Phosphorylation, Glycosylation, and Proteolytic Activity of the 52-kD Estrogen-induced Protein Secreted by MCF_7 Cells

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Abstract. We have studied the posttranslational modifications of the 52-kD protein, an estrogen-regulated autocrine mitogen secreted by several human breast cancer cells in culture (Westley, B., and H. Rochefort, 1980, *Cell*, 20:353–362). The secreted 52-kD protein was found to be phosphorylated mostly (94%) on high-mannose N-linked oligosaccharide chains, and mannose-6-phosphate signals were identified. The phosphate signal was totally removed by alkaline phosphatase hydrolysis. The secreted 52-kD protein was partly taken up by MCF_7 cells via mannose-6-phosphate receptors and processed into 48- and 34-kD protein moieties as with lysosomal hydrolases. By electron microscopy, immunoperoxidase staining revealed most of the reactive proteins in lysosomes. After complete purification by immunoaffinity chromatography, we identified both the secreted 52-kD protein and its processed cellular forms as aspartic and acidic proteinases specifically inhibited by pepstatin. The 52-kD protease is secreted in breast cancer cells under its inactive proenzyme form, which can be autoactivated at acidic pH with a slight decrease of molecular mass. The enzyme of breast cancer cells, when compared with cathepsin D(s) of normal tissue, was found to be similar in molecular weight, enzymatic activities (inhibitors, substrates, specific activities), and immunoreactivity. However, the 52-kD protein and its cellular processed forms of breast cancer cells were totally sensitive to endo-β-*N*-acetylglucosaminidase H (Endo H), whereas several cellular cathepsin D(s) of normal tissue were partially Endo H-resistant. This difference, in addition to others concerning tissue distribution, mitogenic activity and hormonal regulation, strongly suggests that the 52-kD cathepsin D-like enzyme of breast cancer cells is different from previously described cathepsin D(s). The 52-kD estrogen-induced lysosomal proteinase may have important functions in facilitating the mammary cancer cells to proliferate, migrate, and metastasize.

THE mechanism of the control of cell proliferation by intracellular hormones is unknown but can be studied in hormone-responsive human cell lines. The recent discovery of a close relationship between some oncogenes (Bishop, 1983) and growth factors (Waterfield, 1985), some of which act as autocrine signals (Sporn and Todaro, 1980; Heldin and Westermark, 1984), could serve as a guide in finding a new class of growth factors and oncogene products which are regulated by steroid hormones in hormone-responsive cancer. In the estrogen receptor–positive human breast cancer cells (Lippman et al., 1976; Soule et al., 1973), estrogens stimulate the synthesis of several secreted proteins (reviewed in Rochefort et al., 1986) and subsequently increase cell proliferation. We have more specifically studied a glycoprotein, defined according to its apparent molecular mass in SDS PAGE (52,000 daltons, 52 kD), which is secreted into the culture medium when MCF_7 cells are treated with estrogens (Westley and Rochefort, 1980). The protein contains at least two N-linked high-mannose or hybrid oligosaccharide chains (Touïtou et al., 1985) and is processed intracellularly into a 48-kD protein and a more stable 34-kD protein (Morisset et al., 1986*a*). The distribution of this protein in human tissues, as determined with several monoclonal antibodies by using immunoperoxidase staining (Garcia et al., 1985), appears to be relatively specific for epithelial mammary cells, sweat glands, and liver, and to be associated with tumor development and/or cell growth. The protein has been detected in several human mammary cancers but not in the normal resting mammary gland or in endometrium (Garcia et al., 1984). In a study of

125 tissue samples of benign breast disease, the immunostaining was found to be associated with cysts and ductal hyperplasias, both being lesions that increase the risk of developing breast cancer (Garcia et al., 1986).

Several characteristics of the 52-kD protein suggest that it might be an autocrine mitogen (Vignon et al., 1983; Rochefort et al., 1984): (*a*) its increased level in the medium always precedes the stimulation of cell growth by estrogen; (*b*) the protein is not secreted when the wild-type MCF_7 cells are treated by antiestrogens or progestins that block cell growth; (*c*) the 52-kD protein became specifically inducible by antiestrogens in antiestrogen-resistant clones of MCF_7 cells, unlike other regulated proteins (Westley et al., 1984); (*d*) more directly, the purified 52-kD protein (Capony et al., 1986) stimulated the growth of resting MCF_7 cells and transformed the cell surface (Vignon et al., 1986).

In an attempt to identify the function of this protein, we have studied its posttranslational modifications and searched for an enzymatic activity. In a preliminary report, we noticed that the secreted 52-kD protein contains mannose-6-phosphate signals and displays an in vitro acidic proteinase activity (Morisset et al., 1986*b*). We now report a complete characterization of the posttranslational modifications of the secreted and cellular 52-kD protein, and of its cellular localization and processing. The enzymatic activities of the cellular and secreted 52-kD proteins have been characterized and compared to normal human cathepsin D(s).

Materials and Methods

Cell Culture

MCF_7 cells were derived from a metastatic human breast cancer and supplied by the Michigan Cancer Foundation (Detroit). Cells plated out in wells (Nunc 3.5-cm diam) at a concentration of 3×10^5 cells per well were hormone-withdrawn for 6 d, and then stimulated with estradiol (10 nM) for 2 d as previously described (Westley and Rochefort, 1980).

Labeling of Cells

After stimulation, the cells were labeled with [^{35}S]methionine (200 μCi/ml) for 8 h in 500 μl of MEM as previously described (Westley and Rochefort, 1980). To label glycoproteins, cells were labeled in 500 μl of MEM containing 1/10th the normal concentration of glucose plus 0.6 mCi/ml of [2-^{3}H]mannose (54 Ci/mmol, Amersham International, Amersham, UK) or D-[U-^{14}C]glucose (0.3 Ci/mmol, Commissariat à l'Energie Atomique, Saclay, France). For ^{32}P-labeling, the cells were first rinsed twice with 1 ml of MEM containing 1/20th the normal concentration of phosphate and labeled for 7 h in 500 μl of the same MEM plus 2 mCi/ml of [^{32}P]H_3PO_4 (Commissariat à l'Energie Atomique). At the end of the incubation, NaF was added to the ^{32}P-labeled medium at a final concentration of 10 mM, and the cells were rinsed twice in cold PBS containing 10 mM NaF.

Preparation of Medium, NP40 Cell Extracts, and Immunoprecipitation of the Secreted and Cellular 52-kD Proteins

After labeling, the media were collected and centrifuged for 5 min at 1,200 *g*. The cells were lysed directly in the wells in 10 mM Na H_2PO_4, pH 7.4, 10 mM NaCl, 10 mM EDTA, 1% NP-40 (wt/vol), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 kalikrein inhibitors units of trasylol (Sigma Chemical Co.) per ml and centrifuged to give a NP-40 cell lysate. Immunoprecipitation of the cellular 52-kD proteins were carried out in 100 mM Na H_2PO_4, pH 7.4, 2% BSA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS with hybridoma supernatant containing 10 μg/ml of MIG8 monoclonal antibody to the 52-kD protein and 5% of normal BALB/c mouse serum (Garcia et al., 1984). After 18 h at room temperature, sheep anti-mouse antibody was added and the incubation continued for 22 h. Immunoprecipitates were pelleted in an Eppendorf microfuge (Hamburg, Federal Republic of Germany) and washed three times in immunoprecipitation buffer but with 1 mM EDTA and without BSA. Control immunoprecipitations were carried out with a supernatant of parental myeloma strain. Detergents were omitted to immunoprecipitate the secreted 52-kD protein.

