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THE 52-kDa ESTROGEN-INDUCED PROTEIN SECRETED BY MCF_7 CELLS IS A LYSOSOMAL ACIDIC PROTEASE

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An estrogen-induced 52-kDa glycoprotein secreted by human breast cancer cells and able to autostimulate the growth of MCF_7 cells has been purified, using monoclonal antibodies, and characterized. The protein contains mannose 6-phosphate signals on its N-linked high-mannose chains, suggesting that it is a lysosomal enzyme. Both the secreted 52-kDa protein and its processed cellular forms (52-, 48- and 34-kDa) were identified as carboxyl proteinases having an optimal activity at pH 3.5 and being specifically inhibited by pepstatin. This protease is characterized by its inducibility by estrogens and its high concentration in proliferative benign and malignant mammary tissue, when detected by immunohistochemistry. The estrogen-induced secretion of this protease may help to understand how estrogens stimulate mammary tumor growth and/or invasion.

Estrogens stimulate the proliferation and dissemination of hormone-dependent breast cancer cells by unknown mechanisms (1)(2). Several proteins induced by estrogens and secreted into culture medium by the mammary cancer cells in culture have been proposed as mediators of the estrogen effect on tumor growth, acting by autocrine and/or paracrine mechanisms (3)(4)(5).

One of these proteins produced by the human MCF_7 cell line with a molecular weight of 52,000 has been extensively characterized by our laboratory both in its secreted and cellular forms (3)(6). The 52-kDa secreted protein contains at least 2 N-linked Endo-H-sensitive high-mannose chains (7) and corresponds to a precursor of the 48-kDa and 34-kDa cellular proteins (6). We found, by immunohistochemistry, specific staining in some benign and malignant proliferative ductal

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mammary tissue and sweat glands but not in normal mammary cells and other tissues (8)(9). This protein is not induced, but instead inhibited by antiestrogens which block the proliferation of MCF_7 cells (3). Moreover the secreted protein is able to enter estrogen-deprived MCF_7 cells and to stimulate their growth (10).

In order to decipher the mechanism of this mitogenic activity and to approach the biological function of the protein, we have characterized its post translational modification and searched for an enzymatic activity. We identify the 52-kDa protein as an acidic lysosomal protease.

MATERIAL AND METHODS

Cell culture and labeling

MCF_7 cells supplied by the Michigan Cancer Foundation (Detroit), were cultured in medium containing 10 % fetal calf serum. Confluent cells were labeled with $|^{35}S|$methionine (200 µCi/ml) for 8 hours as described (3) or with 0.6 mCi/ml $|2\text{-}6\ ^{3}H|$ mannose (54 Ci/mmole, Amersham). For $|^{32}P|$labeling, the cells were first rinsed twice with 1 ml of MEM containing one-twentieth the normal concentration of phosphate and labeled for 7 hours in 500 µl of the same MEM plus 2 mCi/ml $|^{32}P|H_3PO_4$ (CEA France). Using an anti-52-kDa protein monoclonal antibody (M1G8) (11), the cellular and secreted 52-kDa proteins were immunoprecipitated from NP40 cell lysate, as described (6)(7).

Oligosaccharide chain analysis

The radioactive secreted 52-kDa immunoprecipitates were dissolved in 50 mM NaH_2PO_4, pH 5.4, containing 1 % SDS heated for 1 min at 100°C and diluted ten-fold with water. Endo-H (endo-ß-N-acetylglucosaminidase H, Miles) was added at 25 mU/ml and the samples were incubated for 16-18 hours at 37°C. The proteins were then precipitated with 10 % trichloroacetic acid before SDS-PAGE analysis. The TCA-soluble oligosaccharides were neutralized with 5N NaOH and separated from the bulk of free radioactive label by gel filtration on a G25 column (PD10 Pharmacia) in 0.1 M pyridine-acetic acid pH 5.0. The oligosaccharides were then lyophilized, hydrolyzed or not in 2M trifluororacetic acid, and subjected to high-voltage paper electrophoresis (12). The electrophoretograms were exposed to X.0 mat S films to detect $|^{32}P|$, or cut into 1-cm strips for radioactivity counting. Authentic mannose 6-phosphate was run as an internal control and revealed by ammoniac silver stain reagent (13).

Proteolytic activity assay

Both the secreted and cellular 52-kDa related proteins were purified by a two step affinity procedure as described (10)(11)(14). The purity of these proteins was checked by silver staining of overloaded SDS-PAGE, homogeneity in HPLC gel filtration and by N-terminal amino acid analysis (14). The dialysis buffer following the immuno affinity step was 50 mM acetate, pH 5 - 0.0025 % TW80. The reaction mixture contained 90 ng of purified enzyme, 10,000 cpm of $|^{14}C|$methemoglobin

(NEN), 1 mg/ml non-radioactive hemoglobin, and reaction buffer at appropriate pH in a final volume of 100 µl. Blanks run with dialysis buffer in place of the enzyme were subtracted. After 10 min of incubation at 37°C, the reaction was stopped by adding trichloroacetic acid (TCA). TCA soluble material in 25 µl aliquots was counted for radioactivity.

