dimensional gel electrophoresis (Figs. 1 and 2). The serologically and electrophoretically pure serum component is resolved into more than 12 subclasses, presumably on the basis of carbohydrate heterogeneity. Identical patterns were obtained if the gels were stained with PAS, and no other glycoprotein contaminants were detected. More than 10 labeled subclasses of the immunoprecipitated Achy can be identified with isoelectric points and molecular weights similar to the serum component.

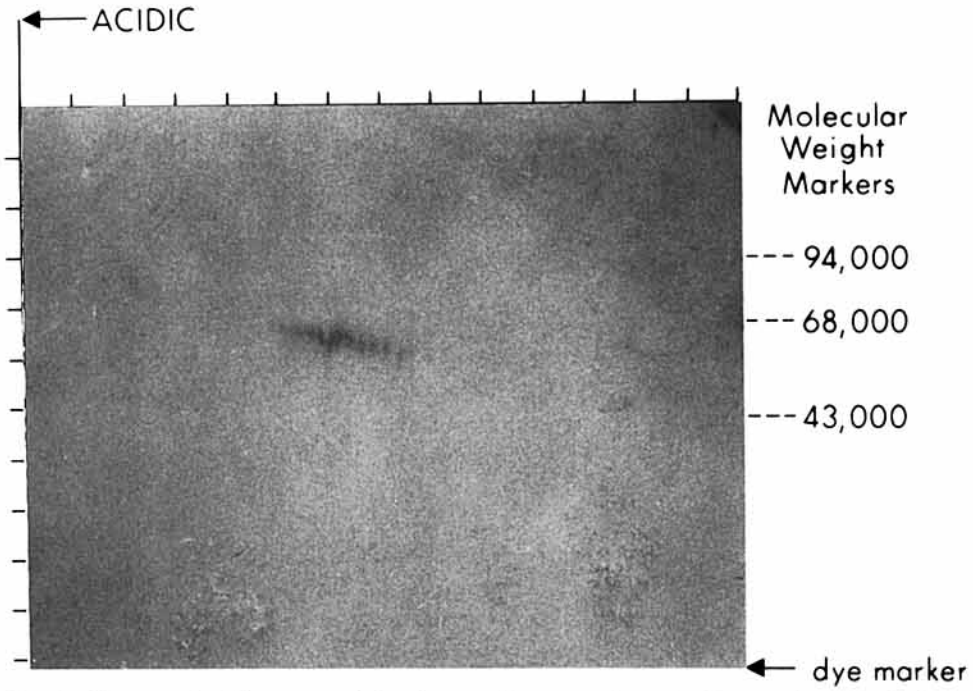

Fig. 1. Fluorograph of immunoprecipitated α-1-antichymotrypsin resolved by two-dimensional gel electrophoresis. Biosynthetically-labeled glycoproteins were obtained from the organ culture supernatant of an infiltrating ductal carcinoma. The precipitate containing 1000 dpm was applied to the gel and exposure time was 3 months. The gradient for isoelectric focusing was from pH 4.0 to 6.0.