Purification of the Secreted and Cellular 52-kD Proteins

Proteins were purified by sequential column chromatographies on concanavalin A (Con A)-Sepharose and anti-52-kD antibody-Sepharose as described (Vignon et al., 1986; Capony et al., 1986). Elution from the immunoaffinity column was performed either at pH 3.0 or 11.0 as indicated when necessary.

Detection of Phosphoamino Acids in Protein

The secreted 52-kD protein was immunoprecipitated and subjected to SDS PAGE. Radioactive bands detected by autoradiography corresponding to the 52-kD proteins were cut out and processed as described by Cooper et al. (1983) for hydrolysis and purification on Dowex AG1-X8 (Dow Corning Corp., Midland, MI). Separation of phosphoamino acids was carried out by cation exchange as previously described (Capony and Demaille, 1983).

Detection of Phosphorylated Oligosaccharides

The radioactive secreted or cellular 52-kD immunoprecipitates were dissolved in 50 mM NaH_2PO_4, pH 5.4, containing 1% SDS heated for 1 min at 100°C, and diluted 10-fold with quartz double-distilled water to dilute the SDS to 0.1%. Endo-β-*N*-acetylglucosaminidase H (Endo H),[1] from Miles Laboratories, Inc. (Elkhart, IN) was added at ≥25 mU/ml and the samples were incubated for 16–18 h at 37°C. The proteins were then precipitated with 10% TCA, and the precipitates were analyzed by SDS PAGE.

The TCA-soluble oligosaccharides were neutralized with 5 N NaOH and the free radioactive label ([^{32}P]- or [^{3}H]mannose) was eliminated by gel filtration on Sephadex G25 (PD10 column, Pharmacia Fine Chemical, Uppsala, Sweden) in 0.1 M pyridine-acetic acid, pH 5.0. Samples were then subjected to high-voltage paper electrophoresis for 15–20 min at 40 V/cm in a Desaga Desaphor electrophorator (Heidelberg, Federal Republic of Germany) on Whatman 3MM paper (Whatman, Inc., Clifton, NJ) saturated with 30 mM $NH_4H\ CO_3$ (Sahagian and Gottesman, 1982). The electropherograms were exposed to X.Omat S films to detect ^{32}P or cut into 1-cm strips for ^{3}H-radioactivity counting.

Sugar Analysis

Endo H-released oligosaccharides labeled with D-[U-^{14}C]glucose were hydrolyzed with 1 N HCl at 100°C for 2 h. The carbohydrates were then analyzed by descending chromatography for 22 h on Whatman 3MM paper in *N*-butanol/pyridine/0.1 N HCl (5:3:2). Radioactivity was counted as above for ^{3}H and sugar standards (Sigma Chemical Co., St. Louis, MO) were detected with aniline oxalate (Broquet et al., 1982).

Identification of Mannose-6-phosphate

The oligosaccharides were lyophylized and hydrolyzed in 2 M trifluoroacetic acid (TFA) in sealed tubes for 2 h at 110° as described elsewhere (Sahagian and Gottesman, 1982). The acid hydrolysates were then dried under nitrogen to eliminate TFA, and the residues were dissolved in 5 mM Tris-HCl, pH 9.0. Aliquots were counted for radioactivity and samples were submitted to high-voltage paper electrophoresis as above. Authentic mannose-6-phosphate was run as external and internal controls (20–50 μg) and revealed by the ammoniacal silver stain reagent (Trevelyan et al., 1950).

Portions of *N*-glycosylated chains or acid hydrolysates containing 200–500 cpm ^{32}P were treated for 2 h at 37°C with or without 0.12 U of *Escherichia coli* alkaline phosphatase (Sigma Chemical Co., type II N) in 20 μl of Tris buffer, pH 9.0. The alkaline phosphatase activity was tested in parallel on 50 μg of authentic mannose-6-phosphate.

QAE-Sephadex Fractionation and Mild Acid Hydrolysis

Endo H-released oligosaccharides were first separated from the polypeptides by chromatography on a Biogel P30 column (Bio-Rad Laboratories,

1. *Abbreviations used in this paper*: Endo H, endo-β-*N*-acetylglucosaminidase H; TFA, trifluoroacetic acid.

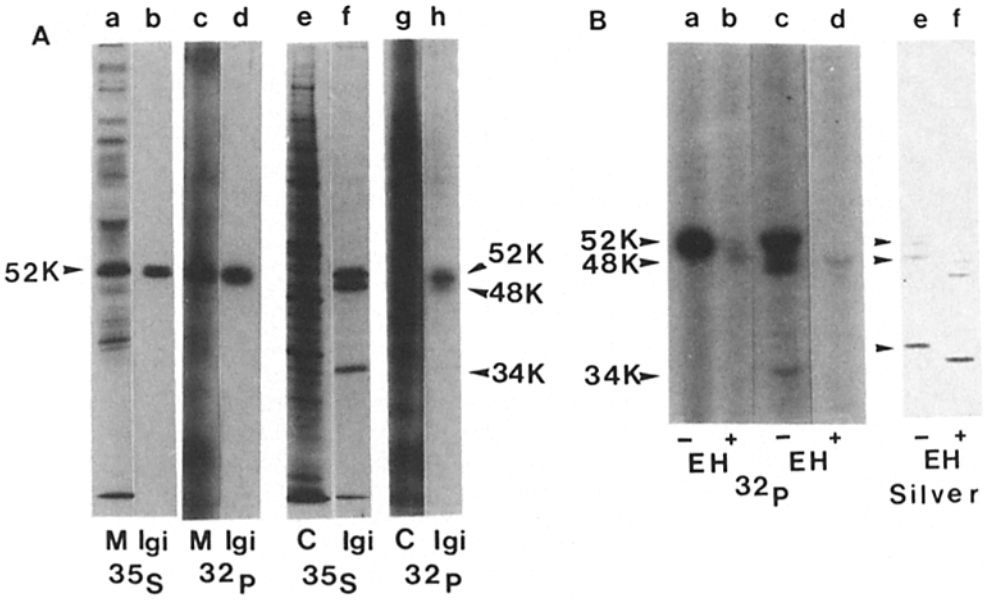

Figure 1. Phosphorylation of the secreted and cellular 52-kD proteins in MCF_7 cells. Estrogen-treated MCF_7 cells were labeled with either [^{35}S]-methionine or [^{32}P]H_3PO_4 as indicated. Media and cell extracts were immunoprecipitated with the M1G8 antibody to the 52-kD protein and analyzed by SDS PAGE as described in Materials and Methods. (*A*) Proteins of media (*M*) (lanes *a* and *c*) and cell extracts (*C*) (lanes *e* and *g*), labeled by ^{35}S and ^{32}P and analyzed before (*M, C*) and after (*IgI*) immunoprecipitation with the anti–52-kD protein antibody (lanes *b, d, f,* and *h*). (*B*) The immunoprecipitated 52-kD proteins were digested (+) or not (−) with Endo H and the TCA-precipitated proteins were electrophoresed and revealed by autoradiography (^{32}P) or silver stained. Lanes *a* and *b*: secreted 52-kD protein. Lanes *c–f*: cellular related proteins. The three immunoreactive proteins are arrowed.

Richmond, CA) (1 × 100 cm) in 1 M pyridine acetic acid, pH 5.0, 0.2% SDS. QAE-sephadex chromatography and mild acid hydrolysis were then performed as described by Tabas and Kornfeld (1980).