Other methods

SDS-polyacrylamide gel electrophoresis was performed with 12.5% acrylamide gel as described (3). Gels containing $|^{3}H|$ and $|^{35}S|$ materials were processed for fluorography ; those containing $|^{32}P|$ were autoradiographed. Unlabeled proteins were stained with a Bio-Rad Silver-stain kit. Radioactive samples were counted in scintillator emulsifier 299 (United Technologies Packard) in an SL30 Intertechnique liquid scintillation spectrometer (France).

RESULTS AND DISCUSSION

I. THE 52-kDa SECRETED PROTEIN CONTAINS MANNOSE 6-PHOSPHATE SIGNALS

In order to characterize the co- and post-translational modifications of the secreted 52-kDa protein, we separately labeled proteins of MCF_7 cells **in vivo** with $|^{32}P|H_3PO_4$, $|^{3}H|$mannose, or $|^{35}S|$methionine, and studied the immunoprecipitated 52-kDa protein by SDS-PAGE. The protein was not only labeled with $|^{35}S|$methionine and $|^{3}H|$mannose as previously described (6) but also phosphorylated (Fig. 1). The

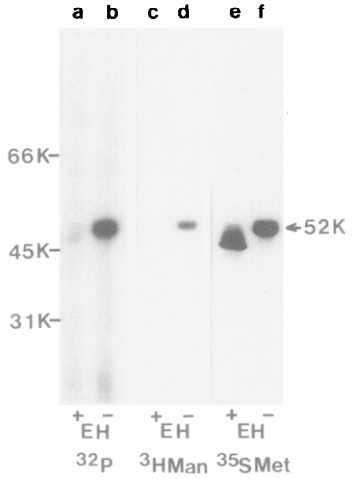

Figure 1: SDS-PAGE of the immunoprecipitated 52 K protein : Effect of Endo-H

The secreted 52-kDa protein was labeled **in vivo** with $|^{32}P|H_3PO_4$, $|^{3}H|$mannose or $|^{35}S|$methionine, and subsequently immunoprecipated with M1G8 mAb. It was then treated with (+) or without (-) Endo-H. The reaction was terminated by adding TCA, and the insoluble proteins were analyzed by SDS-PAGE.

in vivo phosphorylation sites on amino acids were identified as mostly serine and threonine (not shown). However, the proportion of $|^{32}P|$ incorporated into the 52-kDa protein and recovered in phosphoamino acids was low (5 %) compared to the extent of phosphorylation. In fact, endoglycosidase H treatment of the previously labeled 52-kDa protein removed most (94 %) of the $|^{32}P|$ and all of the $|^{3}H|$mannose from the TCA-precipitated material (Fig. 1), while the $|^{35}S|$labeled protein was displaced to lower molecular weights of 50,000 and 48,000, as described (7). The detailed composition of oligosaccharidic chains will be described separately (F. Capony et al., in preparation). The N-linked oligosaccharides were hydrolyzed by trifluoroacetic acid and the monosaccharides were electrophoresed on paper (Fig. 2). Following $|^{3}H|$mannose labeling, twenty percent of the $|^{3}H|$ radioactivity incorporated migrated as an acidic sugar with authentic mannose 6-phosphate run in parallel. The rest of the $|^{3}H|$ radioactivity migrated as $|^{3}H|$mannose (Fig. 2a).

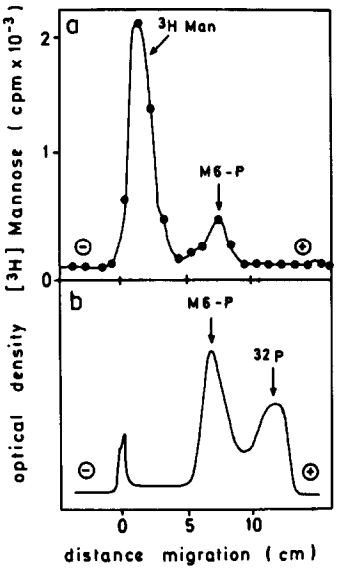

Figure 2: High voltage paper electrophoresis of hydrolyzed 2-$|^{3}H|$mannose (a) and $|^{32}P|$ (b) labeled oligosaccharides

The secreted 52-kDa protein was immunoprecipitated from the culture medium of cells labeled with 2-$|^{3}H|$mannose (a) or with $|^{32}P|$ (b). The oligosaccharidic chains were prepared, hydrolyzed with TFA and analyzed by high-voltage paper electrophoresis as indicated in Materials and Methods.

The positions of authentic mannose 6-phosphate (M-6-P), free $|^{3}H|$mannose or $|^{32}P|$ run on the same electrophoretogram are indicated.

Following $|^{32}P|H_3PO_4$ labeling, most of the radioactivity also migrated as authentic mannose 6-phosphate (Fig. 2b).