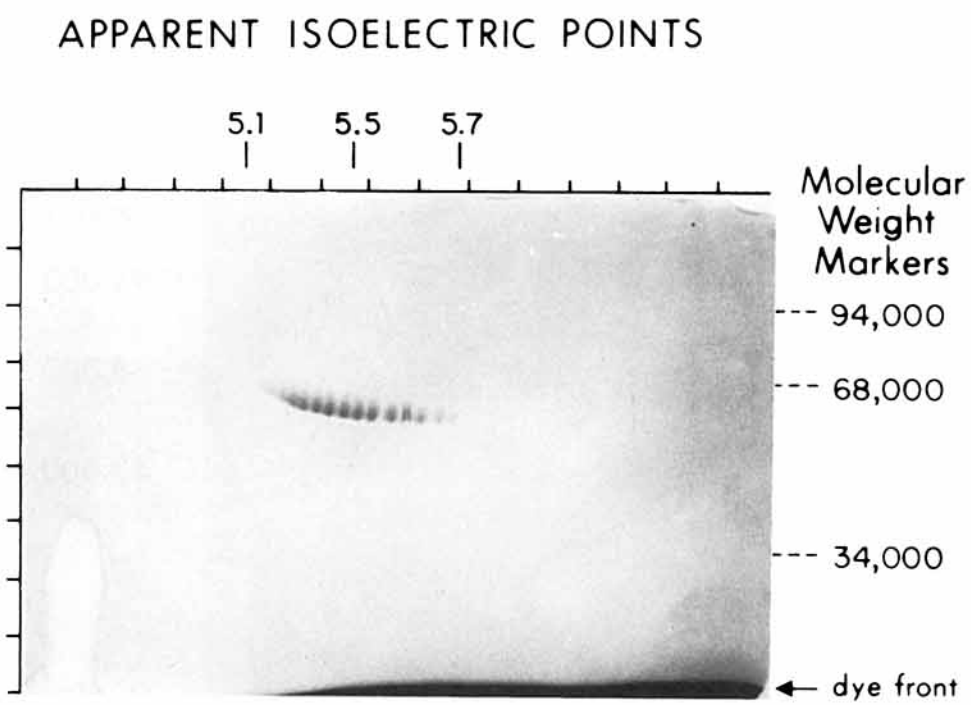

Fig. 2. Two-dimensional gel electrophoretic pattern of α-1-antichymotrypsin which was purified from normal human serum. The gradient for isoelectric focusing was from pH 4.0 to 6.0. Twenty micrograms of protein was applied and the gel was stained with Coomassie Brilliant Blue G. More than 12 Achy subclasses can be identified.

However, more extensive studies are required to unequivocally establish molecular identity or dissimilarity between tumor- and serum-derived Achy.

In order to confirm the epithelial origin of Achy, glycoprotein biosynthesis by MCF-7 cells was monitored by measuring [^{14}C]-glucosamine incorporation into macromolecules. Monolayer cultures of 1×10^6 cells, at densities approaching confluency, released 12,300 dpm of labeled macromolecules into 3.0 ml of supernatant after 72 hr of incubation (Table I). The labeled components are resolved into several major families of glycoproteins by two-dimensional gel electrophoresis and visualized by fluorography (Fig. 3). The component with a molecular weight of 65,000 ± 4000 daltons and with apparent isoelectric points ranging from 4.8 to 5.2 corresponds to the biosynthetically-labeled Achy. This was further confirmed by two-dimensional gel electrophoresis of the immunoprecipitated molecules (not illustrated here). Quantitative immunoprecipitation with anti-Achy accounted for 11.9% of the total labeled glycoproteins (Table I).

Monolayer cultures of MCF-7 and MDA-MB-231 retain human Achy on their surfaces. After extensive washing of these cells with EDTA, MEM media, and PBS, these cells contain Achy as determined by cell surface iodination and specific immunoprecipitation. Of the total incorporated ^{125}I-label, 3.2 to 4.0% was precipitated with rabbit anti-human Achy (Table II). These observations suggest that a portion of the released Achy is retained at the epithelial cell surface and represents a significant percentage of those surface components which cannot be dissociated with EDTA.