Ultrastructural Localization by Immunoperoxidase Staining

MCF_7 cells stimulated by estradiol (10 nM) for 3 d were fixed in 3% paraformaldehyde and 0.05% glutaraldehyde and treated for 30 min with PBS containing 0.05% saponin for membrane permeation. Indirect immunoperoxidase staining was performed as described elsewhere (Garcia et al., 1984). Enzyme activity was revealed using diaminobenzidine (Graham and Karnovsky, 1966). After 1 h of postfixation with osmium tetroxide (1.33%) in collidine buffer, cells were embedded in Epon. The sections were stained with uranyl acetate and examined with a Philips EM 301 microscope (Eindhoven, The Netherlands) at 60 kV.

Proteolytic Activity Assays

Both the secreted and cellular 52-kD related proteins were purified. The final elution was either with citrate buffer, pH 3, followed by dialysis in a 50 mM acetate buffer, pH 5, with 0.0025% Tween 80 (E. Merck, Darmstadt, Federal Republic of Germany) or with lysine buffer, pH 11, without dialysis. The reaction mixture contained routinely 10,000 cpm of [^{14}C]methemoglobin (New England Nuclear, Boston, MA), 100 μg of unlabeled methemoglobin, 10–90 ng of purified enzyme, and reaction buffer at appropriate pH in a final volume of 100 μl. The reaction was initiated by the addition of the enzyme and terminated by adding TCA (final concentration 10%). TCA-soluble material in 25-μl aliquots was counted for radioactivity. Blanks run with dialysis buffer in place of the enzyme were subtracted. At 37°C, the reaction was linear up to 15 min (purification at pH 3) or 60 min (purification at pH 11), and incubation times of 10 min and 30 min were chosen, respectively.

Double-labeled proteoglycans (kindly given by Dr. Mitrovic, INSERM U18, Hôpital Lariboisière, Paris) were also tested as substrate. Briefly, human chondrocyte proteoglycans were labeled in culture with [^{3}H]glycine and $^{35}SO_4$, and purified as described (Mitrovic et al., 1981). 20,000 cpm of $^{35}SO_4$ and 87,000 cpm of ^{3}H-labeled proteoglycans were digested by 80 ng of purified secreted 52-kD protein at different pH in 110 μl of buffer for 45 min at 37°C. After TCA precipitation (10% final) in the presence of 25% FCS, the TCA-soluble material was decanted and counted for ^{3}H and ^{35}S radioactivity using a double-channel program.

Bovine spleen cathepsin D (EC 3.4.23.5) was from Sigma Chemical Co., the human liver cathepsin D (form 34 kD) was prepared according to Barrett (1970). The sheep antiserum to human cathepsin D (SA237) was prepared as described by Dingle et al. (1971).

Other Methods

SDS PAGE was performed by the method of Laemmli (1970) with a 15% acrylamide gel. Samples were prepared as described by Westley and Rochefort (1980). Gels containing ^{3}H and ^{35}S material were processed for fluorography; those containing ^{32}P were autoradiographed. Unlabeled proteins were stained with the Bio-Rad Laboratories silver-stain kit. The molecular mass of proteins was estimated by their mobilities relative to molecular mass protein standards for SDS PAGE (Bio-Rad Laboratories).

Radioactive samples were counted in 4 ml of scintillator emulsifier 299 (United Technologies, Packard Instrument Co., Inc., Zurich, Switzerland) in an SL30 Intertechnique liquid scintillation spectrometer (Intertechnique, Plaisir, France).

Results

Biosynthetic Phosphorylation on N-glycosylated Chains

When confluent MCF_7 cells grown in the presence of estradiol were exposed to [^{32}P]H_3PO_4 for 7 h, in a serum-free medium, several phosphorylated proteins were released into the culture medium. A protein with a molecular mass of

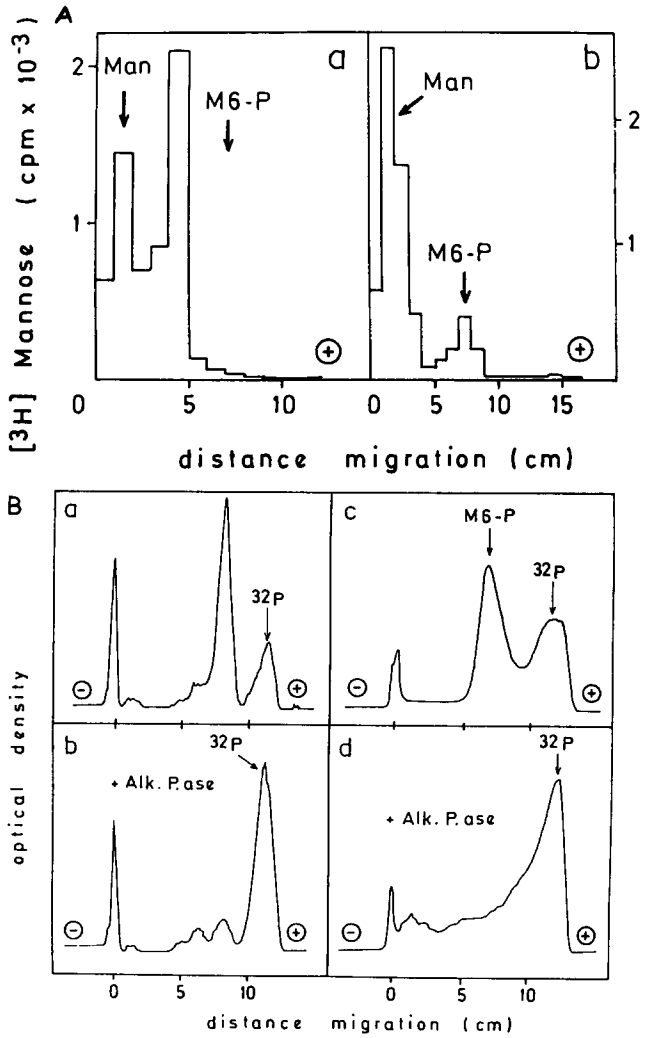

Figure 2. High-voltage paper electrophoresis of (*A*) [2-^{3}H]mannose- or (*B*) ^{32}P-labeled oligosaccharides. The secreted 52-kD protein was immunoprecipitated and digested with Endo H. The cleaved oligosaccharide chains were analyzed before and after hydrolysis with TFA by high-voltage paper electrophoresis with authentic mannose-6-phosphate (*M6P*), [^{3}H]mannose (*Man*), and [^{32}P]H_3PO_4 (^{32}P). (*A*) [^{3}H]mannose labeling. (Panel *a*) intact *N*-glycosylated chains; (panel *b*) TFA hydrolyzed sugars. Radioactivity was detected as described in Materials and Methods. (*B*) ^{32}P-labeling. Intact *N*-glycosylated chains (panels *a* and *b*) and TFA-hydrolyzed sugars (panels *c* and *d*) were digested with alkaline phosphatase (panels *b–d*) or not digested (panels *a–c*) before analysis. The ^{32}P-labeled material was autoradiographed and scanned using a Vernon scanning densitometer.

52,000 daltons was one of the most phosphorylated proteins. It was identified as the estrogen-regulated 52-kD protein by specific immunoprecipitation (Fig. 1 *A*, lanes *c* and *d*). The 52-kD protein immunoprecipitated after [^{35}S]methionine labeling was analyzed in parallel (Fig. 1 *A*, lanes *a* and *b*). No other proteins were recognized by the monoclonal antibodies. Cells deprived of estrogens do not secrete the 52-kD protein and therefore no ^{32}P- or ^{35}S-labeled 52-kD proteins were observed in the medium (results not shown). The cell extract was analyzed similarly. After [^{35}S]methionine labeling, three bands migrating as proteins with molecular mass of 52,000, 48,000, and 34,000 daltons were immunoprecipitated (Fig. 1 *A*, lanes *e* and *f*). The 48- and 34-kD proteins are processed products of the 52-kD protein as shown by pulse-chase experiments (Morisset et al., 1986a). The 34-kD protein is the most abundant and stable of these three proteins. The immunoreactive cellular proteins were labeled by ^{32}P mostly on the 52- and 48-kD forms (Fig. 1 *A*, lanes *g* and *h*). The 34-kD protein was labeled weakly by [^{35}S]methionine and very weakly by ^{32}P (not detected in Fig. 1 *A*).