These results are in agreement with studies showing that the uptake of the secreted 52-kDa protein in MCF_7 cells is specifically inhibited by mannose 6-phosphate (10) and with electron microscopic immunocytochemical studies, localizing the staining in lysosomes-like vesicles (M. Chambon, unpublished experiments). Since these results strongly suggested that the 52-kDa protein is a hydrolase targeted to lysosomes **via** the mannose 6-phosphate receptor (15), we searched for an enzymatic activity.

II. PROTEASE ACTIVITY OF THE PURIFIED SECRETED 52-kDa PROTEIN AND RELATED CELLULAR PROTEINS

Purification of the secreted 52-kDa protein and the related cellular proteins can be achieved by a Con A Sepharose chromatography followed by an immunoaffinity chromatography (10)(14) as shown by silver staining of SDS-PAGE (Fig. 3). While only a 52-kDa protein is found in the culture medium, four immunoreactive proteins of 52-, 48-, 34- and 17-kDa are purified from the cellular extract, the three lower molecular

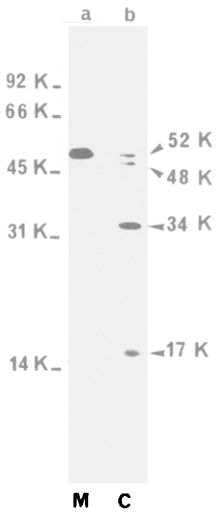

Figure 3: Purity of the 52-kDa proteins
The secreted 52-kDa protein (a) and the related cellular proteins (b) were purified by two successive affinity chromatographies (Con A-Sepharose and anti-52-kDa monoclonal antibodies) in the presence of Tween 80 (0.01 %). 200 ng of each preparation were subjected to SDS-PAGE and silver stained.

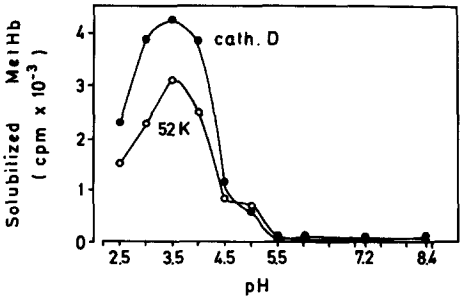

Figure 4: Effect of pH on the proteolytic activity of the 52-kDa protein compared to bovine cathepsin D

Proteolytic activities of 90 ng of purified secreted 52-kDa protein (○) and 90 ng of bovine cathepsine D (●) were assayed using |^{14}C|methemoglobin (MetHb) as substrate, as described in Materials and Methods. The reaction buffers are citrate buffer (pH 2.5 to 4.5), acetate buffer (pH 5 to 6.5), phosphate buffer (pH 7.2) and tris buffer (pH 8.4).

weight proteins being processed from the 52-kDa precursor (6). We found no enzymatic activities of these preparations at physiological pH. However, a strong proteolytic activity was detected on methemoglobin (Fig. 4) for acidic pH, with an optimal pH of 3.5. The nature of the macromolecular substrate (casein and albumin were also hydrolysed) and the total inhibition observed with pepstatin, indicated that these proteins are carboxyl proteinases similar to cathepsin D (16). The molecular weight of the processed cellular proteins (Fig. 3) and their specific activities (Fig. 4) were also similar to those of bovine spleen cathepsin D (17). There was a progressive increase in the specific activity of this protease following the different purification steps of the 52-kDa protein (not shown). Both the secreted 52-kDa protein and the related cellular proteins were similarly active in our routine assay (10 min. incubation). However, we do not exclude autoactivation of the 52-kDa precursor as previously reported for cathepsin D in fibroblasts (17)(18). A complete characterization of the enzymatic activity of the secreted and cellular related proteins and their comparison with human cathepsin D will be reported separately (Capony et al., in preparation).

These data show that the estrogen-regulated 52-kDa protein secreted by human breast cancer cells is a lysosomal carboxyl proteinase. Cathepsins are considered as proteases acting mostly to degrade cellular proteins. The fact that, in breast cancer cells, one of these proteases is secreted

following its induction by estrogens, suggests some important function in mammary cancerogenesis. Estrogens favor the growth and possibly the invasiveness of hormone-dependant breast cancers and these effects may be mediated by estrogen-induced proteases such as the 52-kDa protein. In support to this hypothesis, we found with our monoclonal antibodies that the cellular concentration of this protease was higher in proliferative epithelial mammary cells than in resting mammary cells or other tissues (5)(8). Moreover, the purified secreted 52-kDa precursor is in vitro a mitogen on MCF_7 cells (10) and is already enzymatically or rapidly autoactivable in the culture medium (unpublished results). Several proteases have been suspected to be involved in the process of tumor invasiveness and metastasis (19)(20). Among these proteases, plasminogen activator has been shown previously as being stimulated by both estrogens and progesterone (21)(22). The 52-kDa protein is different (23) and presents additional potential since it is mitogenic.

The relationship, if any, between the proteolytic activity of the 52-kDa protein, and its autocrine mitogenic activity in MCF_7 cells (10), may be of considerable interest in understanding how estrogen stimulates the dissemination and proliferation of breast cancer cells.

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