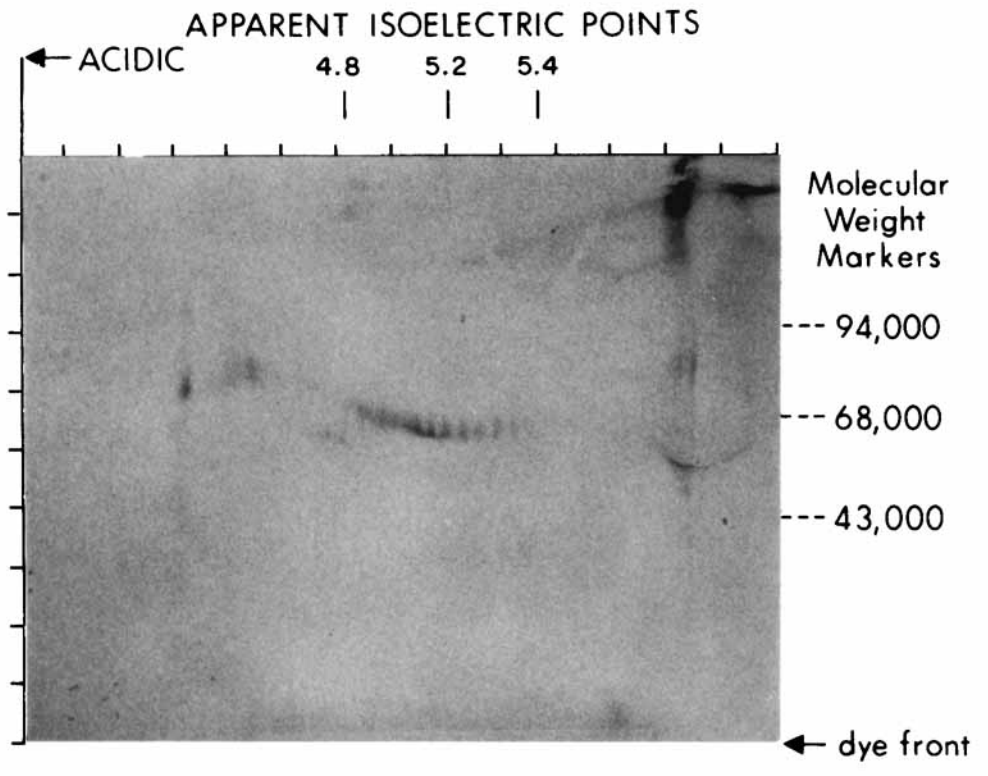

Fig. 3. Fluorograph of [^{14}C]-glucosamine-labeled glycoproteins released by the cell line MCF-7 and separated by two-dimensional gel electrophoresis. Isoelectric focusing was the first dimension, using a gradient from pH 4.0 to 6.0. Marks along the edge represent 1 cm of the original gel.

TABLE II. Immunoprecipitation of ^{125}I-Labeled Cell Surface Components From Established Human Breast Adenocarcinoma Cell Lines

Cell line	Total dpm per 10^6 cell lysate	dpm precipitated with preimmune sera	dpm precipitated with anti-Achy	Percentage of ^{125}I-labeled surface components precipitated as Achy
MCF-7 passage No. 25	318,000	1270	13,990	4.0
MDA-MB-231 passage No. 105	617,000	5550	19,750	3.2

DISCUSSION

Earlier work from our laboratory established that glycoprotein synthesis in organ cultures of breast and prostate surgical specimens can be monitored by the incorporation of isotope-labeled glucosamine [4,11]. Initial incorporation takes place in the Golgi apparatus and can be inhibited by tunicamycin and cycloheximide. The labeled macromolecules gradually migrate toward the apical surface of the epithelial cells. After 48 hr of incubation the majority of newly synthesized glycoproteins accumulate in the lumen. Grain distribution studies on autoradiographs of thin sections revealed that more than 70% of incorporation is by epithelial cells, both in benign and malignant breast specimens. Such preferential synthesis by one of the cell types renders the organ culture system feasible to study glycoprotein synthesis by epithelial cells. In this system tissue integrity and three-dimensional glandular structures are maintained.

Labeled glycoproteins appear in the organ culture supernatants after 3 days of incubation (Table I). These labeled components can be resolved and visualized by fluorography of two-dimensional gel electrophoresis patterns. Five major families of glycoproteins are detected in the supernatants of benign and malignant breast specimens [12,13]. The first group of glycoproteins (Gp I) has molecular weights larger than 200,000 daltons and is located on gels where fibronectin is observed. The second group of labeled components (Gp II) represents an acidic family of glycoproteins with a wide range of molecular weights of 60,000 to greater than 200,000 daltons. The fifth group of glycoproteins (Gp V) can be resolved into four or five spots according to isoelectric points and has molecular weights of 42,000 $\pm$ 3000 daltons. The identities of Gps I, II and V remain uncertain and are the subjects of our current investigations.