We conclude that the 52-kD protein is phosphorylated in vivo in the cellular compartment and remains phosphorylated when secreted into the medium under estrogen stimulation.

The phosphorylation site(s) of the secreted purified 52-kD protein labeled biosynthetically by [^{32}P]H_3PO_4, was (were) then identified. After acid hydrolysis of peptide bonds (Cooper et al., 1983), the phosphoamino acids were separated on an amino acid analyzer and revealed by autoradiography (Capony and Demaille, 1983). Only 3%–5% of the total ^{32}P radioactivity bound to the protein was recovered. It migrated mostly with serine (90%) and to a lesser extent with threonine (9%), and tyrosine was hardly detectable. Because the proportion of ^{32}P incorporated into the 52-kD glycoprotein and recovered into amino acids was low, we suspected that the oligosaccharide moiety was also phosphorylated. In fact, treatment by Endo H, which removes the two *N*-glycosylated chains of the secreted 52-kD protein and displaces the ^{35}S-labeled protein to lower molecular mass of 50- and 48-kD (Touïtou et al., 1985), also removed 94% of the ^{32}P from the 52-kD protein (Fig. 1 *B*, lanes *a* and *b*). This indicated that the majority of the ^{32}P label of the 52-kD protein was incorporated into the high-mannose *N*-glycosylated chains. Endo H had the same effect on the three immunorelated cellular proteins (Fig. 1 *B*, lanes *c–f*). This was confirmed by analyzing the ^{32}P or [^{3}H]mannose-labeled *N*-glycosylated chains released by Endo H treatment. When the *N*-glycosylated chains of the secreted 52-kD protein were labeled by [^{3}H]mannose and analyzed at pH 8 by high-voltage paper electrophoresis, half of them migrated as an acidic component and half as a neutral component (Fig. 2 *A*, panel *a*). When labeled by [^{32}P]H_3PO_4 (Fig. 2 *B*, panel *a*), the cleaved oligosaccharide chains migrated as the acidic compound labeled with [^{3}H]mannose. The bulk of the ^{32}P was removed by alkaline phosphatase and migrated as free H_3PO_4, indicating that most of the phosphate was linked to the *N*-glycosylated chain by a monoester bond and not protected by a terminal sugar (Fig. 2 *B*, panel *b*).

Mannose-6-phosphate Signal and Lysosomal Localization

The [^{3}H]mannose- or [^{32}P]H_3PO_4-labeled oligosaccharides were hydrolyzed with TFA, and the monosaccharides were electrophoresed on paper. 20% of the incorporated ^{3}H radioactivity migrated with the mobility of authentic mannose-6-phosphate run in parallel (Fig. 2 *A*, panel *b*), while most of the ^{3}H radioactivity migrated as [^{3}H]mannose. The ^{32}P incorporated into monosaccharides migrated as authentic mannose-6-phosphate. It was totally liberated as free [^{32}P]-phosphate after alkaline phosphatase treatment (Fig. 2 *B*, panels *c* and *d*).

The *N*-glycosylated chains of the three immunopurified cellular-related 52-kD proteins are also Endo H-sensitive and most of their phosphorylation is removed by this enzymatic digestion (Fig. 1 *B*). After biosynthetic labeling by

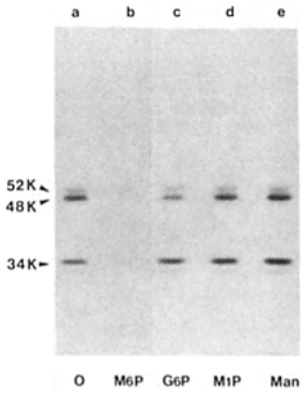

Figure 3. Inhibition by mannose 6-phosphate of the uptake and processing of the 52-kD protein by MCF_7 cells. Confluent MCF_7 cells (1×10^6 cells) maintained in F12/DME containing 1% FCS/dextran-coated charcoal were rinsed twice in F12/DME plus 0.1% BSA. They were then incubated with 400 μl of conditioned media containing [^{35}S]methionine-labeled proteins secreted by MCF_7 cells (300,000 TCA-precipitable counts) for 24 h in F12/DME with (lane *a*) 0.1% BSA alone (*0*), or (lane *b*) 10 mM mannose-6-phosphate (*M6P*), or (lane *c*) 10 mM glucose-6-phosphate (*G6P*), or (lane *d*) 10 mM mannose-1-phosphate (*M1P*), or (lane *e*) 10 mM mannose (*Man*). The labeled cells were then washed three times in F12/DME plus 0.1% BSA, and the cell lysate proteins were immunoprecipitated with the M1G8 anti-52-kD monoclonal antibody. The immunoprecipitates of each series were analyzed by SDS PAGE and revealed by fluorography.

[^{3}H]mannose and by [^{32}P]H_3PO_4, the *N*-glycosylated chains recovered after Endo H digestion showed both neutral and acidic components in electrophoresis. A ^{32}P-labeled compound migrating as mannose-6-phosphate was recovered after acid hydrolysis. However, alkaline phosphatase only partially removed the ^{32}P incorporated into the cellular *N*-glycosylated chains, suggesting that some phosphomannose residues were covered. The behavior of the cellular 52-kD oligosaccharide chains on the QAE-Sephadex column was studied according to Tabas and Kornfeld (1980). Five peaks were eluted by increasing concentrations of NaCl from 0 to 100 mM indicating a large heterogeneity of these *N*-glycosylated chains (not shown). Approximately 20% were neutral whereas the rest had a variable number of negative charges. 80% of the two major peaks (30% each) eluted at 70 and 100 mM were sensitive to alkaline phosphatase, suggesting that they contained accessible mannose-6-phosphate. Sugar analysis after total acid hydrolysis of the secreted and cellular oligosaccharide chains biosynthetically labeled with D-[U-^{14}C]glucose revealed mannose and *N*-acetylglucosamine in an approximate molar ratio of 5:2. No other radiolabeled sugars could be detected.

The presence of mannose-6-phosphate signals on the secreted 52-kD protein was also indirectly demonstrated by incubating the ^{35}S-labeled secreted proteins containing 1 nM concentration of 52-kD protein with unlabeled recipient MCF_7 cells as described by Vignon et al. (1986). Under these conditions, 4% of the 52-kD protein was taken up and processed into 34-kD protein (Fig. 3, lane *a*). When mannose-6-phosphate was added as a competitor to the labeled incubation medium, the uptake and processing of ^{35}S-labeled 52-kD protein was negligible (Fig. 3, lane *b*). In contrast, incubation with glucose 6-phosphate, mannose 1-phosphate, or mannose did not prevent the uptake and processing into a 34-kD protein (Fig. 3, lanes *c–e*). This result indicates that the breast cancer cells have mannose-6-phosphate receptors located on their plasma membrane, and that these receptors facilitate the cellular binding and uptake of the 52-kD protein, as described for other lysosomal enzymes (Creek and Sly, 1984). After incubation, the 52-kD protein remaining in the medium was not processed into the 48- and 34-kD forms, suggesting that the enzyme(s) responsible for this processing is (are) intracellular.

We then used an anti–52-kD monoclonal antibody, which also recognizes the processed cellular 34-kD protein, to localize these proteins in MCF_7 cells, using indirect immunoperoxidase staining and electron-microscopic analysis (Fig. 4). The immunoreactive proteins were exclusively found in the cytoplasm and mostly concentrated in multivesicular bodies and lysosomal vesicles, identified as lysosomes by acid phosphatase activity. Most of the lysosomes containing these proteins were located close to mitochondria and microfilaments. By contrast, only weak staining was observed in Golgi complexes, and no staining was observed with control monoclonal IgG1 mouse antibody (not shown). The ultrastructural localization of the protein was in total agreement with the biochemical characterization of mannose-6-phosphate signals and indicated that the 52-kD protein is the precursor of a lysosomal protein, partly secreted after estrogen stimulation and targeted to lysosomes via mannose-6-phosphate receptors.

Aspartic Proteinase Activity of the Secreted and Cellular Forms of the 52-kD Protein

Our first unsuccessful attempts to find an enzymatic activity of the purified secreted 52-kD protein at neutral pH (our unpublished results) were markedly reoriented on the basis of its lysosomal localization. When the secreted 52-kD protein and the related cellular proteins (52, 48, 34, and 17-kD) were purified to apparent homogeneity by Con A-Sepharose chromatography followed by immunoaffinity chromatography (Vignon et al., 1986; Capony et al., 1986), they displayed a strong proteolytic activity on [^{14}C]methemoglobin, with a maximum at pH 3.5 (Fig. 5 *a*). The substrate was degraded into small peptides, indicating that the enzyme was an endopeptidase. The optimal pH was, however, found to vary according to the substrate tested. For instance, when double-labeled ([^{3}H]glycine and $^{35}SO_4$) human proteoglycans were used as substrates instead of methemoglobin, the purified secreted 52-kD protein displayed a maximal activity at pH 5.5 and was slightly active at pH higher than 6, suggesting that this secreted protease may act extracellularly in vivo (Fig. 5 *a*).

Casein, albumin, and basement membranes were also substrates for this protease (Morisset, unpublished experiments). The initial rate of the reaction was dependent on the amount of the secreted and cellular 52-kD protein, with a linear relationship from 20 to 90 ng of protein (Fig. 5 *b*). The specific activity of the secreted 52 kD protein was identical to that of the related cellular proteins consisting mostly of the 34- and 17-kD enzyme. Pepstatin was the most effective inhibitor, leupeptin and EDTA had some activity at high concentrations, and PMSF was inactive (Fig. 5 *c*). These results indicate that this proteinase is an aspartic proteinase similar to the previously described cathepsin D (Barrett, 1977). The proteinase activity is intrinsic to the 52-kD and related cellular proteins in that its specific activity progressively increased up to 200-fold during purification (Table I). The final specific activity (300–360 cpm solubilized per nanogram of protein in 30 min) was similar whether the protein had

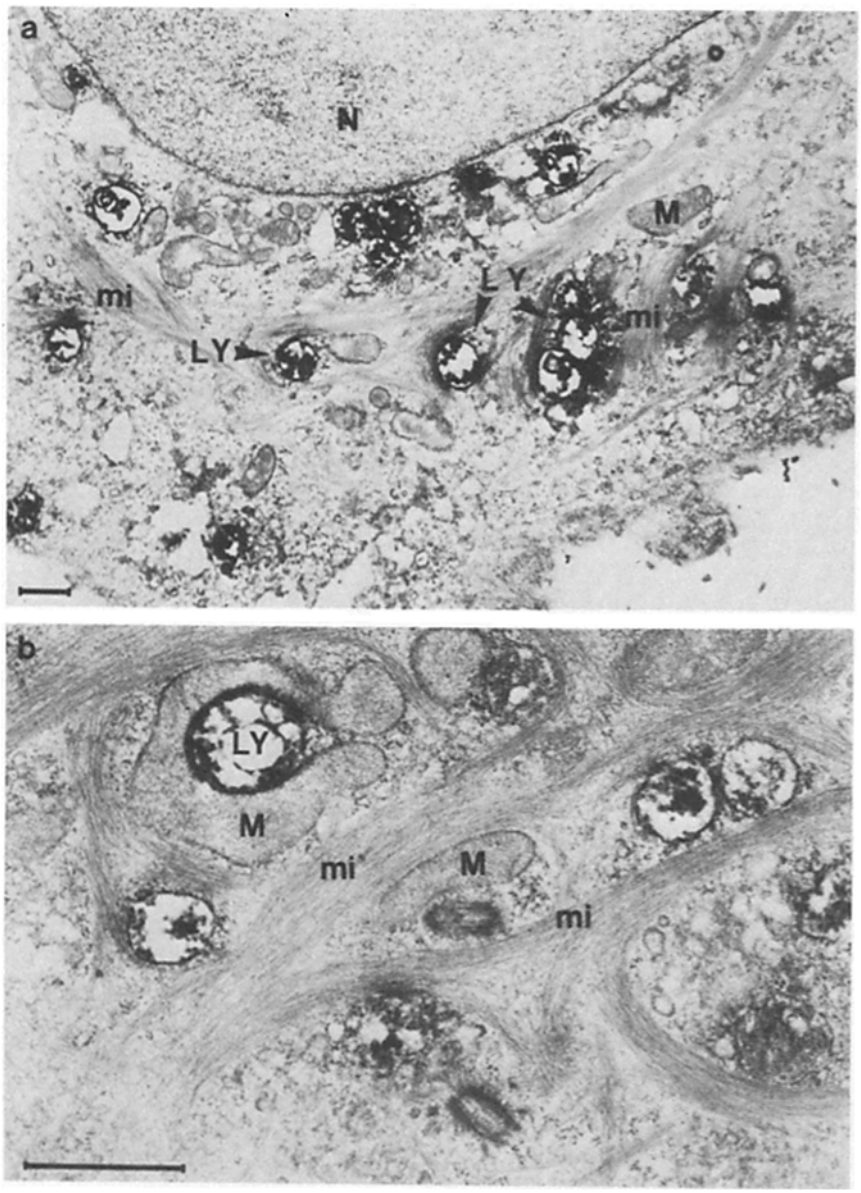

Figure 4. Cellular localization of the 52-kD protein. MCF_7 cells cultured with estradiol were fixed, permeabilized, and incubated with the D7E3 anti-52-kD monoclonal antibody (10 μg/ml). They were immunostained using the indirect peroxidase-antiperoxidase technique as described in Materials and Methods. Staining was mostly observed in lysosomes. (*Ly*) lysosomes; (*N*) nucleus; (*M*) mitochondria; (*mi*) microfilaments. Bars, 1 μm.

Table I. Purification of the Secreted Proteinase Activity

Step	Total protein	Total enzyme activity	Specific activity	Yield	Purification
	μg	*cpm* × 10^{-6}	*cpm per ng protein*	*%*	*-fold*
1. Start medium	6,220	9.6	1.5	100	1
2. Con A eluate	258	6.6	26.4	69	17
3. Ig G eluate	5	1.6	318	16.6	208

The secreted 52 K protein was purified from 80 ml of culture medium conditioned by MCF_7 cells cultured with 10% FCS, as described in Materials and Methods. The final elution of the immunoaffinity column (IgG) was at pH 11. Protein concentrations in the medium and the Con A eluate were determined by the Bradford technique and by scanning the traces of the silver-stained gel in the IgG eluate. Proteinase activity was assayed as described in Materials and Methods and expressed in counts per minute of [^{14}C]methemoglobin solubilized at pH 4.0 for 30 min.

been eluted at acidic or alkaline pH. The purity of the final preparation used for enzymatic studies was shown by finding single silver-stained bands of overloaded SDS polyacrylamide gel (Capony et al., 1986; Morisset et al., 1986*b*) and a single NH_2-terminal amino acid (Leu) of the purified secreted 52-kD protein (Ferrara et al., unpublished results). When the conditioned medium was passed through an immunoaffinity column to remove the 52-kD protein, 96% of the proteolytic activity was retained on the column and recovered after elution of the 52-kD protein.