We have recently identified the third group of glycoproteins (Gp III) as serologically related to serum α-1-acid glycoproteins [13,14]. This component represents from 0.7 to 2.6% of the total released glycoproteins from benign and malignant breast specimens. Gp III may represent the tissue's capacity to modulate the immune system, since α-1-acid glycoprotein is a known inhibitor of lymphoblastogenesis [15].

The fourth group of glycoproteins (Gp IV) is characterized herein as Achy and is present as 0.9 to 7.8% of the total of released components. These percentages are only valid for the released components and may not be applicable to the total amount of glycoproteins synthesized. Since we demonstrate that a portion of Achy is retained on the epithelial cell surface (Table II), the proportion may be significantly higher.

Achy which has been purified from the serum has a molecular weight of 65,000–68,000 daltons. It is composed of a single polypeptide chain and contains from 79 to 89 carbohydrate residues per mole of which 11–14 residues are sialic acid. Based on amino terminal sequences, Achy shows sequence homology to α-1-proteinase inhibitor [10]. Previous work established that liver parenchymal cells are a major site of biosynthesis of Achy [16]. However, the synthetic activities of other tissues have not been studied previously.

There are two further lines of evidence which show that Achy is produced by the epithelial cells. Both benign and malignant epithelia stained positively with rabbit anti-Achy by the immunoperoxidase technique (unpublished observations), although histological stainings and quantitative immunoprecipitations revealed significant variations among different clones of breast adenocarcinomas (Table I). The second line of evidence comes from the biosynthetic studies on the MCF-7 cell line. Under the isoelectric focusing conditions chosen for the two-dimensional gels, the labeled Achy apears as one of the most prominent components on the fluorograph (Fig. 3). The apparent isoelectric points were from 4.8 to 5.4. These appear slightly more acidic than those of normal serum Achy. Whether this is a cell culture artifact or a true reflection of an altered glycosylation by adenocarcinoma cells is not known. However, elevated levels of sialyltransferases in breast adenocarcinomas have been reported which may account for an increase in negative charge [17,18].

As of today no biological function has been assigned to Achy. Our demonstration that it is produced by breast epithelial cells suggests at least two kinds of possible functions. Achy may stabilize the three-dimensional glandular structures in the tissue. During development, epithelial sheets undergo a process of folding and budding [1]. The establishment of a highly branched duct and acinus system requires the continuous deposition, resorption, and rearrangement of the basal lamina. During the process of breast involution, the basement membrane, which represents a layer within the basement lamina, is removed. Immunohistochemical and biochemical studies suggested that there are present involuting breast proteinases which are capable of basement membrane hydrolysis [2]. If Achy were an inhibitor of these unidentified enzymes, it could perform a regulatory function. Both malignant and nonmalignant human breast tissues secrete lysosomal and neutral proteinases [3,19]. Similarly, elevated levels of thiol proteinase have also been found in explants of malignant tissue [19]. Therefore, the gradual breakdown of three-dimensional glandular structures which is associated with neoplasia may be the result of an altered balance in the tissue between proteinases and proteinase inhibitors such as Achy.

Achy has been recognized as a specific inhibitor of chymotrypsin-like proteinases from phagocytic cells (neutrophils, basophils, and tissue mast cells) [20]. However, the nomenclature of this inhibitor may be misleading, since it has a very high affinity for cathepsin G and it is highly unlikely that Achy ever sees chymotrypsin. The association rate for neutrophil-derived cathepsin G is 5.1×10^7 whereas for human pancreatic chymotrypsin it is 1.0×10^4 [10]. In most tissues cathepsin G has a wide spectrum of biological functions. It can act as a promoter of phagocytosis and as a generator of chemotactic activity from serum. It is also involved in the production of factors relating to cytolysis, immune adherence, and histamine release. Both HeLa cells and human newborn fibroblasts become

detached after treatment by granule proteases which include cathepsin G [21]. Therefore, our demonstration that breast tissue produces this inhibitor now raises the possibility that Achy represents the epithelia's attempt to modulate invading leukocytes.

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