The first enzymatic assays were performed with a 52-kD secreted proteinase purified under conditions (final elution step at pH 3.0) which could autoactivate an inactive proenzyme. To see whether the precursor 52-kD protein was secreted in an active or inactive form, we then purified it with a final elution step at pH 11 and tested its proteolytic activity at pH 4.0. The enzymatic activity was very low in the first 8 min of incubation at pH 4.0, and increased thereafter (Fig. 6 *a*). The molecular weight of the secreted 52-kD protein (Fig. 6 *a*, inset *1*) was slightly decreased to 51 kD under these conditions (inset *2*), but no 48- and 34-kD proteins were formed. The activation into the 51-kD protein was inhibited by pepstatin (inset *3*), indicating that the inactive 52-kD precursor undergoes an acid-dependent autoactivation, probably by removal of a short propeptide at the NH_2-ter-

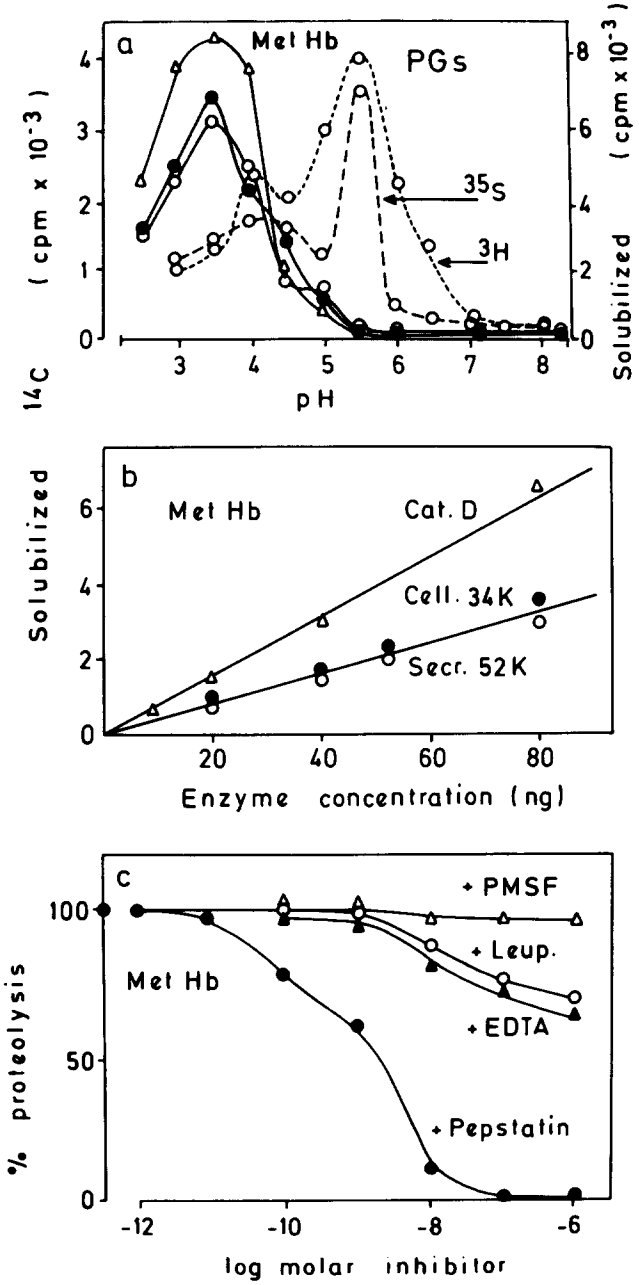

Figure 5. Proteolytic activity of the secreted and cellular related 52-kD proteins and of cathepsin D. Proteolytic activities of purified 52-kD proteins and bovine cathepsin D (see Fig. 6 *A*) were assayed as described in Materials and Methods (10 min at 37°C) using 10,000 cpm of [^{14}C]methemoglobin (*Met Hb*) or ^{3}H- and ^{35}S-labeled proteoglycans (*PGs*) as substrates. TCA-solubilized material was directly counted for radioactivity. Three enzyme preparations were used: secreted 52-kD protein (*open circles*), cellular related protein (*34 K*) (*closed circles*) and bovine cathepsin D (*open triangles*). (*a*) Effect of pH. The reaction buffers are citrate buffer (pH 2.5–4.5), acetate buffer (pH 5–6.5), phosphate buffer (pH 7.2), and Tris buffer (pH 8.4). 90 ng of each enzyme was used. (*b*) Effect of enzyme concentration. Stock solutions (pH 5.0) of cellular-related 52-kD protein containing mostly the 34-kD protein, secreted 52-kD and bovine cathepsin D were diluted in a pH 5.0 acetate buffer and finally assayed for proteolytic activities at a final pH of 4.0. (*c*) Effect of proteinase inhibitors. The proteolytic activity of 80 ng of purified cellular related 52-kD proteins was assayed at pH 4.0 without and with increasing concentrations of the indicated inhibitors. The 100% value corresponded to the noninhibited proteolysis (4,000 cpm released in 10 min).

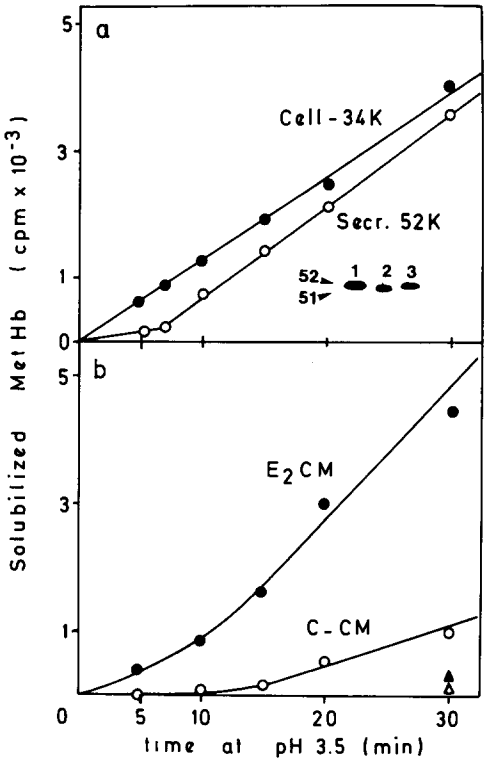

Figure 6. Time-dependent acid activation of proteinase activity. (*a*) The secreted and cellular related 52-kD proteins were purified (final elution at pH 11). 80 ng of each was then incubated at pH 4.0 with [^{14}C]methemoglobin as substrate. The reaction was stopped by adding TCA at the indicated times. 80-ng samples of purified secreted 52-kD protein were incubated for 30 min at 37°C either at pH 11.0 (*1*), or at pH 3.0 without (*2*) or with 1 μM (*3*) pepstatin. They were then compared on an SDS polyacrylamide gel and silver stained. (*b*) Conditioned media from MCF_7 cells treated for 4 d with estradiol (*E_2CM*) (*solid symbols*) or without estradiol (*C-CM*) (*empty symbols*) were prepared as described (Vignon et al., 1983). They contained proteins released for the last 18 h under serum-free conditions in Ham's F12-DME medium. Protein concentration was made equal in E_2-CM and C-CM (105 μg per 700 μl of final volume) by adding Ham's F12-DME medium. Proteinase activity was assayed by adding at time 0 the substrate mixture containing 70,000 cpm of [^{14}C]methemoglobin, 700 μg of unlabeled methemoglobin in citrate buffer (final pH 4.0). 100-μl aliquots were taken from the same batches at different times of incubation and proteolysis was stopped by adding TCA. 30-min samples were also assayed with 10 nM pepstatin (*triangles*).

minal moiety of the molecule. Similar autoactivation has been described for cathepsin D of human fibroblasts (Hasilik et al., 1982) and of bovine spleen (Puizdar and Turk, 1981). By contrast, both the secreted precursor prepared under acidic conditions and the related cellular 52-kD proteins displayed no lag in enzymatic activity (Fig. 6 *a*) and no change in their molecular mass, suggesting that they are fully active in acidic cellular organelles (endosomes, lysosomes). A similar time-dependent activation of the secreted proteinase was observed at acidic pH in the conditioned media containing proteins released by estradiol-treated MCF_7 cells, and was also observed but at a much lower level in conditioned media from control MCF_7 cells (Fig. 6 *b*). Inhibition of the proteolytic activity by pepstatin suggests that this activity is due to the 52-kD protein. This result indicates that the 52-kD protein is active before any purification and that MCF_7 cells

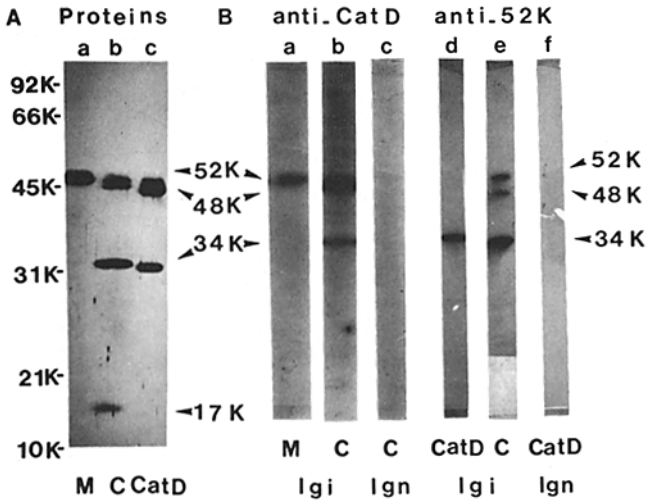

Figure 7. Purity and immunoreactivities of the 52-kD proteins and cathepsin D. (*A*) Purity of the enzymes. (*a*) The secreted and the (*b*) cellular related 52-kD proteins were purified by two chromatography steps with final elution at pH 3.0 (Capony et al., 1986). 200 ng of each preparation and (*c*) of bovine spleen cathepsin D were subjected to SDS PAGE and silver stained. (*B*) Immunoreactivities. (Lanes *a–c*) Immunoprecipitation of the secreted and cellular 52-kD protein by antibodies to human liver cathepsin D. Media (*M*) and cell extracts (*C*) were prepared from MCF_7 cells labeled with [^{35}S]methionine and incubated with an antiserum anti-human liver cathepsin D (Dingle et al., 1971) (lanes *a* and *b*, *Igi*) or an unrelated antiserum (lane *c*, *Ign*). The immunoprecipitates were isolated by protein-A Sepharose as described (Capony et al., 1982), analyzed by SDS PAGE and revealed by fluorography. (Lanes *d–f*) Electrophoretic transfer and immunologic detection of 400 ng each of purified cathepsin D (*Cat D*) and cellular related 52-kD proteins (*C*) were performed with the M1G8 monoclonal antibody to the 52 kD protein (*Igi*) or an unrelated mouse antibody (*Ign*), as described (Garcia et al., 1985).

secrete little if any proteinase inhibitor(s) for this enzyme. Moreover, it shows that the 52-kD protein secreted by human breast cancer cells can act in vitro as a proteinase after its autoactivation at acidic pH, but without being processed into its smaller molecular forms generally present in lysosomes.

Comparison with Cathepsin D from Normal Tissues

We found similarities, but also differences, between the 52-kD protein of MCF_7 cells and other cathepsin D(s) from bovine and normal human tissues. The specific activity of the secreted and related cellular 52-kD proteins were in the same range (55%–80%) as that of cathepsin D, as evaluated on the same substrate under zero-order kinetics (Fig. 5, *a* and *b*; Table I). The molecular mass of the secreted 52-kD protein and of its cellular forms was similar to those of bovine liver (Fig. 7 *A*) and human fibroblast cathepsin D (Gieselmann et al., 1985). These cathepsin D(s), like the mammary 52-kD protein, are processed into active lysosomal 48-kD and 34-kD 17-kD proteinases.

Moreover, polyclonal antibodies to human liver cathepsin D (Dingle et al., 1971) specifically immunoprecipitated the ^{35}S-labeled secreted 52-kD protein and the related cellular proteins of MCF_7 cells (Fig. 7 *B*, lanes *a–c*). In addition, human 34-kD cathepsin D was detected by Western immunoblot using the M1G8 monoclonal antibody to the 52-kD protein (Fig. 7 *B*, lanes *d–f*). Bovine cathepsin D was not detected by our antibodies, confirming their specificity for the human species (Garcia et al., 1985). These results show that the 52-kD-related proteins are antigenically closely related to cathepsin D and very similar to this proteinase. The amino acid composition of the purified 52-kD secreted protein (Capony et al., 1986) and human liver cathepsin D (Barrett, 1977) were found to be similar.

Moreover, the cathepsin D–like enzyme of MCF_7 cells has several characteristics that have not been previously described for normal human cathepsin D: (*a*) it is specifically induced by estrogens but not progesterone (Westley and Rochefort, 1980); (*b*) the secreted 52-kD precursor is mitogenic in MCF_7 cells (Vignon et al., 1986); (*c*) its intracellular concentration, as detected by our monoclonal antibodies, appears to be much higher in proliferative epithelial mammary cells and sweat glands than in other tissues, and is low in fibroblasts and endometrium (Garcia et al., 1986), whereas the distribution of the previously characterized cathepsin D appears more ubiquitous (Barrett, 1977).

Recently, we compared the Endo H sensitivity of the 52-kD–related cellular proteins of MCF_7 cells with that of cellular cathepsin D(s) prepared from several normal tissues and found marked differences. After purification using antibodies to the 52-kD protein, we found that the 34-kD cellular cathepsin D from placenta was different from that of breast cancer tissue or MCF_7 cells in its partial Endo H resistance (Fig. 8 *A*). The three forms in MCF_7 cells (52, 48, 34 kD) and the 34-kD protein of breast cancer, which is the most abundant immunoreactive cellular protein, were totally displaced by Endo H treatment (Fig. 8 *A*, lanes *a–d*), whereas in placenta, the 34-kD and a 28-kD protein, probably corresponding to a proteolytic product, were only partially displaced by this enzymatic treatment (Fig. 8 *A*, lanes *e–f*). Moreover, authentic cathepsin D(s) prepared classically from human liver (Barrett, 1970) or from bovine spleen, were also both partly resistant to Endo H digestion (Fig. 8 *B*). Increasing the concentration of Endo H did not modify the proportion (~40%) of Endo H–resistant chains. These results clearly indicate a difference in glycosylation between the cathepsin D–like enzymes of human breast cancer and those of different normal tissues. Similar partial resistance to Endo H has been described for the human fibroblast cathepsin D (Hasilik and Von Figura, 1981). One possible consequence of the complete Endo H sensitivity of the 52-kD cathepsin in breast cancer cells is the high proportion (up to 50%) of the secretion of its precursor in breast cancer cells (Morisset et al., 1986*a*) compared with normal cells (unpublished results). This difference suggests a defect in the maturation of *N*-glycosylated chains in cancer cells, which may be explained at the level of the structure of the protein, or at another level, such as enzymes involved in the posttranslational modifications of this protease. The sequencing of the MCF_7 52-kD protein from its cloned cDNA and its comparison with the sequence of normal human cathepsin D (Faust et al., 1985) will specify the degree of homology between these proteases.

Discussion

We have shown that the estrogen regulated 52-kD protein secreted by human breast cancer cells is the proenzyme of

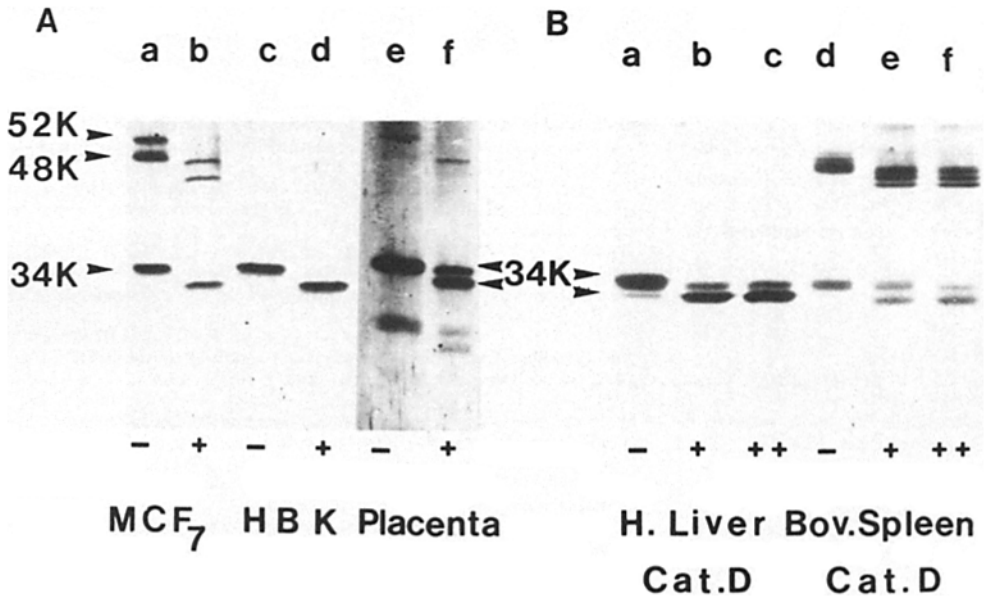

Figure 8. Differences in endo H sensitivity of cellular cathepsin D–like enzymes from breast cancers and normal tissues. (*A*) A pool of six primary breast cancers (*HBK*) and a human placenta were dissected immediately after surgery or delivery and frozen at −80°C. Both tissues were dipped into liquid nitrogen and powdered. The powders were then homogenized at 0–4°C using an Ultraturrax in NP-40 lysis buffer (Capony et al., 1986). The homogenates were centrifuged at 100,000 *g* for 30 min and the supernatants were subjected to the purification protocol used for cellular 52-kD proteins (Capony et al., 1986). Elutions from the 52-kD antibody immunoaffinity columns were carried out at pH 11.0. (*B*) Human liver (lanes *a–c*) and bovine spleen (lanes *d–f*) cathepsin D were obtained as indicated in Materials and Methods. The purified enzymes (6–10 μg/ml) were treated with Endo H as described in Materials and Methods using 50 mU/ml (+) or 200 mU/ml (++) Endo H and an incubation period of 20–24 h at 37°C in the presence of pepstatin. The samples were then run on SDS PAGE and revealed by silver staining as in Fig. 1 *B*.

a lysosomal acid proteinase similar to cathepsin D. The cellular processing of the 52-kD protein was separately shown using pulse chase experiments (Morisset et al., 1986*a*). The protein is partly (40%) secreted into the medium and partly processed intracellularly into 48- and 34-kD proteins. The 34-kD protein is more stable than the other forms and represents 65% of the total immunoreactive 52-kD–related proteins in cells. Lysosomotropic agents (NH_4Cl, monensin) inhibit markedly this intracellular processing in smaller proteins. The secretion of precursor of lysosomal hydrolase first described for I-cell disease (Hickman and Neufeld, 1972) has also recently been reported in mouse transformed fibroblasts (Gal and Gottesman, 1985). In the human MCF_7 cells, estrogens increase both the synthesis of the cellular 52-kD precursor (Morisset et al., 1986*a*), and to a larger extent, its secretion into the medium (Westley and Rochefort, 1980).

Both the 52-kD precursor and the mature 48- and 34-kD proteins are aspartic proteinases similar to the lysosomal cathepsin D on the basis of their molecular mass, inhibitor and substrate specificities, and immunoreactivities. However, there are several differences in the tissue distribution, hormonal regulation, glycosylation, and degree of secretion when compared with the cathepsin D from normal tissue. These differences as well as the mitogenic activity of the protein and its possible action on basement membrane and proteoglycans suggest a role of this secreted protein in the process of tumor growth and/or invasion.

Other proteinases have also been reported to be secreted specifically from transformed cells. The major excreted protein is an activatible acid-proteinase secreted by transformed mouse fibroblasts (Gal and Gottesman, 1985). The MEP and the 52-kD protein have similar optimal pH activity but are clearly different, in that the major excreted protein is a 39-kD mouse cysteine proteinase. Another proteinase "transin" secreted by transformed mouse fibroblasts is analogous to a collagenase (Matrisian et al., 1985). These proteinases may have important functions in the process of cancer cell migration and metastasis. Another characteristic of the 52-kD cathepsin D–like proteinase is its autocrine mitogenic activity on MCF_7 cells (Vignon et al., 1986) by an unknown mechanism. The 52-kD protein is able to bind plasma membrane of MCF_7 cells via mannose-6-phosphate receptors but additional receptors and/or its proteolytic activity may be required to trigger its mitogenic activity as previously discussed for thrombin (Carney and Cunningham, 1978), another mitogenic proteinase. The autocrine mitogenic activity of conditioned media prepared from estrogen-stimulated MCF_7 cells (Vignon et al., 1983) is now confirmed by several groups (Manni et al., 1986; Dickson et al., 1986). The nature of the estrogen-induced proteins or factors primarily responsible for this mitogenic activity in these conditioned media is, however, debated. Classical growth factors activating transmembrane receptors have been detected by their biological and binding activities (Dickson et al., 1986; Salomon et al., 1984). The estrogen-induced lysosomal 52-kD proteinase is another class of autocrine mitogen which may be involved in the production of some of these growth factors from their inactive precursors. Plasminogen activator is also secreted from MCF_7 cells and has been reported to be estrogen stimulated (Butler et al., 1979; Ryan et al., 1984). This serine proteinase is also induced by progestins, its amount appears to be less than the 52-kD cathepsin D–like enzyme and, however, it may act indirectly by activating other proteinases such as collagenase (Liotta, 1986). Moreover, estradiol also induces serine proteinase inhibitors such as the α1 antichymotrypsin (Massot et al., 1985).

In addition to the classical function of cathepsins in intracellular protein degradation, it has been proposed that these proteinases might play a role in the process of tumor invasion (Barrett, 1970; Poole, 1979) by helping cancer cells to migrate, invade adjacent tissue, and metastasize. Our results strongly support this hypothesis. Cloning of the cDNA of this proteinase is in progress to determine the structure of the protein and its possible function in carcinogenesis.